ANTIMICROBIAL RESISTANCE IN AQUATIC ENVIRONMENTS

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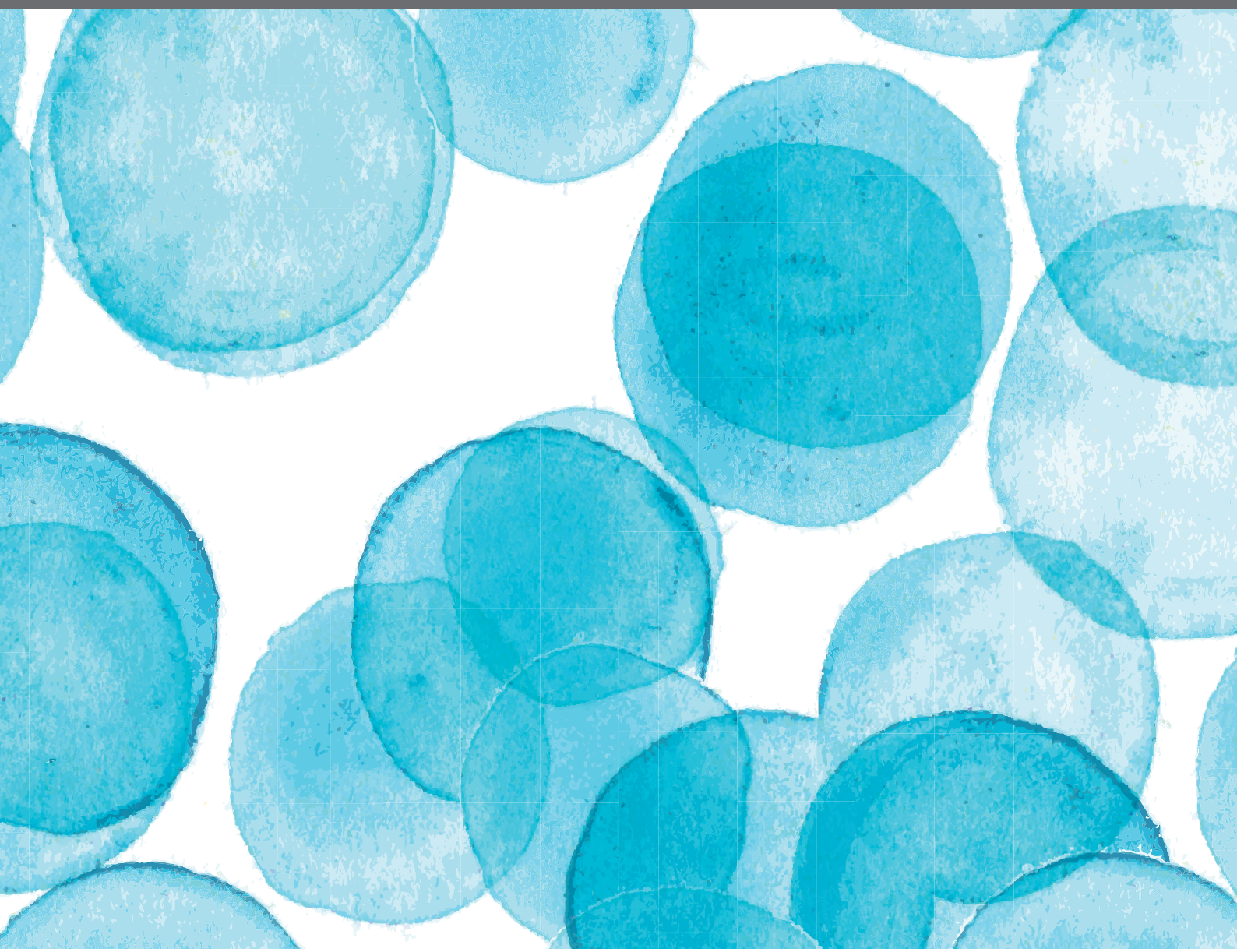

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ANTIMICROBIAL RESISTANCE IN AQUATIC ENVIRONMENTS

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Table of Contents

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EDITORIAL
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Editorial: Antimicrobial Resistance in Aquatic Environments

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Editorial on the Research Topic

Antimicrobial Resistance in Aquatic Environments

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The current SARS-CoV-2 pandemic has exacerbated the rapid diagnosis of infectious diseases outbreaks to take suitable epidemiological measures to minimize negative impacts (Oude Munnink et al., 2021). However, the silent pandemic of antimicrobial resistance (AMR) faces several outstanding questions about its evolution and dissemination. The urgency of an integrated approach involving all ecological compartments, where antimicrobials and antimicrobial resistance genes (ARGs) reservoirs are generated, maintained, and disseminated, is urgently required (Da Silva et al., 2020). Aquatic environments are critical for understanding how the AMR develops and spreads worldwide, considering their role as an endpoint of effluents of wastewater treatment plants (WWTPs) or direct disposition of sewage from human or animal origin (Zheng et al., 2021; Miłobedzka et al., 2022), the runoff of biosolids in the agriculture (Buta et al., 2021), and other anthropogenic factors that contribute to the propagation of antimicrobial resistance determinants.

Therefore, this Research Topic aimed to deliver state-of-the-art knowledge and ideas on aquatic environments' role in selecting, maintaining, and dispersing AMR determinants. Fourteen articles from Europe, Asia, America, and Africa have been published on this topic that complements our knowledge and formulate several questions for the scientific community worldwide.

HOSPITAL WASTEWATER ROLE ON AMR DISSEMINATION

Hospital wastewaters represent a broad reservoir of antibiotic-resistant bacteria (ARB) and ARGs, which include extended-spectrum β-lactamases (ESBLs) and carbapenemase-producing *Enterobacteriaceae* (CPE), for instance (Hassoun-Kheir et al., 2020). However, there are significant knowledge gaps about the proper wastewater treatment technologies to be applied and a lack of protocols and indicators for executing an appropriate risk assessment (Nguyen et al., 2021). This Research Topic includes articles that cover the influence of hospital discharges on their receiving water bodies. In Romania, the study of Popa et al. describes the transmission of multidrug-resistant *Klebsiella pneumoniae* ST101 clone from hospital to wastewater and its persistence after chlorine treatment. The article highlights the risk of inappropriate hospital sewage disposition onto the surface water and their potential implications on the trophic chain. In Brazil, the study of Esposito et al. reports the genomic data and the virulence potential of *Pseudomonas aeruginosa*

that harbor the São-Paulo-Metallo-β-lactamase (SPM-1), carried by high-risk clone ST277 isolated from urban rivers. The authors observed a common resistome and virulome between clinical and environmental SPM-1-producing *P. aeruginosa* strains endemic from Brazil. Additionally, the SNP-based phylogenomics showed a high similarity between clinical and environmental genomes, suggesting that these clones could be disseminated onto water bodies from hospital settings.

METAGENOMICS AS A PROMISING TOOL FOR AMR SURVEILLANCE IN THE ENVIRONMENT

One of the current outstanding questions about the analysis of AMR in the environment is the standardization of genomic and metagenomic assays that could minimize the spatiotemporal variability, the allochthonous ARG levels, the environmental resistome complexity, and the biases about genomic extraction, sequencing, and genomic analyses (Calero-Cáceres et al., 2019; Li et al., 2020). Two articles that highlight the advantages of metagenomics in AMR analysis were included in this Research Topic. First, Perry et al. analyzed the influence of different hospital clinical activities on the abundance of ARGs in hospital wastewater in Scotland, highlighting the advantages of shotgun metagenomics to identify a full range of ARGs that could be used to guide environmental policies about AMR. Additionally, the article of Guo et al., using a high-throughput sequencing-based metagenomic approach, investigated the composition of bacteria and ARGs in wastewater from hospitals in China, suggesting a correlation between the abundance of ARGs and specific bacterial genera and remarking that it is necessary to complement their study including physicochemical analysis for the raw wastewater. Both articles show interesting results and note the necessity to develop an integrative framework that would include omics, physicochemical and epidemiological research to enhance the evaluation of ARGs pollution in environmental sources.

URBAN WWTP INFLUENCE ON AMR DISSEMINATION

Several papers of this special issue analyzed the influence of WWTPs and their discharges on AMR dissemination: In South Korea, Shin et al. characterized an extensively drug-resistant (XDR) *E. coli* isolated from influents of a WWTP. This study suggests that these isolates could be disseminated into the outgoing river from WWTP. The sewage could act as a potential spreader of ARGs, including emerging carbapenemase genes like $bla_{\mathrm{NDM-5}}$. In South Africa, Mbanga et al. characterized isolates of *Enterococcus* spp. from a WWTP and their receiving water bodies that serve as a water source for domestic, agricultural, and recreational purposes. Those isolates harbor a wide plethora of ARGs and virulence factors, showing that the effluents of the WWTP could act as a dissemination vector of multidrug-resistant (MDR) microorganisms. This Research Topic includes a review paper by Uluseker et al. that extensively reviews the current knowledge on sources, spread, and removal mechanisms of ARGs in microbial communities of wastewaters, WWTPs, and downstream recipients. This review includes the basis of antibiotic resistance, an explanation about the dynamics of AMR and antibiotics in WWTPs, and suggestions to be considered for the operation, regulation, and design of WWTPs. These studies suggest the urgent need for regular surveillance and management of water bodies to limit the spread of these isolates.

ANTHROPOGENIC INFLUENCE ON WATER BODIES

Singh et al. analyzed *Escherichia coli* from the river Yamuna (India), a highly polluted river that receives an intense anthropogenic influence from urban and animal origin. Their results showed high AMR profiles, highlighting the presence of CTX-M-15 type ESBLs and the occurrence of class I integrons in their isolates. In Ireland, Sala-Comorera et al. demonstrated the strong impact of different watercourses discharges onto the levels of AMR in both bacterial and bacteriophage fractions in marine bathing waters, which may expose the users to fecal pollution and therefore could increase the probability to be exposed to ARGs. Another outstanding question about AMR in the environment is to demonstrate which levels of AMR are necessary to represent a real environmental danger. Finally, Pallares-Vega et al., shown by *in vitro* assays the role of ecological factors that could hamper conjugative plasmid transfer from gut bacteria once discharged into the environment. Their findings highlight the possibility that the fecal organisms may transfer plasmids in aquatic ecosystems, despite the variable conditions that could occur environmentally.

AMR IMPACT ON THE FOOD CHAIN

The food supply chain connects environmental sources of bacteria with humans and represents another outstanding field in the One Health perspective for understanding the dissemination and evolution of AMR. The article of Montero et al. analyzed ESBL producing *E. coli* isolated from irrigation waters, vegetables, and fruits in Ecuador. These authors detected that the allelic variants of the $bla_{\mathrm{CTX-M}}$ gene found in irrigation channels and vegetables were the same as those observed in commensal *E. coli* from domestic animals, and commensal and pathogenic *E. coli* from humans, suggesting a connection between these different sources. In addition, the article of Cheng et al. analyzed sediments from aquaculture farms in China by constructing network plots based on *16S rRNA* metagenomics, physicochemical analysis, and quantification of ARGs. Their results provide evidence for understanding the environmental risks associated with aquaculture practices. On the other hand, Ye et al. showed in *Edwardsiella tarda,* an important pathogen in aquaculture, that reactive oxygen species (ROS) play a role in bacterial resistance and sensitivity to ceftazidime. They saw a lower ROS production in ceftazidime-resistant *E. tarda* than in a sensitive strain related to the inactivation of the pyruvate cycle. Additionally, their study reveals a new mechanism that increases ROS production, the activation of the pyruvate cycle by Fe^{3+}. These findings

provide tools and knowledge for future new strategies to fight MDR pathogens.

ROLE OF WILDLIFE AS A POTENTIAL RESERVOIR OF AMR

Zeballos-Gross et al. comprehensively reviewed the potential role of gulls as reservoirs and vectors of AMR in the environment, highlighting the current knowledge about related research, the phenotypic and molecular characterization of AMR, limitations about the existing methodologies, and suggestions for improving the derived results.

In summary, this Research Topic provides an excellent update of the role of aquatic ecosystems on the evolution and dissemination of AMR worldwide. Therefore, the editors encourage the scientific community to consider the results and challenges of this special issue.

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WC-C, JO-P, LR-R, and EM contributed to analyzing the reviewed articles and preparing the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

Buta, M., Hubeny, J., Zieliński, W., Harnisz, M., and Korzeniewska, E. (2021). Sewage sludge in agriculture – the effects of selected chemical pollutants and emerging genetic resistance determinants on the quality of soil and crops – a review. *Ecotoxicol. Environ. Saf.* 214, 112070. doi: 10.1016/J.ECOENV.2021.112070

Calero-Cáceres, W., Ye, M., and Balcázar, J. L. (2019). Bacteriophages as environmental reservoirs of antibiotic resistance. *Trends Microbiol.* 27, 570–577. doi: 10.1016/j.tim.2019.02.008

Da Silva, J. B., Espinal, M., and Ramón-Pardo, P. (2020). Antimicrobial resistance: time for action. *Rev. Panam. Salud Pública* 44, e131. doi: 10.26633/RPSP.2020.131

Hassoun-Kheir, N., Stabholz, Y., Kreft, J. U., de la Cruz, R., Romalde, J. L., Nesme, J., et al. (2020). Comparison of antibiotic-resistant bacteria and antibiotic resistance genes abundance in hospital and community wastewater: a systematic review. *Sci. Total Environ.* 743, 140804. doi: 10.1016/J.SCITOTENV.2020.140804

Li, L. G., Huang, Q., Yin, X., and Zhang, T. (2020). Source tracking of antibiotic resistance genes in the environment - Challenges, progress, and prospects. *Water Res.* 185, 116127. doi: 10.1016/J.WATRES.2020.116127

Miłobedzka, A., Ferreira, C., Vaz-Moreira, I., Calderón-Franco, D., Gorecki, A., Purkrtova, S., et al. (2022). Monitoring antibiotic resistance genes in wastewater environments: the challenges of filling a gap in the One-Health cycle. *J. Hazard. Mater.* 424, 127407. doi: 10.1016/J.JHAZMAT.2021.127407

Nguyen, A. Q., Vu, H. P., Nguyen, L. N., Wang, Q., Djordjevic, S. P., Donner, E., et al. (2021). Monitoring antibiotic resistance genes in wastewater treatment: current strategies and future challenges. *Sci. Total Environ.* 783, 146964. doi: 10.1016/J.SCITOTENV.2021.146964

Oude Munnink, B. B., Worp, N., Nieuwenhuijse, D. F., Sikkema, R. S., Haagmans, B., Fouchier, R. A. M., et al. (2021). The next phase of SARS-CoV-2 surveillance: real-time molecular epidemiology. *Nat. Med.* 27, 1518–1524. doi: 10.1038/s41591-021-01472-w

Zheng, D., Yin, G., Liu, M., Chen, C., Jiang, Y., Hou, L., et al. (2021). A systematic review of antibiotics and antibiotic resistance genes in estuarine and coastal environments. *Sci. Total Environ.* 777, 146009. doi: 10.1016/J.SCITOTENV.2021.146009

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ORIGINAL RESEARCH
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Multidrug Resistant *Klebsiella pneumoniae* ST101 Clone Survival Chain From Inpatients to Hospital Effluent After Chlorine Treatment

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In this paper we describe the transmission of a multi-drug resistant *Klebsiella pneumoniae* ST101 clone from hospital to wastewater and its persistence after chlorine treatment. Water samples from influents and effluents of the sewage tank of an infectious diseases hospital and clinical strains collected from the intra-hospital infections, during a period of 10 days prior to wastewater sampling were analyzed. Antibiotic resistant *K. pneumoniae* strains from wastewaters were recovered on selective media. Based on antibiotic susceptibility profiles and PCR analyses of antibiotic resistance (AR) genetic background, as well as whole-genome sequencing (Illumina MiSeq) and subsequent bioinformatic analyses, 11 ST101 *K. pneumoniae* strains isolated from hospital wastewater influent, wastewater effluent and clinical sector were identified as clonally related. The SNP and core genome analyses pointed out that five strains were found to be closely related (with $\leq$18 SNPs and identical cgMLST profile). The strains belonging to this clone harbored multiple acquired AR genes [$bla_{CTX-M-15}$, bla_{OXA-48}, bla_{OXA-1}, $bla_{SHV-106}$, $bla_{TEM-150}$, *aac(3)-IIa*, *aac(6')-Ib-cr*, *oqx*A10, *oqx*B17, *fos*A, *cat*B3, *dfr*A14, *tet*(D)] and chromosomal mutations involved in AR (Δ*mgrB*, Δ*ompK35*, amino acid substitutions in GyrA Ser83Tyr, Asp87Asn, ParC Ser80Tyr). Twenty-nine virulence genes involved in iron acquisition, biofilm and pili formation, adherence, and the type six secretion system – T6SS-III were identified. Our study proves the transmission of MDR *K. pneumoniae* from hospital to the hospital effluent and its persistence after the chlorine treatment, raising the risk of surface water contamination and further dissemination to different components of the trophic chain, including humans.

Keywords: wastewater treatment plant, MDR *Klebsiella pneumoniae*, whole-genome sequencing, hospital wastewater chlorine treatment, hospital sewage

INTRODUCTION

Due to the worldwide use of antibiotics in the treatment of human and animal infectious diseases, but also in livestock and agriculture, a large amount of antibiotics of pharmaceutical origin are found in anthropic environments, such as sewage and wastewater treatment plants (WWTPs) and ends up being discharged in the natural environment (Kraemer et al., 2019; Zhang T. et al., 2019; Zhang Z. et al., 2019). The extensive use of antibiotics leads to the spread of antibiotic resistance (AR), which currently represents a major public health concern. AR rates are particularly high in acute care hospitals driven by selective pressure of antibiotic usage (Hocquet et al., 2016), hospitals being main ecological niches for the selection, accumulation, and spread of antibiotic resistant bacteria (ARB).

Water plays a crucial role in the spreading of AR, through inappropriate release of human and animal effluents in the surface waters through hospital wastewaters, WWTPs, aquaculture farms, surface, and groundwater. Hospital wastewaters are highly complex effluents, carrying a wide range of micro- and macropollutants, including antibiotic compounds, metabolized drugs, disinfectants, patient excrements, and microorganisms. The presence of ARB as well as of antibiotic residues, which could inhibit the growth of susceptible bacteria, are thereby increasing the population of resistant bacteria in the receiving water (Kaur et al., 2020; Rozman et al., 2020).

Hospitals generate a large amount of wastewater per day. The hospital effluents are loaded with pathogenic microorganisms, antibiotics, and other pharmaceutical or toxic substances, which are only partially removed during wastewater treatments, driving the pollution of the natural environments, including selection, and dissemination of AR (Kummerer et al., 2000; Kim and Aga, 2007; Alrhmoun et al., 2014; Laffite et al., 2016). Antibiotic pollutants, as well as heavy metals and even chlorination, could increase the general rates of mutation, recombination, and lateral gene transfer, thus recruiting more genes into the resistome and mobilome, and simultaneously providing the selective force to fix such changes, acting as drivers of bacterial evolution, with potentially adverse consequences for human welfare (Gillings, 2013). To minimize the risk of superbugs selection, the World Health Organization (WHO, 2014) recommends that hospitals have onsite facilities for the pre-treatment of hospital effluent prior to discharge into the general wastewater streaming[1]; the purpose is to eliminate different contaminants, such as bacterial pathogens, antibiotics, disinfectants, radioactive substances, toxic chemicals, etc.

Considering the increasing risk of microbial infections by reclaiming water through processing it in WWTPs, advanced treatment technologies and disinfection process are considered a major tool to control the spread of ARB into the environment (Rizzo et al., 2013), one of the most widely used and accepted ways to achieve it being through chlorine treatment (Huang et al., 2011). Chlorine efficiency depends on the concentration, exposure time and the formulation used. It has been shown that chlorine may also contribute to the selection of bacteria highly resistant to tetracycline, chloramphenicol, trimethoprim, and to the accumulation of various ARGs (such as *amp*C, *aph*A2, bla_{TEM-1}, *tet*A/G, *erm*A/B, plasmids, insertion sequences, and integrons) (Shi et al., 2013). In addition, metagenomic analyses performed on wastewater samples after chlorine treatment, shows that up to 40% of erythromycin resistance genes and 80% of tetracycline resistance genes cannot be removed (Yuan et al., 2015). Furthermore, chlorine treatment may promote conjugation and the ARGs transmission through horizontal gene transfer of mobile genetic elements and also plasmid over-replication and the emergence of multi-drug resistance (MDR) through activation of multi-drug efflux pumps (Shi et al., 2013; Popa et al., 2018; Sanganyado and Gwenzi, 2019; Vrancianu et al., 2020).

Multi-drug resistant *Klebsiella pneumoniae* is a major nosocomial pathogen, causing infections with high morbidity and mortality rates (up to 50%) (Bassetti et al., 2018), caused by limited treatment options. This pathogen harbors a wide resistome that could evolve under antibiotic and biocide selective pressure, leading to the occurrence of extremely drug resistant (XDR) or high-risk (HiR) clones, with great epidemic potential (Navon-Venezia et al., 2017).

As many as 90,000 infections and more than 7,000 deaths in Europe are attributable to *K. pneumoniae* resistant to carbapenems, to colistin or producing extended spectrum β-lactamase (ESBL) (Cassini et al., 2015). In 2018, resistance to carbapenems (last resort antibiotics) in *K. pneumoniae* ranged in various countries from 0 to 63.9%, the highest prevalence being encountered in Greece, followed by Romania (29.5%) and Italy (26.8%) (EARSS-Net), Europe being considered "epidemic" for carbapenemase- producing *K. pneumoniae* (Bassetti et al., 2018). The dissemination of MDR *K. pneumoniae* strains from hospitals to the environment was previously demonstrated (Mahon et al., 2017; Khan et al., 2018; Lepuschitz et al., 2019), highlighting the ability of these strains to survive and persists in environmental conditions. Despite this evidence, primary treatment of hospital wastewaters before their discharge in the urban sewage is not mandatory in many countries (Hocquet et al., 2016; Rozman et al., 2020), these wastewaters ending up being treated in urban wastewater treatment plants. One of the primary treatments of hospital wastewaters used in Romania is represented by chlorine treatment, but the knowledge regarding the effects of chlorination on ARB is scarce. The current literature reports conflicting results, since some studies describe the removal of some ARB by chlorine treatment (Zhang et al., 2015; Lin et al., 2016), while other data suggest that this treatment is ineffective (Yuan et al., 2015).

Previous data from our research team (Surleac et al., 2020) showed that MDR, carbapenemase and ESBL-producing *K. pneumoniae* isolated from clinics, hospital wastewater, and urban WWTPs in different regions of the country exhibit multiple antibiotic and antiseptic resistance, as well as virulence genes, the ST101 clone being the most frequently encountered in all sampling sites. The *K. pneumoniae* ST101 clone seems to be well established in Romanian hospitals (Dortet et al., 2015; Czobor et al., 2016) and wastewaters (Surleac et al., 2020), this determining us to investigate its possible transmission from

[1]https://www.who.int/

hospital to wastewater, aiming to comparatively characterize the *K. pneumoniae* ST101 isolated from an infectious diseases hospital and its wastewaters.

MATERIALS AND METHODS

Isolation of ARB From Clinical and Wastewater Samples

Grab water samples were collected in November (21st and 23rd) 2018, and March (20) 2019 from the influent and the effluent of the hospital collecting sewage tank (**Figure 1**), in which active chlorine solution (0.06 g/L) is intermittently pulverized, according to the hospital standard operating procedure. Water samples were processed following the recommendations of SR EN ISO 9308-2/2014 (coliform bacteria). Briefly, different water volumes and dilutions (undiluted 1 ml, 10 ml, and 30 ml as well as 1 ml and 3 ml out of ten-fold dilutions – 1/10 and 1/100, respectively) were filtered through 0.45 μm pore size membranes (Millipore, France), subsequently inoculated on antibiotic-enriched, chromogenic media (BioMérieux, France), namely ChromID ESBL (for ESBL-producing enterobacteria), ChromID OXA-48 agar and ChromID CARBA agar for carbapenemase (CRE)-producing strains. Up to ten colonies with KESC (*Klebsiella – Enterobacter – Serratia – Citrobacter*) carbapenem-resistance phenotype were randomly selected from each culture media per sample. The isolates were confirmed by subsequent cultivation on the same type of chromogenic media used for their isolation, identified using the MALDI-TOF-MS Bruker system and subsequently introduced in the microbial collection of the Research Institute of the University of Bucharest. In order to evaluate the occurrence of particular clones of MDR *K. pneumoniae* in hospital wastewater, 10 days prior to water sampling, all *K. pneumoniae* strains isolated from positive clinical specimens, were collected by the hospital microbiology laboratory and provided for comparative analysis (**Supplementary Table 1**). The clinical strains were sampled from inpatients, cultured and subsequently isolated on blood agar, and Cystine Lactose Electrolyte Deficient (CLED) agar (Rafila et al., 2015), identified using MALDI-TOF-MS Bruker system, and included in the microbial collection of the Research Institute of the University of Bucharest without any link to personal data regarding the patients.

Antibiotic Susceptibility Testing

The two colonies of *K. pneumoniae* selected from each chromogenic media (ChromID ESBL, OXA-48, and CARBA) and each volume/dilution used for isolation, as well as the clinical *K. pneumoniae* strains were further studied using the disk diffusion method (CLSI, 2019) and the following 16 antibiotics: amikacin (AK), amoxicillin-clavulanic acid (AMC), ampicillin (AMP), aztreonam (ATM), cefepime (FEP), cefoxitin (FOX), ceftriaxone (CRO), cefuroxime (CXM), ciprofloxacin (CIP), ertapenem (ETP), gentamicin (CN), imipenem (IMP), meropenem (MEM), piperacillin (PRL), tetracycline (TET), trimethoprim-sulfamethoxazole (SXT). *Escherichia coli* ATCC 25922 strain was used for quality control.

Screening of β-Lactam Resistance Genes (ARGs)

In order to investigate the genetic support of β-lactam enzymatic resistance (carbapenemases, ESBLs), bacterial DNA was extracted using an adapted alkaline extraction method (Almahdawy et al., 2019). The genetic background of AR was investigated by PCR (PCR Master Mix 2X, Thermo Scientific), using 1 μl of DNA and specific primers for bla_{TEM} (Quinteros et al., 2003), bla_{SHV} (Kim and Lee, 2000), bla_{CTX-M} (Israil et al., 2013), bla_{OXA-48} (Poirel et al., 2011), bla_{VIM} (Shirani et al., 2016), bla_{IMP} (Shirani

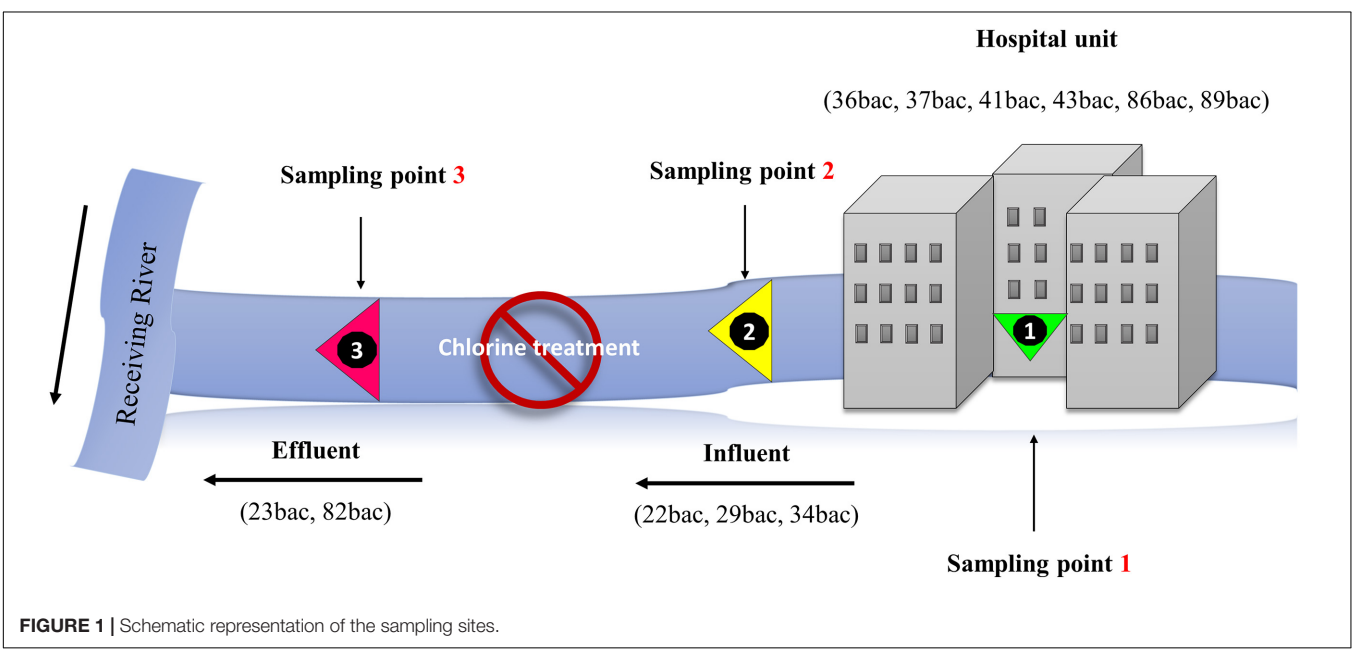

FIGURE 1 | Schematic representation of the sampling sites.

et al., 2016), bla_{NDM} (Nordmann et al., 2011), bla_{SIM} (Qi et al., 2008), bla_{SPM} (Ellington et al., 2007), and bla_{KPC} (Poirel et al., 2011) genes (**Supplementary Table 2**). All reactions were carried out using the temperature of 55°C for primers annealing, except for bla_{TEM} and bla_{IMP}, for which the annealing was performed at 52°C.

WGS and Bioinformatics Setup

The aquatic strains from the three sampling points (n = 23) showing matching AR profiles with the clinical strains (n = 8) (**Supplementary Table 3**) were selected for whole genome sequencing. Total DNA was extracted (DNeasy UltraClean Microbial Kit Qiagen optimized with an additional mechanical and chemical bacterial lysis step and ethanol precipitation) and subjected to Illumina (Nextera DNA Flex Library Prep Kit) sequencing. Both quality (2100 Bioanalyzer, Agilent) and quantity (Qubit 4 Fluorometer, Thermo Fisher Scientific, United States) checks were performed on the DNA pool libraries before starting the paired-end shotgun sequencing on the MiSeq platform (Illumina, United States). MiSeq reagent kit v.3 (600 cycles) was chosen for the high quality of the generated output.

Raw reads were quality-checked using FastQC (Andrews, 2010), assembled using *Shovill* pipeline (Seemann, 2018), and primarily annotated using Prokka (Seemann, 2014). Specific gene profiling was assessed using ABRicate (Seemann, 2020b) software, with specific databases for AR (Feldgarden et al., 2019), virulence (Chen et al., 2016), and plasmid replicons (Carattoli et al., 2014). ARIBA was also used for predicting the resistance genes for each sample (Hunt et al., 2017). Moreover, we also interrogated the database provided by the online CGE platform – http://www.genomicepidemiology.org/- (CGE Platform, 2020), regarding the resistance (Zankari et al., 2012), virulence (Joensen et al., 2014) and the pathogenic potential using Pathogen Finder predictor software (Cosentino et al., 2013). Capsular and LPS antigens (K and O loci) and chromosomal mutations involved in AR were annotated using Kleborate (Wyres et al., 2016; Wick et al., 2018). Strain relatedness was investigated using MLST (Seemann, 2020a), cgMLST (Jolley and Maiden, 2010) Snippy (Seemann, 2015) and kSNP3 (Gardner et al., 2015). Comparative gene analyses were performed using Roary (Page et al., 2015) and the output was used to infer phylogenies using RAxML (Stamatakis, 2014) and visualized using iTOL (Letunic and Bork, 2019).

Strains Selection Based on Phenotypic and Molecular Data

Out of a total of 101 carbapenem resistant *K. pneumoniae* strains, 78 isolated from wastewater and 23 from inpatients, some of them previously characterized for their antibiotic susceptibility profiles (Surleac et al., 2020), 31 were initially selected for the present study, based on their sampling location and MDR phenotype, as defined by Magiorakos et al., 2012 (**Supplementary Table 3**). For all the 31 strains, the MLST profile was inferred from WGS data (Surleac et al., 2020). The MLST profiles revealed by WGS data analyses highlighted that the *K. pneumoniae* ST101 subtype was the most prevalent (n = 11, 36%) in all three sampling points (**Supplementary Figure 2**). Thus, these isolated were further selected for characterization.

RESULTS

For the 31 strains selected, the antibiotic susceptibility profiles, the genetic background of β-lactam resistance and ST-types were compared, (**Supplementary Table 3**) revealing the presence of matching patterns of AR profiles (i.e., resistance to same classes of antibiotics, or to a single antibiotic from an antibiotic class), the presence/absence of ARGs and the abundance of *K. pneumoniae* ST101 The PCR for detection of β-lactam resistance genes revealed the presence of carbapenemases genes, which were, in decreasing frequency order, bla_{OXA-48}, bla_{NDM}, and bla_{KPC} as well as of the bla_{CTX-M} encoding for ESBLs. The bla_{SHV} gene was detected in all isolates (**Supplementary Table 3**).

All these strains were further characterized by whole genome sequencing. The ARGs identified by gene prediction were diverse and encoded resistance to β-lactams, aminoglycosides, quinolones, folate inhibitors, tetracyclines and others; no significant decrease of ARG distribution in the chlorine-treated effluent of vs. untreated influent of the hospital chlorination tank was observed, the majority of the ARGs being present in the aquatic strains isolated from all three sampling points. The most frequent genes encoding for β-lactam resistance were $bla_{CTX-M-15}$, bla_{OXA-1}, bla_{OXA-48}, $bla_{SHV-106}$, bla_{TEM-1}, and $bla_{SHV-158}$, for aminoglycosides were *aac(3)II-a*, *aph(6)-Id, aph(3″)-Ib*, and *aadA2,* while for quinolone resistance the transferable *qnrS1* gene was the most abundant (**Supplementary Figure 1**).

The main characteristics of the selected *K. pneumoniae* ST101 strains were: the presence of the MDR phenotype, three wastewater-sourced strains being resistant to all tested antibiotics (22 bac, 23 bac, 34bac), while the other two were susceptible only to aztreonam (29 bac, 82 bac). All six clinical strains were resistant to all tested antibiotics except for amikacin to which four strains were susceptible (**Supplementary Table 3**).

ST101 *K. pneumoniae* Clones Carry Multiple Antibiotic Resistance, Virulence, and Biocides Resistance Determinants

All *K. pneumoniae* ST101 strains selected for WGS presented the KL17/O1v1 serotype (as predicted by Kleborate software). All of them harbored multiple common ARGs (**Table 1**) encoding for β-lactam (mainly carbapenemase bla_{OXA-48}), quinolone (*oqxA10, oqxB17*) and trimethoprim (*dfrA14*) resistance, as well as chromosomal mutations involved in resistance to quinolones (mutations of *gyrA* and *parC*), colistin (truncation of *mgrB* gene) and linked with the decreased susceptibility to cephalosporins and carbapenems (truncation of porin encoding gene *OmpK35*).

Virulence determinants harbored by the analyzed strains were represented by siderophores, such as enterobactin (*entABCDEF*, *fepABCDG*) and yersiniabactin (*irp1, irp2, ybtSXQPA, fyuA*), as well as the operon *ecpRABCDE*, involved in adherence and biofilm formation. The biocides resistance gene *mdfA* encodes resistance to quaternary-ammonium compounds, sodium

TABLE 1 | Various features of the *K. pneumoniae* ST101 selected strains.

Water sampling date	Source	Chromogenic media	Strain code	Common features					Additional features			
				Serotype	Acquired antibiotic resistance	Chromosomal mutations involved in antibiotic resistance	Virulence genes	Biocides resistance genes	Acquired antibiotic resistance		Chromosomal mutations involved in antibiotic resistance	
21 Nov 2018	Influent	ChromID CARBA	29bac 34bac						bla_{OXA-1}	*aph(6)-Id, aph(3″)-Ib*	*tet(D), sulII, catB3*	OmpK 36GD
23 Nov 2018	Influent	ChromID OXA-48	**22bac**				*entA; entB; entE; entS; fepA; fepB;*		bla_{OXA-1} $bla_{CTX-M-15}$ $bla_{TEM-150}$	*aac (3)-IIa aac (6)-Ib-cr*	*tet(D), catB3*	–
	Effluent	ChromID CARBA	**23bac**				*fepC; fepD; fepG; fimC;*					
Nov 2018	Clinical	–	**36bac**				*fimE; fyuA;*					OmpK 36GD
			37bac		bla_{OXA-48}, $bla_{SHV-106}$	Δ*OmpK35,*	*irp1; irp2; ompA; yagV/ecpE;*	*merA*	bla_{NDM-1}	*rmtC; aph(6)-Id, aph(3″)-Ib*	*dfrA12, sulII,*	OmpK 36GD
			41bac	K17/O1v1	*dfrA14 fosA*	Δ*mgrB,* GyrA-83Y	*yagW/ecpD; yagX/ecpC;*	*mdfA*		*aac (3)-IIa*	*tet(D), catB3*	
			43bac		*oqxA10*	GyrA-87N	*yagY/ecpB; yagZ/ecpA;*		bla_{OXA-1}	*aac (6)-Ib-cr*		OmpK 36GD
20 Mar 2020	Effluent	ChromID CARBA	82bac		*oqxB17*	ParC-80I	*ybtA; ybtE; ybtP; ybtQ;*		$bla_{CTX-M-15}$ $bla_{TEM-150}$	*aac (3)-IIa*		–
March 2020	Clinical	–	86bac 89bac				*ybtS; ybtT; ybtU; ybtX; ykgK/ecpR*			–		

Bold values represents the closely related strains.

hydroxide and other biocides, while *merA* encodes resistance to mercury (**Table 1**).

Additionally, the majority of the strains harbored ESBL ($bla_{TEM-150}$, $bla_{CTX-M-15}$), tetracyclines – *tet(D)*, aminoglycoside- mainly *aac(3)-IIa*, chloramphenicol -*catB3* genes and presented an amino acid substitution in OmpK36 porin, which is known to be associated with carbapenem resistance (**Table 1**).

The pathogenic potential was estimated to 90%, according to Pathogen Finder predictor software (Cosentino et al., 2013). All strains harbored the same plasmid replicons: IncFIA, IncL/M, IncR_1, and Col, respectively.

The mobile genetic platform harboring *aac(6′)-Ib-cr* – bla_{OXA-1} – *catB3* was encountered in all isolates, in three of them, respectively, the clinical strain 36bac and the wastewater isolates 34bac and 29bac being flanked by *IS*26 at both ends. In the other tested strains the *aac(6′)-Ib-cr* gene was truncated (this might be probably due to sequencing limitations) (**Figure 2**).

K. pneumoniae ST101 Clone Survives in Hospital Chlorinated Effluent

Core SNPs analyses revealed that three clinical strains isolated in November 2018 (36bac, 41bac, and 43 bac) are closely related (≤18 SNPs) with two strains isolated on the 23rd November 2018 from influent (22bac) and effluent (23bac), respectively; the wastewater sourced strains presented no SNPs in their core site (**Table 2**). Moreover, cgMLST profiling revealed the same allelic content. Additionally, 99% of the total genes detected in these strains were common (core-genes). This low variability allowed us to hypothesize that the three selected strains belong to the same clone, more as they were tracked in the same temporal sequence, in three spatial points of the hospital-wastewater transmission chain.

These strains, isolated in November 2018, were related with those isolated in March 2019 (**Table 2**), where one clinical strain (89 bac) was found to be closely related with a strain isolated from the hospital effluent (82 bac) and having the same cgMLST profile. All strains presented in **Table 1** are clustered together in the Maximum likelihood phylogeny (**Figure 3**). Two strains from influent (29 bac, 34 bac) and one clinical strain (37 bac) isolated in November 2018, although belonging to ST101 clone, were more distantly related (55–799 SNPs) and belong to a different cluster.

DISCUSSION

Although clinically relevant clones, such as carbapenem producing *K. pneumoniae* have been isolated in different environments, data on the occurrence and characteristics of

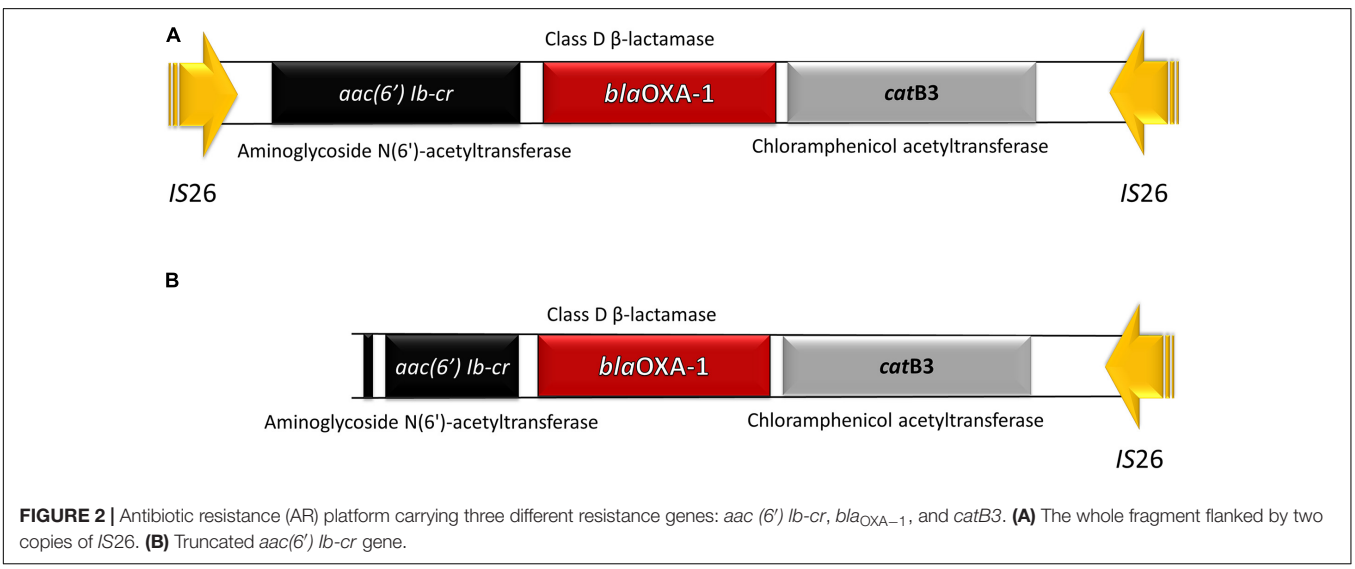

FIGURE 2 | Antibiotic resistance (AR) platform carrying three different resistance genes: *aac (6′) Ib-cr*, bla_{OXA-1}, and *catB3*. **(A)** The whole fragment flanked by two copies of *IS*26. **(B)** Truncated *aac(6′) Ib-cr* gene.

TABLE 2 | Matrix representation of calculated SNPs distances between the closely related strains (≤18 SNPs highlighted in gray).

Strain isolation point-date	Code	Number of SNPs							
IN-23 Nov 2018	22bac	0							
EF-23 Nov 2018	23bac	**0**	0						
CL-Nov 2018	36bac	**4**	**4**	0					
CL-Nov 2018	41bac	**8**	**7**	**18**	0				
CL-Nov 2018	43bac	**5**	**5**	**15**	**14**	0			
EF-20 Mar 2020	82bac	39	40	33	51	193	0		
CL-Mar 2020	86bac	31	38	27	74	194	28	0	
CL-Mar 2020	89bac	38	38	31	50	193	**2**	26	0
		22bac	23bac	36bac	41bac	43bac	82bac	86bac	89bac

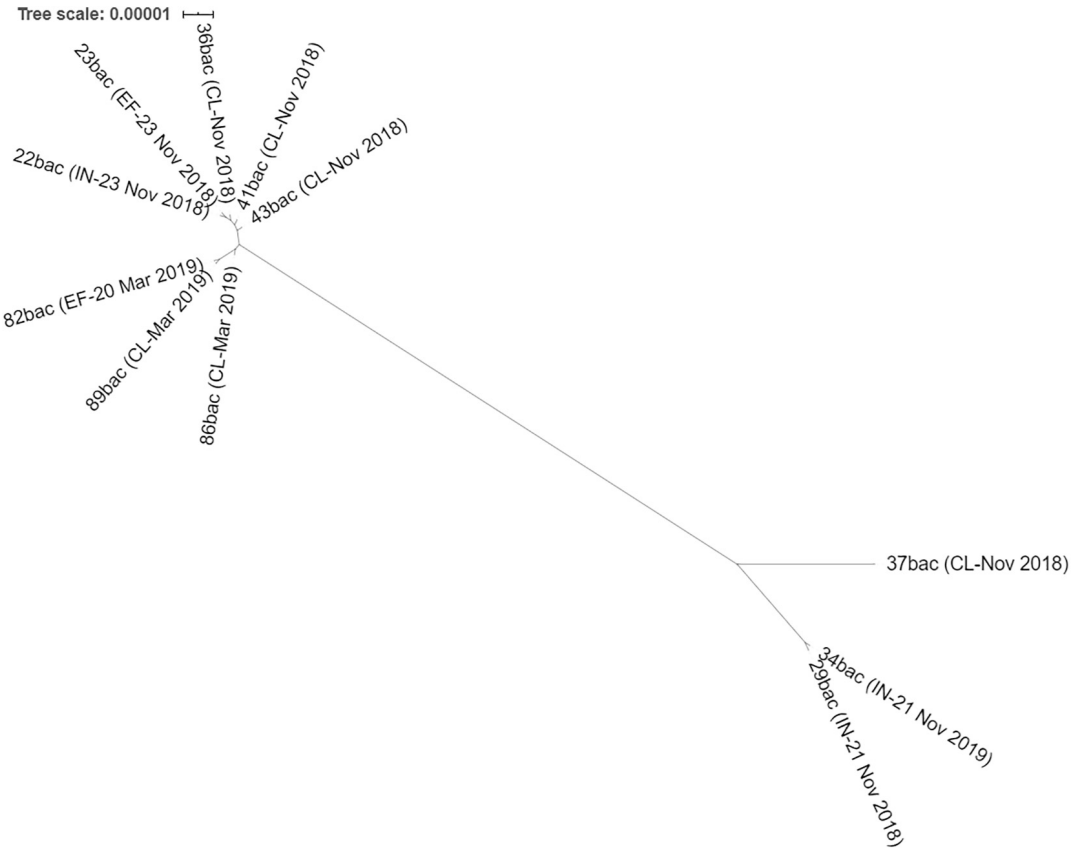

FIGURE 3 | Maximum likelihood phylogeny of all ST101 *K. pneumoniae* isolated from hospital sewage: influent (IN), effluent (EF) of chlorination tank, and from clinical samples (CL) with isolation date.

K. pneumoniae resistant strains in environmental sources are still scarce (Chi et al., 2019). Our study describes the presence of the same clone of *K. pneumoniae* MDR in the hospital wastewater, in the untreated influent as well in the effluent of the chlorination tank, highlighting the inefficiency of the chlorine treatment in removing MDR *K. pneumoniae* from hospital wastewater before being released to urban wastewater collecting system.

In order to prove the clonal dissemination of *K. pneumoniae* from the clinical compartment to the hospital wastewater, a detailed characterization of the ST101 MDR *K. pneumoniae* strains recovered from hospital wastewater before and after chlorination and inpatients was performed. We have demonstrated that five MDR *K. pneumoniae* ST101 strains isolated from intra-hospital infections and two strains isolated from hospital wastewater (hospital WWTP influent and effluent) in November 2018 belong to the same clone, harboring common AR, virulence, and biocides resistance genes, two strains belonging to the same clone being isolated also in March 2019 (isolated from inpatients and from the hospital WWTP effluent, respectively).

The most important feature of the successful *K. pneumoniae* ST101 clone isolated in November 2018 was its resistance to multiple antibiotics, encoded by chromosomal mutations, as well as by resistance genes acquired through horizontal gene transfer, located within mobile genetic elements which could potentially disseminate to commensal bacterial strains. The virulence determinants harbored by this successful clone were represented by siderophores such as enterobactin (*entABCDEF*, *fepABCDG*) and yersiniabactin (*irp1, irp2, ybtSXQPA, fyuA*), their co-presence being associated with an increased risk of respiratory tract infections (Bachman et al., 2011), as well as the operon *ecpRABCDE*, involved in adherence and biofilm formation. The variants of capsular and somatic antigens KL17 and O1v1 were described as strongly associated with *K. pneumoniae* ST101 (Roe et al., 2019). In addition, the O1 antigen has been described as a major contributor to the virulence of pyogenic liver abscess causing *K. pneumoniae* (Hsieh et al., 2012). Additionally, the biocides resistance gene *mdfA* encodes resistance to quaternary-ammonium compounds, sodium hydroxide and other biocides, while *merA* encodes resistance to mercury. Although genes associated with resistance and virulence are usually identified in separate subpopulations of *K. pneumoniae* there are strains harboring both high resistance and virulence (Lam et al., 2019; Wyres et al., 2020b) making them highly pathogenic and almost impossible to treat (Wyres et al., 2020b).

Our data point that the *K. pneumoniae* clone presented here may have the ability to survive in the urban wastewater after

being released in the hospital sewage. The persistence of this clone in the hospital effluent after chlorination indicates its dissemination potential in surface and recreational waters, as previously suggested by other authors (Lepuschitz et al., 2019; Suzuki et al., 2020).

Therefore, multi-level studies are needed for increasing knowledge on ecology, population structure and pathogenicity of resistant *K. pneumoniae* strains and to elucidate the possible transmission of clinical strains into the environment and the subsequent potential risk posed to human and environmental health (Wyres et al., 2020a).

Extended spectrum β-lactamase and carbapenem producing *K. pneumoniae* were previously isolated from different components of the hospital sewage treatment facilities, demonstrating the dissemination of ESBL producers between intra-hospital infections and the final effluent after the treatment process (Prado et al., 2008). The identification of VIM and KPC-producing *Klebsiella* spp. in the treated wastewater of a hospital (Gomi et al., 2018), of OXA-48 producing *K. pneumoniae* from hospital sewage (Zurfluh et al., 2017), or of the presence of the same clone in the hospital sewage and receiving river (Suzuki et al., 2020) raises concerns. Since its first detection in Székely et al. (2013), OXA-48 was the most frequently encountered carbapenemase in Romania (Lixandru et al., 2015; Grundmann et al., 2017; Popescu et al., 2017; Baicus et al., 2018; Surleac et al., 2020).

The IncFII, IncN, IncR, and IncX3 incompatibility groups are most often associated with the horizontal gene transfer of AR genes in *K. pneumoniae* determined mainly by the presence of the following replicons: FIB_K, FII_K, R, Col, FII, FIA (all identified in this study) FIB, X, N, HI1B, AC/2 (Wyres et al., 2020a). Beside these replicons, we have also identified the IncL/M replicon in all isolates. The acquired ARGs in *K. pneumoniae* ST101 isolates are associated with particular mobile elements, e.g., the association of $bla_{CTX-M-15}$ with IncFII plasmids while bla_{OXA-48} was often identified in IncL/M plasmids (Wyres and Holt, 2016), as also revealed by our results.

One might consider a limitation of this study the low number of the selected MDR *K. pneumoniae* strains belonging to the same clone traveling from hospital to WWTP effluents. Further studies, with more sampling campaigns and more isolates are required to provide the epidemiological link from the clinical compartment to the water bodies through hospital wastewater as well as the correlation between the persistence of the clones and different exposure times to chlorination treatment.

CONCLUSION

The survival of ARB in treated hospital wastewater is very alarming and highlights the necessity of an improved surveillance and the need to elucidate the role of the environment in the transmission and dissemination of MDR *K. pneumoniae* strains. The isolation of the same clone from both hospital and WWTP influent and effluent after chlorination suggests the highly adaptive potential of the clone and highlights the need for further studies designed to track the fate of these clones after release from hospital in the aquatic environment. In addition, disinfection strategies for hospital wastewaters should be reconsidered, in the light of such novel epidemiological data.

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 below: https://www.ncbi.nlm.nih.gov/, BioProject PRJNA579879.

AUTHOR CONTRIBUTIONS

LP: conceptualization, data curation, methodology, software, formal analysis, and writing – original draft. IG: conceptualization, data curation, methodology, formal analysis, and writing – original draft. IB: conceptualization, data curation, formal analysis, software, and writing – original draft. MS: data curation, formal analysis, software, and writing – review and editing. SP: conceptualization, methodology, and writing – review and editing. LM, MP, GP, DT, MN, and AnS-C: methodology. AdS-C: conceptualization. DO: conceptualization and writing – review and editing. MC: conceptualization, data curation, formal analysis, funding acquisition project administration, supervision, and writing – review and editing. 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.2020.610296/full#supplementary-material

Supplementary Figure 1 | The distribution of antibiotic resistance (AR) genes to β-lactam antibiotics. **(A)** aminoglycosides and quinolones, **(B)** and other classes of antibiotics, **(C)** predicted from WGS data in the analyzed *K. pneumoniae* strains.

Supplementary Figure 2 | MLST profiles of the *K. pneumoniae* strains isolated from the clinical compartment, influent, and effluent of the chlorination tank.

REFERENCES

Almahdawy, O. T., Pricop, R., Sadik, O., Najee, H., Pircalabioru, G., Marutescu, L., et al. (2019). Description of vancomycin resistance genes in *Enterococcus* sp. clinical strains isolated from Bucharest, Romania. *Rom. Biotechnol. Lett.* 24, 395–399. doi: 10.25083/rbl/24.3/395.399

Alrhmoun, M., Carrion, C., Casellas, M., and Dagot, C. (2014). "Hospital wastewater treatment by membrane bioreactor: performance and impact on the biomasses," in *Proceedings of the International Conference on Biological, Civil and Environmental Engineering, (BCEE-2014) March 17-18*, Dubai (UAE).

Andrews, S. (2010). *FastQC: A Quality Control Tool for High Throughput Sequence Data*. Available online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc (accessed December 18, 2020).

Bachman, M. A., Oyler, J. E., Burns, S. H., Caza, M., Lepine, F., Dozois, C. M., et al. (2011). *Klebsiella pneumoniae* yersiniabactin promotes respiratory tract infection through evasion of lipocalin 2. *Infect. Immun.* 79, 3309–3316. doi: 10.1128/IAI.05114-11

Baicus, A., Lixandru, B., Stroia, M., Cirstoiu, M., Constantin, A., Usein, C. R., et al. (2018). Antimicrobial susceptibility and molecular epidemiology of carbapenem-resistant *Klebsiella pneumoniae* strains isolated in an emergency university hospital. *Rom. Biotechnol. Lett.* 23, 13525–13529.

Bassetti, M., Righi, E., Carnelutti, A., Graziano, E., and Russo, A. (2018). Multidrug-resistant *Klebsiella pneumoniae*: challenges for treatment, prevention and infection control. *Expert Rev. Anti Infect. Ther.* 16, 749–761. doi: 10.1080/14787210.2018.1522249

Carattoli, A., Zankari, E., Garcia-Fernandez, A., Larsen, M. V., Lund, O., Villa, L., et al. (2014). In silico detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. *Antimicrob. Agents Chemother.* 58, 3895–3903. doi: 10.1128/AAC.02412-14

Cassini, A., Hogberg, L. D., Plachouras, D., Quattorocchi, A., Hoxha, A., Simones, G. S., et al. (2015). Attributable deaths and disability-adjusted life-years caused by infections with antibiotic-resistant bacteria in the EU and the European Economic Area in 2015: a population-level modelling analysis. *Lancet Infect. Dis.* 19, 56–66. doi: 10.1016/S1473-3099(18)30605-4

CGE Platform (2020). Available online at: http://www.genomicepidemiology.org/ (accessed June 24, 2020).

Chen, L., Zheng, D., Liu, B., Yang, J., and Jin, Q. (2016). VFDB 2016: hierarchical and refined dataset for big data analysis-10 years on. *Nucleic Acids Res.* 44, D694–D697. doi: 10.1093/nar/gkv1239

Chi, X., Berglund, B., Zou, H., Zheng, B., Borjesson, S., Ji, X., et al. (2019). Characterization of clinically relevant strains of extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* occurring in environmental sources in a rural area of China by using whole-genome sequencing. *Front. Microbiol.* 10:211. doi: 10.3389/fmicb.2019.00211

CLSI (2019). *Performance Standards for Antimicrobial Susceptibility Testing, CLSI Supplement M100*, 29th Edn. Wayne, PA: Clinical and Laboratory Standards Institute.

Cosentino, S., Larsen, M. V., Aarestrup, F. M., and Lund, O. (2013). PathogenFinder – distinguishing friend from foe using bacterial whole genome sequence data. *PLoS One* 8:e77302. doi: 10.1371/journal.pone.0077302

Czobor, I., Novais, A., Chifiriuc, M. C., Mihaescu, G., Lazar, V., and Peixe, L. (2016). Efficient transmission of IncFIIY and IncL plasmids and *Klebsiella pneumoniae* ST101 clone producing OXA-48, NDM-1 or OXA-181 in Bucharest hospitals. *Int. J. Antimicrob. Agents* 48, 223–224. doi: 10.1016/j.ijantimicag.2016.05.004

Dortet, L., Flonta, M., Boudehen, Y.-M., Creton, E., Bernabeu, S., Vogel, A., et al. (2015). Dissemination of carbapenemase-producing *Enterobacteriaceae* and *Pseudomonas aeruginosa* in Romania. *Antimicrob. Agents Chemother.* 59, 7100–7103. doi: 10.1128/AAC.01512-15

Ellington, M. J., Kistler, J., Livermore, D. M., and Woodford, N. (2007). Multiplex PCR for rapid detection of genes encoding acquired metallo-β-lactamases. *J. Antimicrob. Chemother.* 59, 321–322. doi: 10.1093/jac/dkl481

European Centre for Disease Prevention and Control (2020). Available online at: https://www.ecdc.europa.eu/sites/default/files/documents/surveillance-antimicrobial-resistance-Europe-2018.pdf (accessed April 14, 2020).

Feldgarden, M., Brover, V., Haft, D. H., Prasad, A. B., Slotta, D. J., Tolstoy, I., et al. (2019). Validating the AMRFinder tool and resistance gene database by using antimicrobial resistance genotype-phenotype correlations in a collection of isolates. *Antimicrob. Agents Chemother.* 63:e00483-19. doi: 10.1128/AAC.00483-19

Gardner, S. N., Slezak, T., and Hall, B. G. (2015). kSNP3.0: SNP detection and phylogenetic analysis of genomes without genome alignment or reference genome. *Bioinformatics* 31, 2877–2878. doi: 10.1093/bioinformatics/btv271

Gomi, R., Matsuda, T., Yamamoto, M., Chou, P.-H., Tanaka, M., Ichiyama, S., et al. (2018). Characteristics of carbapenemase-producing *Enterobacteriaceae* in wastewater revealed by genomic analysis. *Antimicrob. Agents Chemother.* 62:e02501-17. doi: 10.1128/AAC.02501-17

Gillings, M. R. (2013). Evolutionary consequences of antibiotic use for the resistome, mobilome, and microbial pangenome. *Front. Microbiol.* 4:4. doi: 10.3389/fmicb.2013.00004

Grundmann, H., Glasner, C., Albiger, B., Aanensen, D. M., Tomlinson, C. T., Andrasević, A. T., et al. (2017). Occurrence of carbapenemase-producing *Klebsiella pneumoniae* and *Escherichia coli* in the European survey of carbapenemase-producing *Enterobacteriaceae* (EuSCAPE): a prospective, multinational study. *Lancet Infect. Dis.* 17, 153–163. doi: 10.1016/s1473-3099(16)30257-2

Hocquet, D., Muller, A., and Bertrand, X. (2016). What happens in hospitals does not stay in hospitals: antibiotic-resistant bacteria in hospital wastewater systems. *J. Hosp. Infection.* 93, 395–402. doi: 10.1016/j.jhin.2016.01.010

Hsieh, P. F., Lin, T. L., Yang, F. L., Wu, M.-C., Pan, Y.-J., Wu, S.-H., et al. (2012). Lipopolysaccharide O1 antigen contributes to the virulence in *Klebsiella pneumoniae* causing pyogenic liver abscess. *PLoS One* 7:e33155. doi: 10.1371/journal.pone.0033155

Huang, J.-J., Hu, H.-Y., Tang, F., Li, Y., Lu, S.-Q., and Lu, T. (2011). Inactivation and reactivation of antibiotic-resistant bacteria by chlorination in secondary effluents of a municipal wastewater treatment plant. *Water Res.* 45, 2775–2781. doi: 10.1016/j.watres.2011.02.026

Hunt, M., Mather, A. E., Sánchez-Busó, L., Page, A. J., Parkhill, J., Keane, J. A., et al. (2017). ARIBA: rapid antimicrobial resistance genotyping directly from sequencing reads. *Microb. Genomics* 3:e000131. doi: 10.1099/mgen.0.000131

Israil, A., Chifiriuc, C., Palade, G., and Cotar, A. (2013). Clinical and bacteriological aspects of bacterial infections associated to abdominaş surgical emergencies. *Bucharest: Ars Docendi Publ. House*. 150.

Joensen, K. G., Scheutz, F., Lund, O., Hasman, H., Kaas, R. S., Neilsen, E. M., et al. (2014). Real-time whole-genome sequencing for routine typing, surveillance, and outbreak detection of verotoxigenic *Escherichia coli*. *J. Clin. Micobiol.* 52, 1501–1510. doi: 10.1128/JCM.03617-13

Jolley, K. A., and Maiden, M. C. (2010). BIGSdb: scalable analysis of bacterial genome variation at the population level. *BMC Bioinformatics* 11:595. doi: 10.1186/1471-2105-11-595

Kaur, R., Yadav, B., and Tyagi, R. D. (2020). "Microbiology of hospital wastewater," in *Current Developments in Biotechnology and Bioengineering*, eds G. Mannina, A. Pandey, C. Larroche, H. Y. Ng, and H. H. Ngo (Amsterdam: Elsevier), 103–148. doi: 10.1016/B978-0-12-819722-6.00004-3

Khan, F. A., Hellmark, B., Ehricht, R., Söderquist, B., and Jass, J. (2018). Related carbapenemase-producing *Klebsiella* isolates detected in both a hospital and associated aquatic environment in Sweden. *Eur. J. Clin. Microbiol. Infect Dis.* 37, 2241–2251. doi: 10.1007/s10096-018-3365-9

Kim, J., and Lee, H.-J. (2000). Rapid discriminatory detection of genes coding for SHV b-lactamases by ligase chain reaction. *Antimicrob. Agents Chemother.* 44, 1860–1864. doi: 10.1128/aac.44.7.1860-1864.2000

Kim, S., and Aga, D. S. (2007). Potential ecological and human health impacts of antibiotics and antibiotic-resistant bacteria from wastewater treatment plants. *J. Toxicol. Environ. Health B Crit. Rev.* 10, 559–573. doi: 10.1080/15287390600975137

Kraemer, S. A., Ramachandran, A., and Perron, G. G. (2019). Antibiotic pollution in the environment: from microbial ecology to public policy. *Microorganisms* 7:180. doi: 10.3390/microorganisms7060180

Kummerer, K., Al-Ahmad, A., and Mersch-Sundermann, V. (2000). Biodegradability of some antibiotics, elimination of the genotoxicity and affection of wastewater bacteria in a simple test. *Chemosphere* 40, 701–710.

Laffite, A., Kilunga, P. I., Kayembe, J. M., Devarajan, N., Mulaji, C. K., Giuliani, G., et al. (2016). Hospital effluents are one of several sources of metal, antibiotic

resistance genes, and bacterial markers disseminated in sub-Saharan urban rivers. *Front. Microbiol.* 7:1128. doi: 10.3389/fmicb.2016.01128

Lam, M. M. C., Wyres, K. L., Wick, R. R., Judd, L. M., Fostervold, A., Holt, K. E., et al. (2019). Convergence of virulence and MDR in a single plasmid vector in MDR *Klebsiella pneumoniae* ST15. *J. Antimicrob. Chemother.* 74, 1218–1222.

Lepuschitz, S., Schill, S., Stoeger, A., Pekard-Amenitsch, S., Hululescu, S., Inreiter, N., et al. (2019). Whole genome sequencing reveals resemblance between ESBL-producing and carbapenem resistant *Klebsiella pneumoniae* isolates from Austrian rivers and clinical isolates from hospitals. *Sci. Total Environ.* 662, 227–235. doi: 10.1016/j.scitotenv.2019.01.179

Letunic, I., and Bork, P. (2019). Interactive tree of life (iTOL) v4: recent updates and new development. *Nucleic Acids Res.* 47, W256–W259. doi: 10.1093/nar/gkz239

Lin, W., Zhang, M., Zhang, S., and Yu, X. (2016). Can chlorination co-select antibiotic-resistance genes? *Chemosphere* 156, 412–419. doi: 10.1016/j.chemosphere.2016.04.139

Lixandru, B. E., Cotar, A. I., Straut, M., Usein, C. R., Cristea, D., Ciontea, S., et al. (2015). Carbapenemase-producing *Klebsiella pneumoniae* in Romania: a six-month survey. *PLoS One* 10:e0143214. doi: 10.1371/journal.pone.0143214

Magiorakos, A. P., Srinivasan, A., Carey, R. B., Carmeli, Y., Falagas, M. E., Giske, C. G., et al. (2012). Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin. Microbiol. Infect.* 18, 268–281. doi: 10.1111/j.1469-0691.2011.03570.x

Mahon, B. M., Brehony, C., McGrath, E., Killeen, J., Cormican, M., Hickey, P., et al. (2017). Indistinguishable NDM-producing *Escherichia coli* isolated from recreational waters, sewage, and a clinical specimen in Ireland, 2016 to 2017. *Eurosurveillance* 22:30513. doi: 10.2807/1560-7917.ES.2017.22.15.30513

Navon-Venezia, S., Kondratyeva, K., and Carattoli, A. (2017). *Klebsiella pneumoniae*: a major worldwide source and shuttle for antibiotic resistance. *FEMS Microbiol. Rev.* 41, 252–275. doi: 10.1093/femsre/fux013

Nordmann, P., Poirel, L., Carrer, A., Toleman, M. A., and Walsh, T. R. (2011). How to detect NDM-1 producers. *J. Clin. Microbiol.* 49, 718–721. doi: 10.1128/JCM.01773-10

Page, A. J., Cummins, C. A., Hunt, M., Wong, V. K., Reuter, S., Holden, M. T. G., et al. (2015). Roary: rapid large-scale prokaryote pan genome analysis. *Bioinformatics* 31, 3691–3693. doi: 10.1093/bioinformatics/btv421

Poirel, L., Walsh, T. R., Cuvillier, V., and Nordmann, P. (2011). Multiplex PCR for detection of acquired carbapenemase genes. *Diagn. Microbiol. Infect. Dis.* 70, 119–123. doi: 10.1016/j.diagmicrobio.2010.12.002

Popa, L. I., Barbu, I. C., and Chifiriuc, M. C. (2018). Mobile genetic elements involved in the horizontal transfer of antibiotic resistance genes. *Rom. Arch. Microbiol.* 77, 263–276.

Popescu, C., Popescu, G. A., Dorobat, O., Rafila, A., Tanase, D., Mikula, C., et al. (2017). OXA-48-carbapenemase-producing *Klebsiella pneumoniae* infections – the first cases diagnosed in Romanian National Institute of Infectious Disease. *Rev. Rom. Med. Lab.* 25, 55–61. doi: 10.1515/rrlm-2017-0006

Prado, T., Pereira, W., Silva, D. M., Seki, L. M., Carvalho, A. P. D. 'A., et al. (2008). Detection of extended-spectrum β- lacramase-producing *Klebsiella pneumoniae* in effluents and sludge of hospital sewage treatment plant. *Lett. Appl. Microbiol.* 46, 136–141.

Qi, C., Malczynski, M., Parker, M., and Scheetz, M. H. (2008). Characterization of genetic diversity of carbapenem-resistant *Acinetobacter baumannii* clinical strains collected from 2004 to 2007. *J. Clin. Microbiol.* 46, 1106–1109. doi: 10.1128/JCM.01877-07

Quinteros, M., Radice, M., Gardella, N., Rodriguez, M. M., Costa, N., Korbenfeld, D., et al. (2003). Extended-spectrum B-lactamases in *Enterobacteriaceae* in Buenos Aires, Argentina, public hospitals. *Antimicrob. Agents Chemother.* 47, 2864–2867. doi: 10.1128/AAC.47.9.2864-2867.2003

Rafila, A., Talapan, D., Dorobăţ, O. M., Popescu, G. A., Piţigoi, D., Florea, D., et al. (2015). Emergence of carbapenemase-producing *Enterobacteriaceae*, a public health threat: a Romanian infectious disease hospital based study. *Rev. Rom. Med. Lab.* 23, 295–301. doi: 10.1515/rrlm-2015-0024

Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M. C., et al. (2013). Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: a review. *Sci. Total Environ.* 447, 345–360. doi: 10.1016/j.scitotenv.2013.01.032

Roe, C. C., Vazquez, A. J., Esposito, E. P., Zarrilli, R., and Sahl, J. W. (2019). Diversity, virulence, and antimicrobial resistance in isolates from the newly emerging *Klebsiella pneumoniae* ST101 lineage. *Front. Microbiol.* 10:542. doi: 10.3389/fmicb.2019.00542

Rozman, U., Duh, D., Cimerman, M., and Turk, S. S. (2020). Hospital wastewater effluent: hot spot for antibiotic resistant bacteria. *J. Water Sanit. Hyg. Dev.* 10, 171–178. doi: 10.2166/washdev.2020.086

Sanganyado, E., and Gwenzi, W. (2019). Antibiotic resistance in drinking water systems: occurrence, removal, and human health risks. *Sci. Total Environ.* 669, 785–797. doi: 10.1016/j.scitotenv.2019.03.162

Seemann, T. (2014). Prokka: rapid prokaryotic genome annotation. *Bioinformatics* 30, 2068–2069.

Seemann, T. (2015). *Snippy: Fast Bacterial Variant Calling from NGS Reads (v4.6.0)*. Available online at: https://github.com/tseemann/snippy (accessed May 12, 2020).

Seemann, T. (2018). *Shovill: Faster SPAdes Assembly of Illumina Reads (v1.1.0)*. Available online at: https://github.com/tseemann/shovill (accessed May 12, 2020).

Seemann, T. (2020a). *mlst (v2.19.0) Github*. Available online at: https://github.com/tseemann/mlst (accessed May 12, 2020).

Seemann, T. (2020b). *Abricate, Github (v1.0.1)*. Available online at: https://github.com/tseemann/abricate (accessed May 12, 2020).

Shi, P., Jia, S., Zhang, X.-X., Zhang, T., Cheng, S., and Li, A. (2013). Metagenomic insights into chlorination effects on microbial antibiotic resistance in drinking water. *Water Res.* 47, 111–120. doi: 10.1016/j.watres.2012.09.046

Shirani, K., Ataei, B., and Roshandel, F. (2016). Antibiotic resistance pattern and evaluation of metallo-beta lactamase genes (VIM and IMP) in *Pseudomonas aeruginosa* strains producing MBL enzyme, isolated from patients with secondary immunodeficiency. *Adv. Biomed. Res.* 5:124. doi: 10.4103/2277-9175.186986

Stamatakis, A. (2014). RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 30, 1312–1313. doi: 10.1093/bioinformatics/btu033

Surleac, M., Czobor Barbu, I., Paraschiv, S., Popa, L. I., Gheorghe, I., Marutescu, L., et al. (2020). Whole genome sequencing snapshot of multi-drug resistant *Klebsiella pneumoniae* strains from hospitals and receiving wastewater treatment plants in Southern Romania. *PLoS One* 15:e0228079. doi: 10.1371/journal.pone.0228079

Suzuki, Y., Nazareno, P. J., Nakano, R., Mondoy, M., Nakano, A., Bugayong, M. P., et al. (2020). Environmental presence and genetic characteristics of carbapenemase-producing *Enterobacteriaceae* from hospital sewage and river water in the Philippines. *Appl. Environ. Microbiol.* 86:e01906-19. doi: 10.1128/AEM.01906-19

Székely, E., Damjanova, I., Jánvári, L., Vas, K. E., Molnar, S., Bilca, D. V., et al. (2013). First description of bla(NDM-1), bla(OXA-48), bla(OXA-181) producing *Enterobacteriaceae* strains in Romania. *Int. J. Med. Microbiol.* 303, 697–700. doi: 10.1016/j.ijmm.2013.10.001

Vrancianu, C. O., Popa, L. I., Bleotu, C., and Chifiriuc, M. C. (2020). Targeting plasmids to limit acquisition and transmission of antimicrobial resistance. *Front. Microbiol.* 11:761. doi: 10.3389/fmicb.2020.00761

Wick, R. R., Heinz, E., Holt, K. E., and Wyres, K. L. (2018). Kaptive web: user-friendly capsule and lipopolysaccharide serotype prediction for *Klebsiella* genomes. *J. Clin. Microbiol.* 56:e00197-18. doi: 10.1128/JCM.00197-18

WHO, in Chartier, Y., Emmanuel, J., Pieper, U., Prüss, A., Rushbrook, P., Stringer, R., et al. (Eds.) (2014). *Safe Management of Wastes From Health-care Activities*, 2nd Edn.

Wyres, K. L., and Holt, K. E. (2016). *Klebsiella pneumoniae* population genomics and antimicrobial-resistant clones. *Trends Microbiol.* 24, 944–956. doi: 10.1016/j.tim.2016.09.007

Wyres, K. L., Lam, M. M. C., and Holt, K. E. (2020a). Population genomics of *Klebsiella pneumoniae*. *Nat. Rev. Microbiol.* 18, 344–359. doi: 10.1038/s41579-019-0315-1

Wyres, K. L., Nguyer, T. N. T., Lam, M. M. C., Judd, L. M., van Winh Chau, N., Dance, D. A. B., et al. (2020b). Genomic surveillance for hypervirulence and multi-drug resistance in invasive *Klebsiella pneumoniae* from South and Southeast Asia. *Genome Med.* 12, 11. doi: 10.1186/s13073-019-0706-y

Wyres, K. L., Wick, R. R., Gorrie, C., Jenney, A., Follador, R., Thomson, N. R., et al. (2016). Identification of *Klebsiella* capsule synthesis loci from whole genome data. *Microb. Genom.* 2:e000102. doi: 10.1099/mgen.0.000102

Yuan, Q. B., Guo, M. T., and Yang, J. (2015). Fate of antibiotic resistant bacteria and genes during wastewater chlorination: implication for antibiotic resistance control. *PLoS One* 10:e0119403. doi: 10.1371/journal.pone.0119403

Zankari, E., Hasman, H., Cosentino, S., Vestergaard, M., Rasmyssen, S., Lund, O., et al. (2012). Identification of acquired antimicrobial resistance genes. *J. Antimicrob. Chemother.* 67, 2640–2644. doi: 10.1093/jac/dks261

Zhang, T., Hu, Y., Jiang, L., Yao, S., Lin, K., Zhou, Y., et al. (2019). Removal of antibiotic resistance genes and control of horizontal transfer risk by UV, chlorination and UV/chlorination treatments of drinking water. *Chem. Eng. J.* 358, 589–597. doi: 10.1016/j.cej.2018.09.218

Zhang, Y., Zhuang, Y., Geng, J., Ren, H., Zhang, Y., Ding, L., et al. (2015). Inactivation of antibiotic resistance genes in municipal wastewater effluent by chlorination and sequential UV/chlorination disinfection. *Sci. Total Environ.* 51, 125–132. doi: 10.1016/j.scitotenv.2015.01.028

Zhang, Z., Li, B., Li, N., Sardar, M. F., Song, T., Zhu, C., et al. (2019). Effects of UV disinfection on phenotypes and genotypes of antibiotic-resistant bacteria in secondary effluent from a municipal wastewater treatment plant. *Water Res.* 157, 545–554. doi: 10.1016/j.watres.2019.03.079

Zurfluh, K., Bagutti, C., Brodmann, P., Alt, M., Schulze, J., Fanning, S., et al. (2017). Wastewater is a reservoir for clinically relevant carbapenemase- and 16s rRNA methylase-producing *Enterobacteriaceae*. *Int. J. Antimicrob. Agents* 50, 436–440. doi: 10.1016/j.ijantimicag.2017.04.017

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High Prevalence of Drug Resistance and Class 1 Integrons in *Escherichia coli* Isolated From River Yamuna, India: A Serious Public Health Risk

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Globally, urban water bodies have emerged as an environmental reservoir of antimicrobial resistance (AMR) genes because resistant bacteria residing here might easily disseminate these traits to other waterborne pathogens. In the present study, we have investigated the AMR phenotypes, prevalent plasmid-mediated AMR genes, and integrons in commensal strains of *Escherichia coli*, the predominant fecal indicator bacteria isolated from a major urban river of northern India Yamuna. The genetic environment of $bla_{CTX-M-15}$ was also investigated. Our results indicated that 57.5% of the *E. coli* strains were resistant to at least two antibiotic classes and 20% strains were multidrug resistant, i.e., resistant to three or more antibiotic classes. The multiple antibiotic resistance index of about one-third of the *E. coli* strains was quite high (>0.2), reflecting high contamination of river Yamuna with antibiotics. With regard to plasmid-mediated AMR genes, bla_{TEM-1} was present in 95% of the strains, followed by q*nr*S1 and *arm*A (17% each), $bla_{CTX-M-15}$ (15%), *str*A-*str*B (12%), and *tet*A (7%). Contrary to the earlier reports where $bla_{CTX-M-15}$ was mostly associated with pathogenic phylogroup B2, our study revealed that the CTX-M-15 type extended-spectrum β-lactamases (ESBLs) were present in the commensal phylogroups A and B1, also. The genetic organization of $bla_{CTX-M-15}$ was similar to that reported for *E. coli*, isolated from other parts of the world; and IS*Ecp1* was present upstream of $bla_{CTX-M-15}$. The integrons of classes 2 and 3 were absent, but class 1 integron gene *intI1* was present in 75% of the isolates, denoting its high prevalence in *E. coli* of river Yamuna. These evidences indicate that due to high prevalence of plasmid-mediated AMR genes and *intI1*, commensal *E. coli* can become vehicles for widespread dissemination of AMR in the environment. Thus, regular surveillance and management of urban rivers is necessary to curtail the spread of AMR and associated health risks.

Keywords: commensal *E. coli*, plasmid-mediated antimicrobial resistance genes, integrons, multidrug resistance, horizontal transfer of genes

INTRODUCTION

The gastrointestinal tract of humans and animals is regarded as the primary/natural habitat of *Escherichia coli*. Besides its natural habitat, *E. coli* is also found in secondary habitats like aquatic and terrestrial reservoirs (Méric et al., 2013). Aquatic environments, especially urban water bodies, harbor a heterogeneous collection of microorganisms originating from fecal, hospital, agricultural, and veterinary sources. Moreover, several studies have suggested that aquatic environments serve as genetic reactors that promote transfer of antimicrobial resistance (AMR) and virulence genes among bacteria (Baquero et al., 2008; Hasegawa et al., 2018).

The phylogrouping methods commonly used for population/clonal studies of *E. coli* include multilocus sequence typing (MLST) (Aanensen and Spratt, 2005), multilocus enzyme electrophoresis (MLEE), triplex PCR, etc. Triplex PCR is a widely used rapid and simple technique for phylotyping *E. coli*. In triplex PCR, three genes (*chuA*, *yjaA*, and a gene encoding a fragment of a putative lipase esterase) are PCR-amplified (Clermont et al., 2000). Based on the presence or absence of these three genetic elements, a strain can be assigned to belong to any of the four phylogroups—A, B1, B2, and D (Clermont et al., 2000). Several researchers have studied AMR in *E. coli* isolated from aquatic environments and have reported that these traits are easily transmissible among bacterial species with the help of several mobile genetic elements like integrons, insertion sequences (ISs), plasmids, and transposons (Su et al., 2012; Koczura et al., 2013; Liebana et al., 2013; Pereira et al., 2013; Kaushik et al., 2018). Integrons are regarded as the primary vehicles that disseminate AMR genes among bacterial species (Gillings, 2014) because they can be located on conjugative plasmids, which enhance their horizontal spread.

River Yamuna is a major river of northern India, which is associated with several anthropogenic activities of the population residing in the National Capital Region of India. It gets contaminated with effluents originating from hospital and municipal wastewaters; discharge from livestock, poultry, and agriculture production plants; industries; etc. The high levels of pollutants are expected to provide a positive selection pressure for increasing AMR in bacterial population residing there (Kümmerer, 2009; Tacão et al., 2012). Thus, it is expected to be a crucial reservoir of a diverse *E. coli* populations and an ideal ecological niche for studying strains with diverse phenotypes, genotypes, and AMR. In an earlier study published from our laboratory, we had described the β-lactam susceptibilities and β-lactamase genes in 61 *E. coli* strains of all phylogroups (A, B1, B2, and D) isolated from river Yamuna (Bajaj et al., 2015). Though AMR resistance has been investigated for commensal phylogroups of *E. coli* isolated from veterinary or clinical sources, only a few studies have investigated the AMR phenotypes, genes, and integrons in commensal *E. coli* isolated from urban rivers. The commensal strains of *E. coli* in urban rivers can easily disseminate AMR determinants to pathogenic *E. coli* or other waterborne pathogens via mobile genetic elements. Thus, it is important to study the AMR determinants and integrons in commensal strains residing in water bodies also. Our study is the first report on AMR phenotypes, plasmid-mediated AMR genes, and integrons in the strains of commensal phylogroups (A and B1) of *E. coli* from river Yamuna, India.

MATERIALS AND METHODS

Sample Processing and Isolation of *Escherichia coli*

Two hundred water samples were collected from different sites along the entire stretch of the river Yamuna, which flows through the National Capital Region of India in sterile screw-capped bottles, transported to the laboratory on ice, and processed within 6 h of the sample collection. A schematic figure showing the details of the sampling sites has been published earlier (Bajaj et al., 2015). Enrichment of the samples for isolation of *Escherichia coli* was performed using a published method (Ram et al., 2008). Briefly, 100 ml of water sample was filtered through a 0.45 μm membrane filter (Millipore, MA, United States). The membrane filter was cut into four pieces, and each piece was incubated in 50 ml of MacConkey broth at 37°C, 220 rpm, overnight. The next day, a loopful of the broth culture was streaked on the surface of MacConkey agar plates and incubated at 35°C for 18–20 h. One hundred sixty-two typical *E. coli* colonies were selected and maintained as pure cultures on Luria–Bertani (LB) agar slants at 4°C. Of these, 126 isolates were presumptively identified as *E. coli* using API 20E strips (bioMérieux, France). API 20E is a standardized kit of biochemical tests used to identify members of the family *Enterobacteriaceae* and other non-fastidious Gram-negative rods.

Isolation of Genomic DNA, and PCR Amplification of Gene Encoding 16s rRNA and Phylogrouping Based on Triplex PCR

DNA was extracted from the *E. coli* strains using the boiling lysis method (Rodríguez-Baño et al., 2004). The gene encoding 16S rRNA was PCR-amplified using universal eubacterial forward primer 27F (5′AGAGTTTGATCCTGGCTCAG3′) and reverse primer 1492R (5′ACGGCTACCTTGTTACGACTT3′). The contents of the PCR mixture were 1 × PCR buffer (1.5 $MgCl_2$, 1.5 mM of KCl, 10 mM of Tris–HCl, and 0.1% Triton X-100), 200 μM of the four dNTPs, 1 U of Taq DNA polymerase (New England Biolabs, Ipswich, MA, United States), 10 pmol of forward and reverse primers, and 1 ng of genomic DNA in a final volume of 25 μl. The PCR conditions and methods for purification of PCR amplicons and sequencing have been described earlier (Singhal et al., 2019). Briefly, the PCR amplicons were purified by HiYield^{TM} extraction kit (RBC Bioscience, New Taipei City, Taiwan) and sequenced at a commercial facility using Sanger sequencing (Invitrogen BioServices India Pvt. Ltd., Bangalore, India). The nucleotide sequence homology was analyzed using the nucleotide BLAST (BLASTn) algorithm available at the National Center for Biotechnology Information (NCBI).

The phylogenetic profiles of all the isolates were determined by triplex PCR (Clermont et al., 2000); and 40 strains representing the commensal phylogroups (A and B1) were selected for studying the AMR phenotypes, plasmid-mediated AMR genes, and integrons.

Determining Antimicrobial Susceptibilities, and Extended-Spectrum β-Lactamase and AmpC Production

Antimicrobial susceptibilities of *E. coli* strains for various classes of antibiotics like β-lactams, aminoglycosides, quinolones, and tetracycline were determined by Kirby–Bauer disk diffusion method. The antibiotic disks (Himedia, India) that were used in this study were (charge in μg/disk) as follows: ampicillin (10 μg), piperacillin (100 μg), amoxicillin–clavulanic acid (20/10 μg), cefazolin (30 μg), cefuroxime (30 μg), cefotaxime (30 μg), cefepime (30 μg), streptomycin (10 μg), kanamycin (30 μg), tobramycin (10 μg), netilmicin (30 μg), amikacin (30 μg), nalidixic acid (30 μg), ciprofloxacin (5 μg), ofloxacin (5 μg), and tetracycline (30 μg). The results of antimicrobial susceptibility testing were interpreted following the guidelines of Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute [CLSI], 2018). The multiple antibiotic resistance (MAR) index of each strain was calculated by dividing the number of antibiotics to which a strains was resistant by the number of antibiotics that were tested (Krumperman, 1983). Here, the number of antibiotics to which an *E. coli* strain exhibited resistance was divided by 16 because susceptibility of each strain was tested for 16 antibiotics. The *E. coli* strains were tested for production of extended-spectrum β-lactamases (ESBLs) using a phenotypic confirmatory test recommended by the CLSI (Clinical and Laboratory Standards Institute [CLSI], 2018). Briefly, cefotaxime and ceftazidime disks (30 μg) alone and in combination with clavulanic acid (30/10 μg) were placed on the surface of bacterial lawn spread over Mueller–Hinton agar petri plates. Strains whose zone diameter in the presence of antibiotic–clavulanic acid combination was ≥5 mm were considered as ESBL producers. The strains were tested for phenotypic production of AmpC using AmpC E-test strips (bioMérieux Inc., MO, United States) following the manufacturer's instructions. Strains that showed cefotetan/cefotetan + cloxacillin (CN/CNI) ratio of ≥8 were considered as AmpC producers (Bajaj et al., 2015).

Detection and Analysis of Antimicrobial Resistance Genes

Detection of Genes Encoding β-Lactamases and Genetic Environment of $bla_{CTX-M-15}$

Genes encoding β-lactamases and ESBLs, viz., bla_{TEM}, bla_{SHV}, and bla_{CTX-M}, were detected by PCR amplification of the AMR genes using group-specific primers that amplified the internal coding regions of the genes (Dhanji et al., 2011; Bajaj et al., 2015). The presence of plasmid-encoded AmpC enzymes of the CMY types was determined by PCR amplification of the AMR gene using published primers (Pérez-Pérez and Hanson, 2002). The promoter region and genetic environment of $bla_{CTX-M-15}$ were studied in $bla_{CTX-M-15}$-positive *E. coli* strains by PCR amplification of the corresponding regions using the primers and methods described earlier (Saladin et al., 2002; Dhanji et al., 2011). The primers and the annealing temperatures for amplification of the bla_{TEM}, bla_{SHV}, and bla_{CTX-M} and genetic environment *of* bla_{CTX-M} are described in **Table 1**. The contents of the PCR mixture and methods for purification of the PCR amplicons and sequencing were the same as used for 16s rRNA gene sequencing. The nucleotide sequence homology was analyzed using the nucleotide BLAST (BLASTn) available at NCBI.

PCR Amplification of Genes Encoding Plasmid-Mediated Quinolone Resistance, Aminoglycoside Resistance, and Tetracycline Resistance

The presence of genes encoding plasmid-mediated quinolone resistance (PMQR) was determined by PCR amplification of genes encoding for (i) proteins that protect DNA from quinolone binding (*qnr*A, *qnr*B, *qnr*C, and *qnr*D); (ii) *aac(6')-Ib-cr* acetyltransferase (*aac*), which modifies fluoroquinolones like ciprofloxacin and enrofloxacin; and (iii) active efflux pump (*qep*A).

The presence of aminoglycoside resistance genes was determined by PCR amplification of the genes encoding for linked *str*A-*str*B genes and four types of plasmid-mediated 16S rRNA methylases—*arm*A, *rmt*A, *rmt*B, and *rmt*C— using published primers and annealing temperatures described in **Table 1** (Sunde and Norstrom, 2005; Yamane et al., 2007).

The presence of tetracycline resistance genes was determined by PCR amplification of the tetracycline efflux gene *tet*A using self-designed primers and genes encoding ribosome protective proteins *tet*M and *tet*W using published primers and annealing temperatures (Aminov et al., 2002; **Table 1**). The contents of the PCR mixture and protocols for purification of the PCR amplicons, sequencing of PMQR, and aminoglycoside- and tetracycline-resistance genes and homology search were the same as used for the 16S rRNA genes.

Detection and Analysis of Integrons and Gene Cassettes

The presence and distribution of integrase genes *intI1*, *intI2*, *intI3*, and integron class 1 gene cassette were determined by PCR amplification using published primers (Kraft et al., 1986; Goldstein et al., 2001; White et al., 2001). The variable regions (VRs) of class 1 integrons, which mainly contain an array of gene cassettes, are flanked at 3′ by a conserved segment containing qacEΔ1 and genes coding for quaternary ammonium and sulfonamide, respectively. VRs were investigated as previously reported in all isolates containing class 1 integrons (Guo et al., 2011). The PCR conditions for amplifying the VRs of integrons were the same as for amplifying the 16S rRNA gene except for the primers and annealing temperatures, which have been summarized in **Table 1**. The PCR amplicons were purified and sequenced as

TABLE 1 | Primers and PCR conditions for amplification of antimicrobial resistance genes, integrons and genetic environment of bla_{CTX-M}.

Primers	Nucleotide sequence	Target genes	Amplicon size (bp)	Annealing temperature (°C)	References
TEM1-FTEM-1-R	5′-TCAACAGCGGTAAGATCCTTGA-3′ 5′-TGCAACTTTATCCGCCTCCA-3′	bla_{TEM}	500	60	Bajaj et al., 2015
SHV-fSHV-r	5′-AAATGGATCTGGCCAGCG-3′ 5′-AGCAGCTGCCGTTGCGAA-3′	bla_{SHV}	481	60	Bajaj et al., 2015
ISEcp1/U1MA3	5′-AAAAATGATTGAAAGGTGGT-3′ 5′-ACYTTACTGGTRCTGCACAT-3′	IS*Ecp1*, $bla_{CTX-M-15}$	900	48	Saladin et al., 2002
CTX-MORF477	5′-CCGTTTCCGCTATTACAAAC-3′ 5′-CTGGGACCTACGTGCGCCCG-3′	$bla_{CTX-M-15}$, *orf477*	1050	55	Dhanji et al., 2011
CMY-fCMY-r	5′-AACACACTGATTGCGTCTGAC-3′ 5′- CTGGGCCTCATCGTCAGTTA-3′	bla_{CMY}	1,226	55	Pérez-Pérez and Hanson, 2002
RMTA-FRMTA-R	5′-CTAGCGTCCATCCTTTCCTC-3′ 5′-TTTGCTTCCATGCCCTTGCC-3′	*rmtA*	653	57	Yamane et al., 2007
RMTB-FRMTB-R	5′-GCTTTCTGCGGGCGATGTAA-3′ 5′-ATGCAATGCCGCGCTCGT AT-3′	*rmtB*	173	60	Yamane et al., 2007
RMTC-FRMTC-R	5′-CGAAGAAGTAACAGCCAAAG-3′ 5′-ATCCCAACATCTCTCCCACT-3′	*rmtC*	711	55	Yamane et al., 2007
ARMA-FARMA-R	5′-ATTCTGCCTATCCTAATTGG-3′ 5′-ACC TATACTTTATCGTCGTC-3′	*armA*	315	46	Yamane et al., 2007
str-Fstr-R	5′-TATCTGCGATTGGACCCTCTG-3′ 5′-CATTGCTCATCATTTGATCGGCT-3′	*strA-strB*	538	62	Sunde and Norstrom, 2005
aacC2-FaacC2-R	5′-TAGAGGAGTATCGCGATGC-3′ 5′ATTATCATTGTCGACGGCCT-3′	*aacC2*	861	55	Ho et al., 2010
TetA-FTetA-R	5′-CAACAGACCCCTGATCGTAA-3′ 5′-AAAATTGCTTGCAGCGCC-3′	*tetA*	962	57	This study
TetM-FTetM-R	5′-ACAGAAAGCTTATTATATAAC-3′ 5′-TGGCGTGTCTATGATGTTCAC-3′	*tetM*	171	55	Aminov et al., 2002
TetW-FTetW-R	5′-GAGAGCCTGCTATATGCCAGC-3′ 5′-GGGCGTATCCACAATGTTAAC-3′	*tetW*	168	64	Aminov et al., 2002
QA-FQA-R	5′-TCGCCGCTGCCGCTTTTAT-3′ 5′-TTCGAGGTTGACCCGTCTG-3′	*qnrA*	517	60	Wang et al., 2009
QB-FQB-R	5′-AACCTGAAAGATGCCATT-3′ 5′-AAGGCCTTGTAAATCAAC-3′	*qnrB*	405	50	Wang et al., 2009
QC-FQC-R	5′-GGGTTGTACATTTATTGAATC-3′ 5′-TCCACTTTACGAGGTTCT-3′	*qnrC*	447	50	Wang et al., 2009
QD-FQD-R	5′-CGAGATCAATTTACGGGGAATA-3′ 5′-CGAGATCAATTTACGGGGAATA-3′	*qnrD*	582	57	Cavaco et al., 2009
QS-FQS-R	5′-GACGTGCTAACTTGCGTGAT-3′ 5′-GATCTAAACCGTCGAGTTCG-3′	*qnrS*	456	55	Bajaj et al., 2016
ACC-FACC-R	5′TTGCGATGCTCTATGAGTGGCTA-3′ 5′-CTCGAATGCCTGGCGTGTTT-3′	*aac(6')-Ib*	482	60	Chen et al., 2012
Int1-FInt1-R	5′-CCT CCC GCA CGA TGA TC-3′ 5′-TCC ACG CAT CGT CAG GC-3′	*intI1*	280	60	Kraft et al., 1986
hep58hep59	5′-TCATGGCTTGTTATGACTGT-3′ 5′-GTAGGGCTTATTATGCACGC-3′	Variable region of class 1 integron	Variable	55	White et al., 2000
qacE1-FsuI1-R	5′-AAGTAATCGCAACATCCG-3′ 5′-GGGTTTCCGAGAAGGTGATTGC-3′	*qacEΔ1, sul1*	878	57	Bass et al., 1999; Nandi et al., 2004

described for the 16S rRNA gene, and nucleotide sequence homology was analyzed using the nucleotide BLAST (BLASTn) available at NCBI.

Accession Numbers

Gene sequencing revealed that the $bla_{CTX-M-15}$ genes of the seven $bla_{CTX-M-15}$-positive strains were identical to each other; hence, the partial coding sequence (CDS) of a representative strain (KP20) was submitted to GenBank (NCBI) with the accession number KF040057. The partial CDS of bla_{TEM-1} was also identical to each other; hence, the sequence of one representative strain (IP1N) was submitted to GenBank (NCBI) with the accession number KF055435. The partial CDS of *qnr*S was also identical; hence, the CDS of a representative strain (KP20) was submitted to NCBI GenBank under accession number KF055436. The partial CDS of *tet*A gene of all the three strains (IS47, WB3, and KKC) was submitted under the accession numbers KJ409940–KJ409942.

RESULTS AND DISCUSSION

Molecular Identification and Phylogrouping Based on Triplex PCR

The results of 16S rRNA gene sequencing and homology search using BLAST confirmed that the strains presumptively identified using API 20E strips (bioMérieux, France) were *Escherichia coli*. The results of the triplex PCR and Clermont classification indicated that 50% ($n = 20$) of the strains belonged to phylogroup A [*chuA* (−), *yjaA* (−/+), and TSPE4.C2 (−)] while 50% ($n = 20$) to phylogroup B1 [*chuA* (−), *yjaA* (−), and TSPE4.C2 (+)]. Earlier studies have reported that *E. coli* strains of all phylogroups were present in river Yamuna (Bajaj et al., 2015; Kaushik et al., 2018). Phylogroups A and B1 of *E. coli* represent commensal strains, while phylogroups B2 and D represent pathogenic strains (Herzer et al., 1990; Bingen et al., 1998; Lecointre et al., 1998; Picard et al., 1999). Several studies have indicated that the prevalence of virulence genes in commensal strains of *E. coli* was lesser than in pathogenic strains (Johnson, 1991; Boyd and Hartl, 1998; Lecointre et al., 1998; Picard et al., 1999) but that the commensal strains can easily disseminate AMR determinants to pathogenic *E. coli* or other waterborne pathogens via mobile genetic elements. Thus, AMR determinants and integrons were investigated in these 40 commensal *E. coli* strains.

Phenotypic Testing of Antimicrobial Susceptibilities and Extended-Spectrum β-Lactamase Production

Antibiotic susceptibility testing revealed that 95% ($n = 38$) of the commensal *E. coli* strains were resistant to ampicillin, while 32% ($n = 13$) of the strains were resistant to piperacillin. Among the cephalosporins, 42.5% ($n = 17$) strains were resistant to cefazolin (first-generation cephalosporin), 17.5% ($n = 7$) to cefuroxime (second-generation cephalosporin), 22.5% ($n = 9$) to cefotaxime (third-generation cephalosporin), and 15% ($n = 6$) to cefepime (fourth-generation cephalosporin). The fact that commensal waterborne *E. coli* were less resistant to new-generation cephalosporins than ampicillin is normal because ampicillin was a widely prescribed broad-spectrum penicillin, and over time, bacteria might have developed resistance to this antibiotic. Earlier studies have also reported that ampicillin resistance was highly prevalent in commensal strains of *E. coli* isolated from India and from other parts of the globe like Vietnam, China, Sudan, and Thailand (Dyar et al., 2012; Abdelgader et al., 2018; Lugsomya et al., 2018; Singh A. K. et al., 2018; Purohit et al., 2019). With regard to ESBL production, 17.5% ($n = 7$) of the commensal *E. coli* strains tested positive, while none of the strain tested positive for AmpC production (**Table 2**). All the ESBL-producing strains were resistant to four or more β-lactam antibiotics, and 57% ($n = 4$) of the ESBL producers were resistant to ciprofloxacin. Since, penicillins and cephalosporins are the most frequently used antibiotics in India, it is normal that all the seven ESBL-producing *E. coli* strains were resistant to many antibiotics of these classes. The fact that some ESBL producers were also resistant to ciprofloxacin and streptomycin/kanamycin suggests that besides β-lactam antibiotics, resistance to other antibiotic classes also exhibited co-selection.

With regard to quinolone resistance, 35% ($n = 14$) strains were resistant to older quinolones like nalidixic acid, while 20% ($n = 8$) and 15% ($n = 6$) strains were resistant to newer quinolones like ciprofloxacin and ofloxacin, respectively. In this regard, our results are similar to those of other studies that reported lower ciprofloxacin and ofloxacin resistance in waterborne *E. coli* isolated from other parts of the world (Odonkor and Addo, 2018). Aminoglycoside resistance was observably less prevalent with 12.5% ($n = 5$) strains resistant to older aminoglycosides like streptomycin and less than 7% strains resistant to new aminoglycosides like kanamycin, tobramycin, netilmicin, and amikacin. An earlier study also reported that a low level of aminoglycoside resistance was present in *E. coli* strains isolated from aquatic environments of Kuala Lumpur, Malaysia (Hara et al., 2018). With regard to tetracycline, 27.5% ($n = 11$) of the strains exhibited resistance. The frequent use/misuse of ampicillin, streptomycin, and tetracycline due to frequent prescription, availability, and affordability might be a probable reason for higher bacterial resistance to these antibiotics (Shakya et al., 2013). Additionally, 57.5% ($n = 23$) *E. coli* strains were resistant to at least two antibiotic classes, and 20% ($n = 8$) of the strains were multidrug resistant (MDR), i.e., resistant to three or more antibiotic classes. MDR *E. coli* were defined as bacteria resistant to antibiotics belonging to three or more antimicrobial classes (Magiorakos et al., 2012). An analysis of MAR index revealed that the MAR indexes of 13 MDR *E. coli* strains were quite high (>0.2). Tambekar et al. (2006) reported that bacteria isolated from environments where several antibiotics are used usually show MAR index >0.2. The high MAR index of the *E. coli* strains observed in this study is not surprising because river Yamuna is highly contaminated with effluents originating from hospital and municipal wastewaters; discharge from livestock, poultry, and agriculture production plants; etc.

Antimicrobial Resistance Genes

β-Lactam Resistance and Extended-Spectrum β-Lactamase Encoding Genes

With regard to β-lactam resistance genes, bla_{TEM-1} was present in 95% of the strains ($n = 38$) followed by $bla_{CTX-M-15}$, which was present in 15% ($n = 7$) of the *E. coli* strains. The plasmid-encoded AmpC enzymes (CMY types) were not found in any strain. The presence of bla_{TEM-1} correlated well with ampicillin resistance in all the strains. Similarly, all the $bla_{CTX-M-15}$-positive strains showed phenotypic production of ESBLs. Earlier studies had reported that bla_{TEM-1} was widely present in *E. coli* strains isolated from water bodies of India and other countries like Spain, Australia, France, China, and Poland (Lartigue et al., 2002; Tristram and Nichols, 2006; Garcia-Cobos et al., 2008; Ortega et al., 2012; Liu et al., 2014; Ojdana et al., 2014; Bajaj et al., 2015; Singh N. S. et al., 2018). CTX-M enzymes belong to the family of ESBLs and are the most widely disseminated ESBLs among Enterobacteriaceae all over the globe (Poirel et al., 2002). Among these, $bla_{CTX-M-15}$ is the most widely globally disseminated CTX-M type, which was

TABLE 2 | Detailed information about the commensal strains of *E. coli* isolated from river Yamuna, antimicrobial resistance phenotypes, multiple antibiotic resistance index (MAR index) and antimicrobial resistance genes along with the genetic environment of $bla_{CTX-M-15}$.

Strain designation (Phylogroup)	Antimicrobial resistance and ESBL[a] phenotype	MAR[b] index	Antimicrobial resistance genes	Genetic environment of $bla_{CTX-M-15}$
KKC (A)	PIP, AMP, AMC, STM, TE	0.312	bla_{TEM-1}, *tet*A, *str*A-*str*B,*qnrS1*	–
WB3 (A)	AMP,TE	0.125	bla_{TEM-1}, *tet*A, *str*A-*str*B, *qnrS1*	–
NG23 (A)	AMP, NA	0.125	bla_{TEM-1}, *arm*A	–
IS47 (A)	PIP, AMP, AMC, CTX, S, TE, ESBL	0.375	bla_{TEM-1}, $bla_{CTX-M-15}$, *tet*A, *str*A-*str*B, *qnr*S1	IS*Ecp1*, *orf477*
KP6 (A)	–	–	–	–
IPB (A)	AMP, NA	0.125	bla_{TEM-1}	–
KK5 (A)	PIP, AMP, NA, CIP, OF, STM	0.375	bla_{TEM-1}, *arm*A, *str*A-*str*B	-
KP20 (A)	PIP, AMP, AMC, CZ, CXM, CTX, CPM, STM, TE, ESBL	0.565	bla_{TEM-1}, $bla_{CTX-M-15}$, *arm*A, *str*A-*str*B, *qnrs*S1	IS*Ecp1*, *orf477*
ISF (A)	AMP, AMC, CZ, CXM, CTX, TE,TOB	0.437	bla_{TEM-1}, *arm*A, *str*A-*str*B	–
NG35 (A)	AMP, NA, CIP, OF	0.25	bla_{TEM-1}, *qnrS1*	–
WB2 (A)	PIP, AMP, AMC, CZ, STM	0.312	bla_{TEM-1}, *arm*A, *qnr*S1	–
KK47 (A)	AMP	0.062	bla_{TEM-1}	–
DND24 (A)	AMP, NA	0.125	bla_{TEM-1}	–
IP1N (A)	PIP, AMP, AMC, CZ, CXM, CTX, CPM, NA, CIP, OF,TE, KAN, ESBL	0.75	bla_{TEM-1}, $bla_{CTX-M-15}$, *arm*A	IS*Ecp1*, *orf477*
KK1 (A)	AMP, CZ	0.125	bla_{TEM-1}	–
NeG15 (A)	AMP, CZ	0.125	bla_{TEM-1}	–
NG6 (A)	AMP, NA	0.125	bla_{TEM-1}	–
ISJ (A)	AMP, CZ	0.125	bla_{TEM-1}	–
KK26 (A)	AMP, CZ	0.125	bla_{TEM-1}	–
PA18 (A)	AMP, CZ	0.125	bla_{TEM-1}	–
NG41 (B1)	PIP, AMP, CZ, CXM, CTX, CPM, NA, CIP, ESBL	0.5	bla_{TEM-1}, $bla_{CTX-M-15}$	IS*Ecp1*, *orf477*
NG31(B1)	PIP, AMP, CZ, CXM, CTX, CPM, CIP, NA, ESBL	0.5	bla_{TEM-1}, $bla_{CTX-M-15}$	IS*Ecp1*, *orf477*
DND3(B1)	AMP, CZ, NA	0.187	bla_{TEM-1}, *arm*A	–
KK15(B1)	AMP, CZ	0.125	bla_{TEM-1}	–
PA1(B1)	AMP, CZ	0.125	bla_{TEM-1}	–
KK21(B1)	AMP	0.062	bla_{TEM-1}	–
PA3(B1)	PIP, AMP, TE	0.187	bla_{TEM-1}	–
SVN(B1)	PIP, AMP, AMC, CZ, NA, CIP, OF, TE,TOB,KAN,NET,AK	0.75	bla_{TEM-1}	–
MKNA(B1)	–	–	–	–
DND1(B1)	AMP, AMC	0.125	bla_{TEM-1}	–
PA32(B1)	AMP	0.062	bla_{TEM-1}	–
KK39(B1)	AMP, NA	0.125	bla_{TEM-1}	–
IPK(B1)	AMP	0.062	bla_{TEM-1}	–
WB20(B1)	AMP, CTX,TE	0.185	bla_{TEM-1}	–
NG25(B1)	PIP, AMP, AMC, CZ, CXM, CPM, CTX, CIP, OF, NA, ESBL	0.625	bla_{TEM-1} $bla_{CTX-M-15}$	IS*Ecp1*, *orf477*
DND11(B1)	AMP	0.062	bla_{TEM-1}	–
IS68(B1)	AMP, TE	0.125	bla_{TEM-1}	–
SP13N(B1)	PIP, AMP, AMC, CZ, CXM, CTX, CPM, ESBL	0.437	bla_{TEM-1}, $bla_{CTX-M-15}$, *qnr*S1	IS*Ecp1*, *orf477*
IS45(B1)	PIP, AMP, AMC, CIP, NA, OF, TE, TOB, KAN, NET, AK	0.685	bla_{TEM-1}, *arm*A	–
WB9(B1)	AMP	0.062	bla_{TEM-1}	–

PIP, piperacillin; AMP, ampicillin; AMC, amoxicillin-clavulanic acid; CZ, cefazolin; CXM, cefuroxime; CTX, cefotaxime; CPM, cefepime; NA, nalidixic acid; CIP, ciprofloxacin; OF, ofloxacin; STM, streptomycin; TOB, tobramycin; KAN, kanamycin; NET, netilmycin; AK, amikacin; TE, tetracycline; ESBL[a]: extended-spectrum β*-lactamase; MAR[b] index: multiple antibiotic resistance index.*

first reported from the Indian isolates in 2001 (Karim et al., 2001; Poirel et al., 2002). Later, several studies also reported the prevalence of $bla_{CTX-M-15}$ in aquatic *E. coli* isolated from India (Bajaj et al., 2015; Singh N. S. et al., 2018; Kaushik et al., 2019). Previous studies have associated $bla_{CTX-M-15}$ in aquatic *E. coli* with the pathogenic phylogroups B2 (especially those belonging to the genetic lineage ST131) and D (Nicolas-Chanoine et al., 2008; Coque et al., 2008). However, our study revealed that CTX-M-15 type ESBLs were present in the commensal phylogroups A and B1, also.

Aminoglycoside Resistance Genes

The linked *str*A-*str*B genes are the most widely prevalent streptomycin resistance genes in *E. coli* worldwide and encode for phosphotransferases (Poirel et al., 2018). However, *str*A-*str*B genes were present in only 12% ($n = 5$) of the waterborne *E. coli*. Of these, four strains exhibited phenotypic resistance to streptomycin, while one strain (ISF), despite harboring *str*A-*str*B, was phenotypically susceptible for streptomycin. The 16S rRNA methylases methylate certain amino acid residues of the 16S RNA, resulting in resistance to amikacin, tobramycin, gentamicin, and netilmicin (Griffey et al., 1999). Of the four types of plasmid-mediated 16S rRNA methylase investigated, only *arm*A was found to be present in 17.5% ($n = 7$) of the strains. Earlier studies also reported that a low level of aminoglycoside resistance was present in *E. coli* strains isolated from aquatic environments in Kuala Lumpur, Malaysia (Hara et al., 2018). No correlation was observed in aminoglycoside resistance and presence of *arm*A, except in the *E. coli* strain IS45, which also exhibited phenotypic resistance to amikacin, tobramycin, kanamycin, and netilmicin.

Plasmid-Mediated Quinolone Resistance and Tetracycline Resistance Genes

Of the several PMQR genes (*qnr*A, *qnr*B, *qnr*C, *qnr*D, *qnr*S, *qep*, and *aac*) tested, only *qnr*S was detected in the *E. coli* strains isolated from river Yamuna. The *qnr*S1 was detected as the predominant PMQR gene in about 17% of the aquatic strains ($n = 7$). Earlier studies have also reported that the *qnr*S type gene was the most frequently detected PMQR gene in *E. coli* isolated from environmental *E. coli* worldwide (Bonemann et al., 2006; Cattoir et al., 2008; Rodriguez-Mozaz et al., 2015; Varela et al., 2016; Hara et al., 2018). The presence of *qnr*S1 did not correlate with fluoroquinolone resistance, except in *E. coli* strain NG35. This suggests that the presence of the *qnr*S gene alone might not be a true indicator of fluoroquinolone resistance and the isolate despite that the presence of *qnr*S might exhibit phenotypic susceptibility for fluoroquinolones (Mahmud et al., 2020).

Of the three tetracycline resistance genes, *tet*M and *tet*W were absent and only *tet*A was present in 7.5% ($n = 3$) of the strains. Earlier studies have also indicated that tetracycline efflux-related genes like *tet*A, *tet*B, and *tet*C were more prevalent than ribosomal protection-related genes (like *tet*M and *tet*W) in waterborne *E. coli* (Zhang et al., 2015; Stange et al., 2016). The presence of *tet*A correlated well with phenotypic resistance because the three strains that harbored *tet*A also exhibited tetracycline resistance.

Genetic Environment of $bla_{CTX-M-15}$

Of the 40 *E. coli* strains investigated in this study, only seven strains (15%) harbored the $bla_{CTX-M-15}$ gene. The upstream region of the $bla_{CTX-M-15}$ was analyzed in these seven strains by PCR amplification and sequencing. Gene sequencing revealed that the IS*Ecp1* was present in the upstream region of $bla_{CTX-M-15}$ and *orf477* was present in the downstream region of all the seven strains (**Table 2**). Several investigators have also reported the presence of *orf477* in the downstream region of $bla_{CTX-M-15}$ (Eckert et al., 2006; Dhanji et al., 2011; Wang et al., 2014; Ben Said et al., 2016). IS*Ecp1* is the most common and widely reported IS element (Dhanji et al., 2011; Liu et al., 2014; Upadhyay et al., 2015; Ben Said et al., 2016; Singh N. S. et al., 2018). In the present study, IS sequence IS*Ecp1* was found to be present at 48-bp upstream region of $bla_{CTX-M-15}$. The 42- to 266-bp upstream region has been reported as the preferred insertion site of IS*Ecp1* for different bla_{CTX-M} genes like CTX-M-1, CTX-M-2, and CTX-M-9. An analysis of the −35 and −10 promoter regions of the $bla_{CTX-M-15}$ gene of the seven strains revealed that the −35 (TTGAAA) and −10 (TACAAT) regions were present within 3′ terminus end of IS*Ecp1* and 48 bp away from the $bla_{CTX-M-15}$ start codon. The same organization was previously reported from *E. coli* strains isolated from different countries of the world (Saladin et al., 2002; Boyd et al., 2004; Canton and Coque, 2006; Lavollay et al., 2006; Dhanji et al., 2011;

TABLE 3 | Characteristics of integrons present in commensal *E. coli* strains isolated from river Yamuna, India.

E. coli strains	Class of integron gene	Size of variable gene cassettes (bp)	Gene cassette array
KKC	*intI1*	–	–
DND24	*intI1*	–	–
KP20	*intI1*	–	–
KK5	*intI1*	–	–
WB2	*intI1*	2,800	*aacA4, catB3, dfrA1*
KK26	*intI1*	1,700	*dfrA1, aadA1*
IPB	*intI1*	1,700	*dfrA1, aadA1*
KK1	*intI1*	–	–
PA18	*int1*	–	–
KP6	*intI1*	–	–
NG6	*intI1*	–	–
WB3	*intI1*	–	–
IP1N	*intI1*	–	–
NG35	*intI1*	–	–
NG23	*intI1*	–	–
PA3	*intI1*	–	–
NG41	*intI1*	–	–
NG31	*intI1*	–	–
MKNA	*intI1*	–	–
PA1	*intI1*	–	–
IS47	*intI1*	–	–
SVN	*intI1*	–	–
WB9	*intI1*	–	–
NG25	*intI1*	–	–
WB20	*int1*	1,900	*dhfr12, aadA2*
IS68	*intI1*	–	–
DND11	*intI1*	–	–
IS45	*intI1*	–	–
SP13N	*intI1*	–	–
KK39	*intI1*	–	–

Liu et al., 2014). The presence of IS*Ecp1* along with bla_{CTX-M} has been reported from *E. coli* strains isolated from different parts of the world, indicating that IS*Ecp1* might be evolutionary associated with bla_{CTX-M} (Karim et al., 2001; Saladin et al., 2002; Dhanji et al., 2011; Liu et al., 2014; Wang et al., 2014). IS*Ecp1* can mobilize an adjacent gene as a part of transposition units of varying sizes (Zong et al., 2010). It has also been reported that IS*Ecp1* helps in improving the expression of bla_{CTX-M} in enteric bacteria (Poirel et al., 2003). This is a matter of great concern because the subsequent transfer of ESBL genes from these commensal *E. coli* to pathogenic *E. coli* or other bacteria in aquatic water bodies might pose a serious health challenge (Figueira et al., 2011).

Detection and Analysis of Integrons

The class 1 integron gene *intI1* was detected in 75% ($n = 30$) of the isolates (**Table 3**). None of the strains harbored class 2 and 3 integrase genes *intI2* and *intI3*. Though some strains of *E. coli* reportedly harbored class 2 integrons, mostly class 1 integrons have been reported from *E. coli* isolated from India (Kaushik et al., 2018). Several studies have indicated that integrons of the class 3 were absent in *E. coli* isolated from water bodies across the globe (Laroche et al., 2009; Su et al., 2012; Pereira et al., 2013). Of the 30 *intI1* harboring strains, gene cassette arrays were detected in only 10% ($n = 4$) of the *intI1*-positive *E. coli* strains. Three different types of gene cassette arrays of class 1 were present in the downstream region of *intI1* whose size ranged from 1.7 to 2.8 kb (**Table 2**). Gene sequencing revealed that five gene cassettes of the dihydrofolate reductase (dfr) resistance gene family (*dhfr12* and *dfrA1*), aminoglycoside (*aad*) resistance gene family (*aadA1*, *aadA2*, and *aacA4*), and chloramphenicol (CHL) resistance gene family (*catB3*) were present (**Table 3**). The prevalence of class 1 integron *intI1* in Indian aquatic isolates was quite high (75%) and alarmingly more than that reported for *E. coli* isolated from global aquatic environments (Dolejska et al., 2009; Laroche et al., 2009; Pereira et al., 2013; Ghaderpour et al., 2015; Sidhu et al., 2017). *E. coli* isolates harboring class 1 integrons have been associated with a significantly higher probability for multidrug resistance than those devoid of class 1 integrons (Chen et al., 2011). Moreover, class 1 integron genes *intI1* are accompanied by resistance genes for disinfectants and heavy metals (Partridge et al., 2001) and can also easily horizontally transfer between strains originating from different sources (Nagachinta and Chen, 2008; Zhang et al., 2009). Thus, due to high prevalence of class 1 integron gene *intI1*, the commensal strains of *E. coli* can become vehicles for widespread dissemination of antibiotic resistance to pathogenic *E. coli* and other waterborne bacterial pathogens.

CONCLUSION

Our results indicated a high prevalence of drug resistance in *Escherichia coli* strains of river Yamuna. With regard to plasmid-mediated AMR genes, bla_{TEM-1} was present in 95% strains followed by q*nr*S1 and *arm*A (17% each), $bla_{CTX-M-15}$ (15%), *str*A-*str*B (12%), and *tet*A (7%). Though most of the earlier studies have reported that $bla_{CTX-M-15}$ in waterborne *E. coli* was mostly present in pathogenic phylogroup B2, our study revealed that CTX-M-15 type ESBLs were present in the commensal phylogroups A and B1, also. The genetic organization of $bla_{CTX-M-15}$ was similar to that reported for *E. coli* globally, and IS*Ecp1* was present in the upstream region of $bla_{CTX-M-15}$. Though integrons of classes 2 and 3 were absent, class 1 integron gene *intI* was detected in 75% of the isolates, which indicates its high prevalence in the *E. coli* isolates of the river Yamuna than that reported, globally. The presence of MDR phenotypes, plasmid-mediated AMR genes, and class 1 integron gene *intI1* in *E. coli* is a serious public health risk, because these commensal strains can become potent vehicles for widespread dissemination of AMR determinants to pathogenic *E. coli* and other waterborne pathogens. Thus, our study suggests an urgent need for regular surveillance and management of natural water bodies to curtail the spread of antibiotic resistance in microorganisms.

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

NSS, NS, MK, and JV analyzed the data. NSS and NS wrote the manuscript. JV conceptualized and supervised. All authors contributed to the article and approved the submitted version.

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REFERENCES

Abdelgader, S. A., Shi, D., Chen, M., Zhang, L., Hejair, H. M. A., Muhammad, U., et al. (2018). Antibiotics resistance genes screening and comparative genomics analysis of commensal *Escherichia coli* isolated from poultry farms between China and Sudan. *BioMed. Res. Int.* 2018, 1–9. doi: 10.1155/2018/5327450

Aminov, R. I., Chee-Sanford, J. C., Garrides, N., Teferedegne, B., Krapac, I. J., White, B. A., et al. (2002). Development, validation and application of PCR primers for detection of tetracycline efflux genes of gram negative bacteria. *Appl. Environ. Microbiol.* 68, 1786–1793. doi: 10.1128/aem.68.4.1786-1793.2002

Aanensen, D. M., and Spratt, B. G. (2005). The multilocus sequence typing network: mlst.net. *Nucleic Acids Res.* 33, W728–W733. doi: 10.1093/nar/gki415

Bajaj, P., Kanaujia, P. K., Singh, N. S., Sharma, S., Kumar, S., and Virdi, J. S. (2016). Quinolone co-resistance in ESBL- or AmpC-producing *Escherichia coli* from an Indian urban aquatic environment and their public health implications. *Environ. Sci. Pollut. Res. Int.* 23, 1954–1959. doi: 10.1007/s11356-015-5609-x

Bajaj, P., Singh, N. S., Kanaujia, P. K., and Virdi, J. S. (2015). Distribution and molecular characterization of genes encoding CTX-M and AmpC beta-lactamases in *Escherichia coli* isolated from an Indian urban aquatic environment. *Sci. Total. Environ.* 505, 350–356. doi: 10.1016/j.scitotenv.2014.09.084

Baquero, F., Martinez, J. L., and Canton, R. (2008). Antibiotics and antibiotic resistance in water environments. *Curr. Opin. Biotechnol.* 19, 260–265. doi: 10.1016/j.copbio.2008.05.006

Bass, L., Liebert, C. A., Lee, M. D., Summers, A. O., White, D. G., Thayer, S. G., et al. (1999). Incidence and characterization of integrons, genetic elements mediating multiple-drug resistance, in avian *Escherichia coli*. *Antimicrob. Agents. Chemother.* 43, 2925–2929. doi: 10.1128/aac.43.12.2925

Ben Said, L., Jouini, A., Alonso, C. A., Klibi, N., Dziri, R., Boudabous, A., et al. (2016). Characteristics of extended-spectrum beta-lactamase (ESBL)- and pAmpC beta-lactamase-producing *Enterobacteriaceae* of water samples in Tunisia. *Sci. Total. Environ.* 550, 1103–1109. doi: 10.1016/j.scitotenv.2016.01.042

Bingen, E., Picard, B., Brahimi, N., Mathy, S., Desjardins, P., Elion, J., et al. (1998). Phylogenetic analysis of *Escherichia coli* strains causing neonatal meningitis suggests horizontal gene transfer from a predominant pool of highly virulent B2 group strains. *J. Infect. Dis.* 177, 642–650. doi: 10.1086/514217

Bonemann, G., Stiens, M., Puhler, A., and Schluter, A. (2006). Mobilizable IncQ-related plasmid carrying a new quinolone resistance gene, qnrS2, isolated from the bacterial community of a wastewater treatment plant. *Antimicrob. Agents. Chemother.* 50, 3075–3080. doi: 10.1128/aac.00378-06

Boyd, D. A., Tyler, S., Christianson, S., McGeer, A., Muller, M. P., Willey, B. M., et al. (2004). Complete nucleotide sequence of a 92-kilobase plasmid harbouring the CTX-M-15 extended-spectrum b-lactamase involved in an outbreak in long-term-care facilities in Toronto. Canada. *Antimicrob. Agents Chemother.* 48, 3758–3764. doi: 10.1128/aac.48.10.3758-3764.2004

Boyd, E. F., and Hartl, D. L. (1998). Chromosomal regions specific to pathogenic isolates of *Escherichia coli* have a phylogenetically clustered distribution. *J. Bacteriol.* 180, 1159–1165. doi: 10.1128/jb.180.5.1159-1165.1998

Canton, R., and Coque, T. M. (2006). The CTX-M beta-lactamase pandemic. *Curr. Opin. Microbiol.* 9, 466–475.

Cattoir, V., Poirel, L., and Nordmann, P. (2008). Plasmid-mediated quinolone resistance pump QepA2 in an *Escherichia coli* isolate from France. *Antimicrob. Agents Chemother.* 52, 3801–3804.

Cavaco, L. M., Hasman, H., Xia, S., and Aarestrup, F. M. (2009). qnrD, a novel gene conferring transferable quinolone resistance in Salmonella enterica serovar Kentucky and Bovismorbificans strains of human origin. Antimicrob. Agents Chemother. 53, 603–608. doi: 10.1128/AAC.00997-08

Chen, B., Zheng, W., Yu, Y., Huang, W., Zheng, S., Zhang, Y., et al. (2011). Class 1 integrons, selected virulence genes, and antibiotic resistance in Escherichia coli isolates from the Minjiang River, Fujian Province, China. *Appl. Environ. Microbiol.* 77, 148–155. doi: 10.1128/AEM.01676-10

Chen, X., Zhang, W., Pan, W., Yin, J., Pan, Z., Gao, S., et al. (2012). Prevalence of qnr, aac(6')-Ib-cr, qepA, and oqxAB in *Escherichia coli* isolates from humans, animals, and the environment. *Antimicrob. Agents Chemother.* 56, 3423–3427. doi: 10.1128/aac.06191-11

Clermont, O., Bonacorsi, S., and Bingen, E. (2000). Rapid and simple determination of the *Escherichia coli* phylogenetic group. *Appl. Environ. Microbiol.* 66, 4555–4558. doi: 10.1128/aem.66.10.4555-4558.2000

Clinical and Laboratory Standards Institute [CLSI] (2018). *Performance Standards for Antimicrobial Susceptibility Testing; Approved Standard*, 28th Edn. Wayne, PA: Clinical and Laboratory Standards Institute.

Coque, T. M., Novais, A., Carattoli, A., Poirel, L., Pitout, J., Peixe, L., et al. (2008). Dissemination of clonally related *Escherichia coli* strains expressing extended-spectrum beta-lactamase CTX-M-15. *Emerg. Infect. Dis.* 14, 195–200.

Dhanji, H., Patel, R., Wall, R., Doumith, M., Patel, B., Hope, R., et al. (2011). Variation in the genetic environments of bla(CTX-M-15) in *Escherichia coli* from the faeces of travellers returning to the United Kingdom. *J. Antimicrob. Chemother.* 66, 1005–1012. doi: 10.1093/jac/dkr041

Dolejska, M., Bierosova, B., Kohoutova, L., Literak, I., and Cizek, A. (2009). Antibiotic-resistant *Salmonella* and *Escherichia coli* isolates with integrons and extended-spectrum beta-lactamases in surface water and sympatric black-headed gulls. *J. Appl. Microbiol.* 106, 1941–1950. doi: 10.1111/j.1365-2672.2009.04155.x

Dyar, O. J., Hoa, N. Q., Trung, N. V., Phuc, H. D., Larsson, M., Chuc, N. T. K., et al. (2012). High prevalence of antibiotic resistance in commensal *Escherichia coli* among children in rural Vietnam. *BMC Infect. Dis.* 12:92.

Eckert, C., Gautier, V., and Arlet, G. (2006). DNA sequence analysis of the genetic environment of various blaCTX-M genes. *J. Antimicrob. Chemother.* 57, 14–23. doi: 10.1093/jac/dki398

Figueira, V., Serra, E., and Manaia, C. M. (2011). Differential patterns of antimicrobial resistance in population subsets of *Escherichia coli* isolated from waste- and surface waters. *Sci. Total Environ.* 409, 1017–1023. doi: 10.1016/j.scitotenv.2010.12.011

Garcia-Cobos, S., Campos, J., Cercenado, E., Roman, F., Lazaro, E., Perez-Vazquez, M., et al. (2008). Antibiotic resistance in *Haemophilus influenzae* decreased, except for beta-lactamase-negative amoxicillin-resistant isolates, in parallel with community antibiotic consumption in Spain from 1997 to 2007. *Antimicrob. Agents Chemother.* 52, 2760–2766. doi: 10.1128/aac.01674-07

Ghaderpour, A., Ho, W. S., Chew, L. L., Bong, C. W., Chong, V. C., Thong, K. L., et al. (2015). Diverse and abundant multi-drug resistant *E. coli* in Matang mangrove estuaries, Malaysia. *Front. Microbiol.* 6:977.

Gillings, M. (2014). Integrons: past, present, and future. *Microbiol. Mol. Biol. Rev.* 78, 257–277. doi: 10.1128/mmbr.00056-13

Goldstein, C., Lee, M. D., Sanchez, S., Hudson, C., Phillips, B., Register, B., et al. (2001). Incidence of class 1 and 2 integrases in clinical and commensal bacteria from livestock, companion animals, and exotics. *Antimicrob. Agents Chemother.* 45, 723–726. doi: 10.1128/aac.45.3.723-726.2001

Griffey, R. H., Hofstadler, S. A., Sannes-Lowery, K. A., Ecker, D. J., and Crooke, S. T. (1999). Determinants of aminoglycoside-binding specificity for rRNA by using mass spectrometry. *Proc. Natl. Acad. Sci. USA* 96, 10129–10133. doi: 10.1073/pnas.96.18.10129

Guo, X., Xia, R., Han, N., and Xu, H. (2011). Genetic diversity analyses of class 1 integrons and their associated antimicrobial resistance genes in *Enterobacteriaceae* strains recovered from aquatic habitats in China. *Lett. Appl. Microbiol.* 52, 667–675. doi: 10.1111/j.1472-765x.2011.03059.x

Hara, H., Yusaimi, Y. A., Zulkeffle, S. N. M., Sugiura, N., Iwamoto, K., Goto, M., et al. (2018). Molecular characterization of multi-drug resistant *Escherichia coli* isolates from tropical environments in Southeast Asia. *J. Gen. Appl. Microbiol.* 64, 284–292. doi: 10.2323/jgam.2018.02.003

Hasegawa, H., Suzuki, E., and Maeda, S. (2018). Horizontal plasmid transfer by transformation in *Escherichia coli*: environmental factors and possible mechanisms. *Front. Microbiol.* 9:2365.

Herzer, P. J., Inouye, S., Inouye, M., and Whittam, T. S. (1990). Phylogenetic distribution of branched RNA-linked multicopy single-stranded DNA among natural isolates of *Escherichia coli*. *J. Bacteriol.* 172, 6175–6181. doi: 10.1128/jb.172.11.6175-6181.1990

Ho, P. L., Wong, R. C., Lo, S. W., Chow, K. H., Wong, S. S., and Que, T. L. (2010). Genetic identity of aminoglycoside-resistance genes in *Escherichia coli* isolates from human and animal sources. *J. Med. Microbiol.* 59, 702–707. doi: 10.1099/jmm.0.015032-0

Johnson, J. R. (1991). Virulence factors in *Escherichia coli* urinary tract infection. *Clin. Microbiol. Rev.* 4, 80–128. doi: 10.1128/cmr.4.1.80-128.1991

Karim, A., Poirel, L., Nagarajan, S., and Nordmann, P. (2001). Plasmid-mediated extended-spectrum beta-lactamase (CTX-M-3 like) from India and gene association with insertion sequence ISEcp1. *FEMS Microbiol. Lett.* 201, 237–241. doi: 10.1111/j.1574-6968.2001.tb10762.x

Kaushik, M., Kumar, S., Kapoor, R. K., and Gulati, P. (2019). Integrons and antibiotic resistance genes in water-borne pathogens: threat detection and risk assessment. *J. Med. Microbiol.* 68, 679–692. doi: 10.1099/jmm.0.000972

Kaushik, M., Kumar, S., Kapoor, R. K., Virdi, J. S., and Gulati, P. (2018). Integrons in *Enterobacteriaceae*: diversity, distribution and epidemiology. *Int. J. Antimicrob. Agents* 51, 167–176. doi: 10.1016/j.ijantimicag.2017.10.004

Koczura, R., Mokracka, J., Barczak, A., Krysiak, N., and Kaznowski, A. (2013). Association between the presence of class 1 integrons, virulence genes, and phylogenetic groups of *Escherichia coli* isolates from river water. *Microb. Ecol.* 65, 84–90. doi: 10.1007/s00248-012-0101-3

Kraft, C. A., Timbury, M. C., and Platt, D. J. (1986). Distribution and genetic location of Tn7 in trimethoprim-resistant *Escherichia coli*. *J. Med. Microbiol.* 22, 125–131. doi: 10.1099/00222615-22-2-125

Krumperman, P. H. (1983). Multiple antibiotic resistance indexing of *Escherichia coli* to identify high-risk sources of fecal contamination of foods. *Appl. Environ. Microbiol.* 46, 165–170. doi: 10.1128/aem.46.1.165-170.1983

Kümmerer, K. (2009). The presence of pharmaceuticals in the environment due to human use–present knowledge and future challenges. *J. Environ. Manag.* 90, 2354–2366. doi: 10.1016/j.jenvman.2009.01.023

Laroche, E., Pawlak, B., Berthe, T., Skurnik, D., and Petit, F. (2009). Occurrence of antibiotic resistance and class 1, 2 and 3 integrons in *Escherichia coli* isolated from a densely populated estuary (Seine, France). *FEMS Microbiol. Ecol.* 68, 118–130. doi: 10.1111/j.1574-6941.2009.00655.x

Lartigue, M. F., Leflon-Guibout, V., Poirel, L., Nordmann, P., and Nicolas-Chanoine, M. H. (2002). Promoters P3, Pa/Pb, P4, and P5 upstream from bla(TEM) genes and their relationship to beta-lactam resistance. *Antimicrob. Agents Chemother.* 46, 4035–4037. doi: 10.1128/aac.46.12.4035-4037.2002

Lavollay, M., Mamlouk, K., Frank, T., Akpabie, A., Burghoffer, B., Ben Redjeb, S., et al. (2006). Clonal dissemination of a CTX-M-15 beta-lactamase-producing *Escherichia coli* strain in the Paris area, Tunis, and Bangui. *Antimicrob. Agents Chemother.* 50, 2433–2438. doi: 10.1128/aac.00150-06

Lecointre, G., Rachdi, L., Darlu, P., and Denamur, E. (1998). *Escherichia coli* molecular phylogeny using the incongruence length difference test. *Mol. Biol. Evol.* 15, 1685–1695. doi: 10.1093/oxfordjournals.molbev.a025895

Liebana, E., Carattoli, A., Coque, T. M., Hasman, H., Magiorakos, A. P., Mevius, D., et al. (2013). Public health risks of enterobacterial isolates producing extended-spectrum beta-lactamases or AmpC beta-lactamases in food and food-producing animals: an EU perspective of epidemiology, analytical methods, risk factors, and control options. *Clin. Infect. Dis.* 56, 1030–1037. doi: 10.1093/cid/cis1043

Liu, L., Wang, X., An, S., Zhang, X., Chen, L., Li, Y., et al. (2014). Genetic environment of beta-lactamase genes of extended-spectrum beta-lactamase-producing *Klebsiella pneumoniae* isolates from patients with lower respiratory tract infection in China. *Chin. Med. J.* 127, 2445–2450.

Lugsomya, K., Yindee, J., Niyomtham, W., Tribuddharat, C., Tummaruk, P., Hampson, D. J., et al. (2018). Antimicrobial resistance in commensal *Escherichia coli* isolated from pigs and pork derived from farms either routinely using or not using in-feed antimicrobials. *Mary Ann Liebert* 24, 1054–1066. doi: 10.1089/mdr.2018.0154

Magiorakos, A. P., Srinivasan, A., Carey, R. B., Carmeli, Y., Falagas, M. E., and Giske, C. G. (2012). Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin. Microbiol. Infect.* 18, 268–281. doi: 10.1111/j.1469-0691.2011.03570.x

Mahmud, Z. H., Kabir, M. H., Ali, S., Moniruzzaman, M., Imran, K. M., Nafiz, T. N., et al. (2020). Extended-spectrum Beta-lactamase-producing *Escherichia coli* in drinking water samples Ffrom a forcibly displaced, densely populated community setting in Bangladesh. *Front. Public Health* 8:228.

Méric, G., Kemsley, E. K., Falush, D., Saggers, E. J., and Lucchini, S. (2013). Phylogenetic distribution of traits associated with plant colonization in *Escherichia coli*. *Environ. Microbiol.* 15, 487–501. doi: 10.1111/j.1462-2920.2012.02852.x

Nagachinta, S., and Chen, J. (2008). Transfer of class 1 integron-mediated antibiotic resistance genes from Shiga toxin-producing *Escherichia coli* to a susceptible *E. coli* K-12 strain in storm water and bovine feces. *Appl. Environ. Microbiol.* 74, 5063–5067. doi: 10.1128/aem.00517-08

Nandi, S., Maurer, J. J., Hofacre, C., and Summers, A. O. (2004). Gram-positive bacteria are a major reservoir of class 1 antibiotic resistance integrons in poultry litter. *Proc. Natl. Acd. Sci.* 101, 7118–7122. doi: 10.1073/pnas.0306466101

Nicolas-Chanoine, M. H., Blanco, J., Leflon-Guibout, V., Demarty, R., Alonso, M. P., Canica, M. M., et al. (2008). Intercontinental emergence of *Escherichia coli* clone O25:H4-ST131 producing CTX-M-15. *J. Antimicrob. Chemother.* 61, 273–281. doi: 10.1093/jac/dkm464

Odonkor, S. T., and Addo, K. K. (2018). Prevalence of multidrug-resistant *Escherichia coli* isolated from drinking water sources. *Int. J. Microbiol.* 2018:7204013.

Ojdana, D., Sacha, P. B., and Wieczorek, P. (2014). The occurrence of blaCTX-M, blaSHV, and blaTEM genes in extended-spectrum β-lactamase-positive strains of *Klebsiella pneumoniae*, *Escherichia coli*, and *Proteus mirabilis* in Poland. *Int. J. Antibiot.* 2014:935842.

Ortega, A., Oteo, J., Aranzamendi-Zaldumbide, M., Bartolome, R. M., Bou, G., Cercenado, E., et al. (2012). Spanish multicenter study of the epidemiology and mechanisms of amoxicillin-clavulanate resistance in *Escherichia coli*. *Antimicrob. Agents Chemother.* 56, 3576–3581. doi: 10.1128/aac.06393-11

Partridge, S. R., Brown, H. J., Stokes, H., and Hall, R. M. (2001). Transposons Tn1696 and Tn21 and their integrons In4 and In2 have independent origins. *Antimicrob. Agents Chemother.* 45, 1263–1270. doi: 10.1128/aac.45.4.1263-1270.2001

Pereira, A., Santos, A., Tacao, M., Alves, A., Henriques, I., and Correia, A. (2013). Genetic diversity and antimicrobial resistance of *Escherichia coli* from Tagus estuary (Portugal). *Sci. Total Environ.* 1, 461–462.

Pérez-Pérez, F. J., and Hanson, N. D. (2002). Detection of plasmid-mediated AmpC β-lactamase genes in clinical isolates by using multiplex PCR. *J. Clin. Microbiol.* 40, 2153–2162. doi: 10.1128/jcm.40.6.2153-2162.2002

Picard, B., Garcia, J. S., Gouriou, S., Duriez, P., Brahimi, N., Bingen, E., et al. (1999). The link between phylogeny and virulence in *Escherichia coli* extraintestinal infection. *Infect. Immun.* 67, 546–553. doi: 10.1128/iai.67.2.546-553.1999

Poirel, L., Aires-de-Sousa, M., Kudyba, P., Kieffer, N., and Nordmann, P. (2018). Screening and characterization of multidrug-resistant gram-negative bacteria from a remote African area, São Tomé and Príncipe. *Antimicrob. Agents Chemother.* 62:e01021-18.

Poirel, L., Decousser, J. W., and Nordmann, P. (2003). Insertion sequence ISEcp1B is involved in expression and mobilization of a bla(CTX-M) beta-lactamase gene. *Antimicrob. Agents Chemother.* 47, 2938–2945. doi: 10.1128/aac.47.9.2938-2945.2003

Poirel, L., Kampfer, P., and Nordmann, P. (2002). Chromosome-encoded Ambler class A beta-lactamase of Kluyvera georgiana, a probable progenitor of a subgroup of CTX-M extended-spectrum beta-lactamases. *Antimicrob. Agents Chemother.* 46, 4038–4040. doi: 10.1128/aac.46.12.4038-4040.2002

Purohit, M. R., Lindahl, L. F., Diwan, V., Marrone, G., and Lundborg, C. S. (2019). High levels of drug resistance in commensal E. coli in a cohort of children from rural central India. *Sci. Rep.* 9:6682.

Ram, S., Vajpayee, P., and Shanker, R. (2008). Contamination of potable water distribution systems by multiantimicrobial-resistant enterohemorrhagic *Escherichia coli*. *Environ. Health Perspect.* 116, 448–452. doi: 10.1289/ehp.10809

Rodríguez-Baño, J., Navarro, M. D., Romero, L., Martinez-Martinez, L., Muniain, M. A., Perea, E. J., et al. (2004). Epidemiology and clinical features of infections caused by extended-spectrum beta-lactamase-producing Escherichia coli in nonhospitalized patients. *J. Clin. Microbiol.* 42, 1089–1094. doi: 10.1128/jcm.42.3.1089-1094.2004

Rodriguez-Mozaz, S., Chamorro, S., Marti, E., Huerta, B., Gros, M., Sanchez-Melsio, A., et al. (2015). Occurrence of antibiotics and antibiotic resistance genes in hospital and urban wastewaters and their impact on the receiving river. *Water Res.* 69, 234–242. doi: 10.1016/j.watres.2014.11.021

Saladin, M., Cao, V. T., Lambert, T., Donay, J. L., Herrmann, J. L., Ould-Hocine, Z., et al. (2002). Diversity of CTX-M beta-lactamases and their promoter regions from *Enterobacteriaceae* isolated in three Parisian hospitals. *FEMS Microbiol. Lett.* 209, 161–168. doi: 10.1016/s0378-1097(02)00484-6

Shakya, P., Barrett, P., Diwan, V., Marothi, Y., Shah, H., Chhari, N., et al. (2013). Antibiotic resistance among *Escherichia coli* isolates from stool samples of children aged 3 to 14 years from Ujjain, India. *BMC Infect. Dis.* 13:477.

Sidhu, J. P. S., Jagals, P., Smith, A., and Toze, S. (2017). Comparative prevalence of *Escherichia coli* carrying virulence genes and class 1 and 2 integrons in sub-tropical and cool temperate freshwater. *Environ. Sci. Pollut. Res. Int.* 24, 18263–18272. doi: 10.1007/s11356-017-9497-0

Singh, A. K., Das, S., Singh, S., Gajamer, V. R., Pradhan, N., and Lepcha, Y. D. (2018). Prevalence of antibiotic resistance in commensal *Escherichia coli* among the children in rural hill communities of Northeast India. *PLoS One* 13:e0199179. doi: 10.1371/journal.pone.0199179

Singh, N. S., Singhal, N., and Virdi, J. S. (2018). Genetic environment of blaTEM-1, blaCTX-M-15, blaCMY-42 and characterization of integrons of *Escherichia*

coli isolated from an Indian urban aquatic environment. *Front. Microbiol.* 9:382.

Singhal, N., Maurya, A. K., Mohanty, S., Kumar, M., and Virdi, J. S. (2019). Evaluation of bile salt hydrolases, cholesterol-lowering capabilities, and probiotic potential of enterococcus faecium isolated from rhizosphere. *Front. Microbiol.* 10:1567.

Stange, C., Sidhu, J. P. S., Tiehm, A., and Toze, S. (2016). Antibiotic resistance and virulence genes in coliform water isolates. *Int. J. Hyg. Environ. Health.* 219, 823–831. doi: 10.1016/j.ijheh.2016.07.015

Su, H. C., Ying, G. G., Tao, R., Zhang, R. Q., Zhao, J. L., and Liu, Y. S. (2012). Class 1 and 2 integrons, sul resistance genes and antibiotic resistance in *Escherichia coli* isolated from Dongjiang river, South China. *Environ. Pollut.* 169, 42–49. doi: 10.1016/j.envpol.2012.05.007

Sunde, M., and Norstrom, M. (2005). The genetic background for streptomycin resistance in *Escherichia coli* influences the distribution of MICs. *J. Antimicrob. Chemother.* 56, 87–90. doi: 10.1093/jac/dki150

Tacão, M., Correia, A., and Henriques, I. (2012). Resistance to broad-spectrum antibiotics in aquatic systems: anthropogenic activities modulate the dissemination of bla(CTX-M)-like genes. *Appl. Environ. Microbiol.* 78, 4134–4140. doi: 10.1128/aem.00359-12

Tambekar, D. H., Dhanorkar, D. V., Gulhane, S. R., Khandelwal, V. K., and Dudhane, M. N. (2006). Antibacterial susceptibility of some urinary tract pathogens to commonly used antibiotics. *Afr. J. Biotechnol.* 5:17.

Tristram, S. G., and Nichols, S. (2006). A multiplex PCR for beta-lactamase genes of *Haemophilus influenzae* and description of a new blaTEM promoter variant. *J. Antimicrob. Chemother.* 58, 183–185. doi: 10.1093/jac/dkl150

Upadhyay, S., Hussain, A., Mishra, S., Maurya, A. P., Bhattacharjee, A., and Joshi, S. R. (2015). Genetic environment of plasmid mediated CTX-M-15 extended spectrum Beta-Lactamases from clinical and food borne bacteria in North-Eastern India. *PLoS One* 10:e0138056. doi: 10.1371/journal.pone.0138056

Varela, A. R., Nunes, O. C., and Manaia, C. M. (2016). Quinolone resistant Aeromonas spp. as carriers and potential tracers of acquired antibiotic resistance in hospital and municipal wastewater. *Sci. Total Environ.* 542, 665–671. doi: 10.1016/j.scitotenv.2015.10.124

Wang, J., Stephan, R., Zurfluh, K., Hachler, H., and Fanning, S. (2014). Characterization of the genetic environment of blaESBL genes, integrons and toxin-antitoxin systems identified on large transferrable plasmids in multi-drug resistant *Escherichia coli*. *Front. Microbiol.* 5:716.

Wang, M., Guo, Q., Xu, X., Wang, X., Ye, X., Wu, S., et al. (2009). New plasmid-mediated quinolone resistance gene, qnrC, found in a clinical isolate of Proteus mirabilis. *Antimicrob. Agents Chemother.* 53, 1892–1897. doi: 10.1128/AAC.01400-08

White, P., McIver, C. J., Deng, Y. M., and Rawlinson, W. D. (2000). Characterisation of two new gene cassettes, *aadA5* and *dfrA17*. *FEMS Microbiol. Lett.* 182, 265–269. doi: 10.1111/j.1574-6968.2000.tb08906.x

White, P. A., McIver, C. J., and Rawlinson, W. D. (2001). Integrons and gene cassettes in the *Enterobacteriaceae*. *Antimicrob. Agents Chemother.* 45, 2658–2661. doi: 10.1128/aac.45.9.2658-2661.2001

Yamane, K. J., Wachino, S., Suzuki, H., Kato, K., Shibayama, K., Kimura, K., et al. (2007). 16S rRNA methylase-producing, gram-negative pathogens, Japan. *Emerg. Infect. Dis.* 13, 642–646. doi: 10.3201/eid1304.060501

Zhang, S. H., Lv, X., Han, B., Gu, X., Wang, P. F., Wang, C., et al. (2015). Prevalence of antibiotic resistance genes in antibiotic-resistant *Escherichia coli* isolates in surface water of Taihu Lake Basin, China. *Environ. Sci. Pollut. Res. Int.* 22, 11412–11421. doi: 10.1007/s11356-015-4371-4

Zhang, X. Y., Ding, L. J., and Yue, J. (2009). Occurrence and characteristics of class 1 and class 2 integrons in resistant *Escherichia coli* isolates from animals and farm workers in northeastern China. *Microb. Drug Resist* . 15, 323–328. doi: 10.1089/mdr.2009.0020

Zong, Z., Partridge, S. R., and Iredell, J. R. (2010). ISEcp1-mediated transposition and homologous recombination can explain the context of bla(CTX-M-62) linked to qnrB2. *Antimicrob. Agents Chemother.* 54, 3039–3042. doi: 10.1128/aac.00041-10

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Emergence of High Level Carbapenem and Extensively Drug Resistant *Escherichia coli* ST746 Producing NDM-5 in Influent of Wastewater Treatment Plant, Seoul, South Korea

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High level carbapenem and extensively drug resistant (XDR) *Escherichia coli* strain N7, which produces a variant of New Delhi metallo-β-lactamase (NDM-5), was isolated from the influent of the Jungnang wastewater treatment plant located on Han River, Seoul, South Korea. Phenotypic and genotypic resistances to carbapenem were tested using agar and broth dilution methods, and polymerase chain reaction. Whole-genome sequencing was performed to characterize the genetic structure of strain N7. *E. coli* strain N7, which harbors the bla_{NDM-5} gene, showed high level of carbapenem resistance at concentrations of doripenem (512 mg/L) and meropenem (256 mg/L), and XDR to 15 antibiotics. Based on the genomic sequence analysis, two plasmids, a hybrid IncHI2/N-type and an IncX3 type, were present. The former contains a cluster (bla_{NDM-5}-ble_{MBL}-*trpF*-*dsbD*) bracketed by multi-insertional sequences, IS*3000*, IS*Aba125*, IS*5*, and IS*26*. The latter carries the following resistance genes: bla_{CTX-14}, *aac(3)-IV, aadA1, aadA2, aph(3′)-Ia, aph(4)-Ia, sul1, sul2, sul3, dfrA12, fosA3, oqxA, oqxB, mph(A),* and *floR,* and *cmlA1*. The chromosome, contig3, and contig5 also carry bla_{CTX-64} and *mdf(A), tet(A)*, and *erm(B), tet(M)* and *aadA22,* respectively. Strain N7 also harbors virulence factors such as *fimH*, *flu*, *ecpABCDE*, *sfmA, hlyE,* and *gadA*. This study demonstrates the emergence of high level carbapenem resistant XDR *E. coli* strain N7 containing bla_{NDM-5} in aquatic environment, Seoul, South Korea. Due to the presence of mobile genetic elements, this strain could horizontally transfer resistance genes, including bla_{NDM-5} to environmental bacteria. Thus, it is necessary to conduct continuous surveillance for carbapenem resistance in various aquatic environments.

Keywords: carbapenem resistance, extensively drug resistance, blaNDM gene, wastewater treatment plants, *Escherichia coli*, horizontal gene transfer, aquatic environment

INTRODUCTION

Carbapenem-resistant *Enterobacteriaceae* (CRE) is one of the most critical pathogens, together with carbapenem-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa,* and has been clinically issued with growing concerns in need of new antibiotics (Tacconelli et al., 2017). CRE can produce several enzymes belonging to the class of New Delhi metallo-β-lactamase (NDM) to hydrolyze carbapenems (Doi and Paterson, 2015). Since the first report of NDM (Yong et al., 2009), a series of NDM variants, which possess distinct hydrolytic activity against β-lactams (bla_{NDM}) from NDM-1 to NDM-29, have been identified with the clinical evolution of NDM (Cheng et al., 2018). In particular, NDM-5 producing *Escherichia coli* shows higher level of resistance to carbapenems compared to previously reported NDM-1 producing bacteria (Hornsey et al., 2011).

The first occurrence of NDM-5 producing *E. coli* EC405 was reported in a patient in the United Kingdom in 2011, and it showed a high level of resistance to cephalosporins, carbapenems, aminoglycosides, and quinolones, while being susceptible to colistin and tigecycline (Hornsey et al., 2011). Following this discovery, two carbapenemase-producing *Enterobacteriaceae* (NDM-5 producing *E. coli* and NDM-1 producing *Klebsiella pneumoniae*) showing distinct hydrolytic activity against imipenem were isolated from a traveler from Bangladesh in 2013 and Indonesia in 2014, respectively (Nakano et al., 2014). Subsequently, in South Korea, NDM-9 and NDM-5 producing *Klebsiella variicola* and *E. coli* strains were recovered from a river in 2017 (Di et al., 2017) and patients in 2018 (Jhang et al., 2018), respectively, suggesting that environmental and clinical NDM-producing bacteria are in circulation.

The bla_{NDM} genes have been predominantly found in opportunistic pathogenic bacteria displaying resistance to multiple antimicrobials, particularly, *Enterobacteriaceae*, such as *E. coli*, *Klebsiella* sp., and *Enterobacter* sp. (Bush, 2010). Since the isolation of clinical NDM-1 producing *Acinetobacter* spp. and *Pseudomonas* spp. in 2012 (Bharadwaj, 2012), the occurrence of NDM-producing bacteria has been on the rise in various aquatic environments including river stream, wastewater treatment plants (WWTPs), and tap water (Walsh et al., 2011; Luo et al., 2013; Di et al., 2017). WWTPs have been suggested as potential hot spots for antibiotic resistance (Karkman et al., 2018). Contamination determinants from households, hospitals, farms, and other non-point source pollutions may play a role in selective pressure for the increase in antibiotic resistance, escalating antibiotic resistance that enables the development of multi-drug resistant (MDR), extensively drug resistant (XDR), and/or pan-drug resistant (PDR) bacteria, which make it increasingly difficult to treat infections.

In this study, we report the emergence of pathogenic, and highly carbapenem-resistant and XDR *E. coli* strain N7, isolated from the urban influent of Jungnang WWTP on the Han River located in Seoul, the capital city of South Korea. Whole-genome sequencing analyses of *E. coli* strain N7 indicated that 23 antibiotic resistance genes (ARGs) including bla_{NDM-5}, a variant of NDM, were present in chromosome, plasmids, and contigs. Among them, seventeen were carried on two plasmids, which were formulated structurally in a manner of well-known conserved clusters with either class 1 integron and/or insertional sequences (ISs), suggesting that *E. coli* strain N7 can act as a carrier of ARGs in the aquatic environment.

MATERIALS AND METHODS

Isolation and Identification of Carbapenem-Resistant Bacteria From a WWTP

The influent sample was collected from the Jungnang (JN) WWTP on the Han River, Seoul, South Korea in May of 2018 by using sterile bottles. After collection, the samples were immediately shipped to the laboratory under cool conditions (4°C) and filtered through a 0.22 μm pore size membrane filter (Advantec, Tokyo, Japan). The membranes were suspended in 10 mL of Mueller-Hinton (MH) broth (MBCell, Seoul, South Korea), thoroughly vortexed, and then processed with a serial dilution up to 10^{-3} times (10^{0}, 10^{-1}, 10^{-2}, and 10^{-3}). A 100 μL of sample of the MH broth was spread on mSuperCARBA (CHROMagar, France) agar plates and the plates were incubated at 37°C for 48 h. After incubation, the colonies on the plates were streaked on new MH agar plates containing 8 mg/L of meropenem to obtain a single colony of presumptive carbapenemase-producing bacteria. The isolate grown on the plates were taxonomically identified using 16S rDNA gene sequencing (Macrogen, Seoul, South Korea).

Phenotypic and Genotypic Resistance Test

Eleven carbapenem resistance genes (bla_{IMP}, bla_{VIM}, bla_{NDM}, bla_{SPM}, bla_{AIM}, bla_{DIM}, bla_{GIM}, bla_{SIM}, bla_{KPC}, bla_{BIC}, and bla_{OXA-48}) (Poirel et al., 2011) were screened using PCR detection from the presumptive carbapenemase-producing bacteria. The amplicons were sequenced (Macrogen) and identified using NCBI BLAST[1]. For the screened carbapenemase-producing bacteria, MDR to 16 antibiotics was determined using Kirby-Bauer disk diffusion, and resistance to colistin was determined using broth dilution methods. For MDR, the following antibiotic disks were used: ampicillin-sulbactam (10/10 μg), cefotaxime (30 μg), ceftazidime (30 μg), chloramphenicol (30 μg) ciprofloxacin (5 μg), colistin (2 mg/L), doripenem (10 μg), fosfomycin (200 μg), gentamicin (10 μg), levofloxacin (5 μg), meropenem (10 μg), netilmicin (10 μg), piperacillin (100 μg), tetracycline (30 μg), tobramycin (10 μg), and trimethoprim-sulfamethoxazole (1.25/23.75 μg) (Liofilchem, Roseto degli Abruzzi, Italy). Resistance to the antibiotics was determined according to the Clinical and Laboratory Standards Institute (CLSI) guideline (Clinical Laboratory Standars and Institue, 2016). Subsequently, MICs of 16 antibiotics for *E. coli* strain N7 were evaluated using the broth dilution method (Hasselmann, 2003).

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

Whole Genome Sequencing

The genome was constructed *de novo* using PacBio sequencing data (Pacific Biosciences, Menlo Park, CA, United States). Sequencing analysis was performed at Chunlab Inc. (Seoul, South Korea). PacBio sequencing data were assembled with PacBio SMRT Analysis 2.3.0 using the HGAP2 protocol (Pacific Biosciences). The resulting contigs from PacBio sequencing data were circularized using Circulator 1.4.0 (Sanger Institute, Hinxton, Cambridgeshire, United Kingdom) (Yoon et al., 2017). Circular maps for plasmid structures and linear maps generated by Circulator 1.4.0 and geneCo (Jung et al., 2019), respectively, were manually modified. The chromosomal and plasmid origins of replication were identified using DoriC 5.0 and the plasmid types were determined by PlasmidFinder 1.3, using FASTA file (Carattoli et al., 2014). ARGs were identified using ResFinder (Zankari et al., 2012). Multi-locus sequence type (MLST) was determined by sequences of seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*) according to a previous description (Clermont et al., 2000). The WGS data were deposited in GenBank under the accession JABWPS000000000.

Serotyping and Virulence Determinants

Carbapenemase-producing *E. coli* strain was serotyped with four O-antisera (O26, O111, O146, and O157) (SSI Diagnostica, Hillerød, Denmark) by incubation in MH broth for 16 h, boiling at 95°C for 15 min. Equal volume of the lysate and antisera were mixed in a 96-well culture plate, and then incubated at 52°C overnight. The agglutination of O-antigen and O-antisera was visually checked according to a previously described protocol (SSI Diagnostica). Virulence genes and serotype were determined from WGS data using VirulenceFinder and SerotypeFinder 2.0 (Carattoli et al., 2014; Joensen et al., 2015).

RESULTS

Isolation and Identification of Carbapenem-Resistant Bacteria

Among the 50 isolates from the influent of JN WWTP, 24 isolates were presumptive carbapenem-resistant bacteria. The PCR detection of 11 carbapenemase genes (bla_{IMP}, bla_{VIM}, bla_{NDM}, bla_{SPM}, bla_{AIM}, bla_{DIM}, bla_{GIM}, bla_{SIM}, bla_{KPC}, bla_{BIC}, and bla_{OXA-48}) revealed that only one isolate was positive for the bla_{NDM}. This isolate, N7, was taxonomically identified as *E. coli* by 16S rDNA gene sequencing. MLST revealed that *E. coli* strain N7 belonged to ST746.

Phenotypic Antimicrobial Resistance

Escherichia coli strain N7 showed resistance to ampicillin (MIC, 1,024 mg/L), cefotaxime (MIC, 256 mg/L), ceftazidime (MIC, 512 mg/L), ciprofloxacin (MIC, 1,024 mg/L), colistin (MIC, 8 mg/L), doripenem (MIC, 512 mg/L), fosfomycin (MIC, 1,024 mg/L), gentamycin (MIC, 512 mg/L), imipenem (MIC, 256 mg/L) levofloxacin (MIC, 256 mg/L), meropenem (MIC, 256 mg/L), netilmicin (MIC, 256 mg/L), piperacillin (MIC, 1,024 mg/L), tetracycline (MIC, 512 mg/L), tobramycin (MIC, 256 mg/L), and trimethoprim-sulfamethoxazole (MIC, 4/76 mg/L), but was susceptible to chloramphenicol (**Table 1**). Compared to the CLSI clinical breakpoint, *E. coli* strain N7 exhibited high level of resistance to eight classes of the antibiotics tested, except for trimethoprim-sulfamethoxazole. Regarding the extent of the antibiotic resistance up to 15 of 16 antibiotics tested, strain N7 is likely to be an XDR bacterium.

Determinants for Antimicrobial Resistance and Pathogenicity

WGS data showed that 23 ARGs were present in *E. coli* strain N7 and 21 ARGs were found on two incompatible plasmids and contigs, except for the bla_{CTX-64} and *mdf(A)* genes, which were located on the chromosome (**Table 2**). We found two plasmids in *E. coli* strain N7, identified as an IncX3 plasmid (pKJNI-5), and a hybrid plasmid consisting of IncHI2 and N-type (pKJNI-2).

Figure 1A shows the structure of the IncX3 type plasmid pKJNI-5 containing the bla_{NDM-5} gene. As shown in the figure, the bla_{NDM-5} gene was always followed by a gene cluster composed of bleomycin resistance gene (ble_{MBL}), phosphoribosyl anthranilate isomerase (*trpF*), and protein-disulfide reductase (*dsbD*), as previously reported (Nakano et al., 2014; Zhu et al., 2016; Ho et al., 2018; Yuan et al., 2019). The gene cluster of bla_{NDM-5}-ble_{MBL}-*trpF-dsbD* was also bracketed by IS*3000*-IS*Aba125*-IS*5* in the upstream region and IS*26* in the downstream region (**Figure 1B**), which was also well conserved among diverse bacteria with the *bla*NDM-5 and *bla*NDM-1 genes (Nakano et al., 2014; Zhu et al., 2016; Ho et al., 2018; Yuan et al., 2019). It should be noted that IS*3000* was always found upstream of the gene cluster of bla_{NDM-5}-ble_{MBL}-*trpF-dsbC/D* regions among diverse bacteria. In addition, except in *E. coil* pTK1044, IS*26* is always located downstream of the gene cluster. **Figure 1A** shows the presence of a type IV secretion system (*virD2-virB1-virB4-virB5-virB6-virB8-virB9-virB10-virB11-virD4*) at a site opposite that of bla_{NDM-5} on the plasmid pKJNI-5.

Figure 2A shows the IncHI2/N hybrid-type plasmid pKJNI-2, which carries 16 ARGs {aminoglycosides [*aac(3)-IV, aadA1, aadA2, aph(3′)-Ia,* and *aph(4)-Ia*], β-lactams (bla_{CTX-14}), fosfomycin (*fosA3*), macrolide [*mph(A)*], phenicols (*floR* and *cmlA1*), quinolones (*oqxA* and *oqxB*), sulfonamide (*sul1, sul2,* and *sul3*), and trimethoprim (*dfrA12*)}. Even in the presence of the phenicol resistance gene on the IncHI2/N hybrid plasmid, *E. coli* strain N7 was susceptible to chloramphenicol. IS*257* brackets 15 ARGs except for macrolide [*mph(A)*], and contains a class 1 integron, which carries resistance genes to aminoglycoside (*aadA1* and *aadA2*), chloramphenicol (*cmlA1*), and trimethoprim (*dfrA12*) (**Figure 2B**). The gene cassette associated with class 1 integron of *E. coli* strain N7 was compared with that of previously submitted genomic data of other bacterial strains including *Aeromonas caviae*, *A. baumannii*, *Salmonella* Typhimurium, *P. aeruginosa* (**Figure 2B**). It shows similar patterns of carrying a narrow range of resistance genes to aminoglycoside, β-lactam, chloramphenicol, sulfonamide, and trimethoprim.

In addition, *E. coli* strain N7 carries the following eight virulence factors: adhesion-associated molecules (*fimH*, *flu*,

TABLE 1 | MICs of antimicrobials tested for *E. coli* strain N7 compared with other *E. coli* strains.

No.	Antibiotics	MICs (mg/L) of *E. coli* strains				CLSI clinical breakpoint (mg/L)
		N7	QD28 (Rahman et al., 2014)	QD29 (Rahman et al., 2014)	EC405 (Zhu et al., 2016)	
1	GEN	512	32	≥256	–	16
2	CIP	1024	6	≥32	–	4
3	MEM	256	≥32	≥32	≥32	4
4	SXT	>4/76	–	–	–	4/76
5	CTX	256	≥256	≥256	≥256	4
6	CAZ	512	≥256	≥256	≥256	16
7	AMP	1024	–	–	–	32
8	PIP	1024	–	–	–	128
9	TET	512				16
10	FOF	1024	2	≥1024	–	256
11	NET	256	–	–	–	32
12	DOR	512	–	–	–	4
13	LVX	256	–	–	–	8
14	TOB	256	10	≥256	–	16
15	CHL	S	–	–	–	32
16	CST	8	0.38	0.5	–	2
17	IMP	256	–	–	–	4

GEN: gentamicin; CIP: ciprofloxacin, MEM, meropenem; SXT, trimethoprim-sulfamethoxazole; CTX, cefotaxime; CAZ, ceftazidime; AMP, ampicillin; PIP, piperacillin; TET, tetracycline; FOF, fosfomycin; NET, netilmicin; DOR, doripenem; LVX, levofloxacin; TOB, tobramycin; CHL, chloramphenicol; CST, colistin; IMP, imipenem S, susceptible.

TABLE 2 | Genome features of *E. coli* N7 and its antimicrobial resistance genes.

Sequence type	Replicon	Origin of replication/plasmid incompatibility	Length (bp)	GC (%)	Resistance genes(*n* = 23)	Class of antimicrobials
ST746	Chromosome	*oriC*	4,614,699	50.84	bla_{CTX-64}	β-Lactam
					mdf(A)	Macrolide
	Plasmid pKJNI-2	IncHI2, IncN	255,628	46.75	bla_{CTX-14}	β-Lactam
					aac(3)-IV, aadA1, aadA2, aph(3′)-Ia, aph(4)-Ia	Aminoglycoside
					sul1, sul2, sul3	Sulfonamide
					dfrA12	Trimethoprim
					fosA3	Fosfomycin
					oqxA, oqxB	Quinolone
					mph(A)	Macrolide
					floR, cmlA1	Phenicol
	Plasmid pKJNI-5	IncX3	71,870	47.54	bla_{NDM-5}	β-Lactam
	Contig 3		19,713	52.99	*tet(A)*	Tetracycline
	Contig 5		8,333	57.82	*erm(B)*	Macrolide
					tet(M)	Tetracycline
					aadA22	Aminoglycoside

ecpABCDE, and *sfmA*), and toxins-encoding genes (*hlyE* and *gadA)*. *E. coli* strain N7 belongs to H37 but O-serotype was not determined.

DISCUSSION

In the present study, we report on the emergence of XDR *E. coli* strain N7 which is positive for bla_{NDM-5} and characterization of the genetic context of ARGs, including bla_{NDM-5}. Since the discovery of NDM in a Swedish patient who traveled to India, its variants have grown to 28 different types from diverse bacteria, mostly isolated from clinical samples. In South Korea, NDM-5 producing *Enterobacteriaceae* have been reported only in clinical environments (Park et al., 2016, 2019; Kim et al., 2020), and NDM-9 producing *K. variicola* were found in river (Di et al., 2017).

Escherichia coli strain N7 belonging to ST746 isolated from the urban influent of JN WWTP shows a variant of the NDM, NDM-5 type. From the WGS, we identified

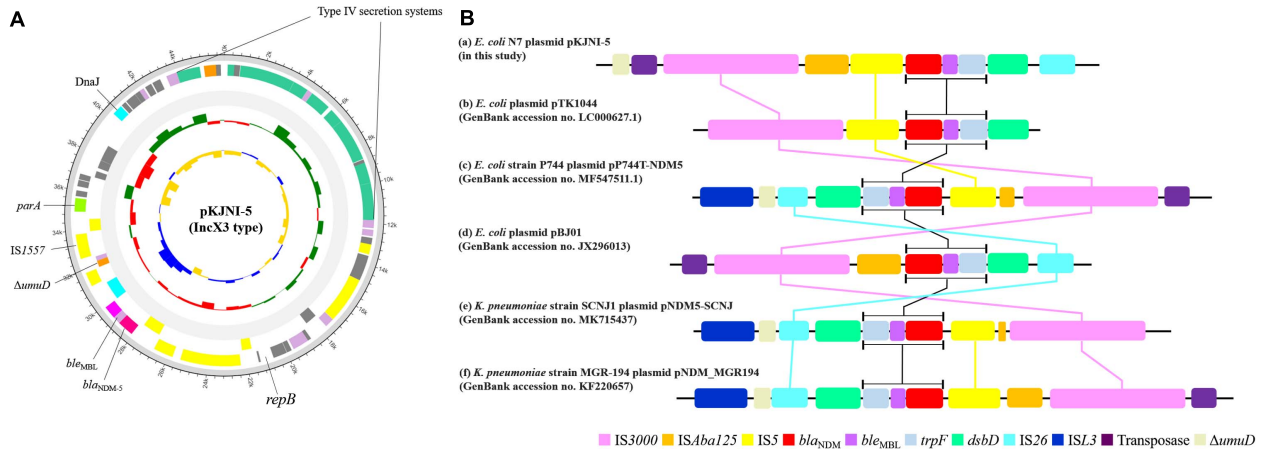

FIGURE 1 | Structure of pKJNI-5 plasmid (IncX3 type) of *E. coli* N7 harboring bla_{NDM-5} **(A)** and comparative sequence analysis of regions of bla_{NDM-5} of *E. coli* N7 with other previously reported genetic structures of bla_{NDM} **(B)**. **(A)** From outermost to innermost ring of plasmid, forward and reverse CDS, track for rRNA and tRNA, GC Skew and GC Ratio was drawn. **(B)** The genetic environment of bla_{NDM-5} of (a) pKJNI-5 isolated in this study, (b) pBJ01, (c) pTK1044, (d) pNDM-MGR194, (e) pP744-T-NDM-5, (f) pNDM5-SCNJ were compared. Indication of each color was described below the genetic structures.

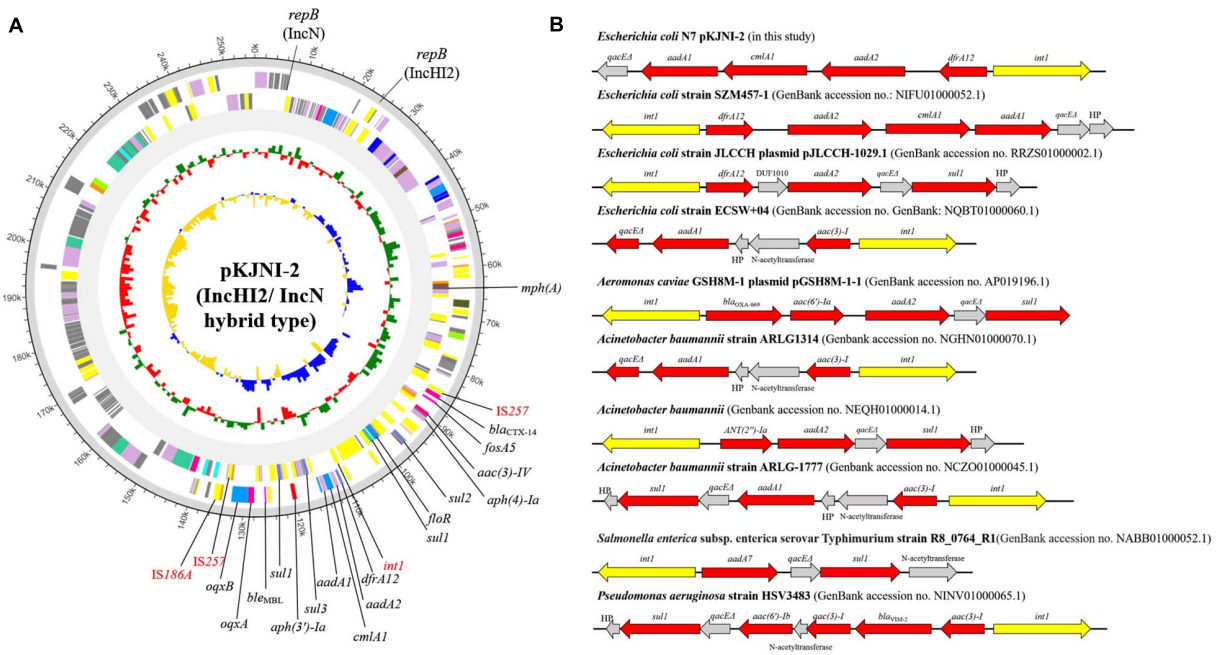

FIGURE 2 | Circular map and comparative sequence analysis of pKJNI-2 plasmid (IncHI2/IncN hybrid type) and comparison of class 1 integron structures. **(A)** From outermost to innermost ring of plasmid, forward and reverse CDS, track for rRNA and tRNA, GC Skew and GC Ratio was drawn. ARGs and mobile genetic elements were indicated in black and red colors, respectively. **(B)** Class 1 integron structure of pKJNI-2 was compared with those previously reported. The *int1* gene and ARGs were colored in yellow and red colors, respectively.

two plasmids such as a narrow host range plasmid IncX3 (Johnson et al., 2012) and a hybrid IncHI2/N. The narrow host range plasmid IncX3 carries a cluster structure of 5′-IS*3000*-DIS*Aba125*-IS*5*-bla_{NDM-5}-ble_{MBL}-*trpF*-*dsbD*-IS*26*-3′ containing the bla_{NDM-5} gene. **Figure 1A** shows the composition of ISs, which cassettes structural genes of 5′-bla_{NDM-5}-ble_{MBL}-*trpF*-*dsbD*-3′ (Liu et al., 2013; Nakano et al., 2014; Zhu et al., 2016; Yuan et al., 2019) with a minor change in the presence and absence of IS*5* and the extent of truncated IS*Aba125* among the analyzed *E. coli* and *K. pneumoniae* strains. The question is still remained why the structural genes of 5′-ble_{MBL}-*trpF*-*dsbD*-3′ with bla_{NDM-5} are always clustered together. In addition, the IncX3 type plasmid in *E. coli* strain N7 also contains a type IV secretion system (*virD2*-*virB1*-*virB4*-*virB5*-*virB6*-*virB8*-*virB9*-*virB10*-*virB11*-*virD4*) located at a site opposite that of bla_{NDM-5}. It should be noted that the type

IV secretion system has also been hypothesized to be involved in horizontal gene transfer between other bacteria (Juhas et al., 2008). Taken together, *E. coli* strain N7 is likely to have a system to transfer recently emerged bla_{NDM-5} gene to other bacteria due to multiple ISs and type IV secretion system, although it contains the narrow host range vector system (Liakopoul et al., 2018).

It is known that *E. coli* ST746 carries extended-spectrum β-lactamase (ESBL) genes from fishes (Sellera et al., 2018) and human patients (Wu et al., 2018). In this study, *E. coli* strain N7 harbored ESBL and eight virulence factors. Surprisingly, *E. coli* strain N7, which showed MIC of meropenem at 256 mg/L, was also resistant to several antibiotics with very high MIC values for the tested antimicrobials (**Table 1**), compared to other NDM-5 producing *E. coli* strains (Hornsey et al., 2011; Rahman et al., 2014; Zhu et al., 2016; Jhang et al., 2018). This XDR pattern can be explained by the presence of several resistance genes located on the broad host range plasmid (**Figure 2**; Zhao et al., 2018). Therefore, the presence of XDR *E. coli*, isolated from the influent of WWTP located in a city, along with the carbapenem-resistance gene raises public health concerns due to the possible dissemination of ARGs to other pathogenic bacteria, and difficulty in treatment of infections. Indeed, XDR pathogenic *E. coli* strains have been reported from human patients (harboring bla_{KPC-2}) (Jeong et al., 2018) and from chickens (co-producing bla_{NDM} and *mcr-1*) (Lv et al., 2018), increasing the likelihood of infectious disease outbreaks. The characteristics of XDR *E. coli* strain N7 can be attributed to the presence of corresponding resistance genes located on two plasmids of an IncX3 and a hybrid IncHI2/N. The occurrence of the IncHI2 plasmid has been frequently reported in *Salmonella* strains with multiple ARGs (Chen et al., 2016). In our experiments, most of the resistance genes were found on the hybrid plasmid IncHI2/N of *E. coli* strain N7, containing diverse resistance determinants, including aminoglycoside [*aac(3)-IV, aadA1, aadA2, aph(3′)-Ia,* and *aph(4)-Ia*], β-lactam (bla_{CTX-64}, bla_{CTX-14}, and bla_{NDM-5}), fosfomycin (*fosA5*), macrolide [*mdf(A)* and *mph(A)*], phenicol (*floR* and *cmlA1*), quinolone (*oqxA* and *oqxB*), sulfonamide (*sul1, sul2,* and *sul3)*, and trimethoprim (*dfrA12*).

CONCLUSION

In conclusion, NDM-5 producing *E. coli* strain N7, which shows a high level of carbapenem resistance and an XDR pattern, was found in the megacity influent of Jungnang WWTP, Seoul, South Korea. Our findings suggest that pathogenic XDR *E. coli* originating from urban activities may be disseminated into the river from WWTP and is a potential carrier or spreader of ARGs, including emerging carbapenemase genes. Thus, we need to focus on the continuous surveillance of carbapenemase-producing bacteria in diverse environments.

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

HS: experiment, data analysis, and manuscript writing. YK: methodology. DH: revision of manuscript. H-GH: overall revision, methodology, and data analysis. All authors contributed to the article and approved the submitted version.

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REFERENCES

Bharadwaj, R. (2012). Prevalence of New Delhi metallo-β-lactamase (NDM-1)-positive bacteria in a tertiary care centre in Pune, India. *Int. J. Antimicrob. Agents* 39, 2011–2012. doi: 10.1016/j.ijantimicag.2011.10.007

Bush, K. (2010). Alarming β-lactamase-mediated resistance in multidrug-resistant Enterobacteriaceae. *Curr. Opin. Microbiol.* 13, 558–564. doi: 10.1016/j.mib.2010.09.006

Carattoli, A., Zankari, E., Garciá-Fernández, A., Larsen, M. V., Lund, O., Villa, L., et al. (2014). In silico detection and typing of plasmids using plasmidfinder and plasmid multilocus sequence typing. *Antimicrob. Agents Chemother.* 58, 3895–3903. doi: 10.1128/AAC.02412-14

Chen, W., Fang, T., Zhou, X., Zhang, D., Shi, X., and Shi, C. (2016). IncHI2 plasmids are predominant in antibiotic-resistant *Salmonella* isolates. *Front. Microbiol.* 7:1566. doi: 10.3389/fmicb.2016.01566

Cheng, Z., Thomas, P. W., Ju, L., Bergstrom, A., Mason, K., Clayton, D., et al. (2018). Evolution of New Delhi metallo-β-lactamase (NDM) in the clinic: effects of NDM mutations on stability, zinc affinity, and mono-zinc activity. *J. Biol. Chem.* 293, 12606–12618. doi: 10.1074/jbc.RA118.003835

Clermont, O., Bonacorsi, S., and Bingen, E. (2000). Rapid and simple determination of the *Escherichia Coli* phylogenetic group. *Appl. Environ. Microbiol.* 66, 4555–4558. doi: 10.1128/AEM.66.10.4555-4558.2000

Clinical Laboratory Standars and Institue (2016). *Clinical and Laboratory Standards Institute: Performance Standards for Antimicrobial Susceptibility Testing Supplement M100S*. Wayne, PA: Clinical and Laboratory Standards Institute.

Di, D. Y. W., Jang, J., Unno, T., and Hur, H. G. (2017). Emergence of klebsiella variicola positive for NDM-9, a variant of New Delhi metallo-β-lactamase, in an urban river in South Korea. *J. Antimicrob. Chemother.* 72, 1063–1067. doi: 10.1093/jac/dkw547

Doi, Y., and Paterson, D. L. (2015). Carbapenemase-producing Enterobacteriaceae. *Semin. Respir. Crit. Care Med.* 36, 74–84. doi: 10.1055/s-0035-1544208

Hasselmann, C. (2003). Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution. *Clin. Microbiol. Infect.* 9, 9–15. doi: 10.1046/j.1469-0691.2003.00790

Ho, P. L., Wang, Y., Liu, M. C. J., Lai, E. L. Y., Law, P. Y. T., Cao, H., et al. (2018). IncX3 epidemic plasmid carrying blaNDM-5 in *Escherichia coli* from swine in multiple geographic areas in China. *Antimicrob. Agents Chemother.* 62:e02295–17. doi: 10.1128/AAC.02295-17

Hornsey, M., Phee, L., and Wareham, D. W. (2011). A novel variant, NDM-5, of the New Delhi metallo-β-lactamase in a multidrug-resistant *Escherichia Coli* ST648 isolate recovered from a patient in the United Kingdom. *Antimicrob. Agents Chemother.* 55, 5952–5954. doi: 10.1128/AAC.05108-11

Jeong, S., Kim, J. O., Yoon, E. J. I, Bae, K., Lee, W., Lee, H., et al. (2018). Extensively drug-resistant *Escherichia Coli* sequence Type 1642 carrying an IncX3 plasmid containing the blaKPC-2 gene associated with transposon Tn4401a. *Ann. Lab. Med.* 38, 17–22. doi: 10.3343/alm.2018.38.1.17

Jhang, J., Wang, H. Y., Yoo, G., Hwang, G. Y., Uh, Y., and Yoon, K. J. (2018). NDM-5 and OXA-48 co-producing uropathogenic *Escherichia coli* isolate: first case in Korea. *Ann. Lab. Med.* 38, 277–279. doi: 10.3343/alm.2018.38.3.277

Joensen, K. G., Tetzschner, A. M. M., Iguchi, A., and Aarestrup, F. M. (2015). Rapid and easy in silico serotyping of *Escherichia coli* isolates by use of whole-genome sequencing data. *J. Clin. Microbiol.* 53, 2410–2426. doi: 10.1128/JCM.00008-15

Johnson, T. J., Bielak, E. M., Fortini, D., Hestbjerg, L., Debroy, C., Nolan, L. K., et al. (2012). Plasmid expansion of the IncX plasmid family for improved identification and typing of novel plasmids in drug-resistant Enterobacteriaceae. *Plasmid.* 68, 43–50. doi: 10.1016/j.plasmid.2012.03.001

Juhas, M., Crook, D. W., and Hood, D. W. (2008). Type IV secretion systems: tools of bacterial horizontal gene transfer and virulence. *Cell. Microbiol.* 10, 2377–2386. doi: 10.1111/j.1462-5822.2008.01187.x

Jung, J., Kim, J. I., and Yi, G. (2019). Genome analysis GeneCo: a visualized comparative genomic method to analyze multiple genome structures. *Bioinformatics* 35, 5303–5305. doi: 10.1093/bioinformatics/btz596

Karkman, A., Do, T. T., Walsh, F., and Virta, M. P. J. (2018). Antibiotic-resistance genes in waste water. *Trends Microbiol.* 26, 220–228. doi: 10.1016/j.tim.2017.09.005

Liakopoul, A., Van Der Goot, J., Bossers, A., Betts, J., Brouwer, M. S. M., Kant, A., et al. (2018). Genomic and functional characterisation of IncX3 plasmids encoding blaSHV-12 in *Escherichia coli* from human and animal origin. *Sci. Rep.* 8:7674. doi: 10.1038/s41598-018-26073-5

Liu, Z., Li, W., Wang, J., Pan, J., Sun, S., Yu, Y., et al. (2013). Identification and characterization of the first *Escherichia coli* strain carrying NDM-1 gene in China. *PLoS One* 8:e66666. doi: 10.1371/journal.pone.0066666

Luo, Y., Yang, F., Mathieu, J., Mao, D., Wang, Q., and Alvarez, P. J. J. (2013). Proliferation of multidrug-resistant New Delhi metallo-β-lactamase genes in municipal wastewater treatment plants in Northern China. *Environ. Sci. Technol. Lett.* 1, 26–30. doi: 10.1021/ez400152e

Lv, L., Zeng, Z., Song, Q., Cao, Y., Wang, J., Li, W., et al. (2018). Emergence of XDR *Escherichia coli* carrying both blaNDM and mcr-1 genes in chickens at slaughter and the characterization of two novel blaNDM-bearing plasmids. *J. Antimicrob. Chemother.* 73, 2261–2263. doi: 10.1093/jac/dky176

Nakano, R., Nakano, A., Hikosaka, K., Kawakami, S., Matsunaga, N., Asahara, M., et al. (2014). First report of metallo-β-lactamase NDM-5-producing *Escherichia coli* in Japan. *Antimicrob. Agents Chemother.* 58, 7611–7612. doi: 10.1128/AAC.04265-14

Kim, S. Y., Seo, J., Shin, J., Chung, Y. J. I, Jeon, Y., Yun, S. J., et al. (2020). Clonal spreading of NDM-5 carbapenemase-producing *Escherichia coli* isolates in a hospital in South Korea. *Diagn. Microbiol. Infect. Dis.* 97, 2016–2018. doi: 10.1016/j.diagmicrobio.2020.115027

Poirel, L., Walsh, T. R., Cuvillier, V., and Nordmann, P. (2011). Multiplex PCR for detection of acquired carbapenemase genes. *Diagn. Microbiol. Infect. Dis* 70, 119–123. doi: 10.1016/j.diagmicrobio.2010.12.002

Rahman, M., Shukla, S. K., Prasad, K. N., Ovejero, C. M., Pati, B. K., Tripathi, A., et al. (2014). Prevalence and molecular characterisation of New Delhi metallo-β-lactamases NDM-1, NDM-5, NDM-6 and NDM-7 in multidrug-resistant *Enterobacteriaceae* from India. *Int. J. Antimicrob. Agents* 44, 30–37. doi: 10.1016/j.ijantimicag.2014.03.003

Sellera, F. P., Fernandes, M. R., Moura, Q., Carvalho, M. P. N., and Lincopan, N. (2018). Extended-spectrum-β-lactamase (CTX-M)-producing *Escherichia coli* in wild fishes from a polluted area in the atlantic coast of South America. *Mar. Pollut. Bull.* 135, 183–186. doi: 10.1016/j.marpolbul.2018.07.012

Tacconelli, E., Magrini, N., Kahlmeter, G., and Singh, N. (2017). *Global Priority List of Antibiotic-Resistant Bacteria to Guide Research, Discovery, and Development of New Antibiotics*. Geneva: World Health Organization, 1–7.

Park, M., Park, S. D., Lee, M. H., Kim, S. H., Lim, K., Lee, G., et al. (2016). The first report of NDM-5-producing uropathogenic *Escherichia coli* isolates in South Korea. *Diagn. Microbiol. Infect. Dis.* 85, 198–199. doi: 10.1016/j.diagmicrobio.2016.02.006

Park, Y., Choi, Q., Kwon, G. C., and Koo, S. H. (2019). Emergence and transmission of New Delhi metallo−β−lactamase−5−producing *Escherichia coli* sequence Type 361 in a tertiary hospital in South Korea. *J. Clin. Lab. Anal.* 34:e23041. doi: 10.1002/jcla.23041

Walsh, T. R., Weeks, J., Livermore, D. M., and Toleman, M. A. (2011). Dissemination of NDM-1 positive bacteria in the New Delhi environment and its implications for human health: an environmental point prevalence study. *Lancet Infect. Dis.* 11, 355–362. doi: 10.1016/S1473-3099(11)70059-7

Wu, L., Chen, J., Wang, L., and Wu, Z. (2018). Whole genome sequence of an MCR-1-carrying, extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* ST746 isolate recovered from a community-acquired urinary tract infection. *J. Glob. Antimicrob. Resist.* 13, 171–173. doi: 10.1016/j.jgar.2018.03.014

Yong, D., Toleman, M. A., Giske, C. G., Cho, H. S., Sundman, K., and Lee, K. (2009). Characterization of a new metallo-β-lactamase gene, blaNDM-1, and a novel erythromycin esterase gene carried on a unique genetic structure in *Klebsiella pneumoniae* sequence Type 14 from India. *Antimicrob. Agents Chemother.* 53, 5046–5054. doi: 10.1128/AAC.00774-09

Yoon, S. H., Ha, S. M., Kwon, S., Lim, J., Kim, Y., Seo, H., et al. (2017). Introducing EzBioCloud: a taxonomically united database of 16S RRNA gene sequences and whole-genome assemblies. *Int. J. Syst. Evol. Microbiol.* 67, 1613–1617. doi: 10.1099/ijsem.0.001755

Yuan, Y., Li, Y., Wang, G., Li, C., Chang, Y. F., Chen, W., et al. (2019). blaNDM-5 carried by a hypervirulent *Klebsiella pneumoniae* with sequence Type 29. *Antimicrob. Resist. Infect. Control* 8:140. doi: 10.1186/s13756-019-0596-1

Zankari, E., Hasman, H., Cosentino, S., Vestergaard, M., Rasmussen, S., Lund, O., et al. (2012). Identification of acquired antimicrobial resistance genes. *J. Antimicrob. Chemother.* 67, 2640–2644. doi: 10.1093/jac/dks261

Zhao, H., Chen, W., Xu, X., Zhou, X., and Shi, C. (2018). Transmissible ST3-IncHI2 plasmids are predominant carriers of diverse complex IS 26-Class 1 integron arrangements in multidrug-resistant *Salmonella*. *Front. Microbiol.* 9:2492. doi: 10.3389/fmicb.2018.02492

Zhu, Y. Q., Zhao, J. Y., Xu, C., Zhao, H., Jia, N., and Li, Y. N. (2016). Identification of an NDM-5-producing *Escherichia coli* sequence Type 167 in a neonatal patient in China. *Sci. Rep.* 6:29934. doi: 10.1038/srep29934

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

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Reactive Oxygen Species-Related Ceftazidime Resistance Is Caused by the Pyruvate Cycle Perturbation and Reverted by Fe^{3+} in *Edwardsiella tarda*

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Reactive oxygen species (ROS) are related to antibiotic resistance and have been reported in bacteria. However, whether ROS contribute to ceftazidime resistance and plays a role in ceftazidime-mediated killing is unknown. The present study showed lower ROS production in ceftazidime-resistant *Edwardsiella tarda* (LTB4-R_{CAZ}) than that in LTB4-sensitive *E. tarda* (LTB4-S), two isogenic *E. tarda* LTB4 strains, which was related to bacterial viability in the presence of ceftazidime. Consistently, ROS promoter Fe^{3+} and inhibitor thiourea elevated and reduced the ceftazidime-mediated killing, respectively. Further investigation indicated that the reduction of ROS is related to inactivation of the pyruvate cycle, which provides sources for ROS biosynthesis, but not superoxide dismutase (SOD) and catalase (CAT), which degrade ROS. Interestingly, Fe^{3+} promoted the P cycle, increased ROS biosynthesis, and thereby promoted ceftazidime-mediated killing. The Fe^{3+}-induced potentiation is generalizable to cephalosporins and clinically isolated multidrug-resistant pathogens. These results show that ROS play a role in bacterial resistance and sensitivity to ceftazidime. More importantly, the present study reveals a previously unknown mechanism that Fe^{3+} elevates ROS production via promoting the P cycle.

Keywords: antibiotic resistance, reactive oxygen species, *Edwardsiella tarda*, the pyruvate cycle, ceftazidime

INTRODUCTION

Edwardsiella tarda is known for causing diseases in both humans and fish, in both of which these diseases can potentially be fatal if untreated. In aquaculture, the bacterium targets at a wide range of fish species and thereby leads to extensive economic losses in the industry (Wang et al., 2011; Abayneh et al., 2013). Various antibiotics are used to prevent and control the infections caused by the bacterium. Unfortunately, the overuse of antibiotics has inadvertently promoted the emergence

and rapid spread of antibiotic-resistant bacteria (Cabello et al., 2016). The emergence of antibiotic-resistant bacteria poses a major challenge for health practitioners and a huge threat to human health and aquaculture since antibiotic-resistant bacteria are insensitive to antibiotics. As the process of developing new pharmaceutical agents to control antibiotic-resistant pathogens is slow and not a viable approach to manage the growing infectious diseases, further understanding of antibiotic resistance mechanisms for control of these antibiotic-resistant pathogens is an important scientific issue and becomes a major research focus (Defoirdt et al., 2011; Blair et al., 2015).

A line of evidences has indicated that microbial metabolic environment confounds antibiotic sensitivity (Peng et al., 2015; Yao et al., 2016; Cheng et al., 2019; Stokes et al., 2019; Jiang et al., 2020a; Li et al., 2020), where reactive oxygen species (ROS) are related to bacterial resistance to antibiotics and antibiotic-mediated killing efficacy (Dwyer et al., 2009; Van Acker and Coenye, 2017; Zhao and Drlica, 2014). The tricarboxylic acid cycle (TCA) cycle plays a crucial role in ROS formation (Van Acker and Coenye, 2017). Therefore, antibiotic-resistant bacteria exhibit the occurrence of reduced or fluctuated TCA cycle and decreased ROS (Ye et al., 2018; Zhang et al., 2020), indicating that low ROS concentrations induce resistance (Van Acker and Coenye, 2017). In the antibiotic-mediated killing mechanisms, antibiotics belonging to different classes activate the TCA cycle, supporting the formation of ROS (superoxide and hydrogen peroxide) via hyperactivation of the electron transport chain (Dwyer et al., 2009; Van Acker and Coenye, 2017). Fe^{3+} causes the activation of the Fenton reaction to generate abundant ROS against methicillin−resistant *Staphylococcus aureus* (MRSA) infection (Song et al., 2020). Our recent publications have showed that the ROS induced by exogenous metabolites elevate aminoglycoside-mediated killing efficacy to EIB202 and *Vibrio alginolyticus* (Ye et al., 2018; Zhang et al., 2020). Reports also indicate that the beta-lactam stress increased the intracellular ROS level (Rosato et al., 2014; Huang et al., 2019), but whether ROS promote beta-lactam-mediated killing is unknown. Therefore, further understanding of ROS role is required for control of beta-lactam-resistant pathogens.

Ceftazidime is a semisynthetic, broad-spectrum, beta-lactam antibiotic, playing a bactericidal action by inhibiting enzymes responsible for cell wall synthesis, primarily penicillin-binding protein 3 (PBP3). The drug is a commonly used antibiotic in clinics. However, with a wide use of cephalosporins in clinics, resistance to cephalosporins including ceftazidime is predominant. Inactivation of β-lactams by β-lactamases, failure in binding to penicillin-binding proteins, and alteration of binding affinity to penicillin-binding proteins are identified as the three common mechanisms of resistance to β-lactams (Peng et al., 2019). However, whether ROS play a role in ceftazidime sensitivity and resistance is largely unknown. Furthermore, information regarding mechanisms for Fe^{3+}-mediated ROS is not available.

In this study, we showed that the intracellular ROS production was lower in LTB4-R_{CAZ} than that in LTB4-S due to a decrease of ROS generation. The decrease of ROS generation was attributed to inactivation of the pyruvate cycle (the P cycle) (Ye et al., 2018). Fe^{3+} promoted the P cycle for elevation of ROS production, thereby elevating ceftazidime-mediated killing. These results are described below.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Edwardsiella tarda LTB4 used in this study was obtained from Professor Xiaohua Zhang, Ocean University of China University. LTB4 was grown at 30°C for 24 h in 50 ml Luria-Bertani (LB) broth in 250-ml flasks.

Measurement of Minimum Inhibitory Concentration

Measurement of minimum inhibitory concentration (MIC) was performed as previously described (National Committee for Clinical Laboratory Standards, 1999). In brief, LTB4 was cultured in LB medium with twofold serially diluted ceftazidime (CAZ, Guangzhou QiYun Biological Technology) ranging from 0.01 to 160 μg/ml. Overnight bacterial cultures were diluted 1:100 in fresh LB medium and cultured at 30°C to an OD600 of 0.5. The tray contained a series of twofold dilutions of antibiotics. Ninety microliters of LB containing CAZ and 10 μl of logarithmic phase cells with 10^7 CFU/ml of LTB4-R_{CAZ} or LTB4-R_{CAZ} were incubated in a microwell plate for 24 h at 30°C. The lowest concentration showing no visible growth was recorded as the MIC. At least three biologic replicates were performed.

Real-Time Quantitative PCR

Real-time quantitative PCR (qRT-PCR) was carried out as previously described (Li et al., 2016). Bacterial cells were harvested at OD600 = 1.0. The total RNA of each sample was isolated with TRIzol (Invitrogen, United States). Reverse transcription-PCR was carried out on a PrimeScript™ RT Reagent Kit with gDNA eraser (Takara, Japan) with 1 μg of total RNA according to manufacturer's instructions. qRT-PCR was performed in 384-well plates, and each well contained a total volume of 10 ml liquid including 5 ml 2X SYBR Premix Ex Taq™, 2.6 μl PCR-grade water, 2 μl cDNA template, and 0.2 μl each pair of primers (10 mM). The primers are listed in **Supplementary Table 1**. All the assays were performed on the LightCycler 480 system (Roche, Germany) according to the manufacturer's instructions, and four independent samples were assayed for both the control group and the test group. The cycling parameters were listed as follows: 95°C for 30 s to activate the polymerase; 40 cycles of 95°C for 10 s; and 60°C for 30 s. Fluorescence measurements were performed at 70°C for 1 s during each cycle. Cycling was terminated at 95°C with a calefactive velocity of 5°C per second, and a melting curve was obtained. To analyze the relative expression level of the target gene, we converted the data to percentages relative to the value of no-treatment group. At least triplicate biological repeats were carried out.

Metabolomics Analysis

Bacterial sample preparation was carried out as previously described (Zhang et al., 2019). In brief, 10 ml OD600 = 1.0 cells were quenched with cold methanol and sonicated for 5 min at 200 W. Samples were centrifuged at 12,000 rpm for 10 min. Supernatant, containing 1 μg/ml ribitol (Sigma–Aldrich) as internal analytical standard, was transferred into a new tube and dried by vacuum centrifugation device (LABCONCO). The dried extracts were then incubated with 80 μl methoxyamine hydrochloride (20 mg/ml, Sigma–Aldrich) in pyridine (Sigma–Aldrich) for 90 min at 37°C and derivatization was done with an identical volume of N-methyl-N-(trimethylsilyl)trifluoroacetamide (Sigma–Aldrich) for another 30 min. Samples were centrifuged at 12,000 rpm for 10 min, and the supernatant was transferred into new tubes. Gas chromatography-mass spectrometry (GC-MS) analysis was performed with an Agilent GC-MS instrument. Spectral deconvolution and calibration were performed using AMDIS and internal standards as previously described. A retention time (RT) correction was performed for all the samples, and then the RT was used as a reference against which the remaining spectra were queried, and a file containing the abundance information for each metabolite in all the samples was assembled. Metabolites from the GC-MS spectra were identified by searching in the National Institute of Standards and Technology (NIST11.L) Mass Spectral Library. Among the detected peaks of all chromatograms, compound peaks were considered as endogenous metabolites and the same metabolite names were merged. The resulting data matrix was normalized by the concentrations of added internal standards and the total intensity. This file was then used for subsequent statistical analyses. The abundance of a metabolite was scaled by total abundance of all metabolites in a sample as its relative abundance for further analysis. Hierarchical clustering was completed in the R platform with the package gplots[1] using the distance matrix. Multivariate statistical analysis included principal component analysis (SIMCA-P 12.0.1), which was used to discriminate sample patterns. GraphPad Prism 7 was used to draw figures.

Measurement of the Activity of Enzymes in the P Cycle

Measurement of enzyme activity was performed as previously described with a few modifications (Zhang et al., 2019; Jiang et al., 2020c). In brief, the harvested cells were collected at OD600 = 1.0 and then re-suspended in sterile saline to OD600 = 1.0 after washing. Samples of 30 ml were collected by centrifugation at 8,000 rpm for 5 min. Pellets were re-suspended in phosphate-buffered saline (PBS) and broke down by sonication for 2 min at a 200-W power setting on ice, and then centrifuged at 12,000 rpm for 10 min to remove insoluble materials. Supernatants containing 200 μg of total proteins were transferred to a pyruvate dehydrogenase (PDH) reaction mix (0.15 mM MTT, 1 mM $MgCl_2$, 0.5 mM PMS, 0.2 mM TPP, 2 mM sodium pyruvate, and 50 mM PBS), an α-ketoglutarate dehydrogenase (KGDH) reaction mix (0.15 mM MTT, 1 mM $MgCl_2$, 0.5 mM PMS, 0.2 mM TPP, 50 mM α-ketoglutaric acid potassium salt, and 50 mM PBS), a succinate dehydrogenase (SDH) reaction mix (0.15 mM MTT, 1 mM PMS, 5 mM sodium succinate, and 50 mM PBS), and a malate dehydrogenase (MDH) reaction mix (0.15 mM MTT, 1 mM PMS, 50 mM PBS, and 50 mM malate) to a final volume of 200 μl in a 96-well plate. Subsequently, the plate was incubated at 37°C for 5 min for PDH/KGDH/SDH/MDH assay and then measured at 566 nm for colorimetric readings. The plate was protected from light during the incubation. The dehydrogenase activity was assayed in quadruplicate.

Detection of Superoxide Dismutase and Catalase Activity

The activity of superoxide dismutase (SOD) and catalase (CAT) was determined by commercial kits (Nanjing Jiancheng, China). The process is as follows: 10 ml of bacteria with OD600 = 1.0 was collected, washed twice with PBS, and suspended in PBS. The suspension was broke down by sonication for 2 min at a 200-W power setting on ice and then centrifuged at 12,000 rpm for 10 min to remove insoluble materials. The supernatant was taken, and the protein concentration was determined. The activity of SOD was measured with 150 μg total protein. The follow-up test was carried out according to the manufacturer's instructions. Then, SOD activity was measured at 450 nm, and CAT activity was measured at 405 nm. The plate was protected from light during the incubation.

Measurement of ATP

Detection of ATP was determined by a BacTiter-Glo^{TM} Microbial Cell Viability Assay (Cat. G8231, Promega, Madison, WI, United States) as previously described (Cheng et al., 2019). In brief, bacterial cells were harvested in the OD600 of 1.0 by centrifugation for 10 min at 12,000 *g* and washed twice by centrifugation with sterile saline. Cells were resuspended with saline solution and adjusted the OD600 to 1.0. Then, 50-μl samples were added to a 96-well plate and mixed with an equal volume of the kit solution. Then, the absorbance was measured using VICTOR X5 (PerkinElmer, Turku, Finland) according to the manufacturer's instructions. The concentration of ATP was calculated according to the standard curve of ATP.

Antibiotic Bactericidal Assays

Antibacterial assay was carried out as described previously (Peng et al., 2015). Overnight bacterial cultures were diluted 1:100 in fresh LB medium and cultured at 30°C to an OD600 of 1.0. Bacterial cells were collected by centrifugation at 8,000 rpm for 5 min. The samples were then washed with sterile saline three times; suspended in M9 minimal media containing 10 mM acetate, 1 mM $MgSO_4$, and 100 μM $CaCl_2$; and diluted to an OD600 of 0.2. Fe^{3+} or/and ceftazidime were added and incubated at 30°C and 200 rpm for 6 h. To determine bacterial count, 100 μl of cultures was obtained and then serially diluted. An aliquot of 10μl of each dilution was plated in TSB agar

[1]https://cran.r-project.org/web/packages/gplots/

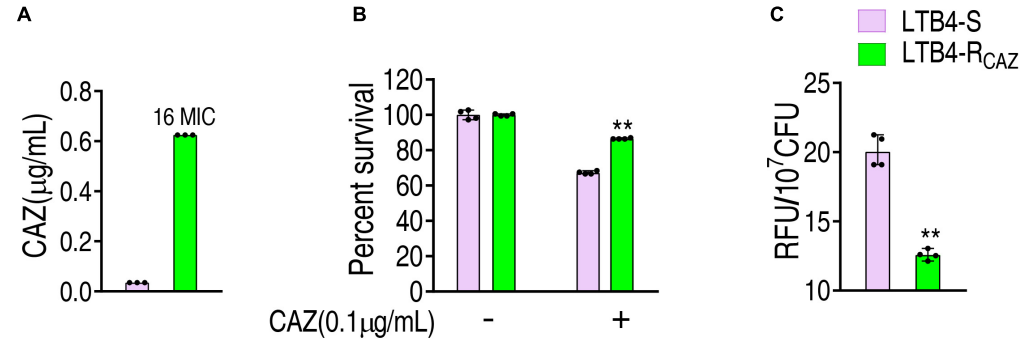

FIGURE 1 | Ceftazidime resistance and reactive oxygen species (ROS) level in LTB4-R_{CAZ}. **(A)** Minimum inhibitory concentration (MIC) of LTB4-S and LTB4-R_{CAZ}. **(B)** Killing efficiency of ceftazidime to LTB4-S and LTB4-R_{CAZ}. **(C)** ROS of LTB4-S and LTB4-R_{CAZ}. Results **(B,C)** are displayed as mean ± SEM, as determined by two-tailed Student's *t*-test. Four biological repeats are carried out. $^{**}P < 0.01$.

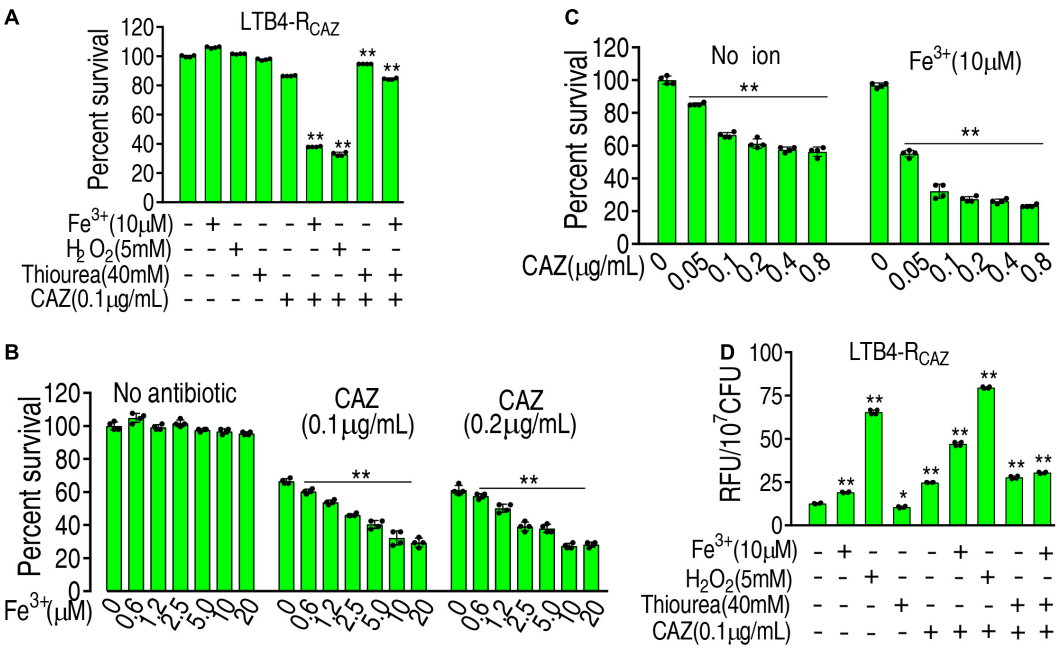

FIGURE 2 | The role of ROS in ceftazidime-mediated killing. **(A)** ROS was quantified in LTB4-R_{CAZ} in the absence or presence of ceftazidime plus Fe^{3+}, H_2O_2, or/and thiourea as indicated by fluorescence. **(B)** Percent survival of LTB4-R_{CAZ} in the presence of ceftazidime and the indicated concentrations of Fe^{3+}. **(C)** Percent survival of LTB4-R_{CAZ} in the presence or absence of Fe^{3+} plus the indicated concentrations of ceftazidime. **(D)** ROS level in the presence or absence of ROS promoter and inhibitor plus ceftazidime. Results are displayed as mean ± SEM, as determined by two-tailed Student's *t*-test. Four biological repeats are carried out. * < 0.05 and ** < 0.01.

plates and incubated at 30°C for 22 h. The plates only with 20–200 colonies were counted, and the colony-forming unit per milliliter was calculated.

Measurement of Membrane Potential

Bacterial membrane potential was measured by BacLight Bacterial Membrane Potential Kit (Invitrogen). In brief, bacteria were diluted to 10^6 CFU/ml and stained with 10 μl of 3 mM $DiOC_2$, followed by incubation for 30 min. Samples were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, United States). The green/red fluorescence was detected with 488–530/610 nm. The membrane potential was determined and normalized as the intensity ratio of the red fluorescence and the green fluorescence. Relative proton motive force (PMF) was determined by test samples compared with control samples.

RESULTS

ROS Is Reduced in LTB4-R_{CAZ}

LTB4 was cultured in LB medium with or without twofold serially diluted ceftazidime, leading to LTB4-R_{CAZ} and LTB4-S,

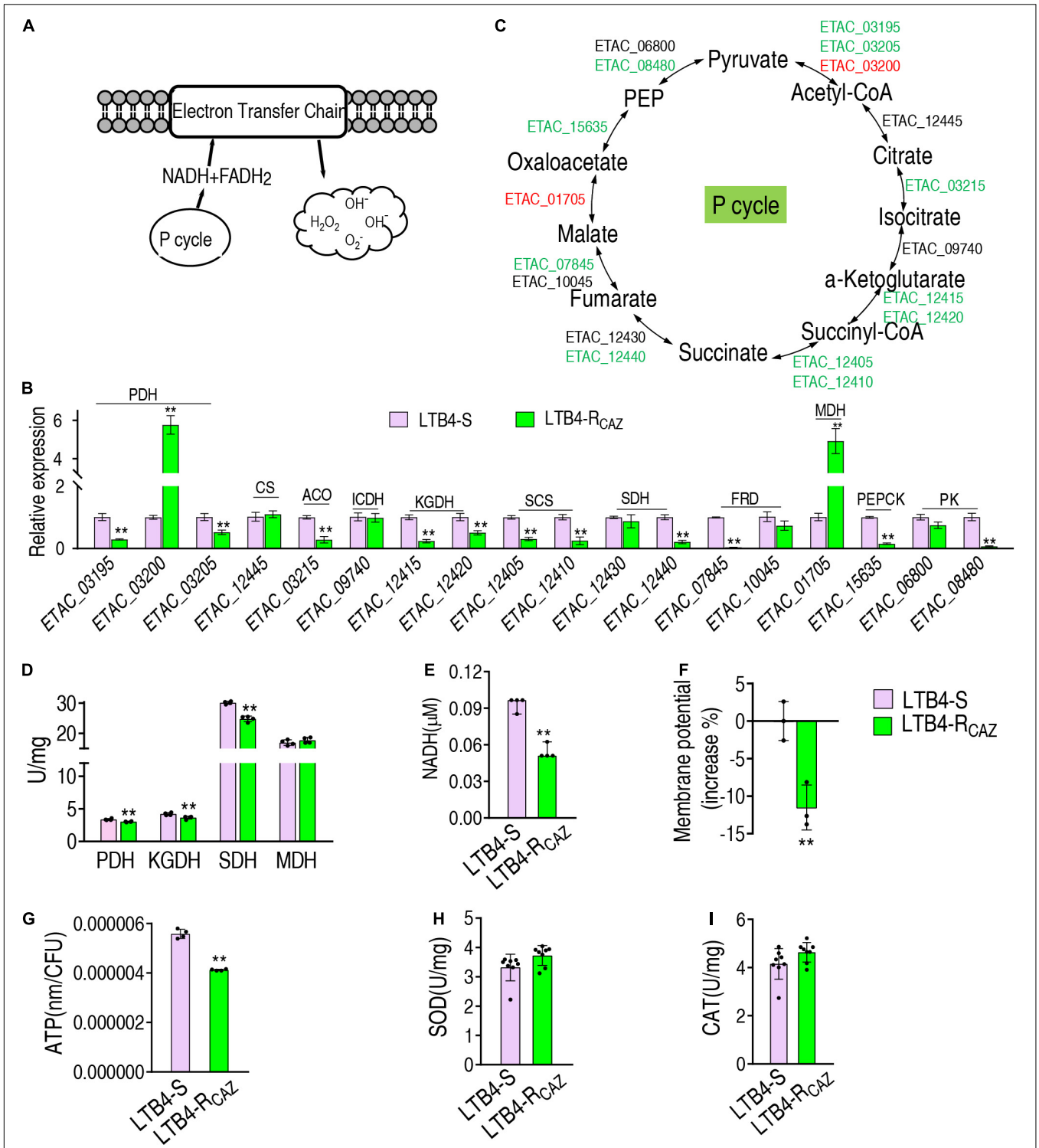

FIGURE 3 | The P cycle, NADH, and ATP in LTB4-S and LTB4-R_{CAZ}. **(A)** Sketch diagram describing the effect of the P cycle on ROS. **(B)** Real-time quantitative PCR (qRT-PCR) for the expression of the P cycle-related genes in LTB4-R_{CAZ} compared to LTB4-S. **(C)** A global view for transcription level of the P cycle genes. Red and blue indicate upregulated and downregulated expression, respectively. **(D)** The activity of PDH, KGDH, SDH, and MDH in the P cycle. NADH **(E)**, membrane potential **(F)**, and ATP **(G)** concentrations in LTB4-S and LTB4-R_{CAZ}. The activity of superoxide dismutase (SOD) **(H)** and catalase (CAT) **(I)** in LTB4-S and LTB4-R_{CAZ}. Results **(B,D–I)** are displayed as mean ± SEM, as determined by two-tailed Student's *t*-test. Four biological repeats are carried out. $^{**}P < 0.01$.

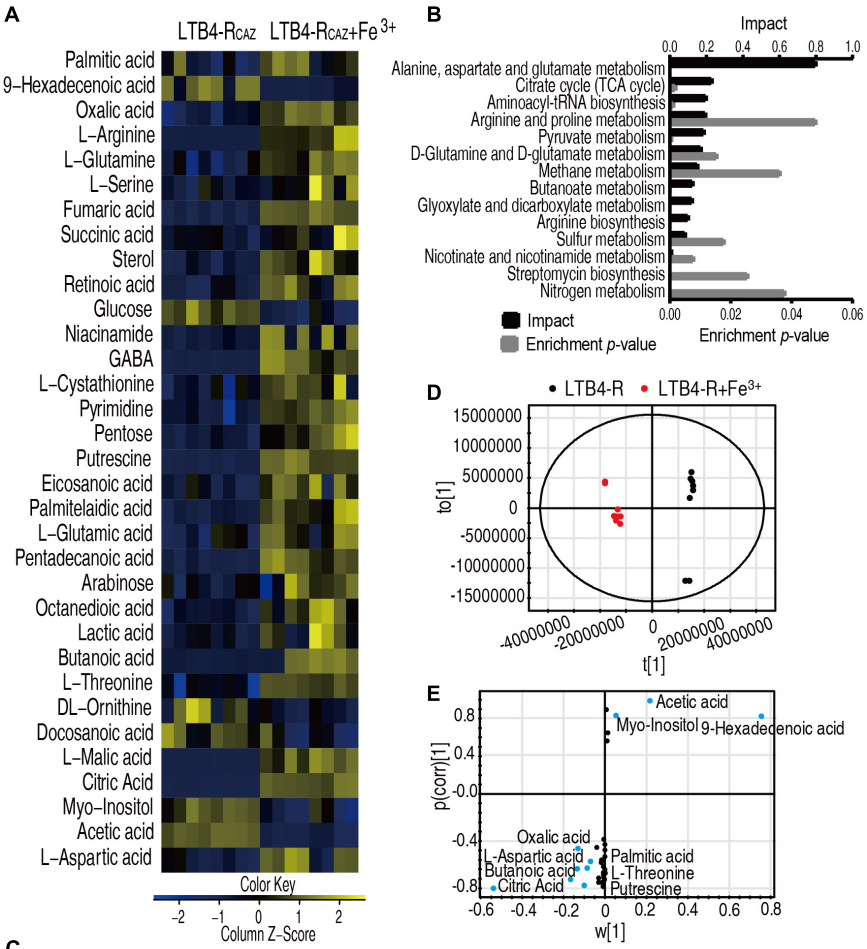

FIGURE 4 | Differential metabolomics of LTB4-R_{CAZ} in response to Fe^{3+}. **(A)** Heat map showing differential abundance of metabolites. Yellow and blue indicate increase and decrease of metabolites relative to the median metabolite level of the control, respectively (see color scale). **(B)** Pathway enrichment of varied metabolites in LTB4-R_{CAZ}. **(C)** Integrative analysis of metabolites in significantly enriched pathways. Yellow and blue indicate increased and decreased metabolites, respectively. **(D)** PCA of LTB4-S and LTB4-R_{CAZ}. Each dot represents the technical replicate analysis of samples in the plot. **(E)** S-plot generated from OPLS-DA. Predictive component *p* [1] and correlation *p* (corr) [1] differentiate LTB4-R_{CAZ} from LTB4-S. Dot represents metabolites, and candidate biomarkers are highlighted in blue.

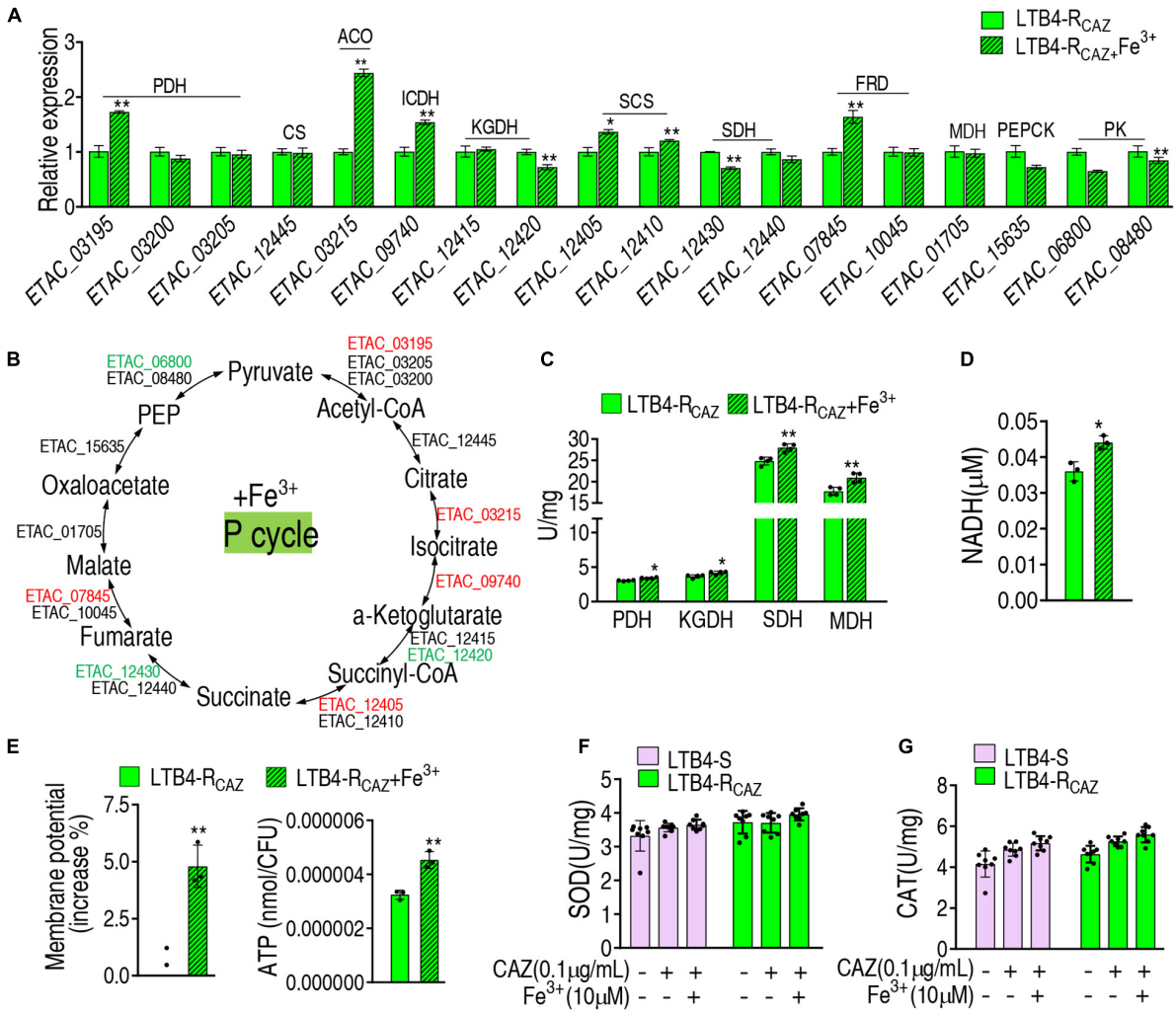

FIGURE 5 | The effect of Fe^{3+} on the P cycle. **(A)** qRT-PCR for expression of the P cycle genes in the presence of Fe^{3+}. **(B)** A global view for transcription level of the P cycle genes. Red and blue indicate upregulated and downregulated expression, respectively. The activity of enzymes of the P cycle **(C)**, NADH level **(D)**, membrane potential **(E)**, and ATP **(F)** of LTB4-R_{CAZ} in the presence or absence of Fe^{3+}. **(G)** The activity of SOD and CAT in the presence or absence of ceftazidime or/and Fe^{3+}. Results **(A,C–G)** are displayed as mean ± SEM, as determined by two-tailed Student's *t*-test. Four biological repeats are carried out. $^{*}p < 0.05$ and $^{**}p < 0.01$.

respectively. The MIC of LTB4-S was 0.039 μg/ml CAZ, while that of LTB4-R_{CAZ} was 0.625 μg/ml CAZ. There was a 16-fold difference between the two strains (**Figure 1A**). Consistently, a higher viability was detected in LTB4-R_{CAZ} than LTB4-S (**Figure 1B**). To explore whether LTB4-R_{CAZ} had a reduced ROS production, ROS production was detected in LTB4-R_{CAZ} and LTB4-S. A lower ROS production was determined in LTB4-R_{CAZ} than LTB4-S (**Figure 1C**). These results indicate that ROS production is reduced in LTB4-R_{CAZ} compared with LTB4-S.

ROS Promoter and Inhibitor Affect Ceftazidime Resistance and Ceftazidime-Mediated Killing

These above results motivated us to speculate that ROS plays an important role in ceftazidime resistance. To demonstrate this, the viability of LTB4-R_{CAZ} was detected in the presence or absence of ROS promoter or/and inhibitor with ceftazidime. There was a stronger resistance to H_2O_2 in LTB4-S than *Escherichia coli* K12 and thereby 5 mM H_2O_2 was used (**Supplementary Figure 1**). The promoters Fe^{3+} and H_2O_2 (positive control) potentiated ceftazidime-mediated killing. However, the inhibitor thiourea not only eliminated the potentiation caused by the promoters but also inhibited the ceftazidime-mediated killing (**Figure 2A**). The effect of Fe^{3+} was increased in a dose-dependent manner and was related to the concentration of ceftazidime used (**Figure 2B**). When the concentration of Fe^{3+} was fixed, the killing efficacy was ceftazidime dose dependent (**Figure 2C**). To validate the role of the ROS promoter or/and inhibitor in the ceftazidime-mediated killing, we measured the ROS production of LTB4-R_{CAZ} in the presence or absence of the ROS promoter or/and inhibitor. ROS production was elevated and reduced in the presence of the

inhibitor thiourea and promoters Fe^{3+}, H_2O_2, or ceftazidime alone, respectively. Comparatively, the elevated ROS ranked as H_2O_2 > ceftazidime > Fe^{3+}. When the synergistic use of ceftazidime with one of the promoters or/and the inhibitor was performed, they promoted and inhibited ROS level, respectively (**Figure 2D**). These results support the conclusion that ROS is related to ceftazidime resistance and promotes ceftazidime-mediated killing.

The P Cycle Is Inactivated and the Activity of Antioxidant Enzymes Is Not Changed in LTB4-R_{CAZ}

To understand why ROS are reduced in LTB4-R_{CAZ}, the P cycle and SOD degradation were investigated. The P cycle is a recently illustrated cycle, which provides respiratory energy in *E. tarda* (Su et al., 2018), and is related to ROS biosynthesis (**Figure 3A**). For the investigation of the P cycle, qRT-PCR was used to measure the expression of genes in the P cycle. Among the 18 genes detected, 11, 2, and 5 were reduced, elevated, and unchanged, respectively. Specifically, the 11 reduced genes encode all enzymes detected except for citrate synthase (CS) and isocitrate dehydrogenase (ICDH). The two elevated genes encode PDH and MDH. The five unchanged genes encode CS, ICDH, SDH, fumarate reductase (FRD), and pyruvate kinase (PK) (**Figures 3B,C**). Consistently, a lower activity of PDH, KGDH, and SDH was detected in LTB4-R_{CAZ} than that in LTB4-S (**Figure 3D**). NADH, membrane potential, and ATP were reduced in LTB4-R_{CAZ} compared with LTB4-S (**Figures 3E–G**). These results indicate that the inactivation of the P cycle forms a characteristic feature in LTB4-R_{CAZ}, which is related to the reduced ROS production. For the investigation of ROS degradation, the activity of ROS degradation enzymes was measured. Among these enzymes working for the ROS degradation, SOD and CAT are widely employed to indicate the antioxidant response. Therefore, the activity of SOD and CAT was detected in LTB4-R_{CAZ} and LTB4-S. A similar activity of SOD and CAT was measured between LTB4-R_{CAZ} and LTB4-S (**Figures 3H,I**). These results indicate that antioxidant response is not changed in LTB4-R_{CAZ}. Taken together, the lower ROS in LTB4-R_{CAZ} is attributed to a reduction of ROS generation instead of an increase of ROS degradation.

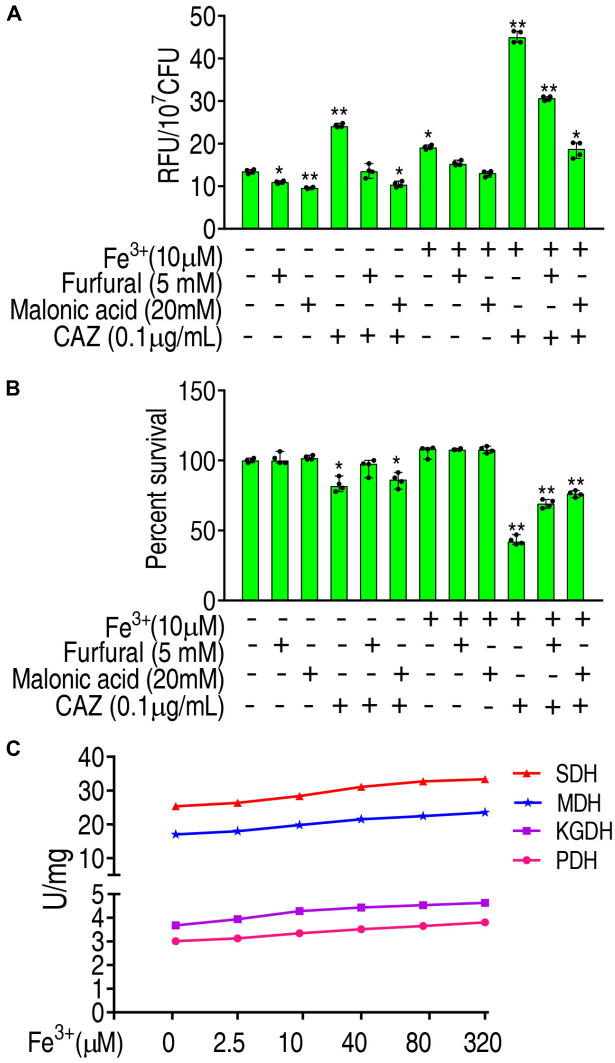

FIGURE 6 | Promotion of Fe^{3+} to the P cycle. **(A)** ROS of LTB4-R_{CAZ} in the presence of Fe^{3+} and CAZ plus inhibitors of the P cycle. **(B)** Percent survival of LTB4-R_{CAZ} in the presence of Fe^{3+} and CAZ plus inhibitors of the P cycle. **(C)** The activity of PDH, KGDH, SDH, and MDH in the presence of Fe^{3+}. Results are displayed as mean ± SEM, as determined by two-tailed Student's *t*-test. Four biological repeats are carried out. $^*p < 0.05$ and $^{**}p < 0.01$.

Fe^{3+} Impacts Metabolic Profile

The above results motivated us to explore whether Fe^{3+} promotes global metabolism including the P cycle to elevate ROS level and potentiate ceftazidime-mediated killing. To do this, GC-MS-based metabolomics was used to compare metabolic profiles between media with or without Fe^{3+}. Four biological and two technical replicates were performed in each group, yielding eight data sets with 65 metabolites in a sample. Among the 65 metabolites, 33 (50.8%) showed differential abundance ($p < 0.05$), with 27 at higher abundance and 6 at lower abundance in the presence of Fe^{3+} (**Figure 4A**). Fourteen pathways were enriched, of which alanine, aspartate, and glutamate metabolism; the TCA cycle; and aminoacyl-tRNA biosynthesis were the top three pathways by impact (**Figure 4B**). Differential metabolites at abundance are listed in **Figure 4C**, where all detected metabolites were elevated in the TCA cycle (**Figure 4C**). Principal component analysis identified two principal components, where component t[1] distinguished LTB4-R_{CAZ} from LTB4-R_{CAZ} + Fe^{3+} (**Figure 4D**). Discriminating variables were identified on an S-plot (**Figure 4E**). Cutoff values were ≥ 0.05 for absolute value of the covariance *p* and ≥ 0.5 for correlation *p* (corr). Nine putative biomarkers were identified, including decreased abundance of 9-hexadecenoic acid, acetic acid, and myo-inositol and elevated abundance of citric acid, oxalic acid, putrescine, butanoic acid, threonine, aspartic acid, and palmitic acid. Among the elevated metabolites, citric acid plays a role in the P cycle.

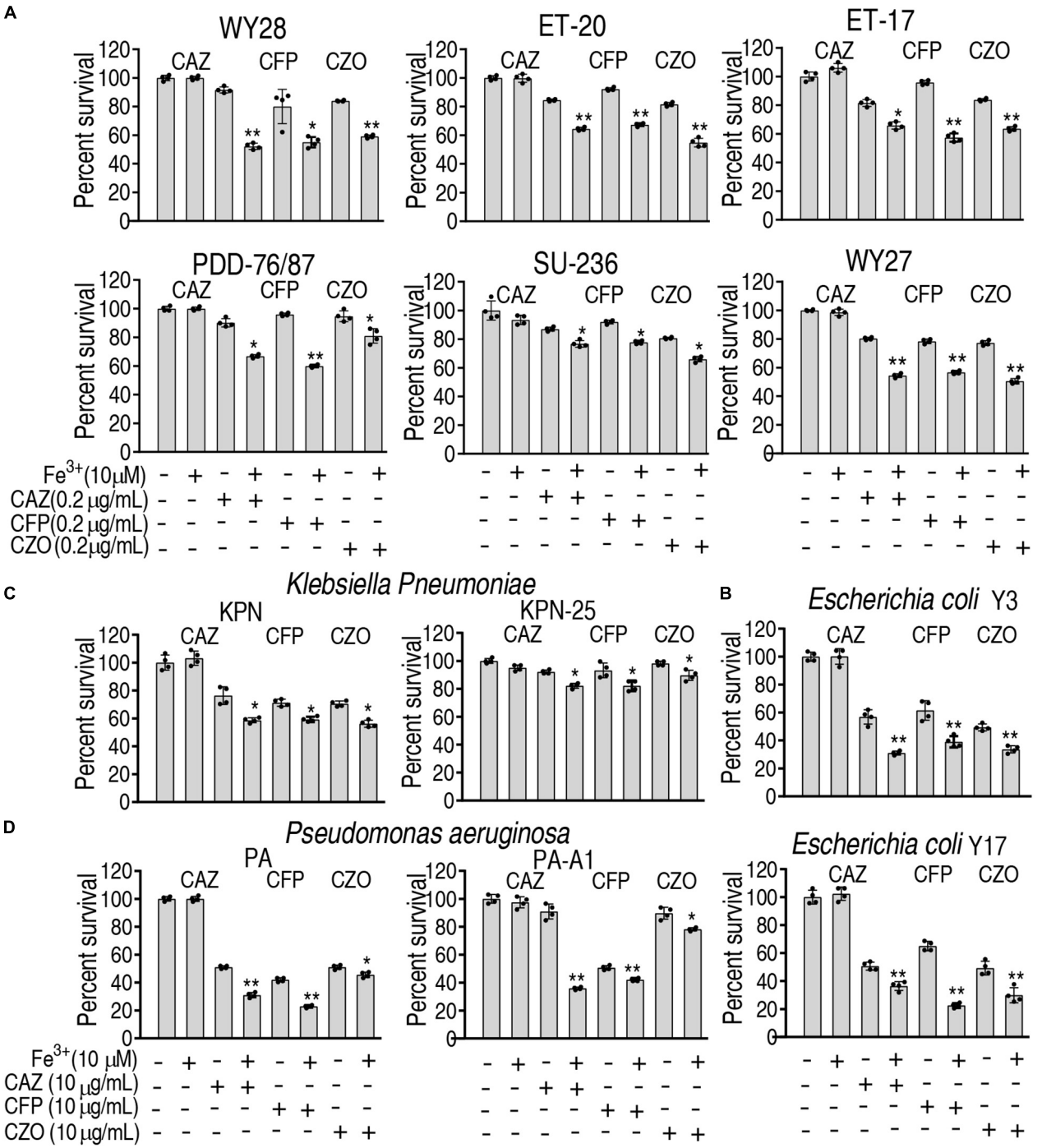

FIGURE 7 | Percent survival of clinically isolated pathogens in the presence of Fe^{3+} and ceftazidime, cefoperazone, or cefazolin. **(A)** *E. tarda*. **(B)** *E. coli*. **(C)** *K. pneumoniae*. **(D)** *P. aeruginosa*.

These results indicate that Fe^{3+} impacts the metabolic profile, where the P cycle is elevated.

Fe^{3+} Activates the P Cycle

To further demonstrate the Fe^{3+}-mediated activation, gene expression and enzyme activity were measured in the P cycle. qRT-PCR analysis showed that out of 18 genes tested, 5, 3, and 10 were elevated, reduced, and unchanged, respectively. In detail, the five elevated genes encode PDH, ACO, ICDH, SCS, and FRD, and the reduced genes encode KGDH, SDH, and PK (these enzymes are encoded by two genes) (**Figures 5A,B**). The activity of PDH, KGDH, SDH,

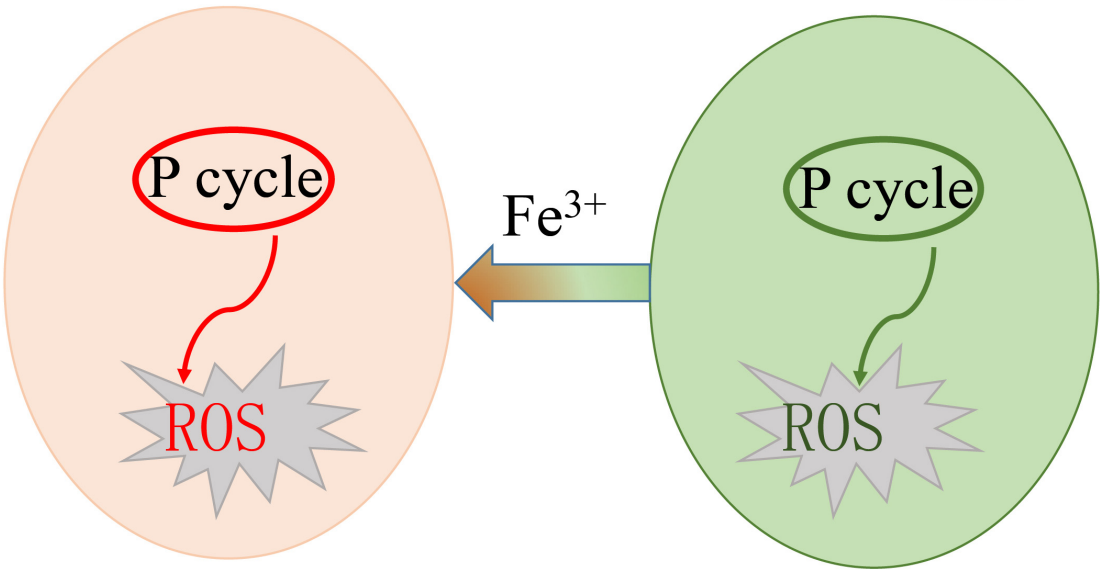

FIGURE 8 | Sketch diagram describing a mechanism by which Fe^{3+} promotes ROS level.

and MDH was elevated (**Figure 5C**), which was further supported by increased NADH, membrane potential, and ATP (**Figures 5D–F**). These results indicate that Fe^{3+} promotes the P cycle but does not affect the activity of SOD and CAT (**Figure 5G**).

Fe^{3+} Promotes ROS via the P Cycle

To further validate that Fe^{3+} promotes ROS via the P cycle, inhibitors of the P cycle were used to investigate whether the inhibition affects ROS and viability of LTB4-R_{CAZ} in the presence of both Fe^{3+} and ceftazidime. Furfural is a non-competitive inhibitor for PDH, and malonic acid is competitive against SDH. Lower ROS were detected in the presence than the absence of furfural or malonic acid, while higher ROS were determined in the medium with than without Fe^{3+} in the presence or absence of ceftazidime and furfural or malonic acid (**Figure 6A**), suggesting that Fe^{3+} partly reverts the inhibition mediated by the two inhibitors. Consistently, a lower viability was detected in the medium with than without Fe^{3+} in the presence of furfural or malonic acid (**Figure 6B**). To understand the mechanisms by which Fe^{3+} promotes the P cycle, we supposed that Fe^{3+} activates the activity of enzymes in the P cycle. To explore this, the activity of PDH, KGDH, SDH, and MDH was measured in a medium with the indicated concentrations of Fe^{3+}. The activity of the four enzymes was increased in a Fe^{3+} dose-dependent manner *in vitro* (**Figure 6C**). These results indicate that Fe^{3+} promotes the activity of PDH, KGDH, SDH, and MDH in the P cycle directly, which is related to the elevation of ROS level in the presence of Fe^{3+}.

Fe^{3+} Promotes the Cephalosporin-Mediated Killing to Clinically Isolated Pathogens

To ensure the Fe^{3+}-induced potentiation is generalizable to cephalosporins and clinically isolated pathogens, three types of cephalosporins (ceftazidime, cefoperazone, and cefazolin) and 12 strains of bacterial pathogens (*E. tarda*, *E. coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*) were used. The 12 strains of pathogens are resistant to at least three classes of antibiotics and thereby belong to multidrug-resistant bacteria. However, Fe^{3+} effectively promotes the three drugs to kill all pathogens (**Figure 7**). These results indicate that the Fe^{3+}-induced potentiation is generalizable to the drugs and bacterial pathogens.

DISCUSSION

The present study first investigated whether ceftazidime resistance is related to ROS level and then explored whether Fe^{3+}-potentiated ROS elevation in ceftazidime-mediated killing is related to the P cycle, which contributes to ROS biosynthesis. For this purpose, ROS level was compared between LTB4-S and LTB4-R_{CAZ}. Lower ROS was detected in LTB4-R_{CAZ} than in LTB4-S. The reduction is accompanied with the inactivation of the P cycle. Since the P cycle is related to ROS generation, the inactivated P cycle should be one reason by which ROS is reduced in LTB4-R_{CAZ}. Moreover, the role of ROS in ceftazidime-mediated killing was demonstrated by the ROS promoter Fe^{3+} and inhibitor

thiourea. They increased and decreased the ceftazidime-mediated killing, respectively, suggesting that the ROS promoter and inhibitor affect the killing via regulation of ROS biosynthesis. These results indicate that the ceftazidime-mediated resistance and killing are regulated by ROS level. We further show that the Fe^{3+}-induced potentiation is generalizable to ceftazidime and other cephalosporins to kill clinically multidrug-resistant pathogens *E. tarda*, *E. coli*, *K. pneumoniae*,and *P. aeruginosa*.

It has been reported that ROS level is a characteristic feature as a consequence of antibiotic resistance and sensitivity (Ma et al., 2016; Ye et al., 2018). Rosato et al. indicated that TCA cycle-mediated generation of ROS is a key mediator for MRSA survival under beta-lactam antibiotic exposure (Rosato et al., 2014). Hayakawa et al. (2019) demonstrated that anti-ROS agent prevents the acquisition of multi-drug resistance in clinical isolates of *P. aeruginosa*. Thomas et al. (2013) showed that a dysfunctional TCA cycle enables *Staphylococcus epidermidis* to resist oxidative stress and alter its cell surface properties, making it less susceptible to beta-lactam antibiotics. In addition, Battán et al. (2004) found that resistance to oxidative stress caused by ceftazidime and piperacillin in a biofilm of *Pseudomonas* is related to bacterial strains. On the other hand, ROS act as an antibiotic sensitizer for the treatment of antibiotic-resistant bacteria involved in infectious diseases (Guo et al., 2018). Recent observations have linked ROS production with bactericidal action of antibiotics, pointing to antibiotic-induced TCA cycle- and respiratory chain-dependent ROS production as playing a role in cell death (Dwyer et al., 2009; Van Acker and Coenye, 2017; Ye et al., 2018). The present study identified that both actions play a role. Specifically, decreased ROS production is a characteristic feature in LTB4-R_{CAZ}, and ceftazidime-induced ROS production is required for ceftazidime-mediated killing in LTB4-R_{CAZ}. These results indicate that the decreased ROS production contributes to the insensitivity to ceftazidime, and thereby reduction of ROS production is a mechanism by which *E. tarda* resist to ceftazidime.

The present study further explored why ROS production is reduced in LTB4-R_{CAZ} based on the P cycle-mediated generation and ROS degradation. It is documented that the reduction of ROS production is attributed to the decreased biosynthesis caused by inactivation of the P cycle instead of the degradation. The understanding of the biosynthesis of ROS is especially important for promoting antibiotic-mediated killing since ROS production contributes to antibiotic resistance. Wang et al. (2014) showed that loss of sigma(s) rendered stationary-phase *E. coli* more sensitive to the bactericidal antibiotic gentamicin due to a weakened antioxidant defense. Our recent publication indicates that alanine enhances aminoglycoside-induced ROS production through promoting ROS biosynthesis pathways and repressing transcription of antioxidant-encoding genes (Ye et al., 2018). Thus, a synergistic use of antibiotics with ROS generation promoter or/and ROS degradation inhibitor will elevate the antibiotic-mediated killing. In addition, metabolites promoting hosts to eliminate bacterial pathogens have been reported (Jiang et al., 2019a,b, 2020b; Gong et al., 2020a,b; Yang et al., 2020), where ROS are involved (Sarr et al., 2018; Gong et al., 2020b). Thus, it is possible to select the promoters or/and inhibitors that simultaneously elevate the ROS level of both hosts and bacteria. This approach will make both the host's anti-infective ability and antibiotic-mediated killing play a role, having the most effect on the elimination of bacterial pathogens via ROS-mediated pathways.

A line of evidence has indicated that Fe^{3+} reduces ROS via the Fenton system (Song et al., 2020). However, information regarding whether Fe^{3+} regulates ROS by other ways is absent in bacteria. The present study showed that Fe^{3+} reverts the inactivity of the P cycle, which elevates ROS biosynthesis. Further evidences include the elevated activity of PDH, KGDH, SDH, and MDH in the presence of Fe^{3+} *in vitro*, suggesting that Fe^{3+} is an activator of these enzymes. Therefore, Fe^{3+} as a ROS promoter plays a role in the P cycle and Fenton system, which is not reported before in bacteria.

In summary, ROS production is decreased as a crucial characteristic of LTB4-R_{CAZ}, which is related to inactivation of the P cycle. Fe^{3+} promotes the activation of the P cycle and thereby elevates ROS level. The elevated ROS potentiates ceftazidime-mediated killing. When ROS inhibitor is used, the killing is reduced (**Figure 8**). These results expand our understanding for the role of Fe^{3+}-induced ROS in antibiotic resistance. They may also provide tools and/or knowledge for future new strategies to stop infections by multidrug-resistant human pathogens.

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

HL conceived the study. JY and YS conducted the experiments. JY, YS, and HL analyzed the data. HL and XP wrote 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.2021.654783/full#supplementary-material

REFERENCES

Abayneh, T., Colquhoun, D. J., and Sørum, H. (2013). *Edwardsiella piscicida* sp. nov. a novel species pathogenic to fish. *J. Appl. Microbiol.* 114, 644–654. doi: 10.1111/jam.12080

Battán, P. C., Barnes, A. I., and Albesa, I. (2004). Resistance to oxidative stress caused by ceftazidime and piperacillin in a biofilm of *Pseudomonas*. *Luminescence* 19, 265–270. doi: 10.1002/bio.779

Blair, J. M., Webber, M. A., Baylay, A. J., Ogbolu, D. O., and Piddock, L. J. (2015). Molecular mechanisms of antibiotic resistance. *Nat. Rev. Microbiol.* 13, 42–51. doi: 10.1038/nrmicro3380

Cabello, F. C., Godfrey, H. P., Buschmann, A. H., and Dölz, H. J. (2016). Aquaculture as yet another environmental gateway to the development and globalisation of antimicrobial resistance. *Lancet Infect. Dis.* 16, e127–e133. doi: 10.1016/S1473-3099(16)00100-6

Cheng, Z. X., Guo, C., Chen, Z. G., Yang, T. C., Zhang, J. Y., et al. (2019). Glycine, serine and threonine metabolism confounds efficacy of complement-mediated killing. *Nat. Commun.* 10:3325. doi: 10.1038/s41467-019-11129-5

Defoirdt, T., Sorgeloos, P., and Bossier, P. (2011). Alternatives to antibiotics for the control of bacterial disease in aquaculture. *Curr. Opin. Microbiol.* 14, 251–258. doi: 10.1016/j.mib.2011.03.004

Dwyer, D. J., Kohanski, M. A., and Collins, J. J. (2009). Role of reactive oxygen species in antibiotic action and resistance. *Curr. Opin. Microbiol.* 12, 482–489. doi: 10.1016/j.mib.2009.06.018

Gong, Q. Y., Yang, D. X., Jiang, M., Zheng, J., and Peng, B. (2020a). L-aspartic acid promotes fish survival against *Vibrio alginolyticus* infection through nitric oxide-induced phagocytosis. *Fish Shellfish Immunol.* 97, 359–366. doi: 10.1016/j.fsi.2019.12.061

Gong, Q. Y., Yang, M. J., Yang, L. F., Chen, Z. G., Jiang, M., et al. (2020b). Metabolic modulation of redox state confounds fish survival against *Vibrio alginolyticus* infection. *Microb. Biotechnol.* 13, 796–812. doi: 10.1111/1751-7915.13553

Guo, L., Xu, R., Zhao, Y., Liu, D., Liu, Z., et al. (2018). Gas plasma pre-treatment increases antibiotic sensitivity and persister eradication in Methicillin-Resistant *Staphylococcus aureus*. *Front. Microbiol.* 9:537. doi: 10.3389/fmicb.2018.00537

Hayakawa, S., Kawamura, M., Sato, T., Hirano, T., Kikuchi, T., et al. (2019). An α-Lipoic acid derivative, and anti-ROS agent, prevents the acquisition of multi-drug resistance in clinical isolates of *Pseudomonas aeruginosa*. *J. Infect. Chemother.* 25, 28–33. doi: 10.1016/j.jiac.2018.10.003

Huang, Y. W., Huang, H. H., Huang, K. H., Chen, W. C., Lin, Y. T., et al. (2019). AmpI functions as an iron exporter to alleviate β-Lactam-mediated reactive oxygen species stress in *Stenotrophomonas* maltophilia. *Antimicrob. Agents Chemother.* 63:e02467-18. doi: 10.1128/AAC.02467-18

Jiang, M., Chen, Z. G., Zheng, J., and Peng, B. (2019a). Metabolites-enabled survival of crucian carps infected by *Edwardsiella tarda* in high water temperature. *Front. Immunol.* 10:1991. doi: 10.3389/fimmu.2019.01991

Jiang, M., Gong, Q. Y., Lai, S. S., Cheng, Z. X., Chen, Z. G., et al. (2019b). Phenylalanine enhances innate immune response to clear ceftazidime-resistant *Vibrio alginolyticus* in Danio rerio. *Fish Shellfish Immunol.* 84, 912–919. doi: 10.1016/j.fsi.2018.10.071

Jiang, M., Kuang, S. F., Lai, S. S., Zhang, S., Yang, J., et al. (2020a). Na(+)NQR confers aminoglycoside resistance via the regulation of L-alanine netabolism. *mBio* 11:e02086-20. doi: 10.1128/mBio.02086-20

Jiang, M., Yang, L., Chen, Z. G., Lai, S. S., Zheng, J., et al. (2020b). Exogenous maltose enhances Zebrafish immunity to levofloxacin-resistant *Vibrio alginolyticus*. *Microb. Biotechnol.* 13, 1213–1227. doi: 10.1111/1751-7915.13582

Jiang, M., Yang, L., Zheng, J., Chen, Z. G., and Peng, B. (2020c). Maltose promotes crucian carp survival against *Aeromonas sobrial* infection at high temperature. *Virulence* 11, 877–888. doi: 10.1080/21505594.2020.1787604

Li, L., Su, Y. B., Peng, B., Peng, X. X., and Li, H. (2020). Metabolic mechanism of colistin resistance and its reverting in *Vibrio alginolyticus*. *Environ. Microbiol.* 22, 4295–4313. doi: 10.1111/1462-2920.15021

Li, W., Yao, Z., Sun, L., Hu, W., Cao, J., et al. (2016). Proteomics analysis reveals a potential antibiotic cocktail therapy strategy for *Aeromonas hydrophila* infection in biofilm. *J. Proteome Res.* 15, 1810–1820. doi: 10.1021/acs.jproteome.5b01127

Ma, L. N., Mi, H. F., Xue, Y. X., Wang, D., and Zhao, X. L. (2016). The mechanism of ROS regulation of antibiotic resistance and antimicrobial lethality. *Yi Chuan* 38, 902–909. doi: 10.16288/j.yczz.16-157

National Committee for Clinical Laboratory Standards (1999). *Methods for Determining Bactericidal Activity of Antimicrobial Agents. Approved Guideline. Document M26-A*. Villanova, Pa: National Committee for Clinical Laboratory Standards.

Peng, B., Li, H., and Peng, X. X. (2019). Proteomics approach to understand bacterial antibiotic resistance strategies. *Expert Rev. Proteomics* 16, 829–839. doi: 10.1080/14789450.2019.1681978

Peng, B., Su, Y. B., Li, H., Han, Y., Guo, C., et al. (2015). Exogenous alanine and/or glucose plus kanamycin kills antibiotic-resistant bacteria. *Cell Metab.* 21, 249–262. doi: 10.1016/j.cmet.2015.01.008

Rosato, R. R., Fernandez, R., Paz, L. I., Singh, C. R., and Rosato, A. E. (2014). TCA Cycle-mediated generation of ROS is a key mediator for HeR-MRSA survival under β-Lactam antibiotic exposure. *PLoS One* 9:e99605. doi: 10.1371/journal.pone.0099605

Sarr, D., Toth, E., Gingerich, A., and Rada, B. (2018). Antimicrobial actions of dual oxidases and lactoperoxidase. *J. Microbiol.* 56, 373–386. doi: 10.1007/s12275-018-7545-1

Song, M., Cheng, Y., Tian, Y., Chu, C., Zhang, C., et al. (2020). Sonoactivated chemodynamic therapy: a robust ROS generation nanotheranostic eradicates multidrug-resistant bacterial infection. *Adv. Funct. Mater.* 30:2003587. doi: 10.1002/adfm.202003587

Stokes, J. M., Gutierrez, A., Lopatkin, A. J., Andrews, I. W., French, S., et al. (2019). A multiplexable assay for screening antibiotic lethality against drug-tolerant bacteria. *Nat. Methods* 16, 303–306. doi: 10.1038/s41592-019-0333-y

Su, Y. B., Peng, B., Li, H., Cheng, Z. X., Zhang, T. T., et al. (2018). Pyruvate cycle increases aminoglycoside efficacy and provides respiratory energy in bacteria. *Proc. Natl. Acad. Sci. U S A.* 115, E1578–E1587. doi: 10.1073/pnas.1714645115

Thomas, V. C., Kinkead, L. C., Janssen, A., Schaeffer, C. R., Woods, K. M., et al. (2013). A dysfunctional tricarboxylic acid cycle enhances fitness of staphylococcus epidermidis during β-Lactam stress. *mBio* 4:e00437-13. doi: 10.1128/mBio.00437-13

Van Acker, H., and Coenye, T. (2017). The role of reactive oxygen species in antibiotic-Mediated killing of bacteria. *Trends Microbiol.* 25, 456–466. doi: 10.1016/j.tim.2016.12.008

Wang, J. H., Singh, R., Benoit, M., Keyhan, M., Sylvester, M., et al. (2014). Sigma S-dependent antioxidant defense protects stationary-phase *Escherichia coli* against the bactericidal antibiotic gentamicin. *Antimicrob. Agents Chemother.* 58, 5964–5975. doi: 10.1128/AAC.03683-14

Wang, Y. M., Wang, Q. Y., Xiao, J. F., Liu, Q., Wu, H. Z., et al. (2011). Genetic relationships of Edwardsiella strains isolated in China aquaculture revealed by rep-PCR genomic fingerprinting and investigation of *Edwardsiella virulence* genes. *J. Appl. Microbiol.* 111, 1337–1348. doi: 10.1111/j.1365-2672.2011.05166.x

Yang, M. J., Xu, D., Yang, D. X., Li, L., Peng, X. X., et al. (2020). Malate enhances survival of zebrafish to *Vibrio alginolyticus* infection in the same manner of taurine. *Virulence* 11, 349–364. doi: 10.1080/21505594.2020.1750123

Yao, Z., Li, W., Lin, Y., Wu, Q., Yu, F., et al. (2016). Proteomic analysis reveals that metabolic flows affect the susceptibility of *Aeromonas hydrophila* to antibiotics. *Sci. Rep.* 6:39413. doi: 10.1038/srep39413

Ye, J. Z., Su, Y. B., Lin, X. M., Lai, S. S., Li, W., et al. (2018). Alanine enhances aminoglycosides-induced ROS production as revealed by proteomic analysis. *Front. Microbiol.* 9:29. doi: 10.3389/fmicb.2018.00029

Zhang, S., Wang, J., Jiang, M., Xu, D., Peng, B., et al. (2019). Reduced redox-dependent mechanism and glucose-mediated reversal in gentamicin-resistant *Vibrio alginolyticus*. *Environ. Microbiol.* 21, 4724–4739. doi: 10.1111/1462-2920.14811

Zhang, S., Yang, M. J., Peng, B., Peng, X. X., and Li, H. (2020). Reduced ROS mediated antibiotic resistance and its reverting by glucose in *Vibrio alginolyticus*. *Environ. Microbiol.* 22, 4367–4380. doi: 10.1111/1462-2920.15085

Zhao, X. L., and Drlica, K. (2014). Reactive oxygen species and the bacterial response to lethal stress. *Curr. Opin. Microbiol.* 21, 1–6. doi: 10.1016/j.mib.2014.06.008

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

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Genomic Analysis of *Enterococcus* spp. Isolated From a Wastewater Treatment Plant and Its Associated Waters in Umgungundlovu District, South Africa

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We investigated the antibiotic resistome, mobilome, virulome, and phylogenomic lineages of *Enterococcus* spp. obtained from a wastewater treatment plant and its associated waters using whole-genome sequencing (WGS) and bioinformatics tools. The whole genomes of *Enterococcus* isolates including *Enterococcus faecalis* (n = 4), *Enterococcus faecium* (n = 5), *Enterococcus hirae* (n = 2), and *Enterococcus durans* (n = 1) with similar resistance patterns from different sampling sites and time points were sequenced on an Illumina MiSeq machine. Multilocus sequence typing (MLST) analysis revealed two *E. faecalis* isolates that had a common sequence type ST179; the rest had unique sequence types ST841, and ST300. The *E. faecium* genomes belonged to 3 sequence types, ST94 (n = 2), ST361 (n = 2), and ST1096 (n = 1). Detected resistance genes included those encoding tetracycline [*tet*(S), *tet*(M), and *tet(L)*], and macrolides [*msr*(C), *msr(D)*, *erm(B)*, and *mef*(A)] resistance. Antibiotic resistance genes were associated with insertion sequences (IS6, ISL3, and IS982), and transposons (Tn3 and Tn6000). The *tet(M)* resistance gene was consistently found associated with a conjugative transposon protein (TcpC). A total of 20 different virulence genes were identified in *E. faecalis* and *E. faecium* including those encoding for sex pheromones (*cCF10, cOB1, cad,* and *came*), adhesion (*ace, SrtA, ebpA, ebpC,* and *efaAfs*), and cell invasion (*hylA* and *hylB*). Several virulence genes were associated with the insertion sequence IS256. No virulence genes were detected in *E. hirae* and *E. durans.* Phylogenetic analysis revealed that all *Enterococcus* spp. isolates were more closely related to animal and environmental isolates than clinical isolates. *Enterococcus* spp. with a diverse range of resistance and virulence genes as well as associated mobile genetic elements (MGEs) exist in the wastewater environment in South Africa.

Keywords: *Enterococcus* spp., whole-genome sequencing, wastewater treatment plant, antibiotic resistance, South Africa

INTRODUCTION

The efficiency of wastewater treatment plants (WWTPs) is critical to preventing the spread of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) into the environment (Karkman et al., 2017; Alexander et al., 2020). Although AMR surveillance in clinical settings and animals is well established in most developed and some developing countries, surveillance in the environment still lags behind (Huijbers et al., 2019). The emergence of ARB and ARGs in the water environment has become an important environmental health issue (Conte et al., 2017; Karkman et al., 2017; Alexander et al., 2020). Dissemination of ARGs is thought to occur in the environment mainly through the transfer of mobile genetic elements (MGEs) such as plasmids, transposons, integrons, gene cassettes, Integrative and conjugative elements (ICE), and insertion sequence common regions between bacterial species (Sanderson et al., 2020). The selection pressure in a given environment is crucial as it influences the spread and accumulation of ARGs some of which may be novel (Bengtsson-Palme et al., 2018). The risk of transfer of ARGs to pathogens increases in environments with a high fecal load and associated fecal bacteria (Huijbers et al., 2019).

Enterococcus species are Gram positive non-sporulating organisms that mainly exist as commensals in the intestinal flora of healthy animals and humans. They can thus be excreted into environmental sources including soil and surface water as fecal matter and are thus commonly used as indicator organisms in water environments (Berendonk et al., 2013; Karkman et al., 2018). Some like *faecalis* and *Enterococcus faecium* are opportunistic pathogens whilst other species such as *Enterococcus hirae* and *Enterococcus durans* are rarely pathogenic in humans (Bourafa et al., 2015; Ryu et al., 2019). *Enterococcus* spp. can easily acquire and disseminate resistance determinants (Medeiros et al., 2014) making them suitable for antibiotic resistance surveillance studies.

Whole-genome sequencing (WGS) is a highly discriminatory technique for studying bacterial species, including Enterococci. However, very few studies have used WGS to study environmental enterococcal isolates (Sanderson et al., 2020; Zaheer et al., 2020). The application of WGS to antibiotic resistance surveillance remains largely confined to clinical and animal settings, with very little attention given to the environment (Hendriksen et al., 2019; Su et al., 2019; WHO, 2020). There is therefore a paucity of data on the role that genomic surveillance plays in understanding the environmental dimensions of antibiotic resistance, particularly in Africa.

In this study, we investigated the antibiotic resistome, mobilome, virulome, and phylogenomic lineages of *Enterococcus* spp. obtained from a WWTP and its associated waters. Additionally, we assayed the role of the water environment in the dissemination of multi-drug resistant *Enterococcus* spp. which could be of clinical or veterinary importance.

MATERIALS AND METHODS

Ethical Consideration

Ethical approval was received from the Biomedical Research Ethics Committee (Reference: BCA444/16) of the University of KwaZulu-Natal. Permission to collect water samples was sought and granted by uMgeni Water which owns and operates the investigated WWTP.

Study Site

Manual grab water samples were collected in sterile 500-mL containers from the influent (29°36′3.70″S 30°25′41.71″E), final effluent (29°35′49.97″S 30°26′19.74″E) of a major WWTP as well as upstream (29°36′10.73″S 30°25′29.97″E) and downstream (29°36′27.54″S 30°27′0.76″E) of its associated receiving water body in uMgungundlovu District, KwaZulu-Natal, South Africa.

The WWTP is the largest in Pietermaritzburg, the provincial capital of KwaZulu-Natal in South Africa. The WWTP discharges its final effluent into the uMsunduzi river, a key water source for domestic, agricultural, and recreational purposes to inhabitants of the several informal settlements along its banks (Moodley et al., 2016).

Bacterial Isolates

Enterococcus spp. were isolated from water samples collected fortnightly over 7 months (May 2018 to November 2018).

Putative identification was accomplished during enumeration using the Enterolert® / Quanti-Tray® 2000 system followed by phenotypic confirmation on Bile Aesculin Azide agar (Merck, Germany) or Slanetz and Bartley agar (Merck, Germany). Samples from, upstream and downstream river water as well as final effluent were diluted 1 mL in 100 mL (0.01 dilution) while the influent with its higher bacterial load was o diluted by 0.05 mL in 100 mL (0.005 dilution) using sterile water. A volume of 100 mL of each sample was analyzed using the Enterolert® Quanti-Tray® 2000 system (IDEXX Laboratories (Pty.) Ltd., Johannesburg, South Africa). *Enterococcus* spp. were obtained from positive quanti-trays, sub-cultured on Bile Aesculin Azide or Slanetz and Bartley agar and incubated at 41°C for 24–48 h. At least ten distinct colonies representing each sampling site were randomly selected from the Bile Aesculin Azide or Slanetz and Bartley agar and further sub-cultured onto the same media, respectively, to obtain pure colonies. Molecular confirmation of *Enterococcus* spp. was done using real-time polymerase chain reaction (rtPCR) of the *tuf* (Elongation factor *tu*) gene (Ke et al., 1999).

Antibiotic susceptibility determination was undertaken using the Kirby-Bauer method on a panel of sixteen commercial antibiotic discs which included: chloramphenicol (CHL, 30 μg), tetracycline (TET, 30 μg), ampicillin (AMP, 10 μg), nitrofurantoin (NIT, 300 μg), ciprofloxacin (CIP, 5 μg), levofloxacin (LVX, 5 μg), imipenem (IPM, 10 μg), linezolid (LZD, 30 μg), erythromycin (E, 15 μg), quinupristin–dalfopristin (Q–D, 15 μg) against *E. faecium* only, tigecycline (TGC, 15 μg), trimethoprim-sulfamethoxazole (SXT, 25 μg), vancomycin

(VAN, 30 μg) and teicoplanin (TEC, 30 μg). Detection of high-level aminoglycoside resistance was ascertained using gentamicin (GEN, 120 μg) and streptomycin (STR, 300 μg) discs. Inhibition zones were measured, and the results were interpreted using the European Committee on Antimicrobial Testing (EUCAST) breakpoint tables (EUCAST, 2020). Breakpoints for chloramphenicol, tetracycline, erythromycin, linezolid, nitrofurantoin, vancomycin, and gentamicin were obtained from the Clinical and Laboratory Standards Institute (CLSI) interpretative charts (CLSI, 2020). *Enterococcus faecalis* ATCC 29212 was used for quality control.

Whole-Genome Sequencing and Analysis

Twelve MDR *Enterococcus* spp. isolates with similar antibiograms obtained from all four sampled sites were selected for WGS. Genomic DNA was extracted using the GenElute Bacterial Genomic DNA kit (Sigma Aldrich, St. Louis, United States) followed by quantification using the 260/280 nm wavelength on a Nanodrop 8000 (Thermo Scientific Waltham, MA, United States). Library preparation was done using the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, United States). WGS was undertaken using an Illumina MiSeq machine (Illumina, San Diego, CA, United States). The raw reads were quality trimming using Sickle v1.33[1] and assembled spontaneously using the SPAdes v3.6.2 assembler. All contiguous sequences were subsequently submitted to GenBank and assigned accession numbers under Bio project PRJNA609064 (**Supplementary Table 1**).

The assembled genomes were analyzed for MLST sequence types on the MLST 1.8 database (Larsen et al., 2012) hosted by the Centre for Genomic Epidemiology (CGE)[2]. Acquired antimicrobial resistance genes and chromosomal point mutations including the DNA gyrase *gyr*A and *parC* genes (quinolone resistance) and the pbp5 gene (ampicillin resistance) were annotated using ResFinder[3] set at default threshold ID (90%) and minimum length (60%) values. Plasmid replicons types were identified using PlasmidFinder 2.1 on the CGE website[4]. Virulence genes were determined using VirulenceFinder 2.0 on the CGE website[5].

The assembled genomes were further analyzed for MGEs, including insertion sequences, using ISFinder[6] (Siguier, 2006), and intact prophages using PHASTER[7] (Zhou et al., 2011; Arndt et al., 2016). ICE and putative integrative and mobilisable elements (IME) were identified using the ICEberg database[8]. RAST SEEDVIEWER[9] was also used to annotate and identify the genomes with integrons, and transposons. The synteny and genetic environment of ARGs and associated MGEs were investigated using the general feature format (GFF3) files from GenBank. The genetic environment of virulence genes detected in the study were also determined using a similar approach. The GFF files were imported into Geneious prime 2020.2[10] for analysis.

Phylogenetic Reconstruction

Whole-genome sequences of the *E. faecalis* and *E. faecium* isolates were compared with isolates curated from the PATRIC website[11] from different African countries including South Africa. The genomes of *E. hirae* and *E. durans* isolates were compared to those of isolates belonging to the respective species curated from the PATRIC website from different countries across the world as there were no/few entries from Africa. Whole-genome sequences of all isolates were uploaded and analyzed on the CSI Phylogeny 1.4 pipeline[12] that recognizes, screens, and validates the location of single nucleotide polymorphisms (SNPs) before deducing a phylogeny based on the concatenated alignment of the high-quality SNPs. SNPs were identified from the alignments using the mpileup module in SAMTools version 0.1.18 (Li et al., 2009). Selection of SNPs was based on default parameters in CSI Phylogeny (Kaas et al., 2014). The following reference genomes were used for each alignment; *E. faecalis,* (*E. faecalis* V583), *E. faecium* (*E. faecium* DO), *E. hirae* (*E. hirae* ATCC 9790), and *E. durans* (*E. durans* ATCC 6056). The phylogenetic tree was constructed using FastTree (Price et al., 2010). The generated phylogenetic trees were viewed, annotated, and edited using the Iterative Tree of Life (iTOL)[13].

RESULTS

Isolate Source and Antibiotic Susceptibility Patterns

A total of 579 *Enterococcus spp*. isolates were obtained from the different sampling points. Of these, 12 isolates were selected for WGS, distributed as follows: three isolates from the upstream site of the WWTP along the receiving river, four from the downstream site, three from the raw influent, and two were from the final effluent of the WWTP (**Supplementary Table 1**). Selected isolates consisted of *E. faecalis* (4 isolates), *E. faecium* (5), *E. hirae* (2), and *E. durans* (1) (**Supplementary Table 1**).

The resistance patterns displayed by the selected isolates from different time points and sampling sites are shown in **Table 1**. The resistance profile TET-SXT-STR was found in two isolates, one *E. faecalis* obtained from raw influent and one *E. durans* isolate from the influent. Two *E. faecalis* isolates from downstream and upstream sites had the same resistance profile ERY-TEC-TET-SXT, other resistance profiles shared by at least two isolates from different sites included *E. faecium* (QD-TET-SXT, QD-TET-SXT-STR), and *E. hirae* (NIT-SXT-STR) isolates.

[1]https://github.com/najoshi/sickle
[2]http://cge.cbs.dtu.dk/services/MLST/
[3]https://cge.cbs.dtu.dk/services/ResFinder/
[4]https://cge.cbs.dtu.dk/services/PlasmidFinder/
[5]https://cge.cbs.dtu.dk/services/VirulenceFinder/
[6]https://isfinder.biotoul.fr/
[7]https://phaster.ca/
[8]http://db-mml.sjtu.edu.cn/ICEberg/
[9]http://rast.nmpdr.org/seedviewer.cgi
[10]https://www.geneious.com
[11]https://www.patricbrc.org/
[12]https://cge.cbs.dtu.dk/services/CSIPhylogeny/
[13]https://itol.embl.de/

TABLE 1 | Distribution of antibiotic resistance genes and mobile genetic elements in environmental *Enterococcus* spp.

Isolate ID (MLST)	Resistance pattern	Date of isolation	Point of isolation	Detected ARG	Insertion sequences	Intact prophages	Putative ICE	Putative IME	Plasmid replicon type
E. faecalis									
°D21 (ST 179)	ERY-TEC-TET-SXT	Jun 12, 2018	Downstream	*dfrG, erm(B), lsa(A), tet(M), aac(6′)-aph(2″), ant(6)-Ia, aph(3′)-III*	ISEfa10, ISEfa11, ISEfa5, ISLsa2	No intact prophage	T4SS type ICE	None detected	repUS43
°U84 (ST 300)	ERY-TEC-TET-SXT	Sep 11, 2018	Upstream	*dfrG, erm(B), lsa(A), tet(L), tet(M)*	ISCac2, ISCysp18, TnBth2, ISMspa1	Entero_phiFL1AEntero_ phiFL3A	None detected	1	repUS43 rep9b
°IN127 (ST 179)	ERY-TEC-LZD-TET-SXT-GEN-STR	Oct 23, 2018	Influent	*erm(B), lsa(A), tet(M)*	ISEfa10, ISEfa11, ISEfa5, ISLsa2	Entero_phiFL1A Entero_phiFL3A	None detected	1	repUS43 rep9c
°IN133 (ST 841)	TET-SXT-STR	Nov 6, 2018	Influent	*lsa(A), msr(C), tet(M), aac(6′)-Ii*	ISMspa1, ISLar7, ISArch1, ISBsp1	Lister_LP_101	T4SS type ICE	2	repUS15 repUS43
E. faecium									
°E21 (ST 1096)	TEC-QD-TET-SXT	Jun 12, 2018	Effluent	*msr(C), tet(M)*	ISEfa10, ISSpn11, ISSmu1, ISLgar1	Lister_B025	T4SS type ICE	1	No hits
°IN91 (ST 361)	QD-TET-SXT	Sep 11, 2018	Influent	*msr(C), tet(M)*	ISGaba2, ISFnu4, ISFnu3, MICBce1	Lister_B025, Bacill_BCJA1c	None detected	1	rep29, repUS43, repUS15
°D95 (ST 94)	QD-TET-SXT-STR	Sep 11, 2018	Downstream	*msr(C), tet(M)*	IS1485, ISLgar4, ISS1W, IS1216V	Entero_vB_IME197	T4SS type ICE	None detected	repUS43
°D98 (ST 94)	QD-TET-SXT	Sep 11, 2018	Downstream	*msr(C), tet(M)*	IS1485, ISEfa12, ISEfa8, ISSag12	Entero_vB_IME197	T4SS type ICE	None detected	repUS43, repUS15
°U129 (ST 361)	QD-TET-SXT-STR	Nov 6, 2018	Upstream	*lsa(A), msr(C), tet(M)*	ISPye7, ISShes9, ISBth14, ISFnu2	Entero_EFC_1	T4SS type ICE (3)	None detected	repUS24, repUS15, repUS43
E. hirae									
°D76	NIT-SXT-STR	Aug 14, 2018	Downstream	*aac(6′)-Iid*	ISEfa11, ISEfa5, IS1251, ISEfa10	No intact prophage	None detected	None detected	No hits
°U73	NIT-SXT-STR	Aug 28, 2018	Upstream	*aac(6′)-Iid*	ISEfa11, ISEfa5, IS1251, ISEfa12	No intact prophage	None detected	None detected	No hits
E. durans									
°E115	TET-SXT-STR	Oct 9, 2018	Effluent	*dfrG, mef(A), msr(D), tet(S), tet(M), aac(6′)-Iih,ant(6)-Ia*	ISEfa11, ISEfa5, ISSsu4, ISDha13	Bacill_BCJA1c Entero_phiFL1A	T4SS type ICE (3)	None detected	repUS15repUS1

TET, tetracycline; NIT, nitrofurantoin; Q–D, quinupristin–dalfopristin; GEN, gentamicin; STR, streptomycin; LZD, Linezolid; ERY, erythromycin; SXT, trimethoprim-sulfamethoxazole; TEC, teicoplanin.

The resistance profiles TEC-QD-TET-SXT, and ERY-TEC-LZD-TET-SXT-GEN-STR were unique to individual isolates (**Table 1**).

Genome Characteristics

The genome and assembly characteristics of the *Enterococcus* spp. sequences are presented in **Supplementary Table 1**. The total assembled genome size ranged from 2.5–3.2 MB, the GC content ranged from 36.6–38.4, the N50, L50; the total number of contigs are also shown in **Supplementary Table 1**.

Antibiotic Resistance Genes

Several ARGs were present in the isolates, with each isolate harboring at least one ARG (**Table 1**). Most of the isolates belonging to all the sub-species harboured macrolides/streptogramins/lincosamides resistance genes *lsa(A)*, *msr(C)*, *msr(D)*, *erm(B)*, and *mef(A)*. Other ARGs included the tetracycline resistance [*tet*(S), *tet*(M), and *tet(L)*], aminoglycoside resistance [*aac(6′)-aph(2″), ant(6)-Ia, aph(3′)-III, aac(6′)-Iid, aac(6′)-Iih*], and trimethoprim resistance (*dfr*G) gene (**Table 1**). In *E. faecalis* macrolide resistance was mediated by the *erm(B)* gene – two isolates from the influent (IN127, ST179), and downstream (D21, ST179) sites had the *erm (B)*, *isa(A)*, and *tet(M)* genes in common. Tetracycline resistance was mediated mainly by the *tet(M)* gene in all the TET resistant 10/12 (83.3%) isolates except for one *E. faecalis* isolate (U84 ST300) from the upstream site that had *tet(M)* and *tet(L)*, as well as an *E. durans,* isolate (E115) from the effluent that had *tet(M)* and *tet(S)*. In the *E. faecium* isolates resistance genes could not be linked to sequence type or source of isolation as all the isolates had the *msr(C)*, and *tet(M)* genes in common (**Table 1**).

The quinolone resistance determinant regions (QRDRs) of the DNA gyrase (*gyr*A) and DNA topoisomerase IV genes (*par*C), were assayed for point mutations in all isolates. The *gyr*A (I259L*, I306V*, N708D*, D759N*, A811V*, G819A*, S820T*, N708D*), and *parC* (I699V*, E707D*, L773I*) showed putatively novel

mutations that were not linked to phenotypic resistance (**Table 2**). Only *E. faecium* isolates harboured mutations in all the assayed genes (**Table 2**). Point mutations in the *pbp5* gene which encodes ampicillin resistance were mostly putatively novel mutations (**Table 2**). No mutations were found in *E. faecalis*, *E. hirae,* and *E. durans*.

Mobile Gene Elements (Plasmids, Insertion Sequences, Intact Prophages, and Integrons)

PlasmidFinder revealed a total of seven different plasmid associated replication genes (repUS15, repUS43, rep9c, rep9b, rep29, repUS24, repUS1). The repUS43 and repUS15 were the most common replicon types occurring in eight (66.7%) and five (41.6%) isolates, respectively (**Table 1**). A total of seven (58.3%) isolates had more than one plasmid replicon; however, no plasmid replicon types were detected in three isolates (one *Enterococcus feacium* and two *E. hirae*) (**Table 1**). There was no unique pattern concerning the replicon type, sequence type, and source of isolation. However, replicon type rep9b/c was only found in *E. faecalis* isolates with rep24 and rep29 being unique to *E. faecium* isolates.

Some ARGs were associated with insertion sequences (IS6, ISL3, and IS982), and transposons (Tn3 and Tn6000) with most of those associated with MGE being plasmid-borne (**Table 3**). However, the majority of ARGs were located on chromosomes and not associated with any MGEs (**Supplementary Table 2**). An *E. faecium* isolate (D95) from the downstream site harboured an efflux pump encoding macrolide resistance gene *msr(A)* that was associated with insertion sequence IS982. The contig carrying the *msr(A)* gene and associated MGE had very high similarity (99–100%) to a target sequence *E. faecium* HB-1 chromosome (CP040878.1) in GenBank (**Table 3**). An *E. faecalis* isolate (D21) from the downstream site had a plasmid-encoded trimethoprim resistance gene *dfrG* whose genetic environment had ISL3. The contig was highly similar to a target sequence in GenBank *E. faecalis* strain 133170041-3 plasmid pAD1 (CP046109.1) confirming carriage of the gene on a plasmid. Another isolate (U84) from the upstream site had a plasmid that co-carried the tetracycline resistance [*tet(M)* and *tet(L)*] and macrolide resistance *erm(B)* genes. The genetic environment of the resistance genes consisted of a recombinase and the Tn3 transposons and the contig was closely related to *E. faecalis* S7316 plasmid Ps7316optrA (LC499744.1) (**Table 3**). The *E. durans* isolate (E115) from the effluent site had an antibiotic resistance genetic island consisting of genes encoding resistance to aminoglycosides [*ant(6)-Ia*], chloramphenicol (*catB*), macrolides [*msr(D)* and *mef(A)*], and trimethoprim (*dfrG*). The resistance island had MGEs including several recombinases and the insertion sequence IS6 (**Table 3**). The resistance island was located on a contig that closely resembled a target sequence in GenBank *E. faecalis* strain transconjugant T4 plasmid pJH-T4 (KY290886.1) implying that it was located on a plasmid. The genetic environment of the tetracycline resistance gene *tet(S)* was associated with the insertion sequence IS6. Interestingly the contig carrying this *tet(S)* gene was highly similar to an *E. faecalis* strain C386 transposon Tn6000 (JN208881.1) (**Table 3**). The *tet(M)* resistance gene was consistently found associated with the tetracycline resistance leader peptide (*tetrLpep*) and a conjugative transposon/transfer protein (*TcpC*), genetic context *tet(M): tetrLpep: TcpC* (IN127, IN133, E21, E115, D21, U129) and the reverse context *TcpC:tetrLpep:tet(M)* in D95, D98, U84). The *TcpC* conjugative TcpC is required for efficient conjugative transfer and mediates tetracycline resistance. Notably, the genetic context was found on contigs with high similarity (98–100%) to *Enterococcus* spp. chromosomal sequences deposited in GenBank except in *E. faecalis* isolate U84 where the genes were co-carried on a plasmid with other ARGs (**Table 3**).

A total of 32 IS families were detected in the genomes (**Table 1**). Ten IS families occurred more than once, with the ISEfa5 (5 isolates), ISEfa11 (5), and ISEfa10 (4) being predominant. ISEfa5 and ISEfa11 occurred in the same five isolates, covering all four species (**Table 1**). The IS did not follow source or sequence type, albeit two *E. faecalis* isolates (IN127, D21) belonging to ST179 had the same four ISs.

TABLE 2 | Point mutation in the *gyrA*, *parC* (quinolone resistance), and *pbp5* (ampicillin resistance) genes in environmental *Enterococcus* spp.

Isolate ID	Pbp5	gyrA	parC
E. faecium			
°IN91	V24A, S27G, K144Q, K2E*, T25A*, S39T*, A73T*, R347C* R390C*, R474G*, Y475C*, K492Q*, K501E*, L573I*, S622N* E646K*, K647E*, V684A*, T25A*, S39T*, D644N*	I259L*, I306V*, N708D*, D759N*, A811V*, G819A*, S820T*	I699V*, E707D*, L773I*
°D95	V24A, S27G, K144Q, T324A, T25A*, S39T*, A73T*, D644N*	I259L*, I306V*, N708D*, D759N*, A811V*, G819A*, S820T*	I699V*, E707D*, L773I*
°D98	V24A, S27G, K144Q, T324A, T25A*, S39T*, A73T*, D644N*	I259L*, I306V*, N708D*, D759N*, A811V*, G819A*, S820T*	I699V*, E707D*, L773I*
°E21	V24A, S27G, R34Q, G66E, E100Q, K144Q, T172A, L177I, A216S, T324A, N496K, A499I, E525D, T25A*, S39T*, A401S*, D644N*	N708D*	
°U129	T25A*, S39T*, D644N*	I259L*, I306V*, N708D*, D759N*, A811V*, G819A*S820T*	I699V*, E707D*, L773I*

**Putatively novel mutations.*
No mutations found in E. faecalis, E. hirae, and E. durans.

TABLE 3 | Mobile genetic elements associated with antibiotic resistance genes in *Enterococcus* spp.

Isolate (MLST)	Contig	Synteny of resistance genes and MGE	Plasmid/chromosomal sequence with closest nucleotide homology (accession number)
E. faecium			
°D95/9 (ST94)	4	*Transposase:::::IS982:msr(A)*	*E. faecium* HB-1 chromosome (CP040878.1)
	39	*TcpC: tetrLpep :tet(M)*	*E. faecium* HB-1 chromosome (CP040878.1)
°D98/9 (ST94)	34	*TcpC:tetrLpep:tet(M)*	*E. faecium HB-1 chromosome (CP040878.1)*
°E21/6 (ST1096)	2	*tet(M): tetrLpep: TcpC*	*E. faecium* isolate e4456 chromosome (LR135482.1)
°U129/11 (ST361)	57	*tet(M): tetrLpep: TcpC*	*E. faecium* HB-1 chromosome (CP040878.1)
E. faecalis			
°IN133/11 (ST841)	3	*tet(M): tetrLpep: TcpC*	*E. faecium isolate* E0139 chromosome (LR132067.1)
°IN127/10 (ST179)	33	*tet(M): tetrLpep: TcpC*	*E. faecalis* strain HA-1 chromosome (CP040898.1)
°D21/6 (ST179)	1	ISL3:::::*dfrG*	*E. faecalis* strain 133170041-3 plasmid pAD1 (CP046109.1)
	23	*tet(M): tetrLpep: TcpC*	*E. faecalis* strain JY32 chromosome (CP045045.1)
	27	*aph(2″)-Ia::: aph(3′)-IIIa*	*E. faecalis* strain TH4125 chromosome (CP051005.1)
°U84/9 (ST300)	2	*TcpC : tetrLpep :tet(M): tet(L)::::::::::recombinase: Tn3:::: Tn3:recombinase:erm(B)*	*E. faecalis* S7316 plasmid Ps7316optrA (LC499744.1)
E. durans			
°E115/10	7	*tet(M): tetrLpep: TcpC*	*E. faecalis 62* chromosome *(CP022712.1)*
	22	*ant(6)-Ia::::catB::msr(D):mef(A):recombinase:recombinase:::recombinase :::IS6:::::::::::::dfrG::recombinase*	*E. faecalis* strain Transconjugant T4 plasmid pJH-T4 (KY290886.1)
	40	*tet(S)*::::IS6	*E. faecalis* strain C386 transposon Tn6000 (JN208881.1)

Intact prophages were found within 9/12 (75%) of the genomes. Three isolates comprising one *E. faecalis* and two *E. hirae* did not possess any intact prophages. A total of seven intact prophages were identified across all the investigated isolates, with Lister_LP_101 and Entero_EFC_1 being unique to individual isolates (**Table 1**). The Entero_phiFL1A was the most common prophage occurring in three different isolates from the upstream, influent, and effluent sites. The Entero_phiFL3A (n = 2) occurred in *E. faecalis* isolates from the upstream and influent site, Lister_B025 (n = 2) occurred in *E. faecium* isolates from the influent and effluent sites. The occurrence of intact prophages was not according to species, as several prophages occurred in different species including Entero_phiFL1A (*E. faecalis* and *E. durans*), Lister_B025 (*E. faecium* and *E. hirae*), and Bacill_BCJA1c (*E. faecium* and *E. durans*). *E. faecalis* and *E. faecium* isolates did not have any intact prophages in common. The intact prophages did not occur according to sequence type, although *E. faecium* isolates (D95, D98) belonging to ST179 had the same prophage Entero_vB_IME197.

Seven isolates had regions encoding the T4SS type ICE, with one *E. faecium* isolate (U129) from the upstream site and the effluent isolate *E. durans* (E115) having three regions each (**Table 1**). The IMEs were detected in five isolates (3 *E. faecalis* and 2 *E. faecium*). Two isolates (one *E. faecalis* and one *E. faecium* isolate) harboured both the ICE and IME. *E. hirae* isolates did not harbour any of the stated MGEs except for insertion sequences implying that these might be central in horizontal gene transfer, however, none of the ARGs in these isolates were associated with MGEs. The genome of environmental *Enterococcus* spp. consists of a rich diversity of MGEs including ISs, transposons, prophages, and plasmids that probably drive genetic exchange within and among these species.

Virulome of *Enterococcus* Isolates

A diversity of virulence genes was found in the *E. faecium* and *E. faecalis* isolates with none identified in *E. hirae* and *E. durans* (**Table 4**). For *E. faecalis*, a total of 20 different virulence genes were identified, including genes encoding sex pheromones, adhesion, cell invasion, aggregation, toxins, biofilm formation, cytolysin production, immunity, antiphagocytic activity, and proteases (**Table 4**). All the *E. faecalis* isolates had eleven of these genes (*cCF10*, *cOB1*, *cad*, *cam*E, *ace*, *SrtA*, *ebpA*, *ebpC*, *efaAfs*, *tpx*, and *gelE*) in common. In *E. faecium*, only four virulence genes were identified and included adhesins (*acm* and *efaAfm*), a sex pheromone (*cad*), and an antiphagocytic factor (*tpx*) (**Table 4**).

The virulence genes in *E. faecium* were mostly devoid of any association with MGEs. Among the *E. faecalis* isolates the *gelE* (protease) was co-carried with the *fsrC* (biofilm formation) virulence gene in a genetic environment that had an integrase and IS256. This occurred in two isolates from the influent (IN127) and downstream (D21) sites with genetic context *gelE:fsrC::::integrase:::: IS256* (**Table 5**). The contigs bearing these virulence genes were highly similar (99 - 100%) to a chromosomal sequence in GenBank *Enterococcus faecalis* strain FDAARGOS_324 chromosome (CP028285.1) implying their carriage in the chromosome. Although, several virulence genes were found to occur together in other *E. faecalis* isolates their genetic environment did not contain any MGEs (**Table 5**). This implies that in addition to MGEs like ISs the transfer of virulence

TABLE 4 | Virulence gene profiles of environmental *Enterococcus* spp.

Isolate ID	Point of isolation	Virulence genes								
		Sex pheromones	Adhesion	Invasin	Aggregation	Cytolytic toxin	Biofilm formation	Antiphagocytic	Immunity	Protease
E. faecalis										
IN133 (ST 841)	Influent	*cCF10, cOB1, cad,camE*	*acm, ace, SrtA, efaAfs, ebpA,ebpC*	*hylB*	*agg*	*cylA, cylL,cylM*	*fsrB*	*tpx*	*ElrA*	*gelE*
IN127 (ST 179)	Influent	*cCF10, cOB1, cad, camE*	*ace, SrtA, ebpA, ebpC, efaAfs*	*hylA*	*agg*	*cylA, cylL, cylM*	–	*tpx*	*ElrA*	*gelE*
D21 (ST 179)	Downstream	*cCF10, cOB1, cad, camE*	*ace, SrtA, efaAfs, ebpA, ebpC*	*hylA*	*agg*	*cylA, cylL, cylM*	–	*tpx*	*ElrA*	*gelE*
U84 (ST 300)	Upstream	*cCF10, cOB1, cad, camE*	*ace, SrtA, ebpA, ebpC, efaAfs*	*hylB*	*agg*	–	*fsrB*	*tpx*	–	*gelE*
E. faecium										
IN91 (ST 361)	Influent	–	*Acm, efaAfm*	–	–	–	–	–	–	–
D95 (ST 94)	Downstream	=	*Acm, efaAfm*	–	–	–	–	–	–	–
D98 (ST 94)	Downstream	–	*acm, efaAfm*	–	–	–	–	–	–	–
E21 (ST 1096)	Effluent	–	*efaAfm*	–	–	–	–	–	–	–
U129 (ST 361)	Upstream	*cad*	–	–	–	–	–	*tpx*	–	–

No virulence genes found in E. hirae and E. duran.

TABLE 5 | Mobile genetic elements associated with virulence genes in *E. faecalis* isolates.

Isolate	Contig	Synteny of virulence genes and MGE	Plasmid/chromosomal sequence with closest nucleotide homology (accession number)
E. faecalis			
IN133/11 (ST841)	13	*SrtC:ebpC:ebpB:epbA*	*Enterococcus faecalis* strain TK-P4B chromosome (CP045598.1)
	45	*CylR2:cylL:cylS*	*Enterococcus faecalis* strain FDAARGOS_528 chromosome (CP033787.1)
	48	*gelE:fsrC::fsrA*	*Enterococcus faecalis* strain L15 chromosome (CP042231.1)
IN127/10 (ST179)	8	*Cyl:::cyL-S:cylL-L:cylR2*	*Enterococcus faecalis* strain JY32 chromosome (CP045045.1)
	22	*gelE:fsrC::::integrase::::IS256*	*Enterococcus faecalis* strain FDAARGOS_324 chromosome (CP028285.1)
	25	*SrtC:ebpC:ebpB:epbA*	*Enterococcus faecalis* strain JY32 chromosome (CP045045.1)
D21/6 (ST179)	16	*gelE:fsrC::::integrase::::IS256*	*Enterococcus faecalis* strain FDAARGOS_324 chromosome (CP028285.1)
	30	*Cyl:::cyL-S:cylL-L:cylR2*	*Enterococcus faecalis* strain JY32 chromosome (CP045045.1)
U84/9 (ST300)	17	*ebpA:ebpB:ebpC:srtC*	*Enterococcus faecalis* strain SF28073 chromosome (CP060804.1)
	17	*gelE:fsrC::fsrA*	*Enterococcus faecalis* strain SF28073 chromosome (CP060804.1)

genes may be moderated by other processes that facilitate genetic exchange e.g., natural transformation.

MLST and Phylogenomics

MLST analysis revealed that two *E. faecalis* isolates had a common sequence type ST179; the rest of the isolates had unique sequence types, ST841, and ST300 (**Table 1**). The *E. faecium* genomes belonged to three sequence types, ST94 ($n = 2$), ST361 ($n = 2$), and ST1096 ($n = 1$).

Phylogenetic analysis of the *E. faecalis* genomes from this study and those from other studies in Africa showed that the isolates were more closely related to animal and environmental isolates than to clinical isolates (**Figure 1**). An isolate obtained from the influent (IN133 and ST841) was more closely related to a Tunisian isolate (1351.1813, ST859) from chicken meat. An

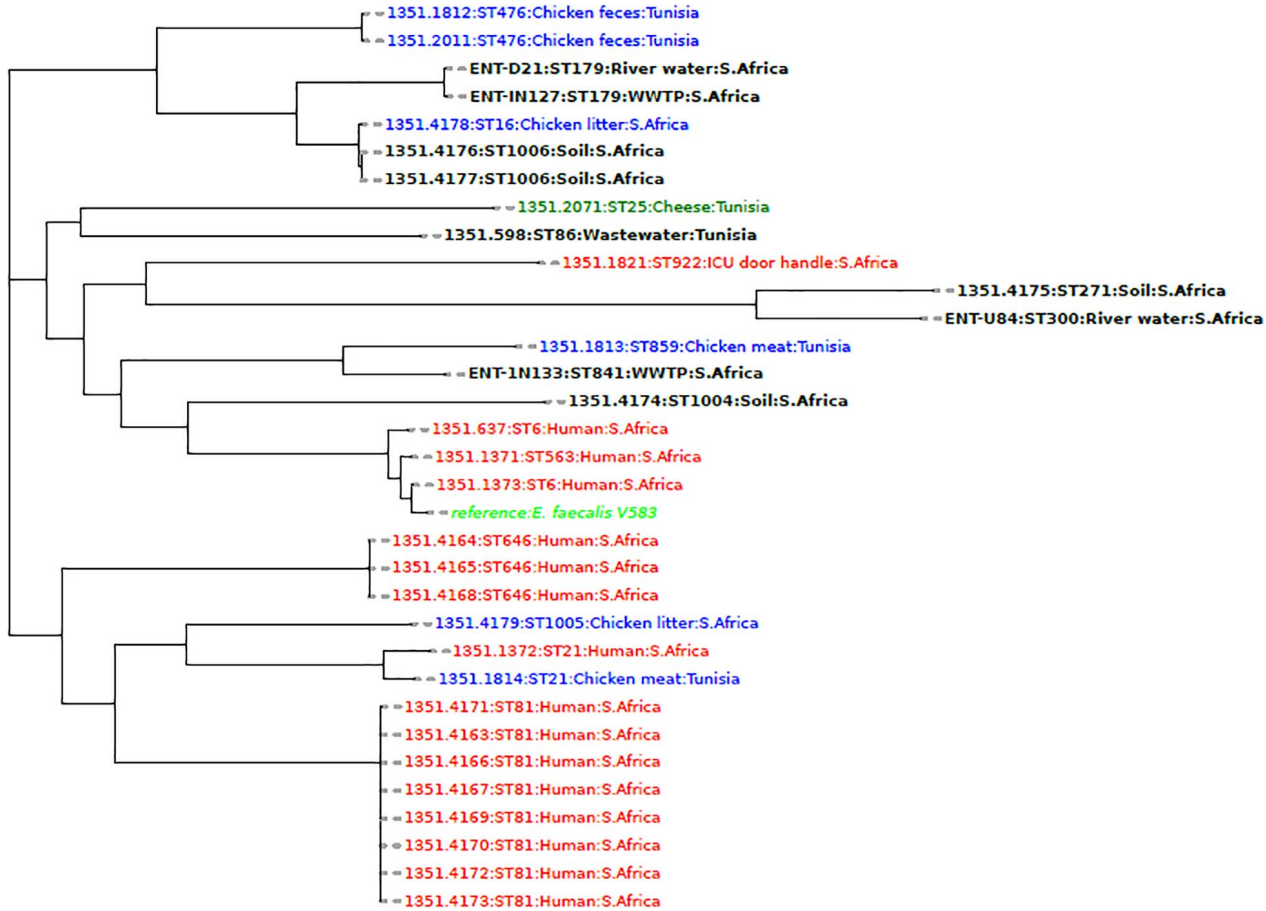

FIGURE 1 | A phylogenomic tree showing the relationship of *E. faecalis* isolates from this study with African isolates from humans (red), animals (blue), and the environment (black) obtained from the PATRIC database (https://www.patricbrc.org/).

isolate (U84, ST300) from the upstream site was closely related to an isolate (1351.4175, ST271) from agricultural soil obtained in the same district of uMgungundlovu in KZN, South Africa. However, the other two isolates (D21, IN127), both ST179, clustered together and were in the same node as environmental isolates obtained from the soil and chicken litter of a sugarcane farm in KZN, South Africa (**Figure 1**).

Comparison of *E. faecium* genomes with other WGS isolates from Africa revealed that the isolates from this study D95, D98 (ST94), and IN91, U129 (ST361) clustered together according to sequence type, but formed a separate clade with isolates obtained from the chicken litter at a sugarcane farm in KZN, South Africa. An isolate E21 (ST1096), was found in a different clade and clustered closely with a South African soil isolate from the same farm in KZN (**Figure 2**).

Phylogenetic analysis of *E. hirae* isolates revealed that the *E. hirae* isolates were more closely related to livestock and environmental isolates. The upstream isolate U73 was closely related and clustered closely with isolates from Goa (Tibetan antelope) fecal matter obtained in China, suggesting that the isolate could be of animal origin. The other isolate (D76) was also closely related and clustered together with isolates from fermented vegetables from Malaysia, signifying that the isolate may be from an agricultural source (**Figure 3**). The *E. durans* isolate clustered closely with a bovine isolate (53345.56) obtained from South Africa and an isolate frim chicken (53345.33) from the United States implying that it is an animal-associated isolate (**Figure 4**).

DISCUSSION

Bioinformatics tools were used to analyze the whole genomes of MDR *Enterococcus* isolates (n = 12) with similar antibiograms obtained from a WWTP and the receiving water bodies at different sampled points and at different timelines. While many ARGs were carried on plasmids, transposable elements, and insertion sequences, most were, carried on chromosomes with no association with MGEs. A few virulence genes were associated with ISs, with most occurring on chromosomes. The abundance of MGEs observed in the Enterococci genomes, however, signifies their importance in gene rearrangements and horizontal gene transfer in these environmental isolates. This study is one of the first studies to explore the resistome, virulome, mobilome,

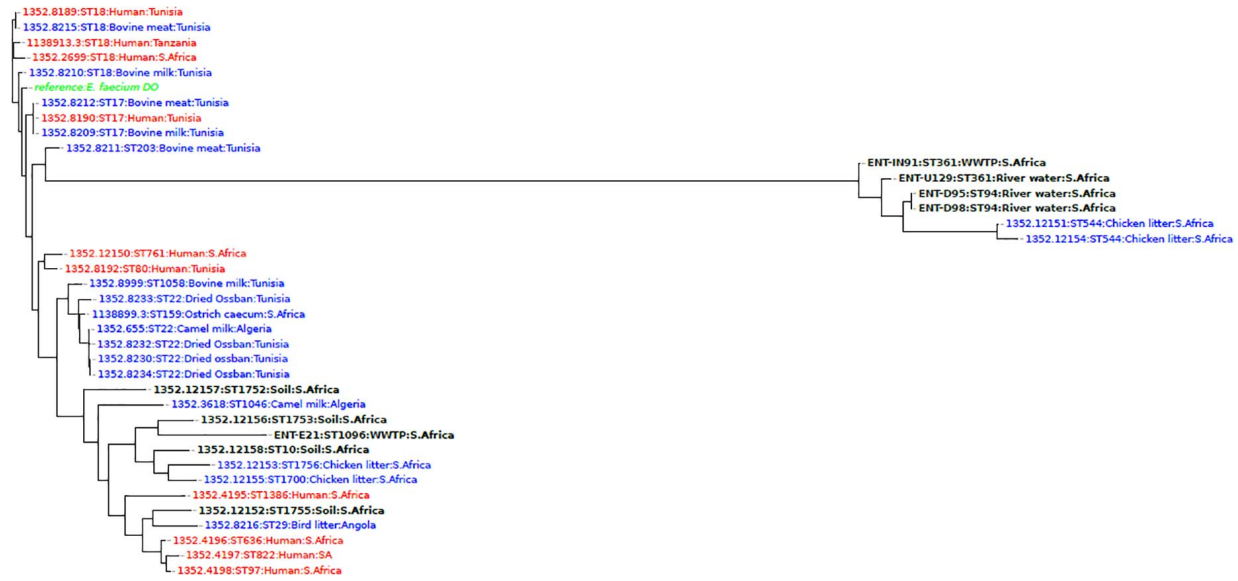

FIGURE 2 | A phylogenomic tree showing the relationship of *E. faecium* isolates from this study with African isolates from humans (red), animals (blue), and the environment (black) obtained from the PATRIC database (https://www.patricbrc.org/).

clonality, and phylogenomics of *Enterococcus* spp. obtained from the water environment in Africa.

Tetracycline resistance genes identified in this study included the *tet(M), tet*(S), and *tet(L)*. The *tet(M)* gene was present in the *E. durans* isolate and in all the *E. faecium* and *E. faecalis* isolates (**Table 1**). The *tet(M)* and *tet(S)* encode for ribosome protection proteins and the *tet(L)* encodes an efflux pump (Tao et al., 2010). There was a high concordance between the phenotypic AST and genotypic data with regards to TET resistance. The genetic context *tet(M):tetrLpep:TcpC* (or its reverse) was found in 9/10 (90%) of isolates that harboured the *tet(M)* gene (**Table 3**). The TcpC gene encodes a conjugative TcpC which is essential for efficient conjugative transfer and has previously been associated with conjugative tetracycline resistance plasmids in *Clostridium perfringens* (Bannam et al., 2006). Most of the *tet(M)* genes were located on chromosomes except for *E. faecalis* isolate U84 where the genetic context was associated with a plasmid. The genes involved in ribosome protection including *tet(M)* are typically found on both plasmids and self-conjugative transposons in chromosomes (Roberts, 1996) as evidenced in this study. Transfer of *tet(M)* in environmental Enterococci is possibly mediated by the conjugative TcpC.

Resistance to macrolides was associated mainly with the *erm(B)* and *msr(C)* genes. The *erm(B)* encodes a ribosomal methylase and is considered to be the most common gene responsible for resistance to erythromycin in enterococci; the methylase can also result in resistance to lincosamides, and streptogramin B (Miller et al., 2015). The rRNA methylases, *erm(A), erm(B),* and *erm(C)* modify specific nucleotides in the 23S rRNA and block macrolide binding (Chancey et al., 2015). Resistance to macrolides may also be caused by mutations in the 23S ribosomal RNA gene or be mediated by efflux pumps (Miller et al., 2015). All isolates that were phenotypically resistant to erythromycin had the *erm(B)* gene (**Table 1**). In *Enterococcus* spp. the *erm(B)* gene is considered the most widespread erythromycin resistance gene (Zaheer et al., 2020). The *msr(C)* gene which encodes an efflux pump was identified in all the *E. faecium* isolates which is consistent with earlier studies that stated that the gene seems to be specific for this species (Zaheer et al., 2020). The genome of the *E. durans* isolate had the efflux pump encoding genes *msr(D)* and *mef(A)* which were unique to this isolate. The macrolide efflux (*mef*) genes were initially identified in *Streptococcus pyogenes* (Sutcliffe et al., 1996) and *S. pneumoniae* (Gay and Stephens, 2001) and have been noted to always occur upstream and to be co-transcribed with an ATP-binding subunit ABC-transporter gene *msr(D)*, functioning as a dual efflux pump (Ambrose et al., 2005). The genes were located on a resistance island consisting of MGEs (recombinase, IS6) together with chloramphenicol, aminoglycoside, and trimethoprim resistance genes (**Table 3**). There is a possibility that this resistance island is transmissible within and across sub-species, although its transferability was not experimentally investigated. Although *E. durans* strains rarely cause infection, the occurrence of these resistance genes implies the importance of these organisms as environmental reservoirs which could potentially mediate the transfer of these genes to pathogens of clinical or veterinary importance.

Enterococci are inherently resistant (low-level) to aminoglycosides, mostly due to the presence of the *aac(6′)-Ii* gene. Some isolates, however, exhibit high-level resistance to gentamicin and streptomycin and are clinically important (Sanderson et al., 2020). The presence of other acquired aminoglycoside resistance genes including *aac*(6′)-Ie–*aph*(2″)-Ia, *aph*(3′)-IIIa, and *ant*(6)-*Ia* confers high-level resistance to various aminoglycosides (Said et al., 2015). Except for *E. durans* isolate E115 none of the aminoglycoside resistance genes were

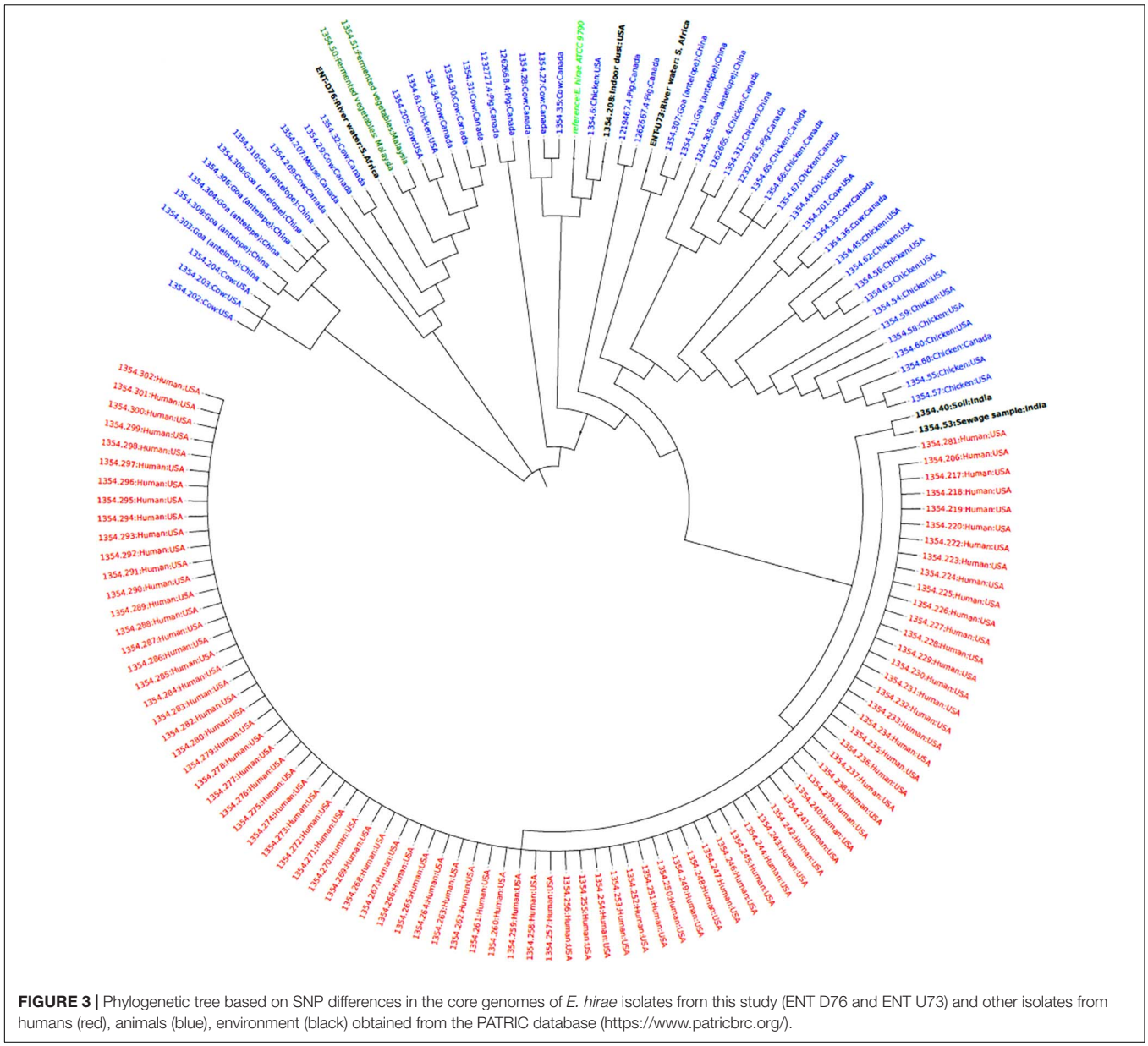

FIGURE 3 | Phylogenetic tree based on SNP differences in the core genomes of *E. hirae* isolates from this study (ENT D76 and ENT U73) and other isolates from humans (red), animals (blue), environment (black) obtained from the PATRIC database (https://www.patricbrc.org/).

associated with MGEs and most were borne on the chromosome (**Table 3**). Isolate E115 had the *ant*(6)-*Ia* gene which formed part of a resistance island that was on a plasmid. The isolate exhibited high-level resistance to streptomycin (**Table 1**).

A diversity of virulence genes was identified in the genomes of the sequenced *E. faecalis* and *E. faecium* isolates (**Table 4**). The *gelE* and *fsr* genes have been shown to occur together in *E. faecalis* isolates from healthy and sick animals (Šeputiene et al., 2012). The *fsr*ABDC operon has been shown to regulate the expression of the *gelE* gene and other virulence genes (Hancock and Perego, 2004). The *gelE* encodes an extracellular zinc endopeptidase that cleaves a broad range of substrates including collagen and gelatin. It accentuates the pathogenesis of endocarditis caused by *E. faecalis* (Thurlow et al., 2010). The *gelE* and *fsr* genes occurred together in several *E. faecalis* isolates including IN133 and U84 that had genetic context *gelE:fsrC::fsrA* (**Table 5**). Isolates D21 and IN127 both had genetic context *gelE:fsrC::::integrase::::IS256* suggesting that IS256 plays a role in the transmission of these virulence genes. The IS256 is prevalent in the genomes of MDR enterococci and staphylococci where it occurs either independently or is associated with ARGs or virulence genes involved in biofilm formation (Hennig and Ziebuhr, 2010; Kim et al., 2019). Other virulence genes were not associated with MGEs suggesting that processes like natural transformation may be important in the transfer of these genes. Generally, the repertoire of virulence genes revealed in this study point to the presence of potentially pathogenic *Enterococcus* spp. in the investigated water environment.

MLST revealed distinct sequence types that are associated with clinical, animal, and agricultural sources. For *E. faecalis*

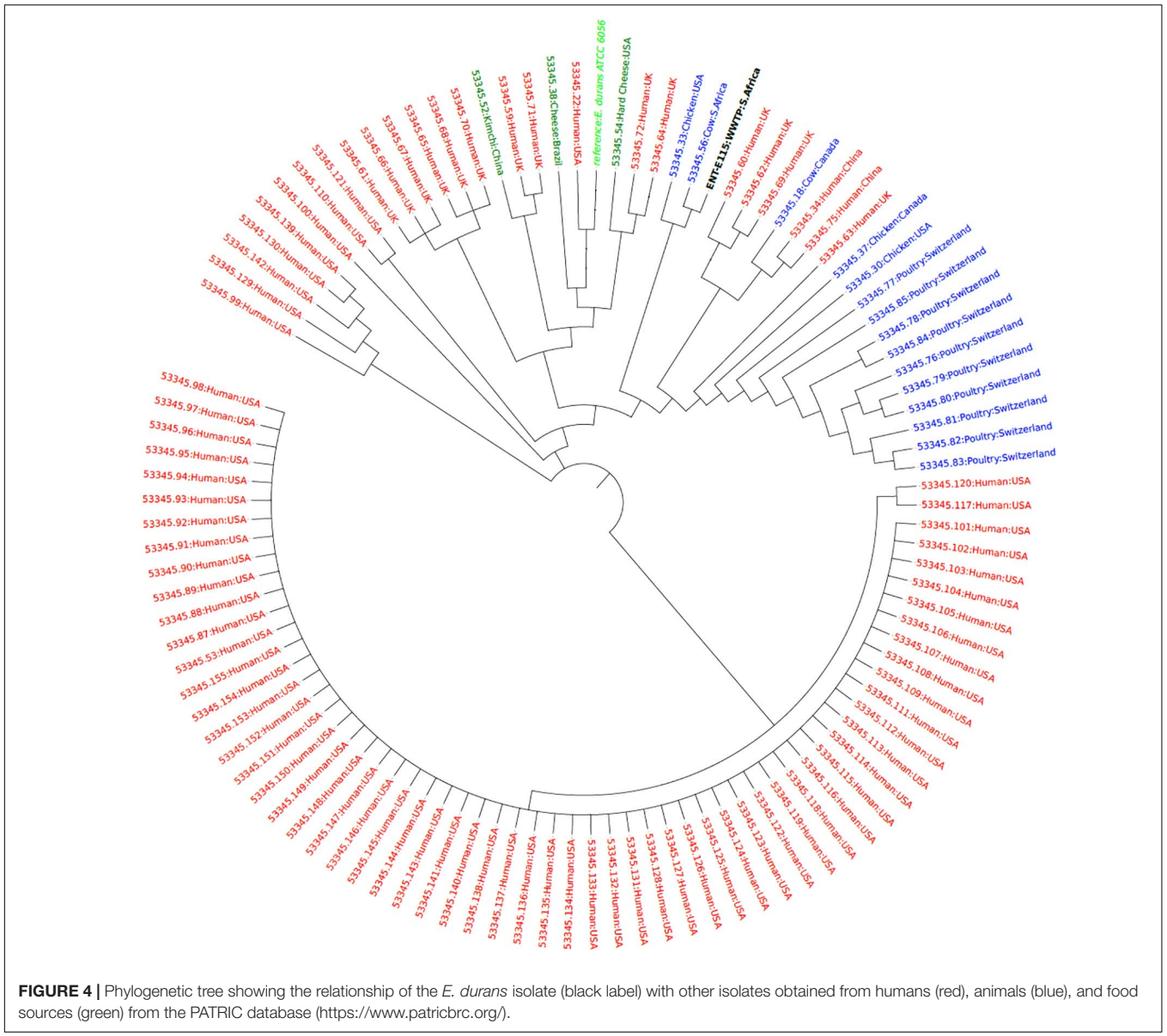

FIGURE 4 | Phylogenetic tree showing the relationship of the *E. durans* isolate (black label) with other isolates obtained from humans (red), animals (blue), and food sources (green) from the PATRIC database (https://www.patricbrc.org/).

isolates, ST179 was the most common sequence type (n = 2) a finding similar to other studies (Zaheer et al., 2020). For the *E. faecium* isolates, ST94 (n = 2) and ST361 (n = 2) were the most prevalent sequence types. In the study by Zaheer et al. (2020) the ST94 was the most abundant ST in the cattle feedlot catch basin and was found in other sources namely, surface water, urban wastewater, and from the clinic. This possibly points to the ubiquitous nature of this sequence type. The ST361 is not one of the notable *E. faecium* STs as it has not been implicated in clinical cases which are mostly attributed to the ST17, ST18 and, ST78 lineages (Palmer et al., 2014). A study from the United Kingdom used WGS to investigate the prevalence of vancomycin-resistant *E. faecium* in 20 WWTPs and reported an *E. faecium* ST361 from a WWTP (Gouliouris et al., 2018).

The phylogenomics of *E. faecalis* and *E. faecium* isolates revealed that all isolates were closely related to environmental or animal isolates, and not clinical isolates (**Figures 1**, **2**). However, the *E. faecalis* influent isolate (IN133, ST841) harbored the cytolysin genes that have been attributed to clinical *E. faecalis* isolates intimating pathogenic potential (Zaheer et al., 2020). Phylogenetic analysis of the *E. hirae* isolates in this study revealed a close association with other animal and environmental isolates (**Figure 3**). *E. hirae* is known to inhabit a variety of animals and plants (Byappanahalli et al., 2012) and has been widely associated with cattle feces, chicken broilers, and associated production systems (Rehman et al., 2018; Zaheer et al., 2020). The *E. durans* isolate was closely related to animal isolates (**Figure 4**), indicating that it may be of animal origin. *E. durans* isolates are known to inhabit humans, animals, and insects and occasionally cause human infections (Byappanahalli et al., 2012). The isolate investigated in this study lacked virulence determinants and is most likely a potential reservoir of ARGs. Although a

small subset of *Enterococcus* spp. isolates were used, this study adds to the limited knowledge of the resistome, virulome, mobilome, and phylogenies in environmental Enterococci in Africa. Future studies should look to use a larger sample size and greater diversity of *Enterococcus* spp. from diverse geographical locations.

CONCLUSION

This is the first report of genomic diversity of *Enterococcus* spp. found in wastewater and associated river water in KwaZulu-Natal, South Africa. *Enterococcus* spp. showed a rich repertoire of ARGs and virulence factors implying that the water environment is a substantive reservoir of MDR microbes which are potential pathogens. Genomic analysis of the Enterococci isolates allowed for the description of the resistome, virulome, and mobilome as well as the determination of phylogenetic relationships with animal, agricultural and environmental isolates. Such work allows a deeper understanding of the potential transmission dynamics related to the spread of antibiotic resistance in the water environment.

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

SE, JM, AA, and DA: co-conceptualized the study. JM: performed the experiments and wrote the manuscript. JM, AA, and DA: analyzed the data. SE, AA, and DA: supervision. SE: funding acquisition. All authors undertook critical revision of the manuscript and reviewed, edited, 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.2021.648454/full#supplementary-material

Supplementary Table 1 | Genome and assembly characteristics of sequenced *Enterococcus* spp. isolates from a wastewater treatment plant and its associated waters.

Supplementary Table 2 | Antibiotic resistance genes not associated with any mobile genetic elements in enterococci.

REFERENCES

Alexander, J., Hembach, N., and Schwartz, T. (2020). Evaluation of antibiotic resistance dissemination by wastewater treatment plant effluents with different catchment areas in Germany. *Sci. Rep.* 10, 1–9. doi: 10.1038/s41598-020-65635-4

Ambrose, K. D., Nisbet, R., and Stephens, D. S. (2005). Macrolide efflux in Streptococcus pneumoniae is mediated by a dual efflux pump (mel and mef) and is erythromycin inducible. *Antimicrob. Agents Chemother.* 49, 4203–4209. doi: 10.1128/AAC.49.10.4203-4209.2005

Arndt, D., Grant, J. R., Marcu, A., Sajed, T., Pon, A., Liang, Y., et al. (2016). PHASTER: a better, faster version of the PHAST phage search tool. *Nucleic Acids Res.* 44, W16–W21. doi: 10.1093/nar/gkw387

Bannam, T. L., Teng, W. L., Bulach, D., Lyras, D., and Rood, J. I. (2006). Functional identification of conjugation and replication regions of the tetracycline resistance plasmid pCW3 from Clostridium perfringens. *J. Bacteriol.* 188, 4942–4951. doi: 10.1128/JB.00298-06

Bengtsson-Palme, J., Kristiansson, E., and Larsson, D. G. J. (2018). Environmental factors influencing the development and spread of antibiotic resistance. *FEMS Microbiol. Rev.* 42, 68–80. doi: 10.1093/femsre/fux053

Berendonk, T. U., Manaia, C. M., Merlin, C., Kassinos, D. F., Cytryn, E., Walsh, F., et al. (2013). Tackling antibiotic resistance: the environmental framework. *Nat. Rev. Microbiol.* 13, 310–317. doi: 10.1038/nrmicro3439

Bourafa, N., Loucif, L., Boutefnouchet, N., and Rolain, J. M. (2015). Enterococcus hirae, an unusual pathogen in humans causing urinary tract infection in a patient with benign prostatic hyperplasia: First case report in Algeria. *N. Microbes N. Infect.* 8, 7–9. doi: 10.1016/j.nmni.2015.08.003

Byappanahalli, M. N., Nevers, M. B., Korajkic, A., Staley, Z. R., and Harwood, V. J. (2012). Enterococci in the Environment. *Microbiol. Mol. Biol. Rev.* 76, 685–706. doi: 10.1128/mmbr.00023-12

Chancey, S. T., Bai, X., Kumar, N., Drabek, E. F., Daugherty, S. C., Colon, T., et al. (2015). Transcriptional attenuation controls macrolide inducible efflux and resistance in Streptococcus pneumoniae and in other gram-positive bacteria containing mef/mel (msr(D)) elements. *PLoS One* 10:1–23. doi: 10.1371/journal.pone.0116254

CLSI (2020). *CLSI M100-ED30:2020 Performance Standards for Antimicrobial Susceptibility Testing, 30th Edition*. Available online at: http://em100.edaptivedocs.net/GetDoc.aspx?doc=CLSI M100 ED30:2020 doi: 10.1016/j.drup.2016.04.003 (accessed May 11, 2020).

Conte, D., Kasuko, J., Nogueira, S., Marenda, T., Lima, R., De, et al. (2017). Characterization of CTX-M enzymes, quinolone resistance determinants, and antimicrobial residues from hospital sewage, wastewater treatment plant, and

river water. *Ecotoxicol. Environ. Saf.* 136, 62–69. doi: 10.1016/j.ecoenv.2016.10.031

EUCAST (2020). *Testing Breakpoint tables for interpretation of MICs and zone diameters*. Available online at: https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_10.0_Breakpoint_Tables.pdf (accessed May 11, 2020).

Gay, K., and Stephens, D. S. (2001). Structure and dissemination of a chromosomal insertion element encoding macrolide efflux in Streptococcus pneumoniae. *J. Infect. Dis.* 184, 56–65. doi: 10.1086/321001

Gouliouris, T., Raven, K. E., Ludden, C., Blane, B., Corander, J., Horner, C. S., et al. (2018). Genomic surveillance of enterococcus faecium reveals limited sharing of strains and resistance genes between livestock and humans in the United Kingdom. *MBio* 9, 1–15. doi: 10.1128/mBio.01780-18

Hancock, L. E., and Perego, M. (2004). The Enterococcus faecalis fsr two-component system controls biofilm development through production of gelatinase. *J. Bacteriol.* 186, 5629–5639. doi: 10.1128/JB.186.17.5629-5639.2004

Hendriksen, R. S., Bortolaia, V., Tate, H., Tyson, G. H., Aarestrup, F. M., and McDermott, P. F. (2019). Using Genomics to Track Global Antimicrobial Resistance. *Front. Public Heal.* 7:00242. doi: 10.3389/fpubh.2019.00242

Hennig, S., and Ziebuhr, W. (2010). Characterization of the transposase encoded by IS256, the prototype of a major family of bacterial insertion sequence elements. *J. Bacteriol.* 192, 4153–4163. doi: 10.1128/JB.00226-10

Huijbers, P. M. C., Flach, C. F., and Larsson, D. G. J. (2019). A conceptual framework for the environmental surveillance of antibiotics and antibiotic resistance. *Environ. Int.* 130:104880. doi: 10.1016/j.envint.2019.05.074

Kaas, R. S., Leekitcharoenphon, P., Aarestrup, F. M., and Lund, O. (2014). Solving the problem of comparing whole bacterial genomes across different sequencing platforms. *PLoS One* 9, 1–8. doi: 10.1371/journal.pone.0104984

Karkman, A., Do, T. T., Walsh, F., and Virta, M. P. (2018). Antibiotic-resistance genes in waste water. *Trends Microbiol.* 26, 220–228. doi: 10.1016/j.tim.2017.09.005

Karkman, A., Do, T. T., Walsh, F., and Virta, M. P. J. (2017). Antibiotic-resistance genes in wastewater. *Trends Microbiol.* 26, 220–228. doi: 10.1016/j.tim.2017.09.005

Ke, D., Picard, O. I. S. J., Martineau, F., Me, C., Roy, P. H., Ouellette, M., et al. (1999). Development of a PCR Assay for Rapid Detection of Enterococci. *J. Clin. Microbiol.* 37, 3497–3503. doi: 10.1128/jcm.37.11.3497-3503.1999

Kim, Y. Bin, Seo, K. W., Shim, J. B., Son, S., Hyun, N. E. B., and Lee, Y. J. (2019). Molecular characterization of antimicrobial-resistant Enterococcus faecalis and Enterococcus faecium isolated from layer parent stock. *Poult. Sci.* 98, 5892–5899. doi: 10.3382/ps/pez288

Larsen, M. V., Cosentino, S., Rasmussen, S., Friis, C., Hasman, H., Marvig, L., et al. (2012). Multilocus Sequence Typing of Total-Genome-Sequenced Bacteria. *J. Clin. Microbiol.* 2012, 1355–1361. doi: 10.1128/JCM.06094-11

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., et al. (2009). The Sequence Alignment/Map format and SAMtools. *Bioinformatics* 25, 2078–2079. doi: 10.1093/bioinformatics/btp352

Medeiros, A. W., Pereira, R. I., Oliveira, D. V., Martins, P. D., d'Azevedo, P. A., Van der Sand, S., et al. (2014). Molecular detection of virulence factors among food and clinical Enterococcus faecalis strains in South Brazil. *Brazilian J. Microbiol.* 45, 327–332. doi: 10.1590/S1517-83822014005000031

Miller, W. R., Munita, J. M., Arias, C. A., Santiago, A., De, and Unit, A. R. (2015). Mechanisms of antibiotic resistance in enterococci. *Expert Rev. Anti Infect. Ther.* 12, 1221–1236. doi: 10.1586/14787210.2014.956092

Moodley, B., Birungi, G., and Ndungu, P. (2016). *Detection and Quantification of Emerging Organic Pollutants in the Umgeni and Msunduzi Rivers*. WRC Report No.2215/1/16, South Africa: Commission Pretoria.

Palmer, K. L., van Schaik, W., Willems, R. J., and Gilmore, M. S. (2014). "Enterococcal genomics," in *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection [Internet]*.

Price, M. N., Dehal, P. S., and Arkin, A. P. (2010). FastTree 2 - Approximately maximum-likelihood trees for large alignments. *PLoS One* 5:9490. doi: 10.1371/journal.pone.0009490

Rehman, M. A., Yin, X., Zaheer, R., Goji, N., Amoako, K. K., McAllister, T., et al. (2018). Genotypes and Phenotypes of Enterococci Isolated From Broiler Chickens. *Front. Sustain. Food Syst.* 2:83. doi: 10.3389/fsufs.2018.00083

Roberts, M. C. (1996). Tetracycline resistance determinants: Mechanisms of action, regulation of expression, genetic mobility, and distribution. *FEMS Microbiol. Rev.* 19, 1–24. doi: 10.1016/0168-6445(96)00021-6

Ryu, B. H., Hong, J., Jung, J., Kim, M. J., Sung, H., Kim, M. N., et al. (2019). Clinical characteristics and treatment outcomes of Enterococcus durans bacteremia: a 20-year experience in a tertiary care hospital. *Eur. J. Clin. Microbiol. Infect. Dis.* 38, 1743–1751. doi: 10.1007/s10096-019-03605-z

Said, B. L., Klibi, N., Lozano, C., Dziri, R., Slama, B. K., Boudabous, A., et al. (2015). Diversity of enterococcal species and characterization of high-level aminoglycoside resistant enterococci of samples of wastewater and surface water in Tunisia. *Sci. Total Environ.* 53, 11–17. doi: 10.1016/j.scitotenv.2015.05.091

Sanderson, H., Ortega-Polo, R., Zaheer, R., Goji, N., Amoako, K. K., Brown, R. S., et al. (2020). Comparative genomics of multidrug-resistant Enterococcus spp. isolated from wastewater treatment plants. *BMC Microbiol.* 20:1–17. doi: 10.1186/s12866-019-1683-4

Šeputiene, V., Bogdaite, A., Ružauskas, M., and Sužiedeliene, E. (2012). Antibiotic resistance genes and virulence factors in Enterococcus faecium and Enterococcus faecalis from diseased farm animals: Pigs, cattle and poultry. *Pol. J. Vet. Sci.* 15, 431–438. doi: 10.2478/v10181-012-0067-6

Siguier, P. (2006). ISfinder: the reference centre for bacterial insertion sequences. *Nucleic Acids Res.* 34, D32–D36. doi: 10.1093/nar/gkj014

Su, M., Satola, S. W., and Read, T. D. (2019). Genome-based prediction of bacterial antibiotic resistance. *J. Clin. Microbiol.* 57, 1–15. doi: 10.1128/JCM.01405-18

Sutcliffe, J., Tait-Kamradt, A., and Wondrack, L. (1996). Streptococcus pneumoniae and Streptococcus pyogenes resistant to macrolides but sensitive to clindamycin: A common resistance pattern mediated by an efflux system. *Antimicrob. Agents Chemother.* 40, 1817–1824. doi: 10.1128/aac.40.8.1817

Tao, R., Ying, G., Su, H., Zhou, H., and Sidhu, J. P. S. (2010). Detection of antibiotic resistance and tetracycline resistance genes in *Enterobacteriaceae* isolated from the Pearl rivers in South China. *Environ. Pollut.* 158, 2101–2109. doi: 10.1016/j.envpol.2010.03.004

Thurlow, L. R., Thomas, V. C., Narayanan, S., Olson, S., Fleming, S. D., and Hancock, L. E. (2010). Gelatinase contributes to the pathogenesis of endocarditis caused by Enterococcus faecalis. *Infect. Immun.* 78, 4936–4943. doi: 10.1128/IAI.01118-09

WHO (2020). *GLASS whole-genome sequencing for surveillance of antimicrobial resistanc*. Geneva: WHO.

Zaheer, R., Cook, S. R., Barbieri, R., Goji, N., Cameron, A., Petkau, A., et al. (2020). Surveillance of Enterococcus spp. reveals distinct species and antimicrobial resistance diversity across a One-Health continuum. *Sci. Rep.* 10, 1–16. doi: 10.1038/s41598-020-61002-5

Zhou, Y., Liang, Y., Lynch, K. H., Dennis, J. J., and Wishart, D. S. (2011). PHAST: A Fast Phage Search Tool. *Nucleic Acids Res.* 39, 347–352. doi: 10.1093/nar/gkr485

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Analysis of Antibiotic Resistance Genes, Environmental Factors, and Microbial Community From Aquaculture Farms in Five Provinces, China

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The excessive use of antibiotics speeds up the dissemination and aggregation of antibiotic resistance genes (ARGs) in the environment. The ARGs have been regarded as a contaminant of serious environmental threats on a global scale. The constant increase in aquaculture production has led to extensive use of antibiotics as a means to prevent and treat bacterial infections; there is a universal concern about the environmental risk of ARGs in the aquaculture environment. In this study, a survey was conducted to evaluate the abundance and distributions of 10 ARGs, bacterial community, and environmental factors in sediment samples from aquatic farms distributed in Anhui (AP1, AP2, and AP3), Fujian (FP1, FP2, and FP3), Guangxi (GP1, GP2, and GP3), Hainan (HP1, HP2, and HP3), and Shaanxi (SP1, SP2, and SP3) Province in China. The results showed that the relative abundance of total ARGs was higher in AP1, AP2, AP3, FP3, GP3, HP1, HP2, and HP3 than that in FP1, FP2, GP1, GP2, SP1, SP2, and SP3. The *sul1* and *tetW* genes of all sediment samples had the highest abundance. The class 1 integron (*intI1*) was detected in all samples, and the result of Pearson correlation analysis showed that the *intI1* has a positive correlation with the *sul1*, *sul2*, *sul3*, bla_{OXA}, *qnrS*, *tetM*, *tetQ*, and *tetW* genes. Correlation analysis of the bacterial community diversity and environmental factors showed that the Ca^{2+} concentration has a negative correlation with richness and diversity of the bacterial community in these samples. Of the identified bacterial community, Proteobacteria, Firmicutes, Chloroflexi, and Bacteroidota were the predominant phyla in these samples. Redundancy analysis showed that environmental factors (TN, TP, Cl^-, and Ca^{2+}) have a positive correlation with the bacterial community (AP1, GP1, GP2, GP3, SP1, SP2, and SP3), and the abundance of ARGs (*sul1*, *tetW*, *qnrS*, and *intI1*) has a positive correlation with the bacterial community (AP2, AP3, HP1, HP2, and HP3). Based on the network analysis, the ARGs (*sul1*, *sul2*, bla_{CMY}, bla_{OXA}, *qnrS*, *tetW*, *tetQ*, *tetM*, and *intI1*) were found

to co-occur with bacterial taxa from the phyla Chloroflexi, Euryarchaeota, Firmicutes, Halobacterota, and Proteobacteria. In conclusion, this study provides an important reference for understanding the environmental risk associated with aquaculture activities in China.

Keywords: aquaculture, sediment, antibiotic resistance genes, environmental factors, bacterial community

INTRODUCTION

Antibiotics are extensively used to prevent and control bacterial infections in medical care, livestock husbandry, and aquaculture (Kümmerer, 2009; Luo et al., 2010). Some of them are also used as growth promoters in aquaculture activities (Chen H. et al., 2018). However, excessive antibiotics and their metabolites would enter the environment, and they might be further absorbed into soil particles and eventually accumulated in sediments because aquatic animals cannot take full advantage of these antibiotics (Kümmerer, 2009). It is worth noting that the abundance of antibiotic resistance genes (ARGs) in soil was associated with the amount of antibiotic residues in the environment (Fahrenfeld et al., 2014), and the ARGs combining with minerals and humus from the environment might exist for a long time (Dang et al., 2017; Hurst et al., 2019; Ma et al., 2019). It is well known that the ARGs have unique biological characteristics, and they could spread by horizontal gene transfer among bacteria of different species and self-amplify among the same species (Guo et al., 2017; Kumar et al., 2017).

The sediments were regarded as an important plot for accumulation and transmission of ARGs (Marti et al., 2014). Shen et al. (2020) reported that several ARGs (*sul1*, *tetG*, *tetW*, *tetX*, and *intl1* gene) were detected in water and sediment of aquaculture farms in Jiangsu Province, China. Chen B. et al. (2018) explored the ARGs in the sediments from bullfrog farms and confirmed that these identified ARGs were able to encode resistance to over 10 categories of antibiotics, such as aminoglycosides, beta-lactams, chloramphenicols, fluoroquinolones, macrolides, polypeptides, sulfonamides, and tetracyclines.

There is a universal concern that the presence of ARGs in sediments is a potential environmental threat (Wang et al., 2015). The antibiotic-resistant bacteria have constituted a huge repository of ARGs in sediments (Martínez, 2008). Once these ARGs have been transferred into the human symbiotic microbes, they would cause great risks of the ecological environment and human health (Smillie et al., 2011; Forsberg et al., 2012). Currently, a study about ARGs in the environment suggested that the existing forms of ARGs largely determine the ways in which these genes are acquired and disseminated among bacterial hosts (Mao et al., 2014). The conception of integron was first proposed by Stokes in 1989 (Stokes and Hall, 1989). It is a key pathway for bacteria to acquire ARGs, which influenced the removal and transfer of ARGs in the bacterial community (Gaze et al., 2011). As one of the most important mobile genetic materials, the integron could capture, rearrange, and express mobile gene cassettes responsible for the spread of ARGs (Zhang X. et al., 2020) and further accelerate the prevalence and transmission of ARGs in the environment (Martinez-Freijo et al., 1998; Cambray et al., 2010).

Previous studies have suggested that the nutrients also promote directly or indirectly the ARGs propagation (Zhao et al., 2017; Zhang J. et al., 2020). Furthermore, the long-time input of nitrogen and phosphorus not only changed the composition of the bacterial community but also drove the propagation of ARGs (Pan et al., 2020). Total organic carbon (TOC) and total dissolved nitrogen (TDN) were potentially important environmental factors, which affected the abundance and diversity of ARGs in urban river systems (Zhou et al., 2017). Moreover, some researches have indicated that bacterial communities shaped the distribution and abundance of ARGs (Huerta et al., 2013; Xiong et al., 2015). These findings suggested that the distribution and prevalence of ARGs are not only related to the use of antibiotics but also affected by many environmental factors. In this study, we aimed to (1) evaluate the relative abundance of 10 ARGs in sediment samples from different aquaculture farms; (2) elucidate the correlation between environmental factors, ARGs abundance, and bacterial community in different aquaculture farms; (3) identify the co-occurrence patterns between ARGs and bacterial taxa.

MATERIALS AND METHODS

Sample Collection

A total of 15 sediment samples were collected from aquaculture ponds distributed in five Chinese provinces, including Anhui (Wuwei, Freshwater aquaculture farm), Fujian (Zhangzhou, Mariculture farm), Guangxi (Qinzhou, Mariculture farm), Hainan (Haikou, Freshwater aquaculture farm), and Shaanxi (Heyang, Freshwater aquaculture farm) between September and October 2019 (**Supplementary Figure 1**). Three ponds were selected in every aquaculture farm. These aquaculture ponds could produce aquatic products with average 4,000 kg or more per year. Due to the high stocking density, different antibiotics, including sulfonamides, tetracyclines, beta-lactams, and quinolones were used for prophylactic purposes on these farms. No bacterial infections occurred in the sampled ponds in the past year according to our investigation.

Each pond has an area of approximately 900–1,200 m^2 with a depth of approximately 150–200 cm. The sediments of all sampled ponds have not been cleaned for at least 1 year to ensure that the samples meet the requirements. The samples were collected from water inlets, water outlets, and center areas of each pond (collected the top 10 cm of the sediment) using the CN-100 bottom sampler (Ruibin, China), and the samples of each pond were completely mixed to avoid heterogeneous differences

caused by single sampling. After mixing, the sample was sealed in a sterile plastic bag and transported at 4°C to the laboratory. All the samples were divided into two parts and stored at −80°C for further analysis.

DNA Extraction and Qualitative PCR of Antibiotic Resistance Genes

The genomic DNA was extracted from 0.25 g lyophilized sediment samples using the TIANamp Soil DNA kit (Tiangen, China). All the operations were performed with the product instructions. The quality of DNA was detected using Ultramicro nucleic acid analyzer (Allsheng, China). The PCR amplification was performed to test 10 ARGs (*sul1*, *sul2*, *sul3*, *tetM*, *tetQ*, *tetW*, *qnrB*, *qnrS*, bla_{OXA}, and bla_{CMY}) and class 1 integron integrase gene (*intl1*) based on the investigation of antibiotic use in the aquaculture farms of this study. The primers of target genes were synthesized by Sangon Biotech (Shanghai, China), and the primer sequences are shown in **Supplementary Tables 1, 2**. The PCR conditions were as follows: pre-denaturation at 95°C for 3 min, then 35 cycles of denaturation at 95°C for 30 s, annealing at the specified temperature (**Supplementary Table 1**) for 30 s, extension at 72°C for 45 s, and a final extension at 72°C for 5 min. PCR products were subsequently detected by agarose gel electrophoresis analysis, and the results are shown in **Supplementary Figure 2**.

High-Throughput Sequencing

In order to further analyze the bacterial community composition in the sediment samples, the high-throughput sequencing of bacterial community was analyzed with an Illumina Mi Seq platform at Novogene (Beijing, China). The V3–V4 regions of bacterial 16S rRNA genes were amplified using the primer pair 341F and 806R. The sequencing analysis was processed using QIIME software for 16S rRNA datasets described in a previous literature (Caporaso et al., 2010).

Quantification of Antibiotic Resistance Genes

PCR products of the target gene were purified with the Universal DNA Purification Kit (Tiangen, China) and ligated into pGM-T vector (Tiangen, China). Subsequently, the pGEM-T vector carrying target gene was transformed into *Escherichia coli* DH5α (Tiangen, China), and the positive clones were acquired after PCR amplification and sequence analysis. Moreover, the recombinant plasmids with target gene were extracted with TIANprep Mini Plasmid Kit (Tiangen, China) and searched for homolog identity with NCBI Blast program. The concentration of recombinant plasmids was checked by Ultramicro nucleic acid analyzer (Allsheng, China), and the standard curves of recombinant plasmids were built with 10-fold serial diluted. The amplification efficiency of all primers ranged from 91.07 to 106.64% with $R^2 > 0.99$ (**Supplementary Table 3**). The target gene copy numbers of the sediment samples were calculated with the CT values according to a previous study (Yuan et al., 2019).

The real-time quantitative PCR (Q-PCR) was performed on a LightCycler® 96 instrument (Roche Ltd., Italy) by utilizing SYBR® Green Pro Taq HS Premix (AG, China) according to the manufacturer's protocol for further analysis. The 10-μl qPCR reaction system contained 2 × SYBR® Green Pro Taq HS Premix (5 μl), 10 μM primer (0.2 μl for each primer, Sangon Biotech, China), RNase-free water (4.1 μl), and DNA samples or standard plasmid (0.5 μl). The amplification condition of qPCR was as follows: initial enzyme activation at 95°C for 30 s, then 40 cycles of at 95°C for 5 s and at 60°C for 30 s.

Analysis of Environmental Factors

The contents of Ca^{2+}, Mg^{2+}, and Cl^- were determined by ethylene diamine tetraacetic acid (EDTA) volumetric method (Wang et al., 2020). The concentrations of total nitrogen (TN) and total phosphorus (TP) were determined by spectrophotometric method (SEPA, 2002; Trolle et al., 2009), and the standard curve of TN and TP is shown in **Supplementary Table 4**.

Statistical Analysis

Pearson correlation analysis was used to analyze the correlation between environmental factors (TN, TP, Cl^-, Ca^{2+}, and Mg^{2+}) with the relative abundance of bacterial community and ARGs. Non-metric multidimensional scaling (NMDS) analysis was used to evaluate the difference of bacteria between different sampling sites. Redundancy analysis (RDA) was employed to assess the effects of environmental factors and ARGs on the bacterial community. The co-occurrence between abundance of ARGs and bacterial taxa was analyzed using network analysis based on the Pearson correlation (Li et al., 2015).

Statistical analysis was performed using SPSS 19.0 (IBM, Chicago, IL, United States). The RDA, Mantel test, Pearson correlation, and heatmap were performed in RStudio (v1.2.5019) with several packages, including vegan and pheatmap packages. Co-occurrence networks were constructed by Gephi software (0.9.2; Gephi, WebAtlas, France).

RESULTS

Environmental Factors in the 15 Sediment Samples

To study the effects of environmental factors on bacterial community in the sediments, the concentrations of five factors (TN, TP, Cl^-, Ca^{2+}, and Mg^{2+}) at different sample sites were detected (**Supplementary Table 5**). Among the sampling sites, TN concentrations ranged from 48.841 ± 0.158 to 193.679 ± 0.45 mg/kg (**Figure 1A**). The lowest concentration of TN appeared in the sample of HP3 and the highest in the sample of GP2. The TP concentrations were relatively lower in other sampling sites, except GP1 (118.757 ± 0.956 mg/kg), GP3 (48.064 ± 1.373 mg/kg), and SP1 (48.938 ± 0.323 mg/kg) (**Figure 1B**). The concentration of Cl^- has a higher correlation with the aquaculture environment of sampling sites. The Cl^- concentration in the sample sites from mariculture farms was generally higher than that of other sampling sites (**Figure 1C**). The Ca^{2+} concentration of GP3 and FP2 was the highest

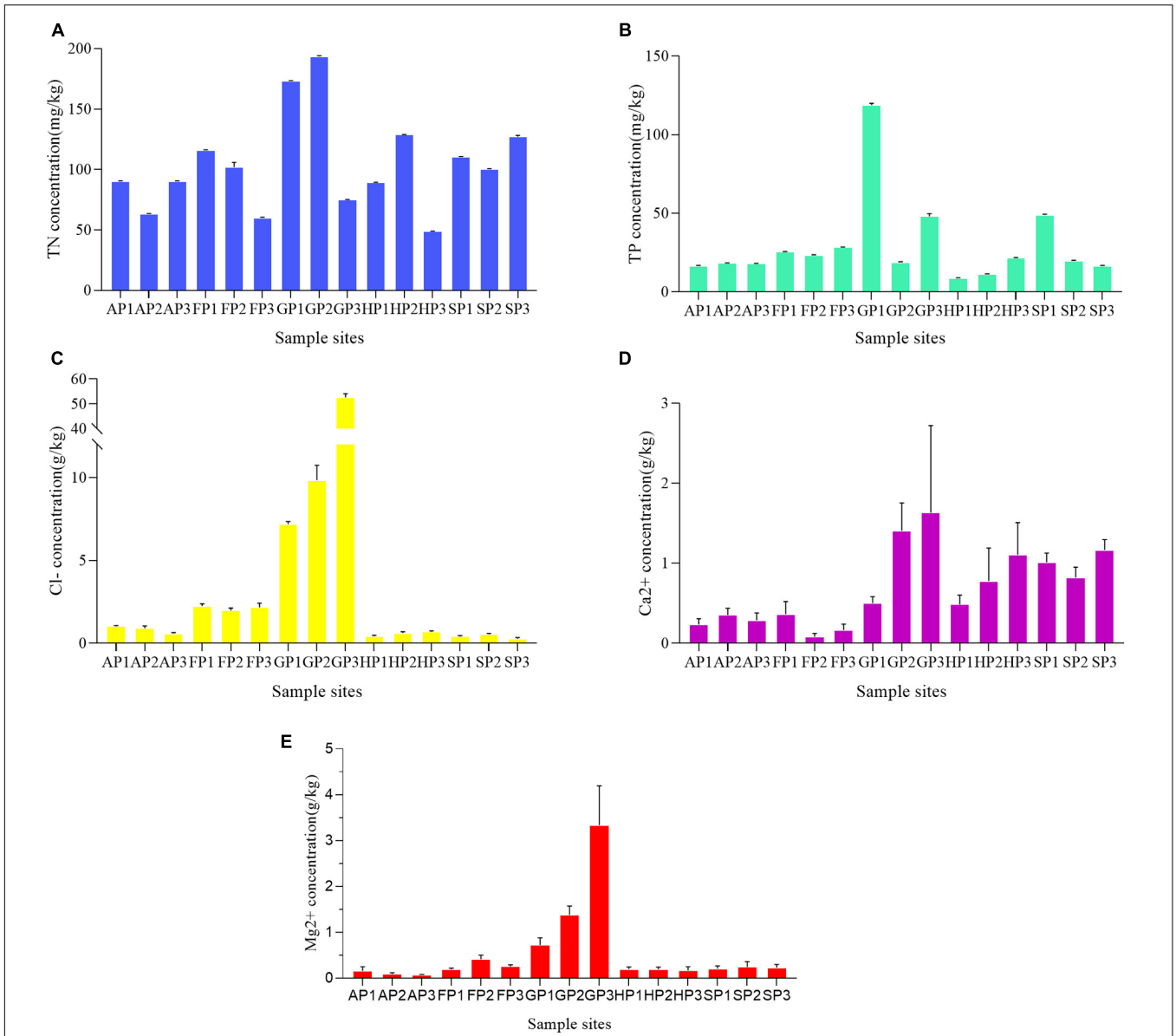

FIGURE 1 | The TN, TP, Cl^-, Ca^{2+}, and Mg^{2+} concentrations **(A–E)** in the 15 sediment samples.

(1.632 ± 0.889 g/kg) and lowest (0.0800 ± 0.0346 g/kg), respectively (**Figure 1D**). The concentrations of Mg^{2+} of all sampling sites were generally lower, with the exception of GP1 (0.720 ± 0.128 g/kg), GP2 (1.377 ± 0.159 g/kg), and GP3 (3.334 ± 0.703 g/kg) (**Figure 1E**).

Bacterial Community in the 15 Sediment Samples

Diversity and Composition of Bacterial Community

With the sequencing of 16S rRNA gene, a total of 61,871 operational taxonomic units (OTUs) were identified from all the 15 sediment samples (**Supplementary Table 6**). The Rank Abundance curves of OTUs were saturated with all samples (**Supplementary Figure 3**), which indicated that the abundance and evenness of bacterial community were similar. The indices of ACE, Chao1, Shannon, and Simpson revealed the richness and diversity of the bacterial communities (**Supplementary Table 6**). The NMDS analysis based on the OTU abundance also indicated that there was no obvious geographic cluster of bacterial communities in different sediment samples (**Supplementary Figure 4**).

At the phylum level, four predominant phyla (Proteobacteria, Firmicutes, Chloroflexi, and Bacteroidota) were detected in all sediment samples (**Figure 2A**). The Cyanobacteria has minor abundance, accounting for 0.30–9.39% of total bacterial 16S rRNA sequence libraries. At the genus level, the 16S rRNA sequence libraries detected 30 predominant bacterial

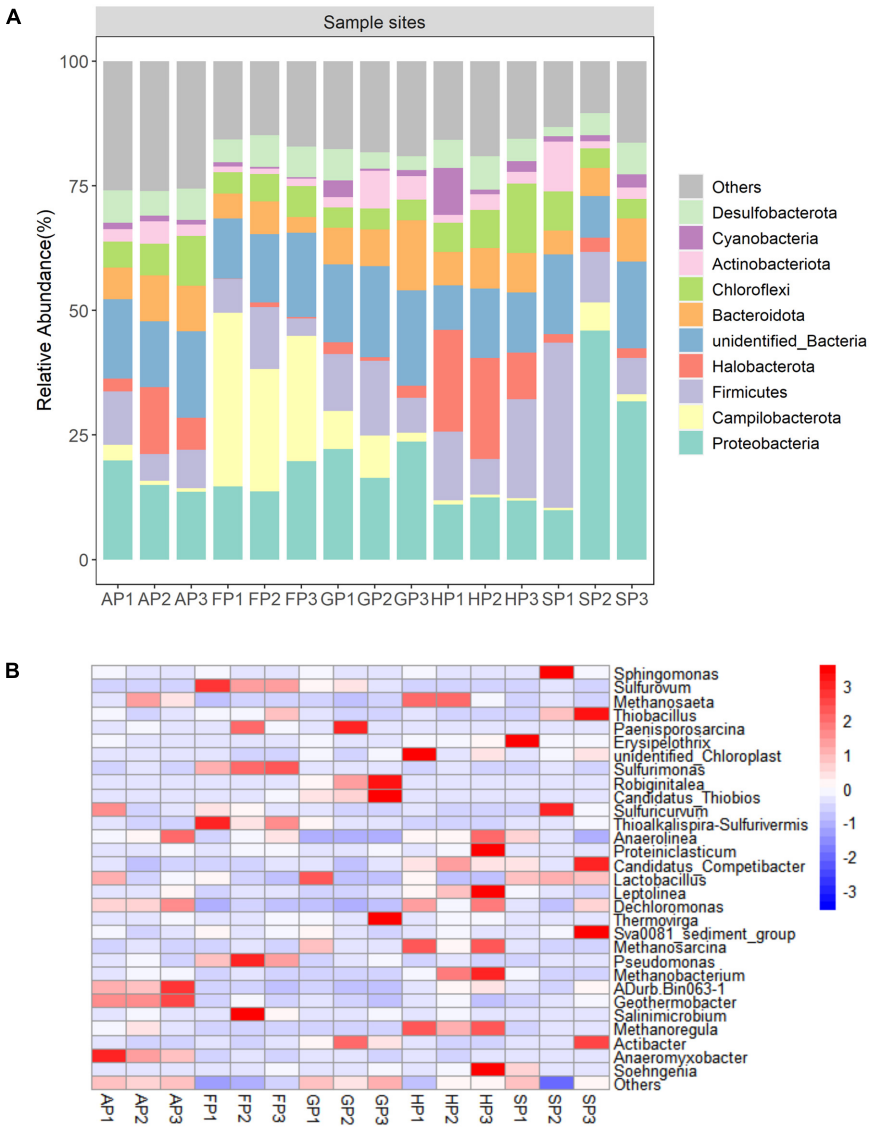

FIGURE 2 | (A) Relative abundance of top 10 phyla in the 15 sediment samples. **(B)** The heatmap at the genus level of the 15 sediment samples.

genes from the 15 sediment samples. The bacterial community mainly includes *Methanosaeta*, *Sphingomonas*, *Sulfurovum*, and *Thiobacillus* (**Figure 2B**). In the phylum Proteobacteria, *Dechloromonas*, *Pseudomonas*, *Sphingomonas*, and *Thiobacillus* were the predominant genera of the bacterial community. In the sediment of FP1, FP2, and FP3, *Sulfurovum* and *Sulfurimonas* have a higher abundance (**Supplementary Figure 5**).

Effect of Environmental Factors on the Diversity of the Bacterial Community

The Pearson correlation analysis was used to evaluate the effects of environmental factors on the bacterial community structure. The results indicated that there was no significant correlation between the concentration of TN, TP, Cl^{-}, and Mg^{2+} with the richness and the diversity of bacterial community. However, the Ca^{2+} concentration had a significantly negative correlation with the richness and diversity of the bacterial community [OTU ($r = -0.61$, $p < 0.05$), ACE ($r = -0.65$, $p < 0.05$), and Chao1 index ($r = -0.62$, $p < 0.05$)].

Abundance and Distribution of Antibiotic Resistance Genes

To analyze the ARGs distribution in different aquaculture farms, 10 tested ARGs and 16S rRNA were investigated. The ARGs abundance was normalized to 16S rRNA genes to compare the difference of ARGs in different samples (Chen et al., 2019). As shown in **Figure 3A**, the ARGs were classified into four categories (sulfonamide, tetracycline, beta-lactam, and fluoroquinolone resistance genes) and integron. The higher abundance of sulfonamide resistance genes was detected in all

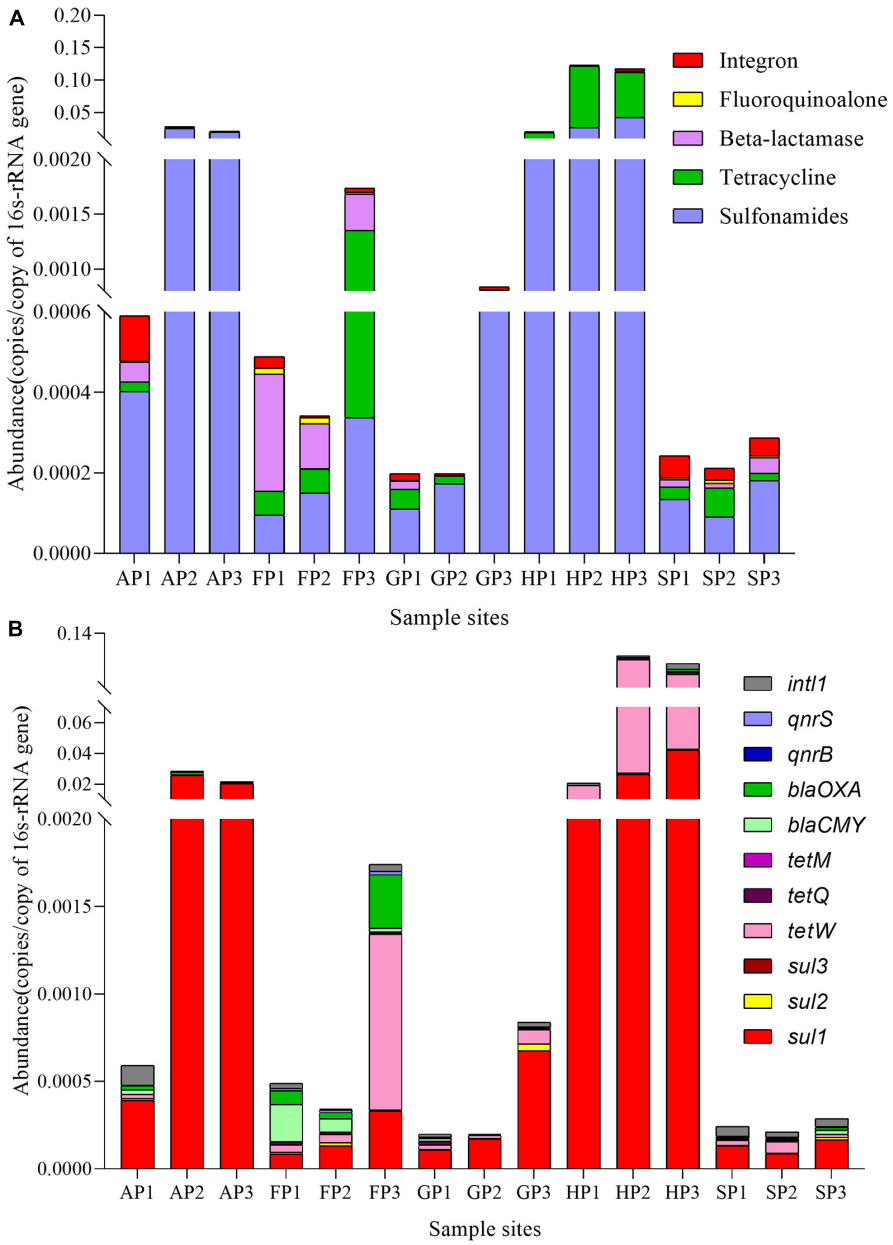

FIGURE 3 | The abundance of antibiotic resistance genes (ARGs) in the 15 sediment samples. **(A)** Five categories. **(B)** 11 kinds.

these samples, but higher abundance of tetracycline resistance genes was detected in the samples of FP3, HP2, and HP3. The highest abundance of total ARGs was detected in the samples of HP (HP1, HP2, and HP3). The beta-lactam resistance genes have the highest abundance in the samples of FP (FP1, FP2, and FP3).

The distribution of ARGs is shown in **Figure 3B**. *Sul1*, *sul2*, and *tetM* genes were the highest abundance in all sediment samples, while *sul3* and *qnrS* genes were only detected in the sediments from HP (HP1, HP2, and HP3) and SP (SP1, SP2, and SP3), respectively. Overall, the ARGs levels in different sample sites were obviously different, which could be associated with the different types of antibiotics used in different aquaculture farms.

Correlations Among Bacterial Community, Antibiotic Resistance Genes, and Environmental Factors

The RDA analysis was performed to further explore the correlation between the bacterial communities of the 15 sediment samples, ARGs abundance, and environmental factors (**Figure 4**). The weights for variables making up canonical axes of RDA are summarized in **Supplementary Table 7**. We found that the TN, TP, Cl^{-}, Ca^{2+}, *sul1*, bla_{CMY}, *intl1*, *qnrS*, and *tetW* have a significant correlation with the bacterial community in the 15 sediment samples (permutations = 999, $p < 0.05$), which

explained 61.82% of overall variation in the bacterial community. The RDA1 and RDA2 explained 44.36 and 17.46% of the total variance, respectively. Positive correlation was found between environmental factors detected in this study and the bacterial community (AP1, GP1, GP2, GP3, SP1, SP2, and SP3), and the bacterial community of the sampling sites showed a negative correlation with ARGs. Moreover, the abundance of ARGs (*sul1*, *tetW*, *qnrS*, and *intl1*) has a much higher correlation with the bacterial community (AP2, AP3, HP1, HP2, and HP3), while bla_{CMY} has a stronger correlation with the bacteria of FP (FP1, FP2, and FP3).

Furthermore, the correlation analysis of 10 ARGs showed that there were significant correlations among multiple ARGs (**Supplementary Figure 6**). Similarly, the *intl1* gene was correlated with the *sul1*, *sul2*, *sul3*, bla_{OXA}, *qnrS*, and *tetM*, *tetQ*, and *tetW* genes. A Mantel test was also performed to demonstrate whether there were high correlations between the total ARGs and *intl1*. Results showed that a significant correlation (permutations = 999, $r = 0.8013$, $p < 0.01$) was found among the total ARGs and *intl1*.

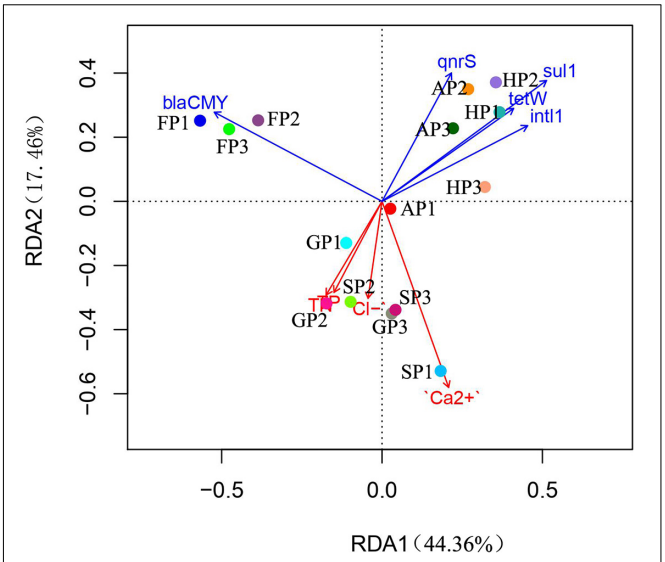

FIGURE 4 | The redundancy analysis (RDA) of environmental factors, antibiotic resistance genes (ARGs), and bacterial communities.

Co-occurrence of Bacterial Community and Antibiotic Resistance Genes in Sediment Samples

The co-occurrence patterns between ARGs and bacterial community were further analyzed with the network analysis (**Figures 5A,B**). As shown in **Figure 5B**, the *intl1* gene was found to co-occur with eight genera of bacteria taxa, followed by *sul1* (7), *sul2* (6), *tetQ* (6), *tetM* (6), *qnrS* (4), *tetW* (3), bla_{CMY} (2), and bla_{OXA} (2).

At the phylum level of the bacterial community, the main phyla potentially carrying the target ARGs were Chloroflexi, Euryarchaeota, Firmicutes, Halobacterota, and Proteobacteria (**Figure 5A**). There was a significant co-occurrence pattern in the bacterial taxa of Chloroflexi and eight subtype ARGs (*sul1*, *sul2*, bla_{OXA}, *qnrS*, *tetW*, *tetQ*, *tetM*, and *intl1*) and a significant co-occurrence pattern in the bacterial taxa of Firmicutes and seven subtype ARGs (*sul1*, *sul2*, bla_{OXA}, *qnrS*, *tetQ*, *tetM*, and *intl1*) ($p < 0.05$) (**Figure 5B**). At the genus level of the bacterial community, *Proteiniclasticum*, *Leptolinea*, *Methanobacterium*, and *Methanoregula* were the main potential hosts of ARGs (**Figure 5B**). Among them, the *Leptolinea* was found to have the most diverse connections with ARGs, including *sul1*, *sul2*, bla_{OXA}, *qnrS*, *tetW*, *tetQ*, *tetM*, and *intl1* ($p < 0.05$). *Proteiniclasticum* was also detected to carry seven ARGs (*sul1*, *sul2*, bla_{OXA}, *qnrS*, *tetQ*, *tetM*, and *intl1*) ($p < 0.05$). In addition, *Sulfurovum* and *Sulfurivermis* have simply co-occurred with the bla_{CMY} gene encoding resistance.

DISCUSSION

Aquaculture ponds are regarded as a major reservoir for antibiotic resistant bacteria and ARGs due to the overreliant use of antibiotics (Boran et al., 2013). However, excessive antibiotics and their metabolites have been released into the environment due to the abuse and misuse of antibiotics (Bu et al., 2013; Liu and Wong, 2013). Relevant studies have indicated that antibiotic contamination could lead to the emergence of ARGs in the environment (Qiao et al., 2018; Yang et al., 2018). In this study, we explored the correlation between environmental factors, ARGs, and the bacterial community in different aquatic environments.

Sulfonamides and tetracyclines were used widely in aquatic farms (Luo et al., 2010), the abundance of *sul* and *tet* genes was significantly correlated with the use of the corresponding antibiotics (Gao et al., 2012). Generally, the establishment of ARGs requires selective pressure on antibiotics over a long period. However, once the selective pressure was established, the ARGs would persist and difficult to be eliminated even if the pressure is removed (Pei et al., 2006; Xiao et al., 2016). In this study, a higher abundance of *sul* and *tet* genes in sediment samples was detected, and it was consistent with previous studies that *sul* and *tet* genes were the dominant ARGs in aquaculture water environments (Hoa et al., 2008; Tamminen et al., 2011). Remarkably, the abundance of *sul* gene was higher than that of *tet* gene, except for the sample from FP3. A previous study also indicated that the *sul* gene persists longer than the *tet* gene (McKinney et al., 2010). The *intl1* was one of the mobile genetic element genes and widely existed in Gram-positive and Gram-negative bacteria (Zeng et al., 2019), and it was regarded as an important pollution genetic marker caused by human activity (Gillings et al., 2015). The abundance of *Intl1* gene is a proxy for anthropogenic pollution among many other factors are that they are linked to genes conferring resistance to antibiotics, and *intl1* gene was also closely related to multidrug resistance (MDR). Our study revealed that the abundance of ARGs (*sul1*, *sul2*, *sul3*, *tetW*, *tetQ*, *tetM*, bla_{OXA}, and *qnrS*) was significantly correlated with the abundance of *Intl1*. It was indicated that *intl1* may play a key role in ARGs proliferation and diffusion from the sediment of aquaculture farms. Moreover, the previous study indicated that there were the co-occurrence patterns among many ARGs in pig

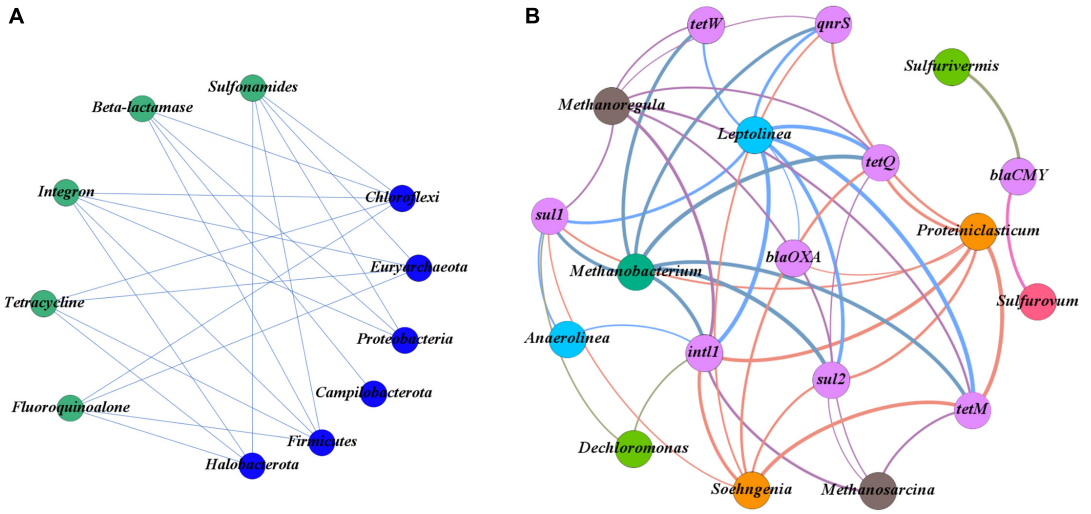

FIGURE 5 | (A) Network plots between antibiotic resistance genes (ARGs) and the phyla of the bacterial community. **(B)** Network plots between ARGs and the genera of the bacterial community.

farm wastewater (Yang et al., 2020). Correlation analysis in the study also showed that there was a significant positive correlation among the different types of ARGs. The bacteria carrying multiple ARGs could easily obtain the resistance to antibiotics (Trudel et al., 2016); therefore, the potential environmental risk of ARGs should be given attention.

In this study, a significant difference was observed in bacterial communities among different aquatic farms. The dominant phyla were Proteobacteria and Firmicutes in all sediment samples. Similar results were found in pig farms and the sediment of a shrimp farm (Yang et al., 2020; Zeng et al., 2020). Within the Proteobacteria phylum, the *Sphingomonas* was the main compositions of the genus. It was reported that the genome of *Sphingomonas* contains multiple efflux pumps (Jia et al., 2019), suggesting that *Sphingomonas* might better exist in the sediments of aquatic environments. A previous study implied that the physiochemical properties of the environment may influence the bacterial community by affecting the nutrient availability or physiological activity (Li et al., 2019). The present study found that the bacterial communities from AP1, GP1, GP2, GP3, SP1, SP2, and SP3 were significantly correlated with environmental factors. In addition, the concentrations of Mg^{2+}, Ca^{2+}, and Cl^{-} in the environment influence the composition of the bacterial community (Xu et al., 2018). It is worth noting that the Ca^{2+} has a significant negative correlation with the richness and diversity of bacterial communities. Interestingly, the calcium carbonate was widely used in aquaculture farms, which led to the high accumulation of Ca^{2+} in the sediments. Therefore, the excessive use of calcium carbonate might lead to a decrease in the diversity and richness of bacterial communities in the environment.

The aquatic environment gradually becomes a reservoir of antibiotic-resistant bacteria because of the use and abuse of antibiotics in aquatic farms (Huang et al., 2017). Previous studies certified that some bacterial taxa from Firmicutes were the dominant ARGs-carrying bacteria (Zhang et al., 2021). We also found that the *Proteiniclasticum* and *Soehngenia* from Firmicutes might be the main potential hosts of ARGs, which have strong co-occurrence with *sul1*, *sul2*, bla_{OXA}, *qnrS*, *tetQ*, *tetM*, and *intl1* genes. Similarly, the *Anaerolinea* and *Leptolinea* from Chloroflexi have strong co-occurrence with ARGs (*sul1*, *sul2*, bla_{OXA}, *qnrS*, *tetW*, *tetQ*, *tetM*, and *intl1*). However, the *Sulfurovum* from Campilobacterota only has a co-occurrence with bla_{CMY}, and the Campilobacterota was the dominant phylum in the samples of FP (FP1, FP2, and FP3). Furthermore, there was a stronger co-occurrence between *tetM* gene and six bacterial taxa in these samples. A previous study revealed the similar results in the soil of swine feedlots (Li et al., 2019). It is worth noting that the *tetM* gene was regarded as a detection tool to track and monitor ARGs transport in agricultural systems (Cadena et al., 2018). Our research found that the ARGs have a complex co-occurrence correlation with the bacterial taxa in sediment, which indicated that some bacterial taxa could be resistant to multiple antibiotics in the sediments. Overall, this study indicated that the ARGs in the sediments of aquaculture farms have an impact on the environment and bacterial communities, and we must pay more attention to and take preventive measures.

CONCLUSION

The present study indicated that the sulfonamides and tetracycline resistance genes were the predominant ARGs in the sediments of the investigated aquatic farms. Some bacterial taxa from the phyla Chloroflexi, Euryarchaeota, Firmicutes, Halobacterota, and Proteobacteria might be the main potential hosts of ARGs in these aquatic farms. Moreover,

the excessive Ca^{2+} might inhibit the diversity and richness of bacterial communities.

DATA AVAILABILITY STATEMENT

The sequence raw datasets in this study can be found in the NCBI repository (http://www.ncbi.nlm.nih.gov/bioproject/708165).

AUTHOR CONTRIBUTIONS

XC and HL played an important role in the conception of the study. CL and YS finished the part of the experiment. RZ and HX organized the original data. YL and XS performed the data analysis. XC wrote the first manuscript. HL edited the final 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.2021.679805/full#supplementary-material

REFERENCES

Boran, H., Terzi, E., Altinok, I., Capkin, E., and Bascinar, N. (2013). Bacterial diseases of cultured mediterranean horse mackerel (trachurus mediterraneus) in sea cages. *Aquaculture* 396-399, 8–13. doi: 10.1016/j.aquaculture.2013.02.025

Bu, Q., Wang, B., Huang, J., Deng, S., and Yu, G. (2013). Pharmaceuticals and personal care products in the aquatic environment in China: a review. *J. Hazard. Mater.* 262, 189–211. doi: 10.1016/j.jhazmat.2013.08.040

Cadena, M., Durso, L. M., Miller, D. N., Waldrip, H. M., Castleberry, B. L., Drijber, R. A., et al. (2018). Tetracycline and Sulfonamide Antibiotic Resistance Genes in Soils from Nebraska Organic Farming Operations. *Front. Microbiol.* 9:1283. doi: 10.3389/fmicb.2018.01283

Cambray, G., Guerout, A. M., and Mazel, D. (2010). Integrons. *Annu. Rev. Genet.* 44, 141–166. doi: 10.1146/annurev-genet-102209-163504

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Chen, B., Lin, L., Fang, L., Yang, Y., Chen, E., Yuan, K., et al. (2018). Complex pollution of antibiotic resistance genes due to beta-lactam and aminoglycoside use in aquaculture farming. *Water Res.* 134, 200–208. doi: 10.1016/j.watres.2018.02.003

Chen, H., Bai, X., Li, Y., Jing, L., Chen, R., and Teng, Y. (2019). Characterization and source-tracking of antibiotic resistomes in the sediments of a peri-urban river. *Sci. Tot. Environ.* 679, 88–96. doi: 10.1016/j.scitotenv.2019.05.063

Chen, H., Liu, S., Xu, X. R., Diao, Z. H., Sun, K. F., Hao, Q. W., et al. (2018). Tissue distribution, bioaccumulation characteristics and health risk of antibiotics in cultured fish from a typical aquaculture area. *J. Hazard. Mater.* 343, 140–148. doi: 10.1016/j.jhazmat.2017.09.017

Dang, B., Mao, D., Xu, Y., and Luo, Y. (2017). Conjugative multi-resistant plasmids in Haihe River and their impacts on the abundance and spatial distribution of antibiotic resistance genes. *Water Res.* 111, 81–91. doi: 10.1016/j.watres.2016.12.046

Fahrenfeld, N., Knowlton, K., Krometis, L. A., Hession, W. C., Xia, K., Lipscomb, E., et al. (2014). Effect of manure application on abundance of antibiotic resistance genes and their attenuation rates in soil: field-scale mass balance approach. *Environ. Sci. Technol.* 48, 2643–2650. doi: 10.1021/es404988k

Forsberg, K. J., Reyes, A., Wang, B., Selleck, E. M., Sommer, M. O., and Dantas, G. (2012). The shared antibiotic resist one of soil bacteria and human pathogens. *Science* 337, 1107–1111. doi: 10.1126/science.1220761

Gao, P., Mao, D., Luo, Y., Wang, L., Xu, B., and Xu, L. (2012). Occurrence of sulfonamide and tetracycline-resistant bacteria and resistance genes in aquaculture environment. *Water Res.* 46, 2355–2364. doi: 10.1016/j.watres.2012.02.004

Gaze, W. H., Zhang, L., Abdouslam, N. A., Hawkey, P. M., Calvo-Bado, L., Royle, J., et al. (2011). Impacts of anthropogenic activity on the ecology of class 1 integrons and integron-associated genes in the environment. *ISME J.* 5, 1253–1261. doi: 10.1038/ismej.2011.15

Gillings, M. R., Gaze, W. H., Pruden, A., Smalla, K., Tiedje, J. M., and Zhu, Y. G. (2015). Using the class 1 integron-integrase gene as a proxy for anthropogenic pollution. *ISME J.* 9, 1269–1279. doi: 10.1038/ismej.2014.226

Guo, J., Li, J., Chen, H., Bond, P. L., and Yuan, Z. (2017). Metagenomic analysis reveals wastewater treatment plants as hotspots of antibiotic resistance genes and mobile genetic elements. *Water Res.* 123, 468–478. doi: 10.1016/j.watres.2017.07.002

Hoa, P. T. P., Nonaka, L., Viet, P. H., and Suzuki, S. (2008). Detection of the sul1, sul2, and sul3 genes in sulfonamide-resistant bacteria from wastewater and shrimp ponds of north Vietnam. *Sci. Tot. Environ.* 405, 377–384. doi: 10.1016/j.scitotenv.2008.06.023

Huang, L., Xu, Y. B., Xu, J. X., Ling, J. Y., Chen, J. L., Zhou, J. L., et al. (2017). Antibiotic resistance genes (ARGs) in duck and fish production ponds with integrated or non-integrated mode. *Chemosphere* 168, 1107–1114. doi: 10.1016/j.chemosphere.2016.10.096

Huerta, B., Marti, E., Gros, M., López, P., Pompêo, M., Armengol, J., et al. (2013). Exploring the links between antibiotic occurrence, antibiotic resistance, and bacterial communities in water supply reservoirs. *Sci. Tot. Environ.* 45, 161–170. doi: 10.1016/j.scitotenv.2013.03.071

Hurst, J. J., Oliver, J. P., Schueler, J., Gooch, C., Lansing, S., Crossette, E., et al. (2019). Trends in Antimicrobial Resistance Genes in Manure Blend Pits and Long-Term Storage Across Dairy Farms with Comparisons to Antimicrobial Usage and Residual Concentrations. *Environ. Sci. Technol.* 53, 2405–2415. doi: 10.1021/acs.est.8b05702

Jia, S., Wu, J., Ye, L., Zhao, F., Li, T., and Zhang, X. X. (2019). Metagenomic assembly provides a deep insight into the antibiotic resistome alteration induced by drinking water chlorination and its correlations with bacterial host changes. *J. Hazard. Mater.* 379:120841. doi: 10.1016/j.jhazmat.2019.120841

Kumar, M., Prasad, R., Sharma, S., Varma, A., and Kumar, V. (2017). Dissemination Mechanism of AntibioticResistance Genes in Environment. *Soil Biol.* 51, 191–205. doi: 10.1007/978-3-319-66260-2_10

Kümmerer, K. (2009). Antibiotics in the aquatic environment–a review–part I. *Chemosphere* 75, 417–434. doi: 10.1016/j.chemosphere.2008.11.086

Li, B., Yang, Y., Ma, L., Ju, F., Guo, F., Tiedje, J. M., et al. (2015). Metagenomic and network analysis reveal wide distribution and co-occurrence of environmental antibiotic resistance genes. *ISME J.* 9, 2490–2502. doi: 10.1038/ismej.2015.59

Li, N., Zhu, C., Liu, C., Zhang, X., Ding, J., Zandi, P., et al. (2019). The persistence of antimicrobial resistance and related environmental factors in abandoned and working swine feedlots. *Environ. Pollut.* 255(Pt 1):113116. doi: 10.1016/j.envpol.2019.113116

Liu, J. L., and Wong, M. H. (2013). Pharmaceuticals and personal care products (PPCPs): a review on environmental contamination in China. *Environ. Int.* 59, 208–224. doi: 10.1016/j.envint.2013.06.012

Luo, Y., Mao, D., Rysz, M., Zhou, Q., Zhang, H., Xu, L., et al. (2010). Trends in antibiotic resistance genes occurrence in the Haihe River, China. *Environ. Sci. Technol.* 44, 7220–7225. doi: 10.1021/es100233w

Ma, L., Li, B., and Zhang, T. (2019). New insights into antibiotic resistome in drinking water and management perspectives: A metagenomic based study of small-sized microbes. *Water Res.* 152, 191–201. doi: 10.1016/j.watres.2018.12.069

Mao, D., Luo, Y., Mathieu, J., Wang, Q., Feng, L., Mu, Q., et al. (2014). Persistence of extracellular DNA in river sediment facilitates antibiotic resistance gene propagation. *Environ. Sci. Technol.* 48, 71–78. doi: 10.1021/es404280v

Marti, E., Variatza, E., and Balcazar, J. L. (2014). The role of aquatic ecosystems as reservoirs of antibiotic resistance. *Trends Microbiol.* 22, 36–41. doi: 10.1016/j.tim.2013.11.001

Martínez, J. L. (2008). Antibiotics and antibiotic resistance genes in natural environments. *Science* 321, 365–367. doi: 10.1126/science.1159483

Martinez-Freijo, P., Fluit, A. C., Schmitz, F. J., Grek, V. S., Verhoef, J., and Jones, M. E. (1998). Class I integrons in Gram-negative isolates from different European hospitals and association with decreased susceptibility to multiple antibiotic compounds. *J. Antimicrob. Chemother.* 42, 689–696. doi: 10.1093/jac/42.6.689

McKinney, C. W., Loftin, K. A., Meyer, M. T., Davis, J. G., and Pruden, A. (2010). tet and sul antibiotic resistance genes in livestock lagoons of various operation type, configuration, and antibiotic occurrence. *Environ. Sci. Technol.* 44, 6102–6109. doi: 10.1021/es9038165

Pan, X., Lin, L., Zhang, W., Dong, L., and Yang, Y. (2020). Metagenome sequencing to unveil the resistome in a deep subtropical lake on the Yunnan-Guizhou Plateau, China. *Environ. Pollut.* 263(Pt B):114470. doi: 10.1016/j.envpol.2020.114470

Pei, R., Kim, S. C., Carlson, K. H., and Pruden, A. (2006). Effect of river landscape on the sediment concentrations of antibiotics and corresponding antibiotic resistance genes (ARG). *Water Res.* 40, 2427–2435. doi: 10.1016/j.watres.2006.04.017

Qiao, M., Ying, G. G., Singer, A. C., and Zhu, Y. G. (2018). Review of antibiotic resistance in China and its environment. *Environ. Int.* 110, 160–172. doi: 10.1016/j.envint.2017.10.016

SEPA (2002). *Water and wastewater monitoring and analysis method*, 4th Edn. Beijing: China Environmental Science Press.

Shen, X., Jin, G., Zhao, Y., and Shao, X. (2020). Prevalence and distribution analysis of antibiotic resistance genes in a large-scale aquaculture environment. *Sci. Tot. Environ.* 711:134626. doi: 10.1016/j.scitotenv.2019.134626

Smillie, C. S., Smith, M. B., Friedman, J., Cordero, O. X., David, L. A., and Alm, E. J. (2011). Ecology drives a global network of gene exchange connecting the human microbiome. *Nature* 480, 241–244. doi: 10.1038/nature10571

Stokes, H. W., and Hall, R. M. (1989). A novel family of potentially mobile DNA elements encoding site-specific gene-integration functions: integrons. *Mol. Microbiol.* 3, 1669–1683. doi: 10.1111/j.1365-2958.1989.tb00153.x

Tamminen, M., Karkman, A., Lõhmus, A., Muziasari, W. I., Takasu, H., Wada, S., et al. (2011). Tetracycline resistance genes persist at aquaculture farms in the absence of selection pressure. *Environ. Sci. Technol.* 45, 386–391. doi: 10.1021/es102725n

Trolle, D., Zhu, G., Hamilton, D., Luo, L., Mcbride, C., and Zhang, L. (2009). The influence of water quality and sediment geochemistry on the horizontal and vertical distribution of phosphorus and nitrogen in sediments of a large, shallow lake. *Hydrobiologia* 627, 31–44. doi: 10.1007/s10750-009-9713-0

Trudel, M. V., Vincent, A. T., Attéré, S. A., Labbé, M., Derome, N., Culley, A. I., et al. (2016). Diversity of antibiotic-resistance genes in Canadian isolates of Aeromonas salmonicida subsp. salmonicida: dominance of pSN254b and discovery of pAsa8. *Sci. Rep.* 6:35617. doi: 10.1038/srep35617

Wang, A., Kong, X., Song, X., Ju, B., and Li, D. (2020). Study on exchangeable cation determining base saturation percentage of soil in south china. *Agricult. Sci.* 11, 17–26. doi: 10.4236/as.2020.111002

Wang, R. X., Wang, J. Y., Sun, Y. C., Yang, B. L., and Wang, A. L. (2015). Antibiotic resistance monitoring in Vibrio spp. isolated from rearing environment and intestines of abalone Haliotis diversicolor. *Mar. Pollut. Bul.* 101, 701–706. doi: 10.1016/j.marpolbul.2015.10.027

Xiao, K. Q., Li, B., Ma, L., Bao, P., Zhou, X., Zhang, T., et al. (2016). Metagenomic profiles of antibiotic resistance genes in paddy soils from South China. *FEMS Microbiol. Ecol.* 92:fiw023. doi: 10.1093/femsec/fiw023

Xiong, W., Sun, Y., Ding, X., Wang, M., and Zeng, Z. (2015). Selective pressure of antibiotics on ARGs and bacterial communities in manure-polluted freshwater-sediment microcosms. *Front. Microbiol.* 6:194. doi: 10.3389/fmicb.2015.00194

Xu, Z., Woodhouse, J. N., Te, S. H., Yew-Hoong Gin, K., He, Y., Xu, C., et al. (2018). Seasonal variation in the bacterial community composition of a large estuarine reservoir and response to cyanobacterial proliferation. *Chemosphere* 202, 576–585. doi: 10.1016/j.chemosphere.2018.03.037

Yang, Y., Chen, Y., Cai, Y., Xing, S., Mi, J., and Liao, X. (2020). The relationship between culturable doxycycline-resistant bacterial communities and antibiotic resistance gene hosts in pig farm wastewater treatment plants. *Ecotoxicol. Environ. Safety* 206:111164. doi: 10.1016/j.ecoenv.2020.111164

Yang, Y., Song, W., Lin, H., Wang, W., Du, L., and Xing, W. (2018). Antibiotics and antibiotic resistance genes in global lakes: A review and meta-analysis. *Environ. Int.* 116, 60–73. doi: 10.1016/j.envint.2018.04.011

Yuan, K., Wang, X., Chen, X., Zhao, Z., Fang, L., Chen, B., et al. (2019). Occurrence of antibiotic resistance genes in extracellular and intracellular DNA from sediments collected from two types of aquaculture farms. *Chemosphere*. 234, 520–527. doi: 10.1016/j.chemosphere.2019.06.085

Zeng, Q., Sun, J., and Zhu, L. (2019). Occurrence and distribution of antibiotics and resistance genes in greenhouse and open-field agricultural soils in China. *Chemosphere* 224, 900–909. doi: 10.1016/j.chemosphere.2019.02.167

Zeng, S., Khoruamkid, S., Kongpakdee, W., Wei, D., Yu, L., Wang, H., et al. (2020). Dissimilarity of microbial diversity of pond water, shrimp intestine and sediment in Aquamimicry system. *AMB Express* 10:180. doi: 10.1186/s13568-020-01119-y

Zhang, J., Buhe, C., Yu, D., Zhong, H., and Wei, Y. (2020). Ammonia stress reduces antibiotic efflux but enriches horizontal gene transfer of antibiotic resistance genes in anaerobic digestion. *Bioresour. Technol.* 295:122191. doi: 10.1016/j.biortech.2019.122191

Zhang, R. M., Liu, X., Wang, S. L., Fang, L. X., Sun, J., Liu, Y. H., et al. (2021). Distribution patterns of antibiotic resistance genes and their bacterial hosts in pig farm wastewater treatment systems and soil fertilized with pig manure. *Sci. Tot. Environ.* 758:143654. doi: 10.1016/j.scitotenv.2020.143654

Zhang, X., Li, X., Wang, W., Qi, J., Wang, D., Xu, L., et al. (2020). Diverse Gene Cassette Arrays Prevail in Commensal *Escherichia coli* From Intensive Farming Swine in Four Provinces of China. *Front. Microbiol.* 11:565349. doi: 10.3389/fmicb.2020.565349

Zhao, Z., Wang, J., Han, Y., Chen, J., Liu, G., Lu, H., et al. (2017). Nutrients, heavy metals and microbial communities co-driven distribution of antibiotic resistance genes in adjacent environment of mariculture. *Environ. Pollut.* 220(Pt B), 909–918. doi: 10.1016/j.envpol.2016.10.075

Zhou, Z. C., Zheng, J., Wei, Y. Y., Chen, T., Dahlgren, R. A., Shang, X., et al. (2017). Antibiotic resistance genes in an urban river as impacted by bacterial community and physicochemical parameters. *Environ. Sci. Pollut. Res. Int.* 24, 23753–23762. doi: 10.1007/s11356-017-0032-0

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Temperature and Nutrient Limitations Decrease Transfer of Conjugative IncP-1 Plasmid pKJK5 to Wild *Escherichia coli* Strains

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Plasmid-mediated dissemination of antibiotic resistance among fecal *Enterobacteriaceae* in natural ecosystems may contribute to the persistence of antibiotic resistance genes in anthropogenically impacted environments. Plasmid transfer frequencies measured under laboratory conditions might lead to overestimation of plasmid transfer potential in natural ecosystems. This study assessed differences in the conjugative transfer of an IncP-1 (pKJK5) plasmid to three natural *Escherichia coli* strains carrying extended-spectrum beta-lactamases, by filter mating. Matings were performed under optimal laboratory conditions (rich LB medium and 37°C) and environmentally relevant temperatures (25, 15 and 9°C) or nutrient regimes mimicking environmental conditions and limitations (synthetic wastewater and soil extract). Under optimal nutrient conditions and temperature, two recipients yielded high transfer frequencies (5×10^{-1}) while the conjugation frequency of the third strain was 1000-fold lower. Decreasing mating temperatures to psychrophilic ranges led to lower transfer frequencies, albeit all three strains conjugated under all the tested temperatures. Low nutritive media caused significant decreases in transconjugants (−3 logs for synthetic wastewater; −6 logs for soil extract), where only one of the strains was able to produce detectable transconjugants. Collectively, this study highlights that despite less-than-optimal conditions, fecal organisms may transfer plasmids in the environment, but the transfer of pKJK5 between microorganisms is limited mainly by low nutrient conditions.

Keywords: horizontal gene transfer, antimicrobial resistance, synthetic wastewater, soil extract agar, environmental conditions, AMR

INTRODUCTION

Antimicrobial resistance (AMR) is considered as one of the most significant challenges to global public health (O'Neill, 2016). The spread of antimicrobial resistance genes (ARGs) via horizontal gene transfer (HGT) between bacteria is a growing concern because it facilitates the dissemination of resistance across a wide variety of microorganisms. Understanding the dynamics of plasmid dissemination in the environment is fundamental to contain and mitigate the AMR challenge.

Horizontal gene transfer (HGT) is an effective ecological trait that shapes bacterial evolution (Ochman et al., 2000). Conjugative plasmids are relevant vectors for HGT (Smillie et al., 2010) and dissemination of AMR (Carattoli, 2013). Gut bacteria from both animal and human origin comprise an important source of AMR-conjugative plasmids (Hu et al., 2013; Ceccarelli et al., 2019). Gut bacteria are released into the environment through manure application to agricultural soils and wastewater discharges, ultimately resulting in the introduction of their ARGs, and plasmids in the environment. Despite having limited survivability, once introduced in the environment, gut bacteria might be able to transfer their AMR determinants to the natural bacterial community. *Escherichia coli* is widely accepted as primary indicator of fecal contamination. Although most *E. coli* strains cause only mild infections, their presence is indicative of the potential presence of other more pathogenic organisms which may be relevant for human health.

Monitoring of environmental HGT remains challenging mainly due to cultivation bias [only 1% of indigenous bacteria are estimated to be cultivable (Amann et al., 1995)]. Fluorescently labeled strains and plasmids comprise a promising methodology to study horizontal gene transfer in complex environments by culture independent methods (Sørensen et al., 2005). Due to donor-recipient incompatibilities and detection limits of the methodology, the experimental design often require a compromise to guarantee the detection of transconjugants (Sørensen et al., 2005; Pinilla-Redondo et al., 2018). As a result, studies addressing environmental dissemination of AMR plasmids usually apply conditions that are optimal for bacterial transmission, namely high bacterial densities, optimal growth temperatures, and/or high nutrient availability (Bellanger et al., 2014a; Jacquiod et al., 2017). Although being relevant for specific scenarios such as mesophilic anaerobic digesters, greenhouses or wastewater in low latitude countries (Al Qarni et al., 2016; Fan et al., 2019), these settings do not reflect the usual average conditions of manured soils, water bodies and wastewater (Abis and Mara, 2006; Barrios-Hernández et al., 2020; Osińska et al., 2020). Such discrepancies in the experimental design might lead to an overestimation of plasmid transfer frequencies and dissemination potential in the environment. Therefore, better insights into how environmental parameters affect plasmid transfer are needed.

The aim of this study was to evaluate *in vitro* the role of environmental factors that could potentially hamper conjugative plasmid transfer from gut bacteria once discharged into the environment. A conjugative broad host range IncP-1plasmid (pKJK5) was used as vector. Most importantly, IncP-1 plasmids have comparatively high conjugation rates and thus allow for analysis of conjugation frequency also under suboptimal conjugation conditions. IncP-1 plasmids often carry clinically relevant ARGs (Rozwandowicz et al., 2018), are abundant in (waste)water (Pallares-Vega et al., 2021), manure (Binh et al., 2008), and soil environments (Shintani et al., 2020) and can potentially disseminate among a wide diversity of phylogenetic groups (Popowska and Krawczyk-Balska, 2013). Furthermore, IncP-1 plasmids (i.e., RP4, pB10 and pKJK5) comprise the predominant plasmids in studies addressing transfer events in environmental settings (Inoue et al., 2005; Bellanger et al., 2014b; Klümper et al., 2015; Li et al., 2018). Solid-surface filter matings were conducted to study HGT between *Escherichia coli* strains (as both donor and recipients, with animal *E. co*li strains harboring extended spectrum beta-lactamase resistance genes on known plasmid types as recipients representative of *E. coli* introduced with animal manure). The transfer was evaluated under different (i) donor-to-recipient cell proportions, (ii) mating temperatures, or (iii) nutritional compositions. The criteria to select the used conditions was based on the presumable main abiotic challenges that gut bacteria face when discharged into the environment, namely nutrient limitations and close-to psychrophilic conditions. The donor-to-recipient cell proportions were tested to assess the limit of the system while aiming for a natural proportion of donor and recipient cells in the mating. By using the same species and a broad-host-range plasmid, potential host-vector and interspecies incompatibilities were discarded as factors. *E. coli* was chosen as a model system for bacteria of public health relevance that can potentially move between anthropogenic related and natural environments, and it was hypothesized that lower temperatures and lower nutrient concentrations would limit plasmid transfer.

MATERIALS AND METHODS

Selection and Characterization of Strains and Plasmids

Three extended-spectrum beta-lactamase (ESBL) carrying *E. coli* strains (09.54, 38.27, and 39.62) isolated from fecal samples of calves or poultry were used as recipients during the mating experiments (**Table 1**). These strains were part of a database from the Dutch national veterinarian institute (Wageningen Bioveterinary Research, WBVR), studying the prevalence of ESBLs in plasmids. The strains qualify for this work because of their species, diverse plasmid content, and because they had been sequenced under the scope of WBVR projects. A genetically engineered *E. coli* strain previously described by Klümper et al. (2015) was selected as donor for the broad-host-range plasmid of the incompatibility group IncP-1. The donor strain (*E. coli* K-12 MG1655:*lacI*^{q}-*pLpp-mCherry-Km*^{R}) is commonly used in dual-labeling fluorescence reporter-gene approaches coupled with fluorescence-activated cell sorting (Pinilla-Redondo et al., 2018) due to the conditionally expressible green fluorescent proteins (GFP) in its IncP-1 plasmid (pKJK5). The IncP-1 plasmid carries a kanamycin resistance determinant and lacI^{q} repressible promoter upstream the *gfpmut3* gene (Sengeløv et al., 2001; Bahl et al., 2007; Klümper et al., 2015).

In order to fully characterize the used strains, whole-genome sequencing using paired-end Illumina was performed, as previously described by Rozwandowicz et al. (2020). The annotation of the sequences was performed with Prokka version 1.12 (Seemann, 2014) and the corresponding sequence type a was conducted with the Multi Locus Sequence Typing online tool MLST 2.0 (Larsen et al., 2012), using the two available schemes (Wirth et al., 2006; Jaureguy et al., 2008). For typing the donor strain and relate the natural recipient strains to

TABLE 1 | Bacterial strains of *E. coli* used as donor and recipient of broad-host-range IncP-1 plasmid, and their characteristics.

Agent	*ST*	Role	Origin	Resistance profile	Plasmids	Source
E. coli MG1655:lacI^q-pLpp-mCherry-Km^R	ST10/ST262	Donor	Laboratory strain	AMP^R, SMX^R, KAN^R, mCherry pKJK5:Km^R	pKJK5 $P_{A1/04/03}$-gfpmut3 (IncP)	Klümper et al. (2015)
E. coli 09.54	ST21/ST481	Recipient	Veal calf	AMP^R, CTX^R, SMX^R, TET^R	IncK	This study
E. coli 38.27	ST10/ST2	Recipient	Poultry	AMP^R, CTX^R, SMX^R, TET^R	IncFI, IncH1, IncI1, p0111	This study
E. coli 39.62	ST101/ST88	Recipient	Poultry	AMP^R, CTX^R, SMX^R, TET^R	IncFIB/FII IncK	This study

ST, Sequence type.

the donor, a reference sequence of *E. coli* MG1665 (accession number: NC_000913.3) from GenBank was used. In addition, the existence of plasmid replicons within the strains was analyzed with PlasmidFinder (Carattoli et al., 2014) applying an identity cut-off equal or greater than 98%. The annotated sequences are deposited in GenBank, BioProject PRJNA661180 under the accession no. JADPVO000000000 (09.54), JADPVP000000000 (38.27) and JADPVQ000000000 (39.62) A core and accessory genome analysis of the donor and recipient strains was conducted with Roary version 13.0 (Page et al., 2015) in Galaxy version 21.01[1]. A maximum likelihood tree based on nucleotide sequence was built with FastTree version 2.1.10 (Price et al., 2010) in Galaxy and graphic visualization of the core and accessory genome was achieved with Phandango (Hadfield et al., 2018).

To identify suitable selective conditions for the identification of transconjugants, the antimicrobial susceptibility profile for each strain was determined by disc diffusion test, according to EUCAST guidelines (EUCAST Disk Diffusion Method for Antimicrobial Susceptibility Testing – version 6.0; available at https://www.eucast.org/). The results were interpreted based on the EUCAST-defined Breakpoints tables for interpretation of MICs and zone diameters (version 8.0) and are summarized in **Table 1** in **Supplementary Information**. **Figure 1** displays this study's schematic of the experimental design and procedure.

Culture Media and Growth Curves

Luria-Bertani (LB), synthetic wastewater (SWW), and soil extract (SE) were used as culture media for the filter matings. Pure bacterial cultures were prepared and maintained in LB broth or plates (tryptone 10 g L^{-1}, yeast extract 5 g L^{-1}, sodium chloride 5 g L^{-1}, and agar 15 g L^{-1}) prior to the experiments, and for the selection of donor, recipients, and transconjugants after the matings, the LB plates were enriched with kanamycin (100 μg mL^{-1}; Sigma Aldrich), tetracycline (16 μg mL^{-1}; Sigma Aldrich), and both kanamycin and tetracycline (100 and 16 μg mL^{-1}), respectively.

The SWW aimed to mimic the average conditions and nutrient proportions of conventional domestic wastewater. The composition was based on that of Boeije et al. (1999), and ISO 11733 guideline, and adjusted to a theoretical COD:N:P concentration and molar ratio close to that of Dutch wastewater (100:9.1:1.4, **Supplementary information Table 2**). The SWW solution contained of 0.07 g L^{-1} urea, 0.011 g L^{-1} NH_4Cl, 0.015 g L^{-1} peptone P (Oxoid, United Kingdom), 0.015 g L^{-1} Lab Lemco (Oxoid, United Kingdom), 0.05 g L^{-1} starch, 0.04 g L^{-1} glycerol that was sterilized by autoclaving. After sterilization, the mix was completed with 0.25 g L^{-1} sodium acetate, 0.12 g L^{-1} skimmed milk powder (Sigma Aldrich, NL), 0.05 g L^{-1} glucose, 0.025 g L^{-1} $FeSO_4$, 0.005 $CaCl_2$ g L^{-1}, 0.025 g L^{-1} $NaHCO_3$ and 0.02 g L^{-1} $MgHPO_4{\cdot}3H_2O$, 0.016 g L^{-1} L $K_3PO_4{\cdot}H_2O$ (unless indicated otherwise, the components were purchased at VWR, NL). These solutions were separately autoclaved, or filter sterilized prior to their aseptic addition to the final solution. SWW media was finally supplemented with the addition of 0.1% (v/v) of trace metal solution which contained 0.280 g L^{-1} NaEDTA, 0.180 g L^{-1} $ZnCl_2$, 1.144 g L^{-1} H_3BO_3, 0.025 g L^{-1} $CoCl_2{\cdot}6H_2O$, 0.589 g L^{-1} $MnCl_2{\cdot}2H_2O$, 0.120 g L^{-1} $CuCl_2{\cdot}2H_2O$, 0.068 g L^{-1} $NiCl_2{\cdot}6H_2O$, 0.025 g L^{-1} $Na_2MoO4{\cdot}5H_2O$, and 0.212 g L^{-1} $KCr(SO_4)_2{\cdot}12H_2O$. The pH was adjusted to 6.8 ± 0.1 with NaOH 1M to match the values found in wastewater [6.5 – 8.5 (Prot et al., 2020)]. When needed, agar (15 g L^{-1}) was added for solid media preparation.

Soil samples for SE medium preparation were collected in the late fall of 2019, from a local dairy farm (Friesland, Netherlands) that uses the field for pasture (grassland) and had not been recently subjected to manure application. In total, 7 kg of sandy loam soil were collected from the field and homogenized. The collected soil was air-dried for 3 days and stored in 500 g zip bags at 4°C until being used. The SE media was prepared as described by Musovic et al. (2010). Briefly, 500 g of dried soil was mixed with 500 mL of demineralized water. Then, the mixture was shaken horizontally, for 3 h, and left for passive settling of the particles, for 5 h. After the 5 h, the supernatant was pipetted and autoclaved (for 15 min, at 121°C) and stored at 4°C, up to one month. The pH values were not adjusted and were kept at its original values (5.0 – 5.3), and no buffer solutions were used to maintain the pH in the different culture media because they could introduce potential nutrients (e.g., phosphate). When needed, agar was added as aforementioned.

The general chemical compositions of the LB, SWW, and SE media were determined by ion chromatography (IC), and inductively coupled plasma (ICP-OES). The determination of the chemical oxygen demand (COD), and the total nitrogen (TN) was achieved with commercially available kits (LCK 514 and LCK 338; Hach). The determination of the total organic carbon (TOC) was achieved with Shimadzu TOC-L_{CPH} analyzer. The composition of the different media used is displayed in **Table 2**.

To quantify the effect of the temperature change in the growth, an inoculum volume of 0.2% (final volume) of overnight culture of each strain was transferred to fresh LB, and incubated

[1]https://usegalaxy.eu/

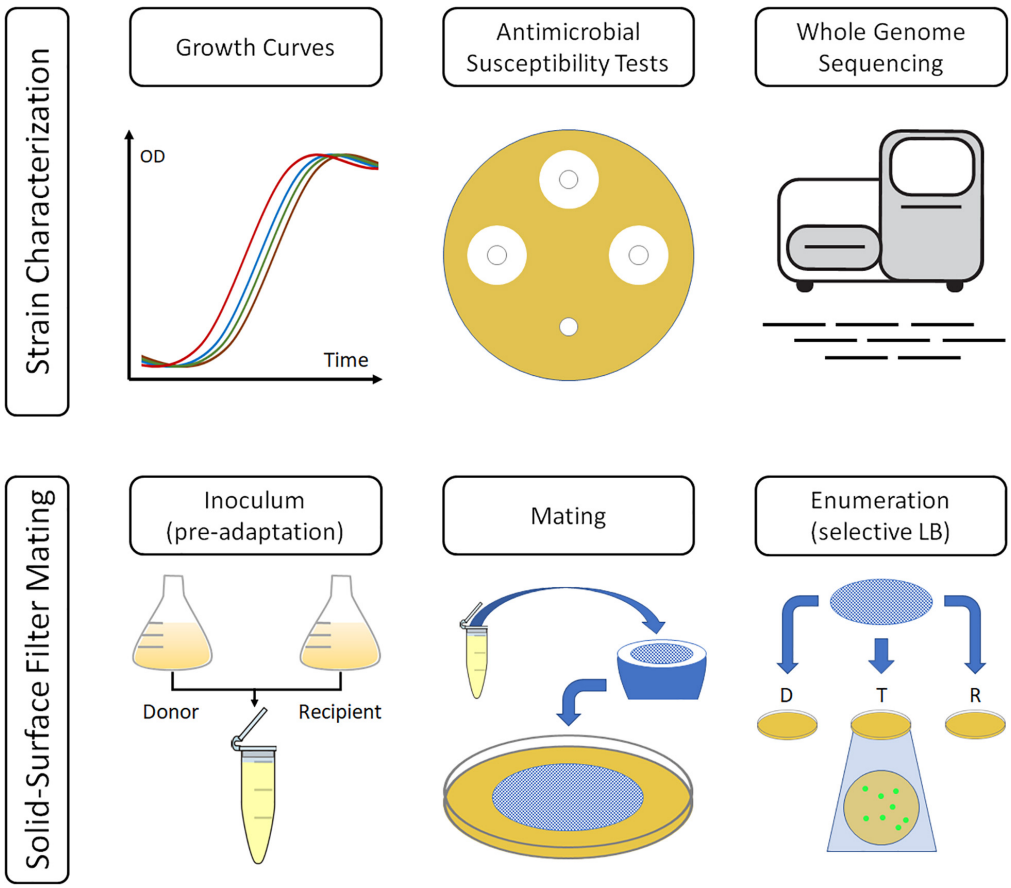

FIGURE 1 | Overview of the procedure to quantify transconjugants. Donors and recipients were grown separately before being mixed, filtered, and incubated for 2 h, at different temperatures or at different media. In the end, bacteria were recovered, and enumerated, in LB containing antibiotic combinations specific for donors, recipients, or transconjugants.

at 9, 15, 25, or 37°C. The Pathogen Modeling Program (PMP) online model (available at: https://pmp.errc.ars.usda.gov/default.aspx) was used to predict the incubation time range to measure bacterial density. To determine the effect of the nutrient composition, inoculums of 0.2% (final volume) overnight culture of each strain were transferred to SWW or SE media, and monitored up to three days. The optical density, at 600 nm (OD_{600}), was measured in a UV-Vis Spectrophotometer (Shimadzu Corp). Colony forming units (CFUs) were determined after preparing 10-fold serial dilutions with saline solution (NaCl; 0.85%), plating in LB agar, and incubating at 37°C, overnight. Measurements were performed in biological triplicates.

Solid Surface Filter Matings: Standard Conditions

Conjugation is a process that requires cell proximity and stable spatial conditions during the mating time (ca 3–5 min). Although these conditions can occur in the liquid phase, they are more likely in "surface-like" configurations (Zhong et al., 2010) occurring in soil grains, sludge flocs or biofilms. Bearing this in mind, filter mating was chosen to study the plasmid transfer.

The conjugation assays were performed by mixing 150 μL of fresh culture of the donor and recipient, and vacuum filtered through mixed-cellulose ester filters (0.45 μm; Millipore) in a Millipore filtration system. Prior to mixing, the cultures were grown for approximately 3 h in LB at 37°C to achieve a density of ca. 2×10^8 CFU mL^{-1}, as experimentally defined by the growth curves. After filtration, the mixed cultures were transferred to plates containing LB and cells were then incubated at 37°C. Following the incubation period, the cells were detached from the filter by vortexing in 1 mL of sterile LB broth, for 5 min. Subsequently, serial decimal dilutions were prepared in sterile saline solution, and 100 μL was spread on LB plates containing kanamycin (donors), tetracycline (recipients) and a combination of both (transconjugants). The results were observed after a 24-h incubation period (total counts), at 37°C, and another 24-h incubation period (colored colonies), at 4°C. The incubation at 4°C was performed to enhance the visualization of the GFP protein (Scott et al., 2006) and to count the green colonies, the plates were observed in a blue-light transilluminator (Safe Imager 2.0; Invitrogen). To confirm the validity of each assay, matings with only the donor or the recipient were also

TABLE 2 | Media composition of the culture media used in the matings with either Luria-Bertani (LB), synthetic wastewater (SWW) or soil extract (SE) medium.

(mg L^{-1})	LB		SWW		SE	
Compound	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
TOC	6,820	80	219	1.0	45	-
COD	21,450	2,450	529	37	173	1
TN	2,050	20	48	2	7	0.4
TP	151	1	7.2	0.2	4	0.0
Ca^{2+}	9	1	3.6	0.0	104	1
K^{+}	272	2	11.5	0.1	21	9.9
Mg^{2+}	7	0.1	37.6	0.1	5	0.6
$Fe^{2+/3+}$	0	0.0	4.5	0.0	<0.05	-
S	127	0	38	1	67	2
NH_4^{+}	60	0	6.2	0.0	<0.10	-
NO_3^{-}	4	0.0	<0.10	-	10	0.0
PO_4^{3-}	259	1	>20	-	12	0.0
SO_4^{2-}	96	9	11	0	191	7

Total organic carbon (TOC), chemical oxygen demand (COD), total nitrogen (TN), total phosphorus (TP).

performed. Each mating was performed in biological triplicates on alternative days.

Solid Surface Filter Matings: Modified Conditions

When different proportions of donor-to-recipient ratios (D/R) were tested, the donor cultures harvested until 10^8 CFU mL^{-1} were serially diluted (10 and 100-fold) in LB and 150 μL was mixed with 150 μL recipient culture to reach the corresponding ratios D/R of 1:10 and 1:100. A total volume of 200 μL of the mixtures were then filtered, and the mating and incubation were performed as aforementioned. The approximate cell density in the filters was 8.9×10^6 CFU cm^{-2}. The effect of temperature in transfer frequency was assessed by following the standard condition procedure, but incubating the filters at 25, 15 and 9°C in LB plates pre-conditioned to the corresponding temperatures. To assess the influence of nutrient availability in the transfer frequency, matings conducted in SWW and SE media were compared to standard nutrient-rich media LB. For SWW matings, donor and recipient cell cultures were pre-adapted to low nutrient conditions by growing them in SWW media (1% overnight inoculum) for approximately 4 h with 180 rpm agitation until a cell density of ca. 2×10^8 CFU mL^{-1} was achieved. Then, cell cultures were mixed and filtered as aforementioned in the standard conditions, and filters were placed in SWW agar plates. Plates were incubated at 37°C for 2 h. For SE matings, no pre-growth from donor nor recipients could be obtained in SE broth, as indicated by the corresponding growth curves (data not shown). Instead, late log phase LB cultures of both donor and recipients ca. 2×10^8 were centrifuged and washed twice in saline solution, and the pellet was finally resuspended in 10 mL of SE broth and incubated overnight at 37°C. Before incubation, an aliquot of the resuspended cells was serially diluted in saline solution, plated in LB and incubated overnight at 37°C. Following the incubation and based on the cell counts of the suspensions, the cell density of both donor and recipient SE cultures were adjusted to approximately 2×10^8 CFU mL^{-1}, mixed in 1:1 ratio and filtered as indicated in the standard procedure. Filters were then placed on SE media and incubated at 37°C for 24 h. In all modified filter matings, cell recovery and subsequent plating were performed as mentioned in the standard conditions.

Genetic Characterization of Donor, Recipient, and Transconjugants

To confirm the strain identity (donor, recipient, and transconjugants), five to ten isolates per mating were collected randomly from each of the media containing the antibiotics, and PCR was performed on the crude cell extracts. Reactions targeting the 16S rRNA gene, mCherry, and *gfpmut3* were prepared in 25-μL reactions containing PCR buffer (1x), (Invitrogen, NL) $MgCl_2$ (3.0 mM), (Invitrogen, NL), dNTPs (0.2 mM) (Promega, NL), forward and reverse primers (0.4 μM; **Supplementary Table 3**), *Taq* polymerase (1.25 U) (Invitrogen, NL), and 1 μL of DNA. The PCR reactions were carried out in a T100 Thermal Cycler (BioRad), following similar denaturation conditions (95°C for 30 s), but specific annealing and elongation conditions (57, 55, or 60°C for 30 s; and 30 – 90 s at 72°C for the 16S rRNA, *gfpmut3*, and mCherry genes, respectively), in 30 cycles. The specificity of the PCR products was confirmed by visualization in 1.5% agarose gel stained with ethidium bromide.

Data Analysis

One-way analysis of variance (ANOVA) was conducted to detect differences in the conjugation frequencies, between strains, temperatures, and culture media. The ANOVA tests were followed by TukeyHSD *post hoc* analysis, and homogeneity of variance was confirmed with Levene's test. Data normality was confirmed with Shapiro-Wilk's method, and when normality was not achieved, group comparison was performed using the equivalent non-parametric test (Kruskal-Wallis). A significance score of $p < 0.05$ was considered to be statistically relevant. These analyses were performed with R version 3.5.1 (R Core Team, 2018) and RStudio (Version 1.1.456[2]). Used software packages consisted of *reshape* (Wickham, 2007) and *tidyverse* (Wickham et al., 2019), a set of packages designed for data cleaning, trimming, and visualization; of *Rcmdr* (Fox, 2005), *PMCMRplus* (Thorsten, 2020), and *car* (Fox and Weisberg, 2019) for ANOVA and Levene's test.

RESULTS

Effect of Donor-to-Recipient (D/R) Ratios

Before the temperature and nutrients assays, the D/R ratios were tested to assess the limit of the system while aiming for a natural proportion of donor and recipient cells in the mating.

Under optimal conditions and 1:1 D/R ratio (37°C and LB, 8.9×10^6 CFU cm^{-2}), two out of three *E. coli* strains

[2]https://www.rstudio.com/

(38.27 and 39.62) yielded high transconjugant numbers (10^9 CFUs mL^{-1}) and transfer frequency (5×10^{-1}) of IncP-1 plasmids. On the other hand, the mating with strain 09.54 produced 10^6 CFU mL^{-1} (transfer frequency of 10^{-3}). The transfer frequency, measured as the transconjugants-to-donors ratio (T/D), resulted in a slight increase in the 1:10 and 1:100 D/R proportions in comparison with the 1:1 proportion in all strains (except for one replicate of strain 09.54; **Figure 2**). Contrarily, the transconjugants-to-recipients ratio (T/R) decreased with the different D/R ratios, approximately −0.7 logs and −1.8 logs in the 1:10 and 1:100 proportions, respectively (strains 38.27 and 39.62). A stronger effect of D/R was observed for strain 09.54, where the T/R decreased 1–3 logs and 3–4 logs in the 1:10 and 1:100 proportion, respectively. Similar results were found for the absolute numbers of transconjugants (**Figure 1** in **Supplementary information**). No transconjugants were recovered for one replicate in the mating of the strains 09.54 (1:100; **Figure 1**). At both 1:10 and 1:100 proportions, transconjugant numbers reached approximately 10^3 CFUs mL^{-1} for at least one of the replicates, which was close to the detection limit (10^2 CFUs mL^{-1}).

Role of Temperature on Conjugative Transfer

Conjugation efficiency among ESBL *E. coli* strains was assessed at temperatures ranging from the optimal laboratory (37°C), room (25°C) and relevant environmental (15°C, 9°C) conditions.

Overall, lower temperatures significantly reduced the number of conjugation events ($p < 0.01$; **Figure 3**). Both T/D and T/R decreased with decreasing temperatures, with a more pronounced reduction in strain 09.54 than in the other two strains (**Figure 3**). The highest number of transconjugants was obtained at 37°C, and at 25°C, and the number of

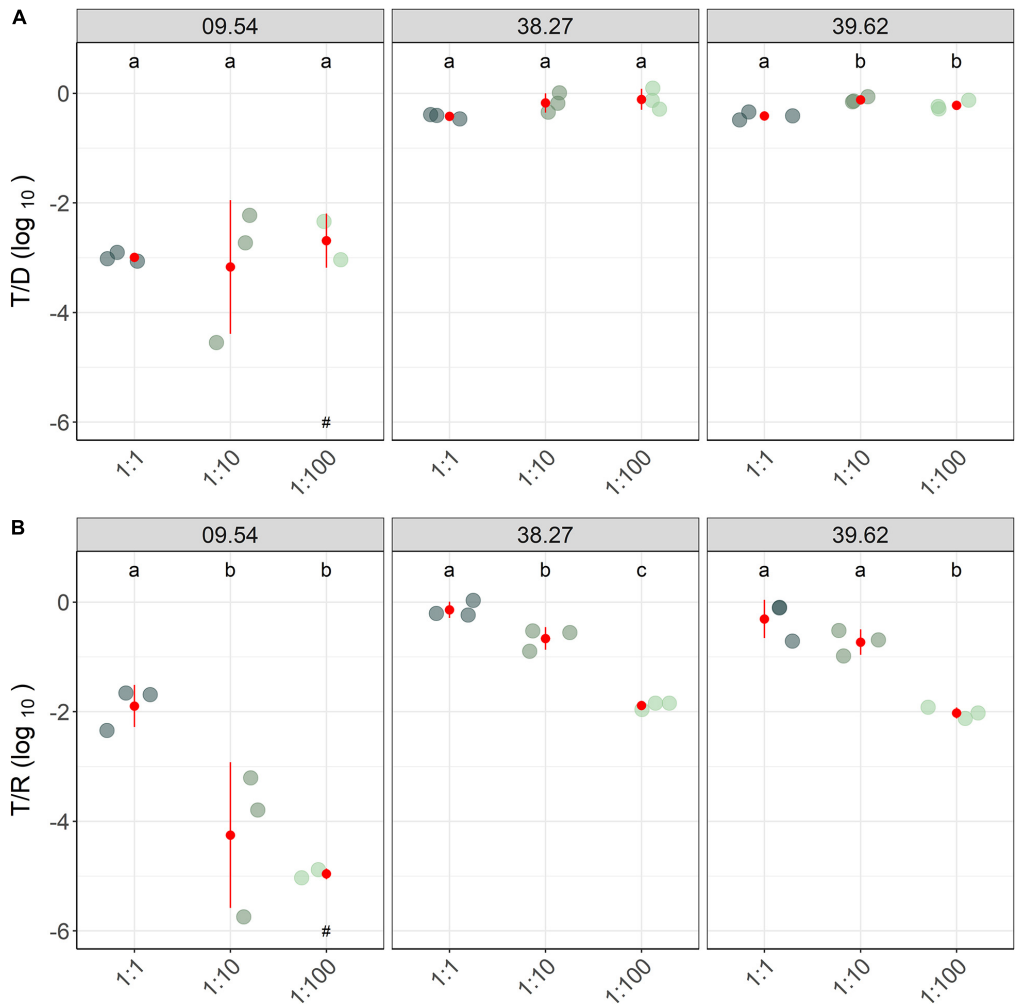

FIGURE 2 | Donor-to-recipient proportions had significant effects on plasmid transfer. Depending on the indicator and strain used, the donor concentration increased or decreased, the transfer frequency. Relative counts of transconjugant-to-donor (T/D; **A**) and transconjugant-to-recipient (T/R; **B**) ratios, after 2-h matings performed at three donor-to-recipient ratios (1:1, 1:10, 1:100) are shown together with average and standard deviation values (in red). Different colors depict distinct donor-to-recipient ratios. ^{a,b,c} Indicate significantly different groups in the transfer frequency between ratios (*Post hoc* Tukey test, $p < 0.05$), and replicates with no detected transconjugants are highlighted ($^{\#}$).

transconjugants decreased roughly 1 log (strains 38.27 and 39.62) or 2 logs (strain 09.54), depending on the strain. With further temperature reduction, lower transconjugant numbers were observed, and at 9°C, conjugation still occurred in all tested strains.

The lowest number of transconjugants was obtained at 9°C for strains 38.27 and 39.62. In strain 09.54, the minimum transconjugant number was already reached at 15°C and maintained at 9°C. However, higher variability among replicates was noticeable with strain 09.54 (**Supplementary Figure 2**), and one replicate did not yield detectable transconjugants (**Supplementary Figure 2**).

Role of Nutrient Concentrations on Conjugative Transfer

Differences in plasmid transfer under diverse nutrient regimes were assessed by comparing conjugation yields and transfer frequencies between rich nutrient media (LB) and common surrogates for natural conditions such as SWW and SE media.

In all tested strains, the decrease in the nutrient concentration of the media resulted in a substantial decrease in conjugation events (**Figure 4**). In comparison with the matings performed in LB, SWW resulted in the reduction of conjugation events by roughly 2 logs. In SE, a 4-log reduction was observed for strain 39.62 (compared to LB; 4), but no transconjugants were recovered for other strains, despite several attempts.

The decline in transconjugant numbers was particularly severe for strain 09.54, which presented the lower number of transconjugants in LB. Its transconjugants were only recovered in one out of three matings performed in SWW, and when SE was used, a further decrease in the number of transconjugants was observed. While matings with strain 39.62 yielded 1.3×10^3 CFUs mL^{-1} transconjugants (3 and 6 logs lower than in SWW and LB, respectively; **Supplementary Figure 3**), the strains

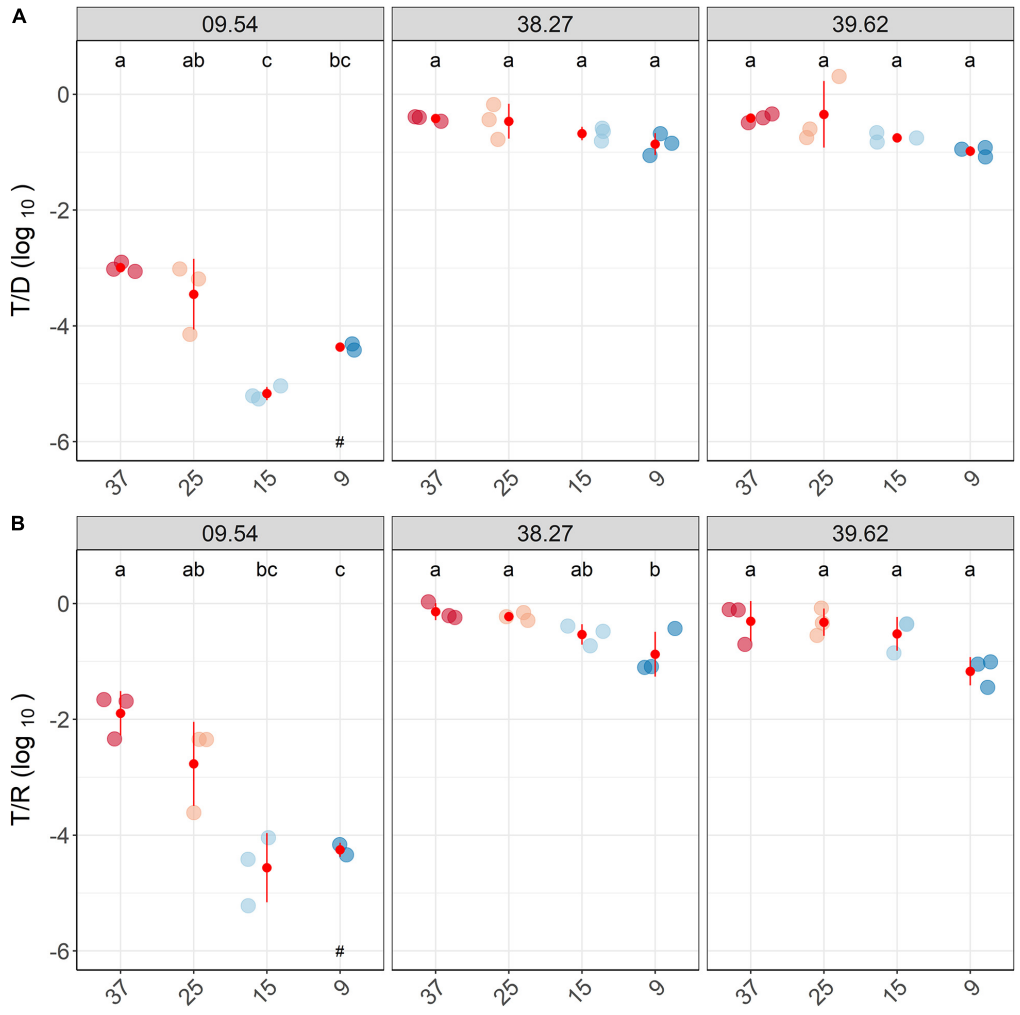

FIGURE 3 | Lower temperature reduced the number of conjugation events. Relative counts of transconjugant-to-donor (T/D; **A**) and transconjugant-to-recipient (T/R; **B**) after 2h-matings performed, at diverse temperatures (37 – 9°C), are shown together with average and standard deviation values (in red). Different colors depict distinct temperatures. ^{a,b,c} Indicate significantly different groups in the transfer frequency between temperatures (*PostHoc* Tukey test, $p < 0.05$), and replicates with no detected transconjugants are highlighted ($^{\#}$).

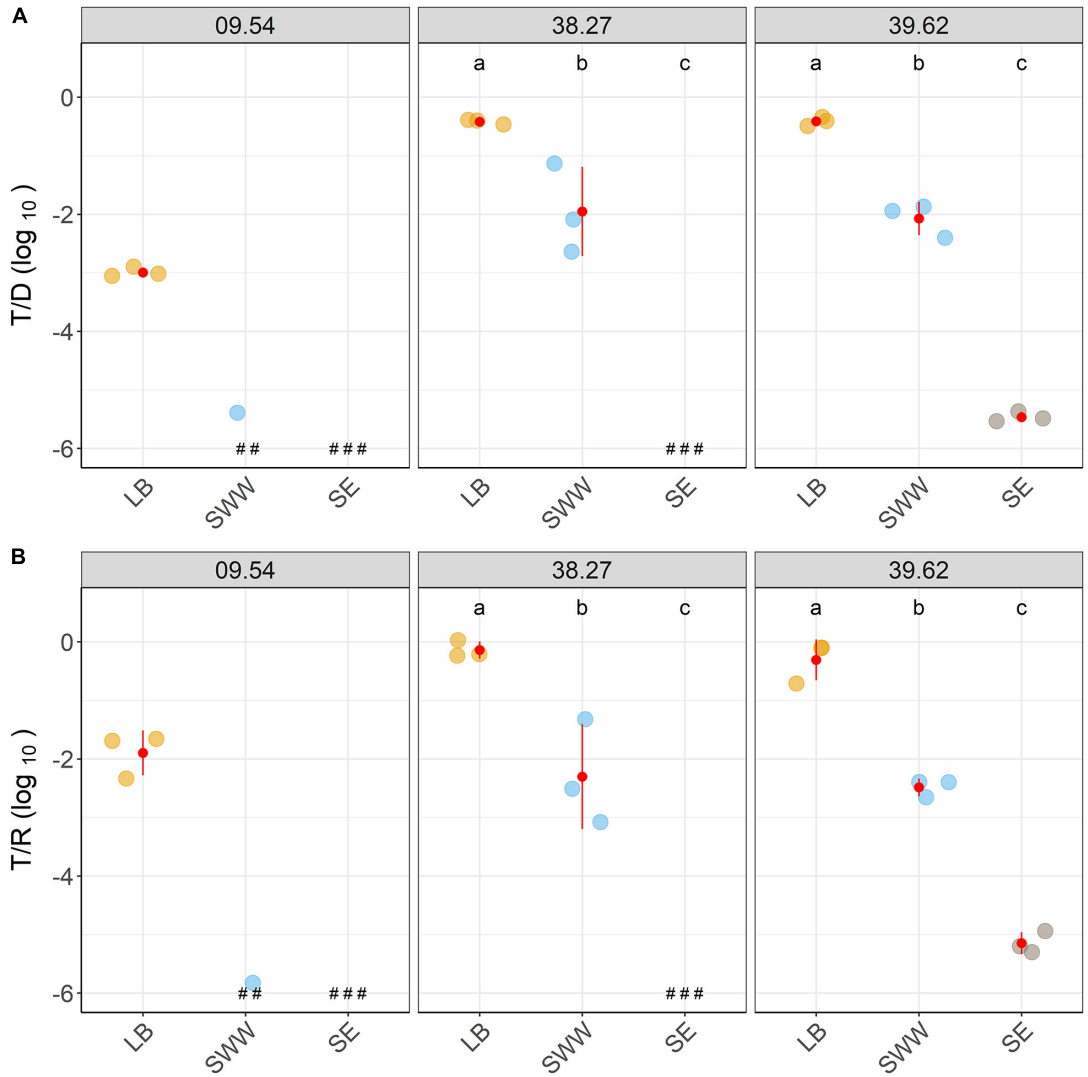

FIGURE 4 | Decrease in nutrient concentration reduced conjugation events. Relative counts of transconjugant-to-donor (T/D; **A**) and transconjugant-to-recipient (T/R; **B**) after 2h-matings performed, at diverse nutrient conditions (Luria-Bertani, LB; synthetic wastewater, SWW; and soil extract, SE), are shown together with average and standard deviation values (in red). Different colors depict distinct media. ^{a,b,c} Indicate significantly different groups of transfer frequency between culture media (*PostHoc* Tukey test, $p < 0.05$), and replicates with no detected transconjugants are highlighted ($^{\#}$).

09.54 and 38.27 did not produce detectable transconjugants (**Supplementary Figure 3**).

DISCUSSION

The effects of temperature and nutrient abundance during mating of an IncP-1 plasmid were evaluated in three natural ESBL *E. coli* recipient strains by monitoring both total amounts of transconjugants and transfer frequencies. The results confirmed that psychrophilic temperatures during mating, as well as nutrient limitation, resulted in the reduction of transfer events. The decrease in the number of transconjugants was more prominent with lower nutrients than with lower temperatures.

Transfer Efficiency Varied Across Strains

Under optimal physiological conditions for the growth of the three *E. coli* strains 09.54, 38.27, and 39.62 tested (rich LB medium, higher mesophilic temperature of 37°C), the conjugative transfer of plasmid significantly differed among the recipients. Two strains showed a high frequency of transfer (5×10^{-1}), while the third (strain 09.54) had 2 logs less. High frequency of transfer is common among IncP-1 plasmids (Thomas and Smith, 1987), which are naturally derepressed (Bradley et al., 1980). Similar transfer frequencies (10^{-2}) have been described before for the pKJK5 plasmid in soil microcosms (Musovic et al., 2006). The difference of transfer frequency among strains from the same species can relate to strain-specific characteristics or repression of silencing systems that either

avoid or limit the expression of the new acquire genes in the recipient cell (Frost and Koraimann, 2010). The plasmid stability and replication depend heavily on complex coordination and synchronicity between the vector and host (Novick, 1987). In the present study, only one bacterial species (*E. coli*) was used to minimize potential genetic incompatibilities between donor and recipients. However, even when the same species are used, variable transfer frequencies are often reported. For instance, Dimitriu et al. (2019) observed a difference up to 5 orders of magnitude in the transfer frequencies of an IncF and IncP-1 among naturally co-occurring *E. coli* isolates. These significant differences are likely linked to the genetic diversity within species. Here, the accessory genes in the used strains corresponded to roughly 50% of the genomic content (**Supplementary Figure 5**). However, which of these accessory traits can be the cause of variation remains a matter of discussion. Dimitriu et al. (2019) found no preferential transfer among isolates sharing serotype or closely related phylogeny. Instead, they proposed that conjugal transfer was favored by clone-relationship, derived from similar restriction-modification systems. Contrarily, a recent study evaluating the transfer of ESBL plasmids among clinical *E. coli* isolates could not find such a relationship Benz et al. (2021).

In addition to host-recipient dynamics, plasmid to plasmid interactions could also affect the transfer dynamics. The stability of a newly acquired plasmid can be strongly influenced by the presence of other plasmids inside the cell (i.e., incompatibility). Here, we prevented the possible incompatibility issues by using strains with plasmids belonging to distinct Inc groups. Still, alternative effects of co-resident plasmids have been proposed recently. Enhanced transfer frequency of IncP-1 plasmids toward recipient cells hosting IncF plasmids has been observed (Gama et al., 2017). Although the mechanism of action is not entirely clear, the authors suggest that this is not a cooperative process but rather opportunistic use of the IncF transfer machinery by IncP-1 plasmids (Gama et al., 2017). In our experiments, we observed that the two strains with higher transfer frequency contained natural IncF plasmids (among others), whereas 09.54 harbored an IncK plasmid. However, further analysis would be necessary to confirm the role of co-existing plasmids in the recipient cell.

Reducing Input of Donors Reduced Overall Transfer Frequency

A lower D/R proportion resulted in a decreased number of transconjugants, suggesting that the relative proportion of donors to recipients can limit HGT.

Receiving environmental compartments typically contain high cell densities, for instance, activated sludge usually contains between 10^9 and 10^{10} CFU mL^{-1} (Manti et al., 2008) and topsoil (the first 10–15 cm) contain between 10^{14} and 10^{15} cells/m^3 (Bickel and Or, 2020). However, exogenous bacteria that enter the system (potential donors) might not be as numerous. For example, assuming a soil density of 1.5, it results in having 10^8 – 10^9 cells/g soil, while the manure from cattle and pigs contains roughly 10^5 *E. coli* cells/g (Schmitt et al., 2019), at least a 1,000-fold difference. This means that the proportion of potential donors is quite small considering the receiving community. This proportion may depend on multiple factors, including sewage flows or manure application rates, but it is reasonable to expect that the potential donors will be a minority in the compartment to which they were introduced.

During conjugation assays, high cell densities (8.9×10^6 CFU cm^{-2}) would mirror natural systems. Conversely, the use of D/R ratios lower than 1:1 (i.e., 1:10 and 1:100) would presumably reflect more accurately the conditions found in anthropogenically impacted environments. However, to observe differences in conjugation rates under varied conditions, the number of donors should be sufficient to produce a detectable amount of transconjugants with a wide margin from the limit of detection (3 to 4 logs) in the matings performed under optimal conditions. Goodman et al. (1993) and Rochelle et al. (1989) observed that a minimum of 10^4 CFU cm^{-2} of donors and recipients were necessary to observe transconjugants. Here, conjugation occurred at donor densities as low as 10^4 CFU cm^{-2} yielding a high amount of transconjugants (10^8) for two of the strains (38.27 and 39.62), but not for the third one (strain 09.54). For this last strain, transconjugants were undetectable or close to the limit of detection with initial donor densities of 10^4 or 10^5 CFU cm^{-2} (D/R of 1:100 and 1:10, respectively). Considering that low D/R could prevent the monitoring of conjugation events for at least one of the strains, the subsequent experiments were conducted with a D/R ratio of 1:1. Similar cell densities and ratios have been previously advised to observe changes in conjugal transfer across a range of (presumably) unfavorable conditions (Fernandez-Astorga et al., 1992).

Lower Temperature Inhibited Plasmid Transfer, but not Entirely

The highest number of transconjugants was obtained at 37°C, which is also the optimal growth temperature for *E. coli*. However, growth of donors and recipients was observed between their concentrations at the start of the experiment and in the controls (approximately 1 log, in all strains; **Supplementary Figure 2**). Together with growth curve data (data not shown), this suggests that, at 37°C, part of the transconjugant numbers originated from clonal expansion rather than a new transfer event. Conversely, at other temperatures, the number of transconjugants observed reflected more accurately the real number of conjugation events, as the 2-h mating time concurred with the lag phase, and, consequently, clonal expansion can assume to be negligible.

Fluctuations in temperature are known to greatly affect the growth and metabolic functions of microorganisms (Trevors et al., 2012). Yet, the effect of a wide range of temperatures on conjugative AMR-related plasmids has seldom been addressed (Bale et al., 1988; Inoue et al., 2005; Banerjee et al., 2016). Although cold conditions are predominantly found around the planet (Rodrigues and Tiedje, 2008) and in relevant environments for AMR spread (**Supplementary Table 4**), studies addressing the environmental dissemination of AMR plasmids in microcosms often used rather warm (>25°C) settings. Warm temperatures (25–30°C) are also common for *in vitro* studies that focus on either capturing environmental plasmids or addressing the

microbial community permissiveness of a given plasmid, because high conjugation rates are required for detecting a high diversity of transconjugants (Jacquiod et al., 2017; Li et al., 2020, 2018).

Conjugation occurred at environmental temperatures (i.e., 15°C), which are average temperatures found in wastewater and soil worldwide (**Supplementary Table 4**), but it also occurred at 9°C. Typically, most wastewater treatment plants do not operate at temperatures below 9°C (because of nitrification failure), but in some countries, particularly northern countries, they can operate at temperatures close to 0°C (Delatolla et al., 2012; Hoang et al., 2014). The use of different strains emphasized that the effect of temperature on the transfer frequency is recipient-dependent and probably not affected just by chromosomally encoded factors, but also by resident plasmids in the recipient. The different outcomes observed between strains highlights the difficulty of inferring results that can be applicable to all putative recipient strains, even when they belong to the same species.

Lower Nutrient Composition Hindered Conjugation

A stronger effect on the transfer frequency was observed in matings performed with lower nutrient concentrations, where the frequency of conjugation was proportional to the nutrient richness of the culture media (LB > SWW > SE). In some cases, it was not possible to recover transconjugants in SE. Some authors suggest that plasmid transfer is related to cell growth and does not occur in non-growing cells (Seoane et al., 2011; Kohyama and Suzuki, 2019), others consider that it happens after cell division and right before entering a non-growing phase (Headd and Bradford, 2020). We observed conjugation in SE media for at least one of the conjugation pairs, despite cell growth was not observed for either donor or recipients in this media.

Comparatively, the SE and SWW media used in this study contained 40 to 300-fold (SE), and 20- to 40-fold (SWW) lower basic nutrients (carbon, nitrogen and phosphorus) concentrations than the classical nutrient-rich media (LB; **Table 2**). Conjugation requires energy and cellular resources to occur, and thus, one could expect that low nutrient conditions would hamper plasmid transfer (Goodman et al., 1993). Interestingly, the effect of nutrient deprivation on conjugation is seldom documented. Fernandez-Astorga et al. (1992) addressed the effect of available TOC in liquid media, finding transconjugants even at 1 mg L^{-1} of TOC. Inoue et al. (2005) observed decreasing transconjugants in media with a decreasing amount of dissolved organic carbon (DOC) (6'636 to 21.6 mg L^{-1}), including LB, synthetic, and real wastewater. However, in the two aforementioned studies and elsewhere (Grabow et al., 1975; O'Morchoe et al., 1988; MacDonald et al., 1992; Headd and Bradford, 2018), donor and recipient cells were pre-grown in a nutrient-rich media and then subjected to conjugation in the low nutrient media. Extra energy and nutrients stored in the cells during this pre-growth phase may allow bacteria to undergo conjugation in an earlier stage of the mating, potentially masking the effect of lower nutrition conditions on conjugation (Curtiss et al., 1969). To bypass this bias, Goodman et al. (1993) starved donors and recipients in minimal media (low amount of salts and no carbon source) prior to the conjugation. They found that, despite the lack of nutrients, conjugation occurred after the donors were starved up to 3 or 20 days, when *E. coli* or *Vibrio* sp. were the donors, respectively. In the current study, when addressing conjugal transfer in low nutrient media, cells were also pre-incubated in the corresponding low-nutrient media (SWW or SE) to avoid the influence of intracellular nutrient reservoirs.

Then again, carbon concentration is likely not the only nutrient that can limit conjugation. In their work, Inoue et al. (2005) observed that transconjugants and transfer rates were 2.5 logs higher in SWW than in 16-fold diluted LB, while both contained similar DOC content (410 mg L^{-1}). Possibly, higher concentration of other nutrients (nitrogen, phosphorus or specific cations) in the SWW allowed an increase in conjugation frequencies and/or clonal expansion of the transconjugants. Pre-growth in media lacking casamino acids delayed *pili* formation after nutritional conditions are restored (Curtiss et al., 1969). As *pili* formation is protein-dependent, nitrogen-compounds are required for plasmid transfer. Despite being an essential nutrient, the role of phosphate or inorganic phosphorus deprivation in conjugation has not been explored yet. Phosphorus is known to be a limiting factor of cell growth and metabolism in oligotrophic environments (Smith and Prairie, 2004). In *E. coli*, phosphorus starvation induces a wide range of metabolic changes including cell surface modification and increase of cell adhesion characteristics (adhesins and fimbria), which could affect the interaction between cells and ultimately the conjugation rates. Finally, the concentration of other micronutrients as divalent cations might also influence conjugation. Recently, Sakuda et al. (2018) observed that the addition of divalent cations to low nutrient media (Ca^{2+} and Mg^{2+}) increased the conjugation frequency of IncP-7 plasmids among *Pseudomonas* strains. Yet, the molecular mechanisms of this effect remain unclear.

Moreover, in the present study, the pH values of the different media were not maintained or adjusted, except in SWW. In SWW, the pH was adjusted to 6.8 close to the ones observed in wastewater [6.5–8.5 (Prot et al., 2020)] while the pH from SE was kept at its original value (5.0 – 5.3), which was representative of Dutch soils of this texture (Römkens and Oenema, 2004). Soil was kept at ambient pH to maintain solubility of soil nutrients. As pH can affect bacterial growth, it could have also contributed to the decrease of transconjugants in this study observed for soil. Indeed, it has been shown that pH values in this range (5.0 – 5.3) can decrease conjugation (Richaume et al., 1989), but it only resulted in a maximum of 3-fold reduction (0.5 logs) when compared to conjugation occurring at neutral pH. In the context of the present study, it is difficult to discriminate what was the effective contribution of pH in decreasing plasmid transfer in SE. However, given the several log decrease in transconjugants, it is reasonable to say that the lower nutrient content had a more important contribution in SE.

Extrapolation of the Results and Limitations of the Study

This study addresses the influence of temperature and nutrient conditions on a specific system based on *E. coli* strains and an IncP-1 broad-host range plasmid. Probably, the impact of

the factors addressed here would differ per species. Bacteria better suited to thrive under typical environmental conditions will most likely be less affected by low temperatures and nutrient conditions, as observed by a longer ability (+13 days) for conjugal transfer when using pre-starved *Vibrio* spp. as donor instead of *E. coli* (Goodman et al., 1993). In addition, the plasmid characteristics (e.g., size, incompatibility group) obviously determine absolute transfer rates. Thus, further research addressing other combinations of donors-recipients will be desirable.

CONCLUSION

When moving from laboratory conditions to environmentally relevant conditions for soils and WWTPs, both lower temperature and lower nutrient concentrations showed to reduce conjugal transfer of an IncP-1 plasmid significantly. The effect lower nutrient concentrations on the number of transconjugants was stronger than the effect of lower temperatures. While nutritional conditions appear critical, the role of single nutrients, such as nitrogen and phosphorus, is not entirely clear and deserves further follow-up research. Furthermore, the transfer potential was recipient-dependent and varied within ESBL *E. coli* strains of the same species.

To conclude, although abiotic factors can hamper plasmid transfer, measurable conjugation between *E. coli* still occurred under conditions that mimicked those commonly found in the wastewater and soil environment (9 – 25°C). Despite conjugation being observed between strains of the same species, this study shows that fecal indicator bacteria were capable of donating an IncP-1 plasmid in less-than-optimal contexts, and consequently, can be a source of transferable AMR traits once they reach the environment.

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

RP-V, GM, LH, and HS conceived and designed the study. RP-V and GM performed the experiments and analyzed the data. MB and RP-V performed the analysis of the next-generation sequencing data. MB, LH, DM, DH, ML, DW, and HS supervised the study. RP-V and GM wrote the manuscript. MB, LH, PM, ML, DW, DM, DH, and HS reviewed and edited the manuscript. All authors read 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.2021.656250/full#supplementary-material

REFERENCES

Abis, K. L., and Mara, D. (2006). Temperature measurement and stratification in facultative waste stabilisation ponds in the UK climate. *Environ. Monit. Assess.* 114, 35–47. doi: 10.1007/s10661-006-1076-7

Al Qarni, H., Collier, P., O'Keeffe, J., and Akunna, J. (2016). Investigating the removal of some pharmaceutical compounds in hospital wastewater treatment plants operating in Saudi Arabia. *Environ. Sci. Pollut. Res.* 23, 13003–13014. doi: 10.1007/s11356-016-6389-7

Amann, R. I., Ludwig, W., and Schleifer, K.-H. (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol. Mol. Biol. Rev.* 59, 143–169. doi: 10.1128/mmbr.59.1.143-169.1995

Bahl, M. I., Hansen, L. H., and Sørensen, S. J. (2007). Impact of conjugal transfer on the stability of IncP-1 plasmid pKJK5 in bacterial populations. *FEMS Microbiol. Lett.* 266, 250–256. doi: 10.1111/j.1574-6968.2006.00536.x

Bale, M. J., Fry, J. C., and Day, M. J. (1988). Transfer and occurrence of large mercury resistance plasmids in river epilithon. *Appl. Environ. Microbiol.* 54, 972–978. doi: 10.1128/aem.54.4.972-978.1988

Banerjee, G., Ray, A. K., and Kumar, R. (2016). Effect of temperature on lateral gene transfer efficiency of multi-antibiotics resistant bacterium, alcaligenes faecalis. *Sains Malays.* 45, 909–914.

Barrios-Hernández, M. L., Pronk, M., Garcia, H., Boersma, A., Brdjanovic, D., van Loosdrecht, M. C. M., et al. (2020). Removal of bacterial and viral indicator organisms in full-scale aerobic granular sludge and conventional activated sludge systems. *Water Res.* 6:40. doi: 10.1016/j.wroa.2019.100040

Bellanger, X., Guilloteau, H., Bonot, S., and Merlin, C. (2014a). Demonstrating plasmid-based horizontal gene transfer in complex environmental matrices: a practical approach for a critical review. *Sci. Total Environ.* 493, 872–882. doi: 10.1016/j.scitotenv.2014.06.070

Bellanger, X., Guilloteau, H., Breuil, B., and Merlin, C. (2014b). Natural microbial communities supporting the transfer of the IncP-1β plasmid pB10 exhibit a

higher initial content of plasmids from the same incompatibility group. *Front. Microbiol.* 5:637. doi: 10.3389/fmicb.2014.00637

Benz, F., Huisman, J. S., Bakkeren, E., Herter, J. A., Stadler, T., Ackermann, M., et al. (2021). Plasmid- and strain-specific factors drive variation in ESBL-plasmid spread in vitro and in vivo. *ISME J.* 15, 862–878. doi: 10.1038/s41396-020-00819-4

Bickel, S., and Or, D. (2020). Soil bacterial diversity mediated by microscale aqueous-phase processes across biomes. *Nat. Commun.* 11:116. doi: 10.1038/s41467-019-13966-w

Binh, C. T. T., Heuer, H., Kaupenjohann, M., and Smalla, K. (2008). Piggery manure used for soil fertilization is a reservoir for transferable antibiotic resistance plasmids. *FEMS Microbiol. Ecol.* 66, 25–37. doi: 10.1111/j.1574-6941.2008.00526.x

Boeije, G., Corstanje, R., Rottiers, A., and Schowanek, D. (1999). Adaptation of the CAS test system and synthetic sewage for biological nutrient removal. *Chemosphere* 38, 699–709. doi: 10.1016/S0045-6535(98)00311-7

Bradley, D. E., Taylor, D. E., and Cohen, D. R. (1980). Specification of surface mating systems among conjugative drug resistance plasmids in *Escherichia coli* K-12. *J. Bacteriol.* 143, 1466–1470. doi: 10.1128/jb.143.3.1466-1470.1980

Carattoli, A. (2013). Plasmids and the spread of resistance. *Int. J. Med. Microbiol.* 303, 298–304. doi: 10.1016/j.ijmm.2013.02.001

Carattoli, A., Zankari, E., Garciá-Fernández, A., Larsen, M. V., Lund, O., Villa, L., et al. (2014). In Silico detection and typing of plasmids using plasmidfinder and plasmid multilocus sequence typing. *Antimicrob. Agents Chemother.* 58, 3895–3903. doi: 10.1128/AAC.02412-14

Ceccarelli, D., Kant, A., Van Essen-Zandbergen, A., Dierikx, C., Hordijk, J., Wit, B., et al. (2019). Diversity of plasmids and genes encoding resistance to extended spectrum cephalosporins in commensal *Escherichia coli* From Dutch livestock in 2007-2017. *Front. Microbiol.* 10:76. doi: 10.3389/fmicb.2019.00076

Curtiss, R., Caro, L. G., Allison, D. P., and Stallions, D. R. (1969). Early stages of conjugation in *Escherichia coli*. *J. Bacteriol.* 100, 1091–1104. doi: 10.1128/JB.100.2.1091-1104.1969

Delatolla, R., Tufenkji, N., Comeau, Y., Gadbois, A., Lamarre, D., and Berk, D. (2012). Effects of long exposure to low temperatures on nitrifying biofilm and biomass in wastewater treatment. *Water Environ. Res.* 84, 328–338. doi: 10.2175/106143012x13354606450924

Dimitriu, T., Marchant, L., Buckling, A., and Raymond, B. (2019). Bacteria from natural populations transfer plasmids mostly towards their kin. *Proc. R. Soc. B Biol. Sci.* 286:20191110. doi: 10.1098/rspb.2019.1110

Fan, X.-T., Li, H., Chen, Q.-L., Zhang, Y.-S., Ye, J., Zhu, Y.-G., et al. (2019). Fate of antibiotic resistant *Pseudomonas* putida and broad host range plasmid in natural soil microcosms. *Front. Microbiol.* 10:194. doi: 10.3389/fmicb.2019.00194

Fernandez-Astorga, A., Muela, A., Cisterna, R., Iriberri, J., and Barcina, I. (1992). Biotic and abiotic factors affecting plasmid transfer in *Escherichia coli* strains. *Appl. Environ. Microbiol.* 58:2708. doi: 10.1128/aem.58.8.2708-2708.1992

Fox, J. (2005). The R commander: a basic statistics graphical user interface to R. *J. Stat. Softw.* 14, 1–42.

Fox, J., and Weisberg, S. (2019). *An R Companion to Applied Regression*, 3 Edn. Thousand Oaks CA: Sage.

Frost, L. S., and Koraimann, G. (2010). Regulation of bacterial conjugation: balancing opportunity with adversity. *Future Microbiol.* 5, 1057–1071. doi: 10.2217/fmb.10.70

Gama, J. A., Zilhão, R., and Dionisio, F. (2017). Conjugation efficiency depends on intra and intercellular interactions between distinct plasmids: plasmids promote the immigration of other plasmids but repress co-colonizing plasmids. *Plasmid* 93, 6–16. doi: 10.1016/j.plasmid.2017.08.003

Goodman, A. E., Hild, E., Marshall, K. C., and Hermansson, M. (1993). Conjugative plasmid transfer between bacteria under simulated marine oligotrophic conditions. *Appl. Environ. Microbiol.* 59, 1035–1040. doi: 10.1128/aem.59.4.1035-1040.1993

Grabow, W. O. K., Prozesky, O. W., and Burger, J. S. (1975). Behaviour in a river and dam of coliform bacteria with transferable or non-transferable drug resistance. *Water Res.* 9, 777–782. doi: 10.1016/0043-1354(75)90134-7

Hadfield, J., Croucher, N. J., Goater, R. J., Abudahab, K., Aanensen, D. M., and Harris, S. R. (2018). Phandango: an interactive viewer for bacterial population genomics. *Bioinformatics* 34, 292–293. doi: 10.1093/bioinformatics/btx610

Headd, B., and Bradford, S. A. (2018). Physicochemical factors that favor conjugation of an antibiotic resistant plasmid in non-growing bacterial cultures in the absence and presence of antibiotics. *Front. Microbiol.* 9:2122. doi: 10.3389/fmicb.2018.02122

Headd, B., and Bradford, S. A. (2020). The conjugation window in an *Escherichia coli* K-12 strain with an IncFII plasmid. *Appl. Environ. Microbiol.* 86, 1–18. doi: 10.1128/AEM.00948-20

Hoang, V., Delatolla, R., Abujamel, T., Mottawea, W., Gadbois, A., Laflamme, E., et al. (2014). Nitrifying moving bed biofilm reactor (MBBR) biofilm and biomass response to long term exposure to 1°C. *Water Res.* 49, 215–224. doi: 10.1016/j.watres.2013.11.018

Hu, Y., Yang, X., Qin, J., Lu, N., Cheng, G., Wu, N., et al. (2013). Metagenome-wide analysis of antibiotic resistance genes in a large cohort of human gut microbiota. *Nat. Commun.* 4:2151. doi: 10.1038/ncomms3151

Inoue, D., Sei, K., Soda, S., Ike, M., and Fujita, M. (2005). Potential of predominant activated sludge bacteria as recipients in conjugative plasmid transfer. *J. Biosci. Bioeng.* 100, 600–605. doi: 10.1263/jbb.100.600

Jacquiod, S., Brejnrod, A., Morberg, S. M., Abu Al-Soud, W., Sørensen, S. J., and Riber, L. (2017). Deciphering conjugative plasmid permissiveness in wastewater microbiomes. *Mol. Ecol.* 26, 3556–3571. doi: 10.1111/mec.14138

Jaureguy, F., Landraud, L., Passet, V., Diancourt, L., Frapy, E., Guigon, G., et al. (2008). Phylogenetic and genomic diversity of human bacteremic *Escherichia coli* strains. *BMC Genom.* 9:560. doi: 10.1186/1471-2164-9-560

Klümper, U., Riber, L., Dechesne, A., Sannazzarro, A., Hansen, L. H., Sørensen, S. J., et al. (2015). Broad host range plasmids can invade an unexpectedly diverse fraction of a soil bacterial community. *ISME J.* 9, 934–945. doi: 10.1038/ismej.2014.191

Kohyama, Y., and Suzuki, S. (2019). Conjugative gene transfer between nourished and starved cells of *Photobacterium damselae* ssp. *damselae* and *Escherichia coli*. *Microb. Environ.* 34, 388–392. doi: 10.1264/jsme2.ME19099

Larsen, M. V., Cosentino, S., Rasmussen, S., Friis, C., Hasman, H., Marvig, R. L., et al. (2012). Multilocus sequence typing of total-genome-sequenced bacteria. *J. Clin. Microbiol.* 50, 1355–1361. doi: 10.1128/JCM.06094-11

Li, L., Dechesne, A., He, Z., Madsen, J. S., Nesme, J., Sørensen, S. J., et al. (2018). Estimating the transfer range of plasmids encoding antimicrobial resistance in a wastewater treatment plant microbial community. *Environ. Sci. Technol. Lett.* 5, 260–265. doi: 10.1021/acs.estlett.8b00105

Li, L., Dechesne, A., Madsen, J. S., Nesme, J., Sørensen, S. J., and Smets, B. F. (2020). Plasmids persist in a microbial community by providing fitness benefit to multiple phylotypes. *ISME J.* 14, 1170–1181. doi: 10.1038/s41396-020-0596-4

MacDonald, J. A., Smets, B. F., and Rittmann, B. E. (1992). The effects of energy availability on the conjugative-transfer kinetics of plasmid RP4. *Water Res.* 26, 461–468. doi: 10.1016/0043-1354(92)90046-7

Manti, A., Boi, P., Falcioni, T., Canonico, B., Ventura, A., Sisti, D., et al. (2008). Bacterial cell monitoring in wastewater treatment plants by flow cytometry. *Water Environ. Res.* 80, 346–354. doi: 10.2175/106143007x221418

Musovic, S., Dechesne, A., Sørensen, J., and Smets, B. F. (2010). Novel assay to assess permissiveness of a soil microbial community toward receipt of mobile genetic elements. *Appl. Environ. Microbiol.* 76, 4813–4818. doi: 10.1128/AEM.02713-09

Musovic, S., Oregaard, G., Kroer, N., and Sørensen, S. J. (2006). Cultivation-independent examination of horizontal transfer and host range of an IncP-1 plasmid among gram-positive and gram-negative bacteria indigenous to the barley rhizosphere. *Appl. Environ. Microbiol.* 72, 6687–6692. doi: 10.1128/AEM.00013-06

Novick, R. P. (1987). Plasmid incompatibility. *Microbiol. Rev.* 51, 381–395.

Ochman, H., Lawrence, J. G., and Groisman, E. A. (2000). Lateral gene transfer and the nature of bacterial innovation. *Nature* 405, 299–304. doi: 10.1038/35012500

O'Morchoe, S. B., Ogunseitan, O., Sayler, G. S., and Miller, R. V. (1988). Conjugal transfer of R68.45 and FP5 between *Pseudomonas aeruginosa* strains in a freshwater environment. *Appl. Environ. Microbiol.* 54, 1923–1929. doi: 10.1128/AEM.54.8.1923-1929.1988

Osińska, A., Korzeniewska, E., Harnisz, M., Felis, E., Bajkacz, S., Jachimowicz, P., et al. (2020). Small-scale wastewater treatment plants as a source of the dissemination of antibiotic resistance genes in the aquatic environment. *J. Hazard. Mater.* 381:121221. doi: 10.1016/j.jhazmat.2019.121221

O'Neill, J. (2016). Tackling drug-resistant infections globally: final report and recommendations. *Lancet Infect. Dis.* 16, 767–768.

Page, A. J., Cummins, C. A., Hunt, M., Wong, V. K., Reuter, S., Holden, M. T. G., et al. (2015). Roary: rapid large-scale prokaryote pan genome analysis. *Bioinformatics* 31, 3691–3693. doi: 10.1093/bioinformatics/btv421

Pallares-Vega, R., Hernandez Leal, L., Fletcher, B. N., Vias-Torres, E., van Loosdrecht, M. C. M., Weissbrodt, D. G., et al. (2021). Annual dynamics of antimicrobials and resistance determinants in flocculent and aerobic granular sludge treatment systems. *Water Res.* 190:116752. doi: 10.1016/j.watres.2020.116752

Pinilla-Redondo, R., Cyriaque, V., Jacquiod, S., Sørensen, S. J., and Riber, L. (2018). Monitoring plasmid-mediated horizontal gene transfer in microbiomes: recent advances and future perspectives. *Plasmid* 99, 56–67. doi: 10.1016/j.plasmid.2018.08.002

Popowska, M., and Krawczyk-Balska, A. (2013). Broad-host-range IncP-1 plasmids and their resistance potential. *Front. Microbiol.* 4:44. doi: 10.3389/fmicb.2013.00044

Price, M. N., Dehal, P. S., and Arkin, A. P. (2010). FastTree 2 - approximately maximum-likelihood trees for large alignments. *PLoS One* 5:e09490. doi: 10.1371/journal.pone.0009490

Prot, T., Korving, L., and van Loosdrecht, M. C. (2020). Ionic strength of the liquid phase of different sludge streams in a wastewater treatment plant. *Chem. Rxiv* [Preprint]. doi: 10.26434/chemrxiv.13359437.v1

R Core Team (2018). *R: A Language and Environment for Statistical Computing*. Vienna: R Found. Stat. Comput.

Richaume, A., Angle, J. S., and Sadowsky, M. J. (1989). Influence of soil variables on in situ plasmid transfer from *Escherichia coli* to *Rhizobium fredii*. *Appl. Environ. Microbiol.* 55, 1730–1734. doi: 10.1128/aem.55.7.1730-1734.1989 doi: 10.1128/aem.55.7.1730-1734.1989

Rochelle, P. A., Fry, J. C., and Day, M. J. (1989). Factors affecting conjugal transfer of plasmids encoding mercury resistance from pure cultures and mixed natural suspensions of epilithic bacteria. *J. Gen. Microbiol.* 135, 409–424. doi: 10.1099/00221287-135-2-409

Römkens, P. F. A. M., and Oenema, O. (2004). *Quick Scan Soils in The Netherlands. Overview of the Soil Status With Reference to the Forthcoming EU Soil Strategy. Wageningen, Alterra, Alterra-Rapport 948*. Wageningen: Alterra.

Rodrigues, D. F., and Tiedje, J. M. (2008). Coping with our cold planet. *Appl. Environ. Microbiol.* 74, 1677–1686. doi: 10.1128/AEM.02000-07

Rozwandowicz, M., Brouwer, M. S. M., Fischer, J., Wagenaar, J. A., Gonzalez-Zorn, B., Guerra, B., et al. (2018). Plasmids carrying antimicrobial resistance genes in *Enterobacteriaceae*. *J. Antimicrob. Chemother.* 73, 1121–1137. doi: 10.1093/jac/dkx488

Rozwandowicz, M., Hordijk, J., Bossers, A., Zomer, A. L., Wagenaar, J. A., Mevius, D. J., et al. (2020). Incompatibility and phylogenetic relationship of I-complex plasmids. *Plasmid* 109:102502. doi: 10.1016/j.plasmid.2020.102502

Sakuda, A., Suzuki-Minakuchi, C., Matsui, K., Takahashi, Y., Okada, K., Yamane, H., et al. (2018). Divalent cations increase the conjugation efficiency of the incompatibility P-7 group plasmid pCAR1 among different *pseudomonas* hosts. *Microbiology* 164, 20–27. doi: 10.1099/mic.0.000583

Schmitt, H., Blaak, H., Hoeksma, P., van de Schans, M., Kemper, M., de Rijk, S., et al. (2019). *Antibioticaresistente Bacteriën, Resistentiegenen en Antibioticaresiduen in Mest*. RIVM. doi: 10.21945/RIVM-2019-0112

Scott, K. P., Mercer, D. K., Glover, L. A., and Flint, H. J. (2006). The green fluorescent protein as a visible marker for lactic acid bacteria in complex ecosystems. *FEMS Microbiol. Ecol.* 26, 219–230. doi: 10.1111/j.1574-6941.1998.tb00507.x

Seemann, T. (2014). Prokka: rapid prokaryotic genome annotation. *Bioinformatics* 30, 2068–2069. doi: 10.1093/bioinformatics/btu153

Sengeløv, G., Kristensen, K. J., Sørensen, A. H., Kroer, N., and Sørensen, S. J. (2001). Effect of genomic location on horizontal transfer of a recombinant gene cassette between *Pseudomonas* strains in the Rhizosphere and Spermosphere of barley seedlings. *Curr. Microbiol.* 42, 160–167. doi: 10.1007/s002840010197

Seoane, J., Yankelevich, T., Dechesne, A., Merkey, B., Sternberg, C., and Smets, B. F. (2011). An individual-based approach to explain plasmid invasion in bacterial populations. *FEMS Microbiol. Ecol.* 75, 17–27. doi: 10.1111/j.1574-6941.2010.00994.x

Shintani, M., Nour, E., Elsayed, T., Blau, K., Wall, I., Jechalke, S., et al. (2020). Plant species-dependent increased abundance and diversity of IncP-1 plasmids in the rhizosphere: new insights into their role and ecology. *Front. Microbiol.* 11:590776. doi: 10.3389/fmicb.2020.590776

Smillie, C., Garcillán-Barcia, M. P., Francia, M. V., Rocha, E. P. C., and de la Cruz, F. (2010). Mobility of plasmids. *Microbiol. Mol. Biol. Rev.* 74, 434–452. doi: 10.1128/MMBR.00020-10

Smith, E. M., and Prairie, Y. T. (2004). Bacterial metabolism and growth efficiency in lakes: the importance of phosphorus availability. *Limnol. Oceanogr.* 49, 137–147. doi: 10.4319/lo.2004.49.1.0137

Sørensen, S. J., Bailey, M., Hansen, L. H., Kroer, N., and Wuertz, S. (2005). Studying plasmid horizontal transfer in situ: a critical review. *Nat. Rev. Microbiol.* 3, 700–710. doi: 10.1038/nrmicro1232

Thomas, C. M., and Smith, C. A. (1987). Incompatibility group P plasmids: genetics, evolution, and use in genetic manipulation. *Annu. Rev. Microbiol.* 41, 77–101. doi: 10.1146/annurev.mi.41.100187.000453

Thorsten, T. (2020). *PMCMRplus: Calculate Pairwise Multiple Comparisons of Mean Rank Sums Extended*.

Trevors, J. T., Bej, A. K., Mojib, N., van Elsas, J. D., and van Overbeek, L. (2012). Bacterial gene expression at low temperatures. *Extremophiles* 16, 167–176. doi: 10.1007/s00792-011-0423-y

Wirth, T., Falush, D., Lan, R., Colles, F., Mensa, P., Wieler, L. H., et al. (2006). Sex and virulence in *Escherichia coli*: an evolutionary perspective. *Mol. Microbiol.* 60, 1136–1151. doi: 10.1111/j.1365-2958.2006.05172.x

Wickham, H. (2007). Reshaping data with the reshape package. *J. Stat. Softw.* 21, 1–20. doi: 10.18637/jss.v021.i12

Wickham, H., Averick, M., Bryan, J., Chang, W., McGowan, L., François, R., et al. (2019). Welcome to the tidyverse. *J. Open Source Softw.* 4:1686. doi: 10.21105/joss.01686

Zhong, X., Krol, J. E., Top, E. M., and Krone, S. M. (2010). Accounting for mating pair formation in plasmid population dynamics. *J. Theor. Biol.* 262, 711–719. doi: 10.1016/j.jtbi.2009.10.013

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The Role of Gulls as Reservoirs of Antibiotic Resistance in Aquatic Environments: A Scoping Review

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The role of wildlife with long-range dispersal such as gulls in the global dissemination of antimicrobial resistance (AMR) across natural and anthropogenic aquatic environments remains poorly understood. Antibiotic-resistant bacteria have been detected in resident and migratory gulls worldwide for more than a decade, suggesting gulls as either sentinels of AMR pollution from anthropogenic sources or independent reservoirs that could maintain and disperse AMR across aquatic environments. However, confirming either of these roles remains challenging and incomplete. In this review, we present current knowledge on the geographic regions where AMR has been detected in gulls, the molecular characterization of resistance genes, and the evidence supporting the capacity of gulls to disperse AMR across regions or countries. We identify several limitations of current research to assess the role of gulls in the spread of AMR including most studies not identifying the source of AMR, few studies comparing bacteria isolated in gulls with other wild or domestic species, and almost no study performing longitudinal sampling over a large period of time to assess the maintenance and dispersion of AMR by gulls within and across regions. We suggest future research required to confirm the role of gulls in the global dispersion of AMR including the standardization of sampling protocols, longitudinal sampling using advanced satellite tracking, and whole-genome sequencing typing. Finally, we discuss the public health implications of the spread of AMR by gulls and potential solutions to limit its spread in aquatic environments.

Keywords: marine birds, One Health, seagulls, wildlife, bacteria, antimicrobial resistance, AMR, ESBL

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INTRODUCTION

Antimicrobial resistance (AMR) is a major global health challenge affecting human, animal, and environmental health (FAO and WHO, 2019; WHO, 2019). Thus, a One Health approach is required to understand the dynamics of AMR between humans and animals (Salgado-Caxito et al., 2021). Many studies have reported the presence of antibiotic-resistant bacteria (ARB) in

wild animals, highlighting their potential role in the spread of clinically important bacteria to humans and domestic animals (Wang et al., 2017; Benavides et al., 2018; Dolejska and Literak, 2019). Wildlife such as wild birds, particularly the ones living in proximity to human settings or agriculture fields, can acquire AMR from anthropogenic sources when feeding on landfills and wastewater (Nelson et al., 2008; Wang et al., 2017). Despite several reports of wild birds carrying ARB (Wang et al., 2017), their impact on the dissemination of ARB in aquatic environments remains still poorly understood.

Gulls can impact the spread of ARB of public health concern by acting either as (i) receivers of ARB or antibiotic-resistant genes (ARGs) and acting as sentinels of human environmental pollution to natural ecosystems (Guenther et al., 2011) or as (ii) reservoirs of ARBs and ARGs, capable of dispersing ARB or ARGs to different geographic locations and to other species including humans and domestic animals. In particular, the migratory capacity of several gull species such as the Franklin's gull (*Leucophaeus pipixcan*), migrating across America from Canada to Chile, could result in the dissemination of ARB and ARGs over extensive geographic areas, dispersing AMR from regions with high levels of AMR to less affected areas (Báez et al., 2015; Dolejska and Literak, 2019). Gulls are also present in most urban and rural environments, and their feces are extensively dispersed in the environment (Bonnedahl and Järhult, 2014). Several studies have detected ARGs in gulls (Oravcova et al., 2017; Ahlstrom et al., 2019b; Haenni et al., 2020). In particular, AMR has been detected in several species of seagulls, which have large breeding distributions in urban areas and feed on human waste (Bonnedahl et al., 2015; Stedt et al., 2015; Ahlstrom et al., 2019a). Thus, gulls have been suggested as potential reservoirs of ARB and ARGs, although evidence proving their role as reservoirs has not been provided (Radhouani et al., 2010; Aberkane et al., 2015; Merkeviciene et al., 2018).

In this scoping review, we summarized the current knowledge regarding the global dissemination of ARB and ARGs among gulls and assess whether there is evidence supporting the assumption that gulls can act as reservoirs of AMR. In particular, we aim to provide a comprehensive overview of the geographic location where ARB and ARGs have been found in gulls, the gull and bacteria species involved, as well as the antibiotic families and genes detected. To discuss the public health implications of gulls, we summarized whether bacteria of critical importance according to WHO have been detected in gulls. We also assessed the number of publications that had either identified the origin of AMR found in gulls or tested and concluded that gulls can disperse AMR across the landscape or to other species. Based on this current evidence, we discussed several recommendations aiming to improve our understanding of the role of gulls in the dissemination of AMR.

MATERIALS AND METHODS

We performed a scoping review following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses extension for Scoping Reviews (PRISMA-ScR) checklist (Tricco et al., 2018; **Supplementary Table 1**). All authors defined research questions, objectives, search strategy, and inclusion/exclusion criteria through previous discussions.

Search Strategy

The search was performed in PubMed, Scopus, and Web of Science databases using three general queries: (antibiotic resist* OR antimicrobial resist*), (*Escherichia* OR *Klebsiella* OR *Staphylococcus* OR *Enterococcus* OR *Enterobacter** OR *Salmonella* OR *Pseudomonas*), and (bacteria). Each of them was merged with (marine bird* OR aquatic bird* OR gull* OR *Larus*). Details of the search strategy are available as an additional file (**Supplementary Table 2**). Visualization, duplicate removal, and storing collected data were performed in Microsoft Excel.

Eligibility Criteria

We aimed to identify peer-reviewed studies on AMR in different wild species of gulls (i.e., seagulls) showing the presence and/or potential transmission of ARB and ARGs. Thus, we included only studies providing at least one of the following information: (i) wild gull species where ARB was recovered, (ii) phenotypic resistance to specific antibiotics in bacteria isolated from gulls, and/or (iii) ARGs identified in bacteria isolated from gulls. There were no restrictions related to the year of publication or geographical location. Any type of reviews or studies including *in vivo* experiments, samples of gulls from rehabilitation centers, or containing previously published data were excluded. Details of all inclusion and exclusion criteria are provided in **Supplementary Table 3**.

Identification and Screening of Articles

After the removal of duplicates, we identified a total of 3,475 articles published from 1964 to January 2021, including 3 additional references that were identified from reading these papers. Pre-selection by title and abstract reduced to 227 articles for full-text analysis, and 90 fulfilled the preestablished criteria and were included in the final analysis (**Figure 1**). The remaining 140 articles did not fit our inclusion criteria as they did not include gull samples, did not present AMR information/data, data from gulls were previously published, the study included experimental infection, the study was performed on captive gulls or in rehabilitation centers, the study sampling was conducted postmortem, or the full text of the article was not available.

Data Extraction

Extracted data were independently performed by three authors (DZG, MSC, and ZRS) and verified by other authors. Disagreements were resolved through discussion. The obtained data were entered into a Microsoft Excel template adapted from a previous study (**Supplementary Table 4**; Salgado-Caxito et al., 2021). This file included the title of the article, authorship, year of publication, gull species included in the study and whether the species was migratory or not, the number of sampled individuals, the bacteria species studied, the number of recovered isolates, the antimicrobial susceptibility tests performed, the name and family of the antibiotics tested, and the molecular typing used (i.e., PCR,

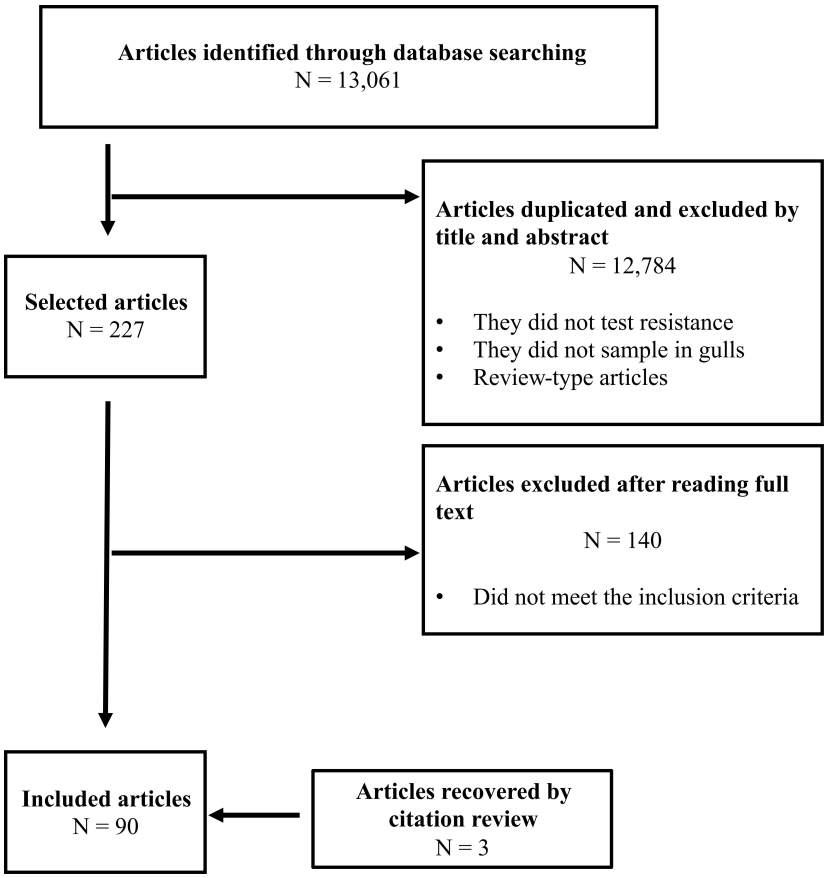

FIGURE 1 | Article search flow diagram.

sequencing, and whole-genome sequencing) when available. To assess the current knowledge on the role of gulls as reservoirs of ARB or ARGs, we also specifically extracted from studies (i) whether the study compared gulls to other animals in the area; (ii) whether the study identified the origin (e.g., anthropogenic source) of the ARB or ARGs found; (iii) if gulls were sampled more than once, particularly in both areas of migration (origin and destination); (iv) if molecular typing of ARB was performed; and (iv) if an individual follow-up and sampling of gulls were performed, along with the method used.

Statistical Analysis

We estimated the proportion of studies filling a given criteria (e.g., studies identifying the presence of ARGs or the origin of ARB) using R. 3.1.6 (R Development Core Team).

RESULTS

Geographic Locations and Gull and Bacteria Species Studied

Our scoping review identified 90 articles published between 1981 and 2020, although only 22% of these studies were published before 2010. The number of studies published on gulls increased from 1 in 1981 to 10 per year in 2020 and peaked in 2017 with 12 articles (**Figure 2B**). Studies were conducted in gulls from all five continents, but the majority of publications were made in Europe (58%) followed by North America (19%) (**Figure 2A**). Studies were conducted in a total of 31 countries, with high-income countries such as the United States (17%), Portugal (12%) and Spain (10%) conducting the highest number of studies (**Figure 2C**). In contrast, in middle- and low-income countries, few publications were conducted (Morocco, 1%; South Africa, 1%; Bangladesh, 1%).

From 100 species of gulls known (IUCN, 2021), ARB or ARGs were recovered from 23 species. Most gulls studied (74%) were migratory species. The number of studies per gull species was highly heterogeneous (**Figure 3B**). The majority of studies focused on the herring gull (*Larus argentatus*, 26%), followed by the laughing gull (*Chroicocephalus ridibundus*, 23%) and the yellow-legged gull (*Larus michahellis*, 19%) (**Figure 3A**). These three species are widely distributed in the northern hemisphere.

Among the 90 studies, 49 ARB species were recovered from gulls. Most studies focused on *Escherichia coli* (59%), *Salmonella enterica* (23%), *Campylobacter jejuni* (8%), and *Klebsiella pneumoniae* (8%) (**Figure 3C**). The temporal trend of publications showed that after 2008, most studies have focused on *E. coli*.

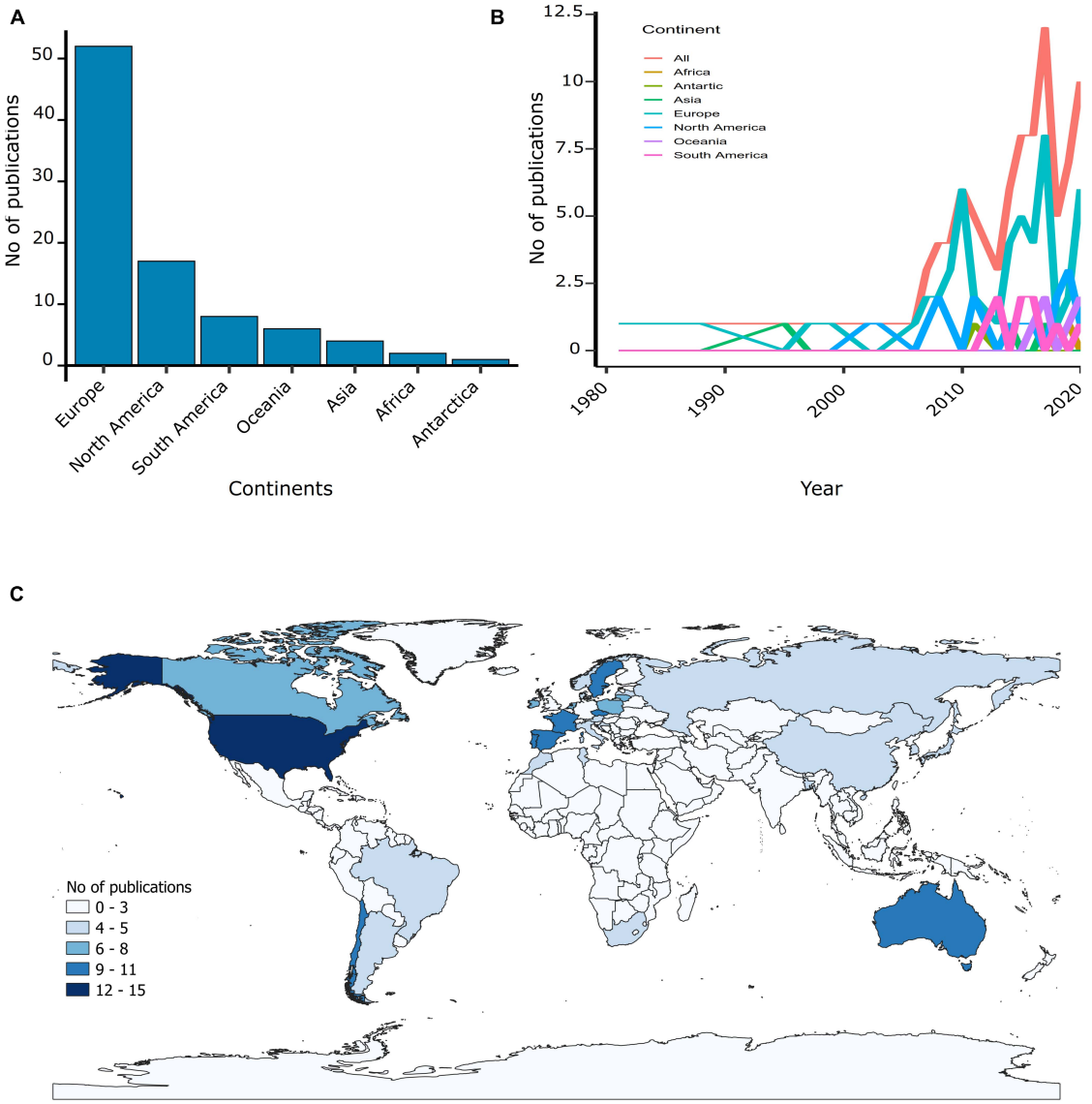

FIGURE 2 | Geographical locations of the AMR studies found in gulls. **(A)** Number of publications per continents. **(B)** Number of publications per continent over the 1980–2020 period. **(C)** Number of publications of AMR in gulls per country in gradient.

Antibiotic Susceptibility in Bacteria From Gulls

Screening of ARB using selective media supplemented with antibiotics before antimicrobial susceptibility tests was performed in 43% of studies. Seventeen percent of studies performed antibiotic susceptibility tests after isolation in non-supplemented media. The remaining 40% of the studies did not present the methodology for recovering isolates. Forty-one publications had information about the number of positive individuals, and 68% of those studies were conducted in Europe. The highest proportion of animals harboring bacteria resistant to at least one antibiotic (referred as positive animals) was estimated in one study in Africa (70%) that included less than 50 individuals. The highest proportion of positive gulls was observed among *Larus dominicanus* (100%), while *Proteus mirabilis* showed the highest proportion of positive individuals (27.9%) (**Table 1**). Given the high heterogeneity in susceptibility methods and antibiotics tested, a comparison of ARB prevalence across studies, defined as the number of positive individuals over the total of sampled animals, could not be performed. Regarding the methodology used to test susceptibility, 68% of studies confirmed phenotypic resistance using the disk diffusion method (CLSI, 2018). Overall, resistance to 79 antibiotic agents from 21 families was tested (**Supplementary Table 5**), including antibiotics used in human medicine such as beta-lactams (i.e., penicillin, cephalosporins, and carbapenems), tetracyclines, fluoroquinolones, sulfonamides,

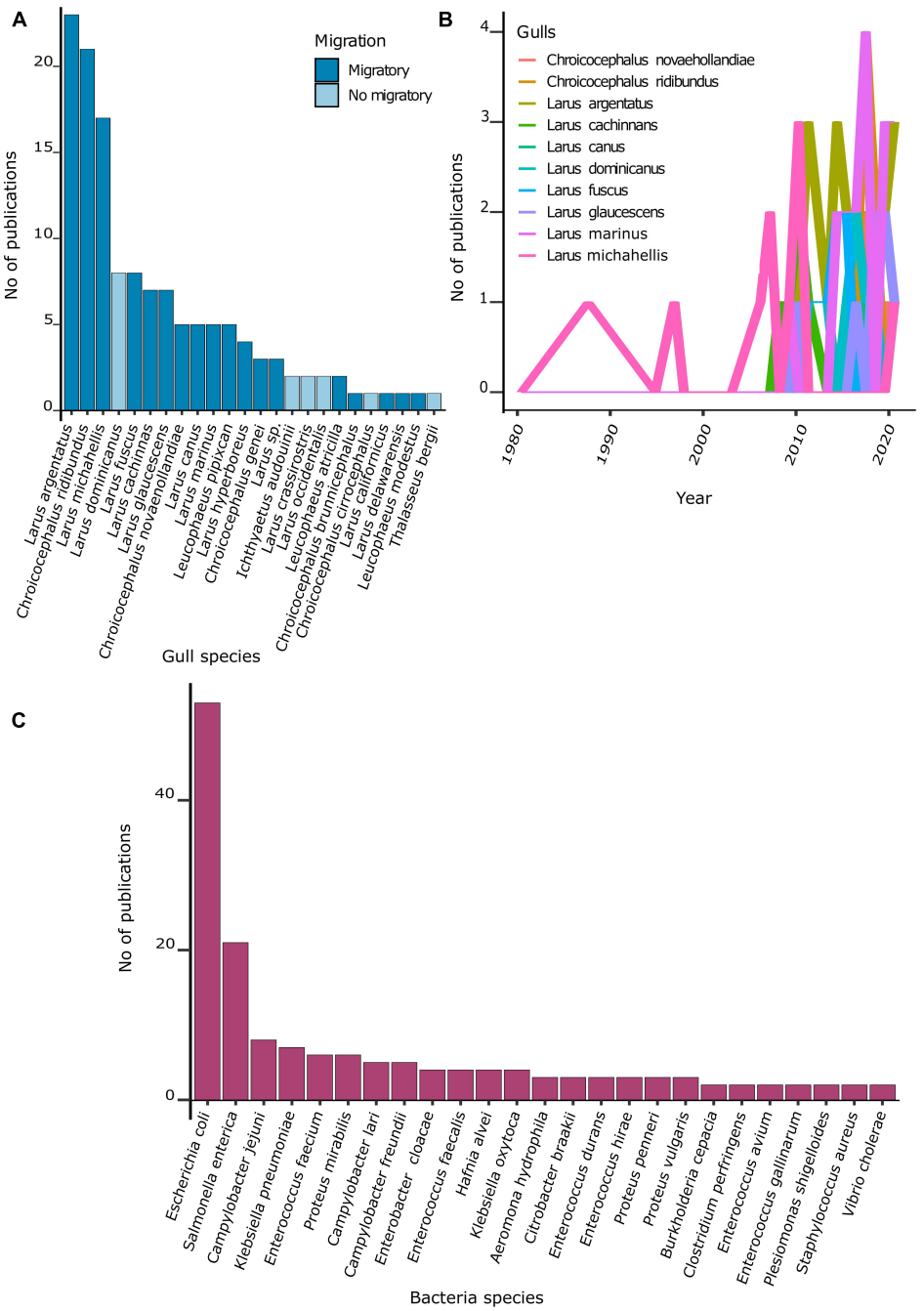

FIGURE 3 | Number of publications per gull and bacteria species. **(A)** Number of publications per gull species. **(B)** Number of publications per gull species over the 1980–2020 period. **(C)** Number of publications per bacteria species.

aminoglycosides, nitrofurans, macrolides, monobactam, polypeptides, glycopeptides, and lincosamides. More than 50% of studies reported at least one bacterial isolate resistant to tetracycline (58%) and ampicillin (52%), followed by chloramphenicol (47%), streptomycin (44%), trimethoprim–sulfamethoxazole (38%), gentamicin (36%), nalidixic acid (35%), and ciprofloxacin (32%) (**Figure 4A**). In particular, broad-spectrum beta-lactams used in human medicine such as amoxicillin with clavulanic acid and ceftazidime were reported in 20% of publications.

Among the four antimicrobial-resistant pathogens considered as a "critical priority" by the WHO (WHO, 2017), all were tested at least once in the reviewed studies. Third-generation cephalosporin-resistant Enterobacterales from gulls were the most reported (41%), followed by carbapenem-resistant Enterobacterales (10%) (**Table 2**). Among "high-priority"

TABLE 1 | AMR bacteria detected in gulls by continent and gull species between 1981 and 2020.

Category	Description	Publications		No. of individuals		% of positive animals
		No.	%	Positives*	Total	
Continent	Africa	1	2.4	28	40	70.0
	Europe	28	68.3	919	6,375	14.4
	North America	5	12.2	50	1,310	3.8
	Oceania	3	7.3	57	1,108	5.1
	South America	4	9.8	164	832	19.7
	Total	*41*		*1,218*	*9,665*	*12.6*
Gull species	*Chroicocephalus novaehollandiae*	2	4.9	4	1,008	0.4
	Chroicocephalus ridibundus	2	4.9	16	1,025	1.6
	Larus argentatus	4	9.8	77	343	22.5
	Larus audouinii	1	2.4	27	111	24.3
	Larus dominicanus	1	2.4	10	10	100.0
	Larus hyperboreus	1	2.4	2	15	13.3
	Larus michahellis	6	14.6	260	814	31.9
	Larus ridibundus	6	14.6	161	2,718	5.9
	Leucophaeus pipixcan	1	2.4	91	124	73.4
	Larus delawarensis	1	2.4	2	32	6.3
	More than one species	16	39.0	568	3,465	16.4
	Total	*41*				
Bacteria species	*Acinetobacter baumannii*	1	2.4	2	741	0.3
	Campylobacter spp.	2	4.9	26	151	17.2
	Enterobacter cloacae	1	2.4	2	15	13.3
	Escherichia coli	21	51.2	805	3,395	23.7
	Proteus mirabilis	2	4.9	98	351	27.9
	Salmonella enterica	9	22.0	130	3,327	3.9
	More than one species	5	12.2	155	1,685	9.2
	Total	*41*				

**Positive individuals represent an individual where at least one resistant bacteria to any antibiotic researched in the study was obtained.*

pathogens, *Campylobacter* spp. (6%) and *Salmonella* spp. (8%) both resistant to fluoroquinolones were the most identified. No "medium-priority" pathogen has been recovered from gulls.

Molecular Characterization of ARGs in Gulls

ARGs were reported in 70% of studies conducted in gulls (**Tables 3**, **4**). Mobile genetic elements (MGE) were identified in 43% of studies, with 35 studies confirming that ARGs were inserted on an MGE. Sixteen percent of studies detected ARGs using PCR alone, or in combination with sequencing (43%). Only 8% of studies characterized bacteria by whole-genome sequencing, and one study used a metagenomic approach (**Figure 4B**).

Most studies detecting ARGs focused on beta-lactamase genes including extended-spectrum beta-lactamases (ESBL), AmpC-type beta-lactamases, and carbapenemases, which were identified in all continents but Antarctica (**Table 3**). Among these beta-lactamases, ESBL were the most identified genes, evenly distributed across continents, particularly the genotype $bla_{\text{CTX-M}}$. Studies detected $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-15}}$ in Asia, Europe, North and South America, and Oceania. Likewise, $bla_{\text{CTX-M-55}}$ was reported in all these continents with the exception of South America. Beta-lactamases $bla_{\text{CMY-2}}$ (AmpC) were reported in Europe, North America, and Oceania, and $bla_{\text{OXA-48}}$ (carbapenemase) were reported in Africa, Europe, North America, and Oceania (**Table 3**). Of the 15 studies that found AmpC-type beta-lactamases, 7 identified that they were inserted on an MGE, 2 identified them on the bacterial core genome, 2 detected both chromosomal and acquired AmpC, and 4 studies did not identify the location of the AmpC gene. Genes conferring resistance to other antibiotics such as fluoroquinolones, aminoglycosides, sulfonamides along with trimethoprim, polypeptides, tetracyclines, chloramphenicol, macrolides, streptogramins, glycopeptides, fosfomycin, and rifamycin were reported in 59 studies (**Table 4**). Asia only reported beta-lactam resistance genes.

Origin of AMR in Gulls

Only 19% of studies suggested a potential origin for the ARB or ARGs detected among gulls. Landfill (41%), places close to gulls nesting, and/or resting areas with high human density (29%), sewage effluents (29%), and contaminated water (6%) were suspected. Suspicions were based on potential contamination sources around the sampling area. However, only one study (Masarikova et al., 2016) carried out sampling to verify whether the gulls acquired the bacteria from a specific contamination source, comparing bacteria from gulls to bacteria isolated from

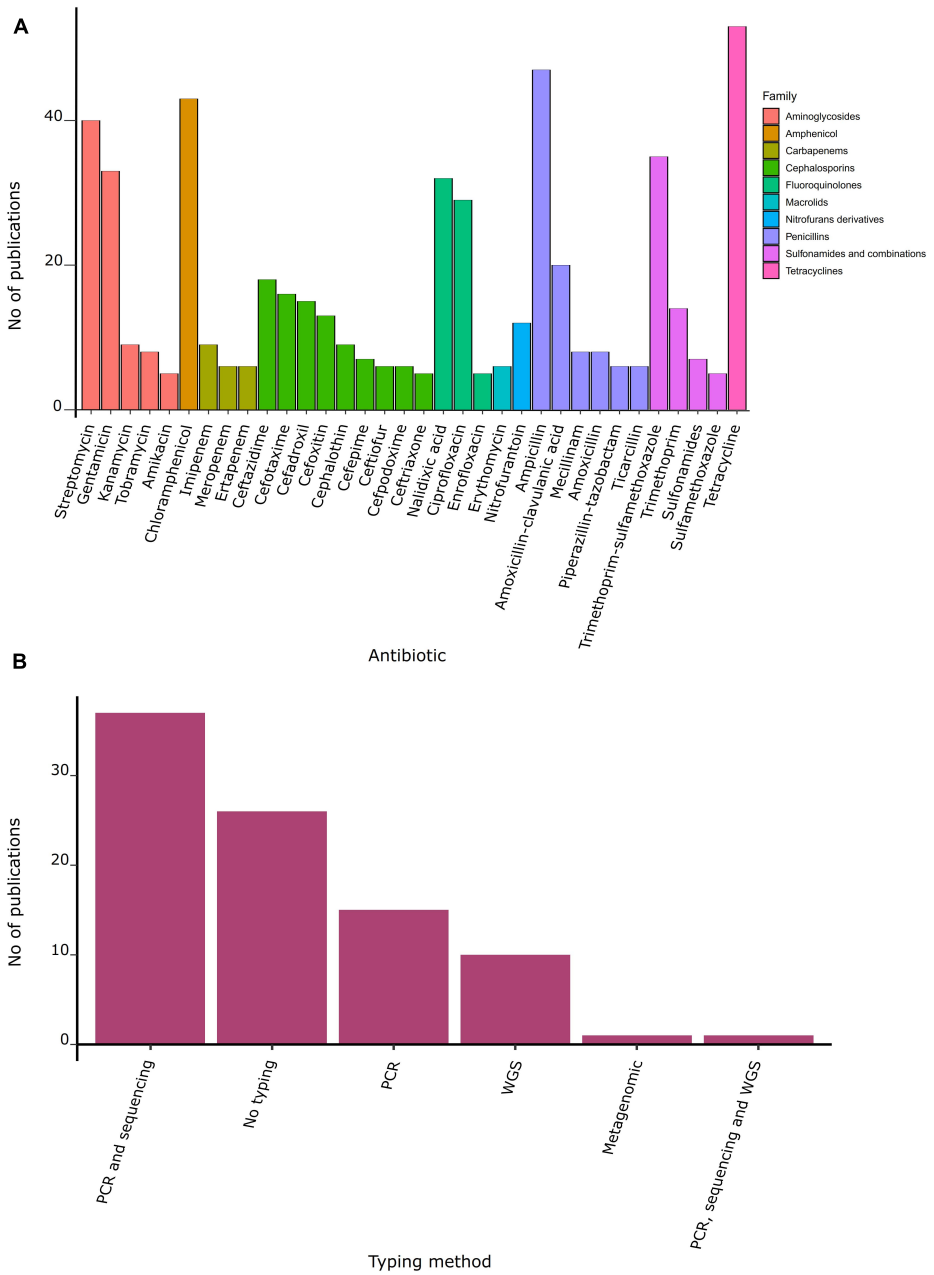

FIGURE 4 | Antibiotic resistance and molecular method used. **(A)** Number of publications by family of antibiotic. **(B)** Number of publications by typing method.

sewage water near their nesting sites. The same AMR phenotypic profiles were obtained in both sample types, and pulsed-field gel electrophoresis (PFGE) detected the same AMR profiles in bacterial clones from wastewater and gulls.

Evidence of Gulls Acting as Reservoirs of ARB or ARGs

Two studies (2%) tagged gulls in both the origin and final movement areas to identify whether they were capable of spreading AMR across the landscape (Palmgren et al., 2006; Ahlstrom et al., 2019a). Ahlstrom et al. (2019a) sampled individual gulls at different periods of time obtaining fecal samples at a landfill and in places where humans and seagulls gathered. Satellite telemetry was used to monitor individuals for up to 3 months, and whole-genome sequencing of bacteria was used to compare *E. coli* isolates between different locations. Their results showed that the prevalence and genetic typing of AMR isolates were highly similar between gulls and a landfill. Palmgren et al. (2006) ringed gulls and sampled 1,047 individuals for up to 3 years. This study failed to detect long-term carriage of antibiotic-resistant *Salmonella* since

TABLE 2 | ARB of the "Global Priority Pathogens" list of the World Health Organization isolated from gulls reported between 1981 and 2020.

Priority category	Pathogens	Antibiotic resistance	No. of publications	Gull species	References
Critical	*Acinetobacter baumannii*	Carbapenem-resistant	1% (1/90)	*C. ridibundus*	Łopińska et al., 2020
	Pseudomonas aeruginosa	Carbapenem-resistant	0% (0/90)	–	–
	Enterobacterales*	Carbapenem-resistant	10% (9/90)	*L. glaucescens, L. argentatus, L. hyperboreus, C. novaehollandiae, L. michahellis, C. genei*	Papagiannitsis et al., 2017; Vergara et al., 2017; Vittecoq et al., 2017; Ahlstrom et al., 2018, 2019a,c; Barguigua et al., 2019; Mukerji et al., 2019; Aires-De-sousa et al., 2020
	Enterobacterales*	Third generation cephalosporin-resistant	41% (37/90)	*L. glaucescens, L. argentatus, L. hyperboreus, L. fuscus, L. michahellis, C. novaehollandiae, L. glaucescens, C. ridibundus, L. marinus, L. canus, L. cachinnans, L. dominicanus, Leucophaeus pipixcan, C. brunnicephalus, L. atricilla*	Poeta et al., 2008; Bonnedahl et al., 2009, 2014; Rose et al., 2009; Hernandez et al., 2010; Simões et al., 2010; Veldman et al., 2013; Hasan et al., 2014; Stedt et al., 2015; Alcalá et al., 2016; Aberkane et al., 2016, 2017; Atterby et al., 2016, 2017; Dolejska et al., 2016; Liakopoulos et al., 2016; Merkeviciene et al., 2017, 2018; Papagiannitsis et al., 2017; Troxler et al., 2017; Vergara et al., 2017; Ahlstrom et al., 2018, 2019a,b, 2021; Mukerji et al., 2019, 2020; Ngaiganam et al., 2019; Aires-De-sousa et al., 2020; Haenni et al., 2020; Zendri et al., 2020
High	*Enterococcus faecium*	Vancomycin-resistant	3% (3/90)	*Chroicocephalus novaehollandiae, L. cachinnans*	Radhouani et al., 2010; Bonnedahl et al., 2014; Oravcova et al., 2017
	Staphylococcus aureus	Methicillin-resistant	2% (2/90)	*L. argentatus*	Merkeviciene et al., 2017; Aires-De-sousa et al., 2020
	Staphylococcus aureus	Vancomycin-intermediate	0% (0/90)	–	–
	Staphylococcus aureus	Vancomycin-resistant	0% (0/90)	–	–
	Helicobacter pylori	Clarithromycin-resistant	0% (0/90)	–	–
	Campylobacter spp.	Fluoroquinolone-resistant	6% (5/90)	*L. michahellis*, *L. audouinii*, *C. ridibundus*, *L. dominicanus*, *Thalasseus bergii*	Merkeviciene et al., 2017; Migura-Garcia et al., 2017; Moré et al., 2017; Troxler et al., 2017; Antilles et al., 2021
	Salmonella spp.	Fluoroquinolone-resistant	8% (7/90)	*L. michahellis*, *L. audouinii, L. dominicanus, C. novaehollandiae*, *C. ridibundus*, *Leucophaeus pipixcan, Leucophaeus modestus*	Fresno et al., 2013; Antilles et al., 2015, 2021; Retamal et al., 2015; Masarikova et al., 2016; Cummins et al., 2020; Tardone et al., 2020
	Neisseria gonorrhoeae	Third generation cephalosporin-resistant	0% (0/90)	–	–
	Neisseria gonorrhoeae	Fluoroquinolone-resistant	0% (0/90)	–	–
Medium	*Streptococcus pneumoniae*	Penicillin-non-susceptible	0% (0/90)	–	–
	Haemophilus influenzae	Ampicillin-resistant	0% (0/90)	–	–
	Shigella spp.	Fluoroquinolone-resistant	0% (0/90)	–	–

**Klebsiella pneumoniae, Escherichia coli, Enterobacter spp., Serratia spp., Proteus spp., Providencia spp., and Morganella spp.*

all positive individuals were negative during the sampling 2 months later.

DISCUSSION

AMR has been detected in resident and migratory gulls worldwide for more than a decade (Fenlon, 1981; Tsubokura et al., 1995; Smith et al., 2002). However, the role of gulls as reservoirs (i.e., having the capacity to disperse and transmit AMR to other species) remains unknown. Our review identified 90 studies on AMR in wild gulls. AMR has been widely detected across all continents including in 23 of 100 species of gulls (IUCN, 2021), 49 bacteria species with 9 of 13 ARB classified as critical priority for human health (WHO, 2017), ARGs from 13 classes, and 47 antibiotic types. Our results show that, with the exception of China, studies in middle- and low-income countries are rare. Similarly, most studies have focused on a few species of gulls from Europe (e.g., *L. argentatus* and *C. ridibundus*) and most on *E. coli* and *Salmonella spp*. Despite ARB and ARGs being widely detected in gulls, our analyses showed that the origin of these AMR remains unknown in 81% of studies, and only two studies followed gulls across time (for up to 3 years), but none has been able to prove that gulls were reservoirs

TABLE 3 | Beta-lactamases genes identified in isolates from gulls reported between 1981 and 2020.

Continent	AmpC	CP	ESBL	Others[a]	References
Africa	NR	bla_{OXA-48}	NR	NR	Barguigua et al., 2019
Antarctica	NR	NR	NR	NR	–
Asia	NR	NR	$bla_{CTX-M-14}$, $bla_{CTX-M-15}$, $bla_{CTX-M-55}$, $bla_{CTX-M-79}$	NR	Hasan et al., 2014
Europe	bla_{CMY-2}, bla_{CMY}, bla_{DHA-1}, bla_{ACT-14}, bla_{ACT-15}, bla_{ACT-23}	bla_{OXA-48}, $bla_{OXA-181}$, bla_{KPC-2}, bla_{KPC-3}, bla_{OXA-71}, $bla_{OXA-208}$, bla_{VIM-1}, bla_{VIM-4}	$bla_{CTX-M-15}$, $bla_{CTX-M-55}$, bla_{SHV-2}, $bla_{CTX-M-1}$, $bla_{CTX-M-14}$, $bla_{CTX-M-27}$, $bla_{CTX-M-9}$, bla_{SHV-12}, bla_{PER}, $bla_{CTX-M-32}$, bla_{CTX-M}, bla_{TEM-84}, $bla_{CTX-M-2}$, $bla_{CTX-M-8}$, $bla_{CTX-M-3}$, $bla_{TEM-52C}$, bla_{TEM-52}, $bla_{CTX-M-14a}$, bla_{PSE-1}	bla_{TEM}, $bla_{OXA-1-like}$, bla_{TEM-1}, bla_{SHV}, bla_{OXA-1}, bla_{OXA-3}, bla_{OXA-5}, bla_{TEM-1b}	Čížek et al., 2007; Dolejska et al., 2007; Poeta et al., 2008; Bonnedahl et al., 2009, 2010; Dolejská et al., 2009; Radhouani et al., 2009; Hernandez et al., 2010; Literak et al., 2010, 2014; Simões et al., 2010; Wallensten et al., 2011; Veldman et al., 2013; Vredenburg et al., 2014; Aberkane et al., 2015, 2016, 2017; Antilles et al., 2015; Stedt et al., 2015; Varela et al., 2015; Carroll et al., 2015; Masarikova et al., 2016; Alcalá et al., 2016; Atterby et al., 2017; Merkeviciene et al., 2017, 2018; Vergara et al., 2017; Vittecoq et al., 2017
North America	bla_{ampC}, bla_{CMY-2}, bla_{CMY-61}, bla_{DHA-1}, bla_{CMY}	bla_{KPC-2}, bla_{OXA-48}, bla_{OXA-9}, bla_{CARB-1}, bla_{CARB-2}, bla_{CARB}	bla_{CTX-M}, $bla_{CTX-M-1}$, $bla_{CTX-M-14}$, $bla_{CTX-M-15}$, $bla_{CTX-M-27}$, $bla_{CTX-M-32}$, $bla_{CTX-M-3}$, $bla_{CTX-M-55}$, $bla_{CTX-M-65}$, $bla_{CTX-M-8}$, $bla_{TEM-141}$, bla_{TEM-52}, bla_{TEM-19}, $bla_{TEM-206}$, $bla_{TEM-214}$, bla_{SHV-12}, bla_{SHV-2}, bla_{SHV-2A}, bla_{SHV-11}, bla_{SHV-14}	bla_{TEM-1A}, bla_{TEM-1B}, bla_{TEM-1C}, bla_{TEM-1D}, bla_{OXA-1}, $bla_{OXA-466}$, bla_{ampH}, bla_{ampC2}, bla_{mrdA}, bla_{ampC1}, bla_{TEM-1}, bla_{SHV-1}, bla_{TEM}	Alroy and Ellis, 2011; Martiny et al., 2011; Bonnedahl et al., 2014, 2015; Atterby et al., 2016; Ahlstrom et al., 2018, 2019a,b, 2021; Gomez-Alvarez et al., 2019
Oceania	bla_{CMY-2}, bla_{CMY-13}, bla_{CMY-42}, bla_{CMY-60}	bla_{OXA-48}, bla_{IMP-4}, bla_{IMP-38}	$bla_{CTX-M-15}$, $bla_{CTX-M-27}$, $bla_{CTX-M-14}$, $bla_{CTX-M-3}$, $bla_{CTX-M-55}$, $bla_{CTX-M-11}$, $bla_{CTX-M-24}$	bla_{TEM-1}, bla_{LAP-2}, bla_{TEM}, bla_{OXA-1}, bla_{SHV}, bla_{OXA-1}, bla_{TEM-1}	Dolejska et al., 2016; Papagiannitsis et al., 2017; Mukerji et al., 2019, 2020; Cummins et al., 2020
South America	NR	NR	$bla_{CTX-M-1}$, $bla_{CTX-M-2}$, $bla_{CTX-M-14}$, bla_{SHV-2A}, bla_{SHV-2}, $bla_{CTX-M-15}$, $bla_{CTX-M-22}$, $bla_{CTX-M-3}$, bla_{TEM-40}, $bla_{TEM-198}$, bla_{SHV-12}	bla_{TEM-1}	Hernandez et al., 2013; Báez et al., 2015; Liakopoulos et al., 2016

AmpC, cephalosporinases; CP, carbapenemases; NR, not reported.
[a]*Corresponds to beta-lactamases that are not classified as ESBL, AmpC, or CP.*

(Palmgren et al., 2006; Ahlstrom et al., 2019a). Therefore, our review highlights the need to increase surveillance of AMR in gulls and design innovative studies aiming to assess their role as reservoirs, which can have major implications for public and conservation measures to limit the global spread of AMR in aquatic systems.

The detection of AMR in gulls across all continents, including critically important antibiotic-resistant pathogens such as ESBL and carbapenemase-producing *E. coli* and *S. enterica*, illustrates the potential of gulls to participate in the alarming global spread of AMR (Dolejska et al., 2016; Ahlstrom et al., 2019c; Aires-De-sousa et al., 2020; Cummins et al., 2020). The migratory capacity of gulls makes them an ideal host to spread ARB and ARGs across landscapes and ecosystems. For example, Ahlstrom et al. (2021) reported that gulls of the *Larus* genus, including *L. argentatus*, can migrate 3,000 km over a week, *Larus fuscus* can migrate from Europe to Africa (Kilpi and Saurola, 1984), while *Leucophaeus pipixcan* migrates from North to South America (Hernandez et al., 2013; Barbieri et al., 2016). In contrast, other gull species such as *L. dominicanus* are resident but also carry ESBL-resistant *E. coli* with ARGs genes such as bla_{CTX-M} and bla_{SHV} and aminoglycoside-resistant *Salmonella enteritidis* with *str* genes (Liakopoulos et al., 2016; Toro et al., 2016). Although these species might not necessarily contribute to the long-range dispersal of AMR, they could participate in local transmission to other species and humans (Vigo et al., 2011; Retamal et al., 2015; Toro et al., 2016). Overall, this review highlights that gulls are at least sentinels of ARB and ARGs spreading in the environment, calling for future research in species and countries where AMR has not yet been studied. In particular, environmental and animal health national and international authorities should consider gulls in the surveillance of AMR within the environment.

Although AMR is widely spread among gulls, there are almost no data on the origin of the observed ARB and ARGs. In fact, less than 20% of studies included in this review mentioned potential sources of AMR contamination. Given that AMR has exponentially increased with antibiotic use in humans and livestock and several gull species feed on human and agricultural waste, most studies suspect a human origin including landfills, places close to gulls nesting, and/or resting areas that have a high human density, sewage effluents, and contaminated water (Bonnedahl et al., 2014; Atterby et al., 2016; Mukerji et al., 2019; Ahlstrom et al., 2021). This is consistent with the overall assumption that wildlife becomes contaminated with AMR from anthropogenic sources in studies suggesting transmission in areas where wildlife lives and feeds (Dolejska and Literak, 2019). However, no study has fully proven the origin of AMR in gulls, and other environmental factors such as co-selection with heavy metals and microplastics can also generate AMR

TABLE 4 | AMR genes identified in isolates from gulls reported between 1981 and 2020.

Continent	FQ	POLY	TET	AMG	CHL	SUL	TMP	MAC	STR	GLY	FOS	RIF	References
Africa	*aac(6′)-Ib-cr*, *qnrS1*, *qnrB1*	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	Barguigua et al., 2019
Antarctica	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	–
Asia	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	–
Europe	*aac(6′)-Ib-cr*, *qnrB*, *gyrA*, *parC*, *qnrA1*, *qnrS*, *qnrB1*, *qnrS1*	*mcr-9*, *mcr-1*,	*tetA*, *tetB*, *tetG*, *tetL*, *tetM*, *tetD*	*aadB*, *aadA*, *aadA1*, *aadA2*, *aadA4*, *aadA5*, *rmtB*, *armA*, *aphA1*, *aacA4*, *aac(3)II*, *strA*, *strB*, *aac(6′)-Ib*, *aph(30′)-IIIa*, *ant(6)-Ia*, *sat*, *aac(3)-IV*, *aac(6′)*, *aadA1a*	*catII*, *catA*, *catA1*, *cmlA*, *cmlA1*, *floR*, *cat*, *catB3*	*sul1*, *sul2*, *sul3*	*dfr1*, *dfr5*, *dfr7*, *dfrA16*, *dfrA1*, *dfrA12*, *dfrA14*, *dfrA17*, *dfrA7*, *dfrA15*	*ermB*	*vatE*, *vatD*	*vanA*	NR	NR	Čížek et al., 2007; Dolejska et al., 2007; Gionechetti et al., 2008; Poeta et al., 2008; Dolejská et al., 2009; Radhouani et al., 2009, 2010, 2011; Bonnedahl et al., 2009, 2010; Hernandez et al., 2010; Literak et al., 2010, 2014; Simões et al., 2010; Wallensten et al., 2011; Veldman et al., 2013; Vredenburg et al., 2014; Aberkane et al., 2015, 2016; Carroll et al., 2015; Aberkane et al., 2017; Stedt et al., 2015; Varela et al., 2015; Antilles et al., 2015; Masarikova et al., 2016; Ruzauskas and Vaskeviciute, 2016; Alcalá et al., 2016; Merkeviciene et al., 2017, 2018; Vergara et al., 2017; Vittecoq et al., 2017; Atterby et al., 2017; Ngaiganam et al., 2019; Ahlstrom et al., 2019b; Haenni et al., 2020; Łopińska et al., 2020; Zendri et al., 2020; Aires-De-sousa et al., 2020
North America	*aac(6′)-Ib-cr*, *gyrA*, *parC*, *parE*, *qnrB4*, *qnrS1*, *oqxB*, *qnrA1*, *qnrB*, *qnrA*	NR	*tetA*, *tetB*, *tetC*, *tetD*, *tetR*	*aac3*, *aac(3)-Iia*, *aac(3)-IId*, *aac(3)-VIa*, *aadA*, *aadA1*, *aadA2*, *aadA2b*, *aadA5*, *ant(2″)-Ia*, *aph(3″)-Ib*, *aph(3′)-Ia*, *aph(6)-Id*, *aph(3′)*, *aac(3)-IIa*, *aph(3′)-IIa*, *strA*, *strB*	*catA1*, *catB3*, *catB4*, *cmlA1*, *floR*	*sul1*, *sul2*, *sul3*	*dfrA1*, *dfrA5*, *dfrA7*, *dfrA8*, *dfrA12*, *dfrA14*, *dfrA15*, *dfrA16*, *dfrA17*, *dfrA5*, *dfrA8*	*ermB*, *mphA*, *mphE*, *ereA*	NR	NR	*fosA3*, *fosA4*, *fosA7*	NR	Alroy and Ellis, 2011; Martiny et al., 2011; Bonnedahl et al., 2014, 2015; Atterby et al., 2016; Ahlstrom et al., 2018, 2019a,c, 2021; Gomez-Alvarez et al., 2019
Oceania	*qnrS*, *qnrB*, *qnrS1*, *qnrB4*, *qnrB6*	*mrc-1*	*tetA*, *tetM*	*strA*, *strB*, *aac(6′)-Iy*, *ant(3″)-IIa*, *aph(3′)-Ia*, *aac(3)-IId*, *aph(3′)-IIIa*, *aac(6′)aph(2″)*	*floR*	*sul2*, *sul3*	*dfrA14*	*mphA*, *ermB*	NR	*vanB*	*fosA7*	*arr-2*	Dolejska et al., 2016; Oravcova et al., 2017; Papagiannitsis et al., 2017; Mukerji et al., 2019, 2020; Cummins et al., 2020
South America	NR	NR	*tetA*	*strA*, *strB*	NR	NR	NR	NR	NR	NR	NR	NR	Hernandez et al., 2013; Báez et al., 2015; Liakopoulos et al., 2016; Toro et al., 2016

FQ, fluoroquinolones; POLY, polypeptides; TET, tetracyclines; AMG, aminoglycosides; CHL, chloramphenicol; SUL, sulfonamides; TMP, trimethoprim; MAC, macrolides; STR, streptogramins; GLY, glycopeptides; FOS, fosfomycin; RIF, rifamycin; NR, not reported.

(Gullberg et al., 2014; Dong et al., 2021). In our review, only one study sampled a potential contamination source to identify the origin of AMR find in gulls, showing that isolates from wastewater and gulls had the same macrorestriction profiles (Masarikova et al., 2016). One possible explanation for the small number of studies trying to identify the origin of AMR in gulls could be that no standard sampling protocol or specific criteria are available to fully determine the origin. Alternatively, logistical challenges such as collecting both wildlife, domestic animals, and human environments at the same time could limit the realization of these studies. Future research could follow methodologies used by studies performed on bacteria susceptible to antibiotics and other wildlife. For example, Nelson et al. (2008) characterized *E. coli* from gulls, garbage, and sewage by ribotyping, finding isolates with > 90% similarity in the band patterns between gulls and sewage. However, this study was not included in this review because it did not test for ARB or ARGs. Similarly, other studies have simultaneously sampled domestic animals and wildlife where contact between species can be frequent (e.g., small-scale farms) to assess potential cross-species transmission of ESBL-*E. coli* (Benavides et al., 2021). Although challenging, identifying the origin of AMR in gulls is essential when planning preventive strategies to limit the spread of AMR in natural ecosystems. Seagulls are characterized by being ubiquitous in most urban and rural environments, and many of them are migratory, so it is assumed that gulls may disperse ARB and ARGs between countries or even continents. Despite this assumption, only two studies included in this review performed longitudinal samplings to test the long-term carriage of ARB or ARGs in gulls (Palmgren et al., 2006; Ahlstrom et al., 2019a), requiring further research to identify their implication as reservoirs of AMR.

The detected ARB and ARGs found in gulls have major implications for both animal and human health. *E. coli* was the most common bacterial species reported followed by *Salmonella*, similarly to other wildlife species (Vittecoq et al., 2016). Both bacterial species are important for public health and are considered a critical priority for human and animal health (Vittecoq et al., 2016; WHO, 2017). *E. coli* and *Salmonella spp.* can also be found at equilibrium as commensal bacteria, so the impact of these bacteria of gull's health is unknown. Other reported pathogens found in gulls, such as *Campylobacter spp.* and *Klebsiella pneumoniae*, are considered as zoonotic pathogens and could represent a threat to human health. Global priority antibiotic-resistant pathogens for human and animal health were increasingly reported among gulls after 2008, particularly those considered as "critical" (WHO, 2017). Broad-spectrum antimicrobial therapies are commonly used to treat bacterial infections in both humans and animals (Bush and Jacoby, 2010). The widespread detection of third-generation cephalosporin-resistant Enterobacterales in addition to resistance to other important antimicrobials, such as carbapenems and fluoroquinolones, could compromise the effective treatment rates representing an important threat to public and veterinary health.

The higher detection of antibiotic-resistant enterobacteria could be explained by the relatively easier collection of fecal samples compared to capturing and sampling gulls to detect other pathogens (e.g., blood bacteria). Thus, the absence of other global-priority ARB in current studies such as *Pseudomonas aeruginosa* carbapenem-resistant (critical priority), *Staphylococcus aureus* vancomycin-intermediate or -resistant (high priority), and *Shigella* spp. fluoroquinolone-resistant (medium priority) could reflect a lack of research and not necessarily that these bacteria are not circulating among gulls. In fact, one study using a metagenomic approach found 31 previously undescribed ARGs, while another detected more than 70 bacterial species and 24 ARGs (Martiny et al., 2011; Merkeviciene et al., 2017). This review identified a high diversity of ARGs including those implicated in bacterial infections of humans and animals such as ESBL and carbapenemases (Bevan et al., 2017; Li et al., 2019). The presence of ARGs inserted in MGE could facilitate the spread of these resistance genes within gulls and between humans and other animals (Loayza et al., 2020). Our review also shows a wide diversity of bacterial clones and ARGs found in gulls. Whole-genome sequencing for bacterial typing was used in 13% of studies since 2011. Thus, the more widespread use of this technique in the following years could increase the detection of ARB clones and ARGs in gulls.

Our review showed that North America and Europe had the most diverse molecular diversity among ARB, which could be associated with more available molecular typing techniques compared to low-income countries. For example, ESBL- *E. coli* ST131, previously associated with nosocomial infections in humans, has been identified in gulls mainly from the United States (Bonnedahl et al., 2014; Ahlstrom et al., 2018, 2019a, 2021) and Portugal (Simões et al., 2010; Vredenburg et al., 2014; Varela et al., 2015). Despite fewer information available, ST131 has also been reported in gulls from low- and middle-income countries (LMICs) such as Bangladesh (Hasan et al., 2014). Future research should also evaluate the pathogenic potential of the detected ARB using whole-genome sequencing to detect virulence factors and other pathogenic genetic material (e.g., biofilms).

CONCLUSION AND FUTURE DIRECTIONS

Our review identified an increasing interest in ARB and ARGs among gulls in the last decade, although there is a considerable lack of information in LMICs, particularly regarding migratory species. Despite the widespread detection and high diversity of ARB and ARGs worldwide, there is no evidence that gulls act as reservoirs of ARB and ARGs. Furthermore, most of the studies could not demonstrate whether ARB and ARGs in gulls came from anthropogenic sources. Finally, we could not compare ARB or ARGs prevalence across studies due to their heterogeneity in the results and methodologies to assess AMR.

Knowledge gaps identified in this review can be overcome by future research. First, the use of whole-genome sequencing

combined with sampling across different species could help assessing cross-species transmission between gulls and humans, domestic animals, or other wild species that do not usually interact with humans but share nesting sites with gulls (e.g., penguins). Secondly, future research should identify if environmental factors such as plastic and heavy metal pollution are also selecting ARB and ARGs in gulls independently of contact with humans. Thirdly, the clinical relevance and conservation implications of the detected ARB for gull's health require further investigation. In particular, there is no evidence that the observed bacteria cause any pathogenicity to the studied gulls nor complicate treatment of gulls with antibiotics in rehabilitation centers. Finally, innovative techniques such as satellite tracking and collaborations across research teams in different countries where gulls migrate (e.g., Canada to Chile for the Franklin's gulls) could help elucidate whether gulls are spreading ARB and/or ARGs across countries and continents during their migration.

AUTHOR CONTRIBUTIONS

JB and DZ-G: conceptualization. DZ-G: data curation. JB, DZ-G, ZR-S, and MS-C: formal analysis, investigation, and methodology. JB: funding acquisition, project administration, resources, software, supervision, validation, and visualization. DZ-G, JB, ZR-S, MS-C, CT, and PP: writing—original draft and writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

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REFERENCES

Aberkane, S., Compain, F., Barraud, O., Ouédraogo, A. S., Bouzinbi, N., Vittecoq, M., et al. (2015). Non-O1/non-O139 *Vibrio cholerae* avian isolate from France cocarrying the blaVIM-1 and blaVIM-4 genes. *Antimicrob. Agents Chemother.* 59, 6594–6596. doi: 10.1128/AAC.00400-15

Aberkane, S., Compain, F., Decré, D., Dupont, C., Laurens, C., Vittecoq, M., et al. (2016). High prevalence of SXT/R391-related integrative and conjugative elements carrying blaCMY-2 in *Proteus mirabilis* isolates from gulls in the South of France. *Antimicrob. Agents Chemother.* 60, 1148–1152. doi: 10.1128/AAC.01654-15

Aberkane, S., Compain, F., Decré, D., Pantel, A., Vittecoq, M., Solassol, J., et al. (2017). Persistence of bla CMY-2 -producing *Proteus mirabilis* in two gull colonies at a 1-year interval in Southern France. *J. Glob. Antimicrob. Resist.* 9, 138–140. doi: 10.1016/j.jgar.2017.05.005

Ahlstrom, C. A., Bonnedahl, J., Woksepp, H., Hernandez, J., Olsen, B., and Ramey, A. M. (2018). Acquisition and dissemination of cephalosporin-resistant E. coli in migratory birds sampled at an Alaska landfill as inferred through genomic analysis. *Sci. Rep.* 8:7361. doi: 10.1038/s41598-018-25474-w

Ahlstrom, C. A., Bonnedahl, J., Woksepp, H., Hernandez, J., Reed, J. A., Tibbitts, L., et al. (2019a). Satellite tracking of gulls and genomic characterization of faecal bacteria reveals environmentally mediated acquisition and dispersal of antimicrobial-resistant *Escherichia coli* on the Kenai Peninsula, Alaska. *Mol. Ecol.* 28, 2531–2545. doi: 10.1111/mec.15101

Ahlstrom, C. A., Ramey, A. M., Woksepp, H., and Bonnedahl, J. (2019b). Early emergence of *mcr-* 1-positive *Enterobacteriaceae* in gulls from Spain and Portugal. *Environ. Microbiol. Rep.* 11, 669–671. doi: 10.1111/1758-2229.12779

Ahlstrom, C. A., Ramey, A. M., Woksepp, H., and Bonnedahl, J. (2019c). Repeated detection of carbapenemase-producing *Escherichia coli* in gulls inhabiting Alaska. *Antimicrob. Agents Chemother.* 63, e758–e719. doi: 10.1128/AAC.00758-19

Ahlstrom, C. A., van Toor, M. L., Woksepp, H., Chandler, J. C., Reed, J. A., Reeves, A. B., et al. (2021). Evidence for continental-scale dispersal of antimicrobial resistant bacteria by landfill-foraging gulls. *Sci. Total Environ.* 764:144551. doi: 10.1016/j.scitotenv.2020.144551

Aires-De-sousa, M., Fournier, C., Lopes, E., de Lencastre, H., Nordmann, P., and Poirel, L. (2020). High colonization rate and heterogeneity of ESBL- and carbapenemase-producing *enterobacteriaceae* isolated from gull feces in Lisbon, Portugal. *Microorganisms* 8, 1–12. doi: 10.3390/microorganisms8101487

Alcalá, L., Alonso, C. A., Simón, C., González-Esteban, C., Orós, J., Rezusta, A., et al. (2016). Wild birds, frequent carriers of extended-spectrum β-lactamase (ESBL) producing *Escherichia coli* of CTX-M and SHV-12 types. *Microb. Ecol.* 72, 861–869. doi: 10.1007/s00248-015-0718-0

Alroy, K., and Ellis, J. C. (2011). Pilot study of antimicrobial-resistant *Escherichia coli* in herring gulls (*Larus argentatus*) and wastewater in the Northeastern United States. *J. Zoo Wildl. Med.* 42, 160–163. doi: 10.1638/2010-0130.1

Antilles, N., García-Bocanegra, I., Alba-Casals, A., López-Soria, S., Pérez-Méndez, N., Saco, M., et al. (2021). Occurrence and antimicrobial resistance of zoonotic enteropathogens in gulls from southern Europe. *Sci. Total Environ.* 763:143018. doi: 10.1016/j.scitotenv.2020.143018

Antilles, N., Garcia-Migura, L., Joensen, K. G., Leekitcharoenphon, P., Aarestrup, F. M., Cerdà-Cúellar, M., et al. (2015). Audouins gull, a potential vehicle of an extended spectrum ß-lactamase producing *Salmonella* Agona. *FEMS Microbiol. Lett.* 362, 1–4. doi: 10.1093/femsle/fnu039

Atterby, C., Börjesson, S., Ny, S., Järhult, J. D., Byfors, S., and Bonnedahl, J. (2017). ESBL-producing *Escherichia coli* in Swedish gulls—a case of environmental pollution from humans? *PLoS One* 12:e0190380. doi: 10.1371/journal.pone.0190380

Atterby, C., Ramey, A. M., Hall, G. G., Järhult, J., Börjesson, S., and Bonnedahl, J. (2016). Increased prevalence of antibiotic-resistant E. coli in gulls sampled in Southcentral Alaska is associated with urban environments. *Infect. Ecol. Epidemiol.* 6:32334. doi: 10.3402/IEE.V6.32334

Báez, J., Hernández-García, M., Guamparito, C., Díaz, S., Olave, A., Guerrero, K., et al. (2015). Molecular characterization and genetic diversity of ESBL-producing *escherichia coli* colonizing the migratory Franklin's Gulls

(*Leucophaeus pipixcan*) in Antofagasta, North of Chile. *Microb. Drug Resist.* 21, 111–116. doi: 10.1089/mdr.2014.0158

Barbieri, E., Alberto Goncalves, C., and Francisco Silveira, L. D. A. (2016). First occurrence of Franklin's Gull (*Leucophaeus pipixcan*, Wagler 1831) in Ilha Comprida beach, Southern coast of São Paulo state, Brazil. *Pan Am. J. Aquat. Sci.* 11:159.

Barguigua, A., Rguibi Idrissi, H., Nayme, K., and Timinouni, M. (2019). Virulence and antibiotic resistance patterns in E. coli, Morocco. *Ecohealth* 16, 570–575. doi: 10.1007/s10393-019-01404-8

Benavides, J. A., Salgado-Caxito, M., Opazo-Capurro, A., González, M. P., Piñeiro, A., Otto, M. M., et al. (2021). ESBL-producing *Escherichia coli* carrying CTX-M genes circulating among livestock, dogs, and wild mammals in small-scale farms of central Chile. *Antibiotics* 10:510. doi: 10.3390/antibiotics10050510

Benavides, J. A., Shiva, C., Virhuez, M., Tello, C., Appelgren, A., Vendrell, J., et al. (2018). Extended-spectrum beta-lactamase-producing *Escherichia coli* in common vampire bats *Desmodus rotundus* and livestock in Peru. *Zoonoses Public Health* 65, 454–458. doi: 10.1111/zph.12456

Bevan, E. R., Jones, A. M., and Hawkey, P. M. (2017). Global epidemiology of CTX-M β-lactamases: temporal and geographical shifts in genotype. *J. Antimicrob. Chemother.* 72, 2145–2155. doi: 10.1093/jac/dkx146

Bonnedahl, J., and Järhult, J. D. (2014). Antibiotic resistance in wild birds. *Ups. J. Med. Sci.* 119, 113–116. doi: 10.3109/03009734.2014.905663

Bonnedahl, J., Drobni, M., Gauthier-Clerc, M., Hernandez, J., Granholm, S., Kayser, Y., et al. (2009). Dissemination of *Escherichia coli* with CTX-M type ESBL between humans and yellow-legged gulls in the South of France. *PLoS One* 4:e5958. doi: 10.1371/journal.pone.0005958

Bonnedahl, J., Drobni, P., Johansson, A., Hernandez, J., Melhus, Å, Stedt, J., et al. (2010). Characterization, and comparison of human clinical and black-headed gull (*Larus ridibundus*) extended-spectrum β-lactamase-producing bacterial isolates from Kalmar, on the southeast coast of Sweden. *J. Antimicrob. Chemother.* 65, 1939–1944. doi: 10.1093/jac/dkq222

Bonnedahl, J., Hernandez, J., Stedt, J., Waldenström, J., Olsen, B., and Drobni, M. (2014). Extended- spectrum β-lactamases in *escherichia coli* and *Klebsiella pneumoniae* in Gulls, Alaska, USA. *Emerg. Infect. Dis.* 20, 897–899. doi: 10.3201/eid2005.130325

Bonnedahl, J., Stedt, J., Waldenström, J., Svensson, L., Drobni, M., and Olsen, B. (2015). Comparison of extended-spectrum β-lactamase (ESBL) CTX-M genotypes in franklin gulls from Canada and Chile. *PLoS One* 10:e0141315. doi: 10.1371/journal.pone.0141315

Bush, K., and Jacoby, G. A. (2010). Updated functional classification of β-lactamases. *Antimicrob. Agents Chemother.* 54, 969–976. doi: 10.1128/AAC.01009-09

Carroll, D., Wang, J., Fanning, S., and Mcmahon, B. J. (2015). Antimicrobial resistance in wildlife: implications for public health. *Zoonoses Public Health* 62, 534–542. doi: 10.1111/zph.12182

Čížek, A., Dolejská, M., Karpíšková, R., Dědičová, D., and Literák, I. (2007). Wild black-headed gulls (*Larus ridibundus*) as an environmental reservoir of *Salmonella* strains resistant to antimicrobial drugs. *Eur. J. Wildl. Res.* 53, 55–60. doi: 10.1007/s10344-006-0054-2

CLSI (2018). *Performance Standards for Antimicrobial Susceptibility Testing*, 28th Edn. Wayne, PA: Clinical and Laboratory Standards Institute.

Cummins, M. L., Sanderson-Smith, M., Newton, P., Carlile, N., Phalen, D. N., Maute, K., et al. (2020). Whole-genome sequence analysis of an extensively drug-resistant *Salmonella enterica* Serovar Agona isolate from an Australian silver gull (*Chroicocephalus novaehollandiae*) reveals the acquisition of multidrug resistance plasmids. *mSphere* 5, 1–14. doi: 10.1128/msphere.00743-20

Dolejska, M., and Literak, I. (2019). Wildlife is overlooked in the epidemiology of medically important antibiotic-resistant bacteria. *Antimicrob. Agents Chemother.* 63, 1–5. doi: 10.1128/AAC.01167-19

Dolejská, M., Bierošová, B., Kohoutová, L., Literák, I., and Čížek, A. (2009). Antibiotic-resistant *Salmonella* and *Escherichia coli* isolates with integrons and extended-spectrum beta-lactamases in surface water and sympatric black-headed gulls. *J. Appl. Microbiol.* 106, 1941–1950. doi: 10.1111/j.1365-2672.2009.04155.x

Dolejska, M., Cizek, A., and Literak, I. (2007). High prevalence of antimicrobial-resistant genes and integrons in *Escherichia coli* isolates from Black-headed Gulls in the Czech Republic. *J. Appl. Microbiol.* 103, 11–19. doi: 10.1111/j.1365-2672.2006.03241.x

Dolejska, M., Masarikova, M., Dobiasova, H., Jamborova, I., Karpiskova, R., Havlicek, M., et al. (2016). High prevalence of *Salmonella* and IMP-4-producing *Enterobacteriaceae* in the silver gull on Five Islands, Australia. *J. Antimicrob. Chemother.* 71, 63–70. doi: 10.1093/jac/dkv306

Dong, H., Chen, Y., Wang, J., Zhang, Y., Zhang, P., Li, X., et al. (2021). Interactions of microplastics and antibiotic resistance genes and their effects on the aquaculture environments. *J. Hazard. Mater.* 403:123961. doi: 10.1016/j.jhazmat.2020.123961

FAO and WHO (2019). *Joint FAO/WHO Expert Meeting in Collaboration with OIE on Foodborne Antimicrobial Resistance: Role of the Environment, Crops and Biocides: Meeting Report*. Rome: FAO.

Fenlon, D. R. (1981). Seagulls (*Larus* spp.) as vectors of *salmonella*e: an investigation into the range of serotypes and numbers of *salmonella*e in gull faeces. *J. Hyg.* 86, 195–202. doi: 10.1017/S0022172400068911

Fresno, M., Barrera, V., Gornall, V., Lillo, P., Paredes, N., Abalos, P., et al. (2013). Identification of diverse *Salmonella* serotypes, virulotypes, and antimicrobial resistance phenotypes in Waterfowl from Chile. *Vector Borne Zoonotic Dis.* 13, 884–887. doi: 10.1089/vbz.2013.1408

Gionechetti, F., Zucca, P., Gombac, F., Monti-Bragadin, C., Lagatolla, C., Tonin, E., et al. (2008). Characterization of antimicrobial resistance and class 1 integrons in *enterobacteriaceae* isolated from mediterranean herring gulls (*Larus cachinnans*). *Microb. Drug Resist.* 14, 93–99. doi: 10.1089/mdr.2008.0803

Gomez-Alvarez, V., Ashbolt, N. J., Griffith, J. F., Santo Domingo, J., and Lu, J. (2019). Whole-genome sequencing of four campylobacter strains isolated from gull excreta collected from Hobie Beach (Oxnard, CA, USA). *Microbiol. Resour. Announc.* 8:e00560-19. doi: 10.1128/mra.00560-19

Guenther, S., Ewers, C., and Wieler, L. H. (2011). Extended-spectrum beta-lactamases producing E. coli in wildlife, yet another form of environmental pollution? *Front. Microbiol.* 2:246. doi: 10.3389/fmicb.2011.00246

Gullberg, E., Albrecht, L. M., Karlsson, C., Sandegren, L., and Andersson, D. I. (2014). Selection of a multidrug resistance plasmid by sublethal levels of antibiotics and heavy metals. *MBio* 5:e01918-14. doi: 10.1128/mBio.01918-14

Haenni, M., Métayer, V., Jarry, R., Drapeau, A., Puech, M. P., Madec, J. Y., et al. (2020). Wide spread of blaCTX–M–9/mcr-9 IncHI2/ST1 plasmids and CTX-M-9-producing *Escherichia coli* and *Enterobacter cloacae* in rescued wild animals. *Front. Microbiol.* 11:601317. doi: 10.3389/fmicb.2020.601317

Hasan, B., Melhus, Å, Sandegren, L., Alam, M., and Olsen, B. (2014). The gull (*Chroicocephalus brunnicephalus*) as an environmental bioindicator and reservoir for antibiotic resistance on the coastlines of the Bay of Bengal. *Microb. Drug Resist.* 20, 466–471. doi: 10.1089/mdr.2013.0233

Hernandez, J., Bonnedahl, J., Eliasson, I., Wallensten, A., Comstedt, P., Johansson, A., et al. (2010). Globally disseminated human pathogenic *Escherichia coli* of O25b-ST131 clone, harbouring blaCTX-M-15, found in Glaucous-winged gull at remote Commander Islands, Russia. *Environ. Microbiol. Rep.* 2, 329–332. doi: 10.1111/j.1758-2229.2010.00142.x

Hernandez, J., Johansson, A., Stedt, J., Bengtsson, S., Porczak, A., Granholm, S., et al. (2013). Characterization and comparison of extended-spectrum β-lactamase (ESBL) resistance genotypes and population structure of *Escherichia coli* isolated from Franklin's gulls (*Leucophaeus pipixcan*) and humans in Chile. *PLoS One* 8:e76150. doi: 10.1371/journal.pone.0076150

IUCN (2021). *Laridae Family*. Available online at: https://www.iucnredlist.org/search/list?taxonomies=22673045&searchType=species (accessed April 12, 2021)

Kilpi, M., and Saurola, P. (1984). Migration and wintering strategies of juvenile and adult *Larus marinus*, L. argentatus and L. fuscus from Finland. *Orn. Fennica* 61, 1–8.

Li, J., Bi, Z., Ma, S., Chen, B., Cai, C., He, J., et al. (2019). Inter-host transmission of carbapenemase-producing *Escherichia coli* among humans and backyard animals. *Environ. Health Perspect.* 127:107009. doi: 10.1289/EHP5251

Liakopoulos, A., Olsen, B., Geurts, Y., Artursson, K., Berg, C., Mevius, D. J., et al. (2016). Molecular characterization of extended-spectrum-cephalosporin-resistant *Enterobacteriaceae* from wild kelp gulls in South America. *Antimicrob. Agents Chemother.* 60, 6924–6927. doi: 10.1128/AAC.01120-16

Literak, I., Dolejska, M., Janoszowska, D., Hrusakova, J., Meissner, W., Rzyska, H., et al. (2010). Antibiotic-resistant *escherichia coli* bacteria, including strains with

genes encoding the extended-spectrum beta-lactamase and QnrS, in waterbirds on the Baltic Sea coast of Poland. *Appl. Environ. Microbiol.* 76, 8126–8134. doi: 10.1128/AEM.01446-10

Literak, I., Manga, I., Wojczulanis-Jakubas, K., Chroma, M., Jamborova, I., Dobiasova, H., et al. (2014). *Enterobacter cloacae* with a novel variant of ACT AmpC beta-lactamase originating from glaucous gull (*Larus hyperboreus*) in Svalbard. *Vet. Microbiol.* 171, 432–435. doi: 10.1016/j.vetmic.2014.02.015

Loayza, F., Graham, J. P., and Trueba, G. (2020). Factors obscuring the role of E. coli from domestic animals in the global antimicrobial resistance crisis: an evidence-based review. *Int. J. Environ. Res. Public Health* 17:3061. doi: 10.3390/ijerph17093061

Łopińska, A., Indykiewicz, P., Skiebe, E., Pfeifer, Y., Trček, J., Jerzak, L., et al. (2020). Low occurrence of *acinetobacter baumannii* in gulls and songbirds. *Polish J. Microbiol.* 69, 85–90. doi: 10.33073/pjm-2020-011

Martiny, A. C., Martiny, J. B. H., Weihe, C., Field, A., and Ellis, J. C. (2011). Functional metagenomics reveals previously unrecognized diversity of antibiotic resistance genes in gulls. *Front. Microbiol.* 2:238. doi: 10.3389/fmicb.2011.00238

Masarikova, M., Manga, I., Cizek, A., Dolejska, M., Oravcova, V., Myskova, P., et al. (2016). *Salmonella enterica* resistant to antimicrobials in wastewater effluents and black-headed gulls in the Czech Republic, 2012. *Sci. Total Environ.* 542, 102–107. doi: 10.1016/j.scitotenv.2015.10.069

Merkeviciene, L., Klimiene, I., Siugzdiniene, R., Virgailis, M., Mockeliunas, R., and Ruzauskas, M. (2018). Prevalence and molecular characteristics of multi-resistant *Escherichia coli* in wild birds. *Acta Vet. Brno* 87, 9–18. doi: 10.2754/avb201887010009

Merkeviciene, L., Ruzauskaite, N., Klimiene, I., Siugzdiniene, R., Dailidaviciene, J., Virgailis, M., et al. (2017). Microbiome and antimicrobial resistance genes in microbiota of cloacal samples from European herring gulls (*Larus argentatus*). *J. Vet. Res.* 61, 27–35. doi: 10.1515/jvetres-2017-0004

Migura-Garcia, L., Ramos, R., and Cerdà-Cuéllar, M. (2017). Antimicrobial resistance of *Salmonella* serovars and *Campylobacter* spp. Isolated from an opportunistic gull species, yellow-legged gull (*Larus michahellis*). *J. Wildl. Dis.* 53, 148–152. doi: 10.7589/2016-03-051

Moré, E., Ayats, T., Ryan, P. G., Naicker, P. R., Keddy, K. H., Gaglio, D., et al. (2017). Seabirds (Laridae) as a source of *Campylobacter* spp., *Salmonella* spp. and antimicrobial resistance in South Africa. *Environ. Microbiol.* 18, 3896–3909. doi: 10.1111/1462-2920.13874

Mukerji, S., Gunasekera, S., Dunlop, J. N., Stegger, M., Jordan, D., Laird, T., et al. (2020). Implications of foraging and interspecies interactions of birds for carriage of *Escherichia coli* strains resistant to critically important antimicrobials. *Appl. Environ. Microbiol.* 86, 1–13. doi: 10.1128/AEM.01610-20

Mukerji, S., Stegger, M., Truswell, A. V., Laird, T., Jordan, D., Abraham, R. J., et al. (2019). Resistance to critically important antimicrobials in Australian silver gulls (*Chroicocephalus novaehollandiae*) and evidence of anthropogenic origins. *J. Antimicrob. Chemother.* 74, 2566–2574. doi: 10.1093/jac/dkz242

Nelson, M., Jones, S. H., Edwards, C., and Ellis, J. C. (2008). Characterization of *Escherichia coli* populations from gulls, landfill trash, and wastewater using ribotyping. *Dis. Aquat. Organ.* 81, 53–63. doi: 10.3354/dao01937

Ngaiganam, E. P., Pagnier, I., Chaalal, W., Leangapichart, T., Chabou, S., Rolain, J. M., et al. (2019). Investigation of urban birds as source of β-lactamase-producing Gram-negative bacteria in Marseille city, France. *Acta Vet. Scand.* 61:51. doi: 10.1186/s13028-019-0486-9

Oravcova, V., Svec, P., and Literak, I. (2017). Vancomycin-resistant enterococci with vanA and vanB genes in Australian gulls. *Environ. Microbiol. Rep.* 9, 316–318. doi: 10.1111/1758-2229.12542

Palmgren, H., Aspán, A., Broman, T., Bengtsson, K., Blomquist, L., Bergström, S., et al. (2006). *Salmonella* in Black-headed gulls (*Larus ridibundus*); prevalence, genotypes and influence on *Salmonella* epidemiology. *Epidemiol. Infect.* 134, 635–644. doi: 10.1017/S0950268805005261

Papagiannitsis, C. C., Kutilova, I., Medvecky, M., Hrabak, J., and Dolejska, M. (2017). Characterization of the complete nucleotide sequences of IncA/C2 plasmids carrying in809-like integrons from *enterobacteriaceae* isolates of wildlife origin. *Antimicrob. Agents Chemother.* 61:e01093-17. doi: 10.1128/AAC.01093-17

Poeta, P., Radhouani, H., Igrejas, G., Gonçalves, A., Carvalho, C., Rodrigues, J., et al. (2008). Seagulls of the Berlengas natural reserve of Portugal as carriers of fecal *Escherichia coli* harboring CTX-M and TEM extended-spectrum beta-lactamases. *Appl. Environ. Microbiol.* 74, 7439–7441. doi: 10.1128/AEM.00949-08

Radhouani, H., Igrejas, G., Pinto, L., Gonalves, A., Coelho, C., Rodrigues, J., et al. (2011). Molecular characterization of antibiotic resistance in enterococci recovered from seagulls (*Larus cachinnans*) representing an environmental health problem. *J. Environ. Monit.* 13, 2227–2233. doi: 10.1039/c0em00682c

Radhouani, H., Poeta, P., Igrejas, G., Gonçalves, A., Vinué, L., and Torres, C. (2009). Antimicrobial resistance and phylogenetic groups in isolates of *Escherichia coli* from seagulls at the berlengas nature reserve. *Vet. Rec.* 165, 138–142. doi: 10.1136/vr.165.5.138

Radhouani, H., Poeta, P., Pinto, L., Miranda, J., Coelho, C., Carvalho, C., et al. (2010). Proteomic characterization of vanA-containing Enterococcus recovered from Seagulls at the Berlengas natural reserve, W Portugal. *Proteome Sci.* 8, 1–12. doi: 10.1186/1477-5956-8-48

Retamal, P., Fresno, M., Dougnac, C., Gutierrez, S., Gornall, V., Vidal, R., et al. (2015). Genetic and phenotypic evidence of the *Salmonella enterica* serotype Enteritidis human-animal interface in Chile. *Front. Microbiol.* 6:464. doi: 10.3389/fmicb.2015.00464

Rose, J. M., Gast, R. J., Bogomolni, A., Ellis, J. C., Lentell, B. J., Touhey, K., et al. (2009). Occurrence and patterns of antibiotic resistance in vertebrates off the Northeastern United States coast. *FEMS Microbiol. Ecol.* 67, 421–431. doi: 10.1111/j.1574-6941.2009.00648.x

Ruzauskas, M., and Vaskeviciute, L. (2016). Detection of the mcr-1 gene in *Escherichia coli* prevalent in the migratory bird species *Larus argentatus*. *J. Antimicrob. Chemother.* 71, 2333–2334. doi: 10.1093/jac/dkw245

Salgado-Caxito, M., Benavides, J. A., Adell, A. D., Paes, A. C., and Moreno-Switt, A. I. (2021). Global prevalence and molecular characterization of extended-spectrum β-lactamase producing- in dogs and cats – a scoping review and meta-analysis. *One Heal.* 12:100236. doi: 10.1016/j.onehlt.2021.100236

Simões, R. R., Poirel, L., Da Costa, P. M., and Nordmann, P. (2010). Seagulls and beaches as reservoirs for multidrug-resistant *Escherichia coli*. *Emerg. Infect. Dis.* 16, 110–112. doi: 10.3201/eid1601.090896

Smith, W. A., Mazet, J. A. K., and Hirsh, D. C. (2002). *Salmonella* in California wildlife species: prevalence in rehabilitation centers and characterization of isolates. *J. Zoo Wildl. Med.* 33, 228–235.

Stedt, J., Bonnedahl, J., Hernandez, J., Waldenström, J., McMahon, B. J., Tolf, C., et al. (2015). Carriage of CTX-M type extended spectrum β-lactamases (ESBLs) in gulls across Europe. *Acta Vet. Scand.* 57, 1–8. doi: 10.1186/s13028-015-0166-3

Tardone, R., Rivera, D., Dueñas, F., Sallaberry-Pincheira, N., Hamilton-West, C., Adell, A. D., et al. (2020). *Salmonella* in raptors and aquatic wild birds in Chile. *J. Wildl. Dis.* 56, 707–712. doi: 10.7589/2019-08-198

Toro, M., Retamal, P., Ayers, S., Barreto, M., Allard, M., Brown, E. W., et al. (2016). Whole-genome sequencing analysis of *Salmonella enterica* serovar enteritidis isolates in chile provides insights into possible transmission between gulls, poultry, and humans. *Appl. Environ. Microbiol.* 82, 6223–6232. doi: 10.1128/AEM.01760-16

Tricco, A. C., Lillie, E., Zarin, W., O'Brien, K. K., Colquhoun, H., Levac, D., et al. (2018). PRISMA extension for scoping reviews (PRISMA-ScR): checklist and explanation. *Ann. Intern. Med.* 169, 467–473. doi: 10.7326/M18-0850

Troxler, S., Hess, C., Konicek, C., Knotek, Z., Barták, P., and Hess, M. (2017). Microdilution testing reveals considerable and diverse antimicrobial resistance of *Escherichia coli*, thermophilic *Campylobacter* spp. and *Salmonella* spp. isolated from wild birds present in urban areas. *Eur. J. Wildl. Res.* 63, 1–11. doi: 10.1007/s10344-017-1125-2

Tsubokura, M., Matsumoto, A., Otsuki, K., Animas, S. B., and Sanekata, T. (1995). Drug resistance and conjugative R plasmids in *Escherichia coli* strains isolated from migratory waterfowl. *J. Wildl. Dis.* 31, 352–357. doi: 10.7589/0090-3558-31.3.352

Varela, A. R., Manageiro, V., Ferreira, E., Guimarães, M. A., Da Costa, P. M., Caniça, M., et al. (2015). Molecular evidence of the close relatedness of clinical, gull and wastewater isolates of quinolone-resistant *Escherichia coli*. *J. Glob. Antimicrob. Resist.* 3, 286–289. doi: 10.1016/j.jgar.2015.07.008

Veldman, K., van Tulden, P., Kant, A., Testerink, J., and Mevius, D. (2013). Characteristics of cefotaxime-resistant *Escherichia coli* from wild birds in The Netherlands. *Appl. Environ. Microbiol.* 79, 7556–7561. doi: 10.1128/AEM.01880-13

Vergara, A., Pitart, C., Montalvo, T., Roca, I., Sabaté, S., Hurtado, J. C., et al. (2017). Prevalence of extended-spectrum-β-lactamase- and/or carbapenemase-producing *Escherichia coli* isolated from yellow-legged gulls from Barcelona, Spain. *Antimicrob. Agents Chemother.* 61:e02071-16. doi: 10.1128/AAC.02071-16

Vigo, G. B., Leotta, G. A., Caffer, M. I., Salve, A., Binsztein, N., and Pichel, M. (2011). Isolation and characterization of *Salmonella enterica* from Antarctic wildlife. *Polar Biol.* 34, 675–681. doi: 10.1007/s00300-010-0923-8

Vittecoq, M., Godreuil, S., Prugnolle, F., Durand, P., Brazier, L., Renaud, N., et al. (2016). REVIEW: antimicrobial resistance in wildlife. *J. Appl. Ecol.* 53, 519–529. doi: 10.1111/1365-2664.12596

Vittecoq, M., Laurens, C., Brazier, L., Durand, P., Elguero, E., Arnal, A., et al. (2017). VIM-1 carbapenemase-producing *Escherichia coli* in gulls from southern France. *Ecol. Evol.* 7, 1224–1232. doi: 10.1002/ece3.2707

Vredenburg, J., Varela, A. R., Hasan, B., Bertilsson, S., Olsen, B., Narciso-da-Rocha, C., et al. (2014). Quinolone-resistant *Escherichia coli* isolated from birds of prey in Portugal are genetically distinct from those isolated from water environments and gulls in Portugal, Spain and Sweden. *Environ. Microbiol.* 16, 995–1004. doi: 10.1111/1462-2920.12231

Wallensten, A., Hernandez, J., Ardiles, K., González-Acuña, D., Drobni, M., and Olsen, B. (2011). Extended spectrum beta-lactamases detected in *Escherichia coli* from gulls in Stockholm, Sweden. *Infect. Ecol. Epidemiol.* 1:7030. doi: 10.3402/iee.v1i0.7030

Wang, J., Ma, Z. B., Zeng, Z. L., Yang, X. W., Huang, Y., and Liu, J. H. (2017). The role of wildlife (wild birds) in the global transmission of antimicrobial resistance genes. *Zool. Res.* 38, 55–80. doi: 10.24272/j.issn.2095-8137.2017.003

WHO (2017). *Global Priority List of Antibiotic-Resistant Bacteria to Guide Research, Discovery, and Development of New Antibiotics*. Geneva: WHO.

WHO (2019). *2019 Antibacterial Agents in Clinical Development: An Analysis of the Antibacterial Clinical Development Pipeline*. Geneva: WHO.

Zendri, F., Maciuca, I. E., Moon, S., Jones, P. H., Wattret, A., Jenkins, R., et al. (2020). Occurrence of ESBL-producing *Escherichia coli* ST131, including the H30 -Rx and C1-M27 subclones, among urban seagulls from the United Kingdom. *Microb. Drug Resist.* 26, 697–708. doi: 10.1089/mdr.2019.0351

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Bacterial and Bacteriophage Antibiotic Resistance in Marine Bathing Waters in Relation to Rivers and Urban Streams

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Fecal pollution of surface water may introduce bacteria and bacteriophages harboring antibiotic resistance genes (ARGs) into the aquatic environment. Watercourses discharging into the marine environment, especially close to designated bathing waters, may expose recreational users to fecal pollution and therefore may increase the likelihood that they will be exposed to ARGs. This study compares the bacterial and bacteriophage ARG profiles of two rivers (River Tolka and Liffey) and two small urban streams (Elm Park and Trimleston Streams) that discharge close to two marine bathing waters in Dublin Bay. Despite the potential differences in pollution pressures experienced by these waterways, microbial source tracking analysis showed that the main source of pollution in both rivers and streams in the urban environment is human contamination. All ARGs included in this study, bla_{TEM}, bla_{SHV}, *qnrS*, and *sul1*, were present in all four waterways in both the bacterial and bacteriophage fractions, displaying a similar ARG profile. We show that nearshore marine bathing waters are strongly influenced by urban rivers and streams discharging into these, since they shared a similar ARG profile. In comparison to rivers and streams, the levels of bacterial ARGs were significantly reduced in the marine environment. In contrast, the bacteriophage ARG levels in freshwater and the marine were not significantly different. Nearshore marine bathing waters could therefore be a potential reservoir of bacteriophages carrying ARGs. In addition to being considered potential additional fecal indicators organism, bacteriophages may also be viewed as indicators of the spread of antimicrobial resistance.

Keywords: antibiotic resistance genes, microbial source tracking, bacteriophages, fecal pollution, rivers, urban streams, bathing waters

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INTRODUCTION

Microbial antibiotic resistance is a severe threat to public health, resulting in failure to treat a range of infections, in extended hospital treatment and in increased healthcare costs (Stewardson et al., 2016; Cassini et al., 2019; Roope et al., 2019; Jit et al., 2020). Furthermore, it is estimated that multidrug resistant pathogens will lead to 10 million deaths by 2050 (OECD, 2018; WHO, 2019). Initially, the problem was mainly approached from a clinical perspective. However, more

recently, the importance of the environment as a major contributor to the spread of antimicrobial resistance in the human and animal population has become increasingly clear (Allcock et al., 2017; Leonard et al., 2018; Hernando-Amado et al., 2020). Successful management strategies and policies to combat the increase in antimicrobial resistance will therefore have to be based on a One Health approach, which recognizes the connectivity between animal, human, and environmental health (Hernando-Amado et al., 2019; Van Bruggen et al., 2019).

Antibiotic resistance in bacteria may arise from chromosomal mutations, however, for most types of antimicrobial resistance, the acquisition of antibiotic resistance genes (ARGs) mediated by horizontal gene transfer mechanisms is a more common mechanism (Andersson and Hughes, 2010; Huddleston, 2014; Baquero et al., 2019). Transduction by bacteriophages is an important mechanism in spreading ARGs within a microbial population (Muniesa et al., 2013b; Balcazar, 2014; Brown-Jaque et al., 2015; Balcázar, 2018; Maganha De et al., 2021). The importance of bacteriophages in spreading ARGs and virulence genes, was recently underscored by the discovery of lateral transduction, which is the basis of genomic hypermobility (Chen et al., 2018; Chiang et al., 2019).

Environments characterized by high levels of fecal matter such as sewage, animal slurry, sludge, and effluent of wastewater treatment plants are hotspots of antibiotic resistant bacteria and phages harboring ARGs, forming an ideal environment for horizontal gene transfer to occur (Calero-Cáceres et al., 2014; Quirós et al., 2014; Ross and Topp, 2015; Calero-Cáceres and Muniesa, 2016; Guo et al., 2017; Yang et al., 2021; Zieliński et al., 2021). It has been shown that transfection and transduction of ARGs from environmental phages conferred resistance to the recipient bacteria (Battaglioli et al., 2011; Colomer-Lluch et al., 2011; Gunathilaka et al., 2017; Wang et al., 2018b; Yang et al., 2021).

The effluent of wastewater treatment plants is an important route by which ARGs may enter the aquatic environment (Rizzo et al., 2013; Marti et al., 2014b; Rodriguez-Mozaz et al., 2015; Lekunberri et al., 2017; Zhou et al., 2020; Nguyen et al., 2021). Sewerage misconnections and leaking septic tanks may be a source of untreated sewage entering rivers and other waterbodies (Kay et al., 2008; Hinojosa et al., 2020; Reynolds et al., 2021). Furthermore, agricultural land run-off may also negatively impact water quality and introduce ARGs into waterbodies (Unc and Goss, 2004; Ballesté et al., 2020). Watercourses discharging into the marine environment, especially close to designated bathing waters, may expose the users to fecal pollution and therefore may increase the likelihood that they will be exposed to ARGs (Molina et al., 2014; Leonard et al., 2018; Ahmed et al., 2020; Reynolds et al., 2020; Sala-Comorera et al., 2021b).

This study focuses on the bacterial and bacteriophage ARG profiles of two rivers entering a large urban environment and two small, completely urban, streams that discharge close to two marine bathing waters. We show that the ARG profiles of these streams and rivers in an urban environment are highly similar and have a strong impact on nearshore marine bathing waters. In contrast to bacterial ARGs, ARGs associated with bacteriophages appear to persist in the nearshore marine environment.

MATERIALS AND METHODS

Site Location

Dublin, the capital of Ireland, is a coastal city on the Irish Sea with 560,000 inhabitants. Near 1,905,000 people live within the greater Dublin area and around sixty watercourses, ranging from major rivers to small streams, which discharge into Dublin Bay. Dublin Bay, which is a UNESCO biosphere, has three designated bathing waters: Dollymount, Sandymount, and Merrion Strands.

In this study, two rivers (River Liffey and River Tolka), two streams (Elm Park Stream and Trimleston Stream), and two bathing waters (Merrion Strand and Sandymount Strand) were selected (**Figure 1** and **Supplementary Table 1**). The River Liffey rises in a pristine area near Kippure in the Wicklow Mountains and flows through agricultural land to reaching Dublin city. The River Liffey has a 125 km course and it is the main river flowing into Dublin Bay (Sweeney, 2017). The River Tolka is 33 km long, and it is the second largest river by flow in Dublin Bay. The river rises near Culmullin Cross Road and flows through agricultural and industrial land into the north of Dublin city (Sweeney, 2017). The Elm Park Stream and the Trimleston Stream catchment is completely urban areas with a population of 40,000 people. The length of the streams are 3.8 and 1.7 km, respectively, and the depth is less than 10 cm. Both streams discharge close to a designated bathing area in Dublin Bay.

Grab samples were collected at the tidal limit at a depth of 10–20 cm, every 5 weeks over 15 months (from September 2018 to November 2019). Bathing water samples were taken during high tide at Merrion and Sandymount Strands. A total of 85 samples collected and stored at 4°C before being processed within 6 h.

Enumeration of Fecal Indicator Organisms

The levels of *Escherichia coli* and intestinal enterococci were determined by membrane filtration. Water samples were filtered through 0.45 μm pore size nitrocellulose membranes (Thermo Scientific) and placed on Tryptone Bile X-Glucuronide agar (Sigma-Aldrich) at 37°C for 4 h, followed by an incubation at 44°C for 18 h to enumerate *E. coli* (ISO, 2001). Intestinal enterococci were enumerated by incubating the membrane on Slanetz and Bartley agar (Oxoid) at 37°C for 48 h. After incubation, membranes were transferred into Bile Aesculin agar at 44°C for 2 h to confirm positive intestinal enterococci colonies (ISO, 2000).

Electron Microscopy

River samples (100 ml) were concentrated by ultrafiltration using 100 kDa Amicon Ultra-15 Centrifugal Filter units (Millipore) and 5 μl of the concentrated samples were pipetted onto a 200-mesh copper grid coated with formvar. Samples were negatively stained with 5 μl of 2% uranyl acetate stain and incubated for 2 min. The grids were imaged using a transmission electron microscope Tecnai G2 (FEI Tecnai) operating at 120 kV. Untreated sewage (50 ml) collected in the influent of a local wastewater treatment plant was used as a positive control.

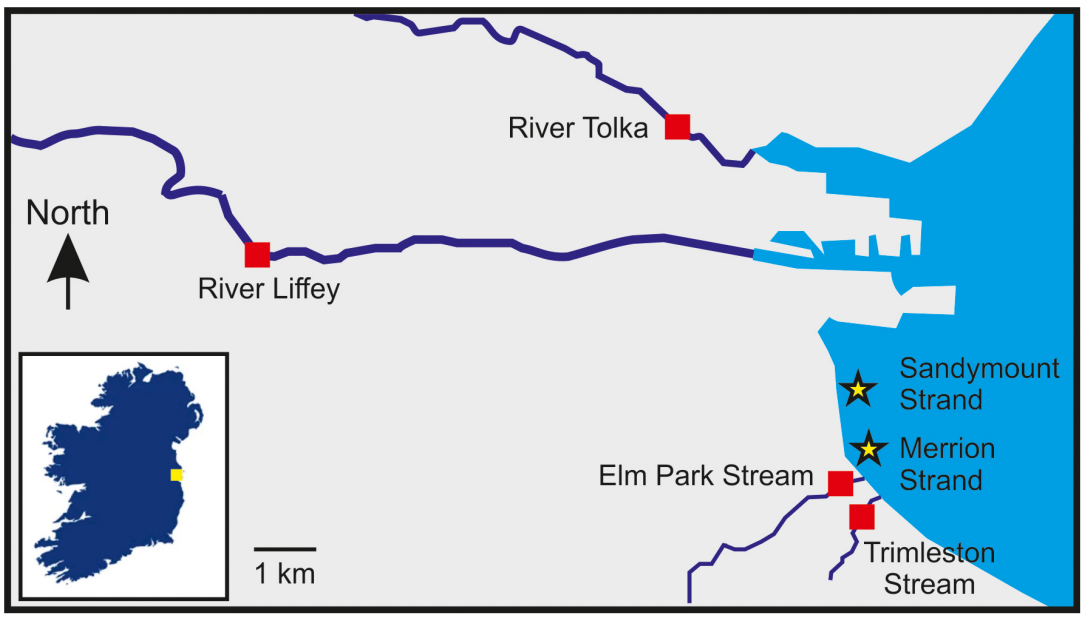

FIGURE 1 | Location of the sampling sites selected in this study. The sampling stations are indicated by red squares (rivers and streams) and stars (bathing waters). The inset box shows the location of Dublin Bay in Ireland indicated by a yellow square.

DNA Extraction From the Bacterial Fraction

DNA of the water samples was extracted after concentrating by filtering 100 ml through 0.22 μm mixed cellulose ester membrane filters. The filters were then transferred in 500 μl of GITC buffer [5 M guanidine thiocyanate, 100 mM EDTA (pH 8), and 0.5% sarkosyl] and stored at −20°C. DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen) with some modifications as reported previously (Gourmelon et al., 2007). The DNA was eluted in a final volume of 70 μl.

DNA Extraction From the Bacteriophage Fraction

DNA of bacteriophages present in water was extracted using the protocol described by Colomer-Lluch et al. (2011). Briefly, 100 ml of water was passed through 0.22 μm low protein binding polyethersulfone filters. The filtrate was concentrated 200-fold using 100 kDa Amicon Ultra-15 Centrifugal Filter units (Millipore), and subjected to chloroform extraction in a 1:1 (v/v) ratio, followed by a DNAse (100 U/ml) treatment at 37°C for 1 h and 10 min at 80°C. At this point, an aliquot of 10 μl was collected as a control to ensure the complete removal of free DNA. Phage particles were subjected to proteinase K digestion (0.2 mg/ml) for 1 h at 56°C followed by phenol/chloroform extraction and ethanol precipitation. The resulting DNA was dissolved into 20 μl water.

Quantification of Gene Target

Primers to amplify the ARGs bla_{TEM}, bla_{SHV}, *qnrS*, and *sul1* and the human (HF183) and ruminant (CF128) microbial source tracking markers, as well as the cycling conditions are described in **Supplementary Table 2**. The MST markers and ARGs were quantified as previously described (Ballesté et al., 2020; Reynolds et al., 2020). Standard curves were generated using linearized cloned standards between 10^0 and 10^6 gene copies to quantify target gene levels in each sample (Ballesté et al., 2020; Reynolds et al., 2020). The limit of detection of each assay was determined as the lowest concentration of DNA target detected in 95% or more of replicates, whereas the limit of quantification was determined as the lowest concentration of DNA quantified within 0.5 SD of the $\log_{10}$ concentration (**Supplementary Table 2**; Blanchard et al., 2012; AFNOR, 2015). All samples and negative controls were analyzed in duplicate in each 96-well plate. The absence of non-packaged DNA in the bacteriophage extraction protocol was verified with 16S rRNA gene amplification by PCR (AllTaq Master Mix Kit, Qiagen) and ARGs genes by qPCR. Only negative samples were used for the subsequent analysis. MST markers and ARGs concentrations were expressed as gene copies per 100 ml (GC/100 ml). The amplification efficiency of each reaction was calculated using the $E = 10^{(1/slope)} - 1$ equation (Rutledge, 2003).

Data Analysis

The non-parametric Mann–Whitney paired-test and Kruskal–Wallis test with Dunn's *post hoc* analysis was used to assess significant differences between microbial source tracking markers and ARGs between waterbodies. The values of the qPCR targets were $\log_{10}$ transformed and Spearman correlation was used to identify relationships between variables. A significance cut-off of $p \leq 0.05$ was used for all analyses. Statistical analysis was carried out using GraphPad Prism 9.1.0. software (GraphPad Software).

RESULTS

Fecal Contamination of Rivers, Urban Streams, and Bathing Waters

All water samples ($n = 85$) were positive for *E. coli* and intestinal enterococci (**Figure 2**). The River Tolka and the River Liffey, which flow through agricultural areas before reaching the city, had similar median levels of fecal indicator organisms as the small urban Elm Park and Trimleston streams. The fecal indicators in the latter varied more than three orders of magnitude. In contrast, the fecal indicator levels were less variable for the River Liffey during this 15-month period, which had the lowest median concentration for the fecal indicators. In general, all rivers and streams received fecal contamination along their course, independently of the catchment area.

The median fecal indicator levels in Merrion and Sandymount Strands were similar with individual values varying by up to four orders of magnitude. Some of the bathing water samples analyzed exceeded the 90th percentile value for sufficient water quality parameters for coastal waters ($\geq$500 CFU/100 ml for *E. coli* and $\geq$185 CFU/100 ml for intestinal enterococci) according to the European bathing water quality Directive (EU, 2006).

Fecal Contamination of Rivers and Streams Is Predominantly Human in Nature

The *Bacteroidales* human (HF183) and ruminant (CF128) markers were deployed to determine the biological origins of pollution (**Figure 3**). The River Liffey catchment is larger than that of the River Tolka and has more agricultural land use. Therefore, as expected, more River Liffey (80%) than River Tolka (66%) samples were positive for the ruminant marker. The levels of the ruminant marker reached values of 5.1×10^5 and 3.4×10^5 GC/100 ml in River Liffey and River Tolka, respectively. In contrast the marker was only sporadically detected in the Elm Park stream (40%) and in only one sample in the Trimleston stream. When the CF128 marker tested positive in Elm Park stream samples, concentrations up to 2.8×10^5 GC/100 ml were reported. The presence of this marker in an urban stream is explained by the run-off from a small pasture grazed by a few heads of cattle on a private property near the Elm Park stream. Similar to the streams, the ruminant CF128 marker was present in 47% of the samples in Merrion Strand and in only one sample in Sandymount Strand.

The human marker was detected in all river and stream samples and in nearly 90% of the bathing water samples. HF183 levels in the streams ranged from 2.3×10^3 to 1.7×10^6 GC/100 ml and from 7.6×10^2 to 4.7×10^4 GC/100 ml in river samples. The median values for rivers and streams differed by less than one order of magnitude. Variations in HF183 levels were observed in both beaches ranging from below the limit of quantification to 6.1×10^4 GC/100 ml for Merrion Strand and 2.2×10^4 GC/100 ml for Sandymount Strand. The median concentrations of the marker in marine samples were lower than those of rivers and streams.

All the rivers and streams were continuously impacted by human pollution. In contrast, the ruminant marker was less prevalent in rivers, streams, and bathing waters. Thus, anthropogenic activities are therefore likely to be the primary driver of fecal pollution at the sampling stations in the different watercourses.

Rivers and Urban Streams Have a Similar ARGs Profile

Bacteriophages may be an important reservoir of ARGs yet are often overlooked. We therefore wanted to analyze water samples for ARGs in both bacteria and bacteriophages. To validate our bacteriophage enrichment procedure, the concentrate was examined using an electron microscope. Bacteriophages with morphologies corresponding to families *Myoviridae*, *Siphoviridae*, and *Podoviridae* (Demuth et al., 1993; Colomer-Lluch et al., 2011) were present (**Figure 4**).

The presence of antibiotic resistance genes in the bacterial and bacteriophage fractions was assessed by selecting four ARGs, bla_{TEM}, bla_{SHV}, *qnrS*, and *sul1*. Bacterial ARGs were found in 73–100% of the river samples and the percentage of positive samples was even higher in the stream samples (87–100%). However, only 20–93% of the bacteriophage fractions from the rivers and 42–100% from the streams contained ARGs (**Figure 5**). In all cases the level of ARGs in the bacteriophage fraction was lower than in the bacterial fraction (**Figure 6**). The bacteriophage DNA preparations did not contain 16S rDNA.

The bla_{TEM} and *sul1* genes the most prevalent in the bacterial fraction, while bla_{TEM} was most abundant in the bacteriophage fraction of the river and stream samples. The bla_{TEM} median levels ranged from 1.1×10^4 to 4.0×10^4 GC/100 ml in the bacterial fraction and from 1.7×10^3 to 1.4×10^4 GC/100 ml in bacteriophage fraction. The *sul1* gene was the most abundant in the bacterial fraction of the River Tolka, River Liffey, and the Trimleston stream with levels ranging from 3.2×10^4 to 7.6×10^4 GC/100 ml. Interestingly, the levels of *sul1* in the bacteriophage DNA were 10- to 100-fold lower.

The median concentrations for the bacterial bla_{SHV} and *qnrS* genes were in the same range (3 $\log_{10}$ GC/100 ml) but were one order of magnitude lower than bla_{TEM} and *sul1*. For the bacteriophage fraction, the median levels for bla_{SHV} and *qnrS* genes observed in rivers were in the same range (2 $\log_{10}$ GC/100 ml) as those found in the urban streams. However, the *qnrS* gene was more commonly found in the urban streams (47–64%) in comparison to the largest rivers (20–40%). Interestingly, an opposite pattern was obtained for bla_{SHV}. Overall, the profiles of the bacterial ARGs in the urban streams and the largest rivers were similar, since no statistical differences for the majority of ARGs were reported between rivers and streams (Kruskal–Wallis, $p > 0.05$, **Supplementary Table 3**).

Spearman correlation analysis was performed to establish the relationship between the levels of ARGs and the human fecal marker. Since there was no significant difference between the ARG profiles of rivers and streams, all samples were treated as a single dataset. The bla_{TEM}, bla_{SHV}, and *qnrS* genes in the bacterial DNA fraction correlated moderately with the

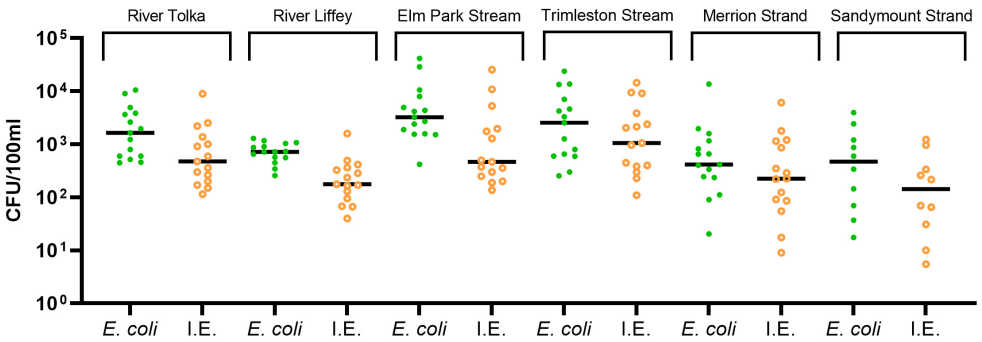

FIGURE 2 | Levels of the fecal indicators *Escherichia coli*, intestinal enterococci (I.E.). The dots show the concentration of each sample and the median are indicated as a horizontal line.

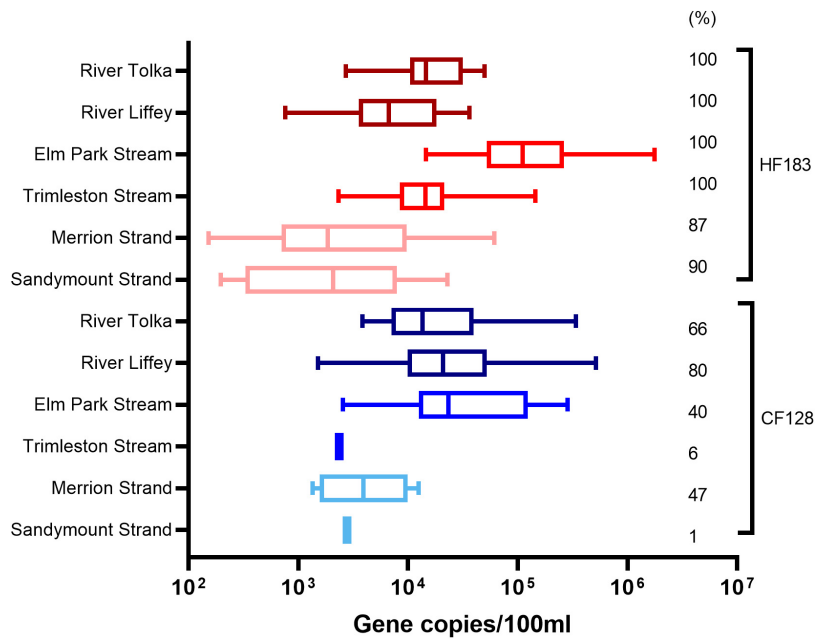

FIGURE 3 | Boxplot representation of the concentrations and percentage of positive samples for the human (HF183) and ruminant (CF128) marker in water samples from rivers (Tolka and Liffey), streams (Elm Park and Trimleston), and bathing waters (Sandymount and Merrion Strands). In the boxplots the lower hinge represents 25% quantile, upper hinge 75% quantile, and center line the median. The whiskers show the maximum and the lowest value. The percentage of samples above the quantification limit is indicated, only values above the quantification limit are plotted.

human fecal marker (Spearman correlation, ρ = 0.292–0.335, $p < 0.05$, **Supplementary Table 4**). However, there was no significant correlation between the human marker and ARGs in the bacteriophage fraction.

ARGs in Bathing Waters Have an Urban Profile

The rivers and streams in this study are mostly impacted by human fecal contamination and have the same ARG profile, despite their substantial difference in size. We hypothesized that the discharge of these streams and rivers into Dublin Bay would affect the nearshore marine environment.

All ARGs present in the streams and rivers were also found in the two bathing waters. The detection frequency profile of the ARGs in the rivers, streams, and strands was very similar (**Figure 5**). The bla_{TEM} and *sul1* genes were the most frequently detected genes, followed by bla_{SHV} and *qnrS*. The levels of individual ARGs in both the bacterial and bacteriophage fractions in Merrion and Sandymount Strand did not differ significantly (Mann–Whitney, $p > 0.05$, **Supplementary Table 5**), which is not surprising, considering that these bathing waters are adjacent to each other. As was observed for the rivers and streams, in the nearshore marine environment bla_{TEM} and *sul1* were the most abundant ARGs in the bacterial fraction, and bla_{TEM} was the most abundant in the bacteriophage fraction (**Figure 6**). The median level of bla_{TEM} ranged from 2.6×10^3 to 6.3×10^3

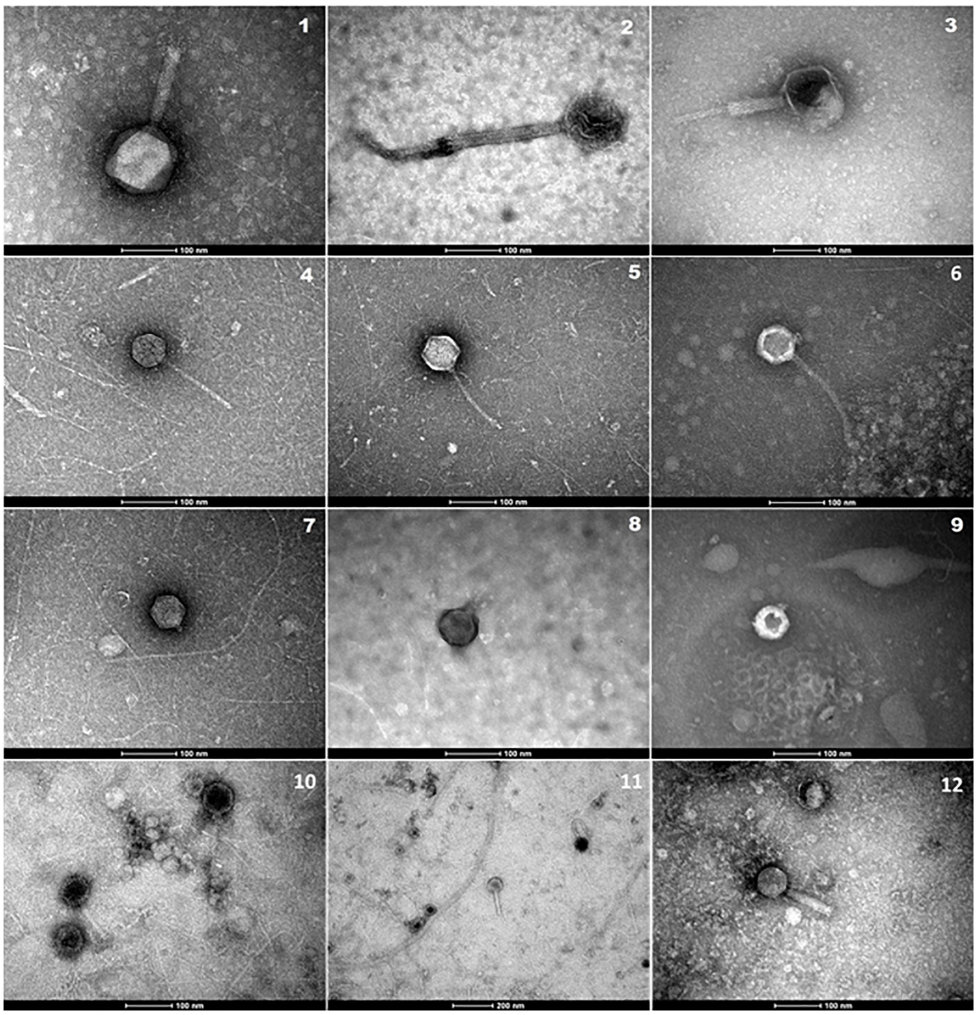

FIGURE 4 | Electron micrographs showing bacteriophages isolated in concentrated water samples from Elm Park Stream (1, 4, 5, 7, 11), River Tolka (2, 8, 10), Trimleston Stream (6, 9), and raw sewage (3, 12). Images 1–3 show *Myoviridae* phages, images 4–6 *Siphoviridae* phages and images 7–9 *Podoviridae* phages. Scale bars represent 100 nm, whereas the scale bar in micrograph-11 represents 200 nm.

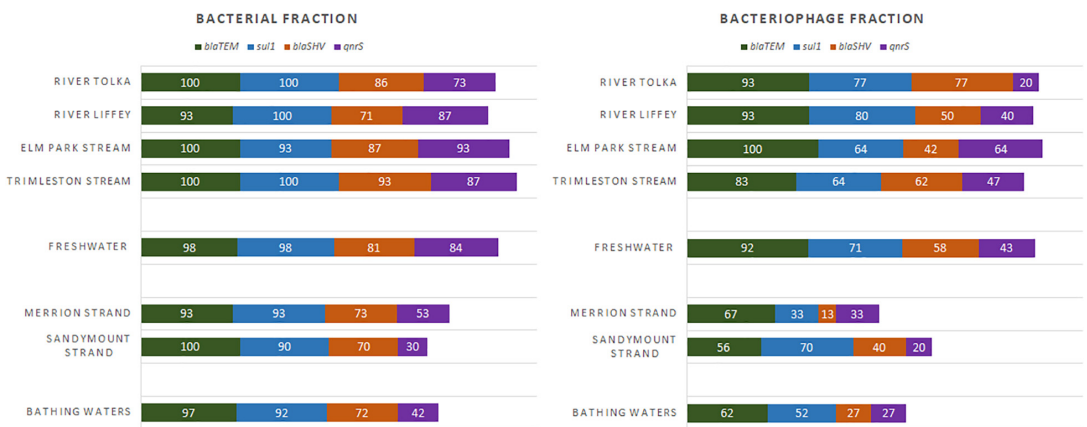

FIGURE 5 | Frequency of detection (%) for the antibiotic resistance genes in freshwater (River Tolka and Liffey, Elm Park, and Trimleston Streams) and bathing waters (Merrion and Sandymount Strands).

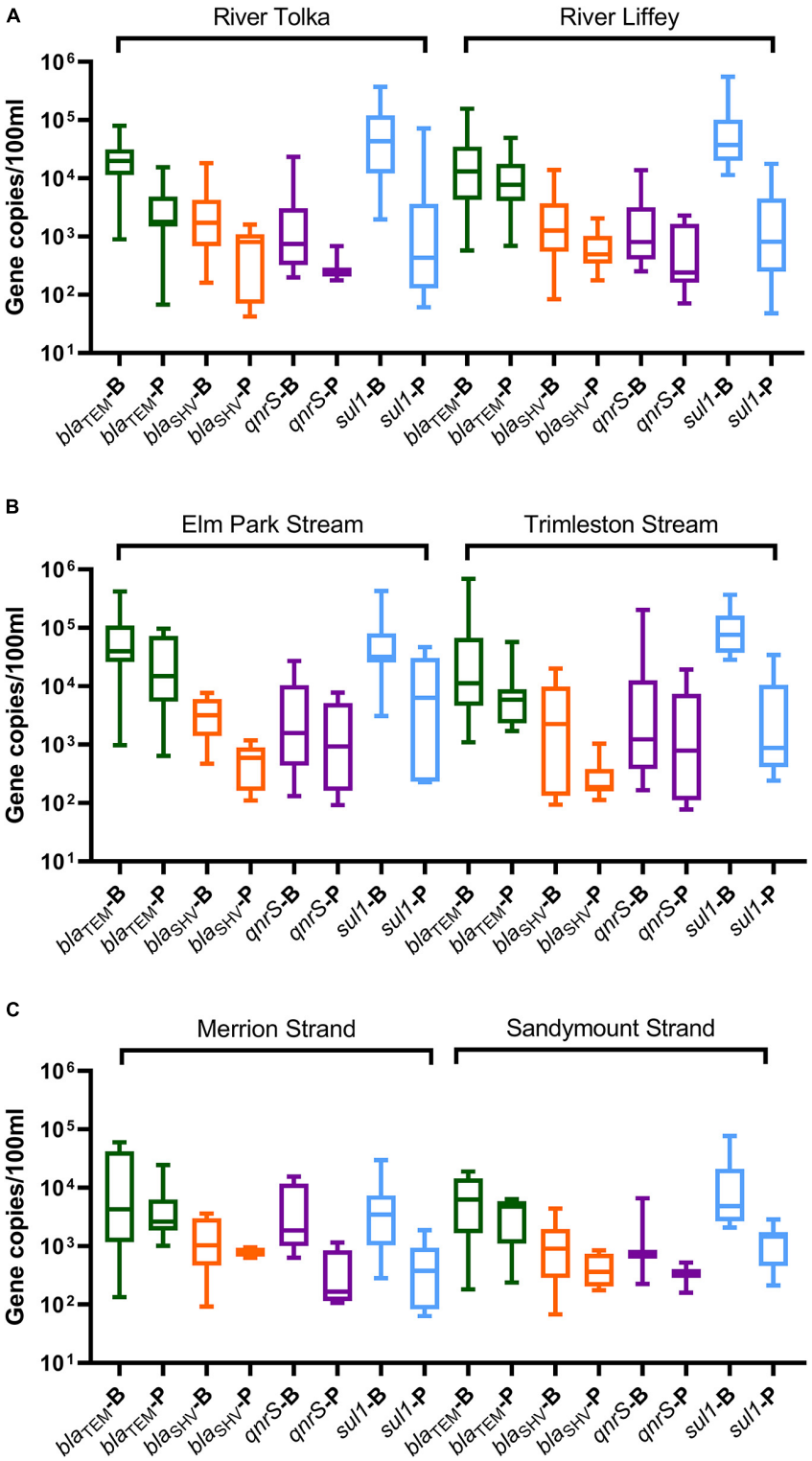

FIGURE 6 | Boxplot representation of the ARGs concentrations in the bacterial (B) and bacteriophage (P) fractions in water samples from **(A)** rivers (Tolka and Liffey), **(B)** streams (Elm Park and Trimleston), and **(C)** bathing waters (Merrion and Sandymount Strands). In the boxplots the lower hinge represents 25% quantile, upper hinge 75% quantile, and center line the median. The whiskers show the maximum and the lowest value. Only values above the quantification limit are plotted.

GC/100 ml and from 3.7×10^2 to 4.8×10^3 GC/100 ml for *sul1*. As was the case for the rivers and streams, the levels of bla_{SHV} and *qnrS* were around 0.5 $\log_{10}$ lower than the two most abundant genes (**Figure 6**).

In comparison to the freshwater samples, the median concentration for bacterial ARGs levels (bla_{TEM}, bla_{SHV} and *sul1*) decreased by significantly two and ninefold in marine bathing waters (Mann–Whitney, p = 0.0001–0.041, **Supplementary Table 5** and **Supplementary Figure 1**), The *qnrS* levels did not change significantly (Mann–Whitney, p = 0.466). In contrast, there was no significant reduction in ARG levels for the phage fraction (Mann–Whitney, p = 0.093–0.653, **Supplementary Table 5** and **Supplementary Figure 1**).

DISCUSSION

The rivers Liffey and Tolka rise outside Dublin and flow through an agricultural area before entering the city and discharging into Dublin Bay. In contrast, the two streams included in this study are completely urban and are therefore experiencing different pollution pressures than the much larger rivers. Despite these potential differences in pollution pressures, microbial source tracking analysis showed that the main source of pollution in both rivers and streams in the urban environment is human contamination, which presumably enters the rivers and streams through for example combined sewer overflows and sewerage misconnections.

The four clinically relevant ARGs included in this study bla_{TEM}, bla_{SHV}, *qnrS*, and *sul1*, were present in all four waterways and occurred in both the bacterial as well as in the bacteriophage fraction. These ARGs selected confer resistance to the most common antibiotics prescribed in Ireland (HSE, 2019). Interestingly, the ARG profiles of the four waterways were very similar, both in terms of the levels of ARGs and the frequency of detection. This is consistent with the urban environment as the main fecal impactor on these waterways. In line with this, a moderate level of correlation between the bacterial bla_{TEM}, bla_{SHV}, and *qnrS* and the HF183 marker was observed, but not for *sul1*. In contrast, there was no correlation between the levels of the four ARGs in bacteriophage fraction and HF183. Partial correlation between ARGs and indicators of fecal contamination has been reported previously (Calero-Cáceres et al., 2017).

In the bacterial fraction, the *sul1* and bla_{TEM} genes were the most prevalent followed by bla_{SHV} and *qnrS*. The bla_{TEM} gene was the most prominent ARG in the bacteriophage fraction, followed by *sul1*, bla_{SHV}, and *qnrS*. Resistance to β-lactam antimicrobial agents by bla_{TEM}, bla_{SHV} are widely distributed in aquatic ecosystems (Colomer-Lluch et al., 2011; Anand et al., 2016; Calero-Cáceres et al., 2017; Zhang et al., 2019), which might indicate that they are particularly amenable to propagation through transduction (Wang et al., 2018a). Sulfonamides are one of the oldest antimicrobial synthetic or semi-synthetic drug classes that have been also used for the treatment of animals (Dasenaki and Thomaidis, 2017) and is an authorized antibiotic for use in aquaculture in Europe (Santos and Ramos, 2018). The *sul1* gene was the second most abundant and variable in concentration in the bacteriophage fraction. This high degree of variability of *sul1* in rivers has also been observed in Mediterranean human-impacted rivers (Calero-Cáceres et al., 2017; Lekunberri et al., 2017) and rivers in China (Yang et al., 2018). Sulfonamide resistance is associated with mobile genetic elements, like class 1 integrons (int1) (Jiang et al., 2019), which may explain the presence in the bacteriophage fraction. Resistance to fluoroquinolones have been associated with clinical *Enterobacteriaceae* isolates as well as in waterborne bacteria, but with lower prevalence in rivers (Poirel et al., 2012; Marti and Balcázar, 2013; Lekunberri et al., 2017).

Aquatic environments are ideally suited for the dispersal of ARGs (Marti et al., 2014a). Rivers and streams may therefore have a lasting effect on the presence of ARGs in the marine environment into which they discharge. In addition, the discharge of wastewater treatment plants will also add to the presence of ARG in the marine environment. The ARG profiles of two bathing waters in Dublin Bay were not significantly different from those of the rivers and streams, although the concentrations and frequency of detection were lower. The bla_{TEM} gene is also the most abundant in the marine environment, which is consistent with the few marine studies in the Mediterranean Sea and the Indian Ocean, where bla_{TEM} gene was the most prevalent and abundant gene in the bacteriophage fraction (Calero-Cáceres and Balcázar, 2019; Blanco-Picazo et al., 2020).

Interestingly, there was a significant two to ninefold reduction in median values of the bacterial bla_{TEM}, bla_{SHV}, and *sul1* genes, whereas the levels of *qnrS* did not change significantly. Freshwater or intestinal bacteria carrying ARGs are likely to die off rapidly in the marine environment, which would account for the decrease of three of the four ARGs. Resistance to fluoroquinolone in marine environments is related to the intrinsic resistance of marine *Vibrionaceae* and *Shewanellaceae* family species which possess chromosome-encoded Qnr-like proteins (Poirel et al., 2005; Cattoir et al., 2007). The decrease of bacterial ARGs in the marine environment was not observed for bacteriophage ARGs. In general, decay rates of bacteriophages in seawater are much lower than those of fecally associated bacteria and therefore persist for prolonged periods of time (Mocé-Llivina et al., 2005; Calero-Cáceres and Muniesa, 2016; Wu et al., 2020; Sala-Comorera et al., 2021a).

Once ARGs have been introduced to the phageome, they may be reintroduced into the bacterial metagenome through horizontal gene transfer mechanisms (Muniesa et al., 2013a). Prolonged persistence of bacteriophages carrying ARGs, may result in transfer of these ARGs to marine microbiota, and eventually find their way into human consumers of seafood. In addition, and perhaps more importantly, ARG carrying bacteriophages may accumulate in filter feeders (e.g., oysters) and enter the food chain in this manner. Furthermore, recreational activities taking place in or on the water may expose people to ARG carrying bacteriophages, which once ingested may transfer these ARGs to the microbiota in the intestinal tract. The EU Bathing Water Directive classifies bathing water solely on the presence of *E. coli* and intestinal enterococci, with a view to prevent gastrointestinal and respiratory disease (EU, 2006). Although the use of bacteriophages as additional

or alternative indicators for fecal contamination has and is being discussed, the data presented here make a case for the inclusion of bacteriophages as indicators for the potential spread of antimicrobial resistance during recreational use of bathing waters.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

LS-C: conceptualization, methodology, validation, formal analysis, data curation, writing—original draft, writing—review and editing, and visualization. TN and LR: investigation and validation. AV, LC, NM, JS, and AG: investigation. GO'H and JO'S: writing—review and editing. WM: conceptualization, supervision, writing—review and editing, supervision, project administration, and 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.2021.718234/full#supplementary-material

REFERENCES

AFNOR. (2015). *Méthodes D'analyse En Santé Animale — Pcr (Réaction De Polymérisation En Chaîne) — Partie 2?: exigences Et Recommandations Pour Le Développement Et La Validation De La Pcr En Santé Animale*. France: AFNOR.

Ahmed, W., Payyappat, S., Cassidy, M., Harrison, N., and Besley, C. (2020). Sewage-Associated Marker Genes Illustrate The Impact Of Wet Weather Overflows And Dry Weather Leakage In Urban Estuarine Waters Of Sydney. *Australia. Sci. Total Environ.* 705:135390. doi: 10.1016/J.Scitotenv.2019.135390

Allcock, S., Young, E. H., Holmes, M., Gurdasani, D., Dougan, G., Sandhu, M. S., et al. (2017). Antimicrobial Resistance In Human Populations: challenges And Opportunities. *Glob. Heal. Epidemiol. Genom.* 2, 1–7. doi: 10.1017/Gheg.2017.4

Anand, T., Bera, B. C., Vaid, R. K., Barua, S., Riyesh, T., Virmani, N., et al. (2016). Abundance Of Antibiotic Resistance Genes In Environmental Bacteriophages. *J. Gen. Virol.* 97, 3458–3466. doi: 10.1099/Jgv.0.000639

Andersson, D. I., and Hughes, D. (2010). Antibiotic Resistance And Its Cost: is It Possible To Reverse Resistance? *Nat. Rev. Microbiol.* 8, 260–271. doi: 10.1038/Nrmicro2319

Balcazar, J. L. (2014). Bacteriophages As Vehicles For Antibiotic Resistance Genes In The Environment. *PLoS Pathog.* 10:E1004219. doi: 10.1371/Journal.Ppat.1004219

Balcázar, J. L. (2018). How Do Bacteriophages Promote Antibiotic Resistance In The Environment? *Clin. Microbiol. Infect.* 24, 447–449. doi: 10.1016/J.Cmi.2017.10.010

Ballesté, E., Demeter, K., Masterson, B., Timoneda, N., Sala-Comorera, L., and Meijer, W. G. (2020). Implementation And Integration Of Microbial Source Tracking In A River Watershed Monitoring Plan. *Sci. Total Environ.* 736:139573. doi: 10.1016/J.Scitotenv.2020.139573

Baquero, F., Coque, T. M., Martínez, J. L., Aracil-Gisbert, S., and Lanza, V. F. (2019). Gene Transmission In The One Health Microbiosphere And The Channels Of Antimicrobial Resistance. *Front. Microbiol.* 10:2892. doi: 10.3389/Fmicb.2019.02892

Battaglioli, E. J., Baisa, G. A., Weeks, A. E., Schroll, R. A., Hryckowian, A. J., and Welch, R. A. (2011). Isolation Of Generalized Transducing Bacteriophages For Uropathogenic Strains Of *Escherichia Coli*. *Appl. Environ. Microbiol.* 77, 6630–6635. doi: 10.1128/Aem.05307-11

Blanchard, P., Regnault, J., Schurr, F., Dubois, E., and Ribière, M. (2012). Intra-Laboratory Validation Of Chronic Bee Paralysis Virus Quantitation Using An Accredited Standardised Real-Time Quantitative Rt-Pcr Method. *J. Virol. Methods* 180, 26–31. doi: 10.1016/J.Jviromet.2011.12.005

Blanco-Picazo, P., Roscales, G., Toribio-Avedillo, D., Gómez-Gómez, C., Avila, C., Ballesté, E., et al. (2020). Antibiotic Resistance Genes In Phage Particles From Antarctic and Mediterranean Seawater Ecosystems. *Microorganisms* 8:1293. doi: 10.3390/Microorganisms8091293

Brown-Jaque, M., Calero-Cáceres, W., and Muniesa, M. (2015). Transfer Of Antibiotic-Resistance Genes Via Phage-Related Mobile Elements. *Plasmid* 79, 1–7. doi: 10.1016/J.Plasmid.2015.01.001

Calero-Cáceres, W., and Balcázar, J. L. (2019). Antibiotic Resistance Genes In Bacteriophages From Diverse Marine Habitats. *Sci. Total Environ.* 654, 452–455. doi: 10.1016/J.Scitotenv.2018.11.166

Calero-Cáceres, W., Melgarejo, A., Colomer-Lluch, M., Stoll, C., Lucena, F., Jofre, J., et al. (2014). Sludge As A Potential Important Source Of Antibiotic Resistance Genes In Both The Bacterial and Bacteriophage Fractions. *Environ. Sci. Technol.* 48, 7602–7611. doi: 10.1021/Es501851s

Calero-Cáceres, W., Méndez, J., Martín-Díaz, J., and Muniesa, M. (2017). The Occurrence Of Antibiotic Resistance Genes In A Mediterranean River and Their Persistence In The Riverbed Sediment. *Environ. Pollut.* 223, 384–394. doi: 10.1016/J.Envpol.2017.01.035

Calero-Cáceres, W., and Muniesa, M. (2016). Persistence Of Naturally Occurring Antibiotic Resistance Genes In The Bacteria and Bacteriophage Fractions Of Wastewater. *Water Res.* 95, 11–18. doi: 10.1016/J.Watres.2016.03.006

Cassini, A., Högberg, L. D., Plachouras, D., Quattrocchi, A., Hoxha, A., Simonsen, G. S., et al. (2019). Attributable Deaths and Disability-Adjusted Life-Years Caused By Infections With Antibiotic-Resistant Bacteria In The Eu and The European Economic Area In 2015: a Population-Level Modelling Analysis. *Lancet Infect. Dis.* 19, 56–66. doi: 10.1016/S1473-3099(18)30605-4

Cattoir, V., Poirel, L., Mazel, D., Soussy, C. J., and Nordmann, P. (2007). Vibrio Splendidus As The Source Of Plasmid-Mediated Qnrs-Like Quinolone Resistance Determinants [1]. *Antimicrob. Agents Chemother.* 51, 2650–2651. doi: 10.1128/Aac.00070-07

Chen, J., Quiles-Puchalt, N., Chiang, Y. N., Bacigalupe, R., Fillol-Salom, A., Chee, M. S. J., et al. (2018). Genome Hypermobility By Lateral Transduction. *Science* 362, 207–212. doi: 10.1126/Science.Aat5867

Chiang, Y. N., Penadés, J. R., and Chen, J. (2019). Genetic Transduction By Phages and Chromosomal Islands: the New and Noncanonical. *PLoS Pathog.* 15:E1007878. doi: 10.1371/Journal.Ppat.1007878

Colomer-Lluch, M., Jofre, J., and Muniesa, M. (2011). Antibiotic Resistance Genes In The Bacteriophage Dna Fraction Of Environmental Samples. *PLoS One* 6:E17549. doi: 10.1371/Journal.Pone.0017549

Dasenaki, M. E., and Thomaidis, N. S. (2017). "Meat Safety: ii Residues and Contaminants" In *Lawrie's Meat Science: eighth Edition*. ed. F. Toldrá. (Netherlands: Elsevier). 553–583. doi: 10.1016/B978-0-08-100694-8.00018-2

Demuth, J., Neve, H., and Witzel, K. P. (1993). Direct Electron Microscopy Study On The Morphological Diversity Of Bacteriophage Populations In Lake Plusssee. *Appl. Environ. Microbiol.* 59, 3378–3384. doi: 10.1128/Aem.59.10.3378-3384.1993

EU. (2006). *Directive 2006/7/Ec Of The European Parliament and Of The Council Of 15 February 2006 Concerning The Management Of Bathing Water Quality And Repealing Directive 76/160/Eec*. EU: EUR-Lex.

Gourmelon, M., Caprais, M. P., Ségura, R., Le Mennec, C., Lozach, S., Piriou, J. Y., et al. (2007). Evaluation Of Two Library-Independent Microbial Source Tracking Methods To Identify Sources Of Fecal Contamination In French Estuaries. *Appl. Environ. Microbiol.* 73, 4857–4866. doi: 10.1128/Aem.03003-06

Gunathilaka, G. U., Tahlan, V., Mafiz, A. I., Polur, M., and Zhang, Y. (2017). Phages In Urban Wastewater Have The Potential To Disseminate Antibiotic Resistance. *Int. J. Antimicrob. Agents* 50, 678–683. doi: 10.1016/J.Ijantimicag.2017.08.013

Guo, J., Li, J., Chen, H., Bond, P. L., and Yuan, Z. (2017). Metagenomic Analysis Reveals Wastewater Treatment Plants As Hotspots Of Antibiotic Resistance Genes and Mobile Genetic Elements. *Water Res.* 123, 468–478. doi: 10.1016/J.Watres.2017.07.002

Hernando-Amado, S., Coque, T. M., Baquero, F., and Martínez, J. L. (2019). Defining And Combating Antibiotic Resistance From One Health And Global Health Perspectives. *Nat. Microbiol.* 4, 1432–1442. doi: 10.1038/S41564-019-0503-9

Hernando-Amado, S., Coque, T. M., Baquero, F., and Martínez, J. L. (2020). Antibiotic Resistance: moving From Individual Health Norms To Social Norms In One Health And Global Health. *Front. Microbiol.* 11:1914. doi: 10.3389/Fmicb.2020.01914

Hinojosa, J., Green, J., Estrada, F., Herrera, J., Mata, T., Phan, D., et al. (2020). Determining The Primary Sources Of Fecal Pollution Using Microbial Source Tracking Assays Combined With Land-Use Information In The Edwards Aquifer. *Water Res.* 184:116211. doi: 10.1016/J.Watres.2020.116211

HSE. (2019). *Aer Antimicrobial Consumption 2018*. Dublin: hse Hpsc.

Huddleston, J. R. (2014). Horizontal Gene Transfer In The Human Gastrointestinal Tract: potential Spread Of Antibiotic Resistance Genes. *Infect. Drug Resist.* 7, 167–176. doi: 10.2147/Idr.S48820

ISO. (2000). *Iso 7899-2:2000 - Water Quality – Detection And Enumeration Of Intestinal Enterococci – Part 2: membrane Filtration Method*. Switzerland: ISO.

ISO. (2001). *Iso 16649-1:2001 - Microbiology Of Food And Animal Feeding Stuffs — Horizontal Method For The Enumeration Of Beta-Glucuronidase-Positive Escherichia Coli — Part 1: colony-Count Technique At 44 Degrees C Using Membranes And 5-Bromo-4-Chloro-3-Indolyl beta-D-glucuronide*. Switzerland: ISO.

Jiang, H., Cheng, H., Liang, Y., Yu, S., Yu, T., Fang, J., et al. (2019). Diverse Mobile Genetic Elements And Conjugal Transferability Of Sulfonamide Resistance Genes (Sul1, Sul2, And Sul3) In *Escherichia Coli* Isolates From Penaeus Vannamei And Pork From Large Markets In Zhejiang. *China. Front. Microbiol.* 10:1787. doi: 10.3389/Fmicb.2019.01787

Jit, M., Ng, D. H. L., Luangasanatip, N., Sandmann, F., Atkins, K. E., Robotham, J. V., et al. (2020). Quantifying The Economic Cost Of Antibiotic Resistance And The Impact Of Related Interventions: rapid Methodological Review, Conceptual Framework And Recommendations For Future Studies. *BMC Med.* 18:38. doi: 10.1186/S12916-020-1507-2

Kay, D., Crowther, J., Stapleton, C. M., Wyer, M. D., Fewtrell, L., Edwards, A., et al. (2008). Faecal Indicator Organism Concentrations In Sewage And Treated Effluents. *Water Res.* 42, 442–454. doi: 10.1016/J.Watres.2007.07.036

Lekunberri, I., Villagrasa, M., Balcázar, J. L., and Borrego, C. M. (2017). Contribution Of Bacteriophage And Plasmid Dna To The Mobilization Of Antibiotic Resistance Genes In A River Receiving Treated Wastewater Discharges. *Sci. Total Environ.* 60, 206–209. doi: 10.1016/J.Scitotenv.2017.05.174

Leonard, A. F. C., Zhang, L., Balfour, A. J., Garside, R., Hawkey, P. M., Murray, A. K., et al. (2018). Exposure To And Colonisation By Antibiotic-Resistant E. Coli In Uk Coastal Water Users: environmental Surveillance, Exposure Assessment, And Epidemiological Study (Beach Bum Survey). *Environ. Int.* 114, 326–333. doi: 10.1016/J.Envint.2017.11.003

Maganha De, A. K. A. C., Borrego, C. M., and Balcázar, J. L. (2021). Antimicrobial Resistance And Bacteriophages: an Overlooked Intersection In Water Disinfection. *Trends Microbiol.* 29, 517–527. doi: 10.1016/J.Tim.2020.12.011

Marti, E., and Balcázar, J. L. (2013). *Comprehensive Analytical Chemistry*. Netherlands: elsevier B.V. 671–684. doi: 10.1016/B978-0-444-62657-8.00019-7

Marti, E., Variatza, E., and Balcazar, J. L. (2014a). The Role Of Aquatic Ecosystems As Reservoirs Of Antibiotic Resistance. *Trends Microbiol.* 22, 36–41. doi: 10.1016/J.Tim.2013.11.001

Marti, E., Variatza, E., and Balcázar, J. L. (2014b). Bacteriophages As A Reservoir Of Extended-Spectrum B-Lactamase And Fluoroquinolone Resistance Genes In The Environment. *Clin. Microbiol. Infect.* 20, O456–O459. doi: 10.1111/1469-0691.12446

Mocé-Llivina, L., Lucena, F., and Jofre, J. (2005). Enteroviruses And Bacteriophages In Bathing Waters. *Appl. Environ. Microbiol.* 71, 6838–6844. doi: 10.1128/Aem.71.11.6838-6844.2005

Molina, M., Hunter, S., Cyterski, M., Peed, L. A., Kelty, C. A., Sivaganesan, M., et al. (2014). Factors Affecting The Presence Of Human-Associated And Fecal Indicator Real-Time Quantitative Pcr Genetic Markers In Urban-Impacted Recreational Beaches. *Water Res.* 64, 196–208. doi: 10.1016/J.Watres.2014.06.036

Muniesa, M., Colomer-Lluch, M., and Jofre, J. (2013a). Could Bacteriophages Transfer Antibiotic Resistance Genes From Environmental Bacteria To Human-Body Associated Bacterial Populations? *Mob. Genet. Elements* 3:E25847. doi: 10.4161/Mge.25847

Muniesa, M., Colomer-Lluch, M., and Jofre, J. (2013b). Potential Impact Of Environmental Bacteriophages In Spreading Antibiotic Resistance Genes. *Future Microbiol.* 8, 739–751. doi: 10.2217/Fmb.13.32

Nguyen, A. Q., Vu, H. P., Nguyen, L. N., Wang, Q., Djordjevic, S. P., Donner, E., et al. (2021). Monitoring Antibiotic Resistance Genes In Wastewater Treatment: current Strategies And Future Challenges. *Sci. Total Environ.* 783:146964. doi: 10.1016/J.Scitotenv.2021.146964

OECD. (2018). *Stemming The Superbug Tide: just A Few Dollars More*. Paris: oECD Publishing.

Poirel, L., Cattoir, V., and Nordmann, P. (2012). Plasmid-Mediated Quinolone Resistance; Interactions Between Human, Animal, and Environmental Ecologies. *Front. Microbiol.* 3:24. doi: 10.3389/Fmicb.2012.00024

Poirel, L., Rodriguez-Martinez, J. M., Mammeri, H., Liard, A., and Nordmann, P. (2005). Origin Of Plasmid-Mediated Quinolone Resistance Determinant Qnra. *Antimicrob. Agents Chemother.* 49, 3523–3525. doi: 10.1128/Aac.49.8.3523-3525.2005

Quirós, P., Colomer-Lluch, M., Martínez-Castillo, A., Miró, E., Argente, M., Jofre, J., et al. (2014). Antibiotic Resistance Genes In The Bacteriophage Dna Fraction Of Human Fecal Samples. *Antimicrob. Agents Chemother.* 58, 606–609. doi: 10.1128/Aac.01684-13

Reynolds, L. J., Martin, N. A., Sala-Comorera, L., Callanan, K., Doyle, P., O'leary, C., et al. (2021). Identifying Sources Of Faecal Contamination In A Small Urban Stream Catchment: a Multiparametric Approach. *Front. Microbiol.* 12:1580. doi: 10.3389/Fmicb.2021.661954

Reynolds, L. J., Sala-Comorera, L., Martin, N. A., Nolan, T. M., Stephens, J. H., Gitto, A., et al. (2020). Correlation Between Antimicrobial Resistance And Faecal Contamination In Small Urban Streams And Bathing Waters. *Sci. Total Environ.* 739:140242. doi: 10.1016/J.Scitotenv.2020.140242

Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M. C., et al. (2013). Urban Wastewater Treatment Plants As Hotspots For Antibiotic Resistant Bacteria And Genes Spread Into The Environment: a Review. *Sci. Total Environ.* 447, 345–360. doi: 10.1016/J.Scitotenv.2013.01.032

Rodriguez-Mozaz, S., Chamorro, S., Marti, E., Huerta, B., Gros, M., Sànchez-Melsió, A., et al. (2015). Occurrence Of Antibiotics And Antibiotic Resistance Genes In Hospital And Urban Wastewaters And Their Impact On The Receiving River. *Water Res.* 69, 234–242. doi: 10.1016/J.Watres.2014.11.021

Roope, L. S. J., Smith, R. D., Pouwels, K. B., Buchanan, J., Abel, L., Eibich, P., et al. (2019). The Challenge Of Antimicrobial Resistance: what Economics Can Contribute. *Science* 364:eaau4679. doi: 10.1126/Science.Aau4679

Ross, J., and Topp, E. (2015). Abundance Of Antibiotic Resistance Genes In Bacteriophage Following Soil Fertilization With Dairy Manure Or Municipal Biosolids, And Evidence For Potential Transduction. *Appl. Environ. Microbiol.* 81, 7905–7913. doi: 10.1128/Aem.02363-15

Rutledge, R. G. (2003). Mathematics Of Quantitative Kinetic Pcr And The Application Of Standard Curves. *Nucleic Acids Res.* 31:e93. doi: 10.1093/Nar/Gng093

Sala-Comorera, L., Reynolds, L. J., Martin, N. A., O'sullivan, J. J., Meijer, W. G., and Fletcher, N. F. (2021a). Decay Of Infectious Sars-Cov-2 And Surrogates

In Aquatic Environments. *Water Res.* 201:117090. doi: 10.1016/J.Watres.2021.117090

Sala-Comorera, L., Reynolds, L. J., Martin, N. A., Pascual-Benito, M., Stephens, J. H., Nolan, T. M., et al. (2021b). CrAssphage As Human Molecular Marker To Evaluate Temporal And Spatial Variability In Faecal Contamination Of Urban Marine Bathing Waters. *Sci. Total Environ.* 789:147828. doi: 10.1016/J.Scitotenv.2021.147828

Santos, L., and Ramos, F. (2018). Antimicrobial Resistance In Aquaculture: current Knowledge and Alternatives To Tackle The Problem. *Int. J. Antimicrob. Agents* 52, 135–143. doi: 10.1016/J.Ijantimicag.2018.03.010

Stewardson, A. J., Allignol, A., Beyersmann, J., Graves, N., Schumacher, M., Meyer, R., et al. (2016). The Health And Economic Burden Of Bloodstream Infections Caused By Antimicrobial-Susceptible And Non-Susceptible *Enterobacteriaceae* And Staphylococcus Aureus In European Hospitals, 2010 And 2011: a Multicentre Retrospective Cohort Study. *Eurosurveillance* 21:30319. doi: 10.2807/1560-7917.Es.2016.21.33.30319

Sweeney, C. L. (2017). *The Rivers Of Dublin - New Revised Edition*. Dublin: irish Academic Press.

Unc, A., and Goss, M. J. (2004). Transport Of Bacteria From Manure And Protection Of Water Resources. *Appl. Soil Ecol.* 25, 1–18. doi: 10.1016/J.Apsoil.2003.08.007

Van Bruggen, A. H. C., Goss, E. M., Havelaar, A., Van Diepeningen, A. D., Finckh, M. R., and Morris, J. G. (2019). One Health - Cycling Of Diverse Microbial Communities As A Connecting Force For Soil, Plant, Animal, Human And Ecosystem Health. *Sci. Total Environ.* 664, 927–937. doi: 10.1016/J.Scitotenv.2019.02.091

Wang, M., Liu, P., Zhou, Q., Tao, W., Sun, Y., and Zeng, Z. (2018a). Estimating The Contribution Of Bacteriophage To The Dissemination Of Antibiotic Resistance Genes In Pig Feces. *Environ. Pollut.* 238, 291–298. doi: 10.1016/J.Envpol.2018.03.024

Wang, M., Xiong, W., Liu, P., Xie, X., Zeng, J., Sun, Y., et al. (2018b). Metagenomic Insights Into The Contribution Of Phages To Antibiotic Resistance In Water Samples Related To Swine Feedlot Wastewater Treatment. *Front. Microbiol.* 9:2474. doi: 10.3389/Fmicb.2018.02474

WHO (2019). *No Time to Wait: securing The Future From Drug-Resistant Infections*. Switzerland: world Health Organization.

Wu, Z., Greaves, J., Arp, L., Stone, D., and Bibby, K. (2020). Comparative Fate Of Crassphage With Culturable And Molecular Fecal Pollution Indicators During Activated Sludge Wastewater Treatment. *Environ. Int.* 136:105452. doi: 10.1016/J.Envint.2019.105452

Yang, Y., Shi, W., Lu, S. Y., Liu, J., Liang, H., Yang, Y., et al. (2018). Prevalence Of Antibiotic Resistance Genes In Bacteriophage Dna Fraction From Funan River Water In Sichuan. *China. Sci. Total Environ.* 626, 835–841. doi: 10.1016/J.Scitotenv.2018.01.148

Yang, Y., Xing, S., Chen, Y., Wu, R., Wu, Y., Wang, Y., et al. (2021). Profiles Of Bacteria/Phage-Comediated Args In Pig Farm Wastewater Treatment Plants In China: association With Mobile Genetic Elements, Bacterial Communities And Environmental Factors. *J. Hazard. Mater.* 404:124149. doi: 10.1016/J.Jhazmat.2020.124149

Zhang, A., Call, D. R., Besser, T. E., Liu, J., Jones, L., Wang, H., et al. (2019). B-Lactam Resistance Genes In Bacteriophage And Bacterial Dna From Wastewater, River Water, And Irrigation Water In Washington State. *Water Res.* 161, 335–340. doi: 10.1016/J.Watres.2019.06.026

Zhou, P., Yang, X., Lou, Wang, X. G., Hu, B., Zhang, L., et al. (2020). A Pneumonia Outbreak Associated With A New Coronavirus Of Probable Bat Origin. *Nature* 579, 270–273. doi: 10.1038/S41586-020-2012-7

Zieliński, W., Korzeniewska, E., Harnisz, M., Drzymała, J., Felis, E., and Bajkacz, S. (2021). Wastewater Treatment Plants As A Reservoir Of Integrase And Antibiotic Resistance Genes – An Epidemiological Threat To Workers And Environment. *Environ. Int.* 156:106641. doi: 10.1016/J.Envint.2021.106641

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Metagenomic Analysis of Antibiotic Resistance Genes in Untreated Wastewater From Three Different Hospitals

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Controlling antibiotic resistance genes (ARGs) is a worldwide intervention to ensure global health. Hospital wastewater is the main pollution source of antibiotic-resistant bacteria and ARGs in the environment. Expanding our knowledge on the bacterial composition of hospital wastewater could help us to control infections in hospitals and decrease pathogen release into the environment. In this study, a high-throughput sequencing-based metagenomic approach was applied to investigate the community composition of bacteria and ARGs in untreated wastewater from three different types of hospitals [the general hospital, traditional Chinese medicine (TCM) hospital, and stomatology hospital]. In total, 130 phyla and 2,554 genera were identified from the microbiota of the wastewaters, with significantly different bacterial community compositions among the three hospitals. Total ARG analysis using the Antibiotic Resistance Genes Database (ARDB) and Comprehensive Antibiotic Resistance Database (CARD) revealed that the microbiota in the wastewaters from the three hospitals harbored different types and percentage of ARGs, and their composition was specific to the hospital type based on the correlation analysis between species and ARG abundance, some ARGs contributed to different bacterial genera with various relationships in different hospitals. In summary, our findings demonstrated a widespread occurrence of ARGs and ARG-harboring microbiota in untreated wastewaters of different hospitals, suggesting that protection measures should be applied to prevent human infections. Concurrently, hospital wastewater should be treated more specifically for the removal of pathogens before its discharge into the urban sewage system.

Keywords: metagenomic, antibiotic resistance genes, hospital wastewater, microbiota, bacterial pathogens

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INTRODUCTION

Antibiotic resistance poses a serious challenge to the treatment of pathogenic infections. However, antibiotic resistance genes (ARGs) are not only the outcome from human clinic settings, but it can also come from the interaction with animals, plants, soil, sea, and environmental samples. Two-thirds of antibiotics are consumed by animal husbandry (Done et al., 2015), and some were used in crops (Taylor and Reeder, 2020), which increase the ARGs in the environment. This may be relevant to human health interventions on food, water, and sewage. Except for the

precision medication in the clinic with antibiotics, the controlling of ARGs need the social system's efforts in the whole world (Zinsstag et al., 2011; Hernando-Amado et al., 2020). Since the COVID-19 outbreak, people from most countries are still being newly infected, which is recognized in the microbiology world as the concept of all humans sharing "One Health" (Ruckert et al., 2020). As most antibiotic resistance (AR) pathogens are released into the natural ecosystems by humans and animals (Karkman et al., 2019), analysis of ARGs in wastewater from hospitals, farms, and wastewater treatment plants is essential (Karkman et al., 2018; Manyi-Loh et al., 2018; Kayali and Icgen, 2020). In hospital wastewater, the levels of AR are different from those in the natural environment (Rodriguez-Mozaz et al., 2015). Hospital effluents are a mixture of different compounds, including pharmaceuticals, diagnostic agents, disinfectants, and metabolites of these compounds. They are highly hazardous because of their infection rate and toxicity. These wastewaters should be treated to reduce transmission of antibiotic resistance bacteria (ARB) to the ecosystem, which is one type of intervention to control resistance (Petrovich et al., 2020). Another intervention is controlling antimicrobials use, such as selecting novel antimicrobials with limited capacity to ARBs or using fewer antimicrobials. Moreover, traditional Chinese medicines (TCMs) were used in the treatment of infectious diseases to avoid antibiotic resistance (Cai et al., 2017; Su et al., 2020).

Some studies have shown a correlation between antibiotics, ARGs, and antibiotic-resistant bacteria in hospital wastewaters (Lira et al., 2020; Lutterbeck et al., 2020; Baraka et al., 2021). There were positive correlations between selected ARGs (sul1, sul2, tetQ, and qnrS) and the concentrations of certain antibiotics in the wastewaters of five hospitals in Xinjiang, China (Li et al., 2016). There were also correlations between antimicrobial residues and bacterial populations as well as between the prevalence of ARGs and bacterial populations in a wastewater treatment plant system of an urban hospital (Varela et al., 2014). However, the types and concentrations of antibiotics in hospital effluents vary. The categories of drugs administered and the duration of administration also vary depending on the type of hospital. In addition, the consumption of antibiotics was found to be seasonally dependent in one city, with no correlation between the seasonal consumption of antibiotics and the total levels of antibiotics in the city's wastewater (Coutu et al., 2013). By examined hospital effluents from four different types of hospitals (university hospital, general hospital, pediatric hospital, and maternity hospital) and the amount of pharmaceuticals in wastewater varied according to their scale (Santos et al., 2013). In fact, antibiotic usage in the treatment of different clinical departments should vary. For the treatment of odontogenic infections, common antibiotics such as amoxicillin, amoxicillin-clavulanic acid, clindamycin, azithromycin ciprofloxacin, metronidazole, gentamycin, and penicillin are used (Poveda-Roda et al., 2007). However, for the primary health sector, the drugs with high concentrations in wastewater were furosemide, ibuprofen, oxytetracycline, and ciprofloxacin (Stuer-Lauridsen et al., 2000). Thus, these factors may lead to differences in bacterial populations and ARG prevalence in wastewaters.

In this study, we evaluated the wastewaters of three hospitals affiliated with Southwest Medical University: Affiliated Hospital of Southwest Medical University (A group), Affiliated Hospital of TCM (B group), and Affiliated Hospital of Stomatology (C group). As drug usage and treatment regimens were dependent on the diseases being treated and the specialists at each hospital, the bacterial populations were expected to be affected, which would result in a significant difference in the prevalence of ARGs in the microbiome. Thus, we aimed to identify the bacterial community composition and prevalence of ARGs in the wastewaters from these three hospitals using high-throughput sequencing analysis. In addition, we attempted to determine special interventions for the protection against infections and the pretreatment of wastewater before its discharge into sewers, which may help reduce ARGs.

MATERIALS AND METHODS

Sample Collection

From each hospital (A, B, and C groups), 500 ml wastewater was collected on October 12, 21, and 30, 2020, that is total nine samples were collected from the outflow of daily medical applications and stored in sterile 500 ml glass bottles. Microbial samples from the wastewater were collected by filtration using filter membranes (0.2 μm in diameter) and stored in sterilized centrifuge tubes at −80°C. Then, the filter membranes were sent to Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China) for DNA extraction and Illumina HiSeq (pair-end library) sequencing.

DNA Extraction and ARG Detection

Total DNA was extracted from the triplet wastewater samples of the three groups using filter membranes with the MP Fast DNA™ Spin Kit for Soil according to the manufacturer's instructions. The concentration of the extracted DNA was determined by spectrophotometry (TBS-380 followed by NanoDrop 2000). The DNA extract quality was assessed using 1% agarose gel electrophoresis.

Genomic DNA was fragmented to 300 bp (average size) using Covaris M220 (Gene Company Limited, China), and a sequencing library was prepared using NEXTFLEX Rapid DNA-Seq Kit (Bioo Scientific, Austin, TX, United States). Sequencing was performed using Illumina MiSeq (Illumina Inc., San Diego, CA, United States) according to the manufacturer's instructions. Sequence data associated with this project have been deposited in the National Center for Biotechnology Information (NCBI) Short Read Archive database (accession no. PRJNA723368).

Sequence Quality Control and Genome Assembly

SeqPrep was used to merge paired reads from the 3' and 5' ends.[1] Low-quality reads (length < 50 bp, quality value < 20, or

[1]https://github.com/jstjohn/SeqPrep

containing N bases) were removed using Sickle.[2] Metagenomics data were assembled using MEGAHIT (Li et al., 2015) with succinct de Bruijn graphs.[3] A contig with a length of 300 bp or more was selected as the final assembling result and used for further gene prediction and annotation.

Gene Prediction, Taxonomy, and Functional Annotation

Open reading frames (ORFs) from each assembled contig were predicted using MetaGene (Noguchi et al., 2006). Of these predicted ORFs, lengths of over 100 bp were retrieved and translated into amino acid sequences using the NCBI translation table.[4]

We clustered all predicted genes with 95% sequence identity (90% coverage) using CD-HIT (Fu et al., 2012). Among the clusters, the longest sequences were selected as representative sequences to construct a non-redundant (NR) gene catalog. For all samples, the reads with quality control were mapped to the representative sequences with 95% identity using SOAPaligner (Li et al., 2008).[5] The gene abundance was then evaluated.

Data Analysis

The NCBI NR database was used to align the representative sequences of the NR gene catalog for taxonomic annotations, with parameter *e*-values ≤1e-5 using BLASTP (Version 2.2.28+; Altschul et al., 1997).[6] BLASTP against the evolutionary genealogy of genes, Non-supervised Orthologous Groups (eggNOG) database (Version 4.5, *e*-value cutoff of $1e^{-5}$), was used for annotating a cluster of orthologous groups of proteins (COG; Tatusov et al., 2003; Jensen et al., 2008). BLASTP against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Xie et al., 2011; *e*-value cutoff of 1e-5) was used for KEGG annotation.[7] BLASTP against the Antibiotic Resistance Genes Database (ARDB)[8] and Comprehensive Antibiotic Resistance Database (CARD)[9] was used for antibiotic resistance gene annotation (*e*-value cutoff of 1e-5). The sequence identity was ≥90%, and the alignment length was ≥30 amino acids. Other analyses were performed using Cloud Majorbio.[10] Kruskal-Wallis H test, FDR correction, and Tukey-Kramer test were used to analyze the differences among multiple groups. Hierarchical clustering and principal coordinate analysis (PCoA) were also performed with a Bray-Curtis distance matrix using the R software package. A value of $p < 0.05$ was considered statistically significant. A co-occurrence network was employed to visualize the correlation between antibiotic types and microbial taxa. A connection indicated a strong ($\rho > 0.5$) and significant (value of $p < 0.05$) Spearman's correlation.

[2]https://github.com/najoshi/sickle
[3]https://github.com/voutcn/megahit
[4]http://www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome.html/index.cgi?chapter=tgencodes#SG1
[5]https://github.com/ShujiaHuang/SOAPaligner
[6]http://blast.ncbi.nlm.nih.gov/Blast.cgi
[7]https://www.genome.jp/kegg/
[8]http://ardb.cbcb.umd.edu/
[9]https://card.mcmaster.ca/
[10]https://cloud.majorbio.com/

RESULTS

Overview of Assembly and Annotation

In total, 847, 368, 612 clean reads were generated, with an average of 94, 152, 068 reads per sample. The statistical information of the contigs is listed in **Supplementary Table S1**. For each DNA dataset, annotation of the protein-coding genes was performed using a BLASTP search against the eggNOG database. Metagenomic assembly, annotations, and predicted ORFs are listed in **Supplementary Table S2**. The gene sequences predicted by the samples were clustered using CD-HIT to construct the NR gene catalog, and the base sequences of the genes in the non-redundant gene catalog were obtained; details are listed in **Supplementary Table S3**.

Bacterial Community Characteristics in Wastewaters From Three Different Hospitals

The bacterial community composition in the wastewaters was determined by the corresponding species and their taxonomic annotation information compared with the NCBI NR database. As shown in **Figure 1A**, there was no difference between the three samples of wastewater collected on different days from each group at the phylum level, indicating that these samples are generally representative of the community abundance of bacteria in the wastewater.

In total, 130 phyla and 2,554 genera were identified from the microbiota of the wastewaters. When comparing the community abundance of bacteria at the phylum level in the wastewaters of the different hospitals, Proteobacteria was the dominant phylum in the C group (89.35–90.03%), while the A and B groups exhibited a relatively low abundance of this phylum (53.42–58.00 and 18.07–24.44%, respectively). For the B group, Firmicutes were the most abundant phylum (49.58–53.44%), while the abundance of this phylum was low in both A and C groups (14.20–20.44 and 0.87–1.44%, respectively). When evaluating data at the genus level, the composition of the microbiota was also different between the three groups (**Figure 1B**). The most abundant genus was *Acinetobacter* (7.80–18.87%) in the A group, *Streptococcus* (6.98–13.78%) in the B group, and *Arcobacter* (14.28–47.90%) in the C group.

There was a significant difference at the phylum and genus levels between the hospitals. Samples from the A and B groups were from general hospitals that treated similar diseases but used different therapeutic regimens and drugs. Thus, these factors resulted in similar bacteria in the wastewaters but significantly different community compositions. Moreover, comparing samples from the A and B groups with the ones from the C group, a significant difference in community composition was observed. The C group hospital specializes in oral diseases, and the pharmaceuticals used for treatments here led to a less diverse bacterial community in the wastewater compared with the bacterial communities of the two general hospitals.

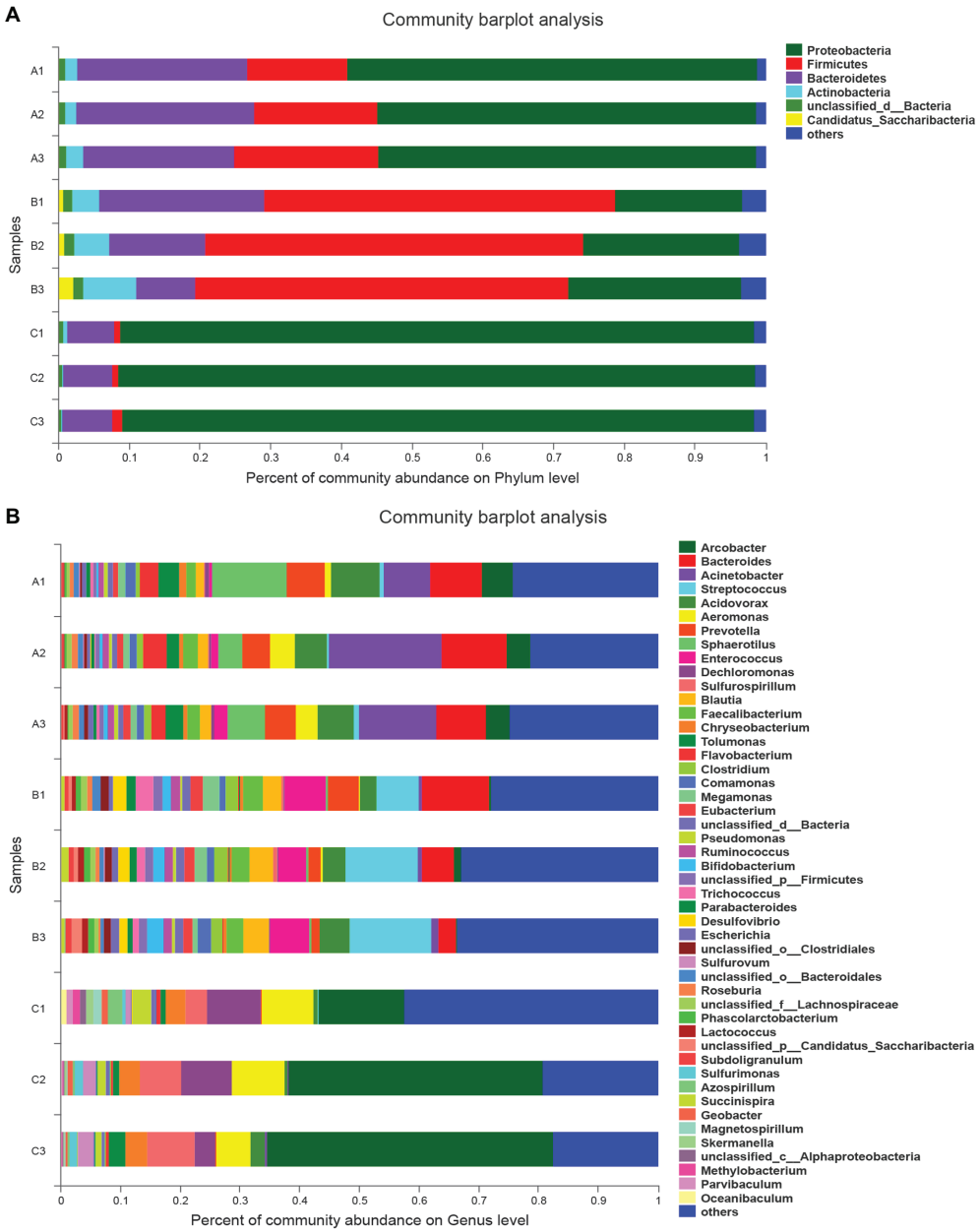

FIGURE 1 | Percent of community abundance of microbiota on Phylum and Genus level in wastewater from hospitals. **(A)** Percent of community abundance on Phylum level; **(B)** Percent of community abundance on Genus level. A group (A1, A2, and A3): wastewater from Affiliated Hospital of Southwest Medical University, B group (B1, B2, and B3): wastewater from Affiliated Traditional Chinese Medicine (TCM) Hospital of Southwest Medical University, C group (C1, C2, and C3): wastewater from Hospital of Stomatology Southwest Medical University.

To verify the differences in the bacterial community at the phylum and genus levels, the Kruskal-Wallis H test was used to analyze the main community abundance. The data are shown in **Figure 2A**. A significant number of sequences affiliated with Proteobacteria and Firmicutes were found in the wastewaters from the three hospitals ($p < 0.05$; **Figure 2A**). The mean proportions differed significantly for nine genera ($p < 0.05$; **Figure 2B**); only *Bacteroides* was not statistically significant. Notably, the composition of *Bacteroides* in the samples from all three hospitals was different, contributing 24.05% of the total microbiome in the A group, 15.43% in the B group, and 6.86% in the C group. According to the cluster tree analysis (**Figure 2C**), the microbiota composition structure was significantly different among the three groups, although, the A and B groups were more similar to each other compared to the C group. The PCoA separated the samples far from the central parallel axis 1 (PC1, 68.12%), indicating that the gene compositions of the wastewaters from these three hospitals also differed significantly (**Figure 2D**).

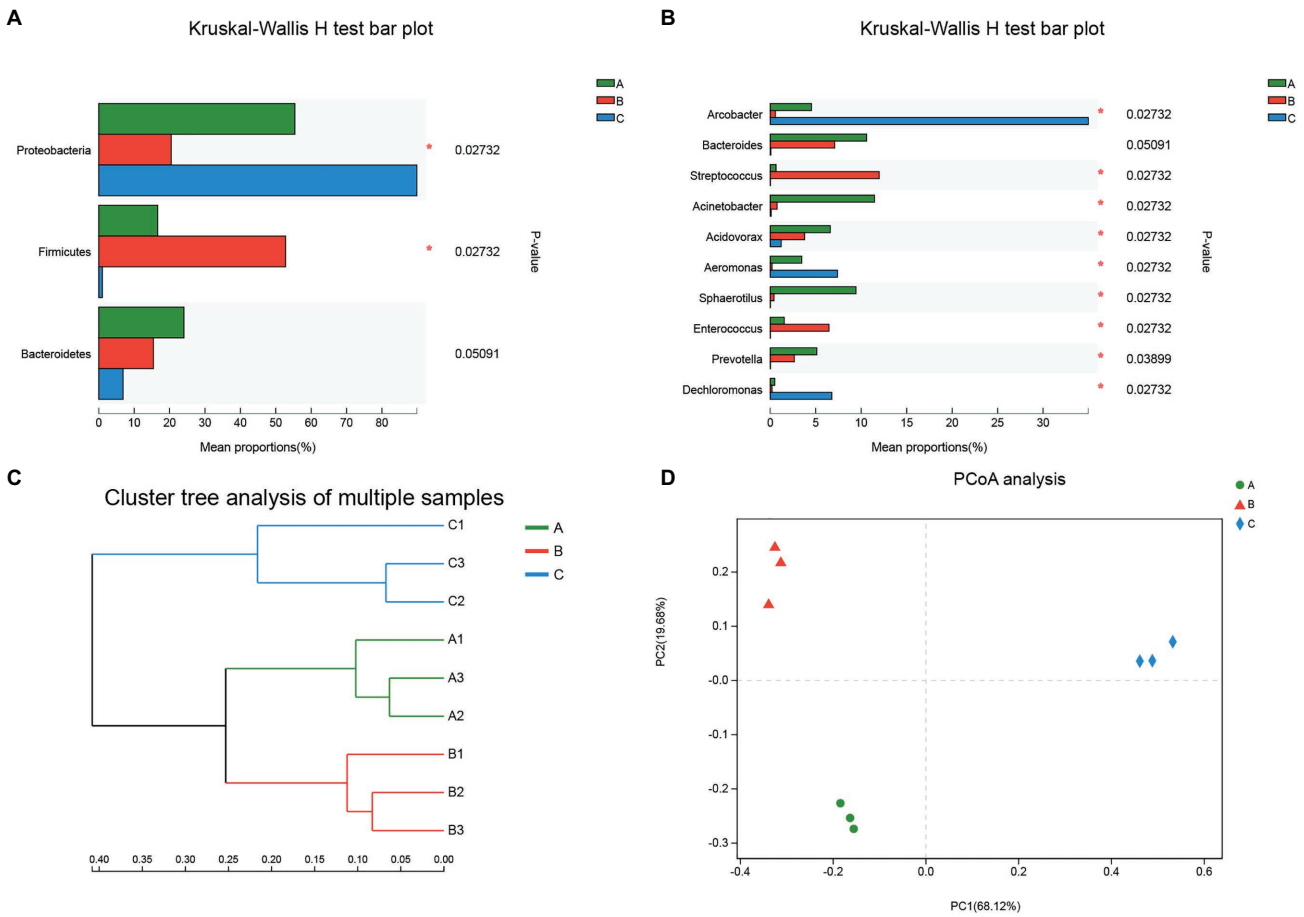

FIGURE 2 | Statistical comparison of the relative abundance. **(A,B)** Microbiota composition difference in Phylum and Genus level; **(C,D)** Relationship of microbiota community between three groups analyzed on cluster tree and principal coordinate analysis (PCoA). Green (bar, line, or circle), A group, samples from Affiliated Hospital of Southwest Medical University; Red (bar, line, or triangle), B group, samples from Affiliated TCM Hospital of Southwest Medical University; Blur (bar, line, or diamond), C group, samples from Hospital of Stomatology Southwest Medical University. Differences were considered statistically significant at * $p < 0.05$ level.

Occurrence of ARGs in Hospital Wastewater Analyzed With ARDB

Using the ARDB, 34 types of ARGs were detected in the wastewaters of the three hospitals wastewater. As shown in **Figure 3A**, ARGs associated with bacitracin were the most abundant resistance genes in the wastewaters from the A and B groups, while the ARGs associated with tetracycline were the most abundant in the wastewater of the C group. The community abundance of ARGs was significantly different in the PCoA analysis; the points of these three groups were located in different quadrants (**Figure 3B**). Depending on the type of ARGs, the diversity of ARGs in the C group was markedly lower than that in the other two groups. There were 438, 474, and 212 types of ARGs in the wastewaters of the A, B, and C groups, respectively.

To verify the community prevalence of general ARGs in hospital wastewater, we used the Kruskal-Wallis H test to analyze the composition of general ARGs. Of the 15 main composition types of ARGs, six types differed in the wastewaters of the three groups (**Figure 3C**; $p < 0.05$). Among the antibiotic-relevant resistant genes, the three most abundant ARGs were associated with bacitracin, tetracycline, and tobramycin. The pairwise comparison of the three most abundant ARGs indicated differences between each two hospitals (**Figures 3D–F**), with the abundance of ARGs for bacitracin being statistically significant ($p < 0.01$). There were no significant differences in the abundance of tetracycline resistance genes among the samples from the three hospitals. The percentage of ARGs associated with tobramycin differed between the A and C groups, while no significant difference was observed between the A and B or B and C groups.

Occurrence of Total Genes of Bacteria Analyzed With Card

The community abundance of bacterial comprehensive antibiotic resistance genes in the wastewater analyzed with CARD differed between the three groups (**Figure 4A**). The percentage of AR genes were the most prevalent class of genes among the wastewaters of the three hospitals, and the composition of antibiotic sensitive (AS) genes and antibiotic target (AT) genes varied. According to the PCoA of the bacterial community

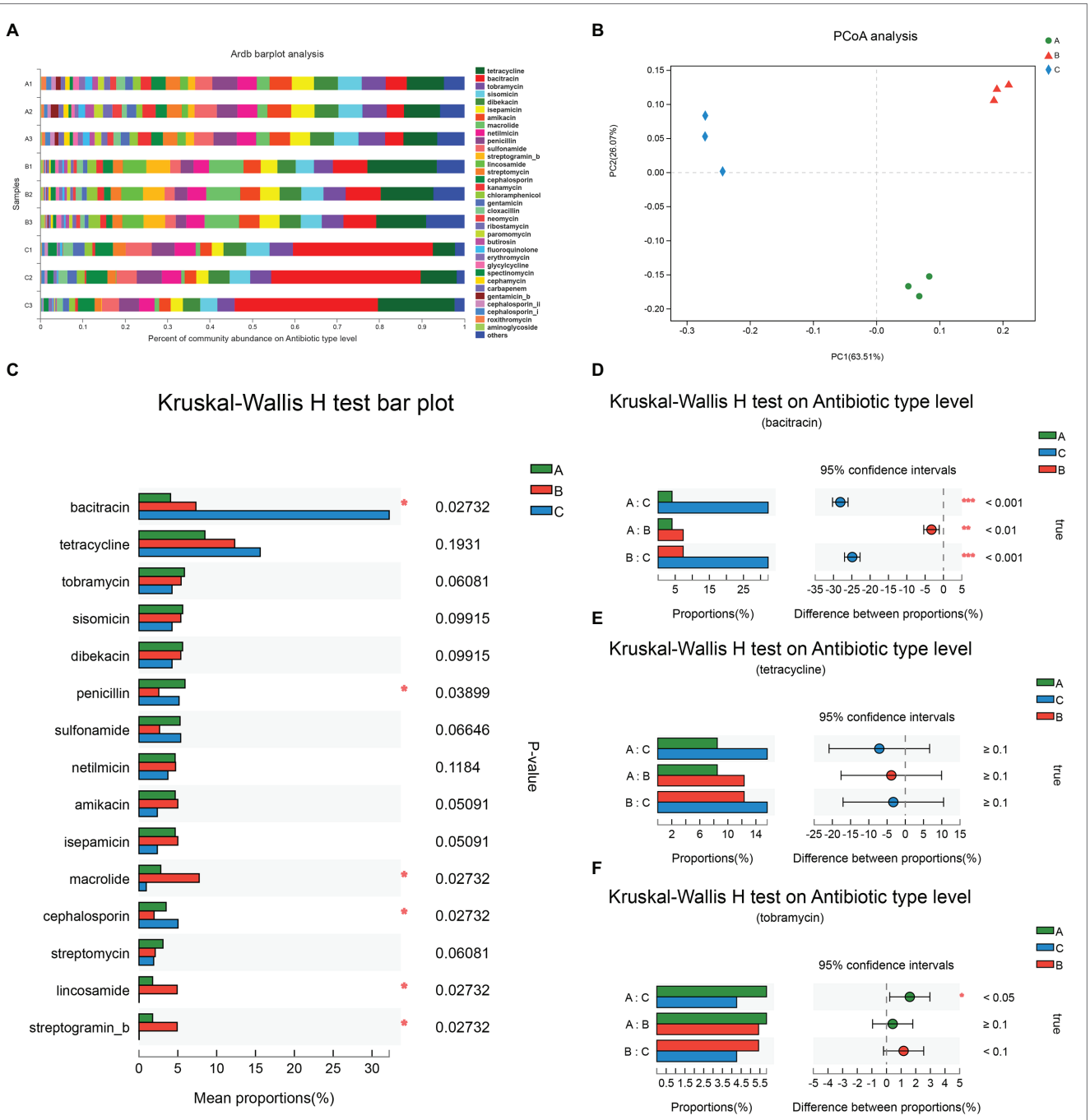

FIGURE 3 | Community abundance of antibiotic resistance genes (ARGs) and statistical comparison on antibiotic type level. **(A)** Percentage of community abundance on antibiotic type level; **(B)** PCoA analysis of community on antibiotic type level; **(C)** The proportions of ARGs in three groups; **(D)** The composition difference of anti-bacitracin genes in pairwise comparison; **(E)** The composition difference of anti-tetracycline genes in pairwise comparison; and **(F)** The composition difference of anti-tobramycin genes in pairwise comparison. Differences were considered statistically significant at $^{*}p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$ level. A group, samples from Affiliated Hospital of Southwest Medical University; Red triangle, B group, samples from Affiliated TCM Hospital of Southwest Medical University; Blur diamond, C group, samples from Hospital of Stomatology Southwest Medical University.

gene class type for the three groups, the points were located in different quadrants and far from the central parallel axis 1 (PC1, 85.79%; **Figure 4B**).

An in-depth analysis of the various class types was analyzed using the Kruskal-Wallis H test. As shown in **Figure 4C**, the proportion of AR, AS, and AT classes in the wastewaters from the three hospitals varied ($p < 0.05$). The differences in comprehensive antibiotic resistance genes for each of the pairwise comparisons at AR, AS, and AT class are shown in **Figures 4D–F**.

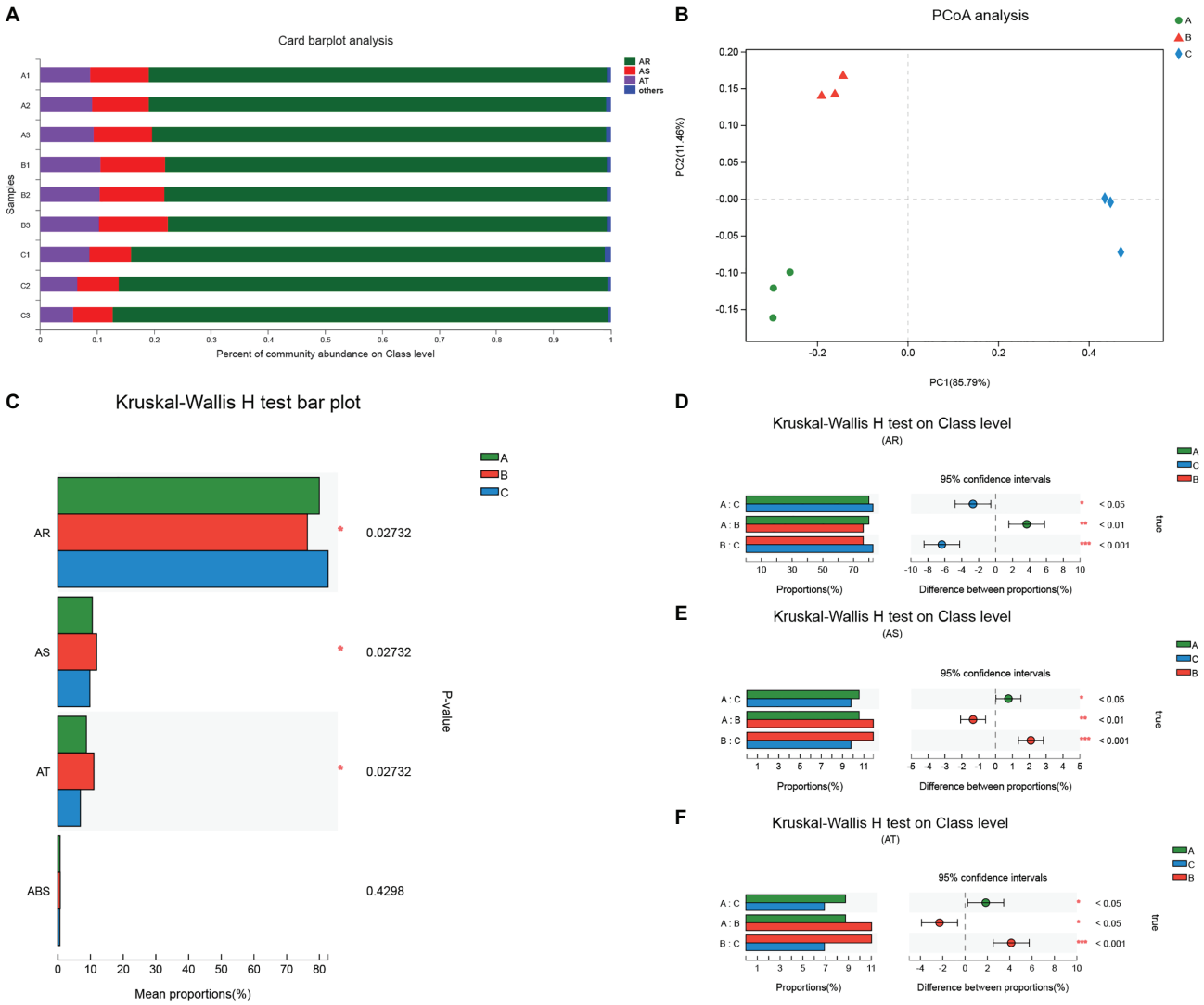

FIGURE 4 | Community abundance of total ARGs and statistical comparison on class type level. **(A)** Percentage of community abundance on antibiotic class type level; **(B)** PCoA analysis of community on antibiotic type level; **(C)** The difference of proportions of ARGs on class level compared in three groups; **(D)** The composition difference of ARGs in pairwise comparison; **(E)** The composition difference of antibiotic sensitive (AS) genes in pairwise comparison; and **(F)** The composition difference of antibiotic target (AT) genes in pairwise comparison. Differences were considered statistically significant at $^{*}p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$ level.

Correlation Analysis Between Species and ARGs Abundance

The possible correlation between ARG types and bacterial genera was assessed under antibiotic type level. As shown in **Figure 5A**, *Aeromonas* contribute 14.50% for tetracycli ARG in C hospital wastewater, while *Enterococcus*, *Bacteroides*, *Streptococcus*, and *Acinetobacter* provided tetracycli ARG with different relative contributions in A and B groups. For bacitracin ARG, several types of bacteria were correlated with different relative abundances, while *Arcobacter* contributed with 47.49% in the C group hospital. *Enterococcus* contributed more than 50% for the tobramycin and sisomicin ARG type in B and C groups, while it contributed 12.80 and 13.31% for tobramycin and sisomicin ARG types in A and B groups, respectively; and they were both only 0.85% in C group. These results indicated that to reduce one type of ARGs in hospital wastewater, different types of bacterial species should be considered.

Then, a network analysis approach was applied to explore the correlation between ARG types and microbial taxa on antibiotic type level (**Figure 5B**). There were 10 antibiotic types ARGs co-occurring with nine genera of bacteria. *Acinetobacter* was the possible host positively associated with six types of ARGs: amikacin, isepamicin, sulforamide, tobramycin, sisomicin, and netilmicin. *Acidovorax* was positively correlated with seven types of ARGs: amikacin, tobramycin, sulfonamide, netilmicin, sisomicin, dibekacin, and isepamicin. In addition, there were positive correlations between six ARGs types and *Bacteroides*.

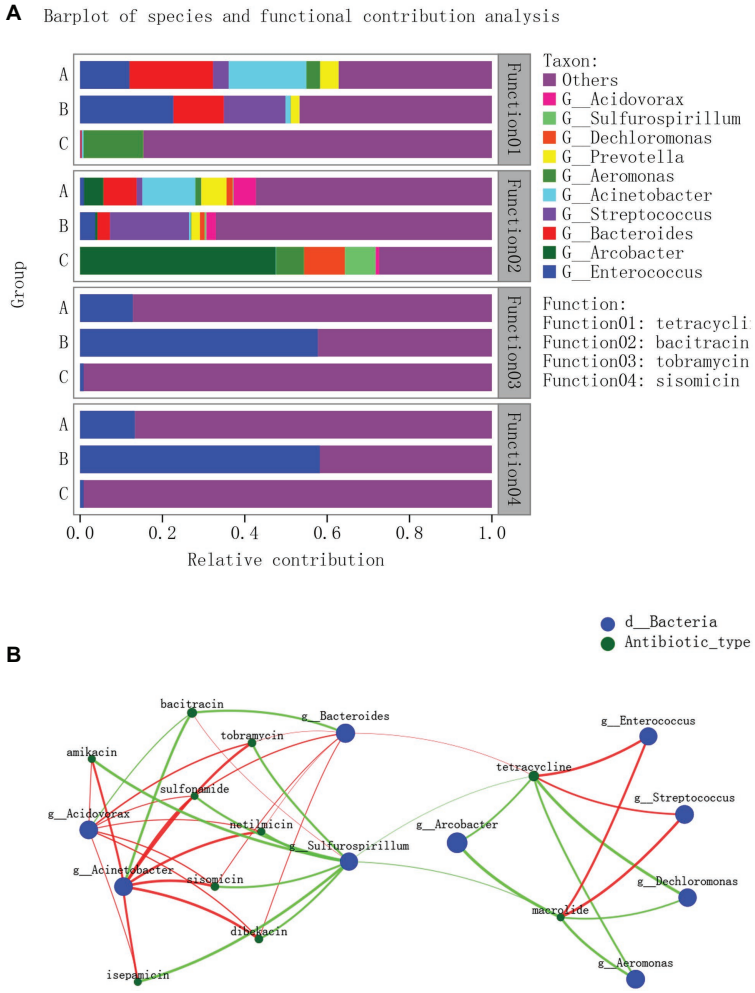

FIGURE 5 | Correlations between species and corresponding ARGs. **(A)** Correlation of microbiota genus and ARGs relative antibiotics type; **(B)** Correlation of microbiota genus and comprehensive ARGs on antibiotic type level. The nodes were colored according to ARG types and genus. A connection represents a strong Spearman's correlation coefficient ($\rho > 0.5$) and significant (value of $p < 0.05$) correlation. The color of the line indicates positive and negative correlation, red indicates positive correlation, and green indicates negative correlation.

Enterococcus and *Streptococcus* were found to be host of tetracycline and macrolide ARGs.

DISCUSSION

Hospital wastewater contains high concentrations of antibiotics and several types of pathogenic bacteria, which are the main source of antibiotic-resistant bacteria and ARGs (Hassoun-Kheir et al., 2020). In this study, we attempted to elucidate the differences in the composition of bacteria in wastewaters from three university-affiliated hospitals in Luzhou city, Sichuan Province, PR China, using high-throughput sequencing analysis. These three hospitals consisted of one general hospital, one general TCM hospital, and one stomatological hospital. By comparing the corresponding species and taxonomic annotation information with the NCBI NR database, our results showed that the community composition of bacteria in the wastewaters was different between hospitals at both the phyla and genera levels with significantly different communities of bacteria in each of them (**Figures 1, 2**). In the wastewater from three comprehensive hospitals with general departments, the community compositions of bacteria were more similar, *Arcobacter*, *Acinetobacter*, and *Dechloromonas* phyla were predominant with different relative abundances (Wang et al., 2018). The bacterial community composition of wastewater from the oncological hospital have shown a higher difference compared to the general hospital (Szekeres et al., 2017). The cluster tree and PCoA analysis for the relationship of community abundance between the three groups in this study also were similar to these results. The composition of bacteria in the wastewaters of the A and B groups was more similar to each other than to that of the C group. This may have occurred because both the A and B groups covered general medical practice, while the C group only focused on stomatology. Herein, relative

protection should be applied as a measure to avoid the spread of infections according to the different bacterial compositions in the wastewater of the hospital. For example, *Arcobacter* was the main contributor to the wastewater from the C group; thus, more resources and attention should be focused on preventing infections and treating the wastewater specifically against *Arcobacter*. In addition, wastewater pretreatments can be targeted to harmful bacteria considering their properties. As Firmicutes live in an anaerobic environment and also are associated with high antibiotic and pollutant levels (Li et al., 2011; Bäumlisberger et al., 2015), monitoring the condition of wastewater may control the abundance of bacteria of this phylum.

Currently, most wastewaters are treated with sewage processing techniques before discharging into the urban sewage, which can remove some antibiotics resistance bacteria (Huang et al., 2012; Majeed et al., 2021). Yet, it still contains higher ARGs than natural water, and one of the main sources of ARGs is hospital effluent (Verlicchi et al., 2010). There were fewer types of ARGs in the wastewater from specialized hospitals compared with general hospitals (Szekeres et al., 2017). In this study, the ARGs in wastewaters from three hospitals were analyzed using the ARDB and CARD. As shown in **Figure 3**, the diversity of the ARGs was the lowest for the C group, however, the ARGs types and percentages in the wastewaters were different between the three groups. Among the 15 main types of ARGs, six types differed in the wastewaters of the three hospitals. For the common antibiotics (bacitracin, tetracycline, and tobramycin), there were significant differences in the levels of bacitracin ARGs among the three groups. In addition, the abundance of bacitracin-resistant bacteria in the C group was significantly more than that in the other two groups. Thus, disinfection and preventive measures used in hospitals that specialize in stomatology should target these specific bacteria.

For the total antibiotic resistance gene analysis using the CARD, the most common class of genes in wastewater from the three hospitals was AR, and together with the other two classes, AS and AT, their compositions were varied in three groups. This result is consistent with the findings of the bacterial community characteristic and ARG type analyses (**Figures 2D, 3B**).

In addition to the correlation between ARG types and bacteria genera, the results indicated that the same ARGs contributed to different bacterial genera with various relatives in the wastewater from three groups. For example, *Enterococcus*, *Bacteroides*, *Streptococcus*, and *Acinetobacter* contributed to tetracycli ARGs in A and B groups, while it comes from *Aeromonas* in the C group. This means that even for the treatment of the same resistance gene, the unique characteristics of species in wastewater should be considered according to different hospitals.

In conclusion, we used high-throughput sequencing to analyze bacterial community composition and ARGs in untreated wastewater samples collected from three university-affiliated hospitals. We found that significant differences in the bacterial community characteristics and ARG composition among the three hospitals and they differed between the types of hospital. Based on the differences in the bacterial communities and ARG compositions between the three types of hospitals, our results suggested that targeted prevention and control measures against related microbiota should be considered, and hospital wastewaters should be treated more specifically for pathogens that are present in it before the discharge into the urban sewage system. However, the wastewater samples were collected in the same season, which cannot cover the bacteria composition during the rest of the year, thus more samples from different seasons may give more information. Otherwise, there were no data about the antibiotic types in raw hospital wastewater, though it gave a negative correlation between the concentrations of antibiotics and ARGs (Wang et al., 2018). Another limitation of this study was that there was no physiochemical analysis for the raw wastewater, which may be affected by the seasons or other environmental conditions. This should be considered in future studies on hospital wastewater. Moreover, special techniques for preventing pathogen infection and release need to be identified according to the medical treatments being offered in a hospital.

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/, PRJNA723368.

AUTHOR CONTRIBUTIONS

CS conceived and designed the experiments. NT collected and processed the samples from the hospital wastewater system. CS and XG analyzed the metagenomics sequence data, created the figures, and wrote the manuscript. HL and LL helped to plan the project and contributed to development of the manuscript. QF and QZ assisted with sample collection and DNA extraction. 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.2021.709051/full#supplementary-material

REFERENCES

Altschul, S. F., Madden, T. L., Schffer, A. A., Zhang, J., Zhang, Z., Webb, M., et al. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25, 3389–3402. doi: 10.1093/nar/25.17.3389

Baraka, A., Traglia, G. M., Montaña, S., Tolmasky, M. E., and Ramirez, M. S. (2021). An acinetobacter non-baumannii population study: antimicrobial resistance genes (ARGs). *Antibiotics* 10:16. doi: 10.3390/antibiotics10010016

Bäumlisberger, M., Youssar, L., Schilhabel, M. B., and Jonas, D. (2015). Influence of a nonhospital medical care facility on antimicrobial resistance in wastewater. *PLoS One* 10:e0122635. doi: 10.1371/journal.pone.0122635

Cai, Y., Zhang, Q., Fu, Y., Li, L., Zhao, N., Lu, A., et al. (2017). Effectiveness of chinese herbal medicine combined with antibiotics for extensively drug-resistant enterobacteria and nonfermentative bacteria infection: real-life experience in a retrospective cohort. *Biomed Res. Int.* 2017:2897045. doi: 10.1155/2017/2897045

Coutu, S., Rossi, L., Barry, D. A., Rudaz, S., and Vernaz, N. (2013). Temporal variability of antibiotics fluxes in wastewater and contribution from hospitals. *PLoS One* 8:e53592. doi: 10.1371/journal.pone.0053592

Done, H. Y., Venkatesan, A. K., and Halden, R. U. (2015). Does the recent growth of aquaculture create antibiotic resistance threats different from those associated with land animal production in agriculture? *AAPS J.* 17, 513–524. doi: 10.1208/s12248-015-9722-z

Fu, L., Niu, B., Zhu, Z., Wu, S., and Li, W. (2012). CD-HIT: accelerated for clustering the next-generation sequencing data. *Bioinformatics* 28, 3150–3152. doi: 10.1093/bioinformatics/bts565

Hassoun-Kheir, N., Stabholtz, Y., Kreft, J.-U., de la Cruz, R., Romalde, J. L., Nesme, J., et al. (2020). Comparison of antibiotic-resistant bacteria and antibiotic resistance genes abundance in hospital and community wastewater: a systematic review. *Sci. Total Environ.* 743:140804. doi: 10.1016/j.scitotenv.2020.140804

Hernando-Amado, S., Coque, T. M., Baquero, F., and Martínez, J. L. (2020). Antibiotic resistance: moving from individual health norms to social norms in one health and global health. *Front. Microbiol.* 11:1914. doi: 10.3389/fmicb.2020.01914

Huang, J. J., Hu, H. Y., Lu, S. Q., Li, Y., Tang, F., Lu, Y., et al. (2012). Monitoring and evaluation of antibiotic-resistant bacteria at a municipal wastewater treatment plant in China. *Environ. Int.* 42, 31–36. doi: 10.1016/j.envint.2011.03.001

Jensen, L. J., Julien, P., Kuhn, M., von Mering, C., Muller, J., Doerks, T., et al. (2008). eggNOG: automated construction and annotation of orthologous groups of genes. *Nucleic Acids Res.* 36, D250–D254. doi: 10.1093/nar/gkm796

Karkman, A., Do, T. T., Walsh, F., and Virta, M. P. J. (2018). Antibiotic-resistance genes in waste water. *Trends Microbiol.* 26, 220–228. doi: 10.1016/j.tim.2017.09.005

Karkman, A., Parnanen, K., and Larsson, D. G. J. (2019). Fecal pollution can explain antibiotic resistance gene abundances in anthropogenically impacted environments. *Nat. Commun.* 10:80. doi: 10.1038/s41467-018-07992-3

Kayali, O., and Icgen, B. (2020). Untreated HWWs emerged as hotpots for ARGs. *Bull. Environ. Contam. Toxicol.* 104, 386–392. doi: 10.1007/s00128-020-02792-2

Li, R., Li, Y., Kristiansen, K., and Wang, J. (2008). SOAP: short oligonucleotide alignment program. *Bioinformatics* 24, 713–714. doi: 10.1093/bioinformatics/btn025

Li, D., Liu, C. M., Luo, R., Kunihiko, S., and Tak-Wah, L. (2015). MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. *Bioinformatics* 31, 1674–1676. doi: 10.1093/bioinformatics/btv033

Li, C., Lu, J., Liu, J., Zhang, G., Tong, Y., and Ma, N. (2016). Exploring the correlations between antibiotics and antibiotic resistance genes in the wastewater treatment plants of hospitals in Xinjiang, China. *Environ. Sci. Pollut. Res.* 23, 15111–15121. doi: 10.1007/s11356-016-6688-z

Li, D., Qi, R., Yang, M., Zhang, Y., and Yu, T. (2011). Bacterial community characteristics under long-term antibiotic selection pressures. *Water Res.* 45, 6063–6073. doi: 10.1016/j.watres.2011.09.002

Lira, F., Vaz-Moreira, I., Tamames, J., Manaia, C. M., and Martínez, J. L. (2020). Metagenomic analysis of an urban resistome before and after wastewater treatment. *Sci. Rep.* 10:8174. doi: 10.1038/s41598-020-65031-y

Lutterbeck, C. A., Colares, G. S., Dell'Osbel, N., Silva, F. P., Kist, L. T., and Machado, Ê. L. (2020). Hospital laundry wastewaters: a review on treatment alternatives, life cycle assessment and prognosis scenarios. *J. Clean. Prod.* 273:122851. doi: 10.1016/j.jclepro.2020.122851

Majeed, H. J., Riquelme, M. V., Davis, B. C., Gupta, S., Angeles, L., Aga, S., et al. (2021). Evaluation of metagenomic-enabled antibiotic resistance surveillance at a conventional wastewater treatment plant. *Front. Microbiol.* 12:657954. doi: 10.3389/fmicb.2021.657954

Manyi-Loh, C., Mamphweli, S., Meyer, E., and Okoh, A. (2018). Antibiotic use in agriculture and its consequential resistance in environmental sources: potential public health implications. *Molecules* 23:795. doi: 10.3390/molecules23040795

Noguchi, H., Park, J., and Takagi, T. (2006). Metagene: prokaryotic gene finding from environmental genome shotgun sequences. *Nucleic Acids Res.* 34, 5623–5630. doi: 10.1093/nar/gkl723

Petrovich, M. L., Zilberman, A., Kaplan, A., Eliraz, G. R., Wang, Y., Langenfeld, K., et al. (2020). Microbial and viral communities and their antibiotic resistance genes throughout a hospital wastewater treatment system. *Front. Microbiol.* 11:153. doi: 10.3389/fmicb.2020.00153

Poveda-Roda, R., Bagán, J. V., Sanchis-Bielsa, J. M., and Carbonell-Pastor, E. (2007). Antibiotic use in dental practice. A review. *Med. Oral Patol. Oral Cir. Bucal.* 12, E186–E192.

Rodriguez-Mozaz, S., Chamorro, S., Marti, E., Huerta, B., Gros, M., Sànchez-Melsió, A., et al. (2015). Occurrence of antibiotics and antibiotic resistance genes in hospital and urban wastewaters and their impact on the receiving river. *Water Res.* 69, 234–242. doi: 10.1016/j.watres.2014.11.021

Ruckert, A., Zinszer, K., Zarowsky, C., Zarowsky, C., Labonté, R., and Carabin, H. (2020). What role for one health in the COVID-19 pandemic? *Can. J. Public Health* 111, 641–644. doi: 10.17269/s41997-020-00409-z

Santos, L. H. M. L. M., Gros, M., Rodriguez-Mozaz, S., Delerue-Matos, C., Pena, A., Barceló, D., et al. (2013). Contribution of hospital effluents to the load of pharmaceuticals in urban wastewaters: identification of ecologically relevant pharmaceuticals. *Sci. Total Environ.* 461–462, 302–316. doi: 10.1016/j.scitotenv.2013.04.077

Stuer-Lauridsen, F., Birkved, M., Hansen, L. P., Lützhøft, H.-C., and Halling-Sørensen, B. (2000). Environmental risk assessment of human pharmaceuticals in Denmark after normal therapeutic use. *Chemosphere* 40, 783–793. doi: 10.1016/S0045-6535(99)00453-1

Su, T., Qiu, Y., Hua, X., Ye, B., Luo, H., Liu, D., et al. (2020). Novel opportunity to reverse antibiotic resistance: to explore traditional Chinese medicine with potential activity against antibiotics-resistance bacteria. *Front. Microbiol.* 11:610070. doi: 10.3389/fmicb.2020.610070

Szekeres, E., Baricz, A., Chiriac, C. M., Farkas, A., Opris, O., Soran, M.-L., et al. (2017). Abundance of antibiotics, antibiotic resistance genes and bacterial community composition in wastewater effluents from different Romanian hospitals. *Environ. Pollut.* 225, 304–315. doi: 10.1016/j.envpol.2017.01.054

Tatusov, R. L., Fedorova, N. D., Jackson, J. D., Jacobs, A. R., Kiryutin, B., Koonin, E. V., et al. (2003). The COG database: an updated version includes eukaryotes. *BMC Bioinformatics* 4:41. doi: 10.1186/1471-2105-4-41

Taylor, P., and Reeder, R. (2020). Antibiotic use on crops in low and middle-income countries based on recommendations made by agricultural advisors. *CABI Agric. Biosci.* 1:1. doi: 10.1186/s43170-020-00001-y

Varela, A. R., André, S., Nunes, O. C., and Manaia, C. M. (2014). Insights into the relationship between antimicrobial residues and bacterial populations in a hospital-urban wastewater treatment plant system. *Water Res.* 54, 327–336. doi: 10.1016/j.watres.2014.02.003

Verlicchi, P., Galletti, A., Petrovic, M., and BarcelÓ, D. (2010). Hospital effluents as a source of emerging pollutants: an overview of micropollutants and sustainable treatment options. *J. Hydrol.* 389, 416–428. doi: 10.1016/j.jhydrol.2010.06.005

Wang, Q., Wang, P., and Yang, Q. (2018). Occurrence and diversity of antibiotic resistance in untreated hospital wastewater. *Sci. Total Environ.* 621, 990–999. doi: 10.1016/j.scitotenv.2017.10.128

Xie, C., Mao, X., Huang, J., Ding, Y., Wu, J., Dong, S., et al. (2011). KOBAS 2.0: a web server for annotation and identification of enriched pathways and diseases. *Nucleic Acids Res.* 39, W316–W322. doi: 10.1093/nar/gkr483

Zinsstag, J., Schelling, E., Waltner-Toews, D., and Tanner, M. (2011). From "one medicine" to "one health" and systemic approaches to

health and well-being. *Prev. Vet. Med.* 101, 148–156. doi: 10.1016/j.prevetmed.2010.07.003

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Genomic Analysis of Carbapenem-Resistant *Pseudomonas aeruginosa* Isolated From Urban Rivers Confirms Spread of Clone Sequence Type 277 Carrying Broad Resistome and Virulome Beyond the Hospital

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The dissemination of antibiotic-resistant priority pathogens beyond hospital settings is both a public health and an environmental problem. In this regard, high-risk clones exhibiting a multidrug-resistant (MDR) or extensively drug-resistant (XDR) phenotype have shown rapid adaptation at the human-animal-environment interface. In this study, we report genomic data and the virulence potential of the carbapenemase, São Paulo metallo-β-lactamase (SPM-1)-producing *Pseudomonas aeruginosa* strains (Pa19 and Pa151) isolated from polluted urban rivers, in Brazil. Bioinformatic analysis revealed a wide resistome to clinically relevant antibiotics (carbapenems, aminoglycosides, fosfomycin, sulfonamides, phenicols, and fluoroquinolones), biocides (quaternary ammonium compounds) and heavy metals (copper), whereas the presence of exotoxin A, alginate, quorum sensing, types II, III, and IV secretion systems, colicin, and pyocin encoding virulence genes was associated with a highly virulent behavior in the *Galleria mellonella* infection model. These results confirm the spread of healthcare-associated critical-priority *P. aeruginosa* belonging to the MDR sequence type 277 (ST277) clone beyond the hospital, highlighting that the presence of these pathogens in environmental water samples can have clinical implications for humans and other animals.

Keywords: critical-priority pathogens, aquatic environments, carbapenemase, *Galleria mellonella*, resistome, virulome, genomic surveillance, One Health

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INTRODUCTION

Carbapenem-resistant *Pseudomonas aeruginosa* are a leading cause of hospital-acquired infections and have become a health priority (Tacconelli et al., 2018). Efforts have been made to prevent colonization, infection, and decrease mortality. Based on that, the WHO proposed a global priority pathogen list of multidrug-resistant (MDR) bacteria to drive research, discovery, and development of new antibiotics. Along with MDR *P. aeruginosa*, the critical pathogens WHO list included *Acinetobacter baumannii* and bacteria from Enterobacterales group (Tacconelli et al., 2018). They were categorized as critical priority through the use of multi-criteria, including being resistant to a large number of antibiotics, such as carbapenems and third generation cephalosporins, the best available options for treating MDR pathogens (Babu et al., 2020). Worryingly, carbapenem-resistant *P. aeruginosa* can cause severe and often deadly infections such as bloodstream infections, pneumonia, and osteomyelitis (Fernández-Barat et al., 2017; Pliska, 2020; Jean et al., 2020; Bobrov et al., 2021; Rosales-Reyes et al., 2021). Carbapenem resistance is usually multifactorial, including overexpression of efflux pumps (i.e., *mexAB-oprM*), deficiency or repression of the porin gene (*oprD*), alterations in the penicillin-binding proteins (PBPs), and chromosomal overexpression of cephalosporinase gene *ampC* (Van Nguyen et al., 2018; Gajdács, 2020; Xu et al., 2020). Moreover, resistance may be acquired by the selection of mutations in chromosomal genes or horizontal uptake of resistance determinants. However, carbapenem resistance has been most associated with production of carbapenemases, which include serine β-lactamases and metallo-β-lactamases (MβLs) (Polotto et al., 2012; Lupo et al., 2018), whereas high-risk global clones have been associated with MDR or extensively drug resistant (XDR) phenotypes. Currently, global *P. aeruginosa* high-risk clones include sequence types (STs) ST235, ST111, ST175, ST233, ST244, ST277, ST298, ST308, ST357, and ST654 (Del Barrio-Tofiño et al., 2020; Kocsis et al., 2021). Specifically, the ST277 has been sporadically reported in Asian, North American, and European countries, whereas in Brazil is highly prevalent (Gales et al., 2003; Hopkins et al., 2016; Del Barrio-Tofiño et al., 2020; Silveira et al., 2020; Kocsis et al., 2021). The success of the Brazilian endemic clone ST277 is associated with carbapenem resistance due to production of the MβL SPM-1 (Gales et al., 2003; Cipriano et al., 2007; da Fonseca et al., 2010; Nascimento et al., 2016; Silveira et al., 2020). Worryingly, SPM-1-producing *P. aeruginosa* have been identified in hospital sewage and hospital wastewater treatment plants (Fuentefria et al., 2009; Miranda et al., 2015), denoting potential to spread throughout the aquatic environment, enabling human exposure and transmission. However, although whole genome sequencing (WGS) of human SPM-1-positive isolates have been performed (Nascimento et al., 2016; Galetti et al., 2019), sequence data from environmental isolates have not been provided for comparative genomic studies. Based on WHO list priority pathogens criteria, which included pathogen mortality, hospital and environment transmissibility and limited treatment options, recognition and genomic characterization of critical priority pathogens is an essential first step to understanding their dynamic of acquisition/dissemination and ultimately to development of preventive intervention strategies (Hendriksen et al., 2019). In this study, we report genomic data and the virulence potential of carbapenem-resistant SPM-1-positive *P. aeruginosa* strains isolated from polluted urban rivers, in Brazil.

MATERIALS AND METHODS

Pseudomonas aeruginosa Strains and Antimicrobial Susceptibility Profiles

During a Brazilian surveillance study (OneBR project) conducted to investigate the burden of antimicrobial resistance in impacted aquatic environments, two *P. aeruginosa* strains [Pa19 (ONE609) and Pa151 (ONE610)] were isolated from two different locations along the Tietê (TIET-04900; S 23° 31' 18", W 46° 37' 52", S 23° 27' 16", and W 46° 54' 36") and Pinheiros (PINH-04900; S 23° 31' 52" and W 46° 44' 54") Rivers in São Paulo, Brazil (Turano et al., 2016). Tietê River stretches through São Paulo state from east to west for approximately 1,100 km, while Pinheiros River is a tributary of the Tietê River that runs 25 km across the city. In this study, both strains were subjected to WGS for investigation and comparative genomic studies using five public sequences from nosocomial SPM-1-positive *P. aeruginosa* strains, previous reported (Silveira et al., 2014, 2020; Nascimento et al., 2016; Galetti et al., 2019). Susceptibility profiles were investigated by disk-diffusion method (CLSI, 2021).

Whole Genome Sequencing and Genomic Analysis

Genomic DNA of Pa19 and Pa151 were extracted using PureLink Quick Gel Extraction & PCR Purification Combo Kit (Life Technologies, Carlsbad, CA). The Illumina paired-end libraries were constructed using a Nextera XT DNA Library Preparation Kit (Illumina Inc.), according to the manufacturer's guidelines. Whole genome sequencing was performed using an Illumina MiSeq platform with 300-bp read lengths. Reads were *de novo* assembled using SPAdes 3.13,[1] and the resulting contigs were automatically annotated by NCBI Prokaryotic Genome Annotation Pipeline (PGAP) version 3.2.[2] Antibiotic resistance genes were predicted using ResFinder 4.1[3] and the Comprehensive Antibiotic Resistance Database (CARD).[4] Multi-locus Sequence Typing prediction was performed using MLST v.2.0.[5] Heavy metal (HM) resistance genes were manually identified using the NCBI database[6] and Geneious Prime version 2020.04 (Biomatters, New Zealand). Additionally, phage prediction was performed by Genome Detective Virus Tool software.[7] The *rmtD* gene was detected and aligned by BLASTn

[1]https://github.com/ablab/spades
[2]https://www.ncbi.nlm.nih.gov/genome/annotation_prok/
[3]https://cge.cbs.dtu.dk/services/ResFinder
[4]https://card.mcmaster.ca/analyze/rgi
[5]https://cge.cbs.dtu.dk/services/MLST/
[6]https://www.ncbi.nlm.nih.gov/Traces/wgs/
[7]https://www.genomedetective.com/app/typingtool/virus/

(Alikhan et al., 2011) against the *rmtD1* allele of the *P. aeruginosa* (PA0905 strain), recovered from a human patient (GenBank accession number. DQ914960). Genetic context analysis of bla_{SPM-1} and *rmtD1* resistance genes of Pa151 were performed with BLASTn algorithm and manually curated using Geneious Prime version 2020.04 (Biomatters, New Zealand).

Moreover, virulence genes, efflux systems, and regulators were determined through the Virulence Factor Database.[8] Serotype was predicted using Past 1.0.[9] SNP-based phylogenetic analysis was performed by using Prokka 1.13.4[10] for pangenome annotation, followed by Roary 3.13.0[11] for core genome analysis. SNP-sites tool[12] was used for SNPs extraction from the core gene alignment; whereas RAxML-NG version 0.9.0[13] for phylogenetic construction and a maximum likelihood tree based on SNP alignment. Additionally, comparative genomic analysis of *P. aeruginosa* sequences was performed by BRIG v.0.95 using the BLASTn algorithm and Island viewer 4.0.

All genomic analysis were based on comparison of sequences of environmental Pa151 (Pinheiros River, GenBank accession number: PHSS00000000.1) and Pa19 (Tietê River, GenBank accession number: PHST01000000) strains, against publically available genome sequences (data obtained by using 300 bp paired-end MiSeq sequencing) of clinical SPM-1-producing *P. aeruginosa* CCBH4851 (catheter tip, GenBank accession number CP021380.2), PA1088 (urine, GenBank accession number CP015001.1), PA11803 (bloodstream, GenBank accession number: CP015003.1), PA12117 (bloodstream, GenBank accession number: LVXB00000000.1) and PA7790 (tracheal aspirate, GenBank accession number: CP014999.1) strains, which were retrieved from NCBI GenBank database.[14] For SNP-based analysis, the genome of the *P. aeruginosa* strain PAO1 (ST549) was used as reference (GenBank accession number: AE004091.2).

Virulence Potential of Carbapenem-Resistant *P. aeruginosa* Strains in the *Galleria mellonella* Larvae Model

The virulence potential of *P. aeruginosa* Pa19 and Pa151 strains was evaluated using the *Galleria mellonella* infection model (Tsai et al., 2016). In brief, groups of *G. mellonella* containing 10 larvae of nearly 0.25–0.35 g (supplied by the Institute of Biomedical Sciences of the University of São Paulo, Brazil) were infected with 10^4 CFU/ml of each strain per larvae, by injecting a 10 µl aliquot in PBS, into the body of the larvae *via* the last left proleg, using a sterile ultra-fine needle syringe (Fuentes-Castillo et al., 2019). Survival was monitored every hour, for 96 h. Two biological replicates and two experimental replicates were performed with a group of 10 larvae per strain, in each replicate. SPM-1-producing *P. aeruginosa* clinical strain PA1088 was used as comparative control (Toleman et al., 2002). Moreover, a control group inoculated with sterile PBS was used in each biological and experimental replication assay, in order to verify that the larvae would not be killed by physical trauma. Survival curves were plotted using the Kaplan-Meier method, whereas statistical analyses were performed by the log rank test with $p < 0.05$ indicating statistical significance (OriginLab Software, Northampton, Massachusetts, United States).

RESULTS

In this study, two carbapenemase (SPM-1)-producing *P. aeruginosa* ST277 (Pa19 and Pa151 strains) isolated from impacted urban rivers in São Paulo, Brazil, were sequenced. As this clone has been endemic in Brazilian hospitals, being also identified in migratory birds (**Figure 1**), we have additionally performed a comparative analysis with publically available genomes obtained from ST277 lineages from human infections.

Genome sequencing yielded a total of 968,818 and 473,825 paired-end reads assembled into 395 and 337 contigs, with 305 and 299x of coverage, to Pa19 and Pa151 strains, respectively. The genome size of Pa19 was calculated at 6,927,007 bp, with a GC content of 67.8%, comprising 6,956 total genes, 60 tRNAs, three rRNAs, four ncRNAs, and 155 pseudogenes (accession number: PHST00000000.1). On the other hand, genome size of Pa151 was calculated at 6,799,801 bp, with a GC content of 66.9%, comprising 6,747 total genes, 59 tRNAs, three rRNAs, four ncRNAs, and 123 pseudogenes (accession number: PHSS00000000.1). Genomic information of *P. aeruginosa* Pa19 and Pa151 strains are available on the OneBR platform[15] under ONE609 and ONE610 ID numbers, respectively.

Environmental Pa19 and Pa151 strains displayed a MDR profile to ticarcillin-clavulanate, cefepime, ceftazidime, imipenem, meropenem, amikacin, gentamicin, nalidixic acid, ciprofloxacin, levofloxacin, and trimethoprim-sulfamethoxazole, and genomic analysis revealed a wide resistome to β-lactams (bla_{SPM-1}, bla_{OXA-56}, $bla_{OXA-396}$, $bla_{OXA-494}$, and bla_{PAO}), aminoglycosides [*aacA4*, *aadA7* and *aph(3')-llb*], fluoroquinolones [*aac(6')lb-cr*, and *gyrA* (T83I) and *parC* (S87L) point mutations], phenicols (*cmx*), sulphonamides (*sul1*), and fosfomycin (*fosA*), which was predicted in agreement with the phenotype. Additionally, Pa151 strain harbored the *rmtD1* and *catB7* genes related to aminoglycosides and chloramphenicol resistance, respectively (**Figure 2**). On the other hand, the *crpP* gene associated with fluoroquinolone resistance, was only identified in the Pa19 genome. Genes associated with resistance to heavy metal [copper (*pcoABD*)], and quaternary ammonium compounds (*qacE*, *qacA*, and *sugE*) were also identified in both environmental *P. aeruginosa* strains (**Figure 2**).

Virulome analysis of environmental Pa19 and Pa151 revealed a wide virulome. In fact, both lineages carried the *quorum sensing* (*lasA*, *lasB*, *lasI*, and *ptxR*), alginate (*alg* cluster), siderophore production (*pvdA*, *pvdF*, and *pvdG*), fimbriae (*cup*

[8]http://www.mgc.ac.cn/VFs/
[9]https://cge.cbs.dtu.dk/services/PAst/
[10]https://github.com/tseemann/prokka
[11]https://github.com/sanger-pathogens/Roary
[12]https://github.com/sanger-pathogens/snp-sites
[13]https://github.com/stamatak/standard-RAxML
[14]https://www.ncbi.nlm.nih.gov
[15]http://onehealthbr.com/

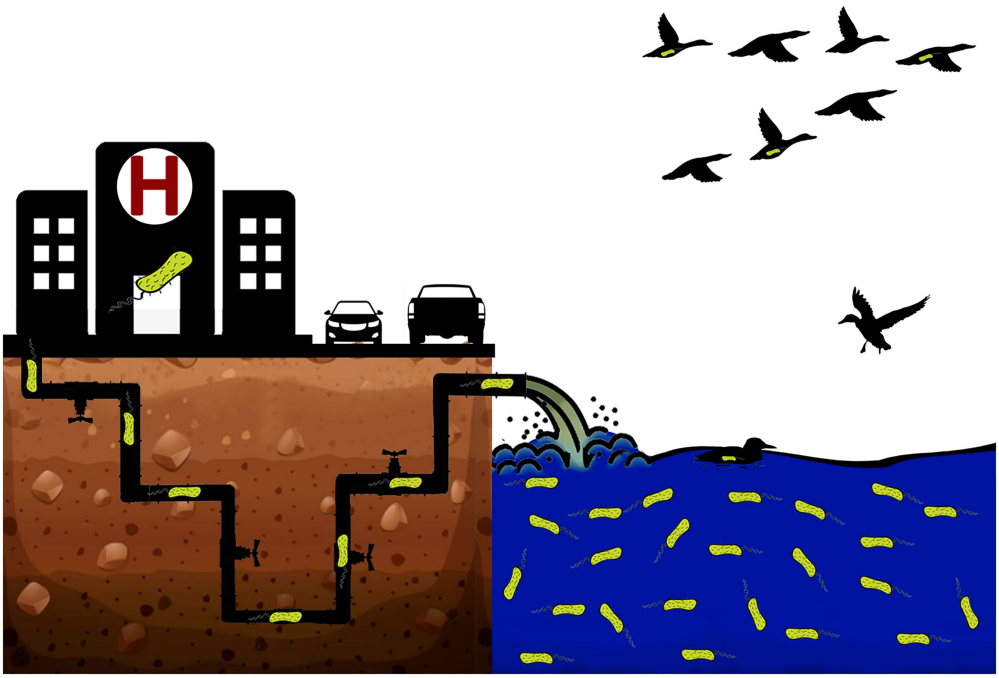

FIGURE 1 | Schematic representation of hypothesis proposed for spread of carbapenemase (SPM-1)-producing *Pseudomonas aeruginosa* clone ST277 beyond the hospital, in Brazil, based on genomic data analyzed in this study.

family), flagellum (*flgABCDEFGHIJLMN*) synthesis, immunity protein (*pyo5*, *imm2*), colicin (*cea*), types II (*gspDEF*), III (*exoYST*), and IV (*vgrD2*/*vgrD4*) secretion systems and exotoxin A (*toxA*) genes; whereas the O2 serotype was identified in both Pa19 and Pa151 environmental strains (**Figure 2**). In this regard, *in vivo* experiments using *G. mellonella* larvae showed that both Pa19 and Pa151 strains killed 100% of the larvae at 24 h post-infection, similarly to what was observed with the clinical SPM-1-producing *P. aeruginosa* PA1088 strain isolated from a case of urinary tract infection (**Figure 3**).

Overall comparison of five human and two environmental ST277 genomes using BLAST Ring Image Generator (BRIG) revealed high nucleotide sequence similarities among *P. aeruginosa* strains, even for aquatic isolates recovered at least 13 years after the first clinical isolate (Toleman et al., 2002). Furthermore, SNP-based phylogenetic analysis revealed that both Pa19 and Pa151 environmental strains were closely related (>94% identity) to all human SPM-1-producing *P. aeruginosa* isolates (**Supplementary Table S1**). However, missing regions at position 5.5 Mbp, named as GI-I, in environmental Pa151 and clinical PA11803 and PA12117 genomes, were identified (**Figure 4**). In this regard, we observed genes encoding the following proteins: integrating conjugative element protein (*pill*), type II secretion system protein, replicative DNA helicase (*dnaB*), nucleoid-associated protein YejK (*yejK*), NADH dehydrogenase (*ndh*), cell division protein ZapE (*zapE*), ParA family protein (*parA*), plasmid stabilization protein ParE (*parE*), integrating conjugative element protein, DNA topoisomerase I (*topA*), pyocin S5 (*pyoS5*), TetR family transcriptional regulator (*tetR*), conjugal transfer protein TraG (*traG*), regulatory protein GemA (*gemA*), conjugative coupling factor TraD (*traD*), his-Xaa-Ser repeat protein HxsA (*hxsA*), his-Xaa-Ser system radical SAM maturase HxsB (*hxsB*), his-Xaa-Ser system radical SAM maturase HxsC (*hxsC*), his-Xaa-Ser system protein HxsD (*hxsD*), chaperone protein ClpB (*clpB*), and genes encoding for membrane proteins, transcriptional regulator, CRISPR-associated proteins, type II secretion system protein, phage tail sheath subtilisin-like, tail fiber protein, phage tail tape measure protein, and phage head morphogenesis protein.

Schematic representations of the genetic context surrounding $bla_{\text{SPM-1}}$ genes in the environmental *P. aeruginosa* PA151 strain is presented in **Figure 5A**. The $bla_{\text{SPM-1}}$ was flanked by a ~4.8 kbp region composed of the IS*91*-$bla_{\text{SPM-1}}$-*groEL*-IS*91* array. The presence of IS elements is related to horizontal gene transfer, whereas the *groEL* encodes for a heat-shock chaperon. Additionally, we also detected the *traG* (encoding a conjugal transfer protein), *eexN* (encoding the entry exclusion protein), *traR* (transcriptional regulator), *bcr1* (bicyclomycin resistance), *virD2* (gene encoding a relaxase), and hypothetical proteins. In **Figure 5B** is presented the genetic context surrounding *rmtD1* gene in PA151 strain. The *rmtD1* was flanked by a ~7.3 kbp region composed of the IS*91*-*rmtD1*-*tgt*-*groEL*-IS*91* array. In addition, *aacA4*, $bla_{\text{OXA-56}}$, *aadA7*, and *qacEΔ1* genes were located on a class 1 integron. Moreover, *cmx* and *sul1* resistance genes, that encodes for chloramphenicol and sulphonamide resistance, respectively, were also identified along with genes encoding hypothetical proteins, transposase, IS*110*, IS*481*, and IS*3* mobile elements.

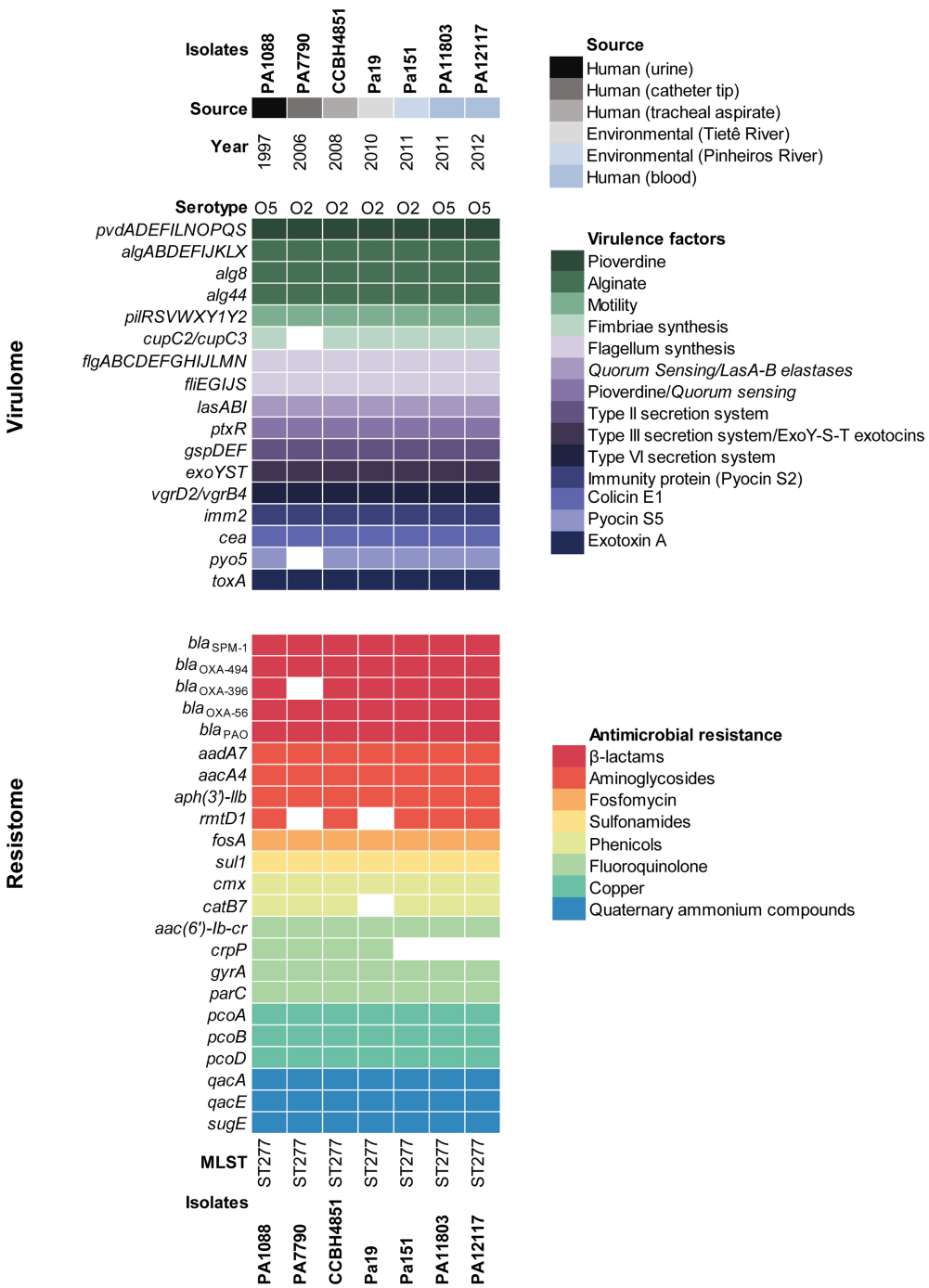

FIGURE 2 | Heatmap showing the distribution of virulence and resistance genes in environmental and clinical SPM-1-positive *P. aeruginosa* strains of ST277 clone. Virulence genes are listed along with their functions and demarcated by colored squares. Resistome is demarcated by colored squares under genes names grouped by their antimicrobial resistance classes. *Pseudomonas aeruginosa* strains are indicated by colors displaying their source and year of isolation.

DISCUSSION

Carbapenem-resistant *P. aeruginosa* are critical-priority pathogens associated with high mortality and morbidity (Georgescu et al., 2016; Tacconelli et al., 2018; Pang et al., 2019). In this regard, one of the major concerns has been the successful expansion and rapid spread of high-risk clones. In Brazil, the metallo-β-lactamase (SPM-1)-producing *P. aeruginosa* ST277 clone has gained significant attention, due to its endemicity status and further identification in migratory birds and polluted environments (Gales et al., 2003; Nascimento et al., 2016; Turano et al., 2016; Martins et al., 2018).

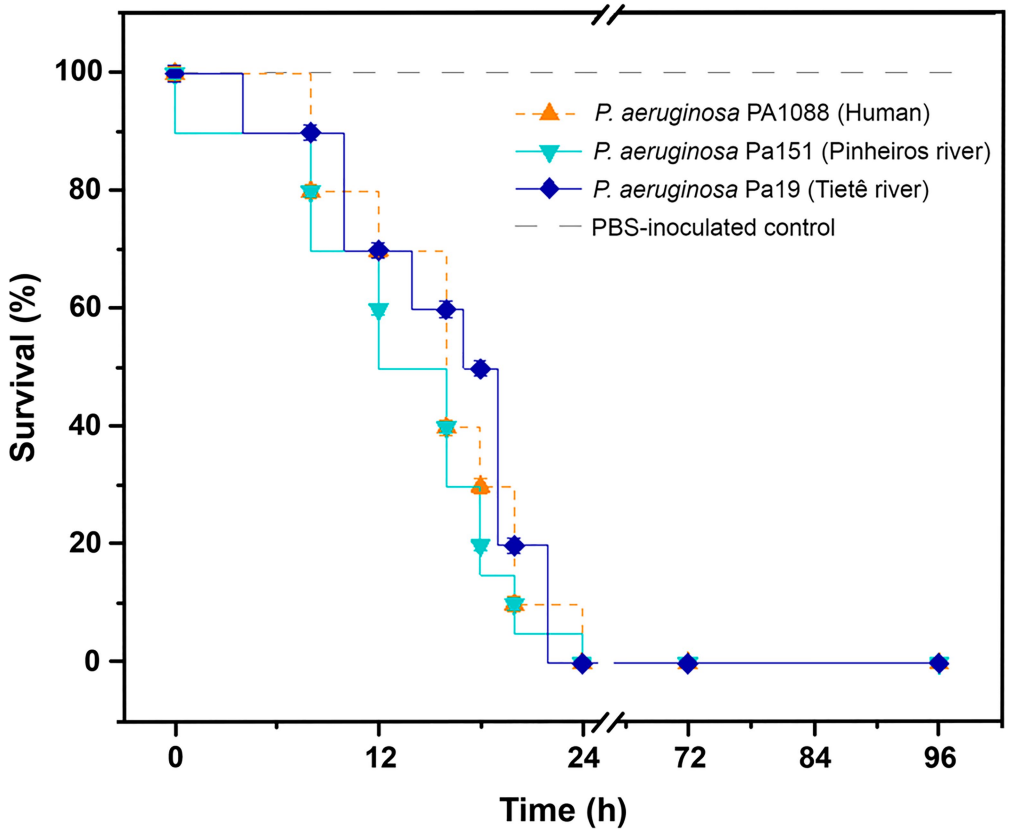

FIGURE 3 | Virulent behavior of SPM-1-producing *P. aeruginosa* isolates. Kaplan-Meier survival curves of *Galleria mellonella* infected with 10^4 CFU/larva of *P. aeruginosa* Pa19 strain (dark-blue line), *P. aeruginosa* Pa151 strain (blue line), and *P. aeruginosa* PA1088 (orange line). Environmental Pa19 and Pa151 strains, and the clinical PA1088 strain killed 100% of larvae at 24 h post-infection. PBS-inoculated control group (light-gray dashed line) presented 100% of survival. For each strain, groups containing 10 *G. mellonella* larvae in each replicate were evaluated in two biological and experimental independent assays.

Worryingly, previous studies have also reported the occurrence of carbapenemase (KPC-2)-producing *Klebsiella pneumoniae* belonging to the clonal group CG258 and OXA-23-positive *A. baumannii* ST79 in the Tietê River (Oliveira et al., 2014; Turano et al., 2016), supporting an anthropogenic trend, most likely due to hospital wastewater discharge and domestic wastewaters effluents (Nascimento et al., 2017; Bartley et al., 2019; Böger et al., 2021; Popa et al., 2021). Therefore, aquatic environment could play an important role in the widespread of critical pathogens (Devarajan et al., 2017). In fact, polluted rivers could be contributing for colonization of local and migratory fauna (Martins et al., 2018; Narciso et al., 2020).

In order to elucidate the genomic aspects associate with the environmental dissemination of healthcare-associated *P. aeruginosa* ST277, we performed a comparative genomic analysis, extracting clinically relevant information (i.e., resistome, virulome, and phylogenomic). Interestingly, although the strains were isolated in different years (1997–2012), we observed that clinical and environmental SPM-1-producing *P. aeruginosa* strains share a common resistome and virulome.

Although, oral antibiotics have been successfully used in the treatment of bacterial infection, for *P. aeruginosa* few therapeutic options are available, being restricted to some fluoroquinolones, including ciprofloxacin, levofloxacin, and prulifloxacin, which are given alone or in combinations with a second intravenously or inhaled anti-pseudomonal antibiotic such as β-lactams (piperacillin/tazobactam, ceftolozane/tazobactam, ceftazidime, cefepime, or carbapenems) and/or aminoglycosides (tobramycin, amikacin, or gentamicin) (Tümmler, 2019; Ibrahim et al., 2020; Nisly et al., 2020). However, under a clinical perspective, even co-resistance to carbapenems and aminoglycosides in ST277 have already been reported and limited therapeutic options. This resistance profile is mediated by bla_{SPM-1} and *rmtD* genes, respectively (Doi et al., 2007). Strikingly, in some ST277, including environmental (Pa19) and human (PA7790) lineages, the *rmtD* gene was not found. On the other hand, the *rmtD1* identified in the environmental Pa151 strain, displayed 100% identity to the *rmtD1* gene from *P. aeruginosa* PA0905 strain, recovered from a human patient in 2005, in Brazil (Doi et al., 2007). The *rmtD1* was subsequently identified in *K. pneumoniae* and other Enterobacterales in Latin America, Europe, and North America (Bueno et al., 2016). Since acquisition of this gene has been linked to transposition events (Doi et al., 2007; Nascimento et al., 2016), most likely genomic plasticity of *P. aeruginosa* has led to the dissemination of *rmtD*$^+$ and *rmtD*-ST277 lineages

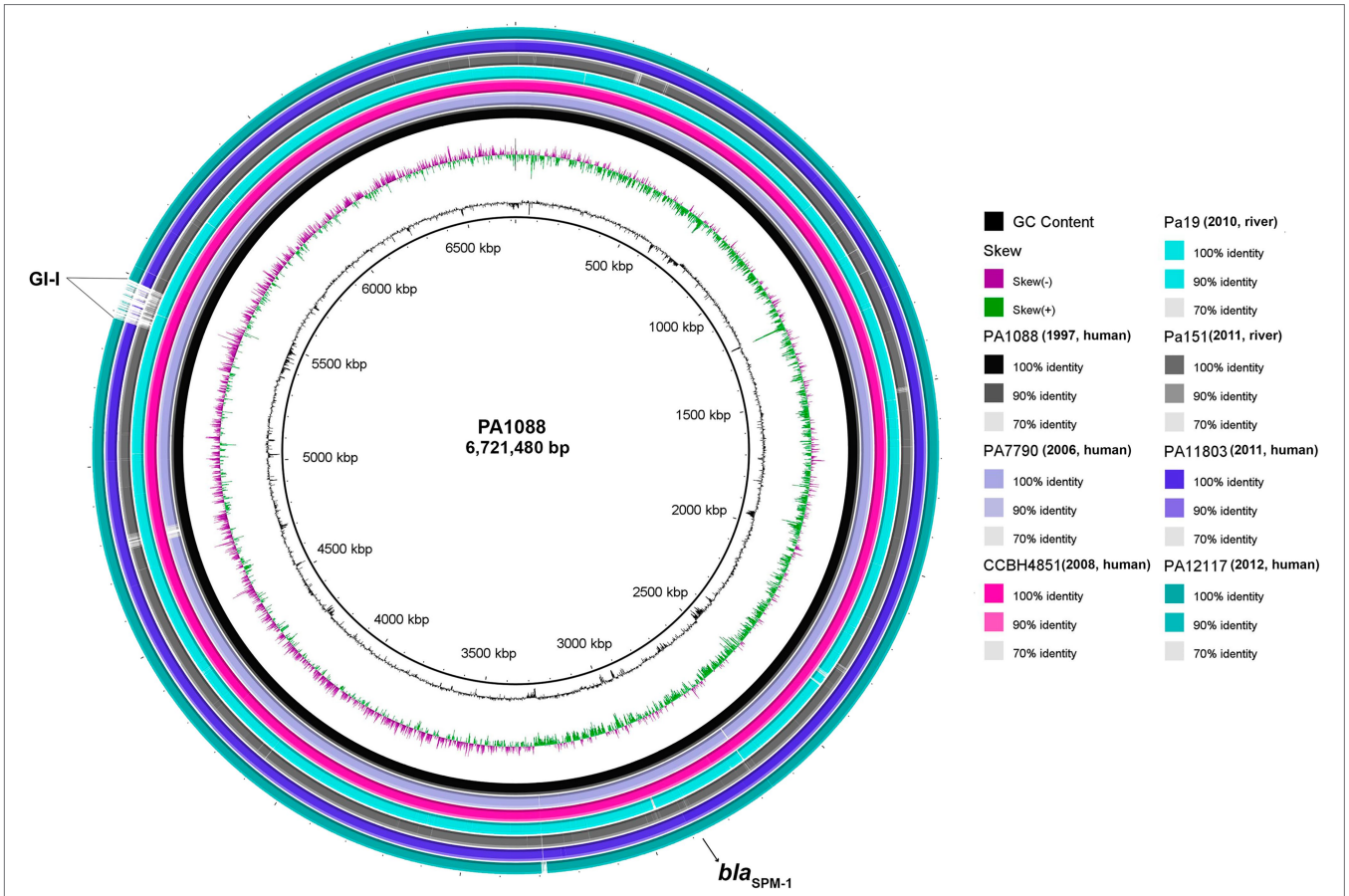

FIGURE 4 | Circular genome maps of SPM-1-producing *P. aeruginosa* belonging to ST277. Circular maps were built by BLAST Ring Image Generator (BRIG) using seven *P. aeruginosa* genomes. All genomes were represented as individual rings and compared against the reference genome PA1088 (GenBank accession number: CP015001.1). The bla_{SPM-1} gene is indicated by a black arrow. Furthermore, several genes associated with DNA replication/repair/regulatory/defense and membrane proteins were identified in the major genomic island, indicated as GI-I.

(Silveira et al., 2020). In Brazil, occurrence of *rmtD* has also been documented in *Escherichia coli* and *K. pneumoniae* (Yamane et al., 2008; Leigue et al., 2015).

The bla_{SPM-1}, IS*91*-bla_{SPM-1}-*groEL*-IS*91* gene array has been previously identified within a Tn*4371*-like integrative and conjugative element (ICE_{Tn4371}6061) considered stable in the chromosome loci of *P. aeruginosa* ST277 strains recovered from humans and animals (Fonseca et al., 2015; Nascimento et al., 2016). Since ICEs are genetic mobile platforms that play an important role during bacterial evolution, they are overlooked as vectors in the spread and resistance emergence in many bacterial species (Fonseca and Vicente, 2016). Moreover, the genetic context of *rmtD1* (IS*91*-*rmtD1*-*tgt*-*groEL*-IS*91*) identified in the environmental strain was similar to previous descriptions, where the presence of the *rmtD* gene in clinical isolates was associated to the Tn*As3* transposon (Fonseca et al., 2015; Nascimento et al., 2016).

In human and aquatic *P. aeruginosa* ST277 isolates the resistome was not restricted to antibiotics, and the presence of genes conferring tolerance to copper and QAC biocides was further detected. Currently, there is a growing concern about biocides that pollute aquatic environments, especially QACs, since these compounds are widely used in domiciliary and hospital settings, as disinfectants, soaps, toothpastes, and mouthwash formulations (Zubris et al., 2017; Fuentes-Castillo et al., 2020). Consequently, ecosystems impacted by HM and biocides could favor the selection and persistence of high-risk clones harboring a broad resistome (Baker-Austin et al., 2006; Zhao et al., 2012; Kim et al., 2018).

Although a limitation of this study was the lack of a known highly virulent *P. aeruginosa* to be used as a positive control in the *in vivo* assay; we observed that the virulent behavior of environmental strains was identical to clinical strains. Indeed, a wide virulome was also predicted in human and environmental *P. aeruginosa* ST277 lineages, denoting a pathogenic potential, as demonstrated in the *G. mellonella* infection model. Lipopolysaccharide (LPS) O-antigen, type IV pili, and flagella are components of the external cell wall structure of *P. aeruginosa* and play important roles in the early stage of colonization, persistence, and bacterial pathogenesis (Hauser, 2011; Behzadi et al., 2021). Furthermore, O-antigen is an important virulence factor in *P. aeruginosa* used for the detection of MDR/XDR high-risk clones (Del Barrio-Tofiño et al., 2019). Strikingly, among clinical strains were identified the serotypes O5 and

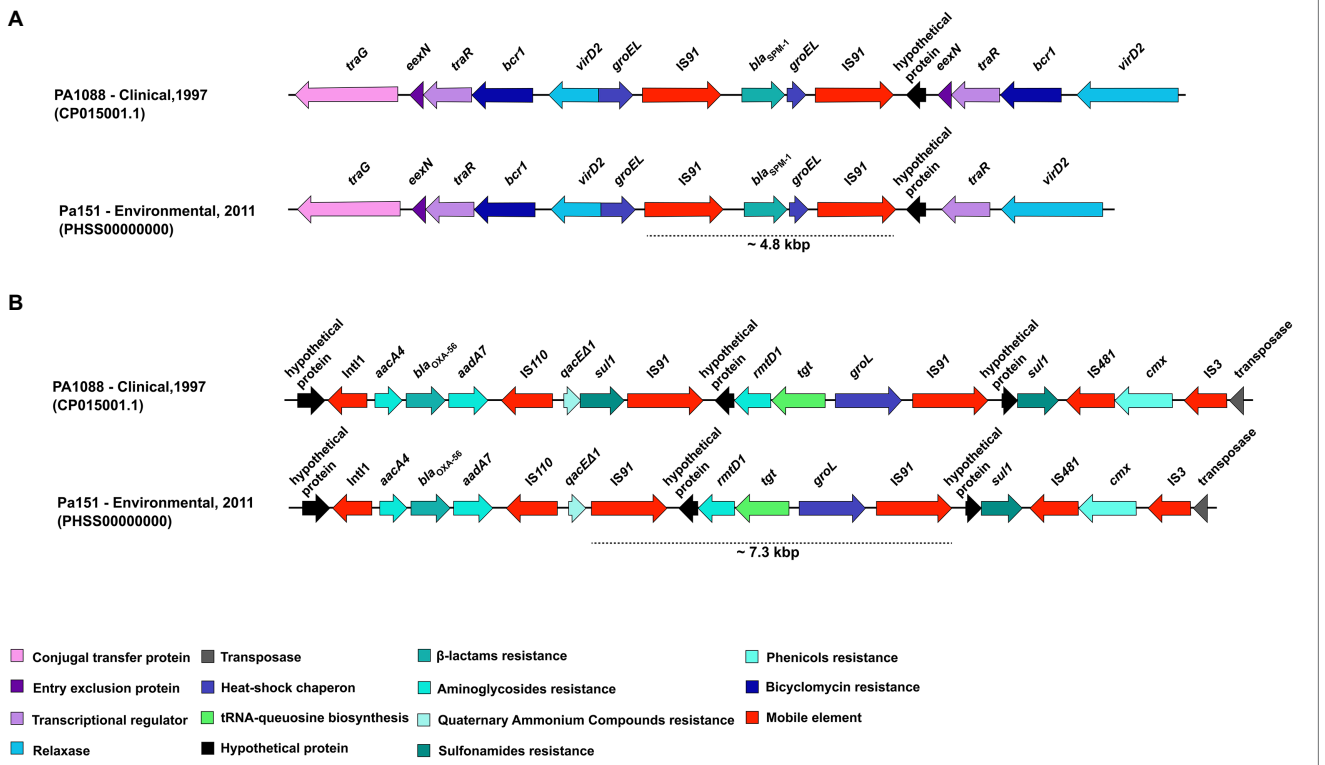

FIGURE 5 | Overall comparison of genetic context of bla_{SPM-1} and *rmtD1* genes carried by clinical and environmental *P. aeruginosa* strains belonging to ST277. **(A)** The bla_{SPM-1} was flanked by a ~4.8 kbp region composed of IS*91*-bla_{SPM-1}-*groEL*-IS*91*. **(B)** The *rmtD1* was flanked by a ~7.3 kbp region composed of the IS*91*-*rmtD1*-*tgt*-*groEL*-IS*91*. In addition, *aacA4*, *aadA7*, bla_{OXA-56}, *qacEΔ1*, *sul1,* and *cmx* resistance genes were also identified along with genes encoding for hypothetical proteins, transposase; as well as IS*110*, IS*481*, and IS*3* mobile genetics elements.

O2. The latter was also identified among environment strains. Both serotypes have been associated with acute and chronic infections (Lu et al., 2014; Li et al., 2018).

Type secretion systems (TSSs) are mechanisms by which bacteria translocate a set of toxins into the cytosol of host cells and/or to the extracellular medium (Abby et al., 2016). *Pseudomonas aeruginosa* is known to have five TSSs, of which Types I (T1SS), II (T2SS), and III (T3SS) are involved in the virulence of this pathogen. Several studies have linked these TSSs with poor outcomes of patients with acute respiratory diseases (i.e., pneumonia), with T3SS being one of the most clinically relevant virulence determinants (Hauser, 2011; McMackin et al., 2019; Sarges et al., 2020). In this context, we detected ExoTSY exotoxins-encoding genes in both clinical and environmental strains. ExoTSY exotoxins are secreted by T3SS and reported to be involved in lung injury, pulmonary-vascular barrier disruption, and end-organ dysfunction in chronic infections, mainly in CF patients; as well as with mortality in animal models (Lu et al., 2014; Sarges et al., 2020; Jurado-Martín et al., 2021). Interestingly, the *toxA* gene (exotoxin A), which is present in the most clinically *P. aeruginosa* strains (Khosravi et al., 2016) was also identified in environmental strains. Exotoxin A has been associated with tissue damage related to poor outcomes of burn patients (Khosravi et al., 2016). In fact, the broad virulome harbored by *P. aeruginosa* ST277 seems to be associated with a remarkable ability to adapt to different human and non-human conditions (Jurado-Martín et al., 2021).

In brief, from comparative analysis, our data revealed that Pa19 and Pa151 environmental strains presented slight variations when compared against clinical strains, suggesting a high degree of genetic conservation, regardless isolation data and exposition to contaminants (antibiotics and biocides residues) present in the polluted aquatic environments.

CONCLUSION

In summary, we report genomic comparative data of antimicrobial-resistant *P. aeruginosa* isolated from aquatic environments in Brazil. The presence of SPM-1-producing *P. aeruginosa* ST277 in urban rivers could be associated with hospital effluents, since SNP-based phylogenomics showed high nucleotide sequence similarity between clinical and environmental genomes. Additionally, wide resistome and virulome have been conserved in environmental isolates, denoting that critical priority *P. aeruginosa* of the high-risk ST277 has successfully expanded beyond the hospital. Therefore, genomic surveillance is essential to rapidly identify and prevent the spread of WHO critical priority clones with One Health implications.

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

FE, BC, HF, and BF performed the data analysis. FE, BC, QM, AC-A, and DF-C conducted the experiments. NL supervised the experiments and designed and coordinated the project. FE, BC, and NL wrote, reviewed, and edited 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.2021.701921/full#supplementary-material

REFERENCES

Abby, S. S., Cury, J., Guglielmini, J., Néron, B., Touchon, M., and Rocha, E. P. (2016). Identification of protein secretion systems in bacterial genomes. *Sci. Rep.* 6:23080. doi: 10.1038/srep23080

Alikhan, N. F., Petty, N. K., Zakour, N. L. B., and Beatson, S. A. (2011). BLAST ring image generator (BRIG): simple prokaryote genome comparisons. *BMC Genomics* 12:402. doi: 10.1186/1471-2164-12-402

Babu, R. N., Mutters, N. T., Marasca, G., Conti, M., Sifakis, F., Vuong, C., et al. (2020). Mandatory surveillance and outbreaks reporting of the WHO priority pathogens for research & discovery of new antibiotics in European countries. *Clin. Microbiol. Infect.* 26, 943.e1–943.e6. doi: 10.1016/j.cmi.2019.11.020

Baker-Austin, C., Wright, M. S., Stepanauskas, R., and McArthur, J. V. (2006). Co-selection of antibiotic and metal resistance. *Trends Microbiol.* 14, 176–182. doi: 10.1016/j.tim.2006.02.006

Bartley, P. S., Domitrovic, T. N., Moretto, V. T., Santos, C. S., Ponce-Terashima, R., Reis, M. G., et al. (2019). Antibiotic resistance in *Enterobacteriaceae* from surface waters in urban Brazil highlights the risks of poor sanitation. *Am. J. Trop. Med. Hyg.* 100, 1369–1377. doi: 10.4269/ajtmh.18-0726

Behzadi, P., Baráth, Z., and Gajdács, M. (2021). It's not easy being green: a narrative review on the microbiology, virulence and therapeutic prospects of multidrug-resistant *Pseudomonas aeruginosa*. *Antibiotics* 10:42. doi: 10.3390/antibiotics10010042

Bobrov, A. G., Getnet, D., Swierczewski, B., Jacobs, A., Medina-Rojas, M., Tyner, S., et al. (2021). Evaluation of *Pseudomonas aeruginosa* pathogenesis and therapeutics in military-relevant animal infection models. *APMIS* doi: 10.1111/apm.13119 [Epub ahead of print]

Böger, B., Surek, M., Vilhena, R. O., Fachi, M. M., Junkert, A. M., Santos, J. M., et al. (2021). Occurrence of antibiotics and antibiotic resistant bacteria in subtropical urban rivers in Brazil. *J. Hazard Mater.* 402:123448. doi: 10.1016/j.jhazmat.2020.123448

Bueno, M. F., Francisco, G. R., de Oliveira Garcia, D., and Doi, Y. (2016). Complete sequences of multidrug resistance plasmids bearing *rmtD1* and *rmtD2* 16S rRNA methyltransferase genes. *Antimicrob. Agents Chemother.* 60, 1928–1931. doi: 10.1128/AAC.02562-15

Cipriano, R., Vieira, V. V., Fonseca, É. L., Rangel, K., Freitas, F. S., and Vicente, A. C. P. (2007). Coexistence of epidemic colistin-only-sensitive clones of *Pseudomonas aeruginosa*, including the bla_{SPM} clone, spread in hospitals in a Brazilian amazon city. *Microb. Drug Resist.* 13, 142–146. doi: 10.1089/mdr.2007.708

CLSI (2021). Performance Standards for Antimicrobial Susceptibility Testing. *31st Edn*. CLSI supplement M100. Clinical and Laboratory Standards Institute.

da Fonseca, É. L., dos Santos Freitas, F., and Vicente, A. C. P. (2010). The colistin-only-sensitive Brazilian *Pseudomonas aeruginosa* clone SP (sequence type 277) is spread worldwide. *Antimicrob. Agents Chemother.* 54:2743. doi: 10.1128/AAC.00012-10

Del Barrio-Tofiño, E., López-Causapé, C., and Oliver, A. (2020). *Pseudomonas aeruginosa* epidemic high-risk clones and their association with horizontally acquired β-lactamases: 2020 update. *Int. J. Antimicrob. Agents* 56:106196. doi: 10.1016/j.ijantimicag.2020.106196

Del Barrio-Tofiño, E., Sánchez-Diener, I., Zamorano, L., Cortes-Lara, S., López-Causapé, C., Cabot, G., et al. (2019). Association between *Pseudomonas aeruginosa* O-antigen serotypes, resistance profiles and high-risk clones: results from a Spanish nationwide survey. *J. Antimicrob. Chemother.* 74, 3217–3220. doi: 10.1093/jac/dkz346

Devarajan, N., Köhler, T., Sivalingam, P., van Delden, C., Mulaji, C. K., Mpiana, P. T., et al. (2017). Antibiotic resistant *Pseudomonas* spp. in the aquatic environment: a prevalence study under tropical and temperate climate conditions. *Water Res.* 115, 256–265. doi: 10.1016/j.watres.2017.02.058

Doi, Y., de Oliveira Garcia, D., Adams, J., and Paterson, D. L. (2007). Coproduction of novel 16S rRNA methylase RmtD and metallo-β-lactamase SPM-1 in a panresistant *Pseudomonas aeruginosa* isolate from Brazil. *Antimicrob. Agents Chemother.* 51, 852–856. doi: 10.1128/AAC.01345-06

Fernández-Barat, L., Ferrer, M., De Rosa, F., Gabarrús, A., Esperatti, M., Terraneo, S., et al. (2017). Intensive care unit-acquired pneumonia due to *Pseudomonas aeruginosa* with and without multidrug resistance. *J. Infect.* 74, 142–152. doi: 10.1016/j.jinf.2016.11.008

Fonseca, E. L., Marin, M. A., Encinas, F., and Vicente, A. C. (2015). Full characterization of the integrative and conjugative element carrying the metallo-β-lactamase bla_{SPM-1} and bicyclomycin *bcr1* resistance genes found in the pandemic *Pseudomonas aeruginosa* clone SP/ST277. *J. Antimicrob. Chemother.* 70, 2547–2550. doi: 10.1093/jac/dkv152

Fonseca, E. L., and Vicente, A. C. (2016). Commentary: clinical utilization of genomics data produced by the international *Pseudomonas aeruginosa* consortium. *Front. Microbiol.* 7:770. doi: 10.3389/fmicb.2016.00770

Fuentefria, D. B., Ferreira, A. E., Gräf, T., and Corção, G. (2009). Spread of metallo-β-lactamases: screening reveals the presence of a bla_{SPM-1} gene in hospital sewage in southern Brazil. *Braz. J. Microbiol.* 40, 82–85. doi: 10.1590/S1517-83822009000100013

Fuentes-Castillo, D., Esposito, F., Cardoso, B., Dalazen, G., Moura, Q., Fuga, B., et al. (2020). Genomic data reveal international lineages of critical priority *Escherichia coli* harbouring wide resistome in Andean condors (*Vultur gryphus* Linnaeus, 1758). *Mol. Ecol.* 29, 1919–1935. doi: 10.1111/mec.15455

Fuentes-Castillo, D., Farfán-López, M., Esposito, F., Moura, Q., Fernandes, M. R., Lopes, R., et al. (2019). Wild owls colonized by international clones of extended-spectrum β-lactamase (CTX-M)-producing *Escherichia coli* and *Salmonella infantis* in the southern cone of America. *Sci. Total Environ.* 674, 554–562. doi: 10.1016/j.scitotenv.2019.04.149

Gajdács, M. (2020). Carbapenem-resistant but cephalosporin-susceptible *Pseudomonas aeruginosa* in urinary tract infections: opportunity for colistin sparing. *Antibiotics* 9:153. doi: 10.3390/antibiotics9040153

Gales, A. C., Menezes, L. C., Silbert, S., and Sader, H. S. (2003). Dissemination in distinct Brazilian regions of an epidemic carbapenem-resistant *Pseudomonas aeruginosa* producing SPM metallo-β-lactamase. *J. Antimicrob. Chemother.* 52, 699–702. doi: 10.1093/jac/dkg416

Galetti, R., Andrade, L. N., Varani, A. M., and Darini, A. L. C. (2019). SPM-1-producing *Pseudomonas aeruginosa* ST277 carries a chromosomal pack of acquired resistance genes: an example of high-risk clone associated with 'intrinsic resistome'. *J. Glob. Antimicrob. Resist.* 16, 183–186. doi: 10.1016/j.jgar.2018.12.009

Georgescu, M., Gheorghe, I., Curutiu, C., Lazar, V., Bleotu, C., and Chifiriuc, M. C. (2016). Virulence and resistance features of *Pseudomonas aeruginosa* strains isolated from chronic leg ulcers. *BMC Infect. Dis.* 16:92. doi: 10.1186/s12879-016-1396-3

Hauser, A. R. (2011). *Pseudomonas aeruginosa*: so many virulence factors, so little time. *Crit. Care Med.* 39, 2193–2194. doi: 10.1097/CCM.0b013e318221742d

Hendriksen, R. S., Munk, P., Njage, P., van Bunnik, B., McNally, L., Lukjancenko, O., et al. (2019). Global monitoring of antimicrobial resistance based on metagenomics analyses of urban sewage. *Nat. Commun.* 10:1124. doi: 10.1038/s41467-019-08853-3

Hopkins, K. L., Meunier, D., Findlay, J., Mustafa, N., Parsons, H., Pike, R., et al. (2016). SPM-1 metallo-β-lactamase-producing *Pseudomonas aeruginosa* ST277 in the UK. *J. Med. Microbiol.* 65, 696–697. doi: 10.1099/jmm.0.000269

Ibrahim, D., Jabbour, J. F., and Kanj, S. S. (2020). Current choices of antibiotic treatment for *Pseudomonas aeruginosa* infections. *Curr. Opin. Infect. Dis.* 33, 464–473. doi: 10.1097/QCO.0000000000000677

Jean, S. S., Chang, Y. C., Lin, W. C., Lee, W. S., Hsueh, P. R., and Hsu, C. W. (2020). Epidemiology, treatment, and prevention of nosocomial bacterial pneumonia. *J. Clin. Med.* 9:275. doi: 10.3390/jcm9010275

Jurado-Martín, I., Sainz-Mejías, M., and McClean, S. (2021). *Pseudomonas aeruginosa*: an audacious pathogen with an adaptable arsenal of virulence factors. *Int. J. Mol. Sci.* 22:3128. doi: 10.3390/ijms22063128

Khosravi, A. D., Shafie, F., Montazeri, E. A., and Rostami, S. (2016). The frequency of genes encoding exotoxin A and exoenzyme S in *Pseudomonas aeruginosa* strains isolated from burn patients. *Burns* 42, 1116–1120. doi: 10.1016/j.burns.2016.02.012

Kim, M., Weigand, M. R., Oh, S., Hatt, J. K., Krishnan, R., Tezel, U., et al. (2018). Widely used benzalkonium chloride disinfectants can promote antibiotic resistance. *Appl. Environ. Microbiol.* 84, e01201–e01218. doi: 10.1128/AEM.01201-18

Kocsis, B., Gulyás, D., and Szabó, D. (2021). Diversity and distribution of resistance markers in *Pseudomonas aeruginosa* international high-risk clones. *Microorganisms* 9:359. doi: 10.3390/microorganisms9020359

Leigue, L., Warth, J. F., Melo, L. C., Silva, K. C., Moura, R. A., Barbato, L., et al. (2015). MDR ST2179-CTX-M-15 *Escherichia coli* co-producing RmtD and AAC(6′)-Ib-cr in a horse with extraintestinal infection, Brazil. *J. Antimicrob. Chemother.* 70, 1263–1265. doi: 10.1093/jac/dku520

Li, H., Du, Y., Qian, C., Li, L., Jiang, L., Jiang, X., et al. (2018). Establishment of a suspension array for *Pseudomonas aeruginosa* O-antigen serotyping. *J. Microbiol. Methods* 155, 59–64. doi: 10.1016/j.mimet.2018.11.006

Lu, Q., Eggimann, P., Luyt, C. E., Wolff, M., Tamm, M., François, B., et al. (2014). *Pseudomonas aeruginosa* serotypes in nosocomial pneumonia: prevalence and clinical outcomes. *Crit. Care* 18:R17. doi: 10.1186/cc13697

Lupo, A., Haenni, M., and Madec, J. Y. (2018). Antimicrobial resistance in *Acinetobacter* spp. and *Pseudomonas* spp. *Microbiol. Spectr.* 6. doi: 10.1128/microbiolspec.ARBA-0007-2017

Martins, W. M. B. S., Narciso, A. C., Cayô, R., Santos, S. V., Fehlberg, L. C. C., Ramos, P. L., et al. (2018). SPM-1-producing *Pseudomonas aeruginosa* ST277 clone recovered from microbiota of migratory birds. *Diagn. Microbiol. Infect. Dis.* 90, 221–227. doi: 10.1016/j.diagmicrobio.2017.11.003

McMackin, E. A. W., Djapgne, L., Corley, J. M., and Yahr, T. L. (2019). Fitting pieces into the puzzle of *Pseudomonas aeruginosa* type III secretion system gene expression. *J. Bacteriol.* 201, e00209–e00219. doi: 10.1128/JB.00209-19

Miranda, C. C., de Filippis, I., Pinto, L. H., Coelho-Souza, T., Bianco, K., Cacci, L. C., et al. (2015). Genotypic characteristics of multidrug-resistant *Pseudomonas aeruginosa* from hospital wastewater treatment plant in Rio de Janeiro, Brazil. *J. Appl. Microbiol.* 118, 1276–1286. doi: 10.1111/jam.12792

Narciso, A. C., Martins, W., Almeida, L., Cayô, R., Santos, S. V., Ramos, P. L., et al. (2020). Healthcare-associated carbapenem-resistant OXA-72-producing *Acinetobacter baumannii* of the clonal complex CC79 colonizing migratory and captive aquatic birds in a Brazilian zoo. *Sci. Total Environ.* 726:138232. doi: 10.1016/j.scitotenv.2020.138232

Nascimento, T., Cantamessa, R., Melo, L., Fernandes, M. R., Fraga, E., Dropa, M., et al. (2017). International high-risk clones of *Klebsiella pneumoniae* KPC-2/CC258 and *Escherichia coli* CTX-M-15/CC10 in urban lake waters. *Sci. Total Environ.* 598, 910–915. doi: 10.1016/j.scitotenv.2017.03.207

Nascimento, A. P. B., Ortiz, M. F., Martins, W. M. B. S., Morais, G. L., Fehlberg, L. C. C., Almeida, L. G. P., et al. (2016). Intraclonal genome stability of the metallo-β-lactamase SPM-1-producing *Pseudomonas aeruginosa* ST277, an endemic clone disseminated in Brazilian hospitals. *Front. Microbiol.* 7:1946. doi: 10.3389/fmicb.2016.01946

Nisly, S. A., McClain, D. L., Fillius, A. G., and Davis, K. A. (2020). Oral antibiotics for the treatment of gram-negative bloodstream infections: a retrospective comparison of three antibiotic classes. *J. Glob. Antimicrob. Resist.* 20, 74–77. doi: 10.1016/j.jgar.2019.07.026

Oliveira, S., Moura, R. A., Silva, K. C., Pavez, M., McCulloch, J. A., Dropa, M., et al. (2014). Isolation of KPC-2-producing *Klebsiella pneumoniae* strains belonging to the high-risk multiresistant clonal complex 11 (ST437 and ST340) in urban rivers. *J. Antimicrob. Chemother.* 69, 849–852. doi: 10.1093/jac/dkt431

Pang, Z., Raudonis, R., Glick, B. R., Lin, T. J., and Cheng, Z. (2019). Antibiotic resistance in *Pseudomonas aeruginosa*: mechanisms and alternative therapeutic strategies. *Biotechnol. Adv.* 37, 177–192. doi: 10.1016/j.biotechadv.2018.11.013

Pliska, N. N. (2020). Pseudomonas aeruginosa as the main causative agent of osteomyelitis and its susceptibility to antibiotics. *Drug Res.* 70, 280–285. doi: 10.1055/a-1150-2372

Polotto, M., Casella, T., de Lucca Oliveira, M. G., Rúbio, F. G., Nogueira, M. L., de Almeida, M. T. G., et al. (2012). Detection of *P. aeruginosa* harboring $bla_{CTX-M-2}$, bla_{GES-1} and bla_{GES-5}, bla_{IMP-1} and bla_{SPM-1} causing infections in Brazilian tertiary-care hospital. *BMC Infect. Dis.* 12:176. doi: 10.1186/1471-2334-12-176

Popa, L. I., Gheorghe, I., Barbu, I. C., Surleac, M., Paraschiv, S., Măruţescu, L., et al. (2021). Multidrug resistant *Klebsiella pneumoniae* ST101 clone survival chain from inpatients to hospital effluent after chlorine treatment. *Front. Microbiol.* 11:610296. doi: 10.3389/fmicb.2020.610296

Rosales-Reyes, R., Vargas-Roldán, S. Y., Lezana-Fernández, J. L., and Santos-Preciado, J. I. (2021). *Pseudomonas aeruginosa*: genetic adaptation, a strategy for its persistence in cystic fibrosis. *Arch. Med. Res.* 52, 357–361. doi: 10.1016/j.arcmed.2020.12.004

Sarges, E. D. S. N., Rodrigues, Y. C., Furlaneto, I. P., de Melo, M. V. H., Brabo, G. L. D. C., Lopes, K. C. M., et al. (2020). Type III secretion system virulotypes and their association with clinical features of cystic fibrosis patients. *Infect. Drug Resist.* 13, 3771–3781. doi: 10.2147/IDR.S273759

Silveira, M., Albano, R., Asensi, M., and Assef, A. P. C. (2014). The draft genome sequence of multidrug-resistant *Pseudomonas aeruginosa* strain CCBH4851,

a nosocomial isolate belonging to clone SP (ST277) that is prevalent in Brazil. *Mem. Inst. Oswaldo Cruz* 109, 1086–1087. doi: 10.1590/0074-0276140336

Silveira, M. C., Rocha-de-Souza, C. M., Albano, R. M., de Oliveira Santos, I. C., and Carvalho-Assef, A. P. D. (2020). Exploring the success of Brazilian endemic clone *Pseudomonas aeruginosa* ST277 and its association with the CRISPR-Cas system type I-C. *BMC Genomics* 21:255. doi: 10.1186/s12864-020-6650-9

Tacconelli, E., Carrara, E., Savoldi, A., Harbarth, S., Mendelson, M., Monnet, D. L., et al. (2018). Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. *Lancet Infect. Dis.* 18, 318–327. doi: 10.1016/S1473-3099(17)30753-3

Toleman, M. A., Simm, A. M., Murphy, T. A., Gales, A. C., Biedenbach, D. J., Jones, R. N., et al. (2002). Molecular characterization of SPM-1, a novel metallo-beta-lactamase isolated in Latin America: report from the SENTRY antimicrobial surveillance programme. *J. Antimicrob. Chemother.* 50, 673–679. doi: 10.1093/jac/dkf210

Tsai, C. J., Loh, J. M., and Proft, T. (2016). *Galleria mellonella* infection models for the study of bacterial diseases and for antimicrobial drug testing. *Virulence* 7, 214–229. doi: 10.1080/21505594.2015.1135289

Tümmler, B. (2019). Emerging therapies against infections with *Pseudomonas aeruginosa*. *F1000Res.* 8:1371. doi: 10.12688/f1000research.19509.1

Turano, H., Gomes, F., Medeiros, M., Oliveira, S., Fontes, L. C., Sato, M. I. Z., et al. (2016). Presence of high-risk clones of OXA-23-producing *Acinetobacter baumannii* (ST79) and SPM-1-producing *Pseudomonas aeruginosa* (ST277) in environmental water samples in Brazil. *Diagn. Microbiol. Infect. Dis.* 86, 80–82. doi: 10.1016/j.diagmicrobio.2016.06.005

Van Nguyen, K., Nguyen, T. V., Nguyen, H. T. T., and Van Le, D. (2018). Mutations in the *gyrA*, *parC*, and *mexR* genes provide functional insights into the fluoroquinolone-resistant *Pseudomonas aeruginosa* isolated in Vietnam. *Infect. Drug Resist.* 11, 275–282. doi: 10.2147/IDR.S147581

Xu, C., Wang, D., Zhang, X., Liu, H., Zhu, G., Wang, T., et al. (2020). Mechanisms for rapid evolution of carbapenem resistance in a clinical isolate of *Pseudomonas aeruginosa*. *Front. Microbiol.* 11:1390. doi: 10.3389/fmicb.2020.01390

Yamane, K., Rossi, F., Barberino, M. G., Adams-Haduch, J. M., Doi, Y., and Paterson, D. L. (2008). 16S ribosomal RNA methylase RmtD produced by *Klebsiella pneumoniae* in Brazil. *J. Antimicrob. Chemother.* 61, 746–747. doi: 10.1093/jac/dkm526

Zhao, H., Xia, B., Fan, C., Zhao, P., and Shen, S. (2012). Human health risk from soil heavy metal contamination under different land uses near dabaoshan mine, southern China. *Sci. Total Environ.* 417–418, 45–54. doi: 10.1016/j.scitotenv.2011.12.047

Zubris, D., Minbiole, K., and Wuest, W. (2017). Polymeric quaternary ammonium compounds: versatile antimicrobial materials. *Curr. Top. Med. Chem.* 17, 305–318. doi: 10.2174/1568026616666160829155805

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Secrets of the Hospital Underbelly: Patterns of Abundance of Antimicrobial Resistance Genes in Hospital Wastewater Vary by Specific Antimicrobial and Bacterial Family

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Background: Hospital wastewater is a major source of antimicrobial resistance (AMR) outflow into the environment. This study uses metagenomics to study how hospital clinical activity impacts antimicrobial resistance genes (ARGs) abundances in hospital wastewater.

Methods: Sewage was collected over a 24-h period from multiple wastewater collection points (CPs) representing different specialties within a tertiary hospital site and simultaneously from community sewage works. High throughput shotgun sequencing was performed using Illumina HiSeq4000. ARG abundances were correlated to hospital antimicrobial usage (AMU), data on clinical activity and resistance prevalence in clinical isolates.

Results: Microbiota and ARG composition varied between CPs and overall ARG abundance was higher in hospital wastewater than in community influent. ARG and microbiota compositions were correlated (Procrustes analysis, $p = 0.014$). Total antimicrobial usage was not associated with higher ARG abundance in wastewater. However, there was a small positive association between resistance genes and antimicrobial usage matched to ARG phenotype (IRR 1.11, CI 1.06–1.16, $p < 0.001$). Furthermore, analyzing carbapenem and vancomycin resistance separately indicated that counts of ARGs to these antimicrobials were positively associated with their increased usage [carbapenem rate ratio (RR) 1.91, 95% CI 1.01–3.72, $p = 0.07$, and vancomycin RR 10.25, CI 2.32–49.10, $p < 0.01$]. Overall, ARG abundance within hospital wastewater did not reflect resistance patterns in clinical isolates from concurrent hospital inpatients. However, for clinical isolates of the family *Enterococcaceae* and *Staphylococcaceae*, there was a positive relationship with wastewater ARG abundance [odds ratio (OR) 1.62, CI 1.33–2.00, $p < 0.001$, and OR 1.65, CI 1.21–2.30, $p = 0.006$ respectively].

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Conclusion: We found that the relationship between hospital wastewater ARGs and antimicrobial usage or clinical isolate resistance varies by specific antimicrobial and bacterial family studied. One explanation, we consider is that relationships observed from multiple departments within a single hospital site will be detectable only for ARGs against parenteral antimicrobials uniquely used in the hospital setting. Our work highlights that using metagenomics to identify the full range of ARGs in hospital wastewater is a useful surveillance tool to monitor hospital ARG carriage and outflow and guide environmental policy on AMR.

Keywords: antimicrobial resistance, metagenomics, hospital waste water, surveillance, environmental risk, resistance dissemination, antibiotic usage

INTRODUCTION

In response to the antimicrobial resistance (AMR) crisis, a challenge for the research and medical communities is understanding the flow of AMR between different environmental niches (Woolhouse et al., 2015) and deciding where to focus surveillance and interventions to inform effective policies and action (Laxminarayan et al., 2016). There is an increasing interest in the contribution of hospital wastewater to AMR in the environment. Sewage treatment does not completely eradicate antimicrobial resistance genes (ARGs) and thus ARGs can enter the food chain through water and the use of sewage sludge in agriculture (Woolhouse and Ward, 2013; Woolhouse et al., 2015). As a complex matrix representing human bodily waste the potential of community sewage as a surveillance tool to monitor the global epidemiology of AMR has recently been explored (Hendriksen et al., 2019; Aarestrup and Woolhouse, 2020).

Hospitals are epidemiologically important nodal points for concentrated antimicrobial consumption and are sources of resistant pathogens (Versporten et al., 2018). Secondary care surveillance, guided by national and international policies, is based on passive reporting of phenotypic and molecular laboratory results for specific pathogens or from screening samples on specific high risk patients (Tornimbene et al., 2018; Department of Health and Social Care, 2019). These methods do not represent the full impact of antimicrobial use and inpatient activity on AMR carriage within a hospital and thus risk of transmission. Nor do they capture all pertinent ARGs. As hospital wastewater contains inpatient bodily waste, we hypothesized that it could be used as a representation of hospital inpatient carriage of AMR and as such may be a useful surveillance tool.

Many previous studies have identified key pathogens and resistant genes in hospital wastewater and attempts have been made to correlate resistance of specific organisms from hospital clinical isolates with hospital wastewater isolates with conflicting results (Talebi et al., 2008; Tuméo et al., 2008; Yang et al., 2009; Santoro et al., 2012; Drieux et al., 2016; Maheshwari et al., 2016). In this study, we apply the technique of metagenomics to hospital waste water (Hendriksen et al., 2019), with cross-sectional sampling of waste water from different hospital departments. The use of metagenomics in hospital waste water is increasingly applied to understand the resistance profile of hospitals (Subirats et al., 2016; Rowe et al., 2017; Ekwanzala et al., 2020; Petrovich et al., 2020; Kutilova et al., 2021). Combining metagenomics and multiple sampling sites allowed us to test hypotheses about what factors may drive patterns in resistance abundance in hospital waste water. We investigated whether clinical activity, such as antimicrobial usage and patient length of stay, impacts resistance abundance in hospital waste water. We also tested our hypothesis that resistance in hospital patients is correlated with the abundance of resistance genes within that department's waste water.

MATERIALS AND METHODS

Sewage Collection and Antibiotic Residue Analysis

Sampling was performed in June 2017 on eight wastewater collection points (CP) in the Western General Hospital, Edinburgh. Each sampling point represented a different clinical departments, identified to capture the effluent from the majority of the hospital (**Supplementary Figure S1**). No treatment was applied to hospital effluent prior to discharge into the main sewerage network. Using composite sampling machines, 100 ml of wastewater was sampled every 15 min over a 24-h period thus aiming to collect a representative sample of waste from the hospital inpatient population. Simultaneously, a 24-h time proportional sample was collected at the inflow site to Seafield community sewage works (hereafter "Seafield"), which serves a population equivalent of 760,000 from Edinburgh and the Lothians. Samples were transported from the site on dry ice and stored at −80°C. Antibiotic residue analysis was performed on 1 L of composite hospital wastewaters and 1 L of domestic sewage using LC-MS/MS as previously described (Berendsen et al., 2015; Hendriksen et al., 2019).

DNA Extraction and Analysis

DNA was extracted from sewage by pelleting using the QIAamp Fast DNA Stool mini kit with an optimized protocol as previously described (Knudsen et al., 2016) and sequenced on the HiSeq4000 platform (Illumina) using 2 × 150 bp paired-end sequencing. The concentrations of gDNA in

nanograms per microliter per sample measured by Qubit can be found in **Supplementary Table S2**. All samples used in this analysis met the minimum quality requirements genomic DNA biomass used by the sequencing firm BGI Genomics. The taxonomic origin of paired reads were assigned using Kraken2 (Wood and Salzberg, 2014) to the standard database, a database of representative bacterial genomes and a database of known vector sequences, UniVec_Core (downloaded 9th April 2019). Taxonomic assignments were summarized at the genus level using kraken-biom (Dabdoub, 2019). One sample, CP2, was heavily contaminated with *Pseudomonas*, likely from the *Pseudomonas fluorescens* species group. In CP2 52.9% of reads aligned to *Pseudomonas* genus OTUs, compared to 7.2% on average for other hospital sites. We therefore removed results from this site from further analysis. We used KMA version 1.2.12 (Clausen et al., 2018) to assign the paired and singleton reads to a database consisting of ResFinder reference genes (Zankari et al., 2012; downloaded 5th of September, 2019). KMA uses *k*-mer seeding followed by the Needleman-Wunsch sequence alignment algorithm to align the rest of the read from these *k*-mer seeds. ResFinder is a reference database of AMR genes. The following flags were used: "-mem_mode -ef -1t1 -cge -nf -shm 1 -t 1." Reads mapping to the human reference genome (GCA_000001405.15) were removed prior to submission to public sequence databases according to the protocol used in the Human Microbiome Project (Sherry, 2011; Human Microbiome Project, 2021).

Data Collection

Data was collected on clinical isolates from the week surrounding the hospital wastewater sampling to represent pathogens in hospital inpatients. All types of clinical isolate were included (including fecal, urine, skin, indwelling plastic, and fluid and tissue) but duplicate samples from the same patient within a 48-h period were excluded. Antimicrobial usage was collated from weekly pharmacy issues to each ward over the 3 months prior to sampling and presented as defined daily dose per 100 occupied bed days (DDD/100OBDs). Pharmacy issues for prescriptions for outpatient use and for theaters were excluded.

Data Analysis

All statistical analysis and plots were produced using R version 3.6.0. The abundance of ARGs and bacterial genera were calculated as Reads Per Kilobase of transcript per Million mapped bacterial reads (RPKM; Munk et al., 2018). This measure is frequently used for metagenomic data, and normalizes the read hit count with respect to the gene length in base pairs and the total number of bacterial reads. Principal coordinate analysis (PCoA; e.g., Borcard et al., 2018) was conducted on Bray-Curtis dissimilarity matrices were determined using Hellinger transformation of the RPKM. Resistance genes from the ResFinder database were grouped into clusters with 90% sequence homology. The top 50 ARGs were visualized using a heatmap and gene-wise and collection point dendrograms as previously described (Hendriksen et al., 2019). Procrustes analysis was used to test the association between the resistome and bacteriome dissimilarities.

Correlation Between Inpatient Activity and ARG Abundance

The source of variance in the abundance of ARGs between the collection points was investigated using a multilevel Poisson model with the dependent variable as counts of ARG reads at each collection point aggregated at the 90% homology cluster level. We used an offset term with the log of the average gene-length per cluster in the ResFinder database, multiplied by the total bacterial reads per collection point. Random effects of collection point, 70% sequence homology cluster, and observation were included in the model, the latter to model the over dispersion inherent to count data (Harrison, 2014).

In the main model, we accounted for co- and cross-resistance by fitting both a measure of direct selection for resistance (effect of department-level usage of antimicrobials on ARGs that confer resistance to those antimicrobials) and indirect selection [effect of total department-level antimicrobial usage (AMU) on ARG abundance]. In a second set of three models, we tested the association between resistance genes and antimicrobial usage of three specific antimicrobials of interest chosen to represent parenteral antimicrobials only used in a hospital setting (carbapenems, vancomycin) and an antimicrobial widely used in both community and hospital (amoxicillin). We use a Bonferroni correction on *p* values of these additional tests to account for increased risk of type I error. We used all antimicrobial resistance phenotypes suggested for any gene in a 90% homology cluster from either the ResFinder or STARAMR (National Microbiology Laboratory, 2021) databases. The average length of stay per department was also used to assess the role of clinical activity on sewage resistance abundance in the main model. The fixed effects structure of the main model was further adjusted using AIC minimizing methods, assessing whether any interaction effect should be included.

To assess the relationship between AMR in clinical isolates and ARG abundance in hospital wastewater a binomial generalized linear mixed effects model was used including random effects for site, the class of the antimicrobial used to test the isolates, and for the species of the isolate to control for inter-species heterogeneity. Two fixed effects were estimated for the log RPKM of all resistance genes in the sewage that had the same resistance phenotype as the isolates: one for isolates that were urinary or fecal, and a second for all other isolate types, due to the different dynamics of inpatient bodily waste being represented in the wastewater system. Using separate binomial regression models, we accounted for heterogeneity between the taxonomic family of the isolates in the relationship between AMR in clinical isolates and sewage ARGs. As some families were rarely tested, the sample size was too small for this heterogeneity to be assessed in a single model. Therefore, the three most frequently isolated families were assessed (*Enterobacteriaceae*, *Enterococcaceae*, and *Staphylococcaceae*), with the log RPKM of phenotypically matched resistance genes as the only model effect. A Bonferroni correction was used to adjust the *p* values of the effects of these models to account

for multiple testing. A similar model was used to evaluate the relationship between AMU and AMR in clinical isolates.

Ethics

This study was conducted following approval from NHS Lothian Research and Development Committee under the sponsorship of University of Edinburgh. There was no direct patient contact and therefore the study did not require ethical board approval.

RESULTS

The hospital departments served by the wastewater collection points differed by pattern of antimicrobial use (**Table 1**; **Supplementary Table S2**) and resistance in the 181 clinical isolates identified in the week surrounding wastewater sampling (**Supplementary Figure S3**).

Metagenomics of Wastewater

An average read pair count of 38.4 million (range 35.7–39.2 million) was obtained with an average of 62% (range 52–73%) of reads allocated to bacteria from the seven hospital wastewater samples and one community sewage sample.[1] An average of 0.25% of reads mapped to ARGs in the seven hospital wastewater samples vs. 0.1% from Seafield (**Supplementary Table S1**).

One thousand, one hundred and fifty-four unique bacterial genera were detected across all samples (range 1,151–1,154 genera per sample; **Supplementary Table S2**). The top 19 genera accounted for >70% of bacterial abundance in all samples (**Figure 1D**). The most predominant genera were *Pseudomonas* and *Acinetobacter*, mainly environmental species such as *Pseudomonas fluorescens*, *Acinetobacter johnsonii*, likely representing bacteria usually present in the hospital pipes. When compared with Seafield, there was a difference in diversity in the hospital samples with a higher predominance of gut associated bacteria including *Faecalibacterium*, *Bacteroides*, *Bifidobacterium*, and *Escherichia* (**Figures 1B,D**).

Antimicrobial resistance gene abundance and composition varied across different hospital collection points and Seafield (**Figures 1A,C, 2**; **Supplementary Figures S4, S6**). Apart from the wastewater collected at CP4, which represents the acute receiving unit with patients directly admitted from the community, ARG abundance from hospital wastewater was higher than ARG abundance in Seafield (**Figure 2**; **Supplementary Figure S4**). ARG composition was strongly correlated with bacterial genus level composition (Procrustes, $p = 0.014$; **Supplementary Figure S6**).

We detected 502 different resistance genes belonging to 10 different antimicrobial classes (**Supplementary Table S3**) but over 65% of the sample resistomes were composed of the 15 most abundant genes (**Supplementary Figure S6**), mainly belonging to the aminoglycoside and macrolide antimicrobial classes (**Figure 1C**). Key ARGs of interest to infection control including *bla*OXA, *bla*IMP, and genes of the *vanA* cluster were identified.

Inpatient Activity and ARG Abundance

No significant relationships were observed between total antimicrobial usage or length of stay and the abundance of ARGs in sewage (**Figure 3**; **Supplementary Table S5**). This result indicates there was no evidence for indirect selection or for the impact of transmission among hospital patients on ARG abundance in sewage when all resistance phenotypes were modeled. There was a significant positive effect of increased phenotypically-matched antimicrobial usage on resistance gene abundance, indicating support for a small role of direct selection (IRR 1.11, CI 1.06–1.16, $p < 0.001$). AIC comparison of fixed effect structures for the model indicated that no interaction effects improved model fit.

We next analyzed data on the association between carbapenem, vancomycin, and amoxicillin usage and ARGs conferring resistance to these specific antimicrobials in three separate models (**Figure 3A**; **Supplementary Table S5**). We found positive associations that were significant between vancomycin ARGs and vancomycin

[1]https://www.ebi.ac.uk/ena/data/view/PRJEB34410

TABLE 1 | Demographics of hospital collection points.

Collection point	Specialties	No. of wards	No. of pts	Average length of stay in days (SD)	Average age in years (SD)	DDD per 100 OBDs	No. of clinical isolates
CP1	Cardiology, Urology	3	46	4.9 (0.8)	62.6 (2.2)	123.7	19
CP3	Oncology, Hematology	7	67	3.7 (2.7)	62.1 (1.0)	200.5	27
CP4	Acute receiving unit	5	35	0.9 (0.7)	70.5 (2.3)	325.8	45
CP5	Neuroscience	3	59	3.3 (1.1)	53.5 (2.1)	73.5	8
CP6	Intensive care, Surgery, Medicine	3	70	7.6 (2.5)	66.6 (1.7)	223.8	17
CP7	Infectious Diseases, Surgery, Medicine	6	105	6.1 (3.2)	63.5 (0.8)	148.1	20
CP8	Respiratory, Medicine for the Elderly, Urology, Surgical High Dependency	6	133	12.8 (9.0)	69.0 (1.0)	116.4	25

SD only represents SD of the average age and length of stay per week. Antimicrobial usage from previous 3 months does not include antibiotics issued for outpatient prescriptions or in theaters. Clinical isolates are from inpatients in the week surrounding wastewater collection. pts, patients; DDD, defined daily dose; OBDs, occupied bed days; and SD, standard deviation.

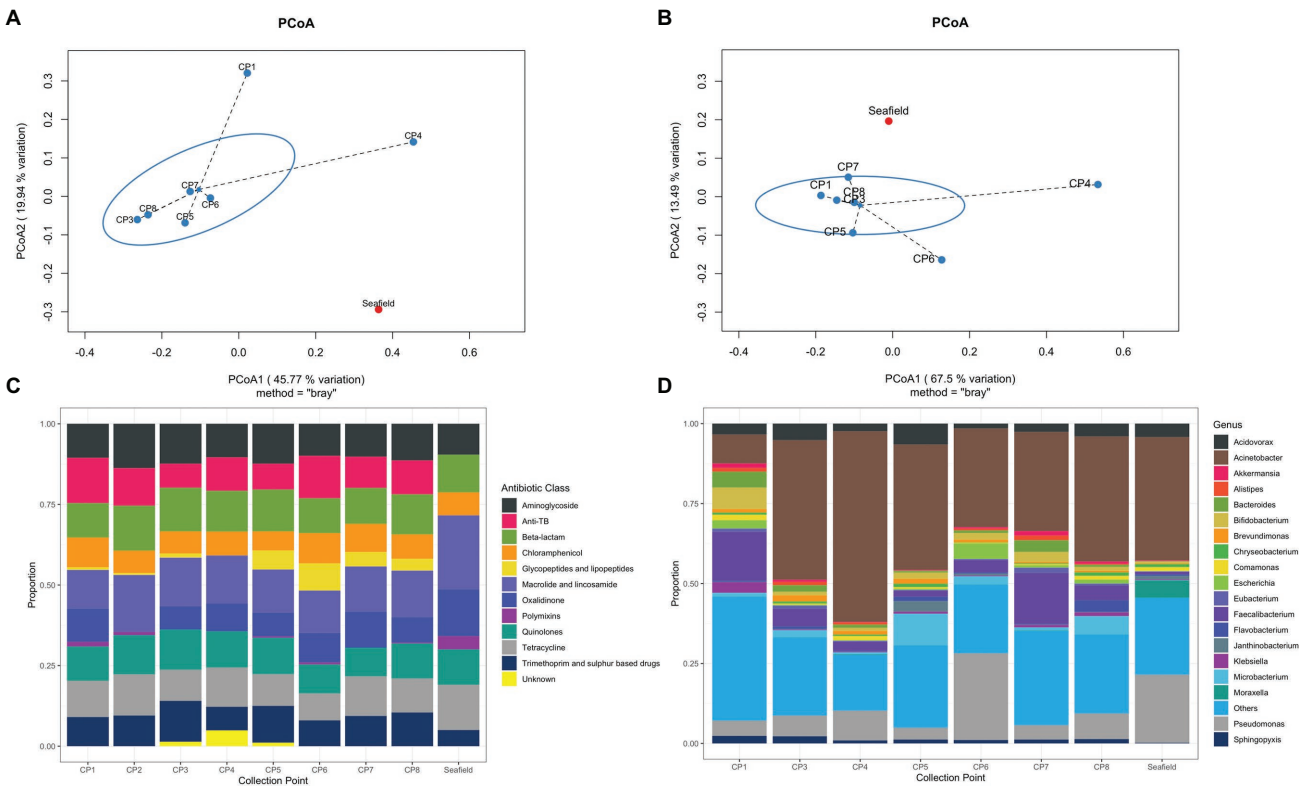

FIGURE 1 | Hospital wastewater and community sewage resistome and microbiome abundance composition. **(A)** Principal coordinate analyses (PCoA) of resistome based on Bray-Curtis dissimilarity. The percentage of variation explained is noted on the axis labels. **(B)** Principal coordinate analyses for the microbiome. **(C)** Relative abundance of antimicrobial resistance genes (ARGs) by antimicrobial class. **(D)** Relative abundance of the 19 most abundant bacterial genera in the wastewater and sewage microbiome. CP, collection point within hospital; Seafield, community sewage works; and TB, tuberculosis.

usage (IRR 10.25, CI 2.32–49.10, $p<0.001$) and showed a trend toward significance between carbapenem ARG abundance and carbapenem antimicrobial usage (IRR 1.91, CI 1.01–3.72, $p=0.07$). No evidence for an association between amoxicillin usage and amoxicillin ARGs was identified. We omitted the observation-level random effect from vancomycin model due to singular model fits, so overdispersion was not accounted for.

Antimicrobial resistance gene abundance at a class level within hospital wastewater did not reflect resistance patterns in clinical isolates when all the data was analyzed in one model (**Figure 3B**; **Supplementary Table S6**). There was no difference between the relationship of isolates from urine and fecal samples with ARG abundance and isolates from other sample types, e.g., skin, which we expect to enter the wastewater system at different rates *via* sinks and showers. We next separately modeled the three most frequently isolated taxonomic families (**Figure 3**; **Supplementary Table S6**). *Enterococcaceae* and *Staphylococcaceae* had a significant positive association with the abundance of ARGs conferring resistance to the same antimicrobial class (OR: 1.62, C.I. 1.32–2.00, $p<0.001$, and OR: 1.65, C.I. 1.21–2.30, $p<0.01$, respectively), but there was no such relationship for resistance levels in Enterobacteriaceae. At an antimicrobial class level, clinical isolate resistance did not reflect the antimicrobial usage of that class in the preceding 3 months (**Supplementary Table S5**).

Analysis of antibiotic residues reflected the high AMU within the hospital compared to the community with an average 12-fold increased residue concentration in hospital effluent (ranging between 4 and 13 μl^{-1}) for the five classes measured (**Supplementary Figure S7**). Our residue data only represents the residue levels from the whole hospital and not individual collection points and thus could not be specifically correlated with ARG abundance.

DISCUSSION

This study identified that hospital AMU impacts ARG abundances in hospital effluent, with implications upstream for infection control in the hospital and downstream for AMR in the environment. Overall, the distribution of bacterial genera and ARGs in our hospital wastewater samples and domestic sewage sample is similar to previously described sewage composition in European regions (Buelow et al., 2018; Hendriksen et al., 2019).

There was a significant positive relationship between inpatient department-level AMU and the abundance of antimicrobial resistance phenotype matched ARGs when all data was considered together. No relationship was found for total department AMU and ARG abundance. This supports a role of direct selection from antimicrobial usage in overall patterns of ARGs in hospital

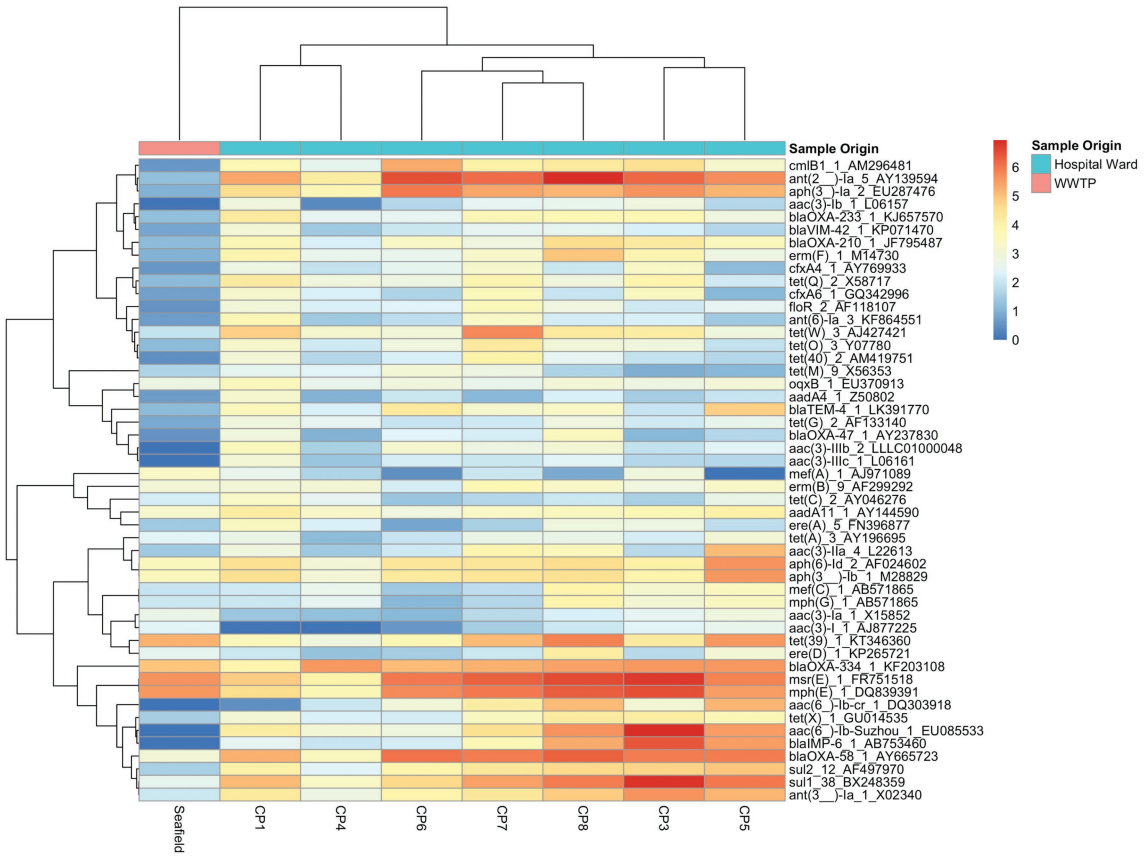

FIGURE 2 | Heat map of 50 most abundant ARGs. Relative abundance of ARGs (RPKM) were log transformed and both ARGs and CPs were clustered using complete-linkage clustering. For ARGs clustering was based on Pearson correlation coefficients, for collection points clustering was based on the BC-dissimilarity matrix (**Figure 1**) which uses all genes.

waste water, but not for indirect selection. Previous studies have found a relationship at a country level between antimicrobial residues and ARG abundance in sewage from the community (Hendriksen et al., 2019). Indeed, our data shows that the hospital antimicrobial residues for ciprofloxacin were around 9,900 µg/L, well above the estimated minimum selection concentration range for *Escherichia coli* and ciprofloxacin resistance of 5–10 µg/L (Kraupner et al., 2018).

The association between phenotype-matched ARGs and AMU was weak. Sewage captures resistance acquired in both the community and in the hospital, but drivers of hospital- and community-acquired resistance differ. For example, amoxicillin is used in both the community and hospitals, and resistance is widespread in the United Kingdom (60% hospital isolates resistant to amoxicillin or ampicillin in 2019; European Centre for Disease Prevention and Control, 2020), suggesting patients be more likely to arrive in hospital with carriage of amoxicillin resistance genes. The acquisition of vancomycin or carbapenem resistance, on the other hand, is associated with prior use of these antibiotics in hospital (Vasilakopoulou et al., 2020; Zhao et al., 2021), and these antibiotics are solely used parenterally in a hospital setting. Factors affecting within-hospital selection for and transmission of resistance, such as hospital antimicrobial usage, may play a stronger role in patterns of ARGs of vancomycin and carbapenems in hospital waste water than the ubiquitously used antibiotic amoxicillin. In support of this theory, we found a positive relationship between AMU and waste water ARGs for vancomycin and carbapenems, but not amoxicillin. Where a particular ward or department consumes high levels of carbapenem or vancomycin then this work demonstrates that there could be high levels of undetected fecal or urinary carriage of carbapenem and vancomycin resistance genes. This could warrant more stringent isolation of these patients, in fitting with concerns about "unsampled transmission chains" in carbapenem-resistant *Enterobacteriaceae* (Cerqueira et al., 2017). In addition, if the 70% renal excretion of unchanged meropenem (Mouton and van den Anker, 1995) selects for resistant organisms in waste water, then procedures for treatment of the bodily waste of patients on meropenem may need to be reconsidered. However, it is important to note that we cannot conclude from this study whether selection for resistance may take place within patients in the hospital or in hospital waste water and whether transfer could be plasmid mediated. Further studies that sample longitudinally from patients and hospital waste water would be required to determine routes and mechanisms of selection for resistance in hospitals and wastewater systems.

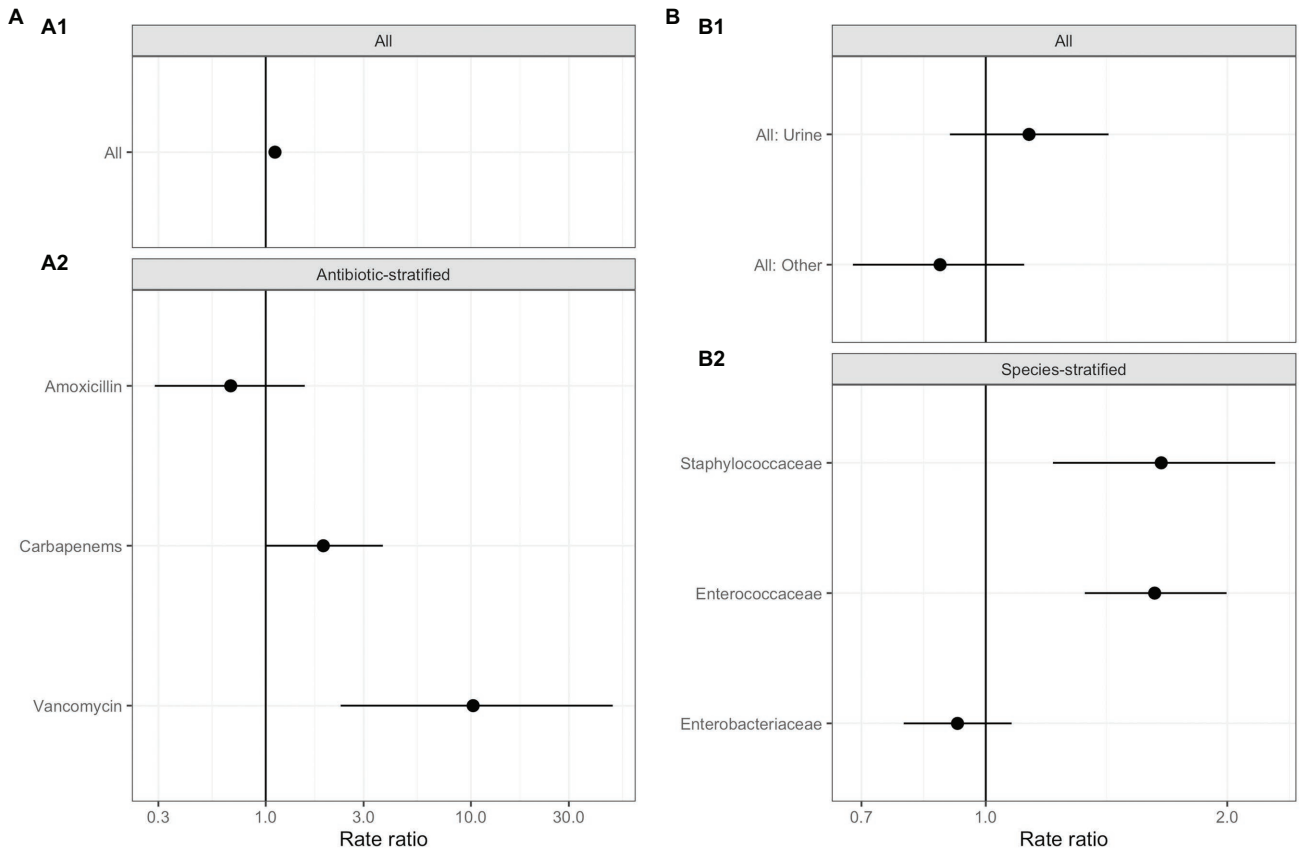

FIGURE 3 | Generalized linear mixed effects models for the relationship between antimicrobial resistance gene abundance, hospital department antibiotic consumption rates, and hospital department rates of resistance in clinical isolates. **(A)** Effect of antimicrobial usage (AMU) measured in defined daily dose per 100 occupied bed days (DDD/100 OBDs) on ARG abundance. **(A1)** The main model, with a single coefficient for all resistance phenotypes. **(A2)** Separate models with coefficients for each antimicrobial. **(B)** Association between antimicrobial resistance gene abundance in the sewage and clinical resistance rates. **(B1)** Main model, with a single coefficient for all clinical isolate taxonomic family, stratified by sample type – urine or fecal samples (All: Urine), and for resistance genes and any other sample source (All: Other). **(B2)** Separate models with coefficients for each isolate taxonomic family.

Length of stay did not impact ARG abundance in this dataset, despite prolonged duration of inpatient stay being a risk factor for carriage and infection with resistant microorganisms in previous studies (Safdar and Maki, 2002; Gupta et al., 2011; Founou et al., 2018). This appears not to support the theory of transmission of antimicrobial resistant organisms among patients and their local environment, including from the hospital water system (Kotay et al., 2017), during their inpatient stay. However, as these data were aggregated at the department-level there were few observations of length of stay, and further research with a greater sample size is needed to investigate this relationship.

Metagenomics can capture ARGs carried by a wide variety of bacterial genera, which is of benefit as the majority of ARGs are carried by non-pathogenic commensal bacteria (Sommer et al., 2009). Although, short-read sequencing cannot conclusively resolve associations between bacteria and ARGs, in our results ARGs are highly correlated with the bacteria identified at that collection point (**Supplementary Figure S7**). This can explain why abundance of ARGs for aminoglycosides, tetracyclines, and macrolides are higher than expected given lower proportions of phenotypic resistance in clinical isolates; the composition of bacterial genera within wastewater may have intrinsic or high levels of resistance to these antimicrobial classes. The potential for transfer of ARGs within the sewage network onto and between human pathogens has been demonstrated indicating the benefit of obtaining a universal view of ARGs (Ludden et al., 2017).

No quantitative relationship was observed between clinical isolates and ARG abundance in hospital wastewater when all data was considered together. In addition, there was no relationship between AMU in the previous 3 months and resistance in clinical isolates. This may be because clinical isolates are not representative enough of carriage of resistance in the inpatient population as there is a low rate of culture positivity. However, when examined separately, there was a positive relationship between resistance in *Enterococcaceae* or *Staphylococcaceae*, but not *Enterobactericeae*, and hospital wastewater ARG abundance. The literature on these relationships is divided (Talebi et al., 2008; Tuméo et al., 2008; Yang et al., 2009; Zarfel et al., 2013; Ory et al., 2016; Hutinel et al., 2019) and future work on antimicrobial usage, specific organisms,

isolate types, and ARG abundance in sewage potentially over a longer time period is required to interrogate these relationships further (Rogues et al., 2007; Mladenovic-Antic et al., 2016).

There was a higher abundance of ARGs in all hospital wastewater samples, bar one (CP4), which represents acute admissions unit, compared to Seafield. The lower abundance in Seafield could be due to dilution, and a decline in the relative abundance of AMR-gene carrying human commensal bacteria in the environment of sewerage system (Pehrsson et al., 2016), or possibly lower exposure to antimicrobial residues in community waste water. Associations between antimicrobial residues in community waste water and ARGs have been found (Hendriksen et al., 2019; Ju et al., 2019), and hospital waste water has been previously shown to have higher antimicrobial residue levels (Booth et al., 2020). Some studies comparing sewage influent in paired communities with and without a hospital have found minimal effect of a hospital on community influent (Buelow et al., 2018; Gouliouris et al., 2019). In other work, comparing resistance in hospital and community waste water has indicated some associations (Rogues et al., 2007; Pehrsson et al., 2016; Ludden et al., 2017), although, not all studies making this comparison have found evidence for a relationship (Paulshus et al., 2019).

Concern has been raised about the impact of hospital wastewater on urban influent and effluent and specific water treatments for hospital wastewater have been called for. This work highlights that physicians could consider prescribing environmentally degradable antimicrobials such as beta-lactams over antimicrobials, which have persistent residues across environmental niches e.g., tetracycline to minimize the impact of antimicrobials on the environmental resistome (Wellington et al., 2013). The ultimate effect of environmental ARGs on human disease is an ongoing important research question (Bürgmann et al., 2018).

The use of metagenomics is a key strength of this study, allowing quantification of resistance genes to a wide range of antibiotics and retrospective investigation if new resistance genes emerge. The 24-h composite samplers provide a representative sample of the hospital (Chau et al., 2020), although hospital staff, outpatients, and visitors will have also contributed to the effluent. In addition, some patients will have moved around the hospital during the sampling period. Although, this study is limited to one hospital site at one time point the variation in antimicrobial use and inpatient characteristics in each department has allowed us to treat them as discrete treatment centers and draw conclusions about factors affecting ARG abundance.

There is little doubt that hospital resistant pathogens can be abundant in wastewater systems (Maheshwari et al., 2016; Ludden et al., 2017; Gouliouris et al., 2019). However, using metagenomic sequencing, we show that resistance in hospital wastewater may quantitatively reflect clinical isolate resistance for some bacterial species (*Enterococcaceae* and *Staphylococcaceae*), although not all. As a surveillance tool this novel technique can represent the burden of AMR carriage in hospital inpatients and hospital pipes for specific resistance genes relating to important parenteral antimicrobials such as carbapenems and vancomycin. It may also aid in identification of emerging patterns of ARG abundance and novel ARGs, and how they may relate to changing patterns of transmission, infection control policies, and antimicrobial usage. Further longitudinal work evaluating the wastewater from multiple hospital sites is needed to establish AMU/ARG relationships, optimal collection points and sampling methods to be able to develop this as a surveillance technique.

In conclusion, we show in a multi-departmental study that the relationships between ARG abundance in hospital wastewater and hospital AMU or clinical resistance levels may vary by antimicrobial type and bacterial species. Our study emphasizes in a novel way the ARG burden from the high antimicrobial consuming and high resistance carriage environment of the hospital and that promoting active antimicrobial stewardship, particularly of key parenteral antimicrobials such as carbapenems and vancomycin, would impact the burden of environmental AMR. Hospital wastewater is an important source of AMR into the environment; this should be considered in environmental policy to reduce the flow of AMR between different environmental reservoirs.

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.ebi.ac.uk/ena, PRJEB34410.

ETHICS STATEMENT

This study was conducted following approval from NHS Lothian Research and Development Committee under the sponsorship of University of Edinburgh. There was no direct patient contact and therefore the study did not require ethical board approval.

AUTHOR CONTRIBUTIONS

MP conceived the project and developed it with input from BB, HL, FA, and MW. MP facilitated sampling and DNA extraction with AW. PK, CP, and BM provided clinical and pharmaceutical databases and input. AH performed antibiotic residue analysis. HL, BB, LM, BW, PM, and MP performed bio-informatics analyses with input from FA and MW. MP and HL drafted the manuscript with input from BB and review and comments from all authors. 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.2021.703560/full#supplementary-material

REFERENCES

Aarestrup, F. M., and Woolhouse, M. E. J. (2020). Using sewage for surveillance of antimicrobial resistance. *Science* 367, 630–632. doi: 10.1126/science.aba3432

Berendsen, B. J. A., Wegh, R. S., Memelink, J., Zuidema, T., and Stolker, L. A. M. (2015). The analysis of animal faeces as a tool to monitor antibiotic usage. *Talanta* 132, 258–268. doi: 10.1016/j.talanta.2014.09.022

Booth, A., Aga, D. S., and Wester, A. L. (2020). Retrospective analysis of the global antibiotic residues that exceed the predicted no effect concentration for antimicrobial resistance in various environmental matrices. *Environ. Int.* 141:105796. doi: 10.1016/j.envint.2020.105796

Borcard, D., Gillet, F., and Legendre, P. (2018). "Unconstrained ordination," in *Numerical Ecology With R Use R!* eds. R. Gentleman, K. Hornik and G. Parmigiani (Cham: Springer International Publishing), 151–201.

Buelow, E., Bayjanov, J. R., Majoor, E., Willems, R. J. L., Bonten, M. J. M., Schmitt, H., et al. (2018). Limited influence of hospital wastewater on the microbiome and resistome of wastewater in a community sewerage system. *FEMS Microbiol. Ecol.* 94:fiy087. doi: 10.1093/femsec/fiy087

Bürgmann, H., Frigon, D., Gaze, W. H., Manaia, C. M., Pruden, A., Singer, A. C., et al. (2018). Water and sanitation: an essential battlefront in the war on antimicrobial resistance. *FEMS Microbiol. Ecol.* 94. doi: 10.1093/femsec/fiy101

Cerqueira, G. C., Earl, A. M., Ernst, C. M., Grad, Y. H., Dekker, J. P., Feldgarden, M., et al. (2017). Multi-institute analysis of carbapenem resistance reveals remarkable diversity, unexplained mechanisms, and limited clonal outbreaks. *Proc. Natl. Acad. Sci. U. S. A.* 114, 1135–1140. doi: 10.1073/pnas.1616248114

Chau, K. K., Barker, L., Sims, N., Budgell, E. B., Harriss, E., and Crook, D. W. (2020). Wastewater surveillance of antimicrobial resistance in human populations: a aystematic review. doi: 10.20944/preprints202010.0267.v1 [Epub ahead of print]

Clausen, P. T. L. C., Aarestrup, F. M., and Lund, O. (2018). Rapid and precise alignment of raw reads against redundant databases with KMA. *BMC Bioinformatics* 19:307. doi: 10.1186/s12859-018-2336-6

Dabdoub, S. (2019). Create BIOM-Format Tables (http://biom-format.org) from Kraken Output (http://ccb.jhu.edu/software/kraken/ and https://github.com/DerrickWood/kraken). Available at: https://github.com/smdabdoub/kraken-biom (Accessed May 01, 2019).

Department of Health and Social Care (2019). Tackling Antimicobiral Resistance 2019–2024: The UK's Five-Year National Action Plan. Available at: https://www.gov.uk/government/publications/uk-5-year-action-plan-for-antimicrobial-resistance-2019-to-2024 (Accessed May 01, 2019).

Drieux, L., Haenn, S., Moulin, L., and Jarlier, V. (2016). Quantitative evaluation of extended spectrum β-lactamase-producing *Escherichia coli* strains in the wastewater of a French teaching hospital and relation to patient strain. *Antimicrob. Resist. Infect. Control* 5, 1–5. doi: 10.1186/s13756-016-0108-5

Ekwanzala, M. D., Dewar, J. B., and Momba, M. N. B. (2020). Environmental resistome risks of wastewaters and aquatic environments deciphered by shotgun metagenomic assembly. *Ecotoxicol. Environ. Saf.* 197:110612. doi: 10.1016/j.ecoenv.2020.110612

European Centre for Disease Prevention and Control (2020). Antimicrobial resistance in the EU/EEA (EARS-Net): Annual Epidemiological Report 2019. Stockholm.

Founou, R. C., Founou, L. L., and Essack, S. Y. (2018). Extended spectrum beta-lactamase mediated resistance in carriage and clinical gram-negative ESKAPE bacteria: a comparative study between a district and tertiary hospital in South Africa. *Antimicrob. Resist. Infect. Control* 7, 1–11. doi: 10.1186/s13756-018-0423-0

Gouliouris, T., Raven, K. E., Moradigaravand, D., Ludden, C., Coll, F., Blane, B., et al. (2019). Detection of vancomycin-resistant *Enterococcus faecium* hospital-adapted lineages in municipal wastewater treatment plants indicates widespread distribution and release into the environment. *Genome Res.* 29, 626–634. doi: 10.1101/gr.232629.117

Gupta, N., Limbago, B. M., Patel, J. B., and Kallen, A. J. (2011). Carbapenem-resistant enterobacteriaceae: epidemiology and prevention. *Clin. Infect. Dis.* 53, 60–67. doi: 10.1093/cid/cir202

Harrison, X. A. (2014). Using observation-level randomeffects to model overdispersion in count data in ecology and evolution. *PeerJ* 2:e616. doi: 10.7717/peerj.616

Hendriksen, R. S., Munk, P., Njage, P., van Bunnik, B., McNally, L., Lukjancenko, O., et al. (2019). Global monitoring of antimicrobial resistance based on metagenomics analyses of urban sewage. *Nat. Commun.* 10:1124. doi: 10.1038/s41467-019-08853-3

Human Microbiome Project (2021). NIH Human Microbiome Project-Tools and Technology. Available at: https://www.hmpdacc.org/hmp/resources/ (Accessed July 30, 2019).

Hutinel, M., Huijbers, P. M. C., Fick, J., Åhrén, C., Larsson, D. G. J., and Flach, C. F. (2019). Population-level surveillance of antibiotic resistance in *Escherichia coli* through sewage analysis. *Euro Surveill.* 24, 1–11. doi: 10.2807/1560-7917.ES.2019.24.37.1800497

Ju, F., Beck, K., Yin, X., Maccagnan, A., McArdell, C. S., Singer, H. P., et al. (2019). Wastewater treatment plant resistomes are shaped by bacterial composition, genetic exchange, and upregulated expression in the effluent microbiomes. *ISME J.* 13, 346–360. doi: 10.1038/s41396-018-0277-8

Knudsen, B. E., Bergmark, L., Munk, P., Lukjancenko, O., Priemé, A., Aarestrup, F. M., et al. (2016). Impact of sample type and DNA isolation procedure on genomic inference of microbiome composition. *mSystems* 1, e00095–e00116. doi: 10.1128/msystems.00095-16

Kotay, S., Chai, W., Guilford, W., Barry, K., and Mathers, A. J. (2017). Spread from the sink to the patient: in situ study using green fluorescent (GFP)-expressing *Escherichia coli* to model bacterial dispersion from hand-washing sink-trap reservoirs. *Appl. Environ. Microbiol.* 83, e03327–e03416. doi: 10.1128/AEM.03327-16

Kraupner, N., Ebmeyer, S., Bengtsson-Palme, J., Fick, J., Kristiansson, E., Flach, C.-F., et al. (2018). Selective concentration for ciprofloxacin resistance in *Escherichia coli* grown in complex aquatic bacterial biofilms. *Environ. Int.* 116, 255–268. doi: 10.1016/j.envint.2018.04.029

Kutilova, I., Medvecky, M., Leekitcharoenphon, P., Munk, P., Masarikova, M., Davidova-Gerzova, L., et al. (2021). Extended-spectrum beta-lactamase-producing *Escherichia coli* and antimicrobial resistance in municipal and hospital wastewaters in Czech Republic: culture-based and metagenomic approaches. *Environ. Res.* 193:110487. doi: 10.1016/j.envres.2020.110487

Laxminarayan, R., Sridhar, D., Blaser, M., Wang, M., and Woolhouse, M. (2016). Achieving global targets for antimicrobial resistance. *Science* 353, 874–875. doi: 10.1126/science.aaf9286

Ludden, C., Reuter, S., Judge, K., Gouliouris, T., Blane, B., Coll, F., et al. (2017). Sharing of carbapenemase-encoding plasmids between enterobacteriaceae in UK sewage uncovered by MinION sequencing. *Microb. Genom.* 3:e000114. doi: 10.1099/mgen.0.000114

Maheshwari, M., Yaser, N. H., Naz, S., Fatima, M., and Ahmad, I. (2016). Emergence of ciprofloxacin-resistant extended-spectrum beta-lactamase-producing enteric bacteria in hospital wastewater and clinical sources. *J. Glob. Antimicrob. Resist.* 5, 22–25. doi: 10.1016/j.jgar.2016.01.008

Mladenovic-Antic, S., Kocic, B., Velickovic-Radovanovic, R., Dinic, M., Petrovic, J., Randjelovic, G., et al. (2016). Correlation between antimicrobial resistance and antimicrobial resistance of *Pseudomonas aeruginosa* in a hospital setting: a 10-year study. *J. Clin. Pharm. Ther.* 41, 532–537. doi: 10.1111/jcpt.12432

Mouton, J. W., and van den Anker, J. N. (1995). Meropenem clinical pharmacokinetics. *Clin. Pharmacokinet.* 28, 275–286. doi: 10.2165/00003088-199528040-00002

Munk, P., Knudsen, B. E., Lukjacenko, O., Duarte, A. S. R., Van Gompel, L., Luiken, R. E. C., et al. (2018). Abundance and diversity of the faecal resistome in slaughter pigs and broilers in nine European countries. *Nat. Microbiol.* 3, 898–908. doi: 10.1038/s41564-018-0192-9

National Microbiology Laboratory (2021). STARAMR. Available at: https://github.com/phac-nml/staramr (Accessed May 01, 2019).

Ory, J., Bricheux, G., Togola, A., Bonnet, J. L., Donnadieu-Bernard, F., Nakusi, L., et al. (2016). Ciprofloxacin residue and antibiotic-resistant biofilm bacteria in hospital effluent. *Environ. Pollut.* 214, 635–645. doi: 10.1016/j.envpol.2016.04.033

Paulshus, E., Kühn, I., Möllby, R., Colque, P., O'Sullivan, K., Midtvedt, T., et al. (2019). Diversity and antibiotic resistance among *Escherichia coli* populations in hospital and community wastewater compared to wastewater at the receiving urban treatment plant. *Water Res.* 161, 232–241. doi: 10.1016/j.watres.2019.05.102

Pehrsson, E. C., Tsukayama, P., Patel, S., Mejia-Bautista, M., Sosa-Soto, G., Navarrete, K. M., et al. (2016). Interconnected microbiomes and resistomes in low-income human habitats. *Nature* 533, 212–216. doi: 10.1038/nature17672

Petrovich, M. L., Zilberman, A., Kaplan, A., Eliraz, G. R., Wang, Y., Langenfeld, K., et al. (2020). Microbial and viral communities and their antibiotic resistance genes throughout a hospital wastewater treatment system. *Front. Microbiol.* 11:153. doi: 10.3389/fmicb.2020.00153

Rogues, A. M., Dumartin, C., Amadéo, B., Venier, A. G., Marty, N., Parneix, P., et al. (2007). Relationship between rates of antimicrobial consumption and the incidence of antimicrobial resistance in *Staphylococcus aureus* and *Pseudomonas aeruginosa* isolates from 47 French hospitals. *Infect. Control Hosp. Epidemiol.* 28, 1389–1395. doi: 10.1086/523280

Rowe, W. P. M., Baker-Austin, C., Verner-Jeffreys, D. W., Ryan, J. J., Micallef, C., Maskell, D. J., et al. (2017). Overexpression of antibiotic resistance genes in hospital effluents over time. *J. Antimicrob. Chemother.* 72, 1617–1623. doi: 10.1093/jac/dkx017

Safdar, N., and Maki, D. G. (2002). The commonality of risk factors for nosocomial colonization and infection with antimicrobial-resistant *Staphylococcus aureus*, enterococcus, gram-negative bacilli, clostridium difficile, and candida. *Ann. Intern. Med.* 136, 834–844. doi: 10.7326/0003-4819-136-11-200206040-00013

Santoro, D. O., Romao, C. M. C. A., and Clementino, M. M. (2012). Decreased aztreonam susceptibility among *Pseudomonas aeruginosa* isolates from hospital effluent treatment system and clinical samples. *Int. J. Environ. Health Res.* 22, 560–570. doi: 10.1080/09603123.2012.678000

Sherry, S. (2011). Human Sequence Removal. National Center for Biotechnology Information. Available at: https://www.hmpdacc.org/hmp/doc/HumanSequenceRemoval_SOP.pdf (Accessed May 01, 2019).

Sommer, M. O. A., Dantas, G., and Church, G. M. (2009). Functional characterization of the antibiotic resistance reservoir in the human microflora. *Science* 325, 1128–1131. doi: 10.1126/science.1176950

Subirats, J., Sànchez-Melsió, A., Borrego, C. M., Balcázar, J. L., and Simonet, P. (2016). Metagenomic analysis reveals that bacteriophages are reservoirs of antibiotic resistance genes. *Int. J. Antimicrob. Agents* 48, 163–167. doi: 10.1016/j.ijantimicag.2016.04.028

Talebi, M., Pourshafie, M. R., Katouli, M., and Mollby, R. (2008). Molecular structure and transferability of Tn1546-like elements in *Enterococcus faecium* isolates from clinical, sewage, and surface water samples in Iran. *Appl. Environ. Microbiol.* 74, 1350–1356. doi: 10.1128/AEM.02254-07

Tornimbene, B., Eremin, S., Escher, M., Griskeviciene, J., Manglani, S., and Pessoa-Silva, C. L. (2018). WHO global antimicrobial resistance surveillance system early implementation 2016-17. *Lancet Infect. Dis.* 18, 241–242. doi: 10.1016/S1473-3099(18)30060-4

Tuméo, E., Gbaguidi-Haore, H., Patry, I., Bertrand, X., Thouverez, M., and Talon, D. (2008). Are antibiotic-resistant *Pseudomonas aeruginosa* isolated from hospitalised patients recovered in the hospital effluents? *Int. J. Hyg. Environ. Health* 211, 200–204. doi: 10.1016/j.ijheh.2007.02.010

Vasilakopoulou, A., Karakosta, P., Vourli, S., Tarpatzi, A., Varda, P., Kostoula, M., et al. (2020). Gastrointestinal carriage of vancomycin-resistant enterococci and carbapenem-resistant gram-negative bacteria in an endemic setting: prevalence, risk factors, and outcomes. *Front. Public Health* 8:55. doi: 10.3389/fpubh.2020.00055

Versporten, A., Zarb, P., Caniaux, I., Gros, M. F., Drapier, N., Miller, M., et al. (2018). Antimicrobial consumption and resistance in adult hospital inpatients in 53 countries: results of an internet-based global point prevalence survey. *Lancet Glob. Health* 6, e619–e629. doi: 10.1016/S2214-109X(18)30186-4

Wellington, E. M. H., Boxall, A. B., Cross, P., Feil, E. J., Gaze, W. H., Hawkey, P. M., et al. (2013). The role of the natural environment in the emergence of antibiotic resistance in gram-negative bacteria. *Lancet Infect. Dis.* 13, 155–165. doi: 10.1016/S1473-3099(12)70317-1

Wood, D. E., and Salzberg, S. L. (2014). Kraken: ultrafast metagenomic sequence classification using exact alignments. *Genome Biol.* 15:R46. doi: 10.1186/gb-2014-15-3-r46

Woolhouse, M. E. J., and Ward, M. J. (2013). Sources of antimicrobial resistance. *Science* 341, 1460–1461. doi: 10.1126/science.1243444

Woolhouse, M. E. J., Ward, M., Van Bunnik, B., and Farrar, J. (2015). Antimicrobial resistance in humans, livestock and the wider environment. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 370:20140083. doi: 10.1098/rstb.2014.0083

Yang, C. M., Lin, M. F., Liao, P. C., Yeh, H. W., Chang, B. V., Tang, T. K., et al. (2009). Comparison of antimicrobial resistance patterns between clinical and sewage isolates in a regional hospital in Taiwan. *Lett. Appl. Microbiol.* 48, 560–565. doi: 10.1111/j.1472-765X.2009.02572.x

Zankari, E., Hasman, H., Cosentino, S., Vestergaard, M., Rasmussen, S., Lund, O., et al. (2012). Identification of acquired antimicrobial resistance genes. *J. Antimicrob. Chemother.* 67, 2640–2644. doi: 10.1093/jac/dks261

Zarfel, G., Galler, H., Feierl, G., Haas, D., Kittinger, C., Leitner, E., et al. (2013). Comparison of extended-spectrum-beta-lactamase (ESBL) carrying *Escherichia coli* from sewage sludge and human urinary tract infection. *Environ. Pollut.* 173, 192–199. doi: 10.1016/j.envpol.2012.09.019

Zhao, S., Perry, M. R., Kennedy, S., Wilson, J., Chase-Topping, M. E., Anderson, E., et al. (2021). Risk factors for carbapenemase-producing organisms among inpatients in Scotland: a national matched case–control study. *Infect. Control Hosp. Epidemiol.* 42, 968–977. doi: 10.1017/ice.2020.1351

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Extended-Spectrum Beta-Lactamase Producing-*Escherichia coli* Isolated From Irrigation Waters and Produce in Ecuador

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In cities across the globe, the majority of wastewater – that includes drug resistant and pathogenic bacteria among other contaminants – is released into streams untreated. This water is often subsequently used for irrigation of pastures and produce. This use of wastewater-contaminated streams allows antibiotic-resistant bacteria to potentially cycle back to humans through agricultural products. In this study, we investigated the prevalence of extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* isolated from produce and irrigation water across 17 provinces of Ecuador. A total of 117 vegetable samples, 119 fruit samples, and 38 irrigation water samples were analyzed. Results showed that 11% of the samples were positive for *E. coli* including 11 irrigation water samples (29%), and samples of 13 vegetables (11%), and 11 fruits (9%). Among the 165 *E. coli* isolates cultured, 96 (58%) had the ESBL phenotype, and 58% of ESBL producing *E. coli* came from irrigation water samples, 11% from vegetables, and 30% from fruits. The $bla_{\text{CTX–M–55}}$, $bla_{\text{CTX–M 65}}$, and $bla_{\text{CTX–M 15}}$ genes were the most frequently found gene associated with the ESBL phenotype and coincided with the $bla_{\text{CTX–M}}$ alleles associated with human infections in Ecuador. Three isolates had the mcr-1 gene which is responsible for colistin resistance. This report provides evidence of the potential role of irrigation water in the growing antimicrobial resistance crisis in Ecuador.

Keywords: fresh produce, irrigation water, ESBL *E. coli*, CTX-M, Extended-spectrum beta-lactamase (ESBL)

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INTRODUCTION

The rise of antimicrobial resistance (AMR) is one of the most serious biological threats facing modern society, and the inability to treat bacterial infections is already occurring in many nosocomial infections (Frieri et al., 2017). The World Health (WHO) has listed extended spectrum β-lactamase-producing Enterobacteriaceae (ESBL-E) as the most critical antimicrobial resistant microorganisms, among the "Highest Priority" pathogens due to the increasing prevalence in humans and livestock (Yassin et al., 2017; Shrivastava et al., 2018; Li et al., 2019; Murray et al., 2021).

Globally, the majority of wastewater produced by urban settlements goes into streams without prior treatment. Only 20% of produced wastewater receives proper treatment (UNESCO, 2012),

and the capacity to treat wastewater often depends on the income level of the country; treatment capacity is 70% of the generated wastewater in high-income countries, compared to ∼8% in low-income countries (Sato et al., 2013). This phenomenon is rising as urban populations grow and developing countries increasingly install pipes to channel wastewater away from communities, even before the development of wastewater treatment plants. The wastewater comes from diverse sources (e.g., homes, hospitals, and animal processing plants, etc.) and contains large quantities of antibiotic resistant bacteria (ARB), often carrying antimicrobial resistance to last-line antimicrobials, such as carbapenems (Lin et al., 2020).

These antimicrobial resistant bacteria (ARB) can cycle back to humans when wastewater-contaminated streams are used to irrigate produce or provide water to food animals (FAO and WHO, 2008; Leff and Fierer, 2013; Pigłowski, 2019); one recent example is the finding of New Delhi metallo-β-lactamases–type carbapenem-resistant *Escherichia coli* in water, domestic food animals, and humans (carbapenem, a last-line drug, is used exclusively in human medicine) (Li et al., 2019; Murray et al., 2021). Many antibiotic-resistant Enterobacterales, members of the intestinal microbiome (including *E. coli*), can survive and multiply in the environment (Vasco et al., 2015; Guerrero et al., 2020) and may colonize humans and domestic animals through the fecal-oral route of transmission. Plasmids and other mobile genetic elements (MGEs) carrying AMR genes promote the dissemination of AMR among intestinal bacteria in the intestine of vertebrates (Bonardi and Pitino, 2019), and this cycle is fundamentally captured in the One Health concept. Produce contamination can happen before pre-harvest (i.e., through contaminated irrigation water or manure fertilization) (Beuchat, 1996; Iwu and Okoh, 2019), as well as post-harvest (i.e., by washing, handling and processing food) with irrigation water (Murray et al., 2017).

Wastewater-impacted irrigation water has been identified as the main source of contamination for fresh produce with pathogenic microorganisms and ARB (Njage and Buys, 2015; Gekenidis et al., 2018a). The fecally contaminated produce can transfer ARB to the consumer especially when the produce is consumed fresh and uncooked (Pesavento et al., 2014; Araújo et al., 2017; Hölzel et al., 2018). Besides contributing to the spread of pathogens, irrigation water may potentially play a leading role in the dissemination of ARB (Moore et al., 2010; Hong et al., 2013; Gekenidis et al., 2018b; Vital et al., 2018).

The production of extended-spectrum β-lactamases (ESBL) is one of the most important mechanisms of antibiotic resistance in Enterobacteriaceae. ESBL genes can be divided into 4 groups: TEM, SHV, OXA, and CTX-M types (Bush and Jacoby, 2010); CTX-M type is the most prevalent of ESBLs described (Rossolini et al., 2008; Bevan et al., 2017). Enterobacteriaceae members are the most common bacterial agents causing foodborne outbreaks associated with the consumption of fresh produce (Cooper et al., 2007; Kilonzo-Nthenge et al., 2018; Al-Kharousi et al., 2019; McDaniel and Jadeja, 2019; Motlagh and Yang, 2019). Pathogenic *E. coli* is a key bacterium in foodborne illnesses, and commensal *E. coli* is a common indicator organism of fecal contamination in aquatic systems (Edberg et al., 2000; Rochelle-Newall et al., 2015; Motlagh and Yang, 2019). *E. coli* is also recognized as an important species in the spread of ARB, mainly due to a high aptitude to acquire genetic information through horizontal gene transfer (Grasselli et al., 2008; Hasegawa et al., 2018; Marlène et al., 2020).

In Ecuador, an upper middle-income country, wastewater is almost entirely released untreated into streams; these streams often serve as irrigation water for produce and food-animal agriculture (Ortega-Paredes et al., 2020a,b). There are few studies about the dissemination of ESBL-*E. coli* from irrigation water to produce (Ben Said et al., 2015; Vital et al., 2018); most of the studies have been carried out in fresh produce from retail centers and groceries (Bhutani et al., 2015; Faour-Klingbeil et al., 2016; Ortega-Paredes et al., 2018; Al-Kharousi et al., 2019; Yang et al., 2019; Colosi et al., 2020; Richter et al., 2020; Song et al., 2020). The aim of this study was to build upon the previous literature to understand the relationship between ARB in irrigation water and ARB on fresh produce obtaining samples from farms and their irrigation water. The study focused on the occurrence of extended spectrum β-lactamase producing *E. coli* in 17 provinces of Ecuador.

MATERIALS AND METHODS

Study Areas

This study was carried out in the following provinces of Ecuador: Manabí, Bolívar, Cañar, Loja, Guayas, Pastaza, Tungurahua, Pichincha, Azuay, Chimborazo, Cotopaxi, Imbabura, Santa Elena, Los Ríos, Morona Santiago, Orellana, and Zamora Chinchipe provinces which are mainly agrarian (**Figure 1**). The samples correspond to those that are collected as part of the national surveillance program that aims to monitor microbiological indicators and pathogens in the food supply ("Programa Nacional de Vigilancia de Microorganismos de Higiene y Control de Microorganismos Patógenos, para la Vigilancia Epidemiológica de Enfermedades Transmitidas por Alimentos de Origen Agrícola y Pecuario del país – PNVCH").

Sampling Fresh Produce

Fresh fruits and vegetables (representing 20 types) were obtained from agricultural farms in 17 provinces of Ecuador, from June to December 2019 (**Figure 1**). In total, 274 samples were analyzed (117 vegetables, 119 fruits were collected from agricultural farms. Among the vegetables consist of lettuce (*Lactuca sativa*, n = 43), onion (*Allium cepa*, n = 31), garlic (*Allium sativum*, n = 21), coriander (*Coriandrum sativum*, n = 17), cabbage (*Brassica oleracea* var. viridis, n = 2), spinach (*Spinacea oleracea*, n = 1), pepper (*Piper nigrum*, n = 1), tomato (*Solanum lycopersicum*, n = 1). The fruit samples correspond to cocoa (*Theobroma cacao*, n = 1), peach (*Prunus persica*, n = 2), strawberry (*Fregaria vesca*, n = 31), melon (*Cucumis melo* var. cantalupensis, n = 7), apple (*Malus domestica*, n = 1), banana (*Musa paradisiaca*, n = 13), blackberry (*Rubus ulmifolius*, n = 31), watermelon (*Citrullus lanatus*, n = 12), grape (*Vitis vinifera*, n = 1), and golden berry (*Physalis peruviana*, n = 20).

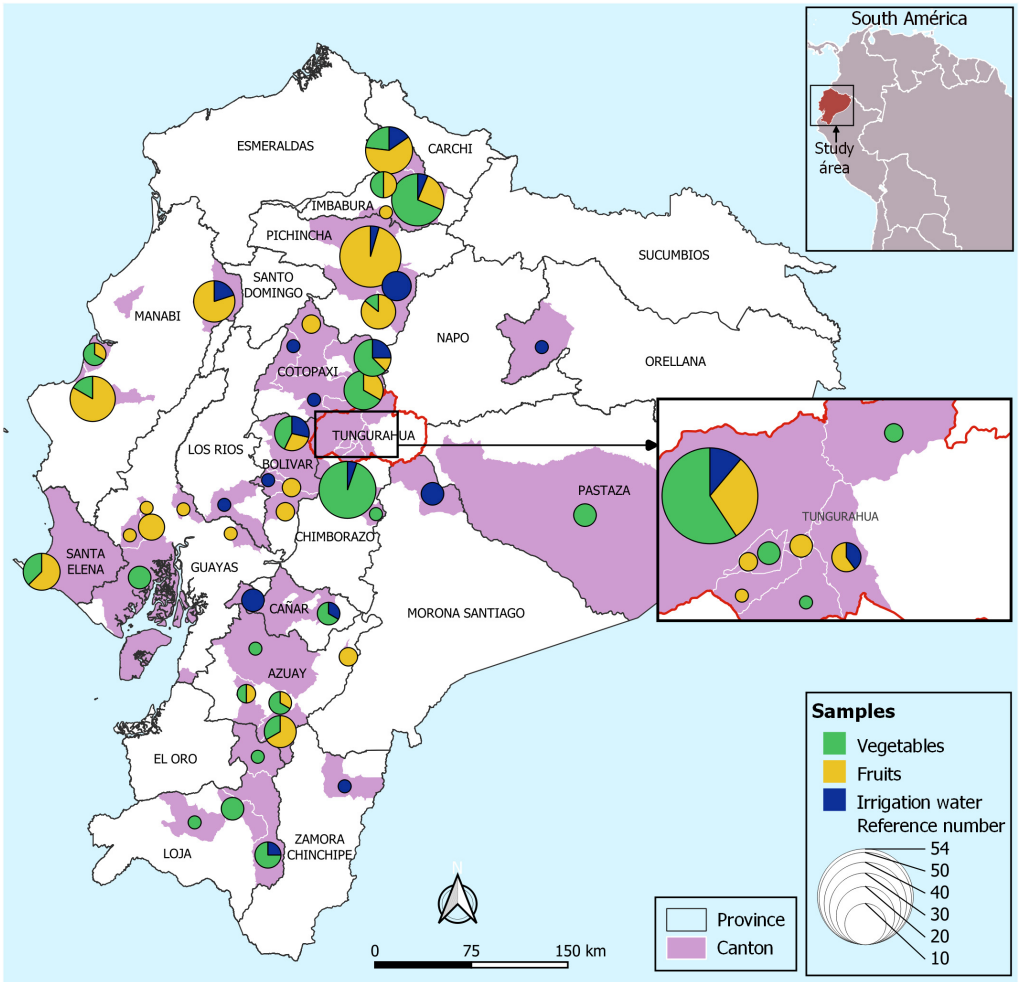

FIGURE 1 | Map of Ecuador showing the sampling locations. Map of sampling locations of irrigation water, fruits and fresh produce. The circles represent the total number of samples according to the color assigned to each sample (vegetables: green, fruits: yellow, and irrigation water: blue) collected in each canton (pink).

Isolation of *Escherichia coli* From Irrigation Water and Produce

The farmers of each crop indicated the irrigation water they used, and this water (n = 37) was collected in sterile bottles and transported to the laboratory at approximately 8°C and processed within 10 h. Five hundred milliliters of water were filtered using a 0.45 μm pore membrane filter (Millipore, United States). The filter was then incubated in Chromocult® coliform agar (Merck, Germany) overnight at 37°C, the apparent *E. coli* colonies were taken and seeded on MacConkey agar (Difco, United States) supplemented with ceftriaxone (2 mg/L) to identify the lactose positive colonies (a maximum of five colonies were picked from each plate) (Richter et al., 2020), colonies of presumptive *E. coli* were then tested for β-glucuronidase activity using Chromocult® medium (Merck, Germany). All *E. coli* confirmed isolates from each sample were kept frozen at −80°C in Tryptic Soy Broth medium (Difco, United States) with 15% glycerol.

The vegetable samples were collected aseptically and refrigerated until analysis (within 12 h). Ten grams of the fresh produce were weighed and placed in a sterile plastic bag and incubated with 90 ml of peptone water (Faour-Klingbeil et al., 2016) for 30 min at room temperature. In the case of fruits such as watermelon and melon, the surface was swabbed, and the swab was placed in peptone water (described above). The next day 100 μl of the liquid was taken and cultured on MacConkey agar (Difco, United States) supplemented with ceftriaxone (2 mg/L) (Botelho et al., 2015). A maximum of five lactose positive colonies were selected from each plate sample and placed on Chromocult coliform agar after 24 h of incubation at 37°C, colonies of presumptive *E. coli*, positive for β-glucuronidase, were selected for additional analyses (Lange et al., 2013). All isolates confirmed to be *E. coli* from each sample were kept frozen at −80°C in Tryptic Soy Broth medium (Difco, United States) with 15% glycerol.

Antimicrobial Susceptibility Testing

Susceptibility tests were performed using the Kirby-Bauer method on Mueller-Hinton agar (Difco, United States)

in accordance with Clinical and Laboratory Standards Institute (CLSI, 2019). Eleven antibiotics were used for testing and included: Cefazolin, CZ (30 μg); Ampicillin, AM (10 μg), Gentamicin, GM (10 μg), Imipenem, IPM (10 μg); Trimethropin-sulfamethoxazole, SXT (1.25/23.75 μg); Ceftazidime, CAZ (30 μg); Cefepime, FEP (30 μg); Ciprofloxacin, CIP (5 μg); Amoxicillin/Clavulanic acid, AmC (20/10μg); cefotaxime, CTX (30μg); and Tetracycline TE (30 μg). After 18 h of incubation, the *E. coli* strains were classified as susceptible, intermediate, or resistant according to the clinical interpretation criteria recommended by CLSI. *E. coli* ATCC 25922 was used as a quality control. To determine the ESBL phenotype, we carried out a diffusion disk method on Mueller Hinton agar as before using antibiotic susceptibility discs (Oxoid, United States) of CTX (30 μg), CAZ (30 μg). Our criterion to determine ESBL was CTX ≤ 27 mm; CAZ ≤ 22 mm (CLSI, 2019). Specifically, ESBL production was confirmed by growth in a medium with discs of ceftazidime (30 mcg) and ceftazidime + clavulanic acid (30 mcg + 10 mcg). An increase of ≥5 mm in zone of inhibition for ceftazidime + clavulanic acid compared to ceftazidime was confirmed as ESBL producers (CLSI, 2019).

PCR Amplification for Detection of β-Lactamase Genes

When samples were positive for ESBL-producing *E. coli*, one to five isolates selected per sample for further analysis. A total of 96 isolates were tested for the following resistance genes: bla_{SHV}, bla_{TEM}, bla_{CTX-M}, and bla_{OXA} (**Table 1**). Bacterial DNA was extracted by boiling (Dashti et al., 2009), and PCR amplification reactions were performed in a volume of 25 μl containing 12.5 μl of 2 × Qiagen Multiplex PCR Master Mix (Qiagen GmbH, Hilden, Germany), 0.2 μM concentrations of each primer, and 2 μl of DNA template. The cycling parameters were as follows: an initial denaturation at 95°C for 15 min; followed by 30 cycles of 94°C for 30 s, 62°C for 90 s, and 72°C for 60 s; and with a final extension at 72°C for 10 min. Amplification products were observed in agarose gel electrophoresis 1.5%, stained with Ethidium bromide at 100V for 45–60 min. The size of the amplified products was compared with the commercial (Invitrogen, United States) 100-bp ladder. The band size (bp) for each gene was: bla_{SHV}, 237; bla_{TEM}, 445; bla_{CTX-M}, 593; and bla_{OXA}: 813 (Fang et al., 2008).

DNA Sequencing and Analysis

Genomic DNA was extracted from the eighty isolates (including isolates of irrigation water, blackberry, strawberry, onion, banana, and garlic) using the Wizard® Genomic DNA Purification (Promega, United States) according to the manufacturer's instructions. The whole genome of isolates was sequenced using Illumina MiSeq. Sequencing was carried out at the University of Minnesota Mid-Central Research and Outreach Center (Willmar, Minnesota) using a single 2 × 250-bp dual-index run on an Illumina MiSeq with Nextera XT libraries to generate approximately 30- to 50-fold coverage per genome. Genome assembly of MiSeq reads for each sample was performed using SPAdes assembler with the careful assembly option and automated k-mer detection (Bankevich et al., 2012). The identification of genus and species of the isolates was carried out using fastANI (Jain et al., 2018) with a percentage greater than 80% of identification. Acquired AMR genes, plasmid types were identified using ABRicate tool (version 0.8.13), Resfinder was the database used for the identification of resistance genes (Zankari et al., 2012); PlasmidFinder database for plasmid replicon identification (Carattoli et al., 2014).

Phylogenetic Analysis

Pan-genome analysis was carried out using Roary, core genes were defined as genes being in at least 99% of isolates analyzed (Page et al., 2015). A maximum-likelihood phylogenetic tree with 1,000 bootstrap replicates based on core genomes of isolates was created using RaxML-NG (Kozlov et al., 2019). The phylogenetic tree was visualized using iTOL (Letunic and Bork, 2019). Additionally, multilocus sequence typing (MLST) (Larsen et al., 2012), based on seven housekeeping genes (*adk, fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*) and core genome (cgMLST) (Hansen et al., 2021) were performed using the Center for Genomic Epidemiology website[1]. The isolates also were characterized by Clermont phylogenetic typing by EzClermont web (Waters et al., 2020).

Sequence Accession Number

Assembled genome contigs have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under the following accession numbers: SAMN20872921, SAMN20872922, SAMN20872998, SAMN20873936, SAMN20873938, SAMN20873941, SAMN20873969, SAMN20873994, SAMN20874637, SAMN20875987, SAMN20875988, SAMN20875992, SAMN20875994, SAMN20875998, SAMN20879008, SAMN20879962, SAMN20879963, SAMN20879975, SAMN20879976, SAMN20880112, SAMN20880135, SAMN20880136, SAMN20881008, SAMN20881023, SAMN20881078, SAMN20881101, SAMN20881102, SAMN20881103, SAMN20881104, SAMN20881105, SAMN20881397, SAMN20881398, SAMN20881399, SAMN20881400, SAMN20882115, SAMN20882121, SAMN20882132, SAMN20882145, SAMN20882146, SAMN20882147, SAMN20882148, SAMN20882149, SAMN20883143, SAMN20883144, SAMN20883145, SAMN20883146, SAMN20883147, SAMN20884528, SAMN20884547, SAMN20884549, SAMN20886717, SAMN20887874, SAMN20887881, SAMN20887882, SAMN20887901, SAMN20887904, SAMN20887915, SAMN20887924, SAMN20887927, SAMN20887932, SAMN20887933, SAMN20888904, SAMN20888908, SAMN20888911, SAMN20888912, SAMN20888913, SAMN20888914, SAMN20888915, SAMN20888916, SAMN20888921, SAMN20888932, SAMN20888933, SAMN20888934, SAMN20888941, SAMN20888958, SAMN20888959, SAMN20888960, SAMN20888962, SAMN20890819, SAMN20891007.

[1]http://www.genomicepidemiology.org/

TABLE 1 | Primers used for detection of different β-lactamase genes in the multiplex PCR.

Genes	Primer sequence (5′ to 3′)	Size (bp)	References
bla_{SHV}	CTT TAT CGG CCC TCA CTCAA AGG TGC TCA TCA TGG GAA AG	237	Fang et al., 2008
bla_{TEM}	CGC CGC ATA CAC TAT TCT CAG AAT GA ACG CTC ACC GGC TCC AGA TTT AT	445	Monstein et al., 2007
bla_{CTX-M}	ATG TGC AGY ACC AGT AAR GTK ATG GC TGG GTR AAR TAR GTS ACC AGA AYC AGC GG	593	Boyd et al., 2004
bla_{OXA}	ACA CAA TAC ATA TCA ACTTCGC AGT GTG TTT AGA ATG GTG ATC	813	Ouellette et al., 1987

RESULTS

Prevalence of *Escherichia coli*

In total, 274 samples were collected, including 117 vegetable samples, 119 fruit samples, and 38 irrigation water samples. Across all samples, a total of 30 (11%) were positive for *E. coli;* 11 of the irrigation water samples had *E. coli* (29%, 11/38), 13 vegetables samples had *E. coli* (11%, n = 13), and 11 fruits (9%, n = 11). In total, 165 isolates of *E. coli* were recovered from 30 samples.

Antimicrobial Susceptibility Testing

Ninety-six isolates (58% n = 96) showed extended-spectrum beta-lactamases (ESBL) phenotype according to the CLSI protocols; 58% of *E. coli* isolates from irrigation water were ESBL-producers, 11% from vegetables, and 30% from fruits. ESBL-*E. coli* were isolated from garlic (2 isolates), onion (9 isolates), strawberry (10 isolates), blackberry (4 isolates), banana (14 isolates), and golden berry (1 isolate).

The rate of resistance was high; more than 80% of recovered *E. coli* isolates were resistant to cefazolin, ampicillin, and cefotaxime. In the case of the *E. coli* isolates from irrigation water, 100% of the isolates were resistant to ampicillin and cefazolin. In addition, these isolates had a high prevalence of resistance to cefotaxime (96%), tetracycline (79%), and cefepime (84%) (**Table 2**).

One hundred percent of the *E. coli* isolates from vegetables and fruits were resistant to ampicillin and cefazolin, cefotaxime, and tetracycline. Ninety-one percent of *E.coli* isolates from vegetables were resistant to cefepime. Two ESBL isolates from irrigation water presented resistance to the critically important class carbapenems, however no carbapenemase gene was detected. Additionally, we observed 33 resistance profiles across all of the extended spectrum beta-lactamase-producing *E. coli* isolates. The resistance profiles with the highest number of isolates are summarized in **Table 3**. In addition, 94% (90 of 96) of the *E. coli* ESBL isolates presented multi-drug resistant (MDR) patterns, with non-susceptible to at least one antibiotic in three or more antimicrobial categories (Magiorakos et al., 2012).

Genotypes of Extended-Spectrum β-Lactamase – *Escherichia coli*

We obtained high-quality genome sequences of 80 ESBL-*E. coli* isolates. MLST analysis using 7 housekeeping genes showed that 80 isolates were assigned to 37 known STs, whereas 7 isolates represented 7 novel STs. ST10 was shared by 14% (n = 11) of isolates from three sources, with a different province of origin: irrigation water (Pichincha), onion (Tungurahua), banana (Manabí), and strawberry (Tungurahua). ST453 (5%, n = 4) and ST224 (8%, n = 6) were shared in two sources and in different provinces of origin of the sample: ST453 (banana = Manabí, irrigation water = Pichincha), ST224 (irrigation water = Pichincha and Zamora Chinchipe, banana = Manabí) (**Table 4**).

The application of a cgMLST scheme showed 55 cgSTs, from which only 2, cgST86226 (banana, Manabí, n = 5; irrigation water Pichincha, n = 1) and cgST135673 (banana Manabí, n = 3; irrigation water, Zamora Chinchipe n = 1) were isolates from two different sources. Several isolates belonging to the same ST (based on 7 genes) were assigned to different cgSTs based on cgMLST and some of the isolates from the same sample had the same cgST. Additionally, we constructed a maximum likelihood tree based on the core genomes to compare the phylogeny of isolates of *E. coli* from the irrigation water, vegetables, and fruits (**Figure 2**). The phylogenetic analysis showed that all isolates with the same cgMLST and obtained from different sources differed in thousands of SNPs indicating that although the isolates were genetically close, they have been evolving apart for many years (**Table 4** and **Figure 2**). The genomes of ESBL-*E. coli* isolates from irrigation and fresh produce did not cluster apart; instead the isolates form different sources seemed to share recent common ancestry (**Figure 2**).

When ESBL-*E.coli* isolates were characterized by Clermont phylogenetic typing, 38% (n = 30) isolates belonged to phylogroup A: irrigation water (n = 21), strawberry (n = 3), onion (n = 4), banana (n = 2). In phylogroup B1 accounted for 35% (n = 28) of isolates: irrigation water (n = 15), banana (n = 7), strawberry (n = 1), blackberry (n = 4), and onion (n = 1). In phylogroup D accounted for 14% of the isolates: irrigation water (n = 4), strawberry (n = 3), garlic (n = 2), onion (n = 1) and banana (n = 1). Phylogroups B2, E and F accounted for 3% (n = 2), 5% (n = 4) and 3% (n = 2) of isolates, respectively. Three (4%) isolates of irrigation water belonged to the cryptic lineage (**Figure 2**).

Detection of β-Lactamase Genes

Ninety-six *E.*coli isolates phenotypically identified as ESBL, were tested by Multiplex PCR for genes encoding SHV, TEM, CTX-M, and OXA enzymes. The CTX-M gene was detected in 98% (94 of 96) of the isolates, followed by TEM 92% (88 of 96), SHV 28% (27 of 96), and OXA 1% (1/96). Additionally, combinations of genes were present: 64% had both CTX-M and TEM; and 26% had CTX-M, TEM, and SHV.

The presence of AMR genes in the genome sequences of 80 *ESBL-E. coli* isolates was investigated by Resfinder. Several ESBL-encoding bla_{CTX-M} gene variants were distributed in

TABLE 2 | Antibiotic susceptibility profiles of isolates ESBL- *E.coli* from irrigation water, vegetables, and fruits.

Antimicrobial categories	Antibiotics	Irrigation water *n* = 56 (frequency/percent)			Vegetables *n* = 11 (frequency/percent)			Fruits *n* = 29 (frequency/percent)		
		R	*S*	I/SDD	*R*	*S*	I/SDD	*R*	*S*	I/SDD
Cephalosporins	Cefazolin	56/100	0/0	0/0	11/100	0/0	0/0	29/100	0/0	0/0
Penicillins	Ampicillin	56/100	0/0	0/0	11/100	0/0	0/0	29/100	0/0	0/0
Aminoglycosides	Gentamicin	17/30	39/70	0/0	7/64	4/36	0/0	15/52	13/45	1/3
Carbapenems	Imipenem	2/4	49/88	5/9	0/0	10/91	1/9	0/0	20/69	9/31
Sulfonamides	Trimethropin/Sulfamethoxazole	36/64	18/32	2/4	10/91	1/9	0/0	21/72	8/28	0/0
Cephalosporins	Ceftazidime	25/45	10/18	21/38	7/64	0/0	4/36	19/66	0/0	10/34
Cephalosporins	Cefepime	47/84	2/4	7/13	10/91	0/0	1/9	22/76	0/0	7/24
Fluoroquinolones	Ciprofloxacin	36/64	10/18	10/18	7/64	2/18	2/18	15/52	9/31	5/17
Aminopenicillin + inhibitor of betalactamase	Amoxicillin/clavulanic acid	17/30	23/41	16/29	6/55	1/9	4/36	22/76	5/17	2/7
Cephalosporins	Cefotaxime	54/96	1/2	1/2	11/100	0/0	0/0	29/100	0/0	0/0
Tetracyclines	Tetracycline	44/79	12/21	0/0	11/100	0/0	0/0	29/100	0/0	0/0

R, resistant; I, intermediate; S, susceptible; SDD, susceptible-dose dependent in the case of cefepime; n, number of isolates tested.

isolates from irrigation water and fresh produce (**Figure 3**). Among the 80 *ESBL-E. coli* isolates, we identified allelic variants of $bla_{\mathrm{CTX-M}}$ in 77 (96%). The most common allelic variants were $bla_{\mathrm{CTX-M-55}}$ in 49 isolates (64%) and the second most common allele was $\mathrm{bla}_{\mathrm{CTX-M-65}}$ in 14 isolates (18%) (**Supplementary Table 1**).

We found some discrepancies in some ESBL- *E.coli* isolates that were positive by PCR for some genes but negative by whole genome sequencing (WGS): 12 isolates for bla_{TEM} gene, 9 isolates for bla_{SHV} genes and $bla_{\mathrm{CTX-M}}$ in one gene. Additionally, 2 isolates showed bla_{SHV} and bla_{TEM} using WGS, but were negative by PCR. The WGS analysis of ESBL-*E. coli* allowed us to identify 2 isolates of *E. coli* from irrigation water and 3 isolates from banana with the presence of the *mcr-1* gene that confers resistance to colistin.

TABLE 3 | The sixteen most common resistance profiles for ESBL-*E. coli* isolated from water, vegetables, and fruits in Ecuador.

Resistance profiles	Produce/Fruits	Irrigation water	Total
CZ-AM-GM-SXT-CAZ-FEP-CIP-AmC-CTX-TE	14	4	18
CZ-AM-FEP-CTX-TE	1	5	6
CZ-AM-SXT-CAZ-FEP-CIP-CTX-TE	0	4	4
CZ-AM-SXT-CAZ-FEP-CIP-AmC-CTX-TE	2	1	3
CZ-AM-GM-SXT-CAZ-FEP-CIP-CTX-TE	0	4	4
CZ-AM-GM-SXT-FEP-CIP-CTX-TE	1	4	5
CZ-AM-SXT-FEP-CIP-CTX-TE	0	4	4
CZ-AM-SXT-FEP-CIP-AmC-CTX-TE	4	2	6
CZ-AM-SXT-CAZ-FEP-CTX-TE	3	0	3
CZ-AM-CAZ-FEP-CTX-TE	1	2	3
CZ-AM-GM-CAZ-CTX-TE	2	0	2
CZ-AM-SXT-FEP-AmC-CTX-TE	2	1	3
CZ-AM-SXT-FEP-CTX-TE	4	0	4
CZ-AM-GM-CAZ-AmC-CTX-TE	4	0	4
CZ-AM-SXT-FEP-AmC-CTX	0	2	2
CZ-AM-SXT-CIP-CTX-TE	0	2	2

CZ, cefazolin; AM, ampicillin; GM, gentamicin; IPM, imipenem; SXT, trimethropin-sulfamethoxazole; CAZ, ceftazidime; FEP, cefepime; CIP, ciprofloxacin; AmC, amoxicillin/Clavulanic acid; CTX, cefotaxime; TE, tetracycline.

DISCUSSION

In this study, we found that irrigation water, fruit, and vegetables were contaminated with ESBL-*E. coli* and the highest percentage was found in irrigation water (58%), which confirms the important and emerging role that irrigation water, contaminated with wastewater, has in the spread of ARB and ESBL *E. coli* and ESBL genes. (Gekenidis et al., 2018a; Vital et al., 2018). The major ESBL gene was the $_{\mathrm{CTX-M}}$ (94 of 96 isolates) followed by bla_{SHV} 28% (27 of 96), and bla_{OXA} 1% (1of 96). The prevalence of $bla_{\mathrm{CTX-M}}$ type ESBL genes in irrigation water *E. coli* was 57%, followed by 15% in banana isolates. Additionally the most abundant allelic variants of bla_{CTX-M} found in vegetables, fruits and irrigation water ($bla_{\mathrm{CTX-M55}}$, $bla_{\mathrm{CTX-M65}}$, and $bla_{\mathrm{CTX-M15}}$) (**Table 4**) are the same alleles found in children and domestic animals in Ecuador (Salinas et al., 2021), in rivers that cross cities (Ortega-Paredes et al., 2020a), and in bacteria from human infections in Ecuador (Cartelle Gestal et al., 2016; Soria Segarra et al., 2018). The presence of the same $bla_{\mathrm{CTX-M}}$ alleles in isolates from different sources provides strong evidence that these sources (irrigation water, domestic animals, and humans) are connected. The allelic variants of $bla_{\mathrm{CTX-M}}$ from isolates obtained from same European country, but from different (unconnected) sources, animal species or time periods, have been shown to be different (Day et al., 2019; Ludden et al., 2019).

Our genomic analysis showed that most strains obtained from irrigation water and produce were genetically different with 3 exceptions (HY1.4.3 and V427.2; HP6.1 and V661.1; HP1.4 and V662.1), however the number of SNPs between thes strains

TABLE 4 | Source and genetic characteristics of ESBL- *E. coli* isolates from different sources in Ecuador.

					Relevant antimicrobial resistance genes				
Sample (*)	**Source**	**Location**	**ST**	**cgST**	**CTX-M**	**TEM**	**SHV**	**OXA**	**mcr-1**
H505	Irrigation	Cañ-La Troncal	937	87149	55	141			
H719	Irrigation	Chim-Riobamba	617	93239	3				
H719	Irrigation	Chim-Riobamba	new7	143498	15	1	187	1	
H726	Irrigation	Imb-Ibarra	155	17156	55	141			
V662	Banana	Man-Portoviejo	10	15007	55	1			
V661.1	Banana	Man-Portoviejo	847	28793	55				
V662	Banana	Man-Portoviejo	6598	39050	8, 55	1			
V662	Banana	Man-Portoviejo	453	86226	8, 55	1			
V662	Banana	Man-Portoviejo	453	86226	55	1			
V662	Banana	Man-Portoviejo	453	86226	55	1	12		
V663 (3)	Banana	Man-Portoviejo	224	135673	55	1			1
V661.3	Banana	Man-Portoviejo	new3	136455	55	1	12		
HY1.3.3	Irrigation	Pich-Yaruquí	6027	2725	55	1			
HY6.5.3	Irrigation	Pich-Yaruquí	522	4492	55	1			
HP1.2	Irrigation	Pich-Yaruquí	10	5994	55,65	141			
HP6.4	Irrigation	Pich-Yaruquí	100	6271	15				
HY8.5.3	Irrigation	Pich-Yaruquí	131	9613			12		
HY3.4.3	Irrigation	Pich-Yaruquí	38	13889	9	1			
HY7.5.3	Irrigation	Pich-Yaruquí	206	17904	65	1			
HP1.4	Irrigation	Pich-Yaruquí	752	21656	65				
HY4.2.2	Irrigation	Pich-Yaruquí	224	29102	55	1			
V727 (2)	Strawberry	Pich-Yaruquí	new4	33815	65		12		
HP6.2	Irrigation	Pich-Yaruquí	1725	34210	55		5		
HY3.5	Irrigation	Pich-Yaruquí	1706	38416	15	1			
HP1.1	Irrigation	Pich-Yaruquí	155	40558	65				
HP4.3	Irrigation	Pich-Yaruquí	7290	43104	8				
HP7.2	Irrigation	Pich-Yaruquí	10	46675	55		12		
HP7.4	Irrigation	Pich-Yaruquí	10	46675	55	141	12		
HY2.4.2	Irrigation	Pich-Yaruquí	new2	79725	15				
HY4.4.2	Irrigation	Pich-Yaruquí	3944	80110	55	1			
HP4.4	Irrigation	Pich-Yaruquí	117	81681	55	141			
HP2.4	Irrigation	Pich-Yaruquí	117	82990	55	141			
HP6.3	Irrigation	Pich-Yaruquí	453	86226	55	141			
HY6 (2)	Irrigation	Pich-Yaruquí	540	96158	15	1			
HY1 (2)	Irrigation	Pich-Yaruquí	540	96158	15	1			
HP7	Irrigation	Pich-Yaruquí	124	96630	65				
HY6	Irrigation	Pich-Yaruquí	9580	96650	55	1			
HY2.3.3	Irrigation	Pich-Yaruquí	10	101136	15			1	
HY8.2.2	Irrigation	Pich-Yaruquí	9962	116134		1	12		
HP6.1	Irrigation	Pich-Yaruquí	1725	117316	55				
HY4.4 (2)	Irrigation	Pich-Yaruquí	205	117479	15	1			
HP6.5	Irrigation	Pich-Yaruquí	10340	117591	3	141			
HP2	Irrigation	Pich-Yaruquí	57	117853	55	141			
HP1.5	Irrigation	Pich-Yaruquí	57	117853	55	141			
V727.4	Strawberry	Pich-Yaruquí	new6	119048	65	176	12		
V727.5	Strawberry	Pich-Yaruquí	4541	119048	65		12		
HY6.5	Irrigation	Pich-Yaruquí	10	134002	55	1			
HY1.3.2	Irrigation	Pich-Yaruquí	2973	135505	55, 65	1			
HP1.3	Irrigation	Pich-Yaruquí	354	137556	55	1			
HY4.3.2	Irrigation	Pich-Yaruquí	224	138183	55	1			
HY1.1.4	Irrigation	Pich-Yaruquí	new1	138274		1			
HY5.2.1	Irrigation	Pich-Yaruquí	155	138689	55	1			

(Continued)

TABLE 4 | (Continued)

Sample (*)	Source	Location	ST	cgST	Relevant antimicrobial resistance genes				
					CTX-M	TEM	SHV	OXA	mcr-1
HY3.5.2	Irrigation	Pich-Yaruquí	155	138689	55	1			
HY1.4.3	Irrigation	Pich-Yaruquí	394	142214	15				
HY6.1.2	Irrigation	Pich-Yaruquí	69	144487	55	1			1
H579.2	Irrigation	Tun-Ambato	206	4018	65				
V696 (4)	Blackberry	Tun-Ambato	5044	32678	55	1			
V698 (3)	Strawberry	Tun-Ambato	10	38518	55	1			
V1140 (2)	Onion	Tun-Ambato	4204	55533	55, 65	1			
V1140	Onion	Tun-Ambato	4204	55533	55	1			
V427.5	Onion	Tun-Ambato	58	60063	55	1			
V469.5	Onion	Tun-Ambato	10	69259	55	1			
V1147 (2)	Garlic	Tun-Ambato	973	118630	3	1			
H579.1	Irrigation	Tun-Ambato	155	138689	55	1			
V427.2	Onion	Tun-Ambato	4368	142214	15				
H430	Irrigation	Zam-Yantzaza	224	135673	55	1			1

**Number of isolates with the same cgST obtained from the same sample. Tun, Tungurahua; Pich, Pichincha; Man, Manabi; Zam, Zamora; Imb, Imbabura; Cañ, Cañar; Chim, Chimborazo.*

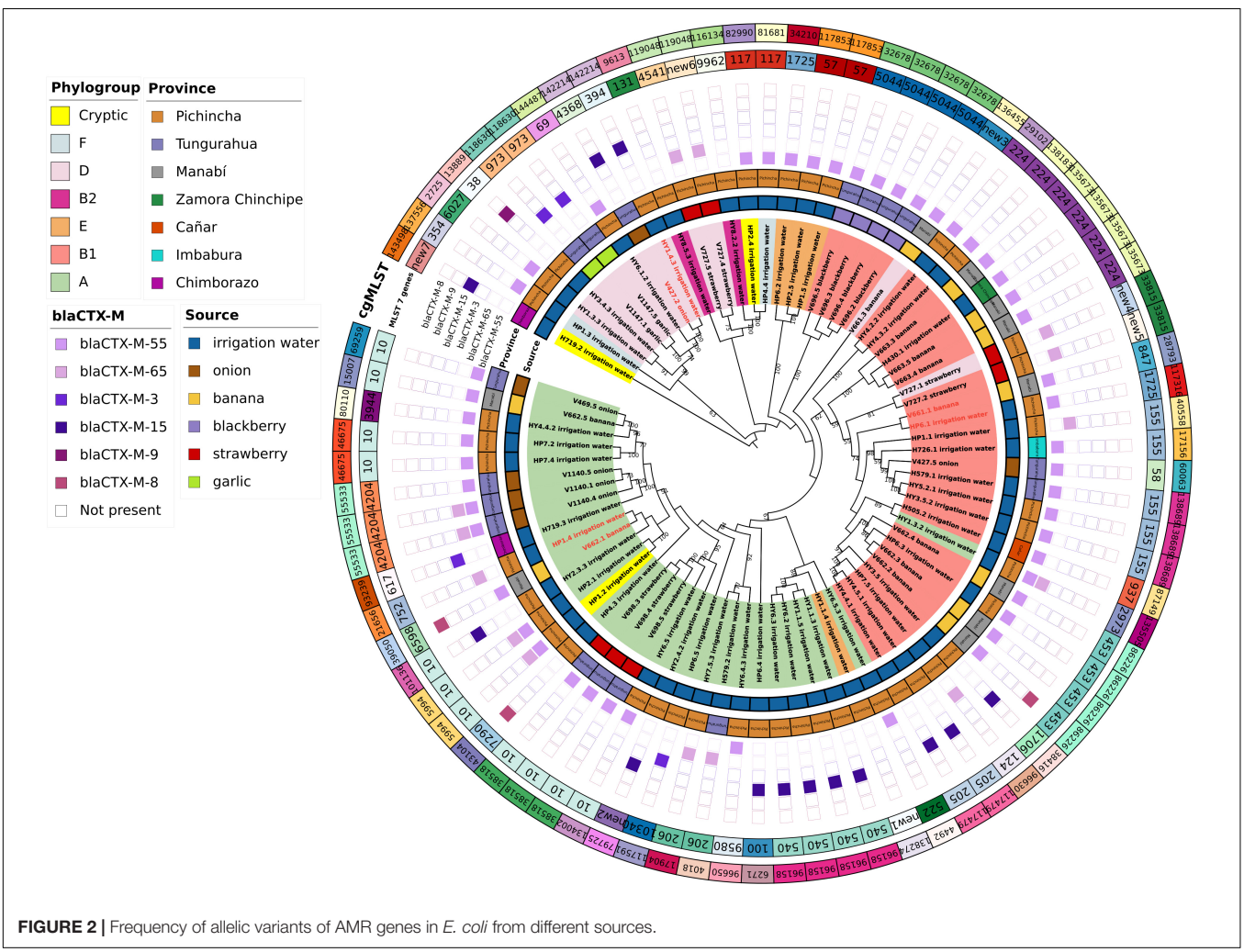

FIGURE 2 | Frequency of allelic variants of AMR genes in *E. coli* from different sources.

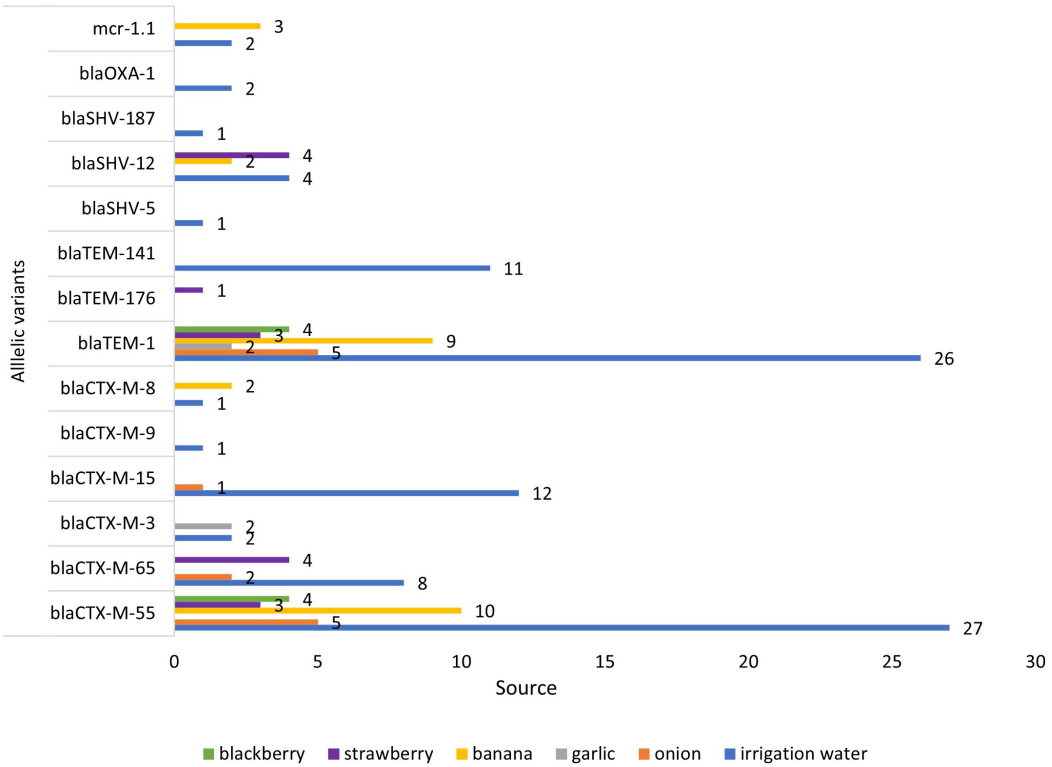

FIGURE 3 | Phylogenetic tree of ESBL-*E.coli* sequences from irrigation water, fruits, and vegetables. Maximum-likelihood phylogenetic tree of core genomes of 80 ESBL- *E.coli* isolates from irrigation water, fruits, and vegetables based. Labels show isolate ID assigned based on host ID, origin of isolate is shown by font colors (irrigation water: blue, onion: brown, banana: yellow, blackberry: purple, strawberry: red, and garlic: green). Background colors in branches indicate the seven phylogroups identified. Numbers represent bootstrap values using 1000 pseudo-replicates.

ranged from 9,332 to 20,310 suggesting that these strains have been evolving apart for many years (**Table 4**). As expected, some isolates from the same vegetable or fruit showed higher level of genetic closeness, for instance: V698.3 and V698.4 had 12 SNP; V663.4 and V663.5, 6 SNPs; V696.2 and V696.4, 13 SNPs; V1147.5 and V1147.1, 2 SNPs). Interstingly, 2 isolates obtaind from the same irrigation channel 1 month appart (HY3.5.2 and HY5.2.1) had 24 SNPs, suggesting that this strain was higly adapted to water. We did not find additional asociation of ESBL-*E.coli* clusters with provinces, which may indicate that different *E. coli* lineages have been widely distributed in the Ecuadorian territory (**Figure 2**).

These findings may indicated that *E. coli* populations in the environment are highly diverse (Day et al., 2019; Ludden et al., 2019) and bla_{CTX-M}-genes are probably diseminating in the environmet mostly by mobile genetic elements and not so much by bacterial clones. The plasmids carrying bla_{CTX-M}-genes disseminate efficiently by conjugation, even between bacteria belonging to different genera (Cantón et al., 2012). Transposable elements (such as IS*Ecp1*) are also very active in bla_{CTX-M}-gene mobilization among different plasmids (Cantón et al., 2012). The activity of these MGEs conceals the source of origin of these antimicrobial resistance genes.

The majority of strains isolated from irrigation water and vegetables belonged to phylogroups A and B1 which are considered more generalists, found in most warm-blooded animals and environmental samples (Touchon et al., 2020). We found that some genetically close *E.coli* isolates, obtained from the same vegetable, had 1 or 2 additional antimicrobial resistance genes which may be a reflection of the dynamic process of antimicrobial resistance gene-turnover in the environment (Barrera et al., 2019).

The bla_{CTX-M} type of ESBL gene is of increasing concern globally (Bevan et al., 2017), and is the predominant ESBL gene in both community and hospital-acquired infections (Manyahi et al., 2017; Fils et al., 2021). A troubling feature of bla_{CTX-M}-bearing plasmids is their ability to capture additional resistance determinants, including carbapenemase genes (Partridge et al., 2012; Potron et al., 2013). Further analysis is necessary to understand whether the plasmids carrying bla_{CTX-M} genes, in bacteria from irrigation water and produce, are the same as those circulating in bacterial isolates from human isolates.

In our study fruits, such as bananas, we hypothesize that their contamination was due to post-harvest processes in which the food is often washed in contaminated water and reused to wash several batches of the product. Although it is true, the skin of the product protects the fruit, the transmission of resistant bacteria can occur through contact and inadequate consumer hygiene (Harris et al., 2003; Hong et al., 2013; Kawamura et al., 2017; Murray et al., 2017; Hölzel et al., 2018).

We also found a higher prevalence of ARB in vegetables in farms than in retail markets in Ecuador (Ortega-Paredes et al., 2018). However, other reports from the Philippines, Lebanon, and Portugal have documented even higher levels (Faour-Klingbeil et al., 2016; Araújo et al., 2017; Vital et al., 2018). In most of the studies, the collection of produce samples has been carried out in groceries and wholesale markets, which makes it difficult to analyze sources of contamination (Bhutani et al., 2015; Yang et al., 2019; Colosi et al., 2020; Richter et al., 2020; Song et al., 2020). In this study, we collected produce and water from farms and their respective irrigation systems, which allowed us to study contamination at the source (i.e., not due to handling, transport, distribution, and processing). We found that MDR isolates were more prevalent in irrigation water isolates compared to fresh produce. Similar results were observed in the Philippines, where 58% of the *E. coli* isolates from irrigation water were MDR (Paraoan et al., 2017). The resistance to these antibiotics was also observed in *E. coli* isolates from irrigation water in other studies (Pignato et al., 2009; Ben Said et al., 2015; Vital et al., 2018).

Our study had some limitations; the number produce and fruit samples obtained in each location may not be representative of produce from other agricultural settings in Ecuador. Additionally, long-read sequencing of plasmids could not be carried out due to budgetary limitations.

We found evidence that fresh produce constitutes an important source of ESBL-*E. coli* and represents a route for the dissemination of resistance genes through the consumption of raw products (Rasheed et al., 2014; Hölzel et al., 2018; Al-Kharousi et al., 2019). We hypothesize that the main source of ABR contamination is irrigation water used for the cultivation of produce, which has been suggested by others as well (Pignato et al., 2009; Gekenidis et al., 2018b). In Ecuador, the lack of sewage treatment may lead to contamination of the food supply with ARB, mainly belonging to the Enterobacteriaceae family (Caicedo-Camposano et al., 2019; Ortega-Paredes et al., 2020a). Antibiotic resistant *E. coli* can transfer antibiotic resistance determinants not only to other strains of *E. coli*, but also to other species of potentially pathogenic bacteria within the gastrointestinal tract (Grasselli et al., 2008; Huddleston, 2014).

CONCLUSION

We found a high prevalence of ESBL-*E. coli* on produce and in irrigation water; $bla_{\mathrm{CTX-M}}$ was the main ESBL gene in these isolates. Allelic variants of the $bla_{\mathrm{CTX-M}}$ gene found in irrigation channels and vegetables were the same as those observed in commensal *E. coli* from domestic animals, and commensal and pathogenic *E. coli* from humans, suggesting connection between these different sources. This paradigm poses the potential risk of further spreading ARB that are resistant to last-line antibiotics such as carbapenems, which are used exclusively in serious infections in hospitals (Sheu et al., 2019). In this case, resistance goes full circle, from humans to vegetables and fruits (potentially meat and dairy), and back to human populations (Murray et al., 2021). Greater investments are needed to support the development and installation of wastewater treatment systems throughout Ecuador, as well as in other low- and middle-income countries.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

JI and LM: isolation of the *Escherichia coli* strains. LM: writing—original draft. JG, PC, and GT: review and editing. GT and LM: study design. 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.2021.709418/full#supplementary-material

REFERENCES

Al-Kharousi, Z. S., Guizani, N., Al-Sadi, A. M., and Al-Bulushi, I. M. (2019). Antibiotic resistance of *Enterobacteriaceae* isolated from fresh fruits and vegetables and characterization of their AmpC b-lactamases. *J. Food Protect.* 82, 1857–1863. doi: 10.4315/0362-028X.JFP-19-089

Araújo, S., Silva, A. T. I, Tacão, M., Patinha, C., Alves, A., and Henriques, I. (2017). Characterization of antibiotic resistant and pathogenic *Escherichia coli* in irrigation water and vegetables in household farms. *Int. J. Food Microbiol.* 257, 192–200. doi: 10.1016/j.ijfoodmicro.2017.06.020

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., et al. (2012). SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol.* 19, 455–477. doi: 10.1089/cmb.2012.0021

Barrera, S., Cardenas, P., Graham, J. P., and Trueba, G. (2019). Changes in dominant *Escherichia coli* and antimicrobial resistance after 24 hr in fecal matter. *Microbiologyopen* 8:e00643. doi: 10.1002/mbo3.643

Ben Said, L., Jouini, A., Klibi, N., Dziri, R., Alonso, C. A., Boudabous, A., et al. (2015). Detection of extended-spectrum beta-lactamase (ESBL)-producing *Enterobacteriaceae* in vegetables, soil and water of the farm environment in Tunisia. *Int. J. Food Microbiol.* 203, 86–92. doi: 10.1016/j.ijfoodmicro.2015.02.023

Beuchat, L. R. (1996). Pathogenic microorganisms associated with fresh produce. *J. Food Protect.* 59, 204–216. doi: 10.4315/0362-028X-59.2.204

Bevan, E. R., Jones, A. M., and Hawkey, P. M. (2017). Global epidemiology of CTX-M β-lactamases: temporal and geographical shifts in genotype. *J. Antimicrob. Chemother.* 72, 2145–2155. doi: 10.1093/jac/dkx146

Bhutani, N., Muraleedharan, C., Talreja, D., Rana, S. W., Walia, S., Kumar, A., et al. (2015). Occurrence of multidrug resistant extended spectrum beta-lactamase-producing bacteria on iceberg lettuce retailed for human consumption. *BioMed Res. Int.* 2015:547547. doi: 10.1155/2015/547547

Bonardi, S., and Pitino, R. (2019). Carbapenemase-producing bacteria in food-producing animals, wildlife and environment: a challenge for human health. *Ital. J. Food Saf.* 8:7956. doi: 10.4081/ijfs.2019.7956

Botelho, L. A. B., Kraychete, G. B., ESilva, J. L. C., Regis, D. V. V., Picão, R. C., Moreira, B. M., et al. (2015). Widespread distribution of CTX-M and plasmid-mediated AmpC β-lactamases in *Escherichia coli* from Brazilian chicken meat. *Mem. Inst. Oswaldo Cruz* 110, 249–254. doi: 10.1590/0074-02760140389

Boyd, D. A., Tyler, S., Christianson, S., McGeer, A., Muller, M. P., Willey, B. M., et al. (2004). Complete nucleotide sequence of a 92-kilobase plasmid harboring the CTX-M-15 extended-spectrum beta-lactamase involved in an outbreak in long-term-care facilities in Toronto, Canada. *Antimicrob. Agents Chemother.* 48, 3758–3764. doi: 10.1128/AAC.48.10.3758-3764.2004

Bush, K., and Jacoby, G. A. (2010). Updated functional classification of β-lactamases. *Antimicrob. Agents Chemother.* 54, 969–976. doi: 10.1128/AAC.01009-09

Caicedo-Camposano, O., Balmaseda-Espinosa, C., Tandazo-Garcés, J., Layana-Bajaña, E., and Sánchez-Vásquez, V. (2019). Water quality for irrigation of San Pablo River, Babahoyo Municipality, Ecuador. *Rev. Cienc. Técnicas Agropecuarias* 28:3.

Cantón, R., González-Alba, J. M., and Galán, J. C. (2012). CTX-M enzymes: origin and diffusion. *Front. Microbiol.* 3:110. doi: 10.3389/fmicb.2012.00110

Carattoli, A., Zankari, E., Garciá-Fernández, A., Larsen, M. V., Lund, O., Villa, L., et al. (2014). In Silico detection and typing of plasmids using plasmidfinder and plasmid multilocus sequence typing. *Antimicrob. Agents Chemother.* 58, 3895–3903. doi: 10.1128/AAC.02412-14

Cartelle Gestal, M., Zurita, J., Paz y Mino, A., Ortega-Paredes, D., and Alcocer, I. (2016). Characterization of a small outbreak of *Salmonella enterica* serovar Infantis that harbour CTX-M-65 in Ecuador. *Braz. J. Infect. Dis.* 20, 406–407. doi: 10.1016/j.bjid.2016.03.007

CLSI (2019). *Performance Standards for Antimicrobial Susceptibility Testing*. Pennisylvania: Clinical and Laboratory standards Institute.

Colosi, I. A., Baciu, A. M., Opriş, R. V., Peca, L., Gudat, T., Simon, L. M., et al. (2020). Prevalence of ESBL, AmpC and Carbapenemase-Producing Enterobacterales Isolated from Raw Vegetables Retailed in Romania. *Foods* 9:1726. doi: 10.3390/foods9121726

Cooper, D., Doucet, L., and Pratt, M. (2007). Outbreaks and factors influencing microbiological contamination of fresh produce. *J. Organ. Behav.* 28, 303–325. doi: 10.1002/j

Dashti, A. A., Jadaon, M. M., Abdulsamad, A. M., and Dashti, H. M. (2009). Heat treatment of bacteria: a simple method of DNA extraction for molecular techniques. *Kuwait Med. J.* 41, 117–122.

Day, M. J., Hopkins, K. L., Wareham, D. W., Toleman, M. A., Elviss, N., Randall, L., et al. (2019). Extended-spectrum β-lactamase-producing *Escherichia coli* in human-derived and foodchain-derived samples from England, Wales, and Scotland: an epidemiological surveillance and typing study. *Lancet Infect. Dis.* 19, 1325–1335. doi: 10.1016/S1473-3099(19)30273-7

Edberg, S. C., Rice, E. W., Karlin, R. J., and Allen, M. J. (2000). *Escherichia coli*: the best biological drinking water indicator for public health protection. *J. Appl. Microbiol. Symp. Suppl.* 88, 106S–116S. doi: 10.1111/j.1365-2672.2000.tb05338.x

Fang, H., Ataker, F., Hedin, G., and Dornbusch, K. (2008). Molecular epidemiology of extended-spectrum β-lactamases among *Escherichia coli* isolates collected in a Swedish hospital and its associated health care facilities from 2001 to 2006. *J. Clin. Microbiol.* 46, 707–712. doi: 10.1128/JCM.01943-07

FAO and WHO (2008). *Microbiological Hazards in Fresh Fruits and Vegetables*. Geneva: World Health Organization.

Faour-Klingbeil, D., Kuri, V., Fadlallah, S., and Matar, G. M. (2016). Prevalence of antimicrobial-resistant *escherichia coli* from raw vegetables in Lebanon. *J. Infect. Dev. Count.* 10, 354–362. doi: 10.3855/jidc.7745

Fils, P. E. L., Cholley, P., Gbaguidi-Haore, H., Hocquet, D., Sauget, M., and Bertrand, X. (2021). ESBL-producing *Klebsiella pneumoniae* in a University hospital: molecular features, diffusion of epidemic clones and evaluation of cross-transmission. *PLoS One* 16:e0247875. doi: 10.1371/journal.pone.0247875

Frieri, M., Kumar, K., and Boutin, A. (2017). Antibiotic resistance. *J. Infect. Public Health* 10, 369–378. doi: 10.1016/j.jiph.2016.08.007

Gekenidis, M. T., Qi, W., Hummerjohann, J., Zbinden, R., Walsh, F., and Drissner, D. (2018a). Antibiotic-resistant indicator bacteria in irrigation water: high prevalence of extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli*. *PLoS One* 13:e0207857. doi: 10.1371/journal.pone.0207857

Gekenidis, M. T., Schöner, U., von Ah, U., Schmelcher, M., Walsh, F., and Drissner, D. (2018b). Tracing back multidrug-resistant bacteria in fresh herb production: from chive to source through the irrigation water chain. *FEMS Microbiol. Ecol.* 94:fiy149. doi: 10.1093/femsec/fiy149

Grasselli, E., François, P., Gutacker, M., Gettler, B., Benagli, C., Convert, M., et al. (2008). Evidence of horizontal gene transfer between human and animal commensal *Escherichia coli* strains identified by microarray. *FEMS Immunol. Med. Microbiol.* 53, 351–358. doi: 10.1111/j.1574-695X.2008.00434.x

Guerrero, T., Calderón, D., Zapata, S., and Trueba, G. (2020). *Salmonella* grows massively and aerobically in chicken faecal matter. *Microb. Biotechnol.* 13, 1678–1684. doi: 10.1111/1751-7915.13624

Hansen, S., Andersen, L., Mette, D., Holm, A., Roer, L., Antoniadis, P., et al. (2021). Using core genome multilocus sequence typing (cgMLST) for vancomycin-resistant Enterococcus faecium isolates to guide infection control interventions and end an outbreak. *J. Global Antimicrob. Resist.* 24, 418–423. doi: 10.1016/j.jgar.2021.02.007

Harris, L. J., Farber, J. N., Beuchat, L. R., Parish, M. E., Suslow, T. V., Garrett, E. H., et al. (2003). Outbreaks associated with fresh produce: incidence, growth, and survival of pathogens in fresh and fresh-cut produce. *Compr. Rev. Food Sci. Food Saf.* 2, 78–141. doi: 10.1111/j.1541-4337.2003.tb00031.x

Hasegawa, H., Suzuki, E., and Maeda, S. (2018). Horizontal plasmid transfer by transformation in *Escherichia coli*: environmental factors and possible mechanisms. *Front. Microbiol.* 9:2365. doi: 10.3389/fmicb.2018.02365

Hölzel, C. S., Tetens, J. L., and Schwaiger, K. (2018). Unraveling the role of vegetables in spreading antimicrobial-resistant bacteria: a need for quantitative risk assessment. *Foodborne Pathog. Dis.* 15, 671–688. doi: 10.1089/fpd.2018.2501

Hong, P. Y., Al-Jassim, N., Ansari, M. I., and Mackie, R. I. (2013). Environmental and public health implications of water reuse: antibiotics, antibiotic resistant bacteria, and antibiotic resistance genes. *Antibiotics* 2, 367–399. doi: 10.3390/antibiotics2030367

Huddleston, J. R. (2014). Horizontal gene transfer in the human gastrointestinal tract: potential spread of antibiotic resistance genes. *Infect. Drug Resist.* 7, 167–176. doi: 10.2147/IDR.S48820

Iwu, C. D., and Okoh, A. I. (2019). Preharvest transmission routes of fresh produce associated bacterial pathogens with outbreak potentials: a review. *Int. J. Environ. Res. Public Health* 16:4407. doi: 10.3390/ijerph16224407

Jain, C., Rodriguez-R, L. M., Phillippy, A. M., Konstantinidis, K. T., and Aluru, S. (2018). High throughput ANI analysis of 90K prokaryotic genomes reveals clear species boundaries. *Nat. Commun.* 9:5114. doi: 10.1038/s41467-018-07641-9

Kawamura, K., Nagano, N., Suzuki, M., Wachino, J., Kimura, K., and Arakawa, Y. (2017). ESBL-producing *Escherichia coli* and Its Rapid Rise among Healthy People. *Food Saf.* 5, 122–150. doi: 10.14252/foodsafetyfscj.2017011

Kilonzo-Nthenge, A., Liu, S., Hashem, F., Millner, P., and Githua, S. (2018). Prevalence of *Enterobacteriaceae* on fresh produce and food safety practices in small-acreage farms in Tennessee, USA. *J. Verbrauch. Lebensm.* 13, 279–287. doi: 10.1007/s00003-018-1172-y

Kozlov, A. M., Darriba, D., Flouri, T., Morel, B., and Stamatakis, A. (2019). RAxML-NG: a fast, scalable and user-friendly tool for maximum likelihood phylogenetic inference. *Bioinformatics* 35, 4453–4455. doi: 10.1093/bioinformatics/btz305

Lange, B., Strathmann, M., and Oßmer, R. (2013). Performance validation of chromogenic coliform agar for the enumeration of *Escherichia coli* and coliform bacteria. *Lett. Appl. Microbiol.* 57, 547–553. doi: 10.1111/lam.12147

Larsen, M. V., Cosentino, S., Rasmussen, S., Friis, C., Hasman, H., Marvig, R. L., et al. (2012). Multilocus sequence typing of total-genome-sequenced bacteria. *J. Clin. Microbiol.* 50, 1355–1361. doi: 10.1128/JCM.06094-11

Leff, J. W., and Fierer, N. (2013). Bacterial Communities Associated with the Surfaces of Fresh Fruits and Vegetables. *PLoS One* 8:e59310. doi: 10.1371/journal.pone.0059310

Letunic, I., and Bork, P. (2019). Interactive Tree of Life (iTOL) v4: recent updates and new developments. *Nucleic Acids Res.* 47, 256–259. doi: 10.1093/nar/gkz239

Li, J., Bi, Z., Ma, S., Chen, B., Cai, C., He, J., et al. (2019). Inter-host transmission of carbapenemase-producing *Escherichia coli* among humans and backyard animals. *Environ. Health Perspect.* 127:107009. doi: 10.1289/EHP5251

Lin, Y., Dong, X., Wu, J., Rao, D., Zhang, L., Faraj, Y., et al. (2020). Metadata analysis of mcr-1-bearing plasmids inspired by the sequencing evidence for horizontal transfer of antibiotic resistance genes between polluted river and wild birds. *Front. Microbiol.* 11:352. doi: 10.3389/fmicb.2020.00352

Ludden, C., Raven, K. E., Jamrozy, D., Gouliouris, T., Blane, B., Coll, F., et al. (2019). One health genomic surveillance of *Escherichia coli* demonstrates distinct lineages and mobile genetic elements in isolates from humans versus livestock. *mBio* 10, e02693–18. doi: 10.1128/mBio.02693-18

Magiorakos, A. P., Srinivasan, A., Carey, R. B., Carmeli, Y., Falagas, M. E., Giske, C. G., et al. (2012). Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin. Microbiol. Infect.* 18, 268–281. doi: 10.1111/j.1469-0691.2011.03570.x

Manyahi, J., Moyo, S. J., Tellevik, M. G., Ndugulile, F., Urassa, W., Blomberg, B., et al. (2017). Detection of CTX-M-15 beta-lactamases in *Enterobacteriaceae* causing hospital- and community-acquired urinary tract infections as early as 2004, in Dar es Salaam, Tanzania. *BMC Infect. Dis.* 17:282. doi: 10.1186/s12879-017-2395-8

Marlène, M., Lee, B., Miller, S., Reyna, Z., Lu, P., Yan, J., et al. (2020). Horizontal Gene Transfer of Antibiotic Resistance from Acinetobacter baylyi to *Escherichia coli* on Lettuce and Subsequent Antibiotic Resistance Transmission to the Gut Microbiome. *mSphere* 5, e00329–20. doi: 10.1128/msphere.00329-20

McDaniel, C., and Jadeja, R. (2019). A Review of Fresh Produce Outbreaks, Current Interventions, Food Safety Concerns and Potential Benefits of Novel Antimicrobial Sodium Acid Sulfate. *MOJ Food Process. Technol.* 7, 59–67. doi: 10.15406/mojfpt.2019.07.00221

Moore, J. E., Moore, P. J. A., Millar, B. C., Goldsmith, C. E., Loughrey, A., Rooney, P. J., et al. (2010). The presence of antibiotic resistant bacteria along the River Lagan. *Agric. Water Manag.* 98, 217–221. doi: 10.1016/j.agwat.2010.09.001

Monstein, H. J., Östholm-Balkhed, Å., Nilsson, M. V., Nilsson, M., Dornbusch, K., and Nilsson, L. E. (2007). Multiplex PCR amplification assay for the detection of blaSHV, blaTEM and blaCTX-M genes in Enterobacteriaceae. *APMIS* 115, 1400–1408. doi: 10.1111/j.1600-0463.2007.00722.x

Motlagh, A. M., and Yang, Z. (2019). Detection and occurrence of indicator organisms and pathogens. *Water Environ. Res.* 91, 1402–1408. doi: 10.1002/wer.1238

Murray, K., Wu, F., Shi, J., Jun Xue, S., and Warriner, K. (2017). Challenges in the microbiological food safety of fresh produce: limitations of post-harvest washing and the need for alternative interventions. *Food Qual. Saf.* 1, 289–301. doi: 10.1093/fqsafe/fyx027

Murray, M., Salvatierra, G., Dávila-Barclay, A., Ayzanoa, B., Castillo-Vilcahuaman, C., Huang, M., et al. (2021). Market Chickens as a Source of Antibiotic-Resistant *Escherichia coli* in a Peri-Urban Community in Lima, Peru. *Front. Microbiol.* 12:635871. doi: 10.3389/fmicb.2021.635871

Njage, P. M. K., and Buys, E. M. (2015). Pathogenic and commensal Escherichiacoli from irrigation water show potential in transmission of extended spectrum and AmpC β-lactamases determinants to isolates from lettuce. *Microb. Biotechnol.* 8, 462–473. doi: 10.1111/1751-7915.12234

Ortega-Paredes, D., Barba, P., Mena-López, S., Espinel, N., and Zurita, J. (2018). *Escherichia coli* hyperepidemic clone ST410-A harboring blaCTX-M-15 isolated from fresh vegetables in a municipal market in Quito-Ecuador. *Int. J. Food Microbiol.* 280, 41–45. doi: 10.1016/j.ijfoodmicro.2018.04.037

Ortega-Paredes, D., Barba, P., Mena-López, S., Espinel, N., Crespo, V., and Zurita, J. (2020a). High quantities of multidrug-resistant *Escherichia coli* are present in the Machángara urban river in Quito, Ecuador. *J. Water Health* 18, 67–76. doi: 10.2166/wh.2019.195

Ortega-Paredes, D., de Janon, S., Villavicencio, F., Ruales, K. J., De La Torre, K., Villacís, J. E., et al. (2020b). Broiler Farms and Carcasses Are an Important Reservoir of Multi-Drug Resistant *Escherichia coli* in Ecuador. *Front. Vet. Sci.* 7:547843. doi: 10.3389/fvets.2020.547843

Ouellette, M., Bissonnette, L., and Roy, P. H. (1987). Precise insertion of antibiotic resistance determinants into Tn21-like transposons: nucleotide sequence of the OXA-1 beta-lactamase gene. *Proc. Natl. Acad. Sci. U.S.A.* 84, 7378–7382. doi: 10.1073/pnas.84.21.7378

Page, A. J., Cummins, C. A., Hunt, M., Wong, V. K., Reuter, S., Holden, M. T. G., et al. (2015). Roary: rapid large-scale prokaryote pan genome analysis. *Bioinformatics* 31, 3691–3693. doi: 10.1093/bioinformatics/btv421

Paraoan, C. E. M., Rivera, W. L., and Vital, P. G. (2017). Detection of Class I and II integrons for the assessment of antibiotic and multidrug resistance among *Escherichia coli* isolates from agricultural irrigation waters in Bulacan, Philippines. *J. Environ. Sci. Health B Pest. Food Contamin. Agric. Wastes* 52, 306–313. doi: 10.1080/03601234.2017.1281647

Partridge, S. R., Paulsen, I. T., and Iredell, J. R. (2012). pJIE137 carrying bla CTX-M-62 is closely related to p271A carrying bla NDM-1. *Antimicrob. Agents Chemother.* 56, 2166–2168. doi: 10.1128/AAC.05796-11

Pesavento, G., Calonico, C., Ducci, B., Magnanini, A., and Lo Nostro, A. (2014). Prevalence and antibiotic resistance of Enterococcus spp. isolated from retail cheese, ready-to-eat salads, ham, and raw meat. *Food Microbiol.* 41, 1–7. doi: 10.1016/j.fm.2014.01.008

Pigłowski, M. (2019). Pathogenic and non-pathogenic microorganisms in the rapid alert system for food and feed. *Int. J. Environ. Res. Public Health* 16:477. doi: 10.3390/ijerph16030477

Pignato, S., Coniglio, M. A., Faro, G., Weill, F. X., and Giammanco, G. (2009). Plasmid-mediated multiple antibiotic resistance of *Escherichia coli* in crude and treated wastewater used in agriculture. *J. Water Health* 7, 251–258. doi: 10.2166/wh.2009.019

Potron, A., Nordmann, P., Rondinaud, E., Jaureguy, F., and Poirel, L. (2013). A mosaic transposon encoding OXA-48 and CTX-M-15: towards pan-resistance. *J. Antimicrob. Chemother.* 68, 476–477. doi: 10.1093/jac/dks397

Rasheed, M. U., Thajuddin, N., Ahamed, P., Teklemariam, Z., and Jamil, K. (2014). Antimicrobial drug resistance in strains of *Escherichia coli* isolated from food sources. *Rev. Inst. Med. Trop. Sao Paulo* 56, 341–346. doi: 10.1590/s0036-46652014000400012

Richter, L., du Plessis, E. M., Duvenage, S., and Korsten, L. (2020). Occurrence, Phenotypic and molecular characterization of extended-spectrum- and AmpC-β-Lactamase producing *Enterobacteriaceae* isolated from selected commercial spinach supply chains in South Africa. *Front. Microbiol.* 11:638. doi: 10.3389/fmicb.2020.00638

Rochelle-Newall, E., Nguyen, T. M. H., Le, T. P. Q., Sengtaheuanghoung, O., and Ribolzi, O. (2015). A short review of fecal indicator bacteria in tropical aquatic ecosystems: knowledge gaps and future directions. *Front. Microbiol.* 6:308. doi: 10.3389/fmicb.2015.00308

Rossolini, G. M., D'Andrea, M. M., and Mugnaioli, C. (2008). The spread of CTX-M-type extended-spectrum β-lactamases. *Clin. Microbiol. Infect.* 14, 33–41. doi: 10.1111/j.1469-0691.2007.01867.x

Salinas, L., Loayza, F., Cárdenas, P., Saraiva, C., Johnson, T. J., Amato, H., et al. (2021). Environmental spread of extended spectrum beta-Lactamase (ESBL) producing *Escherichia coli* and ESBL genes among children and domesticaimals in Ecuador. *Environ. Health Perspect.* 129:7007. doi: 10.1289/EHP7729

Sato, T., Qadir, M., Yamamoto, S., Endo, T., and Zahoor, A. (2013). Global, regional, and country level need for data on wastewater generation, treatment, and use. *Agric. Water Manag.* 130, 1–13. doi: 10.1016/j.agwat.2013.08.007

Sheu, C. C., Chang, Y. T., Lin, S. Y., Chen, Y. H., and Hsueh, P. R. (2019). Infections caused by carbapenem-resistant *Enterobacteriaceae*: an update on therapeutic options. *Front. Microbiol.* 10:80. doi: 10.3389/fmicb.2019.00080

Shrivastava, S. R., Shrivastava, P. S., and Ramasamy, J. (2018). World health organization releases global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. *J. Med. Soc.* 32, 76–77. doi: 10.4103/jms.jms_25_17

Song, J., Oh, S. S., Kim, J., and Shin, J. (2020). Extended-spectrum β-lactamase-producing *Escherichia coli* isolated from raw vegetables in South Korea. *Sci. Rep.* 10:19721. doi: 10.1038/s41598-020-76890-w

Soria Segarra, C., Soria Baquero, E., and Cartelle Gestal, M. (2018). High prevalence of CTX-M-1-Like enzymes in urinary isolates of *Escherichia coli* in Guayaquil, ecuador. *Microb. Drug Resist.* 24, 393–402. doi: 10.1089/mdr.2017.0325

Touchon, M., Perrin, A., de Sousa, J. A. M., Vangchhia, B., Burn, S., O'Brien, C. L., et al. (2020). Phylogenetic background and habitat drive the genetic diversification of *Escherichia coli*. *PLoS Genet.* 16:e1008866. doi: 10.1371/journal.pgen.1008866

UNESCO (2012). *Managing Water Under Uncertainty and Risk, The United Nations World Water Development Report 4*. Paris: United Nations Educational, Scientific and Cultural Organization.

Vasco, G., Spindel, T., Carrera, S., Grigg, A., and Trueba, G. (2015). The role of aerobic respiration in the life cycle of *Escherichia coli*: public health implications. *Av. Cienc. Ingenierías* 7, B7–B9.

Vital, P. G., Zara, E. S., Paraoan, C. E. M., Dimasupil, M. A. Z., Abello, J. J. M., Santos, I. T. G., et al. (2018). Antibiotic resistance and extended-spectrum beta-lactamase production of *Escherichia coli* isolated from irrigation waters in selected urban farms in Metro Manila, Philippines. *Water (Switzerland)* 10:548. doi: 10.3390/w10050548

Waters, N. R., Abram, F., Brennan, F., Holmes, A., and Pritchard, L. (2020). Easy phylotyping of *Escherichia coli* via the EzClermont web app and command-line tool. *Access Microbiol.* 2:acmi000143. doi: 10.1099/acmi.0.000143

Yang, F., Shen, C., Zheng, X., Liu, Y., El-Sayed Ahmed, M. A. E., Zhao, Z., et al. (2019). Plasmid-mediated colistin resistance gene mcr-1 in *Escherichia coli* and *Klebsiella pneumoniae* isolated from market retail fruits in Guangzhou, China. *Infect. Drug Resist.* 12, 385–389. doi: 10.2147/IDR.S194635

Yassin, A. K., Gong, J., Kelly, P., Lu, G., Guardabassi, L., Wei, L., et al. (2017). Antimicrobial resistance in clinical *Escherichia coli* isolates from poultry and livestock, China. *PLoS One* 12:e0185326. doi: 10.1371/journal.pone.0185326

Zankari, E., Hasman, H., Cosentino, S., Vestergaard, M., Rasmussen, S., Lund, O., et al. (2012). Identification of acquired antimicrobial resistance genes. *J. Antimicrob. Chemother.* 67, 2640–2644. doi: 10.1093/jac/dks261

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REVIEW
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A Review on Occurrence and Spread of Antibiotic Resistance in Wastewaters and in Wastewater Treatment Plants: Mechanisms and Perspectives

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This paper reviews current knowledge on sources, spread and removal mechanisms of antibiotic resistance genes (ARGs) in microbial communities of wastewaters, treatment plants and downstream recipients. Antibiotic is the most important tool to cure bacterial infections in humans and animals. The over- and misuse of antibiotics have played a major role in the development, spread, and prevalence of antibiotic resistance (AR) in the microbiomes of humans and animals, and microbial ecosystems worldwide. AR can be transferred and spread amongst bacteria *via* intra- and interspecies horizontal gene transfer (HGT). Wastewater treatment plants (WWTPs) receive wastewater containing an enormous variety of pollutants, including antibiotics, and chemicals from different sources. They contain large and diverse communities of microorganisms and provide a favorable environment for the spread and reproduction of AR. Existing WWTPs are not designed to remove micropollutants, antibiotic resistant bacteria (ARB) and ARGs, which therefore remain present in the effluent. Studies have shown that raw and treated wastewaters carry a higher amount of ARB in comparison to surface water, and such reports have led to further studies on more advanced treatment processes. This review summarizes what is known about AR removal efficiencies of different wastewater treatment methods, and it shows the variations among different methods. Results vary, but the trend is that conventional activated sludge treatment, with aerobic and/or anaerobic reactors alone or in series, followed by advanced post treatment methods like UV, ozonation, and oxidation removes considerably more ARGs and ARB than activated sludge treatment alone. In addition to AR levels in treated wastewater, it examines AR levels in biosolids, settled by-product from wastewater treatment, and discusses AR removal efficiency of different biosolids treatment procedures. Finally, it puts forward key-points and suggestions for dealing with and preventing further increase of AR in WWTPs and other aquatic environments, together with a discussion on the use of mathematical

models to quantify and simulate the spread of ARGs in WWTPs. Mathematical models already play a role in the analysis and development of WWTPs, but they do not consider AR and challenges remain before models can be used to reliably study the dynamics and reduction of AR in such systems.

Keywords: antibiotics, antibiotic resistance genes, antibiotic resistant bacteria, spread mechanisms, wastewater treatment plants

INTRODUCTION

Antibiotic substances are by far the most powerful tools available for the treatment of infectious diseases by inhibition of bacterial cell growth. In addition to being used for the treatment of infections in human patients and farm animals, antibiotics are also routinely given to healthy farm animals to promote growth and proactively prevent disease outbreaks. Antibiotic resistance (AR) is the ability of bacteria to overcome and resist exposure to antibiotic substances, this is made possible by the acquisition of antibiotic resistance genes (ARGs) (Davison et al., 2000; Wright, 2010). Extensive use of antibiotics since the successful purification and mass production of penicillin in the middle of the twentieth century until today has led to an increase in antibiotic resistance, compromising the effectiveness of antibiotics (Davies and Davies, 2010).

Antibiotic resistance is a global and challenging issue (Walsh, 2003; Deurenberg and Stobberingh, 2008; Livermore, 2012). The risk it poses needs to be tackled in a context that combines environmental, and human health, which focuses on the mechanisms that drive biological (growth and exchange) and physiochemical (transport and conversion) spread. Considering human health issues like AR in a context that combines human, animal and environmental factors is the essence of the One Health initiative's perspective, endorsed by the World Health Organisation (WHO) (One Health Initiative, 2020) and AR has large implications for half a dozen of the United Nation's (UN's) sustainable development goals (WHO, 2020). Part of the issue is to understand how ARGs spread in different environments [wastewater, wastewater treatment plants (WWTPs), soil, and receiving aquatic eco-system] to prevent the spread existing and the development of new ARGs (Brooks et al., 2008).

For more than 100 years, the Activated Sludge Process has been and still is among the most widespread wastewater treatment technologies used for the removal of key pollutants from municipal wastewater (Stensel and Makinia, 2014; van Loosdrecht and Brdjanovic, 2014). By bacterial uptake and metabolic conversions of organics and nutrients, cellular growth provides for an auto-catalytical removal process which is further enhanced by settling and recirculation of active biomass as originally proposed by Ardern and Lockett (1914). While bacterial densities in wastewater are normally in the range of 10^5–10^8 cells per ml (Tchobanoglous et al., 2014), the enhancement of biomass in modern biological WWTPs increases the bacterial density in the bioreactors by 3 orders of magnitude and selection by sedimentation results in dense bacterial aggregates. Additionally, depending on operating conditions and temperature, there can be very high material turnover (up to 90%) and much higher specific heterotrophic growth rates (up to 13.2 day^{-1}) in WWTPs bioreactors than in natural water systems. In WWTP bioreactors, microbial diversity and interactions are ubiquitous and frequent (Daims et al., 2006; Nielsen and McMahon, 2014). High abundance, density, diversity, activity, and interactions in the activated sludge bioreactors would suggest an increased rate of gene transfer, including horizontal and vertical exchange of ARG. Mechanisms and rates at which exchange occur in the microbiome of these systems are now under intense study, and resistomes (all ARG's in a microbial community) of activated sludge systems are currently being mapped (Manaia et al., 2018).

Established effluent standards set the quality of WWTP effluents based on environmental effect parameters such as the chemical and biochemical oxygen demand, the amount of suspended solids, total nitrogen, number of coliform bacteria, etc. (Directive 91/271/Eec, 1991). It is not known how, or whether at all, these parameters indicate the prevalence of antibiotic resistant bacteria (ARB) and ARGs. Lately, more attention has been paid to examine detection and elimination techniques for ARB and ARGs, in addition to removal techniques for other micropollutants like detergents, pesticides, pharmaceuticals, and personal care products (Łuczkiewicz et al., 2010; Luo et al., 2014). Some WWTPs use extra disinfection units at the end of the biological treatment process, which include chlorination, UV radiation and ozonation, or quaternary advanced treatment techniques such as advanced oxidation processes (AOPs) or membrane filtration. Such unit processes can as this review will show reduce the number of ARB and possibly also ARGs in the WWTP effluent but are costly to operate and may not be as effective as observed in laboratory studies (Auerbach et al., 2007; Zhang et al., 2015, 2016a; Zhuang et al., 2015). Biological removal of organic material from wastewater is linked to the fast growth of microorganisms in the WWTP, and since the biomass builds up some is continuously discarded as excess biological sludge. ARB and ARGs that are present in the biomass of the reactor will also be present in the sludge, therefore further treatment processes of excess sludge need to be considered. We will in the last part of section four of this review go through the current knowledge of how effective the different sludge treatment methods are able in reducing ARB and ARGs.

This review will present the major groups of antibiotics, the major groups of mechanisms for antibiotic resistance in bacteria, and the general bacterial mechanisms for genetic exchange, but only briefly as other reviews already have covered this in general (Wright, 2010, 2011; Pazda et al., 2019; Zhu et al., 2021) and in the context of wastewater treatment. More space is instead given to go through what is known about which wastewater sources

show the high occurrence of antibiotic resistance, how antibiotic resistance persists and spreads through WWTPs, and what contributes to this persistence. Previous works in the literature already focused on complete lists of every specific type of ARGs that have been found in WWTPs (Pazda et al., 2019), and on the strength and weaknesses of different methods used to measure and analyze ARB and ARGs content in wastewater (Manaia et al., 2018). Therefore, this review will focus on documenting what is known about the removal efficiency of different treatment processes or technologies, i.e., what are the reported elimination efficiencies of ARGs and ARB for different treatment technologies for both wastewater and sludge, and whether the reported results are consistent. Moreover, in this work special efforts have been put into gathering and reviewing results from studies of elimination of ARGs and ARB in different sludge and biosolids treatment processes, as this has been more or less overlooked in other reviews (Barancheshme and Munir, 2018; Pazda et al., 2019; Bairán et al., 2020; Zhu et al., 2021).

In essence, this systematic review aims to describe the factors that affect the persistence and spread of antibiotic resistance in wastewater treatment and to evaluate current and emerging treatment technologies. For completeness this review documents removal efficiencies for antibiotic substances for different treatment technologies, but it does not aim to discuss the pathways and mechanisms for the breakdown of these substances at length, which has been addressed in a recent review by Zhu et al. (2021). Additionally, this review will also discuss how mathematical models can be used to better understand the dynamics of antibiotic resistance spread in WWTPs. It has been suggested that mathematical modeling can help to quantify and simulate the spread of ARGs in WWTPs, but as this review will show only a few models have been proposed and even fewer have been sufficiently parameterized and validated. It will discuss why, and which challenges remain to be tackled before mathematical models can be used to their full potential. Finally, this review concludes with future directions and some key points that should be prioritized for improving the current state of antibiotic resistance in WWTPs.

ANTIBIOTIC RESISTANCE: MECHANISMS, SOURCES, AND TRANSFER

Antibiotics are classified into five major groups, according to their mode of action (**Figure 1**): (i) Cell wall synthesis inhibition (*vancomycin*, *cephlosporins*, β*–lactams*, *bacitracin*); (ii) Protein synthesis inhibition (*aminoglycosides*, *chloramphenicol*, *tetracycline*, *linezolid*); (iii) Nucleic acid synthesis inhibition (*rifampin*, *metronidazole*, *quinolones*, *fluoroquinolones*); (iv) Antimetabolites (*trimethoprim*, *dapsone*, *sulphonamide*) and; (v) Cell membrane disintegration (*polymyxin*, *daptomycin*). Note that some sources use a coarser division into only four groups (Calderón and Sabundayo, 2007; Kapoor et al., 2017) whereas other use a bit finer division into six (Wanger et al., 2017).

Bacteria have developed four main types of resistance mechanisms against antibiotics (**Figure 1**; Zhang et al., 2009; Wright, 2010): (i) Efflux pumps, which effectively excrete antibiotics from the cell (Wright, 2011). There are five efflux protein families: ATP-binding cassette (ABC), multidrug and toxic compound extrusion (MATE), major facilitators (MFs), resistance nodulation cell division (RND), and small multidrug resistance (SMR) (Nishino and Yamaguchi, 2001). (ii) Inactivation of antibiotics occurs when the activity of the antibiotic substance is directly hindered by hydrolysis, or by conversion of functional groups etc. (Wright, 2005; Diaz et al., 2014). (iii) Target by-pass: strategies for target by-pass includes creating new pathways to circumvent the originally targeted enzyme, overproduction of the target compound (Munita and Arias, 2016), structural changes in the cell wall (Vila et al., 2007), and prevention of the antibiotic to bind to its target (Wright, 2010). (iv) Target modification: occurs through modification of the antibiotic targets themselves (Wright, 2010). Multiple types of resistance mechanisms may simultaneously confer resistance against the same family of antibiotics (de Sousa Oliveira et al., 2016). Conversely, one type of resistance mechanism can also confer resistance against more than one type of antibiotics.

Wastewater from hospitals and wastewater and waste from animal husbandry together with runoff from manure amended fields are essential ARB and ARGs sources in aquatic ecosystems (Marti and Balcazar, 2013). Hospital wastewaters have especially been shown to contain many ARGs (Zhang et al., 2009; Rowe et al., 2017). One of the first reports dates back to the early 1970s, Grabow and Prozesky (1973) studied the presence of resistant coliforms in hospital wastewater in Pietermaritzburg in South Africa. They found that 26% of coliform bacteria in hospital wastewater had transferable resistance while only 4% of coliform bacteria in municipal wastewater had transferable resistance (Grabow and Prozesky, 1973). The same trend is seen today. Based on several studies done in Europe and Asia, the total ARGs and ARB concentrations in hospital wastewater were 2–9 orders of magnitude higher than municipal wastewater (Li et al., 2015; Lamba et al., 2017; Hutinel et al., 2019). Rowe et al. (2017) showed that the normalized abundance of ARGs in hospital wastewater samples from the Cambridge University Hospitals was 9-folds greater than in samples collected from the effluent lagoon of the University of Cambridge dairy farm and 34-folds greater than in samples from the River Cam source water, which served as background samples for the environment. Another detailed study of wastewater from three different hospitals in Romania showed the presence of genes encoding for resistance for *tetracyclines*, *aminoglycosides*, *chloramphenicol*, β*-lactams*, *sulphonamides*, *quaternary ammonium*, and *macrolide-lincosamide-streptogramin B* antibiotics with abundance levels in as high ranges as 0.01–0.1 copies per 16S rRNA gene copies measured by qPCR (Szekeres et al., 2017). Moreover, in a recent review by Hassoun-Kheir et al. (2020), 37 studies on the occurrence of AR in hospital wastewaters were examined. The review found that 30 (81%) of the studies reported that hospital wastewater contains higher amounts of AR than community wastewater. Furthermore, in a subset of studies where the impact of hospital wastewater on the dissemination of AR in the environment was considered, 25 out of 32 (78%) studies held

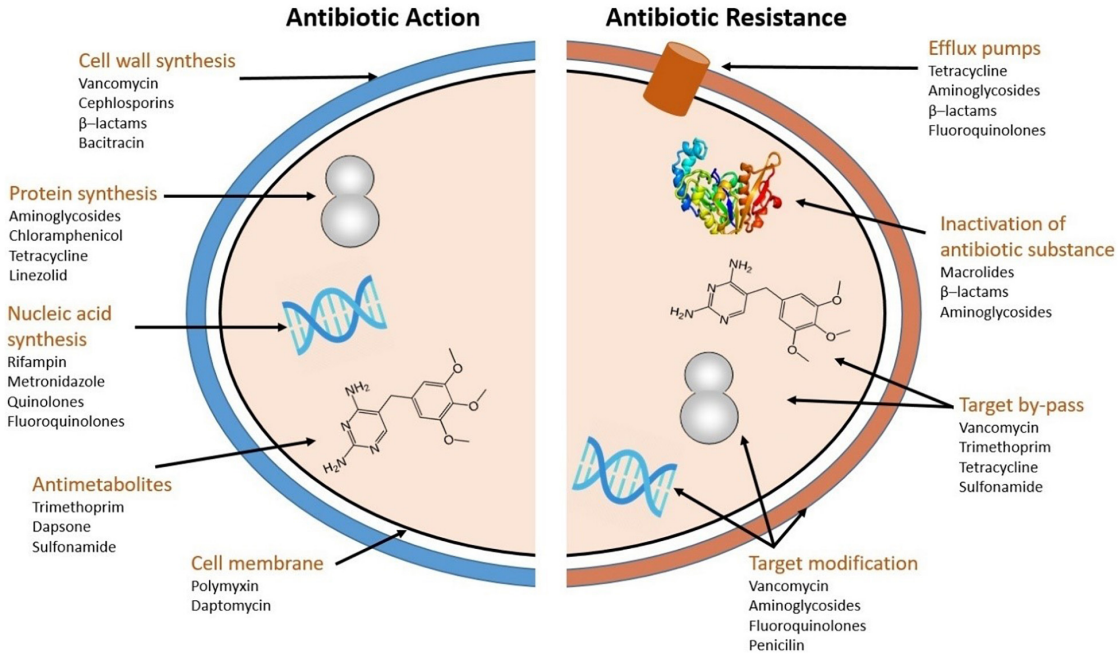

FIGURE 1 | Antibiotic action and resistance mechanisms (adapted from Wright, 2010).

that hospital wastewaters had an important role as a source of AR to the environment.

Apart from the vertical inheritance, antibiotic resistance can be obtained in two ways, through mutation or by horizontal gene transfer (HGT) (Jury et al., 2010). The latter is the most concerning regarding the spread of antibiotic resistance in WWTPs since ARGs can potentially be transferred between organisms effectively and much faster than resistance development through mutations. HGT is a non-reproductive intra- and inter-species transfer of genetic information by means of mobile genetic elements (MGE), such as plasmids and transposons (Barlow, 2009; Huang et al., 2017a). The movement of genes from chromosomes to and between MGEs are mostly facilitated by integrons (Mazel, 2006; Davies and Davies, 2010; Gillings, 2014). There are three different HGT mechanisms for the spread of MGEs. (i) Conjugation: transfer mechanism that requires cell-to-cell contact, where a recipient bacterium acquires genetic material from a donor bacterium, usually in the form of a plasmid (Madigan et al., 2006; **Figure 2**). (ii) Transformation: intra- and inter-species exchange of genetic information by uptake of, free extracellular suspended DNA, which can only be received by a competent bacterium. Following uptake and translocation to the cytoplasm, it is incorporated into the recipient's chromosome or into a plasmid (Madigan et al., 2006; Heuer and Smalla, 2007). Finally; (iii) Transduction: involves bacteriophages that transport different types of genetic elements from their host to the receiver (Cano and Colomé, 1988; Snyder and Champness, 2007; Modi et al., 2013), whereupon this is incorporated into the genome of the new host by recombination (**Figure 2**). There are two types of this mode of transfer, namely generalized and specialized transductions. In generalized transduction, only a segment of bacterial DNA is randomly packed into the bacteriophage head, and bacterial host DNA becomes a part of the DNA of the phage whereas in specialized transduction, both phage and specific bacterial DNA are packed into the head (Chiang et al., 2019). Transduction may also occur *via* gene transfer agents (GTAs), which are DNA carrying structures that resemble bacteriophages, but which do not self-replicate. Although GTAs exact impact has not yet been determined, their potential to act as carriers of resistance in the environment continues their attention (von Wintersdorff et al., 2016).

OCCURRENCE AND SPREAD OF ANTIBIOTIC RESISTANCE GENES IN WASTEWATER TREATMENT PLANTS

Although antibiotic resistance (AR) occurs naturally at low levels in most ecosystems, the occurrence of ARB and ARGs at high levels is associated with anthropogenic activities. **Table 1** shows an overview of resistance genes found in bacteria from wastewater effluents and in aquatic ecosystems. ARGs are frequently detected in WWTPs (Chen and Zhang, 2013; Novo et al., 2013; Rizzo et al., 2013; Manaia, 2014; **Table 1**), and studies have shown that the ARGs found in wastewaters often reside in clinically relevant pathogenic bacteria (Figueira et al., 2011; Marti et al., 2013a; Hembach et al., 2017). Samples from three different stages of a WWTP in Poland showed that approximately 22, 5, and 9% of *Enterobacteriaceae* strains isolated from (i) the raw sewage in the primary sedimentation tank, (ii) the aeration tank, and (iii) from the effluent, respectively, carried the *intI*

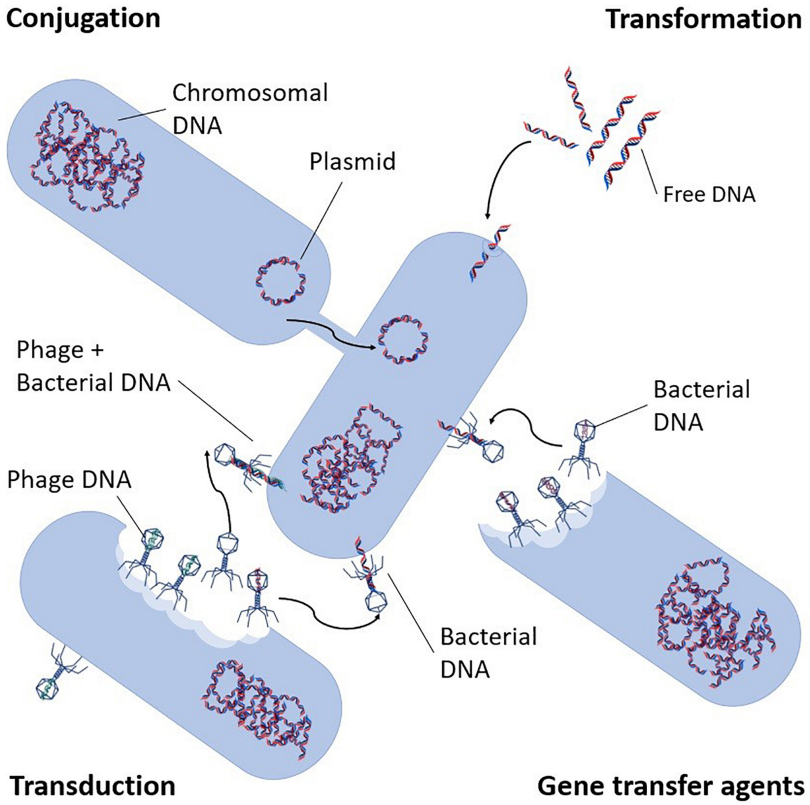

FIGURE 2 | Antibiotic resistance transport mechanisms (adapted from von Wintersdorff et al., 2016).

integron; and that all strains which carried this integron were resistant to at least three unrelated antibiotics (Mokracka et al., 2012). Note that, although a significant fraction of bacteria in the effluent of this WWTP were still resistant, the above percentages must be interpreted with care as the total number of culturable coliform bacteria in the effluent was reduced with a factor of as much as 10^3 in the effluent as compared to in the raw sewage (Mokracka et al., 2012). Many of the *Enterobacteriaceae* isolated from a wastewater treatment plant in the study by Amador et al. (2015) were also found to be resistant, and even multi-resistant. The isolates showed resistance against β-*lactam* group antibiotics, including *cefoxitin*, *amoxicillin*, *cefotaxime*, and non β-*lactam* groups antibiotics, including *trimethoprim/sulfamethoxazole*, *ciprofloxacin*, and *tetracycline*. Other studies (Mokracka et al., 2012; Szekeres et al., 2017; Karkman et al., 2018) have also shown that resistance genes against antibiotics, including *tetracycline*, *methicillin* and *sulphonamide* are present in WWTPs. Based on a review of many studies, *tetracycline* (*tet*) resistance genes have been found to be one of the most commonly occurring ARGs in wastewater treatment systems in many countries (Zhang et al., 2009).

Hospital wastewater is a particular risk as it may contain not only pathogenic single- and multi-drug resistant (MDR) bacteria, as detailed in the previous section but also relatively high concentrations of antibiotic compounds. A high percentage of administered antibiotics are not metabolized in humans and are thus excreted into the sewerage (Sabri et al., 2018). Rodriguez-Mozaz et al. (2015) analyzed a broad range of antibiotics including β-*lactams*, *lincosamides*, *macrolides*, *quinolones/fluoroquinolones*, *sulfonamides*, *tetracyclines*, *dihydrofolate reductase inhibitors*, and *nitroimidazoles* and ARGs released from hospitals and urban wastewaters, their removal by a WWTP effluent and their influence on a receiving river. The results show that antibiotics were detected at high concentrations in downstream river samples with antibiotics such as *ofloxacin* reaching concentrations up to 131.0 ng/L while not being detected upstream of the WWTP discharge. Moreover, *ciprofloxacin* and *sulfamethoxazole* had almost 10-fold higher concentrations in downstream than upstream of the WWTP discharge. Studies indicate that the presence of incompletely degraded antibiotic compounds may exert biological selection pressure for the development of ARGs and provide a breeding ground in WWTPs for HGT between bacteria (Zhang et al., 2009; McKinney and Pruden, 2012; Bouki et al., 2013; Sharma et al., 2014) and propagation of resistance genes (Davies and Davies, 2010).

During wastewater treatment, antibiotics, other pharmaceutical residues, and heavy metals present in the wastewater are in continuous contact with bacteria, leading to the potential selection pressure for resistance genes (Zhang et al., 2009; Ding and He, 2010; Bouki et al., 2013). It is difficult to determine a safe concentration of antibiotics in wastewater as

TABLE 1 | Overview of antibiotic resistance genes (ARGs) found in influent, effluent, and activated sludge in wastewater treatment plants (WWTPs) and if their concentrations increase (↑) or decrease (↓) from influent to effluent [adapted from Pazda et al. (2019) and shortened to only include studies that have measured changes in concentration from influent to effluent].

Antibiotics	Antibiotic resistance genes (ARGs)	Sample				Country	References
		Influent	effluent	activated sludge			
β–Lactams	*ampR*		+	↓	N.A.	Canada	Biswal et al., 2014
	bla_{AmpC}	+	+	↑	N.A.	Germany	Alexander et al., 2015
	bla_{CMY-13}	+	+	↑	N.A.	Sweden	Bengtsson-Palme et al., 2016
	bla_{CTX-M}	+	+	↓	N.A.	Canada	Neudorf et al., 2017
	bla_{CTX-M}	+	+	↓	N.A.	Canada	Neudorf et al., 2017
	$bla_{CTX-M-1}$	+	+	↑	N.A.	Portugal	Amador et al., 2015
	$bla_{CTX-M-9}$	+	−	↓	+	Poland	Korzeniewska et al., 2013
	$bla_{CTX-M-12}$	+	+	↑	+	Canada	Biswal et al., 2014
	$bla_{CTX-M-32}$	+	+	↓	N.A.	Denmark	Laht et al., 2014
	bla_{FOX}	+	+	↑	N.A.	Portugal	Amador et al., 2015
	bla_{OXA}	+	+	↓	+	China	Yang et al., 2014
	bla_{OXA}	+	+	↑	N.A.	Portugal	Amador et al., 2015
	bla_{OXA-10}	+	+	↓	+	China	Yang et al., 2014
	bla_{OXA-46}	+	+	↓	+	China	Yang et al., 2014
	bla_{OXA-58}	+	+	↓	N.A.	Denmark	Laht et al., 2014
	bla_{OXA-58}	+	+	↓	N.A.	Finland	Hultman et al., 2018
	bla_{SHV-5}	+	−	↓	+	Poland	Korzeniewska et al., 2013
	bla_{TEM}	+	+	↑	N.A.	Canada	Biswal et al., 2014
	bla_{TEM}	+	+	↑	N.A.	Portugal	Amador et al., 2015
	bla_{TEM}	+	+	↑	N.A.	Spain	Rodriguez-Mozaz et al., 2015
	bla_{VIM-1}	+	+	↑	N.A.	Germany	Alexander et al., 2015
	bla_{VIM}-11	+	+	↓	+	China	Yang et al., 2014
Quinolone	*gyrA*	+	+	↓	+	China	Xu et al., 2015
	parC	+	+	↓	+	China	Xu et al., 2015
	gnrC	+	+	↑	+	China	Xu et al., 2015
	gnrD	+	+	↓	+	China	Xu et al., 2015
	gnrS	+	+	↑	N.A.	Canada	Neudorf et al., 2017
	gnrS	+	+	↑	N.A.	Spain	Rodriguez-Mozaz et al., 2015
	gnrS1	+	+	↓	N.A.	Canada	Biswal et al., 2014
Macrolide	*ereA*	+	+	↑	N.A.	Canada	Biswal et al., 2014
	ereB	+	+	↑	N.A.	Canada	Biswal et al., 2014
	ermB	+	+	↓	+	China	Yang et al., 2014
	ermB	+	+	↓	N.A.	Canada	Neudorf et al., 2017
	ermB	+	+	↓	N.A.	Germany	Alexander et al., 2015
	ermB	+	+	↑	N.A.	Spain	Rodriguez-Mozaz et al., 2015
	ermF	+	+	↓	+	China	Yang et al., 2014
	macB	+	+	↓	+	China	Yang et al., 2014
	mef	+	+	↓	+	China	Yang et al., 2014
	mph(A)	+	+	↑	N.A.	Canada	Biswal et al., 2014
Tetracycline	*tetA*	+	+	↓	+	China	Xu et al., 2015
	tetB	+	+	↓	N.A.	Canada	Biswal et al., 2014
	tetB	+	+	↓	+	China	Xu et al., 2015
	tetB(P)	+	+	↑	+	Sweden	Bengtsson-Palme et al., 2016
	tetC	+	+	↓	N.A.	Denmark	Laht et al., 2014
	tetE	+	+	↑	+	China	Xu et al., 2015
	tetG	+	+	↓	+	China	Yang et al., 2014
	tetM	+	+	↓	N.A.	Finland	Hultman et al., 2018
	tetM	+	+	↓	+	China	Yang et al., 2014
	tetO	+	+	↓	+	China	Yang et al., 2014
	tetQ	+	+	↓	+	China	Yang et al., 2014
	tetV	+	+	↑	+	China	Yang et al., 2014
	tetW	+	+	↓	+	China	Yang et al., 2014
	tetX	+	+	↓	+	China	Yang et al., 2014
	tetZ	+	+	↑	+	China	Xu et al., 2015
	tet32	+	+	↓	+	China	Yang et al., 2014
	dfrA3	+	+	↑	N.A.	Sweden	Bengtsson-Palme et al., 2016
	dfrA20	+	−	↓	N.A.	Canada	Biswal et al., 2014
	dhfrXV	+	−	↓	N.A.	Canada	Biswal et al., 2014
	sulI	+	+	↓	N.A.	Canada	Neudorf et al., 2017
	sulI	+	+	↑	N.A.	Canada	Biswal et al., 2014
	sulI	+	+	↓	N.A.	Denmark	Laht et al., 2014
	sulI	+	+	↓	+	China	Xu et al., 2015
	sulI	+	+	↑	+	China	Yang et al., 2014
	sulI	+	−	↓	N.A.	United States	Bergeron et al., 2015
	sulII	+	+	↑	N.A.	Canada	Biswal et al., 2014
	sulII	+	+	↓	+	China	Yang et al., 2014
	sulIII	+	+	↓	+	Canada	Biswal et al., 2014
Multidrug efflux pump genes	*mdtF*	+	+	↓	+	China	Zhang et al., 2011
	mdtG	+	+	↓	+	China	Zhang et al., 2011
	mdtH	+	+	↓	+	China	Yang et al., 2014
	mdtN	+	+	↓	+	China	Yang et al., 2014
	mexB	+	+	↓	+	China	Yang et al., 2014
	mexD	+	+	↓	+	China	Yang et al., 2014
	mexF	+	+	↓	+	China	Yang et al., 2014

N.A., not analyzed; N.D, no difference.

results disagree on whether or not antibiotic concentrations lower than the minimum inhibitory concentrations (MIC) cause selection of ARGs. Gullberg et al. (2011) competed for resistant strains against susceptible strains in monoculture with different antibiotic concentrations. The result showed that the resistant strains have a selection advantage even in subminimal inhibitory concentrations and outperform the

susceptible strains (Gullberg et al., 2011; Andersson and Hughes, 2014). On the other hand, a recent study by Klümper et al. (2019) suggests that a diverse bacterial community in a mixed culture may select against resistance. Resistant and non-resistant (otherwise isogenic) focal strains (Escherichia *coli*) cultivated together with a pig fecal community, exhibited more than one order of magnitude higher minimal selection concentration for *gentamicin* or *kanamycin*. For the *gentamicin* resistant focal strain, reduced growth was observed due to higher fitness cost for a range of *gentamicin* concentrations (0–10 μg/ml), indicating that resource limitations have a stronger impact on resistant phenotypes (Gómez and Buckling, 2011; Wale et al., 2017; Klümper et al., 2019). However, at very high gentamicin concentrations (100 μg/ml) only resistant strains could grow. The same behavior was observed under intermediate *kanamycin* concentrations (0–20 μg/ml), the susceptible strain did again show improved growth compared to the resistant strain when co-cultured with the pig fecal community. These findings are in accordance with results from the study of Galera-Laporta and Garcia-Ojalvo (2020), where susceptible Bacillus *subtilis* and *E. coli* were cultivated exposed to *ampicillin* separately and in mixed culture. Cultivated separately, *B. subtilis* was able to grow after a lag phase, while *E. coli* died. Cultivated in a mixed culture the two strains displayed reversed reactions to *ampicillin*. The protective effect of the community might play a role and further experimental effort to evaluate the risk of sub-minimal inhibitory concentrations are required.

Heavy metals and some organic compounds, such as quaternary ammonium compounds (QAC), monoaromatic hydrocarbons (MACH), anti-fouling agents and detergents can increase the selective pressure for ARGs through co-selection (Schlüter et al., 2007; Tuckfield and McArthur, 2008; Di Cesare et al., 2016). Two mechanisms for co-selection are normally distinguished: Co-resistance and cross-resistance. Co-resistance refers to the presence of resistance against more than one class of antibiotics in the same bacterial strain. It occurs due to the physical link between different resistance genes, that are placed together, for example on a plasmid, where the selection of resistance to one of the genes leads to resistance to others. Heavy metal resistance genes (HMRGs), especially against zinc and copper, have been shown to increase the rate of AR dissemination by co-resistance (Yazdankhah et al., 2014). Another example is the co-resistance of *qac* genes encoding for efflux pumps against QAC and MACH; the *qac* genes are typically located on MGEs (plasmids and transposons), often together with ARGs (Jiao et al., 2017). In cross-resistance, however, one single resistance mechanism confers resistance to an entire class of compounds, antibiotics and/or other toxicants (Baker-Austin et al., 2006). For example, if two different antimicrobials are present and both have a common strategy to attack the cell, resistance developed against one will be effective against both i.e., the resistance gained for one compound confers resistance for another compound. An example of cross resistance is multi-drug resistance pumps that can export both metals and antibiotics (Baker-Austin et al., 2006). Thus, co-selection is a plausible explanation for the persistence of some ARGs even when antibiotics are not present (Zhang et al., 2018) and both co- and cross-resistance have an important impact on the antibiotic resistance selection in different environments (Stepanauskas et al., 2005; Knapp et al., 2017).

ANTIBIOTIC RESISTANCE GENE REMOVAL IN WASTEWATER TREATMENT PLANTS

There are many treatment techniques used in WWTPs that have varying potential to remove organic matter, nitrogen, phosphorous, pollutants and pathogens from wastewater. However, the mechanisms and efficacy of these techniques to remove antibiotics, ARB and ARGs remain mostly unexplored. This section aims to look at the existing situation for the removal of ARB and ARGs from wastewater and sludge in WWTPs.

Removal From Wastewater

The operation of redox gradient aerobic, anoxic and anaerobic activated sludge reactors and their sequence in a WWTP affects the removal of ARB and ARGs (Christgen et al., 2015; Du et al., 2015; Szekeres et al., 2017). Du et al. (2015) found that anoxic and anaerobic treatment reduced the concentration of ARGs in wastewater, whereas aerobic treatment increased the concentration. The same has been observed by Pei et al. (2007) who proposed that the difference is related to lower growth rates in anaerobic and anoxic compartments compared to aerobic. However, Christgen et al. (2015) have compared three different wastewater treatment strategies; anaerobic, aerobic, and anaerobic–aerobic sequence bioreactors (AAS) in terms of energy use, treatment performance, and ARG abundance. They reported an opposite effect that aerobic bioreactors and AAS bioreactors had higher ARG removal efficiencies than anaerobic bioreactors alone. The AAS bioreactors showed higher removal of ARGs (>85%), compared to separate aerobic (83%) and anaerobic (62%) treatment systems (Christgen et al., 2015). The authors concluded that even though none of these systems were perfect for ARG removal aerobic and AAS were superior to anaerobic bioreactors. Additionally, results suggested that due to lower energy consumption (32% less) AAS systems were seen to be a promising treatment alternative. Moreover, temperature also plays a role in the removal of ARGs showing higher removal at 20°C than at 4°C (Pei et al., 2007), and aerobic treatment may remove more of some types of ARGs than anaerobic at 20°C.

Membrane bioreactors (MBRs) are potentially much better at removing ARB and ARGs than traditional activated sludge reactors. This is because MBRs are better at removing bacteria in general, due to the extra filtration of the effluent through the membrane (Pauwels et al., 2006). The previously mentioned study by Du et al. (2015) reported that the concentration of ARGs throughout a sequence of treatment steps declined proportionally more in the final treatment in an MBR than it did in any of the prior treatment steps in aerobic and anoxic/anaerobic reactors. The MBR showed more than 5 $\log_{10}$ units gene copies/100 ml removal of *tetG*, *tetW*, *tetX*, and *sulI* resistance genes, mostly due to filtration (pore size 0.1–0.4 μm) (Du et al., 2015; Hiller et al., 2019). Research by Kappell et al. (2018) has similarly shown the

effectiveness of anaerobic MBRs with ARG removals of up to 3.6 $\log_{10}$.

Schwermer et al. (2018) investigated the efficiency of two WWTPs in the removal of *ampicillin*, *sulfamethoxazole*, *ciprofloxacin*, and *tetracycline* resistant *E. coli*. The two WWTPs employed a biofilm process and a conventional activated sludge treatment process, respectively. By physical and chemical treatment strategies in WWTP, the percentage of resistant *E. coli* was reduced but full disinfection was not achievable. However, in both conventional activated sludge and the biofilm processes, the percentage of cultivable resistant *E. coli* did not show a considerable decrease in addition to the physical and chemical treatment steps. Moreover, the effluents were also subjected to ultrafiltration (UF) and the total removal effectiveness of *E. coli* in both WWTPs with UF was >4.2 log. Although the ability of DNA to pass through membranes was mentioned by the authors, they stated that membrane filtration processes can provide an additional barrier and post-treatment alternative for the effluent in order to reduce ARB and ARG release by WWTP effluents. Other membrane filtration processes that can be used as post treatment methods include microfiltration (MF) and reverse osmosis (RO). While the effectiveness of MF efficiency against ARB and ARGs has been studied (Riquelme Breazeal et al., 2013), the application of RO, alone or combined with other methods, has yet to be investigated in detail (Schwermer et al., 2018).

Constructed wetlands (CWs) are engineered aquatic systems with very diverse microbial communities and are used to treat wastewater by the same biogeochemical processes dominant in natural wetlands (Doherty et al., 2015; Lv et al., 2017). They are, however, mostly relevant for cases where the total amount of wastewater is relatively low, or for wastewater with lower amounts of organic matter, e.g., urban and agricultural runoff or post treatment of effluents from conventional treatment plants, rather than raw sewage (Zhang et al., 2018; Liu et al., 2019). Their ability to remove ARB and ARGs have brought CWs to attention. CW's removal mechanisms are dependent on different conditions such as phyta and substrate types together with the physical design of the CW itself (Liu et al., 2019). Li et al. (2019) investigated removal efficiencies for antibiotic and ARG in riverine constructed wetlands. Their results showed that one constructed wetland had 46 and 80% removal efficiency for antibiotics and ARGs, respectively, while another wetland had 70 and 88% removal efficiency, respectively. The difference in efficiencies was associated with antibiotic concentrations in the influent into both wetlands and the scale of the wetland, indicating that the presence of sub-inhibitory levels of antibiotics increases the selective pressure for resistance (Li et al., 2019). In a different study, Chen et al. (2019) designed four different hybrid constructed wetlands. Two horizontal sub-surface flow (HSSF) CWs, one with and one without artificial aeration, and two vertical sub-surface flow (VSSF) CWs, again one with and one without artificial aeration. These four CWs were tested for their ability to remove antibiotics and ARGs. Efficiencies between 87 and 95% for total antibiotic removal, and between 88 and 99% for total ARG removal, were reported. The authors found that the hybrid constructed wetlands with artificial aeration compared to CWs without artificial aeration had higher removal efficiencies of ARB and ARGs, together with higher removal rates of organic carbon, ammonia, nitrogen, and phosphorous.

Several recent studies have investigated the removal of ARGs in WWTPs by chemical disinfection processes, such as chlorination and advanced oxidation processes (AOPs) including ozonation and UV. It showed that these processes can significantly decrease the occurrence of ARGs and pathogenic microorganisms in WWTP effluents (Luczkiewicz et al., 2011; Zhuang et al., 2015; Hiller et al., 2019). Zhuang et al. (2015) reported chlorine disinfection resulted in 1.654–2.28 $\log_{10}$ reduction, and UV irradiation resulted in 0.80–1.21 $\log_{10}$ reduction of ARGs under economically suitable operational conditions. Although ozonation disinfection achieved 1.68–2.55 $\log_{10}$ reduction of ARGs, the authors in the same study advised against the use of this process due to excessive operational costs. Contrary to this, Alexander et al. (2016) indicated that even though ozone treatment can reduce the *erythromycin* resistance gene (*ermB*) by 2 orders of magnitude, ARGs against *vancomycin (vanA)* and *imipemem (blaVIM)* increased within the surviving wastewater bacterial population. Luczkiewicz et al. (2011) showed that ultrafiltration, ozonation, and UV irradiation can reduce the amount of fecal coliform bacteria in wastewater by more than 99%, but there was a slightly higher percentage of ARGs containing bacteria among the bacteria surviving disinfection. They found that of coliforms grown from water samples taken before and after disinfection, 47–60% of *E. coli* isolates were resistant after disinfection compared to 42–50% of isolates before and that 68–90% of *Enterrococus* spp. isolates had resistance after treatment compared to 68–85% before. Recently, using a the combination of two or more AOPs (like Fenton's oxidation reaction, UV/H_2O_2, solar/H_2O_2, photo-Fenton process, TiO_2 photocatalyst and ionizing radiation) have been shown to be effective in the removal of refractory organic compounds (like antibiotics) in secondary effluents (Rizzo et al., 2013; Zhang et al., 2016b). Karaolia et al. (2014) investigated a solar-driven Fenton oxidation that may eliminate ARB. Mccullagh et al. (2007) reported that the utilization of a UV-TiO_2 photocatalyst AOP inactivated a diverse array of bacterial, viral, and protozoal organisms from water and wastewater. While AOPs represent a potential way to remove antibiotics and thus prevent antibiotic resistance, they are not widely used due to their operational costs (Qiao et al., 2018). **Table 2** shows different treatment techniques and their effectiveness in removing different ARGs and pathogens from wastewater under different conditions. Additionally, **Table 3** summarizes the antibiotic elimination efficiencies in different wastewater treatment units. Both tables include information on different treatment techniques categorized in physical, biological, and chemical processes.

All the studies conducted in the literature together with the information presented in **Tables 2**, **3**, suggest that the wastewater to be treated should be analyzed for antibiotics and ARGs, in addition to the standard wastewater characterization parameters. The WWTP should be designed using this characterization specially tailored for the needs of the specific wastewater ensuring the removal of antibiotics and ARGs to avoid the spread of

TABLE 2 | Antibiotic resistance gene (ARG) and pathogen elimination efficiencies of different treatment technologies.

Treatment technologies	Target ARGs	ARG elimination efficiency	Pathogen elimination efficiency	References
Physical processes				
Membrane separation	*floR*, *sulI*, and *sulII*	∼98%	99.9%	Ren et al., 2018
Soil aquifer treatment	bla_{TEM} and *qnrS*	>2 logs	1.2–6.9 logs	Sharma and Kennedy, 2017; Elkayam et al., 2018
Biological processes				
Anaerobic–aerobic seq. bioreactor (AAS)	*Sulfonamide*, *chloramphenicol*, *aminoglycoside*, *tetracycline*, β-*lactam resistance genes*	>85%		Christgen et al., 2015; Thwaites et al., 2018
Aerobic bioreactor		83%		Christgen et al., 2015
Anaerobic bioreactors		62%	18%	Christgen et al., 2015; Zhang et al., 2017
Membrane bioreactor	*bl*and*M-1*, *blaCTX-M-15*, *and blaOXA-48*	2.76–3.84 logs	2.7–5.6 logs	Cheng and Hong, 2017; Harb and Hong, 2017
	sulI, *sulII*, *tetC*, *tetX*, *ereA*, and *int1*	0.5–5.6 logs	–	Zhu et al., 2018
	sul1, *tetG*, *tetW*, and *tetX*	5 log/100 ml	–	Du et al., 2015
	ermB, *tetO*, *sulI*, and *intI1*	≤3.6 log	–	Kappell et al., 2018
CW-surface flow	*sulI*, *sulII*, *sulIII*, *tetA*, *tetB*, *tetC*, *tetE*, *tetH*, *tetM*, *tetO*, *tetW*, *qnrB*, *qnrS*, and *qepA*	77.8% in summer, 59.5% in winter	0.96–4.46 logs	Fang et al., 2017; Shingare et al., 2019
CW-horizontal subsurface flow	*intI1*, *sulI*, *sulII*, *dfrA*, *aac6*, *tetO*, *qnrA*, *blaNMD1*, *blaKPC*, *blaCTX*, and *ermB*	-145.6 to 98.9%	0.7–5.51 logs	Yi et al., 2017; Chen et al., 2019; Shingare et al., 2019
CW-vertical subsurface flow	*tet genes* and *intI1*	33.2–99.1%	0.5–2.84 logs	Huang et al., 2017b; Shingare et al., 2019
CW	*sulI*, *sulII*, *tetA*, *tetC*, *dfrA1*, *dfrA12*, *dfrA13*, *ermB*, and bla_{PSE-1}	80.2 and 87.5%	–	Li et al., 2019
Hybrid CWs	*sulI*, *sulII*, *tetG*, *tetO*, *ermB*, *qnrS*, *qnrD*, *cmlA*, and *floR*	87.8–99.1%	0.71–4.8 logs	Chen et al., 2019; Shingare et al., 2019
Chemical processes				
Chlorination	*sulI*, *tetG*, and *intI1*	1.65–2.28 logs	∼3 logs	Zhuang et al., 2015; Furst et al., 2018
	sulI, *tetX*, *tetG*, and *intI1*	1.20–1.49 logs	–	Zhang et al., 2015
	tetA, *tetB*, *tetC*, *sulI*, *sulII*, *sulIII*, *ampC*, *aph(2″)-Id*, *katG*, and *vanA*	Enhancement	–	Liu et al., 2018
UV	*sulI*, *tetG*, and *intI1*	0.80–1.21 logs	30 min, 254 nm, 2.0 ± 0.3 logs	Zhuang et al., 2015; Sousa et al., 2017
	tetW	0.00–1.89 logs	–	Sullivan et al., 2017
	tetX, *sulI*, *tetG*, and *intI1*	0.36–0.58 logs	–	Zhang et al., 2015
Ozonation	*tet* genes and *sul* genes	<49.2 and <34.5%	30 min, 2.1 ± 0.5 logs	Sousa et al., 2017; Zheng et al., 2017
Photocatalytic oxidation	*sul1*, *tetX*, and *tetG*	2.63–3.48 logs (pH = 3.0) 1.55–2.32 logs (pH = 7.0)	2–3 logs	Zhang et al., 2016b; Moreira et al., 2018
Fenton's oxidation reaction	*sul1*, *tetX*, and *tetG*	2.58–3.79 logs (pH = 3.0) 2.26–3.35 logs (pH = 7.0)	<LOQ*	Zhang et al., 2016b; Moreira et al., 2018

**LOQ, Limit of Quantification.*

antibiotic resistance in the receiving water body. However, even though the removal efficiency of disinfection processes is very high it is not possible to avoid secondary treatment to cut cost, since the organic matter in the wastewater act as precursors of disinfection by-products. Additionally, the secondary treatment also decreases the suspended solids concentration, which is a key parameter for UV disinfection. As a further treatment step, membrane filtration systems might be used to remove the remaining ARG and ARB, and CW can be considered as a post treatment step for effluents in smaller settings. Finally, MBRs that combine biological treatment and membrane filtration make good alternatives for ARB and ARG removal.

Removal From Sludge and Biosolids

Biological wastewater treatment relies on the growth of bacteria and other microorganisms and subsequent flocculation and settling of aggregated biomass. At a steady state, excessive biomass is removed (so called sludge wasting usually done through the underflow from secondary clarifiers) together with other solids that are collected in skimmers and primary clarifiers.

TABLE 3 | Antibiotic elimination efficiencies in different treatment units.

Treatment technologies	Antibiotics	Antibiotic elimination efficiency	References
Physical processes			
Membrane separation	SA, ML	5–28%	Sahar et al., 2011
Soil aquifer treatment	SA	68.2–88.9%	Qin et al., 2020
	ML	90.1%	Fang et al., 2017
Biological processes			
Anaerobic–aerobic seq. bioreactor (AAS)	–	–	–
Aerobic bioreactor	SA	>95%	Qian et al., 2020
Anaerobic bioreactors	SA	~2%	Qian et al., 2020
	TC, SA	>90%, 30–98%	Cheng et al., 2020
Membrane bioreactor	SA	87.4%	Song et al., 2017
	FQ	81.1%	
	TC	83.8%	
	ML	14.3%	
CW-surface flow	BL, SA, FQ, TC, ML	–67 to 100%	Liu et al., 2019
CW-horizontal subsurface flow	BL, SA, FQ, TC, ML	–46 to 100%	Liu et al., 2019
CW-vertical subsurface flow	BL, SA, FQ, TC, ML	20–100%	Liu et al., 2019
Hybrid CWs	SA, FQ, TC, ML	43 ± 32%	Ávila et al., 2014
Chemical processes			
Chlorination	BL	97–100%	Li and Zhang, 2011
	SA	73–100%	
	FQ	50–74%	
	TC	39–83%	
	ML	43–53%	
UV-254 nm	SA	51%	De la Cruz et al., 2012
	FQ	48–65%	
	ML	0%	
	SA, FQ	>99%, 90 min	Michael et al., 2020
Ozonation	BL, SA, FQ, ML	100%, O3 = 14–42 mg	Paucar et al., 2019
	TC	86.4–93.6%, O3 = flow rate 0.5 L/min	Wang et al., 2018
Photocatalytic oxidation	SA	~46%, 300 min, Q_{UV} = 42 kj/L	Michael et al., 2020
	FQ	>99%, 60 min, Q_{UV} = 8 kj/L	
	SA, FQ, TC	100%, 90–100%, 100%	Palominos et al., 2008; Kansal et al., 2014; Espíndola et al., 2019; Sandikly et al., 2019
Fenton's oxidation reaction	BL	100%, H_2O_2/Fe^{2+} = 2–150 μM, pH = 2–4	Elmolla and Chaudhuri, 2009
	SA	74%, H_2O_2/Fe^{2+} = 2.9 μM, pH = 3–6	Qian et al., 2020
	FQ, ML	With citric acid 95%, H_2O_2/Fe^{2+} = 1.75 μM, pH = 3	Macku'ak et al., 2015

BL, β-lactam; SA, sulphonamide; FQ, fluoroquinolones; TC, tetracyclines; ML, macrolides.

Several unit operations reduce water content, stabilize, and treat the discarded sludge before it is disposed or recycled. Biosolids from WWTPs are typically applied to agricultural land as fertilizer, disposed of to landfills, or incinerated (United States Environmental Protection Agency, 2003; Tchobanoglous et al., 2014; Collivignarelli et al., 2019).

Unsurprisingly, most of the resistant bacteria and resistance genes that arrives with the sewage and that grows and propagates through a treatment plant end up in the settled sludge (Munir et al., 2011; Calero-Cáceres et al., 2014; Yuan et al., 2019). Studies examining municipal WWTPs without advanced sludge treatment in the United States (Munir et al., 2011; Gao et al., 2012) and in China (Chen and Zhang, 2013; Wen et al., 2016; Yuan et al., 2019) have shown that although the plants are able to reduce the abundance of resistance genes and resistant bacteria in their effluents by 2–4 orders of magnitude, the amount of resistance genes and resistant bacteria in the biosolids from these plants are of the same order of magnitude as in the inflow sewage (around 10^8–10^{10} copies of *tetW* and *tetO* resistance genes per 100 ml sample and 10^6–10^8 CFU of *tetracycline* resistant bacteria per 100 ml sample).

Supplementary Table 1 gives an overview of reported levels of bacteria, resistant bacteria, and resistance genes in biosolids after sludge treatment at WWTPs around the world. The table also includes extended information about plant type, treatment process, sludge sources, and final application of the biosolids. Conventional sludge treatment methods that simply thickens and dewaters sludge by gravity thickening, belt pressing, centrifugation, or other mechanical methods are not effective at removing resistance genes (**Supplementary Table 1**). Further anaerobic or aerobic digestion of the sludge is also in many cases not enough to substantially reduce the number of resistant bacteria and genes.

Heat drying, which involves reducing the moisture content to below 10% by direct or indirect contact with hot gases (Tchobanoglous et al., 2014), and advanced lime stabilization, which involves the addition of alkali (lime) to increase pH in combination with other treatments like pasteurization or heat drying, are more effective at removing resistance. Heat drying and advanced lime stabilization reduce the density of bacteria in biosolids, and thus also the density of resistant bacteria, to levels similar to and in most cases below what is typical for soil (**Supplementary Table 1**). This is due to the high temperatures

and/or pH that are reached in the processes. The density of resistance genes is on the other hand in many cases still higher than what is typical for unfertilized soil. However, it has been suggested and observed that the genes have lower stability after treatment as many are trapped within dead microorganisms (Lau et al., 2017; Murray et al., 2019). In a study that measured the density of resistance genes in the soil directly after application of biosolids, the authors found that the abundance of resistance genes 2 h after application was remarkably low in soil amended with heat-dried biosolids (Lau et al., 2017). Similar results have also been found for soil amended with biosolids pasteurized at more than 70°C for a period of 30 min in a lab scale experiment (Burch et al., 2017). It seems that many of the resistance genes are rapidly destroyed when they come in contact with soil and moisture.

The N-rich biosolids produced through the N-Viro treatment used at Thorold, Ontario (**Supplementary Table 1**) have particularly low levels of resistance genes. Murray et al. (2019) found that out of 41 selected genes associated with resistance and HGT, 38 were below the detection limit and the remaining 3 were below the quantification limit. The reason is that the pH is so high that double stranded DNA denatures (Murray et al., 2019).

Pyrolysis is another treatment method that consistently reduces the density of resistance genes in biosolids to below what is found in pristine soil in nature. Pyrolysis is not a common biosolid treatment technique today and has many of the same disadvantages as incineration. It has high capital and operating cost, and requires highly skilled operating and maintenance staff, compared to the simpler dewatering, stabilization, and heat drying methods (Tchobanoglous et al., 2014; Carey et al., 2016). It is also energy intensive, but it can potentially be used as a refinement step in treatment plants that already use heat drying, as the added energy cost of pyrolysis is reported to be low compared to the energy already invested in drying the biosolids (McNamara et al., 2016). The benefit of pyrolysis over incineration is that more organic content and nutrients remain in biochar than in incinerated ash, giving biochar a higher fertilizer potential—biochar has an NKP content of 6-13-0 vs. 0-6-2 for incinerated ash (Carey et al., 2016). They both, however, have the risk of containing high levels of heavy metals, which are concentrated in the product during the production process (Carey et al., 2016).

From the results combined in **Supplementary Table 1**, it is worth noting that the density of remaining resistance genes after a specific biosolids treatment method can vary with more than an order of magnitude between facilities (**Supplementary Table 1**). This may be due to differences in the sludge loading or their operation, but also because the methods and protocols for quantification have different sensitivities and efficiencies for extracting and measuring the absolute concentration of genes (Feinstein et al., 2009; Taylor et al., 2019; Yuan et al., 2019).

The trend seen from the numbers in **Supplementary Table 1** is that further treatment beyond digestion is needed to reduce the density of resistant bacteria to levels comparable to or below what is found in soils. The trend coincides comparatively well with the grouping of sludge treatment methods used in the biosolids regulation of the United States (40 CFR Part 503) (United States Environmental Protection Agency, 1994, 2003). Treatment processes are categorized into "processes to significantly reduce pathogens" (PSRP) and "processes to further reduce pathogens" (PFRP). PSRP includes the first set of treatment methods after thickening/dewatering, i.e., aerobic digestion, anaerobic digestion, air drying, composting, and limes stabilization, with specific requirements to process parameters such as time, temperature, and pH (United States Environmental Protection Agency, 1994, 2003). PFRP includes further treatments that use heat or radiation to purposefully kill pathogens, i.e., heat drying, heat treatment, pasteurization, beta- or gamma-ray irradiation, and also composting and thermophilic digestion if the temperature is kept over 55°C for a specified number of days (United States Environmental Protection Agency, 1994, 2003). The PSRP and PFRP grouping are in the United States are used together with bacteria density limits (fecal coliforms or Salmonella) to regulate land application of biosolids. However, the current regulations only require PSRP treatment or an average fecal coliform density below $2{\cdot}10^6$ CFU/g for agricultural use (class B biosolids), and there is no specific mention of either resistant bacteria or resistance genes (United States Environmental Protection Agency, 1994, 2003). Similarly, there are currently no specific limits on resistant bacteria or resistance genes for biosolids in the European Union (Eur-Lex, 2018; Collivignarelli et al., 2019). The European Union directive 86/278/EEC (2018), which regulates the application of biosolids in the EU, does not specify any limits on pathogen content, but several member states have national regulations with limit values for indicator bacteria [typically Salmonella and some type(s) of fecal bacteria] (Collivignarelli et al., 2019).

More and more studies are linking the application of biosolids to higher levels of resistant bacteria and genes in agricultural soil (Ross and Topp, 2015; Gondim-Porto et al., 2016; Burch et al., 2017; Lau et al., 2017; Murray et al., 2019). However, the resistance levels decrease with time after application (Marti et al., 2014; Rahube et al., 2014; Ross and Topp, 2015; Burch et al., 2017; Lau et al., 2017; Murray et al., 2019), and the current evidence for gene transfer to crops and animals remains inconclusive (Marti et al., 2013b; Rahube et al., 2014; Lau et al., 2017; Murray et al., 2019; You et al., 2020). Current regulations in the US and the EU do include time restrictions from application to harvesting and/or grazing (United States Environmental Protection Agency, 1994, 2003; Eur-Lex, 2018; Collivignarelli et al., 2019). Implementation of limits for the density of resistant bacteria and resistance genes to the regulations for biosolids should also be considered. Limits on the density of resistance genes can be difficult to implement, as measurement methods for gene amounts have varying sensitivity and accuracy (Feinstein et al., 2009; Taylor et al., 2019; Yuan et al., 2019). The density of resistance genes can furthermore be an inconsistent factor for risk alone because of the difference in stability and transfer potential between genes in living bacteria, genes in dead bacteria, and free and adsorbed genes outside of bacteria. Significant risk reduction can be achieved merely by stricter limits on the general density of bacteria, e.g., as for biosolids of class A today (Murray et al., 2019). Treatment operations that consistently reach these limits are already implemented technologies at many WWTPs. Stricter limits must, however, be weighed against the implications they will have for the overall use of biosolids as fertilizer and

soil improvement. Moreover, limits and regulations for biosolids must be harmonized with other biological fertilizers such as manure, which is also known to contain high levels of resistance (Marti et al., 2013b, 2014; Ross and Topp, 2015; Nõlvak et al., 2016). Stricter regulations can lead to more incineration and less reuse, an effect that cannot be disregarded in the context of a sustainable and circular economy.

MODELING ANTIBIOTIC RESISTANCE IN WASTEWATER TREATMENT PLANTS

Mathematical models formulated from a mechanistic or holistic understanding of microbial and biogeochemical interactions in aquatic systems have advanced our understanding of dynamics in technical and natural systems (Simon et al., 2002; Benedetti et al., 2013). Mathematical models describing the processes involved in the treatment and biodegradation of wastewater have already successfully been developed and established as standard, well used tools within the WWTP community (Gernaey et al., 2004; Solon et al., 2019). Such models are, as mathematical models in general, functional tools for *a priori* model and hypothesis testing, and *a posteriori* data analysis and performance evaluation. The standard WWTP models are the so-called activated sludge models (ASM1, ASM2, ASM2d, ASM3, and variants), which have been applied for research and process performance evaluations, as well as for the design of new WWTPs (Henze et al., 2000; Van Loosdrecht et al., 2015). These models include the major WWTP processes of biomass growth, carbon oxidation, nitrification, denitrification, and phosphorus removal. None of the standard models, however, include the occurrence or spread of antibiotic resistance among bacteria in WWTPs.

Several mathematical models for the spread of antibiotic resistance in bacteria populations have been proposed, although mainly in theoretical, or simplified, environmental settings (Birkegård et al., 2018). This includes models of the spread of resistance in axenic cultures of bacteria (Tremblay and Rose, 1985; Imran and Smith, 2007; Svara and Rankin, 2011), and a few models that include spread through more than a single strain (Clewlow et al., 1990). There are also models that deal with the dynamics of antibiotic resistance in relation to antibiotic concentrations and distinguish the type of resistance mechanism (Bootsma et al., 2012; Krzyzanski and Rao, 2017); and finally, models that deal with the spread between hosts of bacteria, i.e., in an epidemiological setting (Spicknall et al., 2013; Levin et al., 2014).

The development of mathematical models that combine the biodegradation processes and population dynamics of microorganisms in a WWTP with the presence of antibiotic compounds, ARGs, and the spread of antibiotic resistance in the populations through HGT is still in its early stages. Attempts to combine the use of WWTP and ARG models are few and limited to early-stage developments. There have, however, been attempts to combine antibiotic degradation dynamics with the traditional activated sludge models (ASM-X by Polesel et al., 2016) to assess degradation kinetics of antibiotics and other pharmaceuticals in WWTPs. Few examples exist of models that have been set up to address the effect of antibiotic resistance in realistic environments that are partly similar to WWTPs (Hellweger et al., 2011; Hellweger, 2013; Baker et al., 2016). Baker et al. (2016) modeled the spread of antimicrobial resistance in a slurry tank that collects and stores fecal and urinary waste from cows at a dairy farm. Their model includes most processes that should be considered to capture both population dynamic and resistance spread, i.e., cellular growth and death processes, HGT, segregation loss, antibiotic concentration (to capture selection pressure), slurry inflow and fitness cost. We think that this model structure with the addition of the treatment processes from the ASM models can serve as a basis for a model suitable for a WWTP environment. Baker et al. (2016) parameterized their model based on their experimental data and data from the literature and showed through sensitivity analysis that gene transfer rate is one of the most important parameters for the spread of resistance. Hellweger et al. (2011) and Hellweger (2013) used a mathematical model to test if observed concentrations of antibiotics and densities of *tetracycline* resistant bacteria in the Poudre River in Colorado could be explained by different scenarios for how resistant exogenous bacteria that arrive at the river grow and exchanges genes with indigenous bacteria. They showed that the observed data could not be explained by a scenario with high input of exogenous resistant bacteria to the river without growth in the river itself; their model suggested that there has to be the growth of resistant bacteria and thus maintenance of the resistance gene in the river itself, is most likely due to soft selection pressure from low concentrations of tetracycline (Hellweger et al., 2011).

Wastewater treatment plants are highly complex systems with mixed cultures of microorganisms, and a wide range of modeling approaches, including individual-based models (IbMs), are needed to understand the functioning of such plants from micro to macro scale. Deterministic population-level models, like the classical ASM models, allow for studying the average behavior of systems, e.g., the overall dynamics of populations and concentrations in the plant reactors. However, they may miss some important individual effects on biological processes rates in the bacterial community. Population-level models do not account for individual heterogeneity, local interactions, or adaptive behavior. IbMs do on the other hand treat bacteria as single cells, as discrete entities, and might be better suited to account for the spread of resistance and can potentially overcome these limitations that arise from population model design. HGT is a micro-level process. For example, conjugation of resistance plasmids, as this is a discrete event between individual cells, happens when an individual donor bacterium and a recipient bacterium are close enough in space that a pilus from the donor can attach to the recipient and bring them together (Seoane et al., 2011). IbM of conjugation mechanism allows presenting the intrapopulation variability, to capture the changes that occur during the coupling process (Merkey et al., 2011; Seoane et al., 2011). Moreover, local variations in population density, e.g., flocculation, plays a role in the spread of resistance (Merkey et al., 2011), and the description of actions on the level of the single organism in the model may thus be needed to explain the total population development (Breckling, 2002;

Hellweger et al., 2016). IbMs can determine the relevance of a specific interaction or location for the overall behavior of the biofilm Therefore, IbMs can give insights into the emergence of antibiotic resistance from biofilms to aquatic environments. Moreover, the combination of different level models, population and individual, can provide quantitative analysis of the spread of antibiotic-resistant bacteria.

Although mathematical models can be powerful tools and the ASM models have been very successful for understanding and developing treatment processes, mathematical modeling approaches have so far not yet been able to help improve our understanding of the conditions that drive maintenance, spread or extinction of ARGs or ARB in WWTPs. The main weakness of many of the proposed models of antimicrobial resistance is the lack of experimental data available to parameterize and validate the models (Birkegård et al., 2018). Any extensions of the standard WWTP models to include resistant and non-resistant bacteria, the presence of ARGs, different mechanisms for HGT, and concentrations of antibiotics, should include considerations on how to experimentally measure associated process rates and concentrations. An integrated AR-WWTP model designed -with this in mind- can become a promising tool for theoretical and diagnostic studies of ARG spreading, and it can be of help in identifying which mechanisms and factors that are the most important for the spread of resistance under different circumstances. That is, in evaluating which operational conditions or parameter values that can minimize spread, and which parameters are key drivers.

FUTURE DIRECTIONS AND CONCLUSION

In this review, we assessed the causes and the mechanisms for the spread of ARGs, together with their occurrence, transfer, and potential removal in WWTPs. While the issue of antibiotic resistance could never have completely been prevented, the current universal problem of resistant bacteria is solely due to anthropogenic activities. Moreover, the absence of regulations and strict monitoring regimes have contributed to the escalation of the occurrence of antibiotic resistance in the environment. The research shows that neither conventional nor advanced WWTPs are efficient enough to completely remove ARGs and ARB from water environments, but that more advanced treatment methods perform better. Advanced post treatment methods like UV, ozonation and oxidation of water effluents, and heat drying, lime stabilization and pyrolysis of biosolids, remove considerably more ARGs and ARB than activated sludge treatment alone but are not without disadvantages like more difficult and complex operation and higher cost. Finally, the following key points are proposed to improve current WWTPs and provide guidance for future application:

(i) In order to reduce the threat of antibiotic resistance, it is advisable to set strict threshold limits for antibiotic release from point sources like hospitals and animal husbandries, together with the thresholds for release of metal residues, biocides, and other pharmaceuticals that drive co-selection of resistance.
(ii) Plans for implementation of more advanced treatment processes should consider the economy and ecology of the whole waterway. It may be more cost effective to employ smaller scale treatment plants with disinfection units at point sources than to redesign and rebuild larger municipal WWTPs.
(iii) Efforts should be made to devise and agree upon standard methods to measure and report ARB and ARG levels to make it easier to compare resistance levels between different countries and at different treatment plants. This will also make it easier to evaluate removal efficiencies of treatment methods and to evaluate the performance of already established treatment plants, which can facilitate the decision process of operators and regulatory agencies of whether additional post-treatment steps are necessary.
(iv) Experimental studies should be combined with mathematical modeling to further examine the mechanisms for the spread and population growth of resistant and non-resistant bacteria in wastewater treatment environments. The effect of different treatment methods and plant operation strategies on the spread of resistance genes should be further studied, including the effect of operating conditions (pH, temperature, COD, BOD) on HGT.

These approaches can provide a further understanding of the processes and mechanisms of spread and can therefore help in the design of WWTPs that are less likely to become breeding grounds for antibiotic resistance, and which function better as final barriers.

AUTHOR CONTRIBUTIONS

CU, KMK, and IP-O researched, wrote, and edited the manuscript. KT researched and wrote section "Removal From Sludge and Biosolids" and edited the manuscript. DB researched and designed **Table 3**. SS, MJ, and GK conceived the review outline. RK provided significant input on wastewater treatment and operation of WWTPs and edited the manuscript. All authors reviewed the 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.2021.717809/full#supplementary-material

REFERENCES

Alexander, J., Bollmann, A., Seitz, W., and Schwartz, T. (2015). Microbiological characterization of aquatic microbiomes targeting taxonomical marker genes and antibiotic resistance genes of opportunistic bacteria. *Sci. Total Environ.* 512–513, 316–325. doi: 10.1016/j.scitotenv.2015.01.046

Alexander, J., Knopp, G., Dötsch, A., Wieland, A., and Schwartz, T. (2016). Ozone treatment of conditioned wastewater selects antibiotic resistance genes, opportunistic bacteria, and induce strong population shifts. *Sci. Total Environ.* 559, 103–112. doi: 10.1016/j.scitotenv.2016.03.154

Amador, P. P., Fernandes, R. M., Prudêncio, M. C., Barreto, M. P., and Duarte, I. M. (2015). Antibiotic resistance in wastewater: occcurrence and fate of *Enterobacteriaceae* producers of class A and class C β-lactamases. *J. Environ. Sci. Heal. - Part A Toxic/Hazardous Subst. Environ. Eng.* 50, 26–39. doi: 10.1080/10934529.2015.964602

Andersson, D. I., and Hughes, D. (2014). Microbiological effects of sublethal levels of antibiotics. *Nat. Rev. Microbiol.* 12, 465–478. doi: 10.1038/nrmicro3270

Ardern, E., and Lockett, W. T. (1914). Experiments on the oxidation of sewage without the aid of filters. *J. Soc. Chem. Ind.* 33, 523–539. doi: 10.1002/jctb.5000331005

Auerbach, E. A., Seyfried, E. E., and McMahon, K. D. (2007). Tetracycline resistance genes in activated sludge wastewater treatment plants. *Water Res.* 41, 1143–1151. doi: 10.1016/j.watres.2006.11.045

Ávila, C., Matamoros, V., Reyes-Contreras, C., Piña, B., Casado, M., Mita, L., et al. (2014). Attenuation of emerging organic contaminants in a hybrid constructed wetland system under different hydraulic loading rates and their associated toxicological effects in wastewater. *Sci. Total Environ.* 47, 1272–1280. doi: 10.1016/j.scitotenv.2013.10.065

Bairán, G., Rebollar-Pérez, G., Chávez-Bravo, E., and Torres, E. (2020). Treatment processes for microbial resistance mitigation: the technological contribution to tackle the problem of antibiotic resistance. *Int. J. Environ. Res. Public Health* 17, 1–20. doi: 10.3390/ijerph17238866

Baker, M., Hobman, J. L., Dodd, C. E. R., Ramsden, S. J., and Stekel, D. J. (2016). Mathematical modelling of antimicrobial resistance in agricultural waste highlights importance of gene transfer rate. *FEMS Microbiol. Ecol.* 92:fiw040. doi: 10.1093/femsec/fiw040

Baker-Austin, C., Wright, M. S., Stepanauskas, R., and McArthur, J. V. (2006). Co-selection of antibiotic and metal resistance. *Trends Microbiol.* 14, 176–182. doi: 10.1016/j.tim.2006.02.006

Barancheshme, F., and Munir, M. (2018). Strategies to combat antibiotic resistance in the wastewater treatment plants. *Front. Microbiol.* 8:2603. doi: 10.3389/fmicb.2017.02603

Barlow, M. (2009). "What antimicrobial resistance has taught us about horizontal gene transfer," in *Horizontal Gene Transfer. Methods in Molecular Biology*, eds M. B. Gogarten, J. P. Gogarten, and L. C. Olendzenski (Totowa, NJ: Humana Press), 14151–14154. doi: 10.1007/978-1-60327-853-9

Benedetti, L., Langeveld, J., Comeau, A., Corominas, L., Daigger, G., Martin, C., et al. (2013). Modelling and monitoring of integrated urban wastewater systems: review on status and perspectives. *Water Sci. Technol.* 68, 1203–1215. doi: 10.2166/wst.2013.397

Bengtsson-Palme, J., Hammarén, R., Pal, C., Östman, M., Björlenius, B., Flach, C. F., et al. (2016). Elucidating selection processes for antibiotic resistance in sewage treatment plants using metagenomics. *Sci. Total Environ.* 572, 697–712. doi: 10.1016/j.scitotenv.2016.06.228

Bergeron, S., Boopathy, R., Nathaniel, R., Corbin, A., and LaFleur, G. (2015). Presence of antibiotic resistant bacteria and antibiotic resistance genes in raw source water and treated drinking water. *Int. Biodeterior. Biodegrad.* 102, 370–374. doi: 10.1016/j.ibiod.2015.04.017

Birkegård, A. C., Halasa, T., Toft, N., Folkesson, A., and Græsbøll, K. (2018). Send more data: a systematic review of mathematical models of antimicrobial resistance. *Antimicrob. Resist. Infect. Control* 7:117. doi: 10.1186/s13756-018-0406-401

Biswal, B. K., Mazza, A., Masson, L., Gehr, R., and Frigon, D. (2014). Impact of wastewater treatment processes on antimicrobial resistance genes and their co-occurrence with virulence genes in *Escherichia coli*. *Water Res.* 50, 245–253. doi: 10.1016/j.watres.2013.11.047

Bootsma, M. C. J., van der Horst, M. A., Guryeva, T., ter Kuile, B. H., and Diekmann, O. (2012). Modeling non-inherited antibiotic resistance. *Bull. Math. Biol.* 74, 1691–1705. doi: 10.1007/s11538-012-9731-9733

Bouki, C., Venieri, D., and Diamadopoulos, E. (2013). Detection and fate of antibiotic resistant bacteria in wastewater treatment plants: a review. *Ecotoxicol. Environ. Saf.* 91, 1–9. doi: 10.1016/j.ecoenv.2013.01.016

Breckling, B. (2002). Individual-based modelling: potentials and limitations. *ScientificWorldJournal* 2, 1044–1062. doi: 10.1100/tsw.2002.179

Brooks, B. W., Maul, J. D., and Belden, J. B. (2008). "Antibiotics in aquatic and terrestrial ecosystems," in *Encyclopedia of Ecology*, eds S. E. Jørgensen, B. D. Fath (Academic Press), 210–217. doi: 10.1016/B978-008045405-4.00370-0

Burch, T. R., Sadowsky, M. J., and LaPara, T. M. (2017). Effect of different treatment technologies on the fate of antibiotic resistance genes and class 1 integrons when residual municipal wastewater solids are applied to soil. *Environ. Sci. Technol.* 51, 14225–14232. doi: 10.1021/acs.est.7b04760

Calderón, C. B., and Sabundayo, B. P. (2007). "Antimicrobial classifications: drugs for bugs," in *Antimicrobial Susceptibility Testing Protocols*, eds R. Schwalbe, L. Steele-Moore, and A. C. Goodwin (Boca Raton FL: CRC Press), 7–53.

Calero-Cáceres, W., Melgarejo, A., Colomer-Lluch, M., Stoll, C., Lucena, F., Jofre, J., et al. (2014). Sludge as a potential important source of antibiotic resistance genes in both the bacterial and bacteriophage fractions. *Environ. Sci. Technol.* 48, 7602–7611. doi: 10.1021/es501851s

Cano, R. J., and Colomé, J. S. (1988). *Essentials of Microbiology*. Toronto, ON: Thomson Learning.

Carey, D. E., Yang, Y., McNamara, P. J., and Mayer, B. K. (2016). Recovery of agricultural nutrients from biorefineries. *Bioresour. Technol.* 215, 186–198. doi: 10.1016/j.biortech.2016.02.093

Chen, H., and Zhang, M. (2013). Effects of advanced treatment systems on the removal of antibiotic resistance genes in wastewater treatment plants from Hangzhou. *China. Environ. Sci. Technol.* 47, 8157–8163. doi: 10.1021/es401091y

Chen, J., Deng, W. J., Liu, Y. S., Hu, L. X., He, L. Y., Zhao, J. L., et al. (2019). Fate and removal of antibiotics and antibiotic resistance genes in hybrid constructed wetlands. *Environ. Pollut.* 249, 894–903. doi: 10.1016/j.envpol.2019.03.111

Cheng, D., Ngo, H. H., Guo, W., Chang, S. W., Nguyen, D. D., Liu, Y., et al. (2020). Removal process of antibiotics during anaerobic treatment of swine wastewater. *Bioresour. Technol.* 300:122707. doi: 10.1016/j.biortech.2019.122707

Cheng, H., and Hong, P. Y. (2017). Removal of antibiotic-resistant bacteria and antibiotic resistance genes affected by varying degrees of fouling on anaerobic microfiltration membranes. *Environ. Sci. Technol.* 51, 12200–12209. doi: 10.1021/acs.est.7b03798

Chiang, Y. N., Penadés, J. R., and Chen, J. (2019). Genetic transduction by phages and chromosomal islands: the new and noncanonical. *PLoS Pathog.* 15:e1007878. doi: 10.1371/journal.ppat.1007878

Christgen, B., Yang, Y., Ahammad, S. Z., Li, B., Rodriquez, D. C., Zhang, T., et al. (2015). Metagenomics shows that low-energy anaerobic-aerobic treatment reactors reduce antibiotic resistance gene levels from domestic wastewater. *Environ. Sci. Technol.* 49, 2577–2584. doi: 10.1021/es505521w

Clewlow, L. J., Cresswell, N., and Wellington, E. M. H. (1990). Mathematical model of plasmid transfer between strains of streptomycetes in soil microcosms. *Appl. Environ. Microbiol.* 56, 3139–3145. doi: 10.1128/aem.56.10.3139-3145.1990

Collivignarelli, M. C., Abbà, A., Frattarola, A., Miino, M. C., Padovani, S., Katsoyiannis, I., et al. (2019). Legislation for the reuse of biosolids on agricultural land in Europe: overview. *Sustain* 11, 1–26. doi: 10.3390/su11216015

Daims, H., Taylor, M. W., and Wagner, M. (2006). Wastewater treatment: a model system for microbial ecology. *Trends Biotechnol.* 24, 483–489. doi: 10.1016/j.tibtech.2006.09.002

Davies, J., and Davies, D. (2010). Origins and evolution of antibiotic resistance. *Microbiologia* 74, 417–433. doi: 10.1128/MMBR.00016-10

Davison, H. C., Woolhouse, M. E. J., and Low, J. C. (2000). What is antibiotic resistance and how can we measure it? *Trends Microbiol.* 8, 554–559.

De la Cruz, N., Giménez, J., Esplugas, S., Grandjean, D., De Alencastro, L. F., and Pulgarín, C. (2012). Degradation of 32 emergent contaminants by UV and neutral photo-fenton in domestic wastewater effluent previously treated by activated sludge. *Water Res.* 46, 1947–1957. doi: 10.1016/j.watres.2012.01.014

Deurenberg, R. H., and Stobberingh, E. E. (2008). The evolution of *Staphylococcus aureus*. *Infect. Genet. Evol.* 8, 747–763. doi: 10.1016/j.meegid.2008.07.007

Di Cesare, A., Eckert, E. M., D'Urso, S., Bertoni, R., Gillan, D. C., Wattiez, R., et al. (2016). Co-occurrence of integrase 1, antibiotic and heavy metal resistance genes in municipal wastewater treatment plants. *Water Res.* 94, 208–214. doi: 10.1016/j.watres.2016.02.049

Diaz, L., Tran, T. T., Munita, J. M., Miller, W. R., Rincon, S., Carvajal, L. P., et al. (2014). Whole-genome analyses of Enterococcus faecium isolates with diverse daptomycin MICs. *Antimicrob. Agents Chemother.* 58, 4527–4534. doi: 10.1128/AAC.02686-2614

Ding, C., and He, J. (2010). Effect of antibiotics in the environment on microbial populations. *Appl. Microbiol. Biotechnol.* 87, 925–941. doi: 10.1007/s00253-010-2649-2645

Directive 91/271/Eec (1991). *Council Directive of 21 May 1991 Concerning Urban Waste Water Treatment*. Brussels: European Union.

Doherty, L., Zhao, Y., Zhao, X., Hu, Y., Hao, X., Xu, L., et al. (2015). A review of a recently emerged technology: constructed wetland - microbial fuel cells. *Water Res.* 85, 38–45. doi: 10.1016/j.watres.2015.08.016

Du, J., Geng, J., Ren, H., Ding, L., Xu, K., and Zhang, Y. (2015). Variation of antibiotic resistance genes in municipal wastewater treatment plant with A 2O-MBR system. *Environ. Sci. Pollut. Res.* 22, 3715–3726. doi: 10.1007/s11356-014-3552-x

Elkayam, R., Aharoni, A., Vaizel-Ohayon, D., Sued, O., Katz, Y., Negev, I., et al. (2018). Viral and microbial pathogens, indicator microorganisms, microbial source tracking indicators, and antibiotic resistance genes in a confined managed effluent recharge system. *J. Environ. Eng. (United States)* 144, 5017011–5017013.

Elmolla, E., and Chaudhuri, M. (2009). Optimization of Fenton process for treatment of amoxicillin, ampicillin and cloxacillin antibiotics in aqueous solution. *J. Hazard. Mater.* 170, 666–672. doi: 10.1016/j.jhazmat.2009.05.013

Espíndola, J. C., Cristóvão, R. O., Santos, S. G. S., Boaventura, R. A. R., Dias, M. M., Lopes, J. C. B., et al. (2019). Intensification of heterogeneous TiO2 photocatalysis using the NETmix mili-photoreactor under microscale illumination for oxytetracycline oxidation. *Sci. Total Environ.* 681, 467–474. doi: 10.1016/j.scitotenv.2019.05.066

Eur-Lex (2018). Council Directive of 12 June 1986 on the Protection of the Environment, and in Particular of the Soil, When Sewage Sludge is Used in Agriculture (86/278/EEC). Available online at: https://eur-lex.europa.eu/eli/dir/1986/278/2018-07-04 (accessed September 27, 2021).

Fang, H., Zhang, Q., Nie, X., Chen, B., Xiao, Y., Zhou, Q., et al. (2017). Occurrence and elimination of antibiotic resistance genes in a long-term operation integrated surface flow constructed wetland. *Chemosphere* 173, 99–106. doi: 10.1016/j.chemosphere.2017.01.027

Feinstein, L. M., Woo, J. S., and Blackwood, C. B. (2009). Assessment of bias associated with incomplete extraction of microbial DNA from soil. *Appl. Environ. Microbiol.* 75, 5428–5433. doi: 10.1128/AEM.00120-129

Figueira, V., Vaz-Moreira, I., Silva, M., and Manaia, C. M. (2011). Diversity and antibiotic resistance of *Aeromonas spp*. in drinking and waste water treatment plants. *Water Res.* 45, 5599–5611. doi: 10.1016/j.watres.2011.08.021

Furst, K. E., Pecson, B. M., Webber, B. D., and Mitch, W. A. (2018). Tradeoffs between pathogen inactivation and disinfection byproduct formation during sequential chlorine and chloramine disinfection for wastewater reuse. *Water Res.* 143, 579–588. doi: 10.1016/j.watres.2018.05.050

Galera-Laporta, L., and Garcia-Ojalvo, J. (2020). Antithetic population response to antibiotics in a polybacterial community. *Sci. Adv.* 6:eaaz5108. doi: 10.1126/sciadv.aaz5108

Gao, P., Munir, M., and Xagoraraki, I. (2012). Correlation of tetracycline and sulfonamide antibiotics with corresponding resistance genes and resistant bacteria in a conventional municipal wastewater treatment plant. *Sci. Total Environ.* 421–422, 173–183. doi: 10.1016/j.scitotenv.2012.01.061

Gernaey, K. V., Van Loosdrecht, M. C. M., Henze, M., Lind, M., and Jørgensen, S. B. (2004). Activated sludge wastewater treatment plant modelling and simulation: state of the art. *Environ. Model. Softw.* 19, 763–783. doi: 10.1016/j.envsoft.2003.03.005

Gillings, M. R. (2014). Integrons: past, present, and future. *Microbiol. Mol. Biol. Rev.* 78, 257–277. doi: 10.1128/mmbr.00056-13

Gómez, P., and Buckling, A. (2011). Bacteria-phage antagonistic coevolution in soil. *Science* 332, 106–109. doi: 10.1126/science.1198767

Gondim-Porto, C., Platero, L., Nadal, I., and Navarro-García, F. (2016). Fate of classical faecal bacterial markers and ampicillin-resistant bacteria in agricultural soils under Mediterranean climate after urban sludge amendment. *Sci. Total Environ.* 565, 200–210. doi: 10.1016/j.scitotenv.2016.04.160

Grabow, W. O., and Prozesky, O. W. (1973). Drug resistance of coliform bacteria in hospital and city sewage. *Antimicrob. Agents Chemother.* 3, 175–180. doi: 10.1128/AAC.3.2.175

Gullberg, E., Cao, S., Berg, O. G., Ilbäck, C., Sandegren, L., Hughes, D., et al. (2011). Selection of resistant bacteria at very low antibiotic concentrations. *PLoS Pathog* 7:e1002158. doi: 10.1371/journal.ppat.1002158

Harb, M., and Hong, P. Y. (2017). Molecular-based detection of potentially pathogenic bacteria in membrane bioreactor (MBR) systems treating municipal wastewater: a case study. *Environ. Sci. Pollut. Res.* 24, 5370–5380. doi: 10.1007/s11356-016-8211-y

Hassoun-Kheir, N., Stabholtz, Y., Kreft, J.-U., de la Cruz, R., Romalde, J. L., Nesme, J., et al. (2020). Comparison of antibiotic-resistant bacteria and antibiotic resistance genes abundance in hospital and community wastewater: a systematic review. *Sci. Total Environ.* 5:140804. doi: 10.1016/j.scitotenv.2020.140804

Hellweger, F. L. (2013). Simple model of tetracycline antibiotic resistance in aquatic environment: accounting for metal coselection. *J. Environ. Eng. (United States)* 139, 913–921.

Hellweger, F. L., Clegg, R. J., Clark, J. R., Plugge, C. M., and Kreft, J. U. (2016). Advancing microbial sciences by individual-based modelling. *Nat. Rev. Microbiol.* 14, 461–471. doi: 10.1038/nrmicro.2016.62

Hellweger, F. L., Ruan, X., and Sanchez, S. (2011). A simple model of tetracycline antibiotic resistance in the aquatic environment (with application to the Poudre River). *Int. J. Environ. Res. Public Health* 8, 480–497. doi: 10.3390/ijerph8020480

Hembach, N., Schmid, F., Alexander, J., Hiller, C., Rogall, E. T., and Schwartz, T. (2017). Occurrence of the mcr-1 colistin resistance gene and other clinically relevant antibiotic resistance genes in microbial populations at different municipal wastewater treatment plants in Germany. *Front. Microbiol.* 8:1282. doi: 10.3389/fmicb.2017.01282

Henze, M., Gujer, W., Mino, T., and van Loosdrecht, M. (2000). *Activated Sludge Models ASM1, ASM2, ASM2d and ASM3*. Philippines: I. T. Group.

Heuer, H., and Smalla, K. (2007). Horizontal gene transfer between bacteria. *Environ. Biosafety Res.* 6, 3–13. doi: 10.1051/ebr:2007034

Hiller, C. X., Hübner, U., Fajnorova, S., Schwartz, T., and Drewes, J. E. (2019). Antibiotic microbial resistance (AMR) removal efficiencies by conventional and advanced wastewater treatment processes: a review. *Sci. Total Environ.* 685, 596–608. doi: 10.1016/j.scitotenv.2019.05.315

Huang, W., Tsai, L., Li, Y., Hua, N., Sun, C., and Wei, C. (2017a). Widespread of horizontal gene transfer in the human genome. *BMC Genomics* 18:274. doi: 10.1186/s12864-017-3649-y

Huang, X., Zheng, J., Liu, C., Liu, L., Liu, Y., and Fan, H. (2017b). Removal of antibiotics and resistance genes from swine wastewater using vertical flow constructed wetlands: effect of hydraulic flow direction and substrate type. *Chem. Eng. J.* 308, 692–699. doi: 10.1016/j.cej.2016.09.110

Hultman, J., Tamminen, M., Pärnänen, K., Cairns, J., Karkman, A., and Virta, M. (2018). Host range of antibiotic resistance genes in wastewater treatment plant influent and effluent. *FEMS Microbiol. Ecol.* 94:fiy038. doi: 10.1093/femsec/fiy038

Hutinel, M., Huijbers, P. M. C., Fick, J., Åhrén, C., Larsson, D. G. J., and Flach, C. F. (2019). Population-level surveillance of antibiotic resistance in *Escherichia coli* through sewage analysis. *Eurosurveillance* 24:1800497. doi: 10.2807/1560-7917.ES.2019.24.37.1800497

Imran, M., and Smith, H. L. (2007). "A mathematical model of gene transfer in a biofilm," in *Mathematics for Ecology and Environmental Sciences*, eds Y. Takeuchi, Y. Iwasa, and K. Sato (Berlin: Springer), 93–125.

Jiao, Y. N., Chen, H., Gao, R. X., Zhu, Y. G., and Rensing, C. (2017). *Organic Compounds Stimulate Horizontal Transfer of Antibiotic Resistance Genes in Mixed Wastewater Treatment Systems*. Amsterdam: Elsevier Ltd, doi: 10.1016/j.chemosphere.2017.05.149

Jury, K. L., Vancov, T., Stuetz, R. M., and Khan, S. J. (2010). Antibiotic resistance dissemination and sewage treatment plants. *Curr. Res. Technol. Educ. Appl. Microbiol. Microb. Biotechnol.* 2, 509–519.

Kansal, S. K., Kundu, P., Sood, S., Lamba, R., Umar, A., and Mehta, S. K. (2014). Photocatalytic degradation of the antibiotic levofloxacin using highly crystalline TiO2 nanoparticles. *New J. Chem.* 38, 3220–3226. doi: 10.1039/c3nj01619f

Kapoor, G., Saigal, S., and Elongavan, A. (2017). Action and resistance mechanisms of antibiotics: a guide for clinicians. *J. Anaesthesiol. Clin. Pharmacol.* 33, 300–305. doi: 10.4103/joacp.JOACP

Kappell, A. D., Kimbell, L. K., Seib, M. D., Carey, D. E., Choi, M. J., Kalayil, T., et al. (2018). Removal of antibiotic resistance genes in an anaerobic membrane bioreactor treating primary clarifier effluent at 20 °C. *Environ. Sci. Water Res. Technol.* 4, 1783–1793. doi: 10.1039/c8ew00270c

Karaolia, P., Michael, I., García-Fernández, I., Agüera, A., Malato, S., Fernández-Ibáñez, P., et al. (2014). Reduction of clarithromycin and sulfamethoxazole-resistant Enterococcus by pilot-scale solar-driven Fenton oxidation. *Sci. Total Environ.* 46, 19–27. doi: 10.1016/j.scitotenv.2013.08.027

Karkman, A., Do, T. T., Walsh, F., and Virta, M. P. J. (2018). Antibiotic-resistance genes in waste water. *Trends Microbiol.* 26, 220–228. doi: 10.1016/j.tim.2017.09.005

Klümper, U., Recker, M., Zhang, L., Yin, X., Zhang, T., Buckling, A., et al. (2019). Selection for antimicrobial resistance is reduced when embedded in a natural microbial community. *ISME J.* 13, 2927–2937. doi: 10.1038/s41396-019-0483-z

Knapp, C. W., Callan, A. C., Aitken, B., Shearn, R., Koenders, A., and Hinwood, A. (2017). Relationship between antibiotic resistance genes and metals in residential soil samples from Western Australia. *Environ. Sci. Pollut. Res.* 24, 2484–2494. doi: 10.1007/s11356-016-7997-y

de Sousa Oliveira, K., de Lima, L. A., Cobacho, N. B., Dias, S. C., and Franco, O. L. (2016). "Chapter 2 - mechanisms of antibacterial resistance: shedding some light on these obscure processes?," in *Antibiotic Resistance*, eds K. Kon and M. Rai (Academic Press), 19–35. doi: 10.1016/B978-0-12-803642-6.00002-2

Korzeniewska, E., Korzeniewska, A., and Harnisz, M. (2013). Antibiotic resistant *Escherichia coli* in hospital and municipal sewage and their emission to the environment. *Ecotoxicol. Environ. Saf.* 91, 96–102. doi: 10.1016/j.ecoenv.2013.01.014

Krzyzanski, W., and Rao, G. G. (2017). Multi-scale model of drug induced adaptive resistance of Gram-negative bacteria to polymyxin B. *PLoS One* 12:e0171834. doi: 10.1371/journal.pone.0171834

Laht, M., Karkman, A., Voolaid, V., Ritz, C., Tenson, T., Virta, M., et al. (2014). Abundances of tetracycline, sulphonamide and beta-lactam antibiotic resistance genes in conventional wastewater treatment plants (WWTPs) with different waste load. *PLoS One* 9:e103705. doi: 10.1371/journal.pone.0103705

Lamba, M., Graham, D. W., and Ahammad, S. Z. (2017). Hospital wastewater releases of carbapenem-resistance pathogens and genes in urban India. *Environ. Sci. Technol.* 51, 13906–13912. doi: 10.1021/acs.est.7b03380

Lau, C. H. F., Li, B., Zhang, T., Tien, Y. C., Scott, A., Murray, R., et al. (2017). Impact of pre-application treatment on municipal sludge composition, soil dynamics of antibiotic resistance genes, and abundance of antibiotic-resistance genes on vegetables at harvest. *Sci. Total Environ.* 58, 214–222. doi: 10.1016/j.scitotenv.2017.02.123

Levin, B. R., Baquero, F., and Johnsen, P. J. (2014). A model-guided analysis and perspective on the evolution and epidemiology of antibiotic resistance and its future. *Curr. Opin. Microbiol.* 19, 83–89. doi: 10.1016/j.mib.2014.06.004

Li, B., and Zhang, T. (2011). Mass flows and removal of antibiotics in two municipal wastewater treatment plants. *Chemosphere* 83, 1284–1289. doi: 10.1016/j.chemosphere.2011.03.002

Li, J., Cheng, W., Xu, L., Strong, P. J., and Chen, H. (2015). Antibiotic-resistant genes and antibiotic-resistant bacteria in the effluent of urban residential areas, hospitals, and a municipal wastewater treatment plant system. *Environ. Sci. Pollut. Res.* 22, 4587–4596. doi: 10.1007/s11356-014-3665-3662

Li, S., Zhang, R., Hu, J., Shi, W., Kuang, Y., Guo, X., et al. (2019). Occurrence and removal of antibiotics and antibiotic resistance genes in natural and constructed riverine wetlands in Beijing. China. *Sci. Total Environ.* 664, 546–553. doi: 10.1016/j.scitotenv.2019.02.043

Liu, S. S., Qu, H. M., Yang, D., Hu, H., Liu, W. L., Qiu, Z. G., et al. (2018). Chlorine disinfection increases both intracellular and extracellular antibiotic resistance genes in a full-scale wastewater treatment plant. *Water Res.* 136, 131–136. doi: 10.1016/j.watres.2018.02.036

Liu, X., Guo, X., Liu, Y., Lu, S., Xi, B., Zhang, J., et al. (2019). A review on removing antibiotics and antibiotic resistance genes from wastewater by constructed wetlands: performance and microbial response. *Environ. Pollut.* 254:112996. doi: 10.1016/j.envpol.2019.112996

Livermore, D. M. (2012). Fourteen years in resistance. *Int. J. Antimicrob. Agents* 39, 283–294. doi: 10.1016/j.ijantimicag.2011.12.012

Luczkiewicz, A., Jankowska, K., Bray, R., Kulbat, E., Quant, B., Sokolowska, A., et al. (2011). Antimicrobial resistance of fecal indicators in disinfected wastewater. *Water Sci. Technol.* 64, 2352–2361. doi: 10.2166/wst.2011.769

Łuczkiewicz, A., Jankowska, K., Fudala-Ksiazek, S., and Olańczuk-Neyman, K. (2010). Antimicrobial resistance of fecal indicators in municipal wastewater treatment plant. *Water Res.* 44, 5089–5097. doi: 10.1016/j.watres.2010.08.007

Luo, Y., Guo, W., Ngo, H. H., Nghiem, L. D., Hai, F. I., Zhang, J., et al. (2014). A review on the occurrence of micropollutants in the aquatic environment and their fate and removal during wastewater treatment. *Sci. Total Environ.* 47, 619–641. doi: 10.1016/j.scitotenv.2013.12.065

Lv, T., Carvalho, P. N., Zhang, L., Zhang, Y., Button, M., Arias, C. A., et al. (2017). Functionality of microbial communities in constructed wetlands used for pesticide remediation: influence of system design and sampling strategy. *Water Res.* 110, 241–251. doi: 10.1016/j.watres.2016.12.021

Macku'ak, T., Nagyová, K., Faberová, M., Grabic, R., Koba, O., Gál, M., et al. (2015). Utilization of Fenton-like reaction for antibiotics and resistant bacteria elimination in different parts of WWTP. *Environ. Toxicol. Pharmacol.* 40, 492–497. doi: 10.1016/j.etap.2015.07.002

Madigan, M. T., Bender, K. S., Buckley, D. H., Sattley, W. M., and Stahl, D. A. (2006). *Brock Biology of Microorganisms, Global Edition*. London: Pearson.

Manaia, C. M. (2014). Antibiotic resistance in wastewater: origins, fate, and risks. *Pravent. Gesundheitsforderung* 9, 180–184. doi: 10.1007/s11553-014-0452-453

Manaia, C. M., Rocha, J., Scaccia, N., Marano, R., Radu, E., Biancullo, F., et al. (2018). Antibiotic resistance in wastewater treatment plants: tackling the black box. *Environ. Int.* 115, 312–324. doi: 10.1016/j.envint.2018.03.044

Marti, E., and Balcazar, J. L. (2013). "Antibiotic resistance in the aquatic environment," in *Analysis, Removal, Effects and Risk of Pharmaceuticals in the Water Cycle*, eds M. Petrovic, S. Perez, and D. Barcelo (Chicago, ILL: Wilson & Wilson's), 671–684.

Marti, E., Jofre, J., and Balcazar, J. L. (2013a). Prevalence of antibiotic resistance genes and bacterial community composition in a river influenced by a wastewater treatment plant. *PLoS One* 8:e78906. doi: 10.1371/journal.pone.0078906

Marti, R., Scott, A., Tien, Y. C., Murray, R., Sabourin, L., Zhang, Y., et al. (2013b). Impact of manure fertilization on the abundance of antibiotic-resistant bacteria and frequency of detection of antibiotic resistance genes in soil and on vegetables at harvest. *Appl. Environ. Microbiol.* 79, 5701–5709. doi: 10.1128/AEM.01682-1613

Marti, R., Tien, Y. C., Murray, R., Scott, A., Sabourin, L., and Topp, E. (2014). Safely coupling livestock and crop production systems: how rapidly do antibiotic resistance genes dissipate in soil following a commercial application of swine or dairy manure? *Appl. Environ. Microbiol.* 80, 3258–3265. doi: 10.1128/AEM.00231-214

Mazel, D. (2006). Integrons: agents of bacterial evolution. *Nat. Rev. Microbiol.* 4, 608–620. doi: 10.1038/nrmicro1462

Mccullagh, C., Robertson, J. M. C., Bahnemann, D. W., and Robertson, P. K. J. (2007). The application of TiO 2 photocatalysis for disinfection of water contaminated with pathogenic micro-organisms: a review. *Res. Chem. Intermed.* 33, 359–375. doi: 10.1163/156856707779238775

McKinney, C. W., and Pruden, A. (2012). Ultraviolet disinfection of antibiotic resistant bacteria and their antibiotic resistance genes in water and wastewater. *Environ. Sci. Technol.* 46, 13393–13400. doi: 10.1021/es303652q

McNamara, P. J., Koch, J. D., Liu, Z., and Zitomer, D. H. (2016). Pyrolysis of dried wastewater biosolids can be energy positive. *Water Environ. Res.* 88, 804–810. doi: 10.2175/106143016x14609975747441

Merkey, B. V., Lardon, L. A., Seoane, J. M., Kreft, J. U., and Smets, B. F. (2011). Growth dependence of conjugation explains limited plasmid invasion in biofilms: an individual-based modelling study. *Environ. Microbiol.* 13, 2435–2452. doi: 10.1111/j.1462-2920.2011.02535.x

Michael, S. G., Michael-Kordatou, I., Nahim-Granados, S., Polo-López, M. I., Rocha, J., Martínez-Piernas, A. B., et al. (2020). Investigating the impact of UV-C/H2O2 and sunlight/H2O2 on the removal of antibiotics, antibiotic resistance determinants and toxicity present in urban wastewater. *Chem. Eng. J.* 388:124383. doi: 10.1016/j.cej.2020.124383

Modi, S. R., Lee, H. H., Spina, C. S., and Collins, J. J. (2013). Antibiotic treatment expands the resistance reservoir and ecological network of the phage metagenome. *Nature* 499, 219–222. doi: 10.1038/nature12212.Antibiotic

Mokracka, J., Koczura, R., and Kaznowski, A. (2012). Multiresistant *Enterobacteriaceae* with class 1 and class 2 integrons in a municipal wastewater treatment plant. *Water Res.* 46, 3353–3363. doi: 10.1016/j.watres.2012.03.037

Moreira, N. F. F., Narciso-da-Rocha, C., Polo-López, M. I., Pastrana-Martínez, L. M., Faria, J. L., Manaia, C. M., et al. (2018). Solar treatment (H2O2, TiO2-P25 and GO-TiO2 photocatalysis, photo-Fenton) of organic micropollutants, human pathogen indicators, antibiotic resistant bacteria and related genes in urban wastewater. *Water Res.* 135, 195–206. doi: 10.1016/j.watres.2018.01.064

Munir, M., Wong, K., and Xagoraraki, I. (2011). Release of antibiotic resistant bacteria and genes in the effluent and biosolids of five wastewater utilities in Michigan. *Water Res.* 45, 681–693. doi: 10.1016/j.watres.2010.08.033

Munita, J. M., and Arias, C. A. (2016). Mechanisms of antibiotic resistance. *HHS Public Access* 4, 1–37.

Murray, R., Tien, Y. C., Scott, A., and Topp, E. (2019). The impact of municipal sewage sludge stabilization processes on the abundance, field persistence, and transmission of antibiotic resistant bacteria and antibiotic resistance genes to vegetables at harvest. *Sci. Total Environ.* 651, 1680–1687. doi: 10.1016/j.scitotenv.2018.10.030

Neudorf, K. D., Huang, Y. N., Ragush, C. M., Yost, C. K., Jamieson, R. C., and Truelstrup Hansen, L. (2017). Antibiotic resistance genes in municipal wastewater treatment systems and receiving waters in Arctic Canada. *Sci. Total Environ.* 598, 1085–1094. doi: 10.1016/j.scitotenv.2017.04.151

Nielsen, P. H., and McMahon, K. D. (2014). "Microbiology and microbial ecology of the activated sludge process," in *Activated Sludge - 100 years and Counting*, eds D. Jenkins and J. Wanner (London: IWA Publishing)

Nishino, K., and Yamaguchi, A. (2001). Analysis of a complete library of putative drug transporter genes in *Escherichia coli*. *J. Bacteriol.* 183, 5803–5812. doi: 10.1128/JB.183.20.5803-5812.2001

Nõlvak, H., Truu, M., Kanger, K., Tampere, M., Espenberg, M., Loit, E., et al. (2016). Inorganic and organic fertilizers impact the abundance and proportion of antibiotic resistance and integron-integrase genes in agricultural grassland soil. *Sci. Total Environ.* 562, 678–689. doi: 10.1016/j.scitotenv.2016.04.035

Novo, A., André, S., Viana, P., Nunes, O. C., and Manaia, C. M. (2013). Antibiotic resistance, antimicrobial residues and bacterial community composition in urban wastewater. *Water Res.* 47, 1875–1887. doi: 10.1016/j.watres.2013.01.010

One Health Initiative (2020). *One health*. Available online at: http://www.onehealthinitiative.com (accessed December 10, 2020).

Palominos, R., Freer, J., Mondaca, M. A., and Mansilla, H. D. (2008). Evidence for hole participation during the photocatalytic oxidation of the antibiotic flumequine. *J. Photochem. Photobiol. A Chem.* 193, 139–145. doi: 10.1016/j.jphotochem.2007.06.017

Paucar, N. E., Kim, I., Tanaka, H., and Sato, C. (2019). Ozone treatment process for the removal of pharmaceuticals and personal care products in wastewater. *Ozone Sci. Eng.* 41, 3–16. doi: 10.1080/01919512.2018.1482456

Pauwels, B., Fru Ngwa, F., Deconinck, S., and Verstraete, W. (2006). Effluent quality of a conventional activated sludge and a membrane bioreactor system treating hospital wastewater. *Environ. Technol.* 27, 395–402. doi: 10.1080/09593332708618651

Pazda, M., Kumirska, J., Stepnowski, P., and Mulkiewicz, E. (2019). Antibiotic resistance genes identified in wastewater treatment plant systems – a review. *Sci. Total Environ.* 697:134023. doi: 10.1016/j.scitotenv.2019.134023

Pei, R., Cha, J., Carlson, K. H., and Pruden, A. (2007). Response of antibiotic resistance genes (ARG) to biological treatment in dairy lagoon water. *Environ. Sci. Technol.* 41, 5108–5113. doi: 10.1021/es070051x

Polesel, F., Andersen, H. R., Trapp, S., and Plósz, B. G. (2016). Removal of antibiotics in biological wastewater treatment systems - a critical assessment using the activated sludge modeling framework for xenobiotics (ASM-X). *Environ. Sci. Technol.* 50, 10316–10334. doi: 10.1021/acs.est.6b01899

Qian, M., Yang, L., Chen, X., Li, K., Xue, W., Li, Y., et al. (2020). The treatment of veterinary antibiotics in swine wastewater by biodegradation and Fenton-like oxidation. *Sci. Total Environ.* 710:136299. doi: 10.1016/j.scitotenv.2019.136299

Qiao, M., Ying, G. G., Singer, A. C., and Zhu, Y. G. (2018). Review of antibiotic resistance in China and its environment. *Environ. Int.* 110, 160–172. doi: 10.1016/j.envint.2017.10.016

Qin, K., Chen, Y., Li, J., Xue, C., Wei, L., Song, X., et al. (2020). Removal trends of sulfonamides and their ARGs during soil aquifer treatment and subsequent chlorination: effect of aerobic and anaerobic biodegradation. *Environ. Sci. Water Res. Technol.* 6, 2331–2340. doi: 10.1039/d0ew00270d

Rahube, T. O., Marti, R., Scott, A., Tien, Y. C., Murray, R., Sabourin, L., et al. (2014). Impact of fertilizing with raw or anaerobically digested sewage sludge on the abundance of antibiotic-resistant coliforms, antibiotic resistance genes, and pathogenic bacteria in soil and on vegetables at harvest. *Appl. Environ. Microbiol.* 80, 6898–6907. doi: 10.1128/AEM.02389-2314

Ren, S., Boo, C., Guo, N., Wang, S., Elimelech, M., and Wang, Y. (2018). Photocatalytic reactive ultrafiltration membrane for removal of antibiotic resistant bacteria and antibiotic resistance genes from wastewater effluent. *Environ. Sci. Technol.* 52, 8666–8673. doi: 10.1021/acs.est.8b01888

Riquelme Breazeal, M. V., Novak, J. T., Vikesland, P. J., and Pruden, A. (2013). Effect of wastewater colloids on membrane removal of antibiotic resistance genes. *Water Res.* 47, 130–140. doi: 10.1016/j.watres.2012.09.044

Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M. C., et al. (2013). Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: a review. *Sci. Total Environ.* 447, 345–360. doi: 10.1016/j.scitotenv.2013.01.032

Rodriguez-Mozaz, S., Chamorro, S., Marti, E., Huerta, B., Gros, M., Sànchez-Melsió, A., et al. (2015). Occurrence of antibiotics and antibiotic resistance genes in hospital and urban wastewaters and their impact on the receiving river. *Water Res.* 69, 234–242. doi: 10.1016/j.watres.2014.11.021

Ross, J., and Topp, E. (2015). Abundance of antibiotic resistance genes in bacteriophage following soil fertilization with dairy manure or municipal biosolids, and evidence for potential transduction. *Appl. Environ. Microbiol.* 81, 7905–7913. doi: 10.1128/AEM.02363-2315

Rowe, W. P. M., Baker-Austin, C., Verner-Jeffreys, D. W., Ryan, J. J., Micallef, C., Maskell, D. J., et al. (2017). Overexpression of antibiotic resistance genes in hospital effluents over time. *J. Antimicrob. Chemother.* 72, 1617–1623. doi: 10.1093/jac/dkx017

Sabri, N. A., Schmitt, H., Van der Zaan, B., Gerritsen, H. W., Zuidema, T., Rijnaarts, H. H. M., et al. (2018). Prevalence of antibiotics and antibiotic resistance genes in a wastewater effluent-receiving river in the Netherlands. *J. Environ. Chem. Eng.* 8:102245. doi: 10.1016/j.jece.2018.03.004

Sahar, E., Ernst, M., Godehardt, M., Hein, A., Herr, J., Kazner, C., et al. (2011). Comparison of two treatments for the removal of organic micro-pollutants: conventional Activated Sludge (CAS) Followed by Ultrafiltration (UF) vs. Membrane Bioreactor (MBR). *NATO Sci. Peace Secur. Ser. C Environ. Secur.* 106, 147–161. doi: 10.1007/978-94-007-0280-6_13

Sandikly, N., Kassir, M., El Jamal, M., Takache, H., Arnoux, P., Mokh, S., et al. (2019). Comparison of the toxicity of waters containing initially sulfaquinoxaline after photocatalytic treatment by TiO2 and polyaniline/TiO2. *Environ. Technol. (United Kingdom)* 42, 419–428. doi: 10.1080/09593330.2019.1630485

Schlüter, A., Szczepanowski, R., Pühler, A., and Top, E. M. (2007). Genomics of IncP-1 antibiotic resistance plasmids isolated from wastewater treatment plants provides evidence for a widely accessible drug resistance gene pool. *FEMS Microbiol. Rev.* 31, 449–477. doi: 10.1111/j.1574-6976.2007.00074.x

Schwermer, C. U., Krzeminski, P., Wennberg, A. C., Vogelsang, C., and Uhl, W. (2018). Removal of antibiotic resistant *E. coli* in two Norwegian wastewater treatment plants and by nano- and ultra-filtration processes. *Water Sci. Technol.* 77, 1115–1126. doi: 10.2166/wst.2017.642

Seoane, J., Yankelevich, T., Dechesne, A., Merkey, B., Sternberg, C., and Smets, B. F. (2011). An individual-based approach to explain plasmid invasion in bacterial populations. *FEMS Microbiol. Ecol.* 75, 17–27. doi: 10.1111/j.1574-6941.2010.00994.x

Sharma, S. K., and Kennedy, M. D. (2017). Soil aquifer treatment for wastewater treatment and reuse. *Int. Biodeterior. Biodegrad.* 119, 671–677. doi: 10.1016/j.ibiod.2016.09.013

Sharma, V. K., Siskova, K. M., Zboril, R., and Gardea-Torresdey, J. L. (2014). Organic-coated silver nanoparticles in biological and environmental conditions: fate, stability and toxicity. *Adv. Colloid Interface Sci.* 204, 15–34. doi: 10.1016/j.cis.2013.12.002

Shingare, R. P., Thawale, P. R., Raghunathan, K., Mishra, A., and Kumar, S. (2019). Constructed wetland for wastewater reuse: role and efficiency in removing enteric pathogens. *J. Environ. Manage.* 246, 444–461. doi: 10.1016/j.jenvman.2019.05.157

Simon, M., Grossart, H. P., Schweitzer, B., and Ploug, H. (2002). Microbial ecology of organic aggregates in aquatic ecosystems. *Aquat. Microb. Ecol.* 28, 175–211. doi: 10.3354/ame028175

Snyder, L., and Champness, W. (2007). *Molecular Genetics of Bacteria*. Washington, DC: ASM Press.

Solon, K., Volcke, E. I. P., Spérandio, M., and Van Loosdrecht, M. C. M. (2019). Resource recovery and wastewater treatment modelling. *Environ. Sci. Water Res. Technol.* 5, 631–642. doi: 10.1039/c8ew00765a

Song, X., Liu, R., Chen, L., and Kawagishi, T. (2017). Comparative experiment on treating digested piggery wastewater with a biofilm MBR and conventional MBR: simultaneous removal of nitrogen and antibiotics. *Front. Environ. Sci. Eng.* 11:915. doi: 10.1007/s11783-017-0919-915

Sousa, J. M., Macedo, G., Pedrosa, M., Becerra-Castro, C., Castro-Silva, S., Pereira, M. F. R., et al. (2017). Ozonation and UV254nm radiation for the removal of microorganisms and antibiotic resistance genes from urban wastewater. *J. Hazard. Mater.* 323, 434–441. doi: 10.1016/j.jhazmat.2016.03.096

Spicknall, I. H., Foxman, B., Marrs, C. F., and Eisenberg, J. N. S. (2013). A modeling framework for the evolution and spread of antibiotic resistance: literature review and model categorization. *Am. J. Epidemiol.* 178, 508–520. doi: 10.1093/aje/kwt017

Stensel, D. H., and Makinia, J. (2014). "Activated sludge process development," in *Activated Sludge - 100 Years and Counting*, eds D. Jenkins and W. Jiri (London: IWA Publishing), doi: 10.2166/9781780404943

Stepanauskas, R., Glenn, T. C., Jagoe, C. H., Tuckfield, R. C., Lindell, A. H., and McArthur, J. V. (2005). Elevated microbial tolerance to metals and antibiotics in metal-contaminated industrial environments. *Environ. Sci. Technol.* 39, 3671–3678. doi: 10.1021/es048468f

Sullivan, B. A., Vance, C. C., Gentry, T. J., and Karthikeyan, R. (2017). Effects of chlorination and ultraviolet light on environmental tetracycline-resistant bacteria and tet(W) in water. *J. Environ. Chem. Eng.* 5, 777–784. doi: 10.1016/j.jece.2016.12.052

Svara, F., and Rankin, D. J. (2011). The evolution of plasmid-carried antibiotic resistance. *BMC Evol. Biol.* 11:26–31. doi: 10.1186/1471-2148-11-130

Szekeres, E., Baricz, A., Chiriac, C. M., Farkas, A., Opris, O., Soran, M. L., et al. (2017). Abundance of antibiotics, antibiotic resistance genes and bacterial community composition in wastewater effluents from different Romanian hospitals. *Environ. Pollut.* 225, 304–315. doi: 10.1016/j.envpol.2017.01.054

Taylor, S. C., Nadeau, K., Abbasi, M., Lachance, C., Nguyen, M., and Fenrich, J. (2019). The ultimate qPCR experiment: producing publication quality. *Reproducible Data the First Time. Trends Biotechnol.* 37, 761–774. doi: 10.1016/j.tibtech.2018.12.002

Tchobanoglous, G., Stensel, D. H., Tsuchihashi, R., and Burton, F. L. (2014). *Wastewater Engineering Treatment and Resource Recovery*. New York, NY: McGraw-Hill.

Thwaites, B. J., Short, M. D., Stuetz, R. M., Reeve, P. J., Alvarez Gaitan, J. P., Dinesh, N., et al. (2018). Comparing the performance of aerobic granular sludge versus conventional activated sludge for microbial log removal and effluent quality: implications for water reuse. *Water Res.* 145, 442–452. doi: 10.1016/j.watres.2018.08.038

Tremblay, C., and Rose, M. R. (1985). Population dynamics of gene transfer. *Theor. Popul. Biol.* 28, 359–381. doi: 10.1016/0040-5809(85)90035-90038

Tuckfield, R. C., and McArthur, J. V. (2008). Spatial analysis of antibiotic resistance along metal contaminated streams. *Microb. Ecol.* 55, 595–607. doi: 10.1007/s00248-007-9303-9305

United States Environmental Protection Agency (1994). *A Plain English Guide to the EPA Part 503 Biosolids Rule*. Washington, DC: United States Environmental Protection Agency.

United States Environmental Protection Agency (2003). *Control of Pathogens and Vector Attraction in Sewage Sludge Under 40 CFR Part 503*. Washington, DC: United States Environmental Protection Agency.

van Loosdrecht, M. C. M., and Brdjanovic, D. (2014). Anticipating the next century of wastewater treatment. *Science* 344, 1452–1453. doi: 10.1126/science.1255183

Van Loosdrecht, M. C. M., Lopez-Vazquez, C. M., Meijer, S. C. F., Hooijmans, C. M., and Brdjanovic, D. (2015). Twenty-five years of ASM1: past, present and future of wastewater treatment modelling. *J. Hydroinformatics* 17, 697–718. doi: 10.2166/hydro.2015.006

Vila, J., Martí, S., and Sánchez-Céspedes, J. (2007). Porins, efflux pumps and multidrug resistance in *Acinetobacter baumannii*. *J. Antimicrob. Chemother.* 59, 1210–1215. doi: 10.1093/jac/dkl509

von Wintersdorff, C. J. H., Penders, J., van Niekerk, J. M., Mills, N. D., Majumder, S., van Alphen, L. B., et al. (2016). Dissemination of antimicrobial resistance in microbial ecosystems through horizontal gene transfer. *Front. Microbiol.* 7:173. doi: 10.3389/fmicb.2016.00173

Wale, N., Sim, D. G., Jones, M. J., Salathe, R., Day, T., and Read, A. F. (2017). Resource limitation prevents the emergence of drug resistance by intensifying within-host competition. *Proc. Natl. Acad. Sci. U S A.* 114, 13774–13779. doi: 10.1073/pnas.1715874115

Walsh, C. (2003). Where will new antibiotics come from? *Nat. Rev. Microbiol.* 1, 65–70. doi: 10.1038/nrmicro727

Wang, L., Ben, W., Li, Y., Liu, C., and Qiang, Z. (2018). Behavior of tetracycline and macrolide antibiotics in activated sludge process and their subsequent removal during sludge reduction by ozone. *Chemosphere* 206, 184–191. doi: 10.1016/j.chemosphere.2018.04.180

Wanger, A., Chavez, V., Huang, R. S. P., Wahed, A., Actor, J. K., and Dasgupta, A. (2017). "Chapter 7 - antibiotics, antimicrobial resistance, antibiotic susceptibility testing, and therapeutic drug monitoring for selected drugs," in *Microbiology and Molecular Diagnosis in Pathology* eds A. Wanger, V. Chavez, R. S. P. Huang, A. Wahed, J. K. Actor, and A. Dasgupta (Elsevier), 119–153, doi: 10.1016/B978-0-12-805351-5.00007-7

Wen, Q., Yang, L., Duan, R., and Chen, Z. (2016). Monitoring and evaluation of antibiotic resistance genes in four municipal wastewater treatment plants in Harbin. Northeast China. *Environ. Pollut.* 212, 34–40. doi: 10.1016/j.envpol.2016.01.043

WHO (2020). *Antimicrobial Resistance*. Geneva: WHO.

Wright, G. D. (2005). Bacterial resistance to antibiotics: enzymatic degradation and modification. *Adv. Drug Deliv. Rev.* 57, 1451–1470. doi: 10.1016/j.addr.2005.04.002

Wright, G. D. (2010). Q&A: antibiotic resistance: where does it come from and what can we do about it? *BMC Biol.* 8:123. doi: 10.1186/1741-7007-8-123

Wright, G. D. (2011). Molecular mechanisms of antibiotic resistance. *Chem. Commun.* 47, 4055–4061. doi: 10.1039/c0cc05111j

Xu, J., Xu, Y., Wang, H., Guo, C., Qiu, H., He, Y., et al. (2015). Occurrence of antibiotics and antibiotic resistance genes in a sewage treatment plant and its effluent-receiving river. *Chemosphere* 119, 1379–1385. doi: 10.1016/j.chemosphere.2014.02.040

Yang, Y., Li, B., Zou, S., Fang, H. H. P., and Zhang, T. (2014). Fate of antibiotic resistance genes in sewage treatment plant revealed by metagenomic approach. *Water Res.* 62, 97–106. doi: 10.1016/j.watres.2014.05.019

Yazdankhah, S., Rudi, K., and Bernhoft, A. (2014). Zinc and copper in animal feed – development of resistance and co-resistance to antimicrobial agents in bacteria of animal origin. *Microb. Ecol. Heal. Dis.* 25:25862. doi: 10.3402/mehd.v25.25862

Yi, X., Tran, N. H., Yin, T., He, Y., and Gin, K. Y. H. (2017). Removal of selected PPCPs, EDCs, and antibiotic resistance genes in landfill leachate by a full-scale constructed wetlands system. *Water Res.* 121, 46–60. doi: 10.1016/j.watres.2017.05.008

You, R., Margenat, A., Lanzas, C. S., Cañameras, N., Carazo, N., Navarro-Martín, L., et al. (2020). Dose effect of Zn and Cu in sludge-amended soils on vegetable uptake of trace elements, antibiotics, and antibiotic resistance genes: human health implications. *Environ. Res.* 191:109879. doi: 10.1016/j.envres.2020.109879

Yuan, Q., Bin, Huang, Y. M., Wu, W., Bin, Zuo, P., et al. (2019). Redistribution of intracellular and extracellular free & adsorbed antibiotic resistance genes through a wastewater treatment plant by an enhanced extracellular DNA extraction method with magnetic beads. *Environ. Int.* 131:104986. doi: 10.1016/j.envint.2019.104986

Zhang, J., Zhang, L., Loh, K. C., Dai, Y., and Tong, Y. W. (2017). Enhanced anaerobic digestion of food waste by adding activated carbon: fate of bacterial pathogens and antibiotic resistance genes. *Biochem. Eng. J.* 128, 19–25. doi: 10.1016/j.bej.2017.09.004

Zhang, S., Song, H. L., Yang, X. L., Li, H., and Wang, Y. W. (2018). A system composed of a biofilm electrode reactor and a microbial fuel cell-constructed

wetland exhibited efficient sulfamethoxazole removal but induced sul genes. *Bioresour. Technol.* 256, 224–231. doi: 10.1016/j.biortech.2018.02.023

Zhang, T., Zhang, X. X., and Ye, L. (2011). Plasmid metagenome reveals high levels of antibiotic resistance genes and mobile genetic elements in activated sludge. *PLoS One* 6:e26041. doi: 10.1371/journal.pone.0026041

Zhang, X. X., Zhang, T., and Fang, H. H. P. (2009). Antibiotic resistance genes in water environment. *Appl. Microbiol. Biotechnol.* 82, 397–414. doi: 10.1007/s00253-008-1829-z

Zhang, Y., Geng, J., Ma, H., Ren, H., Xu, K., and Ding, L. (2016a). Characterization of microbial community and antibiotic resistance genes in activated sludge under tetracycline and sulfamethoxazole selection pressure. *Sci. Total Environ.* 571, 479–486. doi: 10.1016/j.scitotenv.2016.07.014

Zhang, Y., Zhuang, Y., Geng, J., Ren, H., Xu, K., and Ding, L. (2016b). Reduction of antibiotic resistance genes in municipal wastewater effluent by advanced oxidation processes. *Sci. Total Environ.* 550, 184–191. doi: 10.1016/j.scitotenv.2016.01.078

Zhang, Y., Zhuang, Y., Geng, J., Ren, H., Zhang, Y., Ding, L., et al. (2015). Inactivation of antibiotic resistance genes in municipal wastewater effluent by chlorination and sequential UV/chlorination disinfection. *Sci. Total Environ.* 51, 125–132. doi: 10.1016/j.scitotenv.2015.01.028

Zheng, J., Su, C., Zhou, J., Xu, L., Qian, Y., and Chen, H. (2017). Effects and mechanisms of ultraviolet, chlorination, and ozone disinfection on antibiotic resistance genes in secondary effluents of municipal wastewater treatment plants. *Chem. Eng. J.* 317, 309–316. doi: 10.1016/j.cej.2017.02.076

Zhu, T., Su, Z., Lai, W., Zhang, Y., and Liu, Y. (2021). Insights into the fate and removal of antibiotics and antibiotic resistance genes using biological wastewater treatment technology. *Sci. Total Environ.* 776:145906. doi: 10.1016/j.scitotenv.2021.145906

Zhu, Y., Wang, Y., Zhou, S., Jiang, X., Ma, X., and Liu, C. (2018). Robust performance of a membrane bioreactor for removing antibiotic resistance genes exposed to antibiotics: role of membrane foulants. *Water Res.* 130, 139–150. doi: 10.1016/j.watres.2017.11.067

Zhuang, Y., Ren, H., Geng, J., Zhang, Y., Zhang, Y., Ding, L., et al. (2015). Inactivation of antibiotic resistance genes in municipal wastewater by chlorination, ultraviolet, and ozonation disinfection. *Environ. Sci. Pollut. Res.* 22, 7037–7044. doi: 10.1007/s11356-014-3919-z

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