ANTIMICROBIALS IN WILDLIFE AND THE ENVIRONMENT

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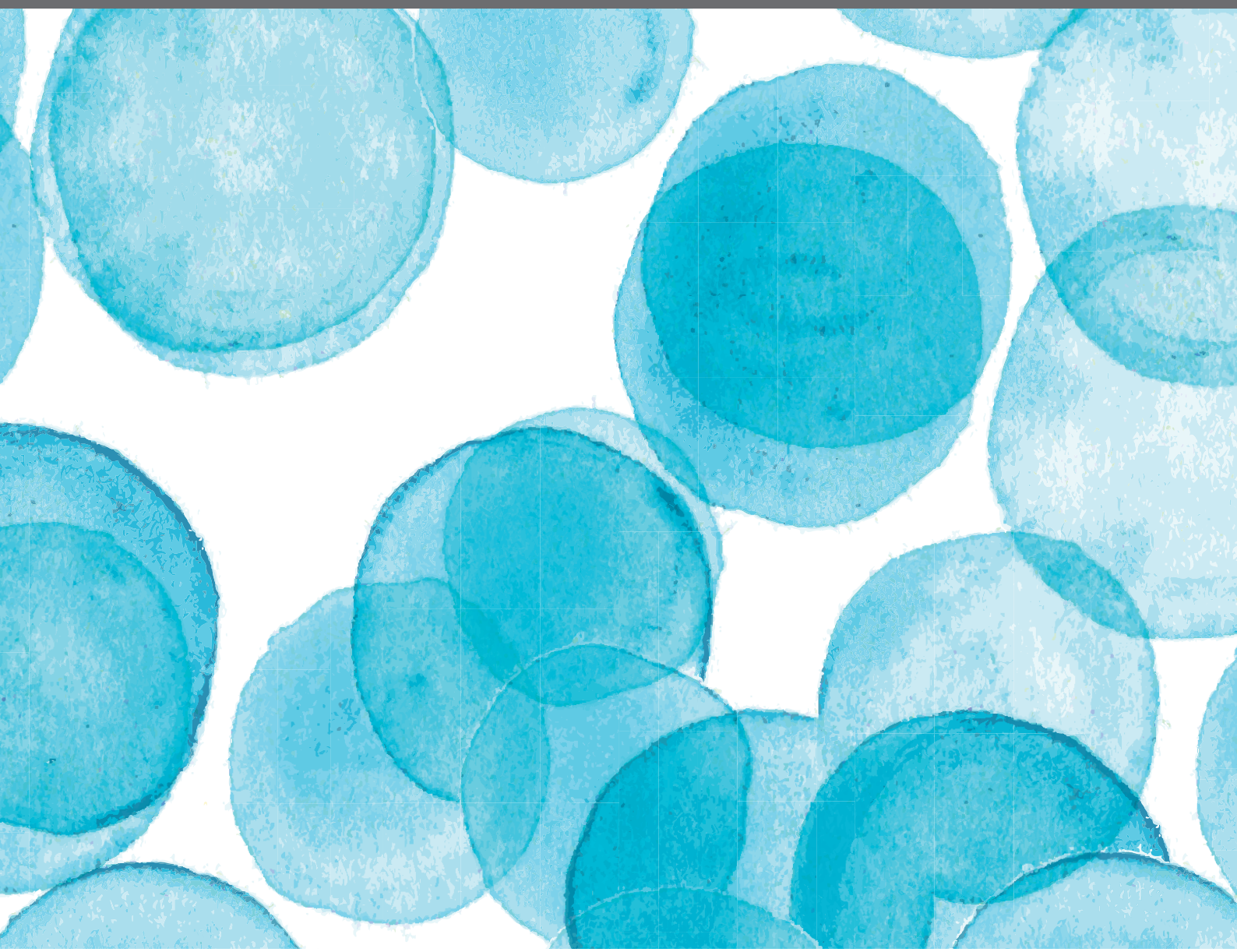

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ANTIMICROBIALS IN WILDLIFE AND THE ENVIRONMENT

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EDITORIAL
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Editorial: Antimicrobials in Wildlife and the Environment

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Keywords: antimicrobial resistance, antimicrobial residues, environment, wildlife, food chain

Editorial on the Research Topic

Antimicrobials in Wildlife and the Environment

Antimicrobial resistance (AMR) is a major threat to both human and animal health since it significantly diminishes the therapeutic options available. Significant amounts of the antimicrobials used in humans and animals are excreted into the environment essentially unchanged, or as metabolites that still retain antimicrobial activity. They reach the environment via direct excretion by pasture animals, from discharges from wastewater treatment plants, via application of manure and sludge to agricultural fields, or direct application of antimicrobials in aquaculture. The release of antimicrobials into the environment can exert a selective pressure on environmental microbiota, leading to the selection of antimicrobial resistance genes (ARGs) in a variety of ecosystems. Thus, the microbiota of plants, wild animals and the environmental microbiota in general can be affected. Another way of dissemination of ARGs into the environmental ecosystems is via AMR bacteria in animal and human faeces. The resulting pool of ARGs in the environment can be considered as self-replicating genetic pollutants, with the possibilities of horizontal transfer, recombination, and generation of a broader diversity of ARGs. From this pool, ARGs can be reintroduced back to human and animal pathogens, thus contributing to the global problem of AMR.

Antimicrobial residues and AMR determinants have been reported in soils, surface and groundwater, glaciers, air, agricultural produce, wildlife and other urban, agricultural and natural environments. The main aim of this Research Topic was to uncover the role of AMR in wildlife and the environment for better understanding of AMR epidemiology in a globalised world. Within this topic, 11 articles have been published that complemented our knowledge on the occurrence and diversity of antimicrobial residues, AMR bacteria and ARGs in the environment and wildlife.

Mbanga et al. characterised genomic diversity of environmental *E. coli* isolates from a wastewater treatment plant and surrounding receiving waters in South Africa using phylogenomic analysis. They concluded that these isolates mainly clustered with clinical isolates, thus highlighting their importance for public health. Continuous surveillance of AMR bacteria in wastewater and associated surface waters could serve as a proxy for local AMR and its dynamic over time. Such a surveillance strategy may be highly relevant in low-resource settings, where clinical surveillance of AMR is too costly. The dissemination of AMR bacteria from river water to vegetables irrigated by this water was studied by Díaz-Gavidia et al. in Central Chile. They isolated Enterobacterales strains that were resistant to four antibiotic classes, with some of them demonstrating multi-drug resistant (MDR) phenotypes. The occurrence of MDR Enterobacterales during the rainy season was less frequent compared to the dry season. Association of AMR frequency with season and vegetable type contributes to a better understanding of a potential public health impact of AMR/MDR bacteria present in irrigation water and vegetables.

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Jauregi et al. evaluated the potential risks associated with antibiotic residues, ARGs and mobile genetic elements (MGEs) introduced into soil and crops via cow manure application. Authors compared different treatments (slurry, fresh, or aged manure from conventional and organic livestock farms) and crops (wheat and lettuce) but no single treatment could be identified as superior to the others to reduce the potential resistome risks. The authors concluded that the treatments should be specific and take into consideration the amendment, soil and crop types.

AMR can be also exchanged between humans and animals through pests and wild animals. The role of common pests in poultry such as the lesser mealworms to serve as a potential reservoir of zoonotic *Salmonella enterica* strains was investigated by Donoso et al. They isolated 15 *S. enterica* strains, 14 of which belonged to the Infantis serotype, with the carriage of pESI plasmid and MDR phenotype.

Wildlife may also contribute to the dissemination of genes conferring resistance to clinically relevant antimicrobials. They could serve as reservoirs of AMR bacteria and also represent epidemiological links between human, livestock and natural environments, especially in the case of long-distance haulers such as migratory birds. Several articles in the Research Topic addressed this issue. Haenni et al. investigated 424 wild birds and 16 wild mammals in a rescue centre in France for the presence of AMR bacteria. They demonstrated a wide dissemination of Enterobacteriaceae with an IncHI2/ST1 plasmid carrying the blaCTX–M−9, blaSHV−12 and mcr-9 genes. Interestingly, these clones are not *bona fide* microbiota of birds but, similarly to nosocomial infections in hospitals, represent dissemination of clones within the rescue centre. These findings suggest a potential dissemination of ESBL-positive clones to the natural environments from rescue centres. Plaza-Rodríguez et al. investigated the occurrence and AMR patterns of several bacterial species in certain wild animals in Germany, including wild boars, roe deer, and wild ducks and geese. In general, the prevalence of AMR bacteria was low. However, resistance was identified against clinically relevant drugs such as third-generation cephalosporins, fluoroquinolones and colistin. Skarzyńska et al. characterised the AMR status of 71 *E. coli* isolates from free-living birds in Poland using phenotypic assessment and WGS. Multiple resistance types were found, including those towards cephalosporins, quinolones, polymyxins, aminoglycosides, as well as fosfomycin. Molecular epidemiological analyses revealed that the *E. coli* strains were of the global lineages ST131, ST10, and ST224 as well as the three novel STs, 11104, 11105, and 11194. O'Hagan et al. investigated the AMR prevalence in *E. coli*, *Salmonella* spp., and methicillin resistant *Staphylococcus aureus* (MRSA) isolates from European badgers and red foxes in Northern Ireland. No MRSA were detected, while ESBLs were detected in 8.90% of badger and 11.53% of fox faecal *E. coli* isolates, which, in addition, also displayed MDR phenotype. AmpC type resistance was found only in the *S. enterica* subsp. *arizonae* isolate. Detection and quantification of ARGs in the gut of kelp gulls and Magellanic penguins revealed that AMR profiles differ between these two bird species (Ewbank et al.), which could be reflective of differential biology, ecology and proximity to human-impacted areas. In another study, the distribution and resistance to third-generation cephalosporins in *E. cloacae* complex members and their relationship between wild anoles and human activities (Pot et al.) was investigated. No relation was identified. The authors suggested that the high level of resistance in wild anoles was probably due to environmental factors that favour the selection of these resistant strains.

Other antibiosis factors like polyene produced by *Streptomyces* that occur naturally in the environment can be active again pathogenic fungi, and constitute an alternative to chemical fungicide treatment and might participate to reduce the burden of phytopharmaceuticals (Li et al.).

In conclusion, it is currently rather difficult to make generalisations because available data are fragmentary and insufficient. More comprehensive and larger-scale studies are necessary to evaluate the occurrence and diversity of antimicrobials and AMR in wildlife and the environment. Within the One Health framework, it is crucial to understand the role played by wildlife and environment in the development and transmission of antimicrobial resistance, and to evaluate its impact.

AUTHOR CONTRIBUTIONS

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

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BRIEF RESEARCH REPORT
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Detection of Antimicrobial Resistant *Salmonella enterica* Strains in Larval and Adult Forms of Lesser Mealworm (*Alphitobius diaperinus*) From Industrial Poultry Farms

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The lesser mealworms (*Alphitobius diaperinus*) constitute a common cosmopolitan pest in poultry flocks and may colonize the litter in adult and larval forms. Previous studies have documented their potential as carriers of enteric pathogens. In this context, *S. enterica* constitutes a prioritized zoonotic agent in the poultry industry due to the sanitary risks and economic losses associated with its presence. The aim of this study is to describe the presence of *S. enterica* strains in larval and adult forms of *A. diaperinus* collected from poultry litter belonging to industrial farms located in the central zone of Chile. A total of 403 specimens (203 adults and 200 larvae) were sampled from three farms and 25 flocks. For bacteriological isolation, beetles were processed to differentiate external and internal contamination. Then, isolates were serotyped according to the Kauffman-White scheme and antimicrobial resistance phenotypes were determined using the disk diffusion method. Gene sequences from the megaplasmid pESI were identified through a PCR based test. These procedures led to the detection of 15 *S. enterica* isolates, belonging to serotypes Infantis (14) and Livingstone (1), from both adults (6) and larval (9) specimens, with a similar external (7) and internal (8) distribution. Furthermore, all *S.* Infantis isolates showed antimicrobial resistance and evidence of megaplasmid pESI carriage, with all possessing multidrug-resistant phenotypes. Our results confirm that *A. diaperinus* constitutes a potential reservoir of zoonotic *Salmonella* strains of sanitary and economic concern for the industry and for public health.

Keywords: poultry, Chile, *Salmonella*, *Alphitobius diaperinus*, drug resistance

INTRODUCTION

The lesser mealworms (*Alphitobius diaperinus*) constitute a common pest in poultry flocks (1), characterized as a scavenger arthropod which colonizes the litter in adult and larval forms. They are able to survive within flocks by consuming feces, food and dead birds, but can also affect residential areas in close proximity to fields treated with manure (2). This insect has been reported to serve as a vector for several enteropathogens, including *E. coli*, *Campylobacter,* and *Salmonella enterica*, among others (3).

S. enterica is an enteric pathogen that is widely distributed in nature and produces a variety of diseases in a range of hosts, including humans, mammals, birds, and reptiles. In addition, insects, plants, and unicellular organisms may also harbor bacteria in the environment (4), leading to the ubiquity and persistence of these bacteria in infecting hosts. More than 2,600 serotypes within the *S. enterica* species have been described, including both host-restricted and host-generalist serotypes (5).

The zoonotic risk of *Salmonella* is mainly associated with its transmission through consumption of contaminated animal and plant-derived foods (6). This usually results in a self-limiting gastroenteritis, although patients with some risk factors, such as infants, immunocompromised individuals and the elderly, can develop extra-intestinal infections that can cause meningitis, sepsis, and even death. In recent years, infection with this bacterium in humans has been among the most common causes of notifiable outbreaks (7). Globally, *S. enterica* serotype Enteritidis (*S. Enteritidis*) and *S. Typhimurium* represent the most common serotypes that cause disease in humans (8). However, several other bacteria, which may be classified as emergent clones or serotypes, have also been responsible for outbreaks in recent years (9, 10). In Chile, the public health service has developed food chain surveillance and food-associated outbreak investigations programs. Together these activities have established Salmonella as the most common pathogen involved in foodborne disease outbreaks with Enteritidis, Infantis, and Typhimurium the most frequently detected serotypes (11). Furthermore, the official veterinary service controls a biosafety program in the poultry industry, with specific indications for prevention and early notification of biological agents, and an official microbiological control program for exported animal products (12).

The progressive increase of antimicrobial resistant bacteria presents a current menace (13), and is cataloged by WHO as one of the most important global threats to public health. For this reason, antimicrobials have been categorized and prioritized in order to preserve their effectiveness (14). In recent years, a gradual increase in drug resistant *Salmonella* strains has been documented in the human food chain, leading to more serious clinical cases and more hospitalizations (15). The purpose of this study is to report and characterize the isolation of antimicrobial-resistant *Salmonella* serotypes in larval and adult forms of *A. diaperinus* collected from poultry litter belonging to industrial farms located in the central zone of Chile.

METHODS

Samples

During December 2018, a total of 403 specimens (203 adults and 200 larvae) were sampled from 25 flocks belonging to three industrial farms located in the central zone of Chile. The insects were collected independently from manure and then stored in sterile 10 mL tubes.

Bacteriological Isolation and Serotyping

Once at the lab, each sample was processed using a two-step procedure for bacteriological isolation in order to differentiate between external and internal contamination. In the first step, the insects were immersed for 10 s in 5 mL of sterile buffered peptone water (Difco BPW broth, Beckton Dicknson, Franklin lakes, NJ, USA) supplemented with 20 μg/mL of novobiocin (Sigma, St. Louis, MO, USA). In the second step, insects were recovered with tweezers and immersed for 1 min in 95% ethanol, air dried and washed with PBS, and homogenized in 1.5 mL tubes using plastic stems. Insect remains were then inoculated into 5 mL of sterile BPW broth supplemented with 20 μg/mL novobiocin. Each inoculate was incubated for 24 h at 37°C. Then 100 uL of each suspension was inoculated into modified semisolid Rappaport Vassiliadis basal medium (Oxoid, Sao Paulo, Brazil) supplemented with 20 μg/mL of novobiocin and incubated at 45.1°C for either 24 or 48 h, depending on whether or not bacterial growth was observed. Cultures were plated onto Xilose Lysine Deoxicholate agar (Difco XLD, broth, Beckton Dicknson, Franklin lakes, NJ, USA) and suspicious colonies were identified using biochemical tests and *invA* gene detection by PCR (16), using the *S.* Enteritidis SARB 16 as a control strain. Finally, *S. enterica* isolates were serotyped according to the Kauffman-White scheme (5).

Disk Diffusion Method

Antimicrobial resistance phenotypes were determined by the disk diffusion method according to the standards recommended by the Clinical Laboratory Standards Institute (17). The following antimicrobials were evaluated: ampicillin (10 μg), amoxicillin + clavulanic acid (20/10 μg), ceftiofur (30 μg), ceftazidime (30 μg); ceftriaxone (30 μg), cefadroxil (30 μg), gentamicin (10 μg), streptomycin (10 μg), azithromycin (15 μg), tetracycline (30 μg), ciprofloxacin (5 μg), enrofloxacin (10 μg), nalidixic acid (30 μg), sulfamethoxazole + trimethoprim (20/5 μg), sulfisoxazole (10 μg), chloramphenicol (30 μg), and fosfomycin (20 μg). *Escherichia coli* ATCC 25922 was used as a control strain. The multi-drug resistance (MDR) condition was determined by the simultaneous resistance to three or more antimicrobial classes (18).

PCR Assays

After bacterial growth was observed, nucleic acids were extracted using the DNA extraction kit (Roche®) according to the manufacturer's instructions. Then, a PCR based test was performed under standard conditions for the identification of the pESI (plasmid for emerging *S.* Infantis) genes *faeAB*, *ipfA*, *merA*, *pemK, ccdAB,* and *traC*, using primers described previously (19).

Statistical Analyses

Sampling variables such as the presence of *Salmonella* in poultry flocks, the bacterial location in the *A. diaperinus* body and its developmental stage, were contrasted with isolation results through a logistic regression analysis, using the INFOSTAT (2010v) software.

RESULTS

Out of the samples analyzed, 10 flocks belonging to the three farms were found to be infected. A total of 15 *S. enterica* isolates

were detected from lesser mealworms, including *S*. Infantis (14) and *S*. Linvingstone (1) serotypes. Additionally, all *S*. Infantis isolates showed multi-drug resistance phenotypes, and the pESI sequences were variably detected in most of the strains, with the exception of *S*. Livingstone (**Table 1**).

Analysis of infection status of flocks, bacterial location and the stage of host development variables, determined that none of them were statistically associated with *Salmonella* detection ($p > 0.05$).

DISCUSSION

Within poultry farms, *S. enterica* contaminates productive units and the food chain through diverse transmission pathways, including environmental sources (20) that can hold bacteria, allowing repeated infection of hosts. In fact, when such reservoirs remain unnoticed, continuous exposure and outbreaks over several years have been documented, both in animal and human settings (21, 22). This study suggests that one such potential reservoir are lesser mealworms, which are common arthropods that live on the manure within poultry facilities (23). On sampled insects, bacterial isolates were indistinctly detected both externally and internally and in larval and adult forms ($p > 0.05$). Although larvae may have a higher capacity to transmit infection to chickens than do the adult forms (23), our results suggest that beetles always present a risk of carrying and spreading *Salmonella* within poultry flock environments, that apparently depends on the bacterial dose to which these animals are exposed (24). It is feasible that *A. diaperinus* directly and indirectly transmits *Salmonella* to animals, since it is consumed by broiler chicks (25, 26) and also disseminates bacteria to the chicken manure (24).

Whether *A. diaperinus* is a reservoir host or simply a mechanical vector of *Salmonella* is an still unknown condition that new studies should address. Whatever the role, the field evidence suggests that this arthropod can survive cleaning and disinfection procedures, which presents a risk for its transmission in poultry pens (24, 27, 28). Furthermore, this insect represents a good protein source for human consumption (29), resulting in an additional public health risk if zoonotic pathogens colonize its body from the environment or through its diet (29).

It has been determined that beetles harboring *Salmonella* in their gut can shed bacteria thorough their feces for an average of 8 days, allowing persistent pathogen dispersal between flock rotations (30). In the sampled farms, routine biosecurity management practices currently incorporate an exhaustive cleaning procedure in which manure and organic matter are removed with pressurized water, and a sanitation procedure in which disinfectants are applied to pens during 14-days empty periods. Despite of these procedures, beetles have not been eradicated and persist in consecutive flocks, as does *S. enterica*, suggesting that insects play a role in the continuous exposure of birds to this bacterium. Personnel from the farms recognize the presence of small cracks and crevices within facilities, in which arthropods may survive and continuously contaminate the surrounding environment.

Regular surveillance is performed by the same farms to detect *Salmonella* infection in poultry and flocks are classified according to their infection status with this bacterium. However, in this study such condition was not a predictor of *Salmonella* detection in beetles ($p > 0.05$), suggesting that a more stringent surveillance sampling is needed, or that a differential risk exists in contamination of arthropods and chickens within pens. In fact, it has been reported that insects may be early indicators of *Salmonella* infection in flocks, with higher detection rates than other samples obtained from these environments (27).

In analyzed specimens, *S*. Infantis and *S*. Livingstone serotypes were detected. *S*. Infantis is an emerging serotype within the poultry industry, which apparently emerged 75 years ago and then expanded globally during industrialization of livestock production (31). S. Livingstone is a wide host range serotype associated with diverse hosts, including cattle (32), pigs (33), poultry (34), and sea lions (35), among others, although it is less frequently linked to disease in humans than is *S*. Infantis (8). The lower frequency of this serotype (and the absence of others) in sampled beetles may be explained by a competitive exclusion phenomenon that characterizes the transmission and colonization of *Salmonella* in poultry (36) and inside the gut of *A. diaperinus* (29).

Despite of causing milder clinical outcomes in humans than other serotypes (37), emergent *S*. Infantis strains have been associated with the acquisition of chromosomal mutations and the transmission of genetic traits, such as plasmids, which confer MDR phenotypes in most of the strains recently isolated from poultry around the globe (38–40). In Chile, antibiotics used in animals account for 95% of all antibiotics imported by the country (41), suggesting that the practice of veterinary medicine could have major impacts on the selection of drug resistant bacteria (15). In 2018, the surveillance of non-typhoidal *Salmonella* carried out by the public health service reported the emergence of *S* Infantis, as the second most frequent serotype in both intestinal and extraintestinal clinical cases, after *S*. Enteritidis. Moreover, in the same year *S*. Infantis showed the highest antimicrobial resistance levels against sulfamethoxazole /trimethoprim, chloramphenicol and ampicillin, with resistance levels ranging between 48 and 58%. In contrast, high susceptibility to ciprofloxacin was still observed, although some extraintestinal isolates (3/18) expressed resistance against this drug (11). In general, such results agree with the phenotypes observed in this study, suggesting that strains detected in lesser mealworms have been subjected to similar selection pressure within poultry environments and belong to the same transmission chains that cause disease in humans.

The *S*. Infantis drug resistance has been associated with the unique pESI megaplasmid which was initially described in Israeli isolates in 2007 (42), and along withwith some pESI-like variants, has since been described in other territories across the world (43–45). This mobile genetic structure can be transferred to other commensal or pathogenic bacteria within the host intestinal environments (19). Although contains conserved and polymorphic segments, the plasmid-associated pattern of resistance includes antimicrobials such as tetracycline, sulfametoxazole and trimethoprim, among others (19, 43), which

TABLE 1 | Description of *Salmonella* isolates detected in lesser mealworms.

***Salmonella* serotype**	**Host data***			**Antimicrobial resistance****																**pESI genes**					
	ID	**Loc**	**Stage**	**AMP*** **	**AMC*** **	**EFT*** **	**CAZ**	**CRO*** **	**CFR**	**CN*** **	**S**	**AZN*** **	**TE**	**CIP*** **	**ENR*** **	**NA*** **	**STX**	**SF**	**C**	**FOT*** **	***faeAB***	***ipfA***	***merA***	***pemK***	***ccdAB***	***traC***
Infantis	3	Int	Adult																							
Infantis	38	Ext	Adult																							
Infantis	50	Ext	Adult																							
Infantis	82	Int	Adult																							
Infantis	90	Int	Larva																							
Infantis	100	Int	Larva																							
Infantis	102	Int	Larva																							
Infantis	124	Ext	Larva																							
Infantis	126	Ext	Larva																							
Infantis	126	Int	Larva																							
Infantis	268	Ext	Larva																							
Infantis	294	Ext	Adult																							
Infantis	394	Int	Adult																							
Infantis	403	Int	Larva																							
Livingstone	356	Ext	Larva																							

*ID, identification; Loc, location; Int, Internal; Ext, External. Gray spaces represent a phenotype or gene detection.

**AMP, Ampicillin; AMC, Amoxicillin/ Clavulanic acid; EFT, Ceftiofur; CAZ, Ceftazidime, CRO, Ceftriaxone; CFR, Cefadroxil; CN, Gentamicin; S, Streptomycin; AZN, Azithromycin; TE, Tetracycline; CIP, Ciprofloxacin; ENR, enrofloxacin; NA, Nalidixic acid; SXT, Sulfamethoxazole /Trimethoprim; SF, Sulfisoxazole; C, Chloramphenicol; FOT, Fosfomycin.

***Critically important antimicrobials (14).

have been subjected to positive selection and spreading of drug resistance as a result of widespread and common use (31). In this study, we found resistances against these same and other antimicrobials, as well as genetic evidence of the pESI presence, with some polymorphisms among bacterial isolates (**Table 1**). The existence of is plasmid might explain the MDR phenotype observed in all *S*. Infantis isolates, which is likely dispersed within local productive farms. Furthermore, these MDR phenotypes have also been associated with enhanced resistance to heavy metals and environmental fitness of the strains in which they are present (46). These characteristics represent bacterial survival mechanisms that challenge the strategies implemented by producers and sanitary authorities to control and prevent salmonellosis. The emergence of *S.* Infantis strains harboring pESI or pESI-like plasmids is a risk to public health and requires exhaustive epidemiological characterizations of the animal and environmental transmission chains so that effective control methods can be implemented.

This study has some limitations. All samples were taken during a single month and belong to industrial farms from the same company, and therefore may not be representative of the epidemiological conditions of other seasons, environments or farms throughout the country. A more extended sampling scheme, involving the collection of samples over a longer period of time and from a wider variety of companies in Chile, would have resulted in a more complete understanding of the *S*. Infantis-*A. diaperinus* relationship. In addition, a higher resolution method is needed for the plasmid description, in order to characterize and compare the pESI structure of Chilean isolates with those reported elsewhere, and for to elucidate virulence functions and risk potentials of these strains. A strength of this study is that the isolation procedure was able to discriminate between internal and external *Salmonella* contamination in adults and larvae from *A. diaperinus*, confirming the ability of this insect for bacterial transmission within flock environments.

In conclusion, there are MDR *Salmonella* strains in lesser mealworms within industrial poultry farms from Chile. These arthropods constitute a host reservoir of this zoonotic pathogen and represent economic and sanitary risks to the food chain of the country. In this regard, this study supports actions for permanent control strategies of *A. diaperinus* populations in animal facilities.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

AD: field sampling, laboratory work, and analyses of results. NP: field sampling, analysis of results, and writing the manuscript. PR: conception, design of the study, analysis, interpretation of results, and writing the manuscript. All authors contributed to the article and approved the submitted version.

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Wide Spread of *bla*$_{CTX-M-9}$/*mcr-9* IncHI2/ST1 Plasmids and CTX-M-9-Producing *Escherichia coli* and *Enterobacter cloacae* in Rescued Wild Animals

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Wildlife has recently been pinpointed as one of the drivers of dissemination of genes conferring resistances to clinically important antimicrobials. The presence of both extended-spectrum beta-lactamase- (ESBL) and carbapenemase-encoding genes has notably been reported in wild birds, that can act as sentinels of antimicrobial resistance (AMR) contamination but also as long-distance spreaders in case of migratory birds. Here, 424 wild birds brought to a rescue center in France were sampled over a 6-month period. These birds encompassed 62 different sedentary or migratory species. A further 16 wild mammals present in the center were also investigated. No carbapenemase-producer was found, but we identified a surprisingly high proportion (24.1%) of ESBL-positive isolates. A total of 144 non-duplicate isolates were collected, including *Escherichia coli* (n = 88), *Enterobacter cloacae* (n = 51), and *Citrobacter freundii* (n = 5), of which 123 carried the *bla*$_{CTX-M-9}$ gene. PFGE, phylogroup, and MLST revealed the presence of a limited number of ESBL-positive clones circulating in these animals, all presenting multiple associated resistances. Next-generation sequencing on a subset of isolates, followed by Southern blot hybridization, showed the wide dissemination of an IncHI2/ST1 plasmid carrying the *bla*$_{CTX-M-9}$, *bla*$_{SHV-12}$ and *mcr-9* genes. In all, our results undoubtedly reflect cross transmissions of ESC-resistance (ESC-R) Enterobacteriaceae within the rescue center – similarly to nosocomial spreads observed at hospital, rather than the true bacterial flora of birds. We also showed that the spread of ESC-R in this rescue center did not only rely on clonal but also on a highly successful plasmidic transmission. Since most animals are intended to get back to nature after a few days or weeks, this is obviously an issue with regard to ESBL dissemination in natural environments.

Keywords: CTX-M, *mcr-9*, wild bird, *E. coli*, *E. cloacae*, IncHI2

INTRODUCTION

The importance of wildlife in disseminating genes conferring resistances to clinically important antimicrobials (CIAs), such as to extended-spectrum cephalosporins (ESC) or carbapenems (CP), has been recurrently highlighted (Wang et al., 2017; Dolejska and Literak, 2019). Antimicrobial resistance (AMR) in wildlife most likely reflects the pollution of natural environments with AMR genes, plasmids or bacterial clones selected in non-wildlife sectors. AMR acquisition most probably occurs through food opportunities – including dejections from domestic animals or polluted lands – and water sources, but the exact origin of AMR in specific wild individuals is usually unknown (Guenther et al., 2011; Mukerji et al., 2020). In most cases, AMR in wildlife has been found incidentally and, to date, most publications refer to wild birds, which encompass a wide diversity of animal species, habits and behaviors.

In wild birds, major *Escherichia coli* clones circulating in humans were detected, such as of sequence type (ST)131, ST410, ST648, or ST38 to only name a few, suggesting that these animals were indirectly contaminated by human sources (Schaufler et al., 2016; Atterby et al., 2017; Guenther et al., 2017; Yang et al., 2019). In rare occasions, AMR epidemiology in wild birds more clearly mirrored the one in humans and/or domestic animals in the same country, thereby supporting the hypothesis of local cross-sectorial transmissions. It was notably exemplified for CTX-M-producing Enterobacteriaceae in Sweden and Canada (Bonnedahl et al., 2015; Atterby et al., 2017) or IMP-4-producing Enterobacteriaceae in Australia (Dolejska et al., 2016). In addition to being sentinels of AMR contamination from other sectors, migratory birds can also spread AMR genes or antimicrobial resistant bacteria over very long distances, whose impacts on public health are suspected but not fully clarified (Guenther et al., 2012; Fuentes-Castillo et al., 2019).

At a global scale, CP- or colistin resistance in wild birds has been much less reported than ESC-resistance (ESC-R). It may however reflect a lack of studies, or the accumulation of case reports that do not accurately reflect the true epidemiology. Indeed, investigations on AMR in wild birds sometimes revealed unexpected reservoirs, such as recently where a high proportion of CP-resistant NDM-5-producing *Klebsiella pneumoniae* was observed in migratory birds in China (Liao et al., 2019). With regard to colistin resistance, a limited number of studies reported the plasmid-mediated *mcr-1* gene in wild birds (Tarabai et al., 2019). In France, ESBL-producing Enterobacteriaceae were detected in 2009 in 17/90 (18.9%) of juvenile yellow-legged gulls (Bonnedahl et al., 2009), while the sporadic detection of a VIM-1-producing *E. coli* was reported in 2012 in the same area (South of France) and the same bird species (Vittecoq et al., 2017). Recently, multi-drug resistant Enterobacteriaceae were again found in yellow-legged gulls in Marseille, France (Ngaiganam et al., 2019).

In all, several works investigating AMR in wild birds resulted from convenient samples from dead animals. Numerous studies were also based on fecal dejections collected in various places where birds live or transit, such as landfills, beaches, urban parks, nests, and other habitats, but associating fecal samples to the right bird species may be challenging. In some situations however, these approaches valuably allowed studying AMR in large cohorts of individuals of the same bird species in their natural environment, as for instance illustrated for black kites (*Milvus milvus*), corvids (*Corvus brachyrhynchos*, *Corvus corax*), gulls (*Larus glaucescens, Larus ridibundus*), pigeons (*Columba livia*) or storks (*Ciconia ciconia*) (Bonnedahl et al., 2010, 2014; Jamborova et al., 2018; Tarabai et al., 2019; Zendri et al., 2020). Here, we adopted a different strategy by systematically sampling all incoming wild birds at a French rescue center over a 6-month period. Such a systematic sampling design, which can for example be set up in rescue centers or during ringing campaigns (Guenther et al., 2010; Schaufler et al., 2016), is still rare in the field of AMR in wild birds. Both sedentary and migratory species were considered and sampled, and AMR genes, plasmids and bacterial clones were further investigated at phenotypic and molecular levels. Wild mammals present in the center were also studied. Our data support interesting hypotheses on the spread of AMR at the interface of wild animals and human communities.

MATERIALS AND METHODS

Bacterial Isolates

Between April and November 2015, 424 migratory and sedentary birds were sampled at an animal rescue center in the Hérault department, South of France. Cloacal sample was taken using Eswab minitip or pernasal flocked (Labelians, Nemours, France), depending to the size of the bird. Sampling was performed in the first days after arrival at the center, ranging from 24 h to 10 days. During the study, and for epidemiological reasons (see section "Results"), 16 mammals which were temporarily hosted in the rescue center were also sampled using the same procedure as for birds. Within 24 h after sampling, samples were plated on Drigalsky agar as a control of growth, as well as on the selective ChromID ESBL, ChromID OXA-48 and CarbaSMART media for the detection of ESC- and CP resistance. One colony per morphology was picked up and identified by MALDI TOF (VITEK MSVersion 3.0, bioMérieux, Marcy L'Etoile, France).

Antimicrobial Susceptibility Testing

Susceptibility testing was performed by disc diffusion on Mueller-Hinton agar (Bio-Rad, Marne-la-Coquette, France), according to the guidelines and clinical breakpoints of the Antibiogram Committee of the French Society for Microbiology[1]. The following discs of human and/or veterinary interest were tested: amoxicillin, amoxicillin + clavulanic acid, cefalotin, cefuroxime, cefotaxime, ceftiofur, ceftazidime, cefoxitin, cefepime, aztreonam, cefquinome, ertapenem, streptomycin, kanamycin, amikacin, apramycin, gentamicin, tobramycin, netilmicin, chloramphenicol, florfenicol, tetracycline, colistin, sulfonamides, trimethoprim, nalidixic acid, and enrofloxacin. *E. coli* ATCC 25922 was used as a quality control. Minimum inhibitory concentration (MIC) for colistin was determined by the broth microdilution method, as recommended by EUCAST (EUCAST, 2016).

[1]https://www.sfm-microbiologie.org/

Identification of β-Lactamase Genes

PCRs were performed using specific primers for the detection of bla_{TEM}, bla_{SHV}, bla_{CTX-M} group 1, group 2, and group 9 (Shibata et al., 2006; Dierikx et al., 2010). For all bla_{CTX-M} group 1 and group 9-positive isolates, additional PCRs were performed using the primers ISEcp1L1/P2D and MA1/MA2, respectively. The bla_{CMY} genes were detected using CF1/CF2 primers (Eckert et al., 2004). All positive amplicons were sequenced (Genewiz, London, United Kingdom). The *mcr-1* to *mcr-5* genes were detected using published primers (Lescat et al., 2018) while detection of the *mcr-9* gene was performed using the primers mcr9_int_for (5′-GAAACTAACCCCCAGGAAGC) and mcr9_int_rev (5′-TTTTGGCGATTTCATCATCA).

Genetic and Molecular Typing of the Strains

Phylogenetic grouping of the *E. coli* isolates was performed using the improved method described by Clermont et al. (2013). PFGE was performed on *Xba*I-digested DNA. Multi-locus sequence typing (MLST) was performed on one representative of each PFGE profile according to the following websites: https://pubmlst.org/bigsdb?db=pubmlst_mlst_seqdef for *E. coli* according to the Achtman scheme, and https://pubmlst.org/ecloacae/ for *E. cloacae*.

Whole Genome Sequencing

Genomic DNA of eight selected isolates (four *E. coli* and four *E. cloacae*) was extracted from an overnight culture using the GmbH & Co. KG – NucleoSpin® Microbial DNA (Macherey Nagel, Germany). Whole genome sequencing was performed using the NovaSeq technology (Illumina). The paired-end reads (average read length of 151 bp) were generated with a 69-fold to 131-fold coverage. After Trimmomatic cleaning, *de novo* assembly was performed using Shovill (version 0.9.0). Resistance genes were searched from the assembled genomes using the ResFinder database (CGE[2]). Pairwise single nucleotide polymorphism (SNP) distances were calculated from core genome alignments generated by Roary using snp-dists[3].

Plasmid Characterization

Plasmids were typed by PCR-based replicon typing (PBRT) according to the PBRT kit scheme described by Carattoli et al. (2005) using a commercial kit (Diatheva, Cartoceto, Italy). PFGE-S1 gels were performed, followed by Southern blot using adequate probes ($bla_{CTX-M-9}$, bla_{SHV-12}, *mcr-9*, IncHI2) according to the manufacturer's protocol (Roche Diagnostics, Meylan, Germany). Plasmid co-localization was assessed by comparison between the bands corresponding to the resistance genes and those corresponding to the Inc type of the plasmid.

Ethics Statement

No ethical approval was needed since this study did not involve any experimentation on animals.

[2]http://www.genomicepidemiology.org/

[3]https://github.com/tseemann/snp-dists

Accession Number(s)

The whole genome shotgun project was deposited in DDBJ/EMBL/GenBank under the BioProject accession number PRJNA659767.

RESULTS

Over the 6-month period, a total of 424 wild birds were sampled, that belonged to 62 different species distributed into 25 sedentary ones and 37 species migrating either inside Europe or to Africa. All of these species were represented by less than 20 individuals, except for black swifts (*Apus apus*, *n* = 74), rock pigeon (*C. livia*, *n* = 55), Turkish turtledove (*Streptopelia decaocto*, *n* = 31), and black-billed magpie (*Pica pica*, *n* = 21). The vast majority of the birds were referred to the rescue center after serious injury (various traumas, such as broken legs or wings) or because they had been found incidentally. In some occasions, related juveniles from the same litter were collected at the same time. Altogether, all birds were confirmed to be devoid of obvious bacterial or viral infection after veterinary examination so that any AMR in the positive animals should be considered as carriage.

A total of 102 birds (102/424, 24.1%) were positive for the presence of at least one ESC-R Enterobacteriaceae, while no CP-resistant isolate was identified (**Supplementary Table 1**). Forty-six positive birds were sedentary (14 different species including ducks, sparrows and gulls) while 56 were migratory (19 different species either migrating in Europe, such as tawny owls or in Africa, such as swallows). No bird species was more represented amongst the positive individuals compared to the negative ones. Different bacterial morphologies were identified on numerous selective plates, but only non-duplicate isolates (based on the PFGE profile, phylogroup, CTX-M-type and the antibiogram) were kept for further analysis. Multiple ESC-R isolates were identified in 36/102 samples (*n* = 35.3%). For each positive animal, one to four different bacterial morphologies were identified, so that a total of 144 different isolates were collected (**Supplementary Table 1**). ESC-R Enterobacteriaceae were identified as *E. coli* (*n* = 88), *Enterobacter cloacae* (*n* = 51), and *Citrobacter freundii* (*n* = 5). One ESC-R *E. coli* and one ESC-R *E. cloacae* were concomitantly identified in 22/102 samples (*n* = 19.6%), and the same CTX-M-9 enzyme was found in concomitant *E. coli* and *E. cloacae* isolates in 20/22 samples.

The $bla_{CTX-M-1}$ gene was found in 21 isolates (20 *E. coli* and 1 *E. cloacae*), while the $bla_{CTX-M-9}$ gene was dominantly detected in the 123 remaining isolates, i.e., in 68 *E. coli*, 50 *E. cloacae* and five *C. freundii* isolates (**Supplementary Table 1**). All isolates presented multiple associated resistances (**Table 1**), the most frequent ones being to sulfonamides (100% in *E. cloacae*; 98.9% in *E. coli*), tetracyclines (78.4 and 85.2%, respectively) and chloramphenicol (90.2 and 72.7%, respectively). *E. cloacae* isolates were also often resistant to gentamicin (64.7%) and enrofloxacin (25.5%). No resistance to amikacin or colistin was detected.

The vast majority of *E. coli* isolates belonged to the phylogroups A (*n* = 32) and B1 (*n* = 53) usually associated to commensal isolates, while only three belonged to the more

TABLE 1 | Phenotypic resistances associated to ESBL-producing *E. coli* and *E. cloacae*.

Antibiotic	*E. coli* (*n* = 88)		*E. cloacae* (*n* = 51)	
	Number	Percentage	Number	Percentage
Streptomycin	22	25.0	14	27.5
Kanamycin	61	69.3	40	78.4
Amikacin	0	0.0	0	0.0
Apramycin	0	0.0	0	0.0
Gentamicin	35	39.8	33	64.7
Tobramycin	61	69.3	45	88.2
Netilmicin	57	64.8	39	76.5
Chloramphenicol	64	72.7	46	90.2
Florfenicol	1	1.1	0	0.0
Tetracycline	75	85.2	40	78.4
Colistin*	0	0.0	0	0.0
Sulfonamides	87	98.9	51	100.0
Triméthoprim	19	21.6	13	25.5
Nalidixc acid	12	13.6	21	41.2
Enrofloxacin	5	5.7	13	25.5

**As determined using the micro-dilution method.*

TABLE 2 | Sequence types (ST) of ESBL-producing *E. coli* and *E. cloacae*.

ST	ESBL enzyme	Phylogroup	Number of isolates	Number of PFGE profiles
E. coli				
746	CTX-M-9	A	30	1
1246	CTX-M-9	B1	26	1
155	CTX-M-1	B1	14	6
40	CTX-M-9	B1	3	1
224	CTX-M-1	B1	3	3
223	CTX-M-9	B1	2	1
4054	CTX-M-9	B1	2	1
10	CTX-M-1	A	1	1
88	CTX-M-1	B1	1	1
136	CTX-M-1	B2	1	1
162	CTX-M-9	B1	1	1
174	CTX-M-9	D	1	1
1643	CTX-M-9	B2	1	1
ND[1]	CTX-M-9	A	1	1
ND	CTX-M-9	A	1	1
E. cloacae				
New (SLV ST714)	CTX-M-9	–	16	1
135	CTX-M-9	–	11	1
78	CTX-M-9	–	10	1
104	CTX-M-9	–	9	1
ND	CTX-M-9	–	1	1
ND	CTX-M-9	–	1	1
ND	CTX-M-9	–	1	1
ND	CTX-M-9	–	1	1
ND	CTX-M-1	–	1	1

[1]*ND, not done.*

virulent B2 (n = 2) and D (n = 1) phylogroups. A total of 22 PFGE profiles and 14 different STs were identified among the *E. coli* isolates (**Supplementary Table 1** and **Table 2**, also see **Supplementary Figures 1**, **2**). The two PFGE profiles A and B were dominant and represented 63.6% (56/88) of the *E. coli* isolates. These two PFGE profiles corresponded to ST746 (n = 30) and ST1246 (n = 27), respectively, and both produced CTX-M-9. ST155 (n = 14) was also recurrently found and produced CTX-M-1. This ST was more heterogeneous than ST746 and ST1246 since six different PFGE profiles were identified. Dynamics of these three main *E. coli* lineages over the 6-month period showed that ST1246 sporadically but regularly occurred between May and July, while ST746 had a more epidemic behavior, with 25 isolates detected between the end of June and mid-July (**Supplementary Figure 3**). For *E. cloacae* as well, most isolates distributed into a limited number of STs and PFGE profiles and also presented a peak of occurrence between the end of June and mid-July. Indeed, *E. cloacae* isolates mainly belonged to the new ST corresponding to a single locus variant of ST714 (n = 16; allelic sequence 2/2/gyrB*/133/51/2/14), ST135 (n = 11), ST78 (n = 10), and ST104 (n = 9), representing 90% (46/51) of all isolates. Contrary to *E. coli*, no *E. cloacae* isolate produced CTX-M-1.

During the 6-month sampling period, a few wild mammals (16) were also healed in the rescue center, using the same facilities as for birds, and that were sampled and analyzed using the same procedures as for birds. Bacterial identification and antimicrobial susceptibility testing proved that eight of these animals carried ESBL-producing *E. coli* isolates (**Table 3** and **Supplementary Table 1**), including one rabbit (*Oryctolagus cuniculus*), two hares (*Lepus europaeus*), one squirrel (*Sciurus vulgaris*), and four foxes (*Vulpes vulpes*). Further molecular analysis of ESBL genes together with PFGE and MLST determination concluded that the rabbit, the two hares and one fox carried an ST223 CTX-M-9-producing *E. coli*. The three other foxes carried an ST155 CTX-M-1-producing *E. coli* while the squirrel carried an ST1246 CTX-M-9-producing *E. coli*. All these CTX-M-producing *E. coli* lineages and corresponding PFGE profiles had also been detected in birds. No *E. cloacae* was detected in wild mammals.

To further characterize the isolates circulating in the rescue center, four couples of CTX-M-9-producing *E. coli*/*E. cloacae* (each couple originating from a single bird) were fully sequenced using Illumina technologies (**Table 4**). The four bird species were a magpie (*P. pica*), a jackdaw (*Corvus modenula*), an eagle owl (*Bubo bubo*), and a crag martin (*Ptyonoprogne rupestris*). Except *P. rupestris* which has a long-distance migratory behavior (Africa), the three other species are considered sedentary. The four *E. coli* isolates were from the two main lineages (ST746 and ST1246) found in this study, with the *B. bubo* and *P. rupestris* harboring a ST746 *E. coli*, and the *P. pica* and *Corvus modenula* harboring a ST1246 *E. coli*. SNP analysis on the core genome proved that isolates from a same ST were genetically highly similar, which differed by respectively, 8 and 21 SNPs. The same genomic similarities were found among the two *E. cloacae* of the same ST in the corresponding birds; which differed by respectively 29 and 63 SNPs.

NGS data also revealed additional information on the gene content, which was coherent with the resistance phenotypes

TABLE 3 | Characteristics of the eight *E. coli* isolates collected from mammals in the rescue center.

Animal number	Strain number	Sampling date	Animal species	Phylogeny	PFGE profile	MLST	CTX-M enzyme
A1_140	40430	15/06/2015	*Oryctolagus cuniculus*	B1	C	ST223	CTX-M-9
A2_143	40431	16/06/2015	*Lepus europaeus*	B1	C	ST223	CTX-M-9
A3_144	40432	16/06/2015	*Lepus europaeus*	B1	C	ST223	CTX-M-9
A4_145	40433	16/06/2015	*Sciurus vulgaris*	B1	B	ST1246	CTX-M-9
A5_166	40440	18/06/2015	*Vulpes vulpes*	B1	C	ST223	CTX-M-9
A6_167	40441	18/06/2015	*Vulpes vulpes*	B1	O	ST155	CTX-M-1
A7_168	40442	18/06/2015	*Vulpes vulpes*	B1	O	ST155	CTX-M-1
A8_169	40443	18/06/2015	*Vulpes vulpes*	B1	O	ST155	CTX-M-1

TABLE 4 | Epidemiological and molecular features of the eight isolates that were fully sequenced.

Strain	Common name (species)	Migratory behavior	Sampling date	Bacterial species	MLST	ESBL genes	CTX-M-carrying plasmid	Resistance genes
40412	Magpie (*Pica pica*)	Sedentary	03/06/2015	*E. coli*	1246	$bla_{CTX-M-9}$, bla_{SHV-12}	IncHI2/ST1	*aac*(6′)-*Ib3*, *aadA2b*, *ant*(2″)-*Ia*, *catA1*, *tet*(A), *sul1*, *mcr-9*, *aac*(6′)-*Ib-cr*, *qnrA1*
40435	Jackdaw (*Corvus modenula*)	Sedentary	16/06/2015	*E. coli*	1246	$bla_{CTX-M-9}$, bla_{SHV-12}	IncHI2/ST1	*aac*(6′)-*Ib3*, *aadA2b*, *ant*(2″)-*Ia*, *catA1*, *tet*(A), *sul1*, *mcr-9*, *aac*(6′)-*Ib-cr*, *qnrA1*
40460	Eagle owl (*Bubo bubo*)	Sedentary	30/06/2015	*E. coli*	746	$bla_{CTX-M-9}$, bla_{SHV-12}	IncHI2/ST1	*aac*(6′)-*Ib3*, *aadA2b*, *ant*(2″)-*Ia*, *catA1*, *tet*(A), *sul1*, *mcr-9*, *aac*(6′)-*Ib-cr*, *qnrA1*
40466	Crag martin (*Ptyonoprogne rupestris*)	Long distance (Africa)	30/06/2015	*E. coli*	746	$bla_{CTX-M-9}$, bla_{SHV-12}	IncHI2/ST1	*aac*(6′)-*Ib3*, *aadA2b*, *ant*(2″)-*Ia*, *catA1*, *tet*(A), *sul1*, *mcr-9*, *aac*(6′)-*Ib-cr*, *qnrA1*
40508	Magpie (*Pica pica*)	Sedentary	03/06/2015	*E. cloacae*	New*	$bla_{CTX-M-9}$, bla_{SHV-12}	IncHI2/ST1	*aac*(6′)-*Ib3*, *aadA2b*, *ant*(2″)-*Ia*, *catA1*, *tet*(A), *sul1*, *mcr-9*, *aac*(6′)-*Ib-cr*, *qnrA1*
40513	Jackdaw (*Corvus modenula*)	Sedentary	16/06/2015	*E. cloacae*	New	$bla_{CTX-M-9}$, bla_{SHV-12}	IncHI2/ST1	*aac*(6′)-*Ib3*, *aadA2b*, *ant*(2″)-*Ia*, *catA1*, *tet*(A), *sul1*, *mcr-9*, *aac*(6′)-*Ib-cr*, *qnrA1*
40522	Eagle owl (*Bubo bubo*)	Sedentary	30/06/2015	*E. cloacae*	78	$bla_{CTX-M-9}$	IncHI2/ST1	*aadA2b*, *ant*(2″)-*Ia*, *sul1*, *mcr-9*, *qnrS1*, *dfrA15*
40527	Crag martin (*Ptyonoprogne rupestris*)	Long distance (Africa)	30/06/2015	*E. cloacae*	78	$bla_{CTX-M-9}$	IncHI2/ST1	*aadA2b*, *ant*(2″)-*Ia*, *sul1*, *mcr-9*, *qnrS1*, *dfrA15*

**New variant which is a SLV of ST712 (allelic profile: 2/2/gyrB*/133/51/2/14).*

observed (**Table 4**). Moreover, in all eight CTX-M-9-producing *E. coli* and *E. cloacae* of the four birds, NGS demonstrated the presence of the IncHI2/ST1 plasmid replicon and the *mcr-9* gene. Southern blot experiments on these eight isolates confirmed that $bla_{CTX-M-9}$ and *mcr-9* co-localized on the IncHI2/ST1 plasmid, together with the bla_{SHV-12} gene when present. The presence of the *mcr-9* and bla_{SHV-12} genes was further assessed by PCR, revealing that bla_{SHV-12} was present in 99 and *mcr-9* in 118 out of the 123 CTX-M-9-producing isolates. Southern blot experiments performed on a representative sub-set of 21 CTX-M-9/SHV-12/MCR-9 positive *E. coli* and *E. cloacae* proved that these three genes systematically co-localized on an IncHI2/ST1 plasmid.

DISCUSSION

In this study, we report a high prevalence of 24.1% of ESC-R in a large collection of 424 wild birds sampled on arrival in an animal rescue center in France over a 6-month period. However, this high prevalence, moreover in a collection encompassing over 50 different bird species, must be cautiously interpreted. Indeed, when considering the delay between entrance and sampling (from 24 h to 10 days) together with the molecular evidence of a limited number of highly prevalent ESC-R clones colonizing the positive animals, this picture most likely reflects cross transmissions of ESC-R within the rescue center, similarly

to nosocomial spreads observed at hospital. This interpretation is further reinforced by the evidence of ESBL carriage in several mammals kept in this center with the same bacterial lineages as observed in birds. The presence of ESBL-producing Enterobacteriaceae in wild mammals strongly suggests that they can potentially act as environmental vectors of resistance genes and/or resistant bacteria. This also alerts on the importance of separating animals (here mammals and birds) that can share AMR determinants before being released in nature. In all, the present situation does obviously not reflect the true prevalence of ESC-R in birds in the wild, but reveals a particular bacterial flora specific to the rescue center. A plausible hypothesis would be that some birds have been collected as naturally positive ESBL carriers, and that bird-adapted bacterial clones then colonized the environment of the rescue center – including aqueous solutions, sinks, cages or aviaries as shown in hospitals – thus favoring their persistence and dissemination (Lowe et al., 2012; Chapuis et al., 2016). In particular, *E. coli* ST746 had already been reported in birds in France in 2009, possibly suggesting specific adaptive properties (Bonnedahl et al., 2009). ST155 has also been reported in wild birds in many occasions (Hernandez et al., 2013; Alcalá et al., 2016).

The high prevalence of ESC-R *E. cloacae* isolates, often in co-occurrence with ESC-R *E. coli*, was another interesting feature of this study. Even though *E. cloacae* alone or as co-contaminant has been sporadically reported in birds (Giacopello et al., 2016), such a high prevalence was an unprecedented situation. It also most probably results from the intra-center spread of a limited number of ESC-R *E. cloacae* lineages, but this suggests that these lineages may be particularly adapted to the avian hosts. While ST104 and ST135 have only been sporadically reported, ST78 is also considered as a high-risk clone for humans and is a major driver of CP-resistance spread, notably in North America (Izdebski et al., 2015; Annavajhala et al., 2019). Whether this clone had a human source in our study or is also adapted to birds cannot be inferred from our results, and investigations on the microbiota of birds are clearly needed to better understand transmission routes of multi-drug resistant bacteria.

Interestingly, the spread of ESC-R in this rescue center did not only rely on clonal but also on plasmidic transmission. Here, we evidenced the wide dissemination of a single IncHI2/ST1 plasmid bearing *mcr-9*, $bla_{\mathrm{CTX-M-9}}$, and in most cases $bla_{\mathrm{SHV-12}}$. This plasmid was equally found in ESC-R *E. coli* and ESC-R *E. cloacae*, strongly suggesting that plasmid spread within the same bird has occurred as well. Of note, all isolates were phenotypically susceptible to colistin, which is a known feature of *mcr-9*, a gene that was first described in 2019 in *Salmonella enterica* from a human patient in the United States and since then has not been shown to confer phenotypic resistance to colistin (Carroll et al., 2019). The study by Chavda et al. (2019) strongly suggested that *mcr-9* was associated with large InHI2/IncHI2A plasmid, which was then corroborated by two other publications reporting *mcr-9*/$bla_{\mathrm{CTX-M-9}}$/$bla_{\mathrm{VIM-4}}$ in *E. cloacae* from a young patient in the United States and *mcr-9*/$bla_{\mathrm{SHV-12}}$ in several Enterobacteriaceae from horses in Sweden on IncHI2 plasmids (Borjesson et al., 2019). The occurrence of *mcr-9* in both horses and birds may argue for an environmental dissemination and for a large epidemic success of these IncHI2/ST1 plasmids, thanks to plasticity and optimal conjugation properties at low temperatures (around 25°C) (Garcia-Fernandez and Carattoli, 2010). However, since *mcr-9* does not confer phenotypic resistance to colistin, its real role remains to be studied.

In terms of CTX-M epidemiology, the over-dominance of $bla_{\mathrm{CTX-M-9}}$ in ESBL-positive animals was unexpected since $bla_{\mathrm{CTX-M-9}}$ is very rare in France, both in domestic animals and humans (Robin et al., 2017). The $bla_{\mathrm{SHV-12}}$ gene was also detected in a significant proportion of isolates, a gene that also remains infrequent in the current ESBL epidemiology in animals, except in some food-producing birds (broilers) in Europe. Therefore, one could argue that the environmental IncHI2 plasmid carrying $bla_{\mathrm{CTX-M-9}}$, *mcr-9* and $bla_{\mathrm{SHV-12}}$ may have been introduced occasionally by wild animals before displaying an epidemiological success in the rescue center. It is therefore to consider that veterinarians and all people in contact were highly exposed to ESC-R in this setting, which constitutes an open door for further ESBL spread in the community. Moreover, most animals in the rescue center are also intended to get back to nature after a few days or weeks, and this is obviously another issue with regard to ESBL dissemination in natural environments.

CONCLUSION

In conclusion, to our best knowledge, this study highlights for the first time the broad dissemination of both ESC-R plasmid and ESC-R *E. coli* and *E. cloacae* clones in a rescue center for wild animals, mainly birds. It also reveals a probable dynamic transmission of ESBL genes between *E. coli* and *E. cloacae*, which can then further disseminate to the environmental settings, but also ultimately to people in contact and to wildlife once birds are released in nature. The exact source of ESC-R in this center is not easy to clarify. The nature of the ESBL genes found, which are rather rare in domestic animals and humans, may argue for an external reservoir but this remains speculative. Importantly however, this study highlights to what extent such settings at the interface between wildlife and non-wildlife sectors may act as critical points in the amplification of ESC-R prevalence. Even though the epidemic success of the IncHI2 plasmid carrying $bla_{\mathrm{CTX-M-9}}$, *mcr-9* and $bla_{\mathrm{SHV-12}}$ can be highlighted, the causes of such a wide dissemination of ESBL-producing bacteria inside the rescue center also remain unknown and may warrant investigation. Antibiotherapy was not common practice in this center (antibiotics were only prescribed in case of open fracture; M-PP, personal communication) but, as demonstrated in a recent study, a single source, such as the use of contaminated disinfectants, may be sufficient for a large and long-term contamination by resistant bacteria (Keck et al., 2020). Other hypotheses include frequent handling of animals, different birds kept in the same cage, difficulties to disinfect surfaces (often in wood) or inadequate hygiene procedures in often crowded settings, such as animal rescue centers. Since that study, the rescue center has implemented measures and procedures to avoid cross contaminations and control intra-center infections.

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/genbank/, PRJNA659767.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because no ethical approval was needed since this study did not involve any experimentation on animals. Samples used in this study were routine samples taken at the entry of wild birds in the recue center.

AUTHOR CONTRIBUTIONS

NK, MH, and J-YM designed the experiments, analyzed the data, and drafted the manuscript. M-PP performed the sampling campaign. RJ and VM performed the experiments. AD performed the NGS analyses. All authors approved the final version of this 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.2020.601317/full#supplementary-material

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Wildlife as Sentinels of Antimicrobial Resistance in Germany?

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The presence of bacteria carrying antimicrobial resistance (AMR) genes in wildlife is an indicator that resistant bacteria of human or livestock origin are widespread in the environment. In addition, it could represent an additional challenge for human health, since wild animals could act as efficient AMR reservoirs and epidemiological links between human, livestock and natural environments. The aim of this study was to investigate the occurrence and the antibiotic resistance patterns of several bacterial species in certain wild animals in Germany, including wild boars (*Sus scrofa*), roe deer (*Capreolus capreolus*) and wild ducks (family Anatidae, subfamily Anatinae) and geese (family Anatidae, subfamily Anserinae). In the framework of the German National Zoonoses Monitoring Program, samples from hunted wild boars, roe deer and wild ducks and geese were collected nationwide in 2016, 2017, and 2019, respectively. Fecal samples were tested for the presence of *Salmonella* spp. (in wild boars and wild ducks and geese), *Campylobacter* spp. (in roe deer and wild ducks and geese), Shiga toxin-producing *Escherichia* (*E.*) *coli* (STEC), commensal *E. coli* and extended-spectrum beta-lactamase- (ESBL) or ampicillinase class C (AmpC) beta-lactamase-producing *E. coli* (in wild boars, roe deer and wild ducks and geese). In addition, the presence of methicillin-resistant *Staphylococcus aureus* (MRSA) was investigated in nasal swabs from wild boars. Isolates obtained in the accredited regional state laboratories were submitted to the National Reference Laboratories (NRLs) for confirmation, characterization and phenotypic resistance testing using broth microdilution according to CLSI. AMR was assessed according to epidemiological cut-offs provided by EUCAST. *Salmonella* spp. were isolated from 13 of 552 (2.4%) tested wild boar fecal samples, but absent in all 101 samples from wild ducks and geese. Nine of the 11 isolates that were submitted to the NRL Salmonella were susceptible to all tested antimicrobial substances. *Campylobacter* spp. were isolated from four out of 504 (0.8%) roe deer fecal samples, but not from any of the samples from wild ducks and geese. Of the two isolates received in the NRL *Campylobacter*, neither showed resistance to any of the substances tested. From roe deer, 40.2% of the fecal samples (144 of 358) yielded STEC compared to 6.9% (37 of 536) from wild boars. In wild ducks and geese, no STEC isolates were found. Of 150 STEC isolates received in the NRL (24 from wild boars and

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126 from roe deer), only one from each animal species showed resistance. Of the 219 isolates of commensal *E. coli* from wild boars tested for AMR, 210 were susceptible to all 14 tested substances (95.9%). In roe deer this proportion was even higher (263 of 269, 97.8%), whereas in wild ducks and geese this proportion was lower (41 of 49, 83.7%). Nevertheless, selective isolation of ESBL-/AmpC-producing *E. coli* yielded 6.5% (36 of 551) positive samples from wild boars, 2.3% (13 of 573) from roe deer and 9.8% (10 of 102) from wild ducks and geese. Among the 25 confirmed ESBL-/AmpC-producing isolates from wild boars, 14 (56.0%) showed resistance up to five classes of substances. This proportion was lower in roe deer (3 of 12, 25%) and higher in wild ducks and geese (7 of 10, 70%). None of the 577 nasal swabs from wild boars yielded MRSA. Results indicate that overall, the prevalence of resistant bacteria from certain wild animals in Germany is low, which may reflect not only the low level of exposure to antimicrobials but also the low level of resistant bacteria in the areas where these animals live and feed. However, despite this low prevalence, the patterns observed in bacteria from the wild animals included in this study are an indicator for specific resistance traits in the environment, including those to highest priority substances such as 3rd generation cephalosporins, fluoroquinolones and colistin. Therefore, also continuous monitoring of the occurrence of such bacteria in wildlife by selective isolation is advisable. Furthermore, the possible role of wildlife as reservoir and disperser of resistant bacteria would need to be assessed, as wild animals, and in particular wild ducks and geese could become spreaders of resistant bacteria given their capacity for long-range movements.

Keywords: monitoring, one health, zoonotic agents, antimicrobial resistance (AMR), wild boar, cervids, wild bird, Germany

INTRODUCTION

The presence of bacteria carrying antimicrobial resistance (AMR) genes is an increasingly serious and complex threat affecting public health worldwide (1). This implies that all underlying economic, social, political, environmental, and biological factors have to be considered in this context (2). Nowadays intensive contact between humans, domestic and wild animals occurs due to the expansion of urban populations and the fragmentation, encroachment and loss of natural habitats. In this scenario, it is of utmost importance to examine AMR through a "One Health" perspective (3–5). This perspective contemplates an integrated and holistic multidisciplinary approach (6), highlighting the importance of a better integration of human, livestock, wildlife and environmental aspects, in order to identify key priorities for combating AMR (2, 5, 7).

Even though wild animals are unlikely of being treated with antibiotics, the overlap between habitats inevitably increases the transmission of resistant bacteria between the different niches (8). Some wild species have been used as bioindicators or sentinels for the spread of resistant bacteria in the environment (9–11). Inadequately treated waste from humans and livestock animals treated with antimicrobial substances promotes the spread of resistant bacteria from animal stables and waste water treatment plants to the environment (12–14), and therefore to the wild fauna. However, despite the fact that many studies affirm that wild animals are reservoirs and dispersers of AMR, this role is less well-established. To make this statement, more in-depth epidemiological analyzes are needed, as the mere fact of being carriers of AMR does not mean that they can be a vehicle of contagion for humans or other animals (15, 16). In consequence, it becomes important to study the presence of AMR genes in wildlife and consider the role of wild animals in the dynamics of AMR (15), as they could represent a major epidemiological link between natural and humanized environments (15, 16). Roe deer (*Capreolus capreolus*) and wild boar (*Sus scrofa*) are the most frequent and widespread wild ungulates in Germany (17), with an estimated number of around 2.4 million individuals of roe deer and one million of wild boars, which represents 24 and 25% of the total European wild boar and roe deer population, respectively (18). As an ecologically adaptable species, both can be found in a wide variety of habitats from natural ones like forests or pastures, to more anthropogenic areas like agricultural landscapes and even urban or peri-urban areas (18, 19). Therefore, they might be prone to have contact to humans and livestock directly (20), as well as indirectly via garbage and sewage. On the other hand, some wild bird populations, including wild ducks and geese belonging to different species within the Anatidae family and the Anatinae and Anserinae subfamilies, have experienced extraordinary growth in the last decades in Germany (21, 22). Among other reasons, this is due to milder winter conditions (21). It is therefore not unusual nowadays to find large groups of wild ducks and geese in crops producing food and feed, or on wetlands and lakes used as source of drinking water for humans

and livestock, or for aquatics (23, 24). Due to their capacity for long-range movements, wild birds like ducks and geese are potential spreaders of bacteria with AMR genes beyond borders (16, 25–27).

Previous studies have demonstrated the presence of AMR and resistance genes in bacteria from a large variety of wildlife species throughout Europe (28–30), including resistances to those substances of highest priority like 3rd generation cephalosporins, fluoroquinolones, colistin or even carbapenems (31, 32).

To the best of our knowledge, in Germany, the availability of studies regarding the presence of resistant bacteria in wild animals is scarce and mostly limited to certain regions (33–35). This makes that the role of wild animals in the dynamics of AMR in Germany is still not fully understood. Based on previous studies it is clear that the presence of distinct bacterial species, their antimicrobial susceptibility, as well as their profiles of resistance genes might be highly variable among different countries (19). Therefore, the aim of the present study was to investigate the occurrence and the antibiotic resistance patterns of *Salmonella* spp., *Campylobacter* spp., Shiga toxin-producing *Escherichia* (*E.*) *coli* (STEC), methicillin-resistant *Staphylococcus aureus* (MRSA), commensal *E. coli*, and extended-spectrum beta-lactamase- (ESBL) or ampicillin class C (AmpC) beta-lactamase-producing *E. coli* in samples collected from wild boars, roe deer and wild ducks and geese in Germany within the National Zoonoses Monitoring Program.

MATERIALS AND METHODS

In the framework of the German National Zoonoses Monitoring Program, 942 samples from hunted wild boars, 573 from roe deer and 100 from wild ducks and geese were collected nationwide in 2016, 2017, and 2019, respectively. Samples from wild ducks and geese mainly originated from cadavers collected for the monitoring of avian influenza, or taken from hunted birds. Fecal samples were tested for the presence of *Salmonella* spp. (in wild boars and wild ducks and geese), *Campylobacter* spp. (in roe deer and wild ducks and geese), STEC, commensal *E. coli*, and ESBL-/AmpC-producing *E. coli* (in wild boars, roe deer and wild ducks and geese) (**Table 1**). In addition, the presence of MRSA was investigated in nasal swabs from wild boars. No sample size was specified for each federal state, as the investigations took place depending on the availability of suitable samples. Samples were provided from all federal states except Hamburg and Bremen.

Primary isolation was carried out by the accredited regional state laboratories using harmonized procedures (**Table 2**). Results of the analysis of samples were reported to the Federal Office of Consumer Protection and Food Safety (BVL) for aggregation and reporting at national level. Isolates obtained were submitted to the National Reference Laboratories (NRLs) at the German Federal Institute for Risk Assessment (BfR) for confirmation, characterization and phenotypic resistance testing.

Isolates from *Salmonella* spp., *Campylobacter* spp., STEC, *E. coli*, and MRSA were confirmed and characterized using the designated, internationally recognized procedures (**Table 2**). For the determination of resistance, broth microdilution method according to CLSI M07-A10 and CLSI M45-A was used (42, 43).

The isolates were subjected to the examination spectrum of antimicrobial substances established at BfR. For this purpose, the ready-made plate formats EUVSEC and EUVSEC2 (*Salmonella* spp. and *E. coli*), EUCAMP2 (*Campylobacter* spp.), and EUST (MRSA) from the company TREK Diagnostic Systems were used (44).

AMR was assessed according to epidemiological cut-offs provided by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and fixed in Commission Implementing Decision 2013/652/EC (45). Technical specifications proposed by EFSA (40) were applied for MRSA.When no epidemiological cut-off values were described, the evaluation was carried out based on EFSA criteria (41). Isolates from the wild-type population in this publication are further called susceptible to the respective agent, those with MIC values above the cut-off resistant. An overview of the antimicrobial substances used, the tested concentration ranges as well as the evaluation criteria can be found in **Supplementary Tables 1–3**.

Prevalence of the zoonotic pathogens in the fecal samples from wild animals as well as the prevalence of resistant bacteria within the isolates were calculated as the proportion of positive samples resp. resistant isolates and with the associated 95% confidence interval shown. The 95% confidence interval was calculated according to the procedure determined by Agresti and Coull (46).

Escherichia coli isolates resistant to third generation cephalosporins were further characterized in regard of the harbored ESBL/pAmpC genes. Therefore, isolates were pre-screened by real-time PCR for the presence of the typical betalactamases TEM, CTX, SHV, and CMY (47). ESBL variant was then determined by Sanger sequencing of PCR products. TEM variant was only determined in case no other ESBL/pAmpC gene was detected, as most *E. coli* harbor the narrow spectrum beta-lactamase $bla_{\mathrm{TEM-1}}$. Isolates which were negative in real-time PCR were additionally screened for the presence of bla_{FOX}, bla_{MOX}, bla_{CIT}, bla_{DHA}, and bla_{EBC} by PCR. As some betalactamase variants differ within the primer regions, we could not distinguish between CTX-M-14 and −17 (CTX-M-14 like), between CTX-M-65 and 90 (CTX-M-65-like), and between CMY-2/-22 and -66 (CMY-2-like).

RESULTS

Salmonella spp.

Salmonella spp. were isolated from 13 of 552 (2.4%) wild boar fecal samples (**Table 3**).

Of the 13 isolates found in fecal samples from wild boars, 11 were submitted to the BfR. Serotyping of these isolates resulted in three *Salmonella* Enteritidis, one *Salmonella* Typhimurium, one *Salmonella* Stanleyville, and six *Salmonella enterica* subspecies I., that could not be further identified by serotyping.

Of the 11 isolates, nine (81.8%) were susceptible to all tested substances (**Table 4**). Just two isolates (18.2%) showed resistance

TABLE 1 | Overview of prevalence and resistance studies carried out for wildlife in the German Zoonoses-Monitoring in 2016, 2017, and 2019.

Year	Animal	Matrix	*Salmonella* spp.	*Campylobacter* spp.	STEC	MRSA	Commensal *E. coli*	ESBL-/AmpC-producing *E. coli*
2016	Wild boar	Feces	X		X		X	X
2016	Wild boar	Nasal swabs				X		
2017	Roe deer	Feces		X	X		X	X
2019	Wild ducks and geese	Feces	X	X	X		X	X

TABLE 2 | Microbiological methods used in the investigation according to microorganism and survey year.

Microorganism	Year	Primary isolation	Confirmation and further typing
Salmonella spp.	2016 2019	ISO 6579:2002 ISO 6579-1:2017	ISO 6579:2002 ISO 6579-1:2017 Serotyping according to the White-Kauffmann-Le Minor scheme (36)
Campylobacter spp.	2017 2019	ISO 10272-1:2006 ISO 10272-1:2017	ASU §64 LFGB, L00.06-32 2013-08
STEC	2016 2017 2019	Suggested methods dependent on the matrix: - ISO/TS 13136:2012 and ISO based method in 2016 - ASU §64 LFGB, L00.00-92 2006-12 - ASU §64 LFGB L07.18-1 2002-05 - Real-time PCR systems for the detection of the Shiga toxin genes *stx1* and *stx2* and the intimin gene eae in 2016 and 2017	Confirmation and typing for virulence genes as described by Tzschoppe et al. (37). Molecular H-typing according to Beutin et al. (38). Verotoxin ELISA (RIDASCREEN Verotoxin enzyme immunoassay #C2201, R-biopharm, Germany) according to the manufacturer
	2019	Suggested methods: - DIN 10118 "Microbiological examination of food—Detection of verotoxins in food of animal origin with an immunological test system" - Protocol for the qualitative detection and isolation of shigatoxin-producing *E. coli* (STEC) - Detection of *E. coli* producing the Stx2f subtype by Real-Time PCR (EU-RL VTEC: Laboratory methods for VTEC detection and typing (https://www.iss.it/documents/20126/1049000/EU_RL_VTEC_Method_10_Rev_0.pdf)	
MRSA	2016 2017 2019	Recommended method of the National Reference Laboratory for staphylococci including *S. aureus* at the Federal Institute for Risk Assessment (39)	In-house multiplex PCR test (39) and broth microdilution method according to CLSI M07-A10 and classification according to EFSA (40)
ESBL-/AmpC-producing *E. coli*	2016 2017 2019	EURL laboratory protocol for the Isolation of ESBL-, AmpC-, and carbapenemase-producing *E. coli* from caecal samples Version 3 in 2016 and 2017 EURL laboratory protocol for the Isolation of ESBL-, AmpC-, and carbapenemase-producing *E. coli* from caecal samples Version 6	Broth microdilution method according to CLSI M07-A10 and classification according to 2013/652/EU and EFSA (41)
Commensal *E. coli*	2016 2017 2019	No specific standardized method is prescribed. It is just recommended to plate a small amount of feces directly on a suitable medium. Confirmation with in-house method.	Cultivation on ENDO-Agar (Thermo Scientific, Germany)

to two or three groups of active ingredients (**Figure 1**), including fluoroquinolones and colistin (**Table 5**).

Salmonella spp. were not found in any of the 101 samples from wild ducks and geese (**Table 3**).

Campylobacter spp.

Campylobacter spp. were isolated from four out of 504 (0.8%) fecal samples from hunted roe deer (**Table 3**). Three isolates were sent to the BfR, but one of them could not be re-cultivated. Of the two remaining isolates (both *Campylobacter jejuni*), neither showed resistance to any of the six substances tested (**Table 4**).

Campylobacter spp. were absent in the 93 fecal samples from wild ducks and geese (**Table 3**).

STEC

Out of 536 fecal samples tested from wild boars, 37 yielded STEC (6.9%) (**Table 3**). In total, 24 STEC isolates were sent to the BfR for further typing and resistance testing. The results of the STEC typing from wild boars are available in **Supplementary Table 4**. From those isolates, three did not produce measurable Shiga toxin. With the exception of one isolate, all isolates had a *stx2* gene; meanwhile just five isolates carried a *stx1* gene. One isolate could not be typed with regard to its O antigen, but

TABLE 3 | Overview of the examined samples and the prevalence and 95% confidence interval (95% CI) of different microorganisms in feces samples (*Salmonella* spp., *Campylobacter* spp., STEC, commensal *E. coli*, and ESBL-/AmpC-producing *E. coli*) and nasal swabs (MRSA) from wild boar, roe deer and wild ducks and geese in 2016, 2017 and 2019, respectively.

	Wild boars (2016)			Roe deer (2017)			Wild ducks and geese (2019)		
	Examined samples	Positive samples	Prevalence (in %) (95% CI)	Examined samples	Positive samples	Prevalence (in %) (95% CI)	Examined samples	Positive samples	Prevalence (in %) (95% CI)
Salmonella spp.	552	13	2.4 (1.3–4.0)				101	0	0.0 (0.0–4.4)
Campylobacter spp.				504	4	0.8 (0.2–2.1)	93	0	0.0 (0.0–4.8)
STEC	536	37	6.9 (5.0–9.4)	358	144	40.2 (35.3–45.4)	95	0	0.0 (0.0–4.7)
MRSA	577	5*	0.0 (0.0–0.8)						
Commensal *E. coli*	538	511	95.0 (92.8–96.6)	573	537	93.7 (91.4–95.4)	102	51	50.0 (40.5–59.5)
ESBL-/AmpC-producing *E. coli*	551	36	6.5 (4.7–8.9)	573	13	2.3 (1.3–3.9)	102	10	9.8 (5.2–17.3)
Total	942			573			100		

**Isolates not confirmed in the reference laboratory.*

TABLE 4 | Overview of the isolates for which a resistance test was carried out and prevalence and 95% confidence interval (95% CI) of resistant isolates.

	Wild boars (2016)			Roe deer (2017)			Wild ducks and geese (2019)		
	Total isolates	Resistant isolates	Prevalence (in %) (95% CI)	Total isolates	Resistant isolates	Prevalence (in %) (95% CI)	Total isolates	Resistant isolates	Prevalence (in %) (95% CI)
Salmonella spp.	11	2	18.2 (4.0–48.8)				0		
Campylobacter spp.				2	0	0.0 (0.0–71.0)	0		
STEC	24	1	4.2 (0.0–21.9)	126	1	0.8 (0.0–4.8)	0		
MRSA	0								
Commensal *E. coli*	219	9	4.1 (2.1–7.7)	269	6	2.2 (0.9–4.9)	49	8	16.3 (8.2–29.3)
ESBL-/AmpC-producing *E. coli*	25	25	100.0 (84.2–100.0)	12	12	100.0 (71.8–100.0)	10	10	100.0 (67.9–100.0)

was serologically rough. The rest of the isolates belonged to 14 different O groups, including the O157 group. The two isolates belonging to this group had both also the H7 antigen and the genes *eae* and *ehxA*, which code for virulence factors. The *eae* gene was also detected in isolates from serogroups O26 and O45. These isolates also carried the *ehxA* gene. The *eae* gene was not found in any other serogroup. The *ehxA* gene was detected in 15 isolates (62.5%).

From the 24 STEC isolates from wild boars tested for resistance, all were completely susceptible except one (95.8%). This isolate showed resistance to six substance classes (**Figure 1**), including the (fluoro-)quinolone nalidixic acid and ciprofloxacin (**Table 5**).

From roe deer, 40.2% (144 of 358) of the fecal samples yielded STEC. One hundred twenty-six STEC isolates from the feces of hunted deer were submitted to the BfR. The results of the STEC typing from roe deer are available in **Supplementary Table 5**. Twenty-five of these 126 isolates did not produce measurable Shiga toxin with the ELISA system used. Most of the isolates had a *stx2* gene ($n = 92$) and 40 isolates carried a *stx1* gene. One hundred fifteen isolates belonged to 19 different O serogroups, and 11 could not be typed. Of the serogroups, O146 was most frequently represented, meanwhile the serogroup O157 was not detected in any of the analyzed isolates. The *eae* gene occurred in one isolate of the serogroup O26. This isolate also carried the *ehxA* gene. The *ehxA* gene was detected in 54 isolates.

Of the 126 STEC isolates tested for resistance, only one (0.8%) showed resistance to gentamicin. As shown in **Figure 1** and **Table 6**, all the other isolates were without exception susceptible to all tested substances.

In wild ducks and geese, no STEC isolates were found.

Commensal *E. coli*

Commensal *E. coli* were isolated from 95% (511 of 538) of the fecal samples from wild boars. Of the 219 isolates of *E. coli* from wild boars tested for AMR, 210 (95.9%) were susceptible to all 14 tested substances (**Table 4**). Among the nine other isolates, seven showed resistance only to one substance class. The other two isolates showed resistance to two or four classes (**Figure 1**). No resistance to 3rd generation cephalosporins or carbapenems was found, but some isolates were resistant to ciprofloxacin and nalidixic acid (0.9% each) and four isolates (1.8%) showed resistance to colistin (**Table 5**).

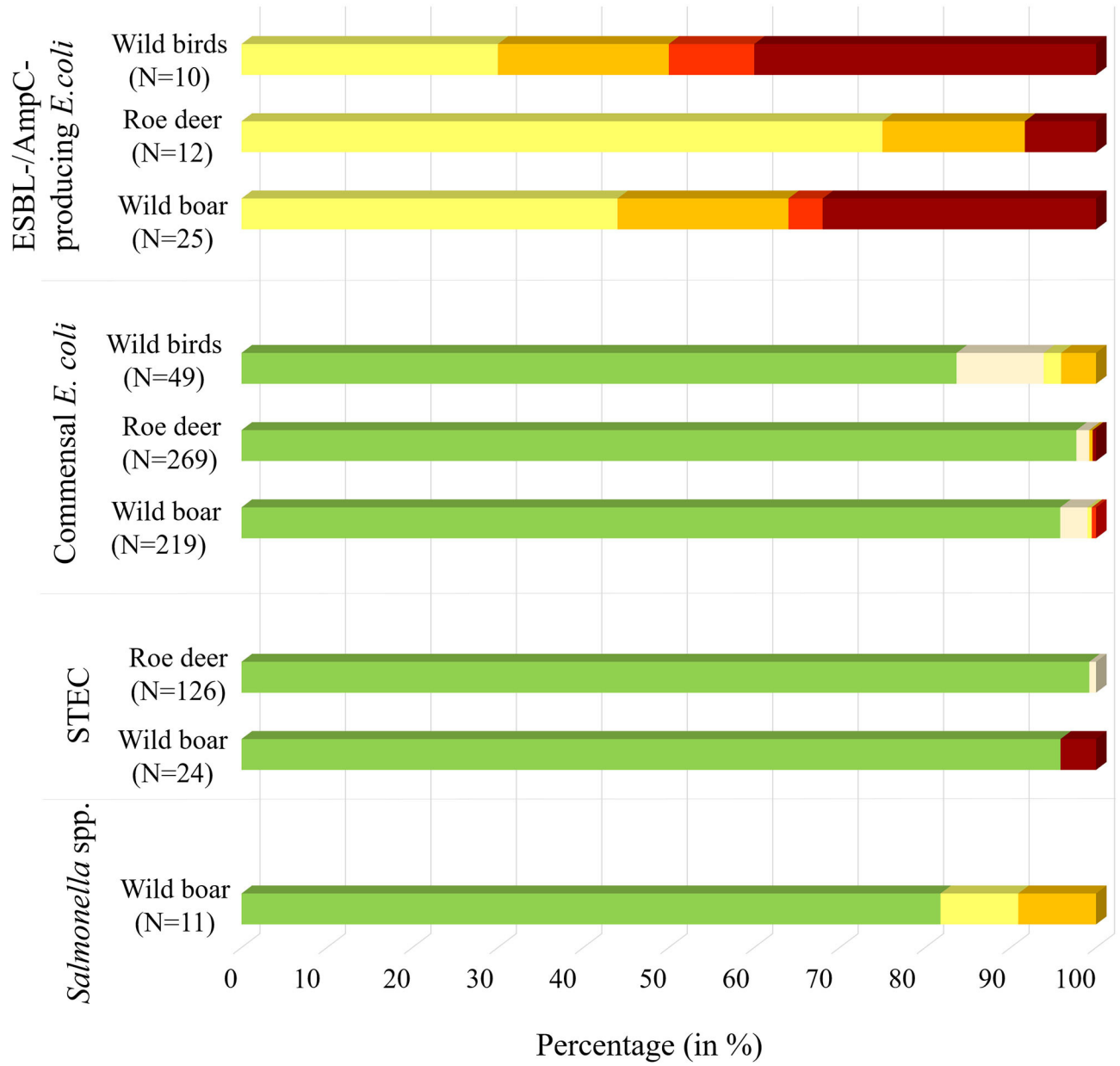

FIGURE 1 | Overview of the isolates found in wild boar, roe deer (excluding *Campylobacter* spp.) and wild ducks and geese, including information on the percentage of samples that were susceptible to al testes substances or resistant to one (1x res.), two (2x res.), three (3x res.), four (4x res.) or more than four classes (> 4x res.) of antibiotic substances.

A total of 93.7% (537 of 573) of the fecal samples from hunted roe deer yielded commensal *E. coli*. Among the 269 isolates, 263 (97.8%) were susceptible to all tested substances, while six (2.2%) displayed resistance to at least one of the tested antimicrobials (**Table 4**). Four of these isolates were resistant to only one substance class and two isolates were resistant to three, resp. five substance classes (**Figure 1**). Resistance to the 3rd generation cephalosporins (cefotaxime and ceftazidime) and to the fluoroquinolone ciprofloxacin were observed (0.4% of the isolates each) (**Table 6**). No colistin or meropenem resistant *E. coli* were observed in isolates from roe deer.

In wild ducks and geese, 50% (51 of 102) of the fecal samples yielded commensal *E. coli*. Of the 49 isolates submitted to the BfR, 41 (83.7%) were sensitive to all tested substances (**Table 7**). Only

TABLE 5 | Number and proportion of tested resistant isolates from wild boars and the number of substance classes to which the isolates were resistant.

	Salmonella spp.		STEC		Commensal *E. coli*		ESBL-/AmpC-producing *E. coli*	
	N	%	N	%	N	%	N	%
No. samples	**11**		**24**		**219**		**25**	
Gentamicin	0	0.0	1.0	4.2	0	0.0	5	20.0
Chloramphenicol	1	9.1	1.0	4.2	2	0.9	4	16.0
Cefotaxime	0	0.0	0.0	0.0	0	0.0	25	100
Ceftazidime	0	0.0	0.0	0.0	0	0.0	24	96.0
Nalidixic acid	1	9.1	1.0	4.2	2	0.9	4	16.0
Ciprofloxacin	1	9.1	1.0	4.2	2	0.9	8	32.0
Ampicillin	1	9.1	1.0	4.2	1	0.5	25	100
Colistin	1	9.1	0.0	0.0	4	1.8	0	0.0
Sulfamethoxazole	0	0.0	1.0	4.2	2	0.9	7	28.0
Trimethoprim	0	0.0	1.0	4.2	2	0.9	6	24.0
Tetracycline	1	9.1	1.0	4.2	1	0.5	9	36.0
Azithromycin	0	0.0	0.0	0.0	0	0.0	2	8.0
Meropenem	0	0.0	0.0	0.0	0	0.0	0	0.0
Tigecycline	0	0.0	0.0	0.0	0	0.0	0	0.0
Susceptible	9	81.8	23.0	95.8	210	95.9	0	0.0
1x resistant	0	0.0	0.0	0.0	7	3.2	0	0.0
2x resistant	1	9.1	0.0	0.0	1	0.5	11	44.0
3x resistant	1	9.1	0.0	0.0	0	0.0	5	20.0
4x resistant	0	0.0	0.0	0.0	1	0.5	1	4.0
>4x resistant	0	0.0	1.0	4.2	0	0.0	8	32.0

TABLE 6 | Number and proportion of tested resistant isolates from roe deer and the number of substance classes to which the isolates were resistant.

	STEC		Commensal *E. coli*		ESBL-/AmpC-producing *E. coli*	
	N	%	N	%	N	%
No. samples	**126**		**269**		**12**	
Gentamicin	1	0.8	0	0.0	1	8.3
Chloramphenicol	0	0.0	0	0.0	0	0.0
Cefotaxime	0	0.0	1	0.4	12	100.0
Ceftazidime	0	0.0	1	0.4	12	100.0
Nalidixic acid	0	0.0	0	0.0	0	0.0
Ciprofloxacin	0	0.0	1	0.4	1	8.3
Ampicillin	0	0.0	4	1.5	12	100.0
Colistin	0	0.0	0	0.0	0	0.0
Sulfamethoxazole	0	0.0	3	1.1	2	16.7
Trimethoprim	0	0.0	2	0.7	2	16.7
Tetracycline	0	0.0	2	0.7	1	8.3
Azithromycin	0	0.0	1	0.4	0	0.0
Meropenem	0	0.0	0	0.0	0	0.0
Tigecycline	0	0.0	0	0.0	0	0.0
Susceptible	125	99.2	263	97.8	0	0.0
1x resistant	1	0.8	4	1.5	0	0.0
2x resistant	0	0.0	0	0.0	9	75.0
3x resistant	0	0.0	1	0.4	2	16.7
4x resistant	0	0.0	0	0.0	0	0.0
>4x resistant	0	0.0	1	0.4	1	8.3

two isolates (4.1%) were resistant to two resp. three substance classes (**Figure 1**). Among the resistant isolates, resistance to 3rd generation cephalosporins, fluoroquinolones and colistin was observed in 2% of the isolates each (**Table 7**).

ESBL-/AmpC-Producing *E. coli*

Selective isolation yielded isolates suspicious of being ESBL-/AmpC-producing *E. coli* in 6.5% (36 of 551) of the samples from wild boars (**Table 3**). Of the 25 isolates confirmed at the BfR, 23 showed an ESBL and two an AmpC phenotype. Among these isolates, that were resistant to cefotaxime, ceftazidime and ampicillin, 11 (44%) showed no other resistance, while 14 (56%) showed resistance to up to five further substance classes (**Figure 1** and **Table 5**). Apart from colistin, tigecycline and meropenem, resistance was observed to all other substances in at least one isolate. Nine isolates (36%) were resistant to tetracycline and eight isolates (32%) to ciprofloxacin. Wild boars showed the highest diversity of resistance determinants among the three groups (**Figure 2**). The most prevalent ESBL gene was $bla_{\mathrm{CTX-M-1}}$ (56%), followed by $bla_{\mathrm{CTX-M-15}}$ (20%) and $bla_{\mathrm{CTX-M-14}}$-like (12%). One of the isolates with an AmpC phenotype harbored a $bla_{\mathrm{CMY-2}}$ like gene, whereas the other one did not harbor one of the genes screened for.

ESBL-/AmpC-producing *E. coli* were detected in 13 of the 573 (2.3%) fecal samples from hunted roe deer (**Table 3**). Of the twelve isolates submitted to the BfR, phenotypically three showed an AmpC and nine and ESBL phenotype. Nine of those twelve isolates (75%) showed only resistance to cefotaxime, ceftazidime and ampicillin. Three isolates (25%) showed additional resistances to trimethoprim, sulfamethoxazole, gentamicin, ciprofloxacin or tetracycline (**Figure 1** and **Table 6**). No resistance was observed to chloramphenicol, colistin, meropenem and tigecycline. There was a similar distribution of isolates harboring the ESBL genes $bla_{\mathrm{CTX-M-1}}$ (33%) and $bla_{\mathrm{CTX-M-15}}$ (25%) and AmpC-producing isolates (25%). As none of the most prevalent pAmpC genes could be detected, an overexpression of chromosomal AmpC was assumed but not further characterized. The remaining two isolates harbored the $bla_{\mathrm{CTX-M-14}}$ gene.

In samples from wild ducks and geese, ESBL-/AmpC-producing *E. coli* were isolated from ten of the 102 (9.8%) fecal samples (**Table 3**). Of the ten isolates submitted to the BfR, eight showed an ESBL phenotype, one an AmpC phenotype, while another one exhibited ESBL and AmpC phenotype. Among the ten confirmed ESBL-/AmpC-producing *E. coli* isolates submitted to the BfR with resistance to cefotaxime, ceftazidime and ampicillin, seven (70%) showed additional resistance up to five classes of substances, including nalidixic acid and ciprofloxacin (in 50% of isolates each). Resistance to colistin or meropenem was not observed (**Table 7**). The most prevalent ESBL was

TABLE 7 | Number and proportion of tested resistant isolates from wild ducks and geese and the number of substance classes to which the isolates were resistant.

	Commensal *E. coli*		ESBL-/AmpC-producing *E. coli*	
	N	%	N	%
No. samples	**49**		**10**	
Gentamicin	1	2.0	2	20.0
Chloramphenicol	0	0.0	0	0.0
Cefotaxime	1	2.0	10	100.0
Ceftazidime	1	2.0	10	100.0
Nalidixic acid	1	2.0	5	50.0
Ciprofloxacin	1	2.0	5	50.0
Ampicillin	5	10.2	10	100.0
Colistin	1	2.0	0	0.0
Sulfamethoxazole	3	6.1	6	60.0
Trimethoprim	1	2.0	2	20.0
Tetracycline	1	2.0	2	20.0
Azithromycin	0	0.0	1	10.0
Meropenem	0	0.0	0	0.0
Tigecycline	0	0.0	0	0.0
Susceptible	41	83.7	0	0.0
1x resistant	5	10.2	0	0.0
2x resistant	1	2.0	3	30.0
3x resistant	2	4.1	2	20.0
4x resistant	0	0.0	1	10.0
>4x resistant	0	0.0	4	40.0

again CTX-M-1 (60%). CTX-M-15 was produced by 30% of the isolates, including the one which showed an ESBL and AmpC phenotype and produced an additional DHA betalactamase. The isolate with the AmpC phenotype alone only harbored a $bla_{\text{TEM}-1}$, indicating an additional resistance mechanism which wasn't detected so far.

MRSA

From the 577 nasal swab samples from wild boars tested, five isolates were found suspicious of being MRSA. However, none of them could be confirmed as MRSA at the BfR (**Table 3**).

DISCUSSION

The examination of the fecal samples from wild animals included in this study revealed low levels of the important zoonotic pathogens *Salmonella* spp., *Campylobacter* spp., and MRSA. In contrast, STEC were frequently found in roe deer (40.2%), but infrequently in wild boars and were absent in wild ducks and geese. The antibiotic resistance patterns found in this study indicate that overall, the prevalence of AMR is low in bacteria from the studied wild animals in Germany. This might reflect not only the low level of exposure of these wildlife species to antimicrobials but also the low level of resistant bacteria in the areas where these animals live and feed (16). These good results could be also interpreted as an indication of the low level of anthropogenic impact in these areas, or of an adequate management of antibiotic residues of human or livestock origin in Germany. However, this interpretation should be done with caution, since this study has also shown that wild boars, roe deer and wild ducks and geese are carriers of bacteria with specific resistance traits including colistin, fluoroquinolones or 3rd generation cephalosporins. These substances are considered highest priority critically important antimicrobials by the World Health Organization (48). The origin of these isolates is not known, but due to the lifestyle of the wild animals tested, uptake of the resistant bacteria via feed or drinking water, or through direct contact with garbage and sewages, is a likely reason for carriage (49). Other factors than geographic distance to humans, livestock or wastes should be considered in future studies (50), as it has been demonstrated that wildlife populations living in remote places with little direct human or livestock contact can also harbor resistant bacteria (51). The possible role of wildlife as reservoir and disperser of resistant bacteria in Germany would need to be further assessed by including adequate epidemiological analysis, as wild animals, and in particular, wild ducks and geese could become spreaders of resistant bacteria given their capacity for long-range movements. Samples included in this study were distributed across the federal states of Germany. Two federal states did not participate in sampling. Both are city-states with only small hunting areas. One federal state took several times the required number of samples in wild boars and in roe deer. The impact of these additional samples on the overall prevalence estimates was considered minimal for the pathogens studied, as the prevalences recorded in this federal state were similar to those obtained without the inclusion of its samples (data not shown). The total number of samples from wild ducks and geese was low. Therefore, the obtained results should be interpreted with caution, and future studies including a higher number of samples, should be carried out to verify that the results obtained in this study can be extrapolated to the general population of wild ducks and geese in Germany.

As available studies have shown that the prevalence of bacteria and the results of the antimicrobial sensitivity analysis could be highly variable among different geographical locations (19), further analyses with respect to regional distribution and genetic traits need to be carried out to examine potential regional hot spots of AMR in wildlife in Germany.

Our results showed that even when *Salmonella* spp. were found in fecal samples from wild boars hunted in Germany, the prevalence is low. This is in accordance with previous reports from Spain, Portugal and Italy that likewise found low prevalence of *Salmonella* isolates from wild boar feces (52–55). However, substantially higher *Salmonella* prevalences have been be found in serum samples, tonsils or lymph nodes (31, 54, 55), or in animals co-habiting with livestock (56). *Salmonella* Enteritidis was the most frequent serotype, which agrees with previous investigations, which also detected *Salmonella* Enteritidis in wild boars (57). However, serovar *Salmonella* Choleraesuis that has been found increasingly in recent years in diseased wild boars in Germany (34, 35) was not detected in our study. A greater diversity of serotypes was recognized in Spain by Navarro-Gonzalez et al. (56) and Gil Molino et al. (55).

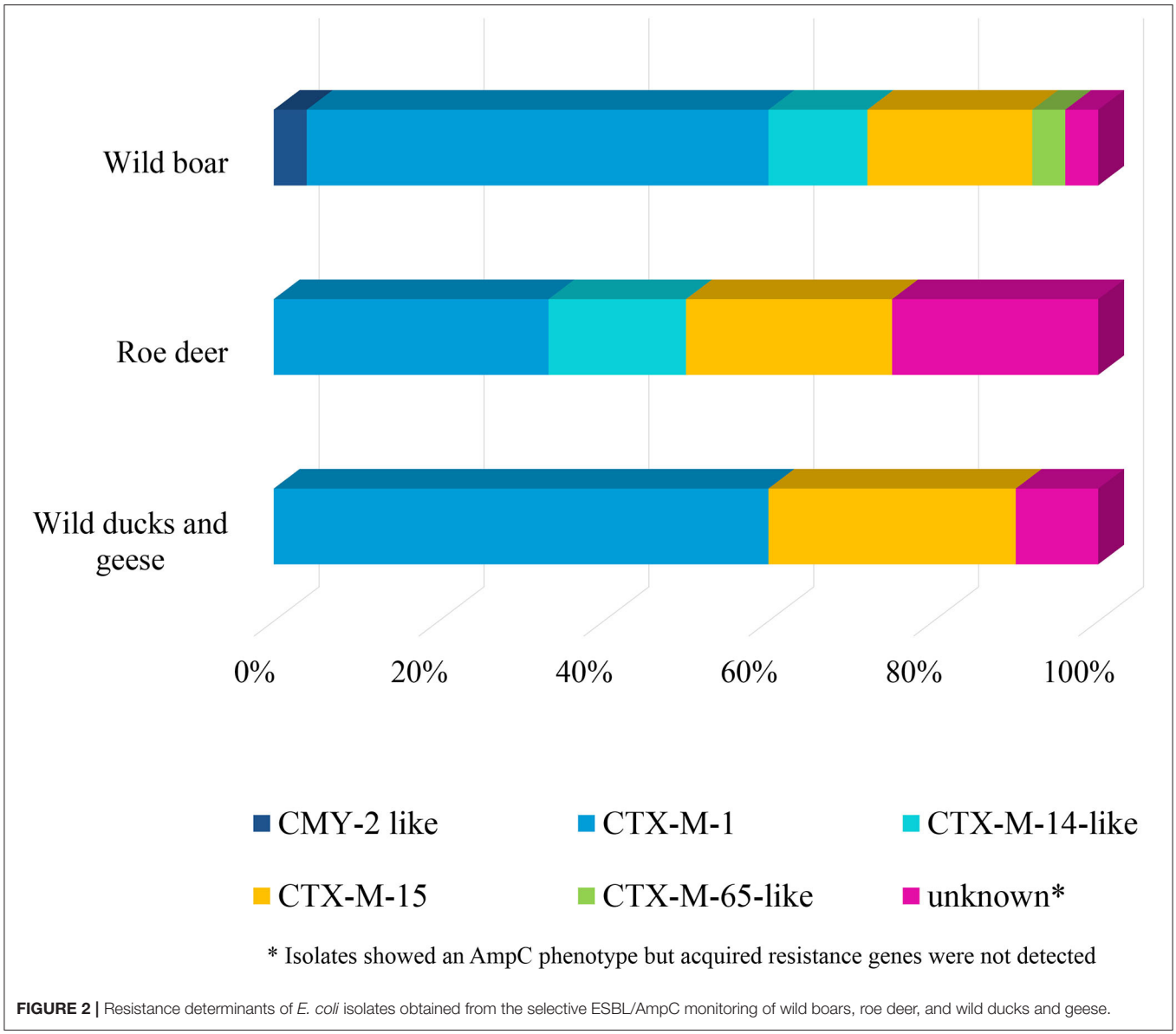

FIGURE 2 | Resistance determinants of *E. coli* isolates obtained from the selective ESBL/AmpC monitoring of wild boars, roe deer, and wild ducks and geese.

As Navarro-Gonzalez et al. (56), we found low resistance rates in the *Salmonella* isolates submitted for testing, with the vast majority of the isolates from wild boars being sensitive to all substances. This differs from previous studies that found higher resistance patterns with almost all isolates resistant to at least one antimicrobial substance (31, 35, 55). Despite the high proportion of fully susceptible isolates found in our study, resistance to ciprofloxacin and colistin were found in one *Salmonella* Enteritidis isolate each in agreement with previous studies (31).

The absence of *Salmonella* spp. in wild ducks and geese is in agreement with previous studies, where predominantly negative results or very low prevalence of *Salmonella* spp. in wild birds has been observed (27, 58–61). Therefore, as other authors hypothesized, the importance of wild birds in spreading *Salmonella* could be limited to those residing in areas that are highly contaminated by human waste or domestic animal manure (60, 61).

In our study, *Campylobacter* spp. were rarely found in roe deer feces. This is consistent with previous studies that suggest that wild cervids, and in particular roe deer, are of limited importance as *Campylobacter* reservoirs (28, 62–64). Although several authors have isolated *Campylobacter* spp. from wild deer, the number of studies that include their resistance profiles is still very limited. Carbonero et al. (65) reported more than 60% of the isolates from roe deer resistant to at least one antimicrobial substance, including streptomycin, tetracycline and ciprofloxacin. In our study, the two *Campylobacter jejuni* submitted to the BfR were susceptible to all tested substances.

Despite the fact that the intestinal tract of wild birds is considered a favorable environment for *Campylobacter* colonization, with reported prevalence ranging from 9.2 to 52.2%

in wild ducks and geese (66, 67), *Campylobacter* spp. were absent in fecal samples from wild ducks and geese analyzed in this study. This absence could be due to loss of *Campylobacter* survival due to extreme temperatures, low water content, or ultra-violet light levels to which fecal content of bird cadavers sampled in this study were subjected.

Prevalence of STEC reported in wildlife in Europe shows a general pattern with a lower prevalence in wild boars (4.8–9%) than in deer (25–42%) (68–72). This is in line with our results. The isolates from wild boars and roe deer submitted to the BfR showed considerable diversity. The most prevalent Shiga toxin gene was *stx2*, whereas *stx1* was detected only in 40 isolates from roe deer and 5 from wild boars. This is also in concordance with previous studies carried out in Europe, which reported higher prevalence of *stx2* than of *stx1* among STEC isolates from wild ungulates (64, 69, 70, 72, 73). Our data reinforce the role of certain wild species as reservoirs of STEC strains that are potentially pathogenic to humans, as two isolates found in wild boars were described as *E. coli* O157:H7 (0.37%). Although there are studies in which this STEC serotype was absent in wild ungulates (28), in other studies prevalences of 0.75–3.41% are described (74, 75). Other clinical relevant serotypes (e.g., O103:H2 and O26:H11) with high similarity to human strains are also described in game meat in Germany (76). The serotype O27:H30, that has been associated with deer previously (71, 77), was found in three isolates from roe deer. Of the 150 STEC isolates analyzed at the BfR only one from each animal species showed resistance. The resistant isolate from wild boars showed resistance to six substance classes, including the (fluoro-)quinolones nalidixic acid and ciprofloxacin. This high percentage of isolates susceptible to the antimicrobial substances observed among STEC strains from wild animals has also been found in previous studies (71, 74).

Despite the fact that some studies suggest that wild birds could act as carriers of STEC, in general zero or low levels of STEC have been described in wild birds (62, 78, 79). This is in line with our findings.

As part of the physiological gut microbiota, commensal *E. coli* have been reported in wild mammals with high prevalence (52, 80, 81). Likewise, *E. coli* were found in our study in almost all the analyzed samples from wild boars and roe deer, but only in 50% (51 of 102) of the samples from wild ducks and geese. This observation is in the range described by previous studies that revealed a large variation in the prevalence of *E. coli* in geese, ranging from below 10–100% (59, 70, 82).

Resistance of commensal *E. coli* from wild animal fecal samples analyzed in this study were typically low. This is in agreement with the available literature, which shows in general low antimicrobial resistance rates among *E. coli* from wild ungulates (28, 80, 83–85) or wild birds (86), compared to livestock animals. To some concern, some isolates from the animal species included in this study exhibited resistance to 3rd generation cephalosporins (cefotaxime and ceftazidime), fluoroquinolones or colistin (in 1.8 and 2% of the isolates in wild boar and wild ducks and geese, respectively). Resistance to fluoroquinolones in wild ungulates has been previously described (81). Colistin resistance genes have been previously found in *E. coli* isolates from wild birds (87, 88), but to the best of our knowledge, this is the first report of colistin resistance in *E. coli* isolates from wild boars.

Our results showed that in Germany wild boars, roe deer and wild ducks and geese are reservoirs of ESBL-/AmpC-producing *E. coli*, which may reflect the general distribution of such bacteria in the environment outside of farm animal husbandry. Indeed, the proportion of positive samples found in wild boars corresponded roughly to the detection rate that was observed in a cross-sectional study in humans in Germany (89). The presence of ESBL-/AmpC-producing *E. coli* in wild animals is in line with previous studies on wild birds (33, 90–93) and wild ungulates (80, 84, 94, 95), which reported prevalences similar to those reported in our study.

Phenotypically grouped in ESBL-producers, AmpC-producers or ESBL+AmpC-producers, the ESBL-producing isolates dominated in all animal species included in this study, which might be linked with contact to human or livestock waste. The proportion of the AmpC- phenotype was higher in the isolates from roe deer (25%). High proportions and modest genetic diversity of ESBLs producing *E. coli* from wild animals have been previously reported (33, 91–93, 96).

Genotypically, CTX-M-1 was the most prevalent ESBL (51%), but in 36% of the isolates harbored a CTX-M-15 or CTX-M-9-group betalactamase. In livestock, CTX-M-1 is the most prevalent ESBL, especially in pigs and cattle, whereas CTX-M-15 and CTX-M-14 are detected only in minor proportions in livestock or meat (97, 98). One the other hand, in humans CTX-M-15 is predominant from clinical ESBL associated infection (99). Nevertheless, in non-clinical settings, CTX-M-1 is also found as the most prevalent ESBL variant (100). Therefore, a clear transmission route can not be derived from these data. Conceivable transmission could be manure fertilized fields, contaminated water sources or waste. Although SHV and CMY-2 is frequently detected in poultry production (101), none of these betalactamases were detected in ducks and geese and only one CMY-2-like isolate was found in wild boars. This might hint to hardly transmission from poultry production into the wild.

Among the confirmed ESBL-/AmpC-producing *E. coli* isolates from wild animals with characteristic resistance to betalactams, a significant percentage presented further resistance to up to five classes of substances, including fluoroquinolones. This percentage was numerically higher in wild ducks and geese, followed by wild boars and finally roe deer. In contrast to the non-selectively isolated commensal *E. coli*, resistance to colistin was not observed in ESBL-/AmpC-producing *E. coli*.

MRSA has been previously found in meat from wild boars in Germany (102). However, in our study it was noticeable that all isolates from wild boars sent to the BfR with suspicion of MRSA were not confirmed as MRSA, but instead turned out to be methicillin-susceptible *Staphylococcus aureus*. It could be assumed that the *S. aureus*, incorrectly identified as MRSA, were able to survive in the selective media because of other resistance mechanisms, such as increased beta-lactamase activity (103). The absence of MRSA in wild boars is in line with previous studies where MRSA were absent or rarely found in nasal swabs taken from wild boars (104–109).

Despite the low levels of resistance found in the animal species studied, our results underline that antimicrobial resistance is less frequent in roe deer, followed by wild boars and finally wild ducks and geese. This can be clearly seen in the resistance profiles of commensal *E. coli* and ESBL-/AmpC-producing *E. coli* (**Figure 1**), where data is available for all three studied animal species. This is in line with available data that suggest that carnivorous and omnivorous species are generally at a higher risk of AMR carriage (16). Particularly low resistance rates have been found in isolates from roe deer, which could demonstrate a lower level of exposure of roe deer to human and animal waste. Wild boars have been reported to carry resistant bacteria to a greater extent than other wild animal species, as in addition to their omnivorous behavior, their increased mobility and their high tolerance to human disturbance (19, 30), brings them to a closer contact with humans and livestock. On the other hand, the higher resistance found in wild ducks and geese might be attributable to a greater contact with wastewater or domestic animal manure containing high levels of bacteria carrying antibiotic resistance. However, we have to take into account that the low number of samples from wild ducks and geese analyzed in this study makes our margin of error larger, as shown by the wide confidence interval, so the actual prevalence of the population may vary. Future studies focusing simultaneously on several animal species living in the same habitat are needed to confirm the observed differences and determine the influencing factors.

One of the major concerns regarding the presence of resistant bacteria in wild animals is the potential contamination of meat with resistant bacteria during game meat production (110). Injuries to the digestive tract caused by gunshots, lower degree of bleeding compared to slaughtered animals and delayed evisceration of game bodies under suboptimal environmental conditions (111, 112), are the main factors that could contribute to such contamination. Since consumer exposure to resistant bacteria is possible through the consumption of contaminated meat (113), careful hygiene practices must be observed during harvesting, processing and marketing of game meat. Special attention should be paid to the presence of bacteria resistant to 3rd generation cephalosporins, fluoroquinolones, colistin or even carbapenems, which pose a serious public health concern. Further studies evaluating the relationship between the prevalence of resistance in feces from wild animals and the presence of resistant bacteria in game meat is needed in Germany in order to evaluate this potential pathway for human exposure to resistant bacteria.

The population size of the wild animals contemplated in this study has been increasing during the last decades in most of the European countries (18). This fact together with the increased fragmentation of natural ecosystems, has led to a greater proximity of these animal species to urban and peri-urban areas. Therefore, it may be advisable to investigate continuously the occurrence of resistant bacteria in wildlife. Additionally, as humans, livestock and environment play a relevant role in the origin of AMR in wild animals, a "One Health" approach would be essential when approaching it (114). Through this approach, efforts should focus on the determination of the role of wildlife in the dynamics of AMR, especially for those resistance traits to high priority substances for human and animal health. As the interpretation of resistance patterns also depends on the sampling techniques, the methodology and laboratory techniques employed to determine the susceptibility to antibiotics, standardization and harmonization need further improvement (19, 115) to allow for the comparison of data on AMR in wildlife between countries. Detailed regional studies will be required to identify factors affecting AMR in wild animals as well as potential pathways from which wildlife is acquiring resistant bacteria. In addition, the identification and evaluation of strategies to reduce the spread of AMR from humans and livestock to the environment and wildlife will be essential (116).

CONCLUSIONS

Wild boars, roe deer and wild ducks and geese can be used as bioindicators or sentinels for the presence of resistant bacteria in the environment. Our results indicate that overall, the prevalence of resistant bacteria in the selected wild animals in Germany is low, which may reflect the low level of exposure of these animals to antimicrobials and the low level of resistant bacteria in the environment. However, the patterns observed in bacteria from the wild animals included in this study are an indicator for specific resistance traits in the environment, including those to highest priority substances such as 3rd generation cephalosporins, fluoroquinolones and colistin. To account for the low prevalence of AMR in wildlife in conjunction with the presence of resistance to critically important antimicrobials use of selective isolation in the continuous monitoring of the AMR in wildlife is advisable. Furthermore, the possible role of wildlife as reservoir of resistant bacteria would need to be assessed, as wild animals, and in particular wild ducks and geese could become spreaders of resistant bacteria given their capacity for long-range movements.

DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: The dataset used in this article belongs to the German National Zoonoses Monitoring Program. Currently these data are not publicly available, however work is currently underway to create a public database that contains this data along with many other data from other programs and years. Requests to access these datasets should be directed to Carolina Plaza-Rodríguez, Carolina.Plaza-Rodriguez@bfr.bund.de.

AUTHOR CONTRIBUTIONS

Conceptualization: CP-R and B-AT. Formal analysis: CP-R, B-AT, and KA. Original draft preparation: CP-R. Review and editing: CP-R, KA, MG, JAH, AI, IS, KS, ES, LW, BP, SN, AK, and B-AT. 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/fvets.2020.627821/full#supplementary-material

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Estimation of the Prevalence of Antimicrobial Resistance in Badgers (*Meles meles*) and Foxes (*Vulpes vulpes*) in Northern Ireland

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Antimicrobial resistant (AMR) bacteria can be shared between humans and animals, through food, water, and the environment. Wild animals are not only potential reservoirs of AMR, but are also sentinels mirroring the presence of AMR zoonotic bacteria in the environment. In Northern Ireland, little is known about levels of AMR in bacteria in wildlife, thus the current study aimed to estimate the prevalence of AMR bacteria in wildlife using wildlife species from two ongoing surveys as a proxy. Nasopharyngeal swabs and faecal samples from European badgers (*Meles meles*) (146 faecal samples; 118 nasal samples) and red foxes (*Vulpes vulpes*) (321 faecal samples; 279 nasal samples) were collected throughout Northern Ireland and were used to survey for the presence of extended spectrum beta lactamase resistant and AmpC-type beta lactamases *Escherichia coli* (ESBL/AmpC), *Salmonella* spp. (only in badgers) and methicillin resistant *Staphylococcus aureus* (MRSA). ESBLs were detected in 13 out of 146 badger faecal samples (8.90%) and 37 out of 321 of fox faecal samples (11.53%), all of them presenting multi-drug resistance (MDR). Fourteen out of 146 (9.59%) badger faecal samples carried *Salmonella* spp. [*S. Agama* ($n = 9$), *S.* Newport ($n = 4$) and *S. enterica* subsp. *arizonae* ($n = 1$)]. Overall, AMR was found only in the *S. enterica* subsp. *arizonae* isolate (1/14, 7.14%). No MRSA were detected in nasopharyngeal swabs from badgers ($n = 118$) and foxes ($n = 279$). This is the first attempt to explore the prevalence of AMR in the two common wildlife species in Northern Ireland. These findings are important as they can be used as a base line for further research exploring the origin of the found resistance. These results should encourage similar surveys where environmental samples are included to bring better understanding of AMR dynamics, and the impact on wildlife, domestic livestock and humans.

Keywords: Antimicrobial Resistance, wildlife, ESBL, AmpC, *Salmonella*, MRSA, badgers, foxes

INTRODUCTION

The phenomenon of microbes becoming resistant, due to a generalised inappropriate use of antimicrobials in humans and animals, is currently happening at a global scale for a broad range of microorganisms (Dolejska and Literak, 2019). The direct consequences of Antimicrobial Resistance (AMR) include longer illnesses, increased mortality and increased costs (World Health Organization, 2015).

AMR can occur spontaneously in nature. AMR occurring naturally in some bacterial species is denominated "intrinsic resistance" (OIE, 2012) defined as the innate ability of a bacteria species to resist the action of an antibiotic due to its structural or functional characteristics. However, high levels of AMR are a result of selective pressure exerted on the bacterial population due to the use of antimicrobial agents (Schwarz et al., 2001). Furthermore, as many antibiotics belong to the same class of medicines, resistance to one specific antibiotic agent can lead to resistance to a whole related class. Moreover, resistance that develops in one organism or location can spread rapidly through, for instance, exchange of genetic mobile material such as plasmids between different bacteria (Davies and Davies, 2010). The importance of these AMR bacteria present in humans and animals, which can be transmissible through food, water, and the environment (Arnold et al., 2016a; Dolejska and Literak, 2019), make them a focus point for a "One Health" approach. Regarding wildlife, antimicrobial resistant bacteria occurrence depends on host interaction with potentially anthropogenic impacted habitats by landfills, draining of insufficiently treated wastewaters and wastes from intensively manage livestock farms (Wellington et al., 2013; Dolejska and Literak, 2019Wellington et al., 2013). However, AMR bacteria in wildlife such as rodents has also been described in areas with low levels of anthropogenic activity (Osterbald et al., 2001). As the continuous exchange of bacteria between environmental niches contributes to their dissemination (Wellington et al., 2013), the role of the environment in relation to AMR is one of the focus areas of the European Union Action Plan (European Commission, 2017).

Little is known about whether AMR is present in the environment and if at all, the levels in zoonotic or commensal bacteria in Northern Ireland. Previous research suggests that the levels of AMR in the British Isles are relatively low compared to other areas such as Africa, parts of South America and Southern Asia and broadly similar to much of continental Europe (Hendriksen et al., 2019). As resistant bacteria are present in wild animals, they can be useful sentinels mirroring the presence of AMR bacteria in an area (Dolejska and Literak, 2019). Wildlife related data can therefore be used to gain a better understanding of the levels of AMR in environmental bacteria (Gilliver et al., 1999; Mo et al., 2018). In the current study, data collected from European badgers (*Meles meles*; order carnivore, omnivorous) (Roper, 1994) and red foxes (*Vulpes vulpes*; order carnivore, omnivorous) (Soe et al., 2017) were therefore used to estimate the prevalence of AMR in these two wildlife species in Northern Ireland.

MATERIALS AND METHODS

Study Population and Design

Wildlife samples were collected as part of two ongoing surveys performed in Northern Ireland. The first of these is a road traffic accident (RTA) survey which is conducted with the aim of estimating the prevalence of bovine tuberculosis, caused by *Mycobacterium bovis*, in European badgers (*Meles meles*) (Courcier et al., 2018). This survey is conducted all year around and has been in place in Northern Ireland since 1998. It involves the collection of up to 350 badger carcasses per year. These carcasses were reported by members of the public and collected by dedicated staff and collection vehicles. Only carcasses deemed suitable for postmortem examination were taken to two veterinary diagnostic laboratories. Detailed procedures for this survey are described previously (Courcier et al., 2018). The second survey aims to establish the geographical epidemiological status of *Echinococcus multilocularis* using red fox (*Vulpes vulpes*) carcasses collected throughout Northern Ireland of which the majority are shot by hunters (for reasons of pest control), while a minority are reported after being killed by a road traffic accident (Courcier et al., 2014). This survey encompasses 325 foxes every year which are tested for *E. multilocularis* in faecal samples in order to demonstrate freedom from this parasite (Commission Delegated Regulation (EU) No 1152/2011 Annex I). Fox carcasses were reported and collected through the same channels as the RTA badger survey involving dedicated reporting systems, collection staff and vehicles. Faecal (badgers, $n = 146$; foxes, $n = 321$) and nasopharyngeal swabs (badgers, $n = 118$; foxes, $n = 279$) were collected from foxes and badgers from September 2018–June 2019. Badgers and foxes were collected within 24–48 h after death in order to prevent autolysis of carcasses. Post mortem procedures were not performed on carcasses in an advanced stage of autolysis.

Samples from badger and fox carcasses were processed for bacteria of interest. These were two Gram negative enterobacteriaceae [Extended spectrum beta lactamase resistant and AmpC-type beta lactamases *Escherichia coli* (ESBL/AmpC), *Salmonella* spp. (only badgers)] and one Gram positive-Methicillin resistant *Staphylococcus aureus* (MRSA).

E. coli (ESBL/AmpC) was tested from the faeces of foxes ($n = 321$) and badgers ($n = 146$), *Salmonella* spp. were tested from faeces of badgers ($n = 146$) and MRSA from nasopharyngeal swabs from foxes ($n = 279$) and badgers ($n = 118$). Fox samples were not tested for the presence of *Salmonella* spp. Both fox and badger carcasses were collected throughout Northern Ireland.

Microbiological Methods

The microbiological methods applied in this study are described below and outlined in **Figure 1**.

Microbiological Confirmation and Minimum Inhibitory Concentration (MIC)

It is well documented that badgers can carry *Salmonella* spp. (Wray et al., 1977; Euden, 1990; Wilson et al., 2003). Although *Salmonella* spp. have been occasionally isolated from foxes (Euden, 1990), at present this wildlife species can be consider

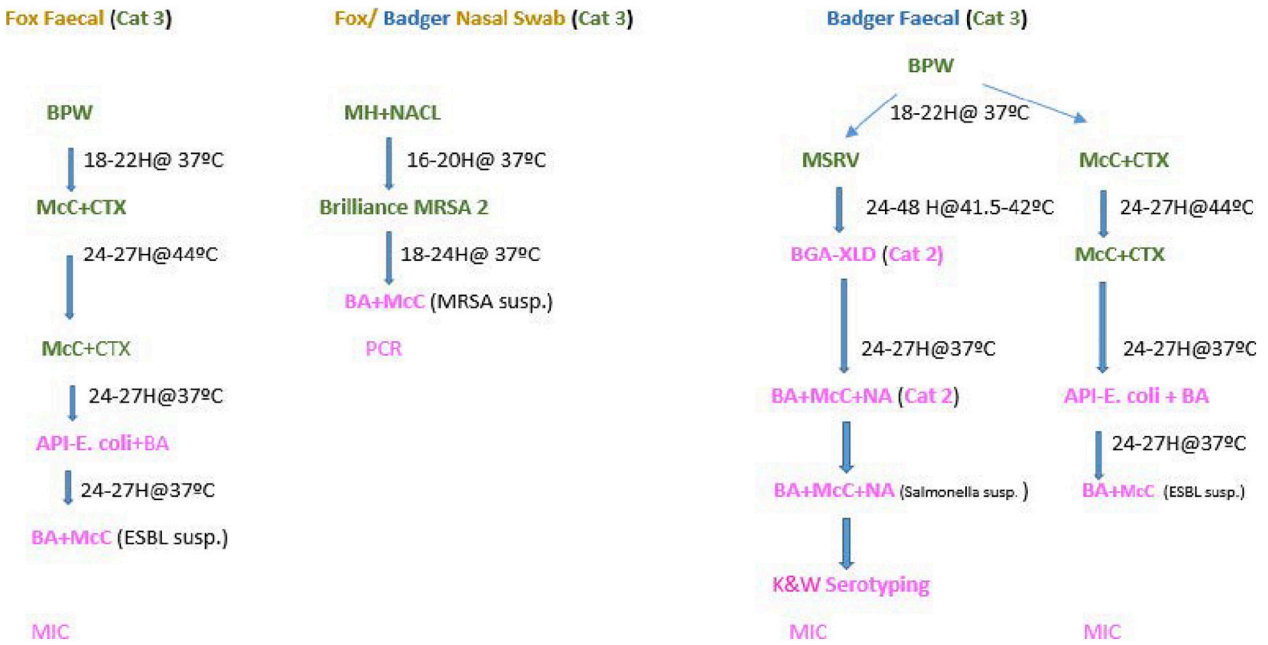

FIGURE 1 | Flow chart- Microbiological processing of faecal and nasopharyngeal swabs collected from foxes and badgers (2018–2019).

an incidental host and is still not considered yet a reservoir of *Salmonella* spp. (Chiari et al., 2014). Hence, it was decided, for logistic and budget limitations, that only faecal samples from badgers would be tested for presence or absence of *Salmonella* spp.

Although the three bacteria of interest fall under hazard category two agents and can be processed in a Category two Laboratory (CL2), *M. bovis* and *E. multilocularis* (the agents that the carcasses are primarily collected for), are classified as hazard three agents. Therefore suspected samples must be processed in a Category 3 Laboratory (CL3), due to serious biosafety impact. Thus faecal and nasopharyngeal swabs were initially processed in a CL3. Once any of the microorganisms of interest were isolated, processing/confirmation continued in a CL2 (see **Figure 1**).

ESBL/AmpC

One gram of faeces was inoculated in Buffer Peptone Water (BPW) and incubated at 37°C for 18–22 h. Thereafter, a 10 µl loop was inoculated onto MacConkey agar containing 1 mg/L cefotaxime (CTX) plate. The CTX plates were incubated for 24–27 h at 44°C. Pure colonies from the CTX were then inoculated again on to MacConkey CTX plates to maintain selective pressure and incubated at 37°C for 24 h. Thereafter Analytical Profile Index (API) tests were carried for identification according to manufacturer specifications[1].

Colistin mcr-1

Polymerase Chain Reaction (PCR) was performed according to Liu et al. (2016) on the only colistin resistant *E. coli* detected, to determine the presence of the plasmid-mediated gene mcr-1.

Salmonella spp.

Salmonella spp. were identified and confirmed as described elsewhere (Porter et al., 2020).

Minimum Inhibitory Concentration (MIC)

Once ESBL and *Salmonella* spp. were identified, individual colonies were set-up in blood and McConkey plates in preparation for MIC. MIC was tested for the recommended set antimicrobials equal for *E. coli* and *Salmonella* spp. specified in Commission Decision EU/652/2013, the MIC technique is described elsewhere (Lahuerta-Marin et al., 2017). The European Committee on Antimicrobial Susceptibility Testing (EUCAST) Epidemiological cut-off values (ECOFFs) were applied as specified in EU Commission Decision EU/652/2013.

The antimicrobials for which the MICs were checked were: ampicillin, azithromycin, ceftazidime, cefepime, cefoxitin, chloramphenicol, ciprofloxacin, colistin, cefotaxime, ertapenam, gentamycin, imipenem, meropenem, nalidixic acid, sulfamethoxazole, temocillin, tetracycline, tigecycline, and trimethoprim. Cefotaxime + clavulanic acid, ceftazidime + clavulanic acid were also included to determine synergy, which allowed to classify them as phenotypic ESBLs and AmpC following growth on CTX media (**Figure 1**). ESBLs and AmpC were classified based on EUCAST guidelines as follows:

"Presumptive ESBL producers" refers to those isolates with MICs > 1 mg/L for cefotaxime and/or ceftazidime and a synergy test positive for any of these antimicrobials and susceptibility to meropenem (MEM ≤ 0.125 mg/L). These isolates

[1]https://www.biomerieux-usa.com/clinical/api

may also harbour other resistance mechanisms (e.g., AmpC-encoding genes).

"Presumptive AmpC producers" refers to isolates with MICs > 1 mg/L for cefotaxime and/or

ceftazidime and cefoxitin MIC > 8 mg/L together with susceptibility to meropenem (MEM ≤ 0.125 mg/L). No distinction between acquired AmpC and natural AmpC was made. These isolates may also harbour other resistance mechanisms (e.g., ESBL-encoding genes).

"Presumptive ESBL + AmpC producers" refers to isolates with the ESBL + AmpC phenotype as described above.

MRSA

The nasopharyngeal swabs were placed in glass universals containing 10 ml aliquots of Muller Hinton (MH) broth and 6.5% NaCl and incubated for 16–20 h at 37°C. Then a 10 μl loop of the broth was spread on Brilliance 2 MRSA agar and incubated for 24 h at 37°C. Subculture presumptive MRSA colonies were put on to blood agar and incubated for 24 h at 37°C. Presumptive colonies were then confirmed by PCR, according to the protocol for PCR Amplification of mecA, mecC, spa, and pvl validated by the European Reference Laboratory for Antimicrobial Resistance (Eurl-Ar, 2012).

Statistical Analyses

Data were described and proportions along with 95% confidence intervals (95% CI) were calculated. Percentages resistance and distributions of MIC values were calculated for every antimicrobial. Significant differences of proportions were calculated applying chi-squared tests. All statistical analyses were conducted using R (version 4.0.1; The R Project for Statistical Computing[2]) and maps were produced using ArcMap (version 10.3.1; ESRI).

RESULTS

Extended Beta Lactamase and AmpC-Type Beta-Lactamases Escherichia Coli (ESBL/AmpC)

A total of 13 out of 146 badger faeces samples (8.90%; 95% CI 5.02–15.04%) carried ESBL. Fourteen of these isolates showed resistance against any of the tested antimicrobials (**Table 1** and **Figure 2**). ECOFFs were based on European Union guidelines (European Union, 2013).

A total of 37 out of 321 fox faeces samples (11.53%; 95% CI 8.35–15.66%) contained ESBL. All of these 37 isolates showed resistance against any of the tested antimicrobials (**Table 1**). **Tables 2**, **3** show the distribution of resistant ESBL/AmpC type *Escherichia coli* found by antimicrobial. Cut off points were based on European Union guidelines (European Union, 2013).

There was no significant difference in the proportion of ESBL/AmpC resistant isolates found in badgers compared to foxes (Chi-squared 0.724, df = 1, $p = 0.395$).

[2]https://www.r-project.org/

One phenotypic ESBL strain was also resistant to colistin. The strain was negative to *mcr-1*.

Salmonella spp.

Salmonella spp. was detected from 14 out of 146 badger faeces samples (9.59%; 95% CI 5.54–15.86%). *S. Agama* was the most prevalent serovar ($n = 9$) followed by *S.* Newport ($n = 4$) and *S. enterica* subsp. *arizonae* ($n = 1$). AMR were only observed in one out of 14 *Salmonella* spp. isolates (7.14%; 95% CI 0.37–35.83%), the *S. enterica* subsp. *arizonae* isolate, which showed resistance against ampicillin (MIC > 64 mg/L), ceftazidime (MIC = 64 mg/L), cefoxitin (MIC > 64 mg/L), cefotaxime (MIC = 32 mg/L), and ertapenam (MIC = 0.12 mg/L) and no synergy with clavulanate (**Table 1** and **Figure 2**). This type of resistance is consistent with AmpC. Overall one of the 146 badger faecal samples collected contained therefore resistant *Salmonella* spp. isolates (0.68%; 95% CI 0.03–4.32).

Methicillin-Resistant Staphylococcus Aureus (MRSA)

No MRSA was detected in any of the nasopharyngeal swabs of badgers ($n = 118$) or foxes ($n = 279$) (**Table 1**).

DISCUSSION

In Northern Ireland, little is known about the prevalence of AMR in wildlife. Therefore, two common wildlife species (European badgers (*Meles meles*) and red foxes (*Vulpes vulpes*)) were used as sentinels to gain insight into this. The current study aimed to survey for the presence of resistance in these two wildlife species in ESBL/AmpC, *Salmonella* spp. (only in badgers) and MRSA.

In the current study 13 out of 146 badger faecal samples contained ESBL with all 13 showing resistance (all against multiple antimicrobials). Furthermore, 37 out of 321 fox faecal samples contained ESBL with all 37 showing resistance (again all against multiple antimicrobials). This is of concern as our results show high correlation between ESBL/AmpC resistance and levels of resistance to other antimicrobials including one isolate resistant to colistin. ESBL is known to be widely distributed in wildlife and is considered to be a key indicator pathogen to trace the evolution of multi-resistant bacteria in the environment and wildlife (Guenther et al., 2011). It was anticipated that ESBL in wildlife would express a multi-resistant phenotype, not due to the nearby use of antimicrobials or antimicrobials in sub-therapeutic concentrations in natural environments, but because distant use had caused a multi-resistant organism to evolve in the first place which subsequently spread to different ecological niches (O'Brien, 2002). The majority of previous research into ESBL prevalence in wildlife has focussed on birds and rodents and prevalence appears to be highest in urbanised areas (Guenther et al., 2011). However, previous research did report the presence of resistant ESBL in foxes (1 out of 7 animals sampled) (Costa et al., 2006) and in badgers (Alonso et al., 2017). It is also documented that plasmids that harbour ESBL and/or pAmpC genes may also carry other resistance genes, meaning

TABLE 1 | Distribution of number of samples, isolates, and AMR positive isolates collected from foxes and badgers (2018–2019).

Bacterium	Badgers			Foxes		
	Number of samples tested	Number (%) of isolates	Number (%) of AMR positive	Number of samples tested	Number (%) of isolates	Number (%) of AMR positive
Escherichia coli (ESBL/AmpC) (faeces)	146	13 (8.90%)	13 (100%)	321	37 (11.53%)	37 (100%)
Salmonella spp. (faeces)	146	14 (9.59%)	1 (7.14%)	0	–	–
Methicillin resistant *Staphylococcus Aureus* (MRSA) (Nasopharyngeal swabs)	118	0 (0.0%)	–	279	0 (0.0%)	–

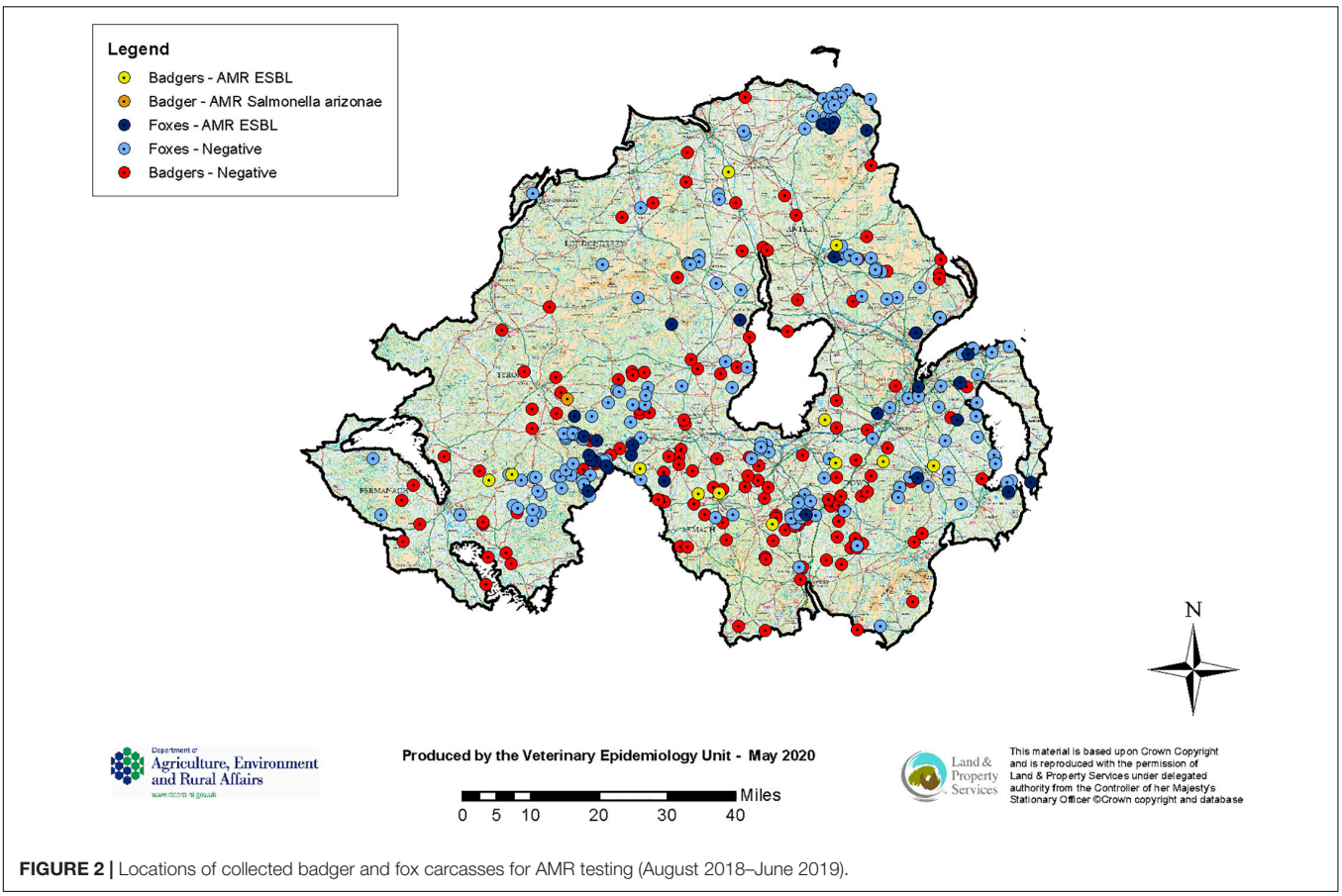

FIGURE 2 | Locations of collected badger and fox carcasses for AMR testing (August 2018–June 2019).

that ESBL/pAmpC-producing pathogens can be resistant to other classes of antimicrobial agents as well (MacVane et al., 2014).

Staphylococcus aureus is a commensal bacterium with the potential to cause severe disease in humans and animals. MRSA, which is resistant to most β-lactam antibiotics, is a major cause of hospital-associated infections. Livestock-associated (LA)-MRSA has also been recognised to cause infections in humans (Köck et al., 2010) and has been detected in pigs and cattle in Northern Ireland (Hartley et al., 2014; Lahuerta-Marin et al., 2016). The first reported isolation of LA-MRSA in Northern Ireland, and indeed in the UK, was detected in a pig from a mixed swine-dairy cattle herd in 2014 (Hartley et al., 2014). Two on-farm investigations followed and environmental and animal samples were collected. LA-MRSA CC398 t034, the most common strain type in livestock in Northern Ireland (Sharma et al., 2016), was isolated from all environmental samples collected from the first infected farm and from the pig samples only. The bacterium was not detected from any other animal samples (cattle, dog, and sheep) collected form the follow-up epidemiological investigation of the index case (unpublished data). These results showed that LA-MRSA CC398 t034 was restricted to the environment and that the main animal hosts were pigs. However, in the current study no MRSA was observed in nasopharyngeal swabs from either badgers or foxes, hence more research is required and perhaps collection of wildlife around infected farms could be an option for future surveys. It is possible that sampling of

TABLE 2 | Minimum inhibitory concentrations (MICs) and antimicrobial resistance in ESBL *Escherichia coli* isolated from faecal samples ($n = 16$) from badgers (*Meles meles*) in Northern Ireland in 2018–2019.

	Resistance	Distribution (%) of MIC values (mg/L)																
Substance	**(%)**	**0.015**	**0.03**	**0.06**	**0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**512**	**1,024**
Ampicilin	100													100				
Azithromycin*									7.7	46.2	38.5	7.7						
Ceftazidime	100							7.7	23.1	38.5	7.7	7.7	15.4					
Cefepime	61.5			38.5					15.4	30.8		15.4						
Cefoxitin	38.5								15.4	23.1	23.1	15.4	23.1					
Chloramphenicol	53.8										61.5			15.4	38.5			
Ciprofloxacin	38.5	61.5				23.1					15.4							
Colistin	0.0							100										
Cefotaxime	100							23.1	15.4	15.4			7.7	38.5				
Ertapenam	0.0	84.6	15.4															
Gentamycin	0.0						76.9	23.1										
Imipenem	0.0				61.5	38.5												
Meropenem	0.0		100.0															
Nalidixic acid	30.8									61.5	7.7				30.8			
Sulphamethoxazole	92.3										7.7							92.3
Temocillin*									7.7	61.5	15.4	7.7						
Tetracycline	92.3								7.7				30.8	61.5				
Tigecycline	0.0					92.3	7.7											
Trimethoprim	69.2					23.1	7.7						69.2					

**No EUCAST ECOFF available. Cefotaxime + Clavulanic acid and Ceftazidime + Clavulanic acid were included for MIC, as described in Decision EU/652/2013, to determine if synergy was present or not.*

TABLE 3 | Minimum inhibitory concentrations (MICs) and antimicrobial resistance in ESBL/AmpC isolated from faecal samples ($n = 39$) from red foxes (*Vulpes vulpes*) in Northern Ireland in 2018–2019.

	Resistance	Distribution (%) of MIC values (mg/L)																
Substance	**(%)**	**0.015**	**0.03**	**0.06**	**0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**512**	**1,024**
Ampicilin	100													100				
Azithromycin*									5.4	48.7	37.9	5.4		2.8				
Ceftazidime	100							8.1	21.7	16.2	19.0	16.2	10.8		2.8			
Cefepime	67.6			24.3	8.1	8.1		2.8	18.9	13.5	24.3							
Cefoxitin	56.8								8.1	24.3	10.8	29.7	10.8	16.2				
Chloramphenicol	40.1										59.5			8.1	32.4			
Ciprofloxacin	51.3	48.6		2.8	8.1	21.6	2.8				16.2							
Colistin	2.8							97.2				2.8						
Cefotaxime	100							13.5	13.5	16.2	16.2	2.8	5.4	32.4				
Ertapenam	2.8	73.0	16.2	8.1		2.8												
Gentamycin	13.5						64.9	21.7			5.4	5.4	2.8					
Imipenem	0.0				62.1	35.1	2.8											
Meropenem	0.0		100.0															
Nalidixic acid	43.2									51.3	5.4			2.8	40.6			
Sulphamethoxazole	62.2										5.4	29.7	2.8					62.2
Temocillin*							2.8		8.1	62.1	24.3	2.8						
Tetracycline	64.9								35.1				8.1	56.8				
Tigecycline	0.0					97.2	2.8											
Trimethoprim	48.7					27.0	24.3						48.7					

**No EUCAST ECOFF available. Cefotaxime + Clavulanic acid and Ceftazidime + Clavulanic acid were included for MIC, as described in Decision EU/652/2013, to determine if synergy was present or not.*

badgers and foxes may not be a good proxy or indicator for environmental contamination with this LA-MRSA type. In that case, wildlife may represent an underestimation of levels of LA-MRSA in the environment (if any), thus, the use of wildlife samples as an environmental proxy could be a limitation for this particular pathogen.

Previous studies have demonstrated that a wide range of *Salmonella* spp. is known to be commonly present in badgers in the UK (Taylor, 1968; Wray et al., 1977; Euden, 1990; Wilson et al., 2003). The reported range of serovars is broad with *S. Agama* being the most commonly isolated serovar (Euden, 1990), as observed here. The only resistant serovar *S. enterica* subsp. *arizonae* detected in the current study is one of the less frequently found subspecies of *Salmonella* spp., most commonly detected in reptiles (especially snakes and tortoises) (Hall and Rowe, 1990; Bertrand et al., 2008; Bruce et al., 2018). Strains of *Salmonella* spp. with resistance to antimicrobial drugs are now widespread in both developed and developing countries (Threlfall, 2006). The only resistant isolate in the study was *S. enterica* subsp. *arizonae* showing AMR against five antimicrobials including 3rd generation of cephalosporins. ESBLs *Salmonella* spp. have been detected in chickens (Dierikx et al., 2010; de Souza et al., 2019) and other livestock (Riano et al., 2006). ESBL Salmonella isolations from wildlife are uncommon but ESBL *S. Infantis* isolated from owls have been reported (Fuentes-Castillo et al., 2019). On the other hand, human salmonellosis due to ESBL non-paratyphi *Salmonella* spp. have been described. The UK reported cases of clinical salmonellosis in humans due to ESBL *Salmonella* spp. (EFSA, 2018, 2019). However, at present, ESBL *Salmonella* spp. are uncommon in livestock in the UK, but have been reported by some European Member States (EFSA, 2018, 2019; Uk-Varss, 2019). Genetic characterisation of the strain will provide more clues about the potential origin. In addition, the results of the current study are very interesting as sampled badgers were carrying relatively low levels of *Salmonella* spp., and not many of the isolates carried any AMR compared to AMR levels observed in livestock (Porter et al., 2020). This suggests exposure and dynamics of infection with *Salmonella* spp. in both domestic livestock and wildlife reservoirs such as badgers are different. Nevertheless the impact of carrying ESBL *Salmonella* spp. may be high particularly regarding treatment, as human infections due to *S. enterica* subsp. *arizonae* can occur and have been described before (Gavrilovici et al., 2017; Lakew et al., 2013).

The reservoir of resistance genes in the environment is known to be due to a mix of naturally occurring resistance, those present in animal and human waste and the selective effects of pollutants, which can co-select for mobile genetic elements carrying multiple resistant genes. Resistance genes can be acquired from any source, but gene flow is probably structured by ecology, with species that share similar niches drawing from similar gene pools (Wellington et al., 2013). Several possible transmission routes exist including direct contact with infected individuals, their tissues or their faeces, water and soil (Vittecoq et al., 2016). Once present in the environment, the resistant bacteria can then be potentially acquired by wild animals and then reintroduced to humans. Therefore, the potential of plasmid transfer between strains of the same species or between different bacterial species or genera creates an environmental reservoir of resistance with potentially far reaching impacts for human health (Carroll et al., 2015). As there is a significant level of resistant ESBL/pAmpC- producing pathogens found in wildlife in the current study, similar to previous studies (Costa et al., 2006; Alonso et al., 2017; Darwich et al., 2019), the sources of these would need further investigation focusing on both farm animal and human related origins (Arnold et al., 2016b; Larsson et al., 2018). Potential associations that could be explored include water sources, human and farm animal population density, and proximity to hospitals and pharmaceutical industries (Arnold et al., 2016b). Further molecular characterisation into ESBL and *Salmonella* spp. would also be useful in order to provide insight into the possible correlation between phenotypic and genotypic patterns of resistance between isolates from wildlife, livestock and humans. The results of further research could help prioritising the development of effective One Health strategies to mitigate the spread of AMR into the environment in targeted areas in Northern Ireland, such as: pre-treatment of manure before use as fertiliser; pre-treatment of waste across the farm to slaughterhouse continuum before discharge into the general sewage system; education to increase awareness of all hospital personnel on hygiene, sanitation and safe disposal practices; insurance of the safe disposal of antimicrobial medicines and hazardous waste; consideration of pre-treatment of hospital waste before discharge into the general sewage system (FAO, 2018). Furthermore, research into the possible correlation of AMR bacteria in wildlife/livestock and their environment would be useful, because monitoring of AMR in wildlife and the environment can be used as an early detection of new AMRs (Martínez, 2009; Radhouani et al., 2014).

This survey is the first step to assess the risk of wildlife as a potential environmental reservoir of antimicrobial resistance for domestic livestock and humans. We expect that these results encourage the Environmental Agency in Northern Ireland to test bacteria isolated from environmental samples- soil, dust and water. Hence this will contribute to a better one health understanding of AMR (European Commission, 2017).

LIMITATIONS OF THE STUDY

Only one ESBL strain also presented resistance to colistin. It was previously described that plasmid-mediated *mcr-1* had resistance to this critically important antibiotic (Liu et al., 2016). This PCR was performed as an initial screening. Since then, 10 plasmids have been described (*mcr-1–10*). There is no validated PCR available to detect presence or absence of all 10 *mcr*s. Further analyses will be conducted in relation to testing for *mcr-1*. In the near future, there are plans to test for *mcr-1–5* following the validated PCR protocol developed by EURL-AR[3]. If the colistin resistant ESBL carry any of *mcr-6–10* plasmids, we will be able to detect it when we perform whole genome sequencing on

[3]https://www.eurl-ar.eu/CustomerData/Files/Folders/21-protocols/396_mcr-multiplex-pcr-protocol-v3-feb18.pdf

this isolate. This was not performed in this initial stage of this survey.

No microbiological isolation of commensal *E. coli* was performed for this study. If the survey is performed again in the near future, it would be desirable to calculate the prevalence of commensal *E. coli* and the levels of resistance of this bacterium carried by wildlife species in Northern Ireland.

Due to the nature of the sampling within the surveys (convenience sampling) and the relatively low numbers of positive samples involved, it is not possible to identify clusters and potentially relate them to features such as rivers or drainage basins. Further research could be conducted to address this.

Wildlife sampling for AMR is important (based on the "One Health" concept), but challenging. Similar to other research (Mo et al., 2018), dead animals were therefore the sample source for this study. This provides difficulties in relation to autolysis. However, in the current study this was prevented as much as possible by collecting and processing the badgers and foxes within 24–48 h after death.

This project has provided the first insight into the prevalence of AMR in wildlife as a proxy for the environment. Results showed that ESBL/AmpC were the most prevalent type of resistance in badgers and foxes. Moreover, the ESBL/AmpC isolates recovered were also resistant to several other antimicrobial agents. AMR levels in *Salmonella* spp. were very low but highly resistant. The resistance pattern was unusual in that resistance against for example, AmpC and MRSA was not detected. These results suggest that the three pathogens may have different dynamics of infection and exposure from the environment into the sampled wildlife species. These findings will form a baseline for further research and are an important first step in our understanding of the levels of AMR bacteria in wildlife and potentially the environment.

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.

ETHICS STATEMENT

Ethical review and approval was not required for this study because the animals were already deceased. Animal carcasses (badgers and foxes) were collected from the roadside after becoming victims of road traffic accidents or, in the case of some of the foxes, were shot by hunters (not for the purpose of the study).

AUTHOR CONTRIBUTIONS

AP-L and AL-M conceived and designed the study. MO'H and AL-M wrote the manuscript, with interpretation of results and discussion inputs from AP-L. JM organised collection of the samples. CC, CH, CB, NS, and RD performed laboratory work. MO'H carried out statistical analyses. RH approved the concept of the study and liaised to initiate the study. All authors read and approved the final manuscript.

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Antibiotic Resistance in Agricultural Soil and Crops Associated to the Application of Cow Manure-Derived Amendments From Conventional and Organic Livestock Farms

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The application of organic amendments to agricultural soil can enhance crop yield, while improving the physicochemical and biological properties of the recipient soils. However, the use of manure-derived amendments as fertilizers entails environmental risks, such as the contamination of soil and crops with antibiotic residues, antibiotic resistance genes (ARGs) and mobile genetic elements (MGEs). In order to delve into these risks, we applied dairy cow manure-derived amendments (slurry, fresh manure, aged manure), obtained from a conventional and an organic farm, to soil. Subsequently, lettuce and wheat plants were grown in the amended soils. After harvest, the abundance of 95 ARGs and MGE-genes from the amended soils and plants were determined by high-throughput qPCR. The structure of soil prokaryotic communities was determined by 16S rRNA amplicon sequencing and qPCR. The absolute abundance of ARGs and MGE-genes differed between treatments (amended vs. unamended), origins of amendment (conventional vs. organic), and types of amendment (slurry vs. fresh manure vs. aged manure). Regarding ARG-absolute abundances in the amendments themselves, higher values were usually found in slurry vs. fresh or aged manure. These abundances were generally higher in soil than in plant samples, and higher in wheat grain than in lettuce plants. Lettuce plants fertilized with conventional amendments showed higher absolute abundances of tetracycline resistance genes, compared to those amended with organic amendments. No single treatment could be identified as the best or worst treatment regarding the risk of antibiotic resistance in soil and plant samples. Within the same treatment, the resistome risk differed between the amendment, the amended soil and, finally, the crop. In other words, according to our data, the resistome risk in manure-amended crops cannot be directly inferred from the analysis of the amendments themselves. We concluded that, depending on the specific question under study, the analysis of the resistome risk should specifically focus on the amendment, the amended soil or the crop.

Keywords: emerging contaminants, mobile genetic elements, organic farming, soil microbial diversity, antibiotic resistance genes

INTRODUCTION

Antibiotics are indispensable tools for the treatment of bacterial infections in human medicine and veterinary medicine. Antibiotics are mainly used for the curative and, to a lesser extent, preventive treatment of bacterial infectious diseases. Besides, they are also used in many countries as growth promoters in animal production farms (1). However, the use of antibiotics for disease prevention is not recommended by the Word Health Organization (2) and the European Union banned the use of antibiotics for animal growth promotion in 2006 [Regulation (EC) No. 1831/2003]. The use, abuse and inappropriate use of antibiotics (i) in livestock farms for animal production purposes, (ii) in human medicine for the treatment of bacterial infections, and (iii) in agriculture for crop production purposes is gradually causing the emergence and dissemination of antibiotic resistant bacteria (some of them show simultaneous resistance to many—*multiresistant*—or even all—*panresistant*—known antibiotics), due to the selective pressure exerted by antibiotics on exposed bacterial populations. Many antibiotics used in veterinary practice are the same used to treat bacterial infections in humans or have the same mode of action or belong to the same antibiotic family (3), leading to the alarming intensification and augmentation of the well-known huge problem of multiresistant bacterial strains currently putting at risk, at a global scale, our capacity to fight and control bacterial human pathogens (4).

Most antibiotics administered to livestock are not fully metabolized and, hence, are released, together with their transformation products, into the environment along with the feces and urine (5). In fact, a considerable percentage (30–90%) of the antibiotic administered to a given animal for veterinary purposes can be directly excreted in the urine and feces (5). Animal manure is therefore a source of antibiotic contamination (antibiotics are nowadays considered emerging contaminants) and a reservoir of antibiotic resistant bacteria (ARB) harboring and potentially spreading antibiotic resistance genes (ARGs) (6). Animal manure is commonly applied to agricultural soil as organic fertilizer. Apart from providing valuable plant nutrients that can enhance crop yield, the application of manure can simultaneously improve soil physicochemical and biological properties, i.e., soil quality (7–9). Regrettably, the agronomic application of manure can also lead to the emergence and dissemination of ARB and ARGs in the amended agricultural soil and, subsequently, in the food crops grown for human consumption (10, 11). To make matters worse, ARB can disseminate ARGs to other bacteria through horizontal gene transfer (HGT) mediated by mobile genetic elements (MGEs), such as integrons, phages, plasmids, integrative conjugative elements, transposons, etc. (12, 13).

Understandably, most of the attention given to the problem of antibiotic resistance (AR) has been directed to hospital settings. Nonetheless, in the last years, more and more awareness is being developed concerning the vastly complex environmental dimension of AR and its central role in the emergence, maintenance and spread of AR at a global scale (14). Undeniably, the emergence and dissemination of AR in agroecosystems, resulting from the application of animal manure as organic fertilizer, begets a potential risk for human health and the environment, being currently an issue of much global concern that, urgently, requires the development and implementation of practices and management measures that mitigate (or, better, eliminate), such a risk (15). Among other measures aimed at enhancing the sustainability of animal production practices, organic livestock farming promotes a considerable reduction of the use of antibiotics for veterinary purposes, compared to conventional livestock systems. In principle, this reduction in antibiotic use implies concomitantly a lower level of selective pressure for bacterial populations to acquire and maintain AR by evolutionary adaptation mechanisms (16). In addition, the composting of animal manure has recurrently been reported as an effective option for the reduction of antibiotic concentrations in animal manure and, to a lesser extent, for the decrease in the abundance of ARGs in these animal-derived organic amendments (17, 18).

On the other hand, the presence of antibiotics and their transformation products (some of these are also bioactive compounds) in animal manure may significantly alter the composition of soil microbial communities when applied to agricultural soil. These antibiotic-induced changes in soil microbial composition frequently have important consequences for the soil resistome and mobilome (19, 20). Relevantly, soil microbial diversity (in terms of richness, evenness, composition, etc.) is regularly used as a biological indicator of the impact of disturbances (e.g., contamination) on soil health (21–23).

Our objective was to study, under controlled microcosm conditions, the emergence and dissemination of AR in agricultural soil and food crops (lettuce and wheat) derived from the application of dairy cow wastes as organic fertilizer. In order to delve into possible management practices that could minimize the resistome risk, we compared the effects of the application of: (i) three types of commonly used amendments: slurry vs. fresh manure vs. aged manure; and (ii) amendments from a conventional livestock farm vs. an organic livestock farm. To quantify the magnitude of the resistome risk in agricultural soil and food crops, we used the following end-points: (i) antibiotic concentrations; (ii) abundance of ARGs and MGE-genes in soils and plants (lettuce and wheat grain); and (iii) observed relationships between the structural diversity of soil prokaryotic communities (from 16S rRNA amplicon sequencing data) and the abundance of ARGs and MGE-genes. We hypothesized that the resistome risk will be higher in soils and plants: (i) amended with dairy cow wastes, compared to non-amended controls; (ii) amended with dairy cow wastes from the conventional livestock farm vs. the organic livestock farm; and (iii) amended with slurry wastes vs. fresh and aged wastes. We also hypothesized that lettuce samples will show a higher resistome risk than wheat grain samples.

MATERIALS AND METHODS

Experimental Design

The amendments used in this study were kindly provided by two dairy cow farms located in the province of Biscay (Spain):

a conventional livestock farm and an organic livestock farm. Three types of amendments (i.e., slurry, fresh manure, aged manure) from these two different origins (i.e., conventional livestock farm and organic livestock farm) were studied. In both farms, representative samples of cow slurry were taken from a pool where the feces and urine from the cows in production were deposited. In contrast, fresh manure samples were taken from the cow bedding (made from feces, urine, and wheat straw) of the non-producing cows: heifers, dry cows and cows undergoing treatment (the latter only in the conventional farm). As for the aged manure, a composite sample was taken from a manure pile that had been stored for ∼6 months. All samplings were carried out on the same day. Fresh and aged manure samples were collected in polyethylene bags, while slurry samples were contained in plastic barrels. All samples were immediately transferred to the laboratory and stored at 4°C until use. The experimental soil was collected from the upper 30 cm layer of a semi-natural grassland field which, to our knowledge, has never been amended with any kind of inorganic or organic fertilizer. Immediately after collection, the soil was sieved to <4 mm. For our microcosm study, experimental pots containing 2 and 4 kg of dry weight (DW) soil were used for lettuce and wheat plants, respectively. The dose of amendment was carefully adjusted in order to provide an equivalent of 100 and 180 kg N ha^{-1} for lettuce and wheat plants, respectively. The amendments were manually incorporated into the soil and thoroughly mixed for homogenization purposes. A 2 week stabilization period was allowed before crop planting (lettuce seedlings) or sowing (wheat seeds). Lettuce (*Lactuca sativa* L. var. Batavia) and hard winter wheat (*Triticum aestivum* L. var. Qualidu) plants were used in this study, since they are most commonly grown in our region for agricultural purposes. Our experiment was carried out in a growth chamber under the following controlled conditions: 14/10 h light/dark cycle, 20/16°C day/night temperature, 70% relative humidity, and a photosynthetic photon flux density of 150 μmol photon m^{-2} s^{-1}. Throughout the experimental period, plants were bottom watered every 2–3 days. Each treatment was replicated four times. Lettuce plants were harvested after 44 days of growth, while wheat plants were harvested after 171 days. For the determination of crop production, lettuce plants (aerial part = shoot biomass) were cut from the base with a scalpel and then freshly weighed. Similarly, in wheat plants, spikes were husked, and wheat grains freshly weighed. Dry weight of lettuce plants and wheat grains was determined by drying in an oven at 70°C until reaching a constant mass. On the other hand, soil samples were collected from the pots at crop harvest time (see below section Effect of Treatments on Biological Parameters Related to the Resistome Risk).

Amendment and Soil Physicochemical Characterization

Before the beginning of the experiment, the dairy cow manure-derived amendments and the experimental semi-natural grassland soil were physicochemically characterized (24) according to the following parameters: pH, organic matter (OM) content, total nitrogen (N), potassium (K^+), and Olsen phosphorus (P). Dry weight of soils was determined by drying in an oven at 30°C until reaching a constant mass. Mineral and pseudo-total metal concentrations were determined by Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) following aqua regia digestion (25). Antibiotic concentrations were determined by Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) in SAILab Instrumental Analytical Solutions (Barcelona, Spain). In particular, the concentration of 57 antibiotics belonging to nine families (aminoglycosides, cephalosporins, macrolides, nitrofurans, penicillins, polypeptides, quinolones, sulphonamides, and tetracyclines) was quantified. For confirmation purposes and in order to assess the rate of degradation of the antibiotics present in the manure and soil samples, a second analysis of antibiotic concentrations was carried out 2 months later. In this second analysis, the antibiotic families that, in the first analysis, exceeded the detection limit of the technique in at least one of the studied antibiotics (i.e., polypeptides and quinolones) were again analyzed.

Effect of Treatments on Biological Parameters Related to the Resistome Risk

For the assessment of the effect of treatments on biological parameters that provide information on resistome risk, at crop harvest time, soil samples were collected from the experimental pots and then sieved to <2 mm. Prior to DNA extraction, soil samples were washed twice in 120 mM K_2PO_4 (pH 8.0) to wash away extracellular DNA (26). DNA was extracted from soil samples (0.25 g DW soil) using the Power Soil™ DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA). Similarly, DNA was extracted from plant samples using the innuPREP Plant DNA Kit (Analytik Jena, Jena, Germany). The concentration of soil and plant DNA was quantified with a NanoDrop spectrophotometer (ND-1000, Thermo Scientific, Wilmington, DE). Soil and plant DNA was stored at −20°C until use.

For the quantification of ARG and MGE-gene abundances, high-throughput real-time PCR (HT-qPCR) reactions were performed using the nanofluidic qPCR BioMark™ HD system, with 48.48 and 96.96 Dynamic Array Integrated Fluidic Circuits (ICFs) (Fluidigm Corporation) following Urra et al. (27). A total of 96 validated primer sets (28) were used, including 85 primer sets targeting ARGs conferring resistance against all major classes of antibiotics [10 aminoglycosides, 14 β-lactamases, 5 FCA (fluoroquinolone, quinolone, florfenicol, chloramphenicol, and amphenicol), 13 MLSB (macrolide, lincosamide, streptogramin B), 5 multidrugs (i.e., those conferring resistance to more than one antibiotic), 4 sulfonamides, 24 tetracyclines, and 10 vancomycines], 10 primers sets targeting MGE-genes (8 genes encoding transposases and 2 genes encoding integrases), and the 16S rRNA as reference gene. DNA samples were pre-amplified with a pool of primers (final concentration for each primer pair = 50 nM; 16 PCR cycles) and then treated with exonuclease I. Subsequently, 1:10 dilutions of specific target amplification reactions were loaded onto the Dynamic Array IFCs, following the Fluidigm's Fast Gene Expression Analysis—EvaGreen® Protocol. The SsoFast™ EvaGreen® Supermix with

Low ROX (Bio-Rad Laboratories, Redmond, WA) was used for amplification (with a final primer concentration, both forward and reverse, of 500 nM). The cycling program consisted of 1 min at 95°C, followed by 30 cycles at 95°C for 5 s and 60°C for 20 s, followed by a melting curve. Four replicates were included for each sample. Measurements were conducted in the Gene Expression Unit of The Genomics Facility of SGIker—University of the Basque Country, Spain. Raw data obtained from the analysis were processed with the Fluidigm Real-Time PCR Analysis Software (v.3.1.3) with linear baseline correction and manual threshold settings. A threshold cycle, C_T value, of 31 was chosen since the highest C_T value obtained in our study was 30.53. A detection of an ARG or MGE-gene was considered positive when 3 out of the 4 technical replicates for each sample were above the detection limit. The value of the detection limit was used for non-amplified genes. Furthermore, a comparative C_T method was used to calculate ARG and MGE-gene relative abundances, normalized to the abundance of the 16S rRNA control gene, expressed as fold-change (FC) (29):

$$\begin{aligned} \Delta C_T &= C_{T(target\ gene)} - C_{T(16S\ rRNA\ gene)} \\ \Delta\Delta C_T &= \Delta C_{T(amended\ sample)} - \Delta C_{T(unamended\ sample)} \\ FC &= 2^{-\Delta\Delta C_T} \end{aligned}$$

Real-time PCR measurements of the abundance of the 16S rRNA gene were performed to estimate total bacterial biomass, following the reaction mixtures and PCR conditions described in Epelde et al. (30). The relative copy number (GR) was calculated as the proportion of the abundance of the ARG or MGE-gene to the abundance of the 16S rRNA gene (31). Absolute ARG and MGE-gene abundances ($GA_{ARG,MGE}$) were calculated as follows (32):

$$\begin{aligned} GR &= 10^{\frac{(31-C_T)}{(10/3)}} \\ GA_{ARG,MGE} &= \frac{GA_{16S} \times GR_{ARG,MGE}}{GR_{16S}} \end{aligned}$$

In order to assess the impact of amendments on soil prokaryotic community composition, the preparation of amplicon libraries was carried out using a dual indexing approach with sequence-specific primers (33) targeting the V4 region of the 16S rRNA gene: primers 519F (CAGCMGCCGCGGTAA) adapted from Øvreås et al. (34) and 806R (GGACTACHVGGGTWTCTAAT) from Caporaso et al. (35). Sequencing was performed with an Illumina MiSeq V2 platform and paired-end sequencing strategy (2 × 250 nt) at Tecnalia, Spain. Read paired ends were merged, quality filtered and clustered into operational taxonomic units (OTUs) as described in Lanzén et al. (33). The taxonomic classification was performed using CREST (36).

Statistical Analysis

One-way ANOVA with Duncan's multiple-range tests was performed to compare absolute abundance values of ARGs and MGE-genes among treatments: *type of amendment* = slurry vs. fresh manure vs. aged manure, and *origin of amendment* = conventional livestock farm vs. organic livestock farm. Identical analyses were performed for crop production data. The effect of the experimental factors (*type* × *origin*) was tested by two-way ANOVA using package *agricolae* of R software (v.3.6.3). R package *vegan* (37) was used to calculate α-diversity indices (i.e., richness, Shannon's, Simpson's, Pielou's) for soil prokaryotic diversity data and 16S rRNA amplicon sequencing data visualization. Principal component analysis (PCA) of ARG and MGE-gene absolute abundances was performed using Canoco 5 (38). Venn diagram was performed to examine the overlapping, in terms of the presence of ARGs and MGE-genes, between soil and plant samples with *venn* package in R. Kendall's rank correlation coefficients, followed by Bonferroni's multiple comparisons test, between soil prokaryotic taxa (at order level) and absolute abundances of ARGs and MGE-genes (grouped by antibiotic family and MGE category) were obtained using R software.

RESULTS

Amendment and Soil Physicochemical Characterization

The soil was characterized as a clay loam, with a pH of 6.2, an OM of 6.3%, a total N content of 0.32%, an Olsen P content of 3.4 mg kg^{-1} DW soil, and a K^+ content of 395 mg kg^{-1} DW soil. Regarding the physicochemical properties of the dairy cow manure-derived amendments (**Table 1**), we observed that: (i) amendments from the conventional farm showed higher OM content than those from the organic farm; (ii) all pH values ranged between 8.2 and 9.4; (iii) slurry samples from both the conventional and organic farm showed higher N content, compared to fresh and aged manure; (iv) Pb, Cr, and Ni concentrations were higher in fresh and aged manure from the organic vs. the conventional farm; and (v) the following metal concentration gradient for Pb, Cr, and Ni was observed in the amendments from both the conventional and organic farm: aged manure > fresh manure > slurry.

Concerning antibiotic concentrations in the amendments and the semi-natural grassland soil (**Table 2**), in the first analysis, colistin was detected in fresh and aged manure from both the conventional and organic farm. Furthermore, marbofloxacin was detected in all the amendments from the conventional farm, as well as in the slurry from the organic farm. In the second analysis carried out 2 months later, only colistin was detected in the fresh manure from the conventional farm (**Table 2**), indicating a possible degradation of marbofloxacin.

Effect of Treatments on Crop Production

Pertaining to lettuce shoot biomass, higher values were found when the soil was amended with aged manure from both the conventional and organic farm, as well as with slurry from the conventional farm, compared to slurry from the organic farm, fresh manure from the conventional farm and the unamended control (**Table 3**).

As far as wheat production is concerned, no statistically significant ($p < 0.05$) differences among treatments were observed. In any case, the highest value of wheat grain weight was found in pots amended with aged manure from the organic farm.

TABLE 1 | Physicochemical properties of the amendments.

	Organic farm			Conventional farm		
	Aged manure	**Fresh manure**	**Slurry**	**Aged manure**	**Fresh manure**	**Slurry**
Dry matter (%)	29.87	21.74	7.30	17.51	18.51	12.12
OM (%)	41.33	67.48	74.62	78.30	80.20	83.41
pH (1:25)	9.41	9.29	8.40	8.48	9.16	8.25
N (%)	2.78	3.45	3.97	1.94	2.32	3.38
Olsen phosphorus (g kg^{-1})	5.61	8.12	6.36	3.65	4.83	6.66
Potassium (g kg^{-1})	37.21	33.56	43.43	16.67	22.06	25.03
Cd (mg kg^{-1} DW)	0.62	0.71	0.40	0.50	0.36	0.15
Pb (mg kg^{-1} DW)	150.88	36.95	17.59	4.14	3.31	1.55
Cr (mg kg^{-1} DW)	51.80	22.32	11.58	17.58	15.23	13.54
Ni (mg kg^{-1} DW)	27.16	11.05	6.07	8.93	8.25	7.69

TABLE 2 | Antibiotic concentrations in the amendments and the semi-natural grassland soil.

	Antibiotic (μg kg^{-1})	Organic farm			Conventional farm			
		Aged manure	**Fresh manure**	**Slurry**	**Aged manure**	**Fresh manure**	**Slurry**	**Soil**
First analysis	Colistin	470	230	<50	223	147	<50	<50
	Marbofloxacin	<5	<5	139	81	245	41.6	<5
Second analysis	Colistin	<50	<50	<50	<50	117	<50	<50

Antibiotics whose concentration did not exceed the detection limit are not included.

Effect of Treatments on Biological Parameters Related to the Resistome Risk

Regarding the absolute abundances of ARGs and MGE-genes in the amendments collected from the livestock farms (**Supplementary Table 1**), higher values were detected for aminoglycoside resistance genes, compared to all the other genes. In turn, lower values were observed for β-lactamase, vancomycin and multidrug resistance genes. No single amendment could be identified as the best or worst amendment according to the absolute abundances of ARGs and MGE-genes (**Supplementary Table 1**).

Out of the 95 ARGs and MGE-genes quantified here, 44 and 64 genes were detected in lettuce plants and lettuce soils, respectively (**Figure 1**). In addition, 5 and 25 genes were exclusively detected in lettuce plants and lettuce soils, respectively (i.e., lettuce plants and lettuce soils shared 39 genes) (**Figure 1**). Those five genes that were only detected in lettuce plants (and not in lettuce soils) encoded resistance to β-lactamase (one gene), MLSB (one gene), vancomycin (one gene) and tetracycline (2 genes). In turn, the 25 genes that were found only in lettuce soils (and not in lettuce plants) encoded resistance to FCA (one gene), tetracycline (3 genes), multidrug (3 genes), β-lactamase (4 genes), MLSB (4 genes), aminoglycosides (5 genes), and vancomycin (5 genes).

Values of ARG absolute abundances in lettuce soils ranged from 3.25×10^8 (for soil amended with slurry from the conventional farm) to 1.81×10^9 (for the unamended control soil) copies g^{-1} DW soil (**Supplementary Table 1**). In these lettuce soils, the absolute abundance of MGE-genes was higher than that of ARGs: from 1.27×10^{10} (for soil amended with fresh manure from the organic farm) to 8.76×10^{10} (for the unamended control soil) copies g^{-1} DW soil. In lettuce soils, integrase-related genes showed the highest absolute abundance values. By contrast, multidrug resistance genes presented the lowest absolute abundance values in lettuce soils (however, differences were not statistically significant). Furthermore, the lettuce unamended (control) soil showed higher absolute abundance values for vancomycin resistance genes, compared to all the other lettuce soils. In relation to the effect of the experimental variables (type and origin of amendment) on absolute abundance values in lettuce soils, the application of aged manure led to significantly higher absolute abundances of aminoglycoside resistance genes, compared to the application of slurry (**Supplementary Table 1**). Moreover, lettuce soils amended with aged manure showed higher absolute abundance values for tetracycline resistance genes, compared to lettuce soils amended with fresh manure or slurry.

In lettuce plants, the absolute abundance of ARGs ranged from 1.08×10^8 (for plants fertilized with slurry from the conventional farm) to 2.56×10^9 (for plants fertilized with fresh manure from the conventional farm) copies g^{-1} DW plant tissue (**Supplementary Table 1**). The absolute abundance of MGE-genes in lettuce plants ranged from 3.53×10^8 (for plants fertilized with slurry from the conventional farm) to 5.59×10^9 (for plants fertilized with fresh manure from the conventional farm) copies g^{-1} DW plant tissue. In lettuce plants, the absolute abundance of ARGs was higher in plants fertilized

TABLE 3 | Effect of treatments on lettuce (shoot biomass) and wheat production (grain weight).

	Organic farm			Conventional farm			
	Aged manure	**Fresh manure**	**Slurry**	**Aged manure**	**Fresh manure**	**Slurry**	**Unamended control**
LETTUCE							
Shoot biomass (g)	138.1 ± 12.7^{ab}	122.0 ± 3.9^{bc}	107.4 ± 15.7^{c}	142.2 ± 6.9^{a}	118.3 ± 14.5^{c}	142.7 ± 11.6^{a}	103.8 ± 14.4^{c}
WHEAT							
Grain weight (g)	6.4 ± 2.9^{ns}	5.6 ± 1.8^{ns}	6.2 ± 3.0^{ns}	5.9 ± 1.6^{ns}	4.3 ± 2.6^{ns}	6.0 ± 1.7^{ns}	4.3 ± 1.2^{ns}

Means (n = 4) and standard errors. Errors with different letters are significantly different ($p < 0.05$) according to Duncan's multiple range test. ns, non-significant.

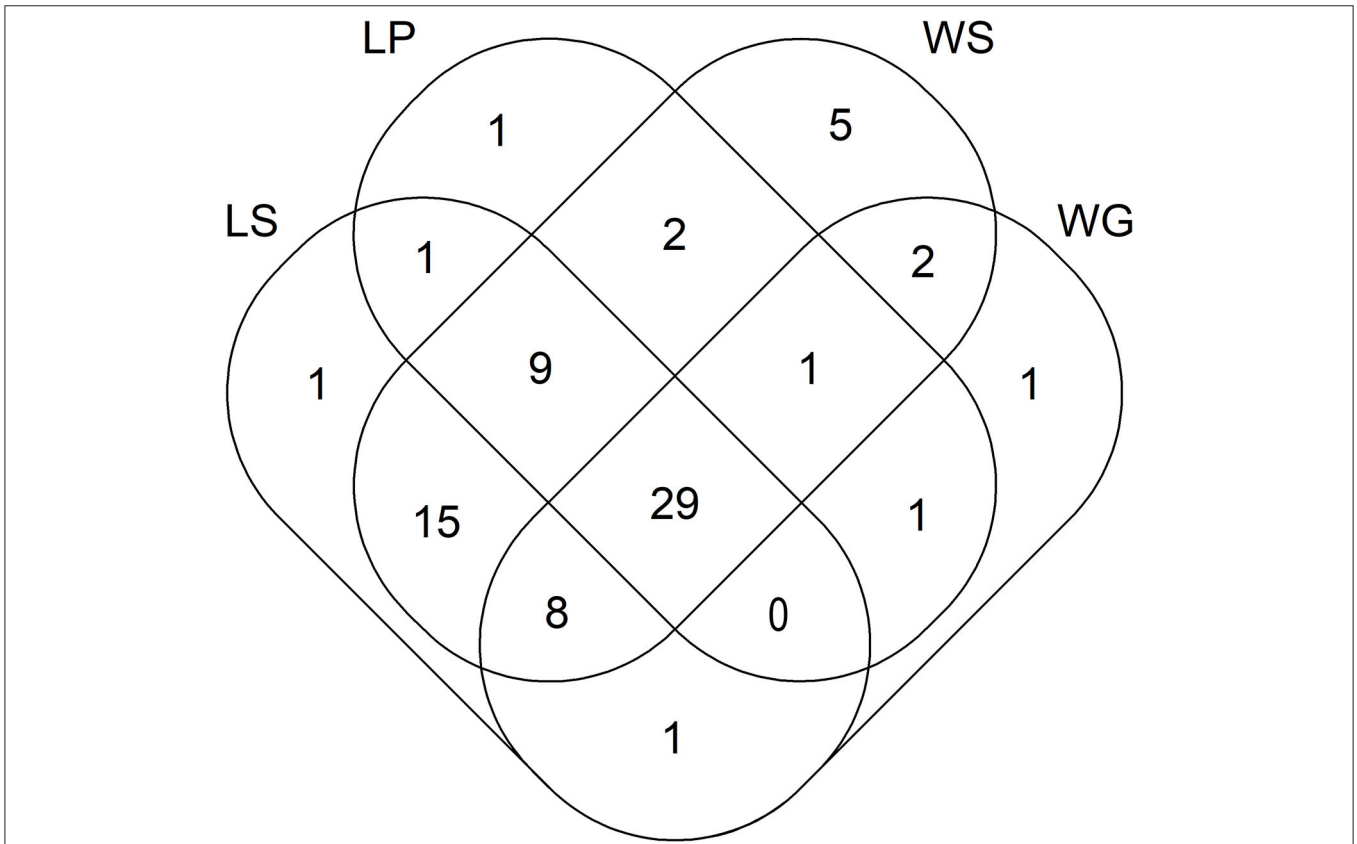

FIGURE 1 | Venn diagram showing the number of ARGs and MGE-genes for lettuce and wheat samples. LS, lettuce soil; LP, lettuce plant; WS, wheat soil; WG, wheat grain.

with fresh manure from the conventional farm, compared to all the other lettuce plants, except for the unamended control (**Supplementary Table 1**). Genes encoding resistance to β-lactamase, FCA, multidrug, tetracycline, and vancomycin showed lower absolute abundance values than genes encoding sulfonamide and transposase in lettuce plants. Lettuce plants fertilized with fresh manure from the conventional farm showed higher absolute abundance values for aminoglycoside resistance, tetracycline resistance and transposase-related genes than lettuce plants from the other treatments (except for aged manure from the conventional farm and the unamended control). Similarly, lettuce plants fertilized with amendments from the conventional farm exhibited higher absolute abundance values of tetracycline resistance and transposase related genes than those fertilized with amendments from the organic farm.

Regarding wheat, out of the 95 ARGs and MGE-genes quantified here, 43 and 71 genes were detected in wheat grains and wheat soils, respectively (**Figure 1**). In addition, 3 and 31 genes were exclusively detected in wheat grains and wheat soils, respectively (i.e., wheat grains and wheat soils shared 40 genes) (**Figure 1**). Specifically, three tetracycline-resistance genes were only detected in wheat grain (and not in wheat soil). In turn, the 31 genes that were found only in wheat soil (and not in wheat grain) encoded resistance to multidrug (2 genes),

aminoglycosides (3 genes), MLSB (3 genes), vancomycin (7 genes), β-lactamase (8 genes), and tetracycline (8 genes). The absolute abundance of ARGs in wheat soils ranged from 1.50×10^{10} (for wheat soil amended with slurry from the conventional farm) to 7.64×10^{10} (for wheat soil amended with fresh manure from the organic farm) copies g^{-1} DW soil (**Supplementary Table 1**). In these wheat soils, the absolute abundance of MGE-genes ranged between 3.03×10^{11} (for wheat soils amended with slurry from the conventional farm) to 1.17×10^{12} (for wheat soils amended with fresh manure from the organic farm) copies g^{-1} DW soil. On the other hand, the absolute abundance of ARGs in wheat grains ranged from 4.04×10^{9} (for wheat fertilized with fresh manure from the conventional farm) to 1.47×10^{10} (for wheat fertilized with slurry from the organic farm) copies g^{-1} DW grain. The absolute abundance of MGE-genes in wheat grains ranged from 8.74×10^{10} (for wheat fertilized with aged manure from the organic farm) to 2.33×10^{11} (for control unamended pots) copies g^{-1} DW grain. Wheat soils amended with fresh manure from both livestock farms showed higher absolute abundance values of aminoglycoside resistance genes, compared to wheat soils amended with slurry (**Supplementary Table 1**). Likewise, higher absolute abundance values of aminoglycoside, MLSB and vancomycin resistance genes were detected in wheat soils supplemented with amendments from the organic vs. conventional farm. Wheat grains grown with amendments from the organic farm exhibited higher absolute abundance values of FCA resistance genes, compared to those from pots treated with amendments from the conventional farm.

Figure 2 represents ARG and MGE-gene absolute abundances grouped by antibiotic family and MGE category for all soil and plant samples. The PCA clearly separated three clusters: (i) wheat soils; (ii) wheat grains; and (iii) lettuce soils and plants. The first axis (PC1) accounted for 75.2% of the total variance and showed negative loadings for the following absolute abundances: aminoglycoside, β-lactamase, FCA, integrase, MLSB, sulfonamide, tetracycline, and transposase genes. In addition, PC2 accounted for 16.1% of the total variance and showed positive loading for multidrug and negative loading for vancomycin genes.

No statistically significant differences were found among treatments for both lettuce and wheat data (soil and plant data) in relation to the relative abundances of ARGs and MGE-genes grouped by antibiotic family and MGE category (**Supplementary Figures 1**, **2**).

Regarding the impact of treatments on soil prokaryotic diversity in lettuce soils, as reflected by Illumina MiSeq sequencing data, 73.1, 53.4, and 22.0% of the reads were taxonomically classified to order, family and genus rank, respectively. Concerning wheat soils, 67.6, 51.1, and 20.4% of the reads were classified to order, family and genus rank, respectively. Statistically significant differences were found in 15 and 3 orders in lettuce and wheat soils, respectively (**Supplementary Table 2**). For lettuce soils, out of these 15 orders, the following belong to the 30 most abundant orders detected in those soils: *Cytophagales*, *SC-I-84*, *Pseudonocardiales*, *Solirubrobacterales*, *C0119*, *KD4-96,* and *Nitrososphaerales* (**Supplementary Figure 3**). Similarly, out of the abovementioned three orders in wheat soils, the following two belong to the 30 most abundant orders: *Rhodospirillales* and *Desulfurellales* (**Supplementary Figure 4**).

Data on the impact of treatments on soil prokaryotic α-diversity are shown in **Table 4**. Lettuce soils amended with slurry from the conventional farm showed higher richness than those amended with slurry from the organic farm (and also higher richness, compared to the untreated control soil). Moreover, Shannon's diversity was lower in soils amended with aged manure for the organic farm and the unamended control soil, compared to all the other soils. In wheat soils, higher richness values were observed in soil amended with aged manure from the conventional farm, compared to soil amended with fresh manure and slurry from the organic farm (**Table 4**).

In lettuce soils, Kendall's rank correlation coefficients showed significant correlations (positive and negative) among 43 orders and 7 ARG and 2 MGE-gene absolute abundances grouped by antibiotic family and MGE category (**Supplementary Table 2**). Among these 43 orders, the following five presented multiresistance: *Micrococcales*, *Pseudonocardiales*, *Rhizobiales, Rubrobacterales,* and *Solirubrobacterales* (**Supplementary Table 2**). The orders *Micrococcales*, *Pseudonocardiales, Rhizobiales,* and *Solirubrobacterales* appeared in the list of the 30 most abundant orders in lettuce soils (**Supplementary Figure 3**). The order *Pseudonocardiales* was positively correlated with genes encoding resistance to MLSB, tetracycline and vancomycin (**Supplementary Table 2**). The lettuce unamended soil showed higher abundance of *Pseudonocardiales* than the other soils (**Supplementary Table 3**). Fifteen orders showed, at least, two negative correlations with ARG and MGE-gene absolute abundances (**Supplementary Table 2**).

In wheat soils, Kendall's rank correlation coefficients showed significant correlations (positive and negative) among 14 orders and 6 ARG and 2 MGE-gene absolute abundances grouped by antibiotic family and MGE category (**Supplementary Table 2**). Among these 14 orders, the following three presented multiresistance: *Limnochordales*, *Tepidisphaerales,* and *WN-HWB-116* (**Supplementary Table 2**).

As far as differences between lettuce and wheat pots, wheat soil and grain samples showed higher absolute abundances of ARGs and MGE-genes than lettuce soil and plant samples (**Supplementary Table 4**). In terms of absolute abundances, the highest number of statistically significant differences between lettuce and wheat soils was observed in soils amended with fresh manure from the organic farm.

DISCUSSION

The incorporation of organic amendments into agricultural soil as fertilizers often increases soil OM content (39) and fertility, and results in an overall improvement of soil quality (8). In particular, organic farming practices promote the maintenance and enhancement of soil OM and fertility by means of the application of farmyard manure and similar

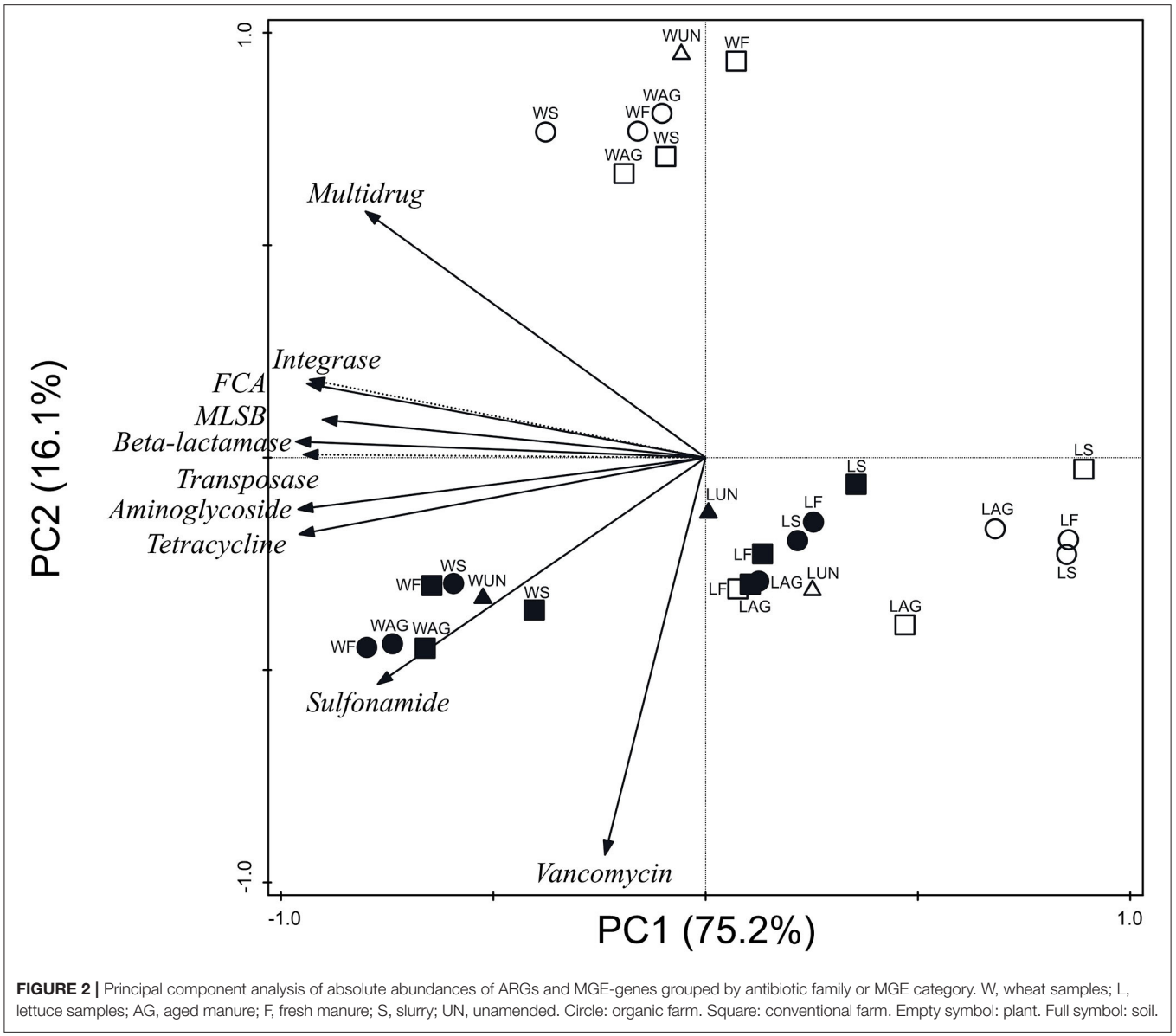

FIGURE 2 | Principal component analysis of absolute abundances of ARGs and MGE-genes grouped by antibiotic family or MGE category. W, wheat samples; L, lettuce samples; AG, aged manure; F, fresh manure; S, slurry; UN, unamended. Circle: organic farm. Square: conventional farm. Empty symbol: plant. Full symbol: soil.

organic amendments. In Europe, the area under organic farming increased from 10.0 million hectares in 2012 to 13.4 million hectares in 2018 (Eurostat Statistics for Organic Farming). Despite the abovementioned well-recognized benefits, there is increasing concern about the use of manure-derived amendments as organic fertilizers since their application entails a variety of environmental risks such as, for instance, the emergence, maintenance and dissemination of AR in agricultural soils and crops (6, 17, 40). The application of manure-derived amendments to agricultural soil can also lead to pronounced changes in the diversity and composition of soil microbial communities (41), with potential concomitant alterations of soil functioning. We hypothesized that the resistome risk would be higher in soils and plants amended with animal wastes from conventional livestock farming vs. organic livestock farming (after all, the administration of antibiotics to animals raised under organic farming is limited by regulations). Nonetheless, such hypothesis is not supported by the results of our study. Actually, even regarding the concentration of antibiotics in the amendments collected from the organic vs. conventional farm, no clear differences were observed, which could be due to the fact that organic farms do apply antibiotics in some specific cases, e.g., during a long-term mastitis.

As described above, a large proportion (30–90%) of the antibiotics administered to livestock are not fully metabolized and are then excreted, together with their transformation products, into the environment along with the feces and urine (5). The amount and rate of antibiotic excretion varies greatly among animal species and age (42, 43), type and dosage of antibiotic, form of administration, etc. (44). As an example, the following concentrations (mg kg^{-1}) have been reported for dairy cow manure: 0.43–2.69 for tetracycline,

TABLE 4 | Effect of treatments on soil prokaryotic diversity.

Lettuce soil	Richness	Shannon's	Simpson's	Pielou's
ORG_AG	3626 ± 200^{cd}	6.78 ± 0.04^{b}	$0.997 \pm 5.2E\text{-}04^{ns}$	0.811 ± 0.015^{ns}
ORG_FRES	3894 ± 48^{ab}	7.03 ± 0.04^{a}	$0.998 \pm 2.9E\text{-}04^{ns}$	0.815 ± 0.005^{ns}
ORG_SLU	3722 ± 65^{bc}	6.99 ± 0.03^{a}	$0.998 \pm 5.2E\text{-}05^{ns}$	0.814 ± 0.003^{ns}
CONV_AG	3774 ± 73^{abc}	6.98 ± 0.02^{a}	$0.998 \pm 9.2E\text{-}05^{ns}$	0.825 ± 0.015^{ns}
CONV_FRES	3784 ± 33^{abc}	7.00 ± 0.03^{a}	$0.998 \pm 1.4E\text{-}04^{ns}$	0.813 ± 0.009^{ns}
CONV_SLU	3941 ± 149^{a}	7.05 ± 0.06^{a}	$0.998 \pm 2.9E\text{-}04^{ns}$	0.815 ± 0.007^{ns}
UNAMEN	3550 ± 95^{d}	6.89 ± 0.04^{bc}	$0.997 \pm 1.1E\text{-}04^{ns}$	0.808 ± 0.002^{ns}
Wheat soil	**Richness**	**Shannon's**	**Simpson's**	**Pielou's**
ORG_AG	4517 ± 85^{ab}	7.04 ± 0.04^{ns}	$0.998 \pm 2.0E\text{-}04^{ns}$	0.817 ± 0.010^{ns}
ORG_FRES	4285 ± 176^{b}	6.95 ± 0.07^{ns}	$0.997 \pm 3.1E\text{-}04^{ns}$	0.810 ± 0.005^{ns}
ORG_SLU	4268 ± 259^{b}	6.91 ± 0.12^{ns}	$0.997 \pm 3.6E\text{-}04^{ns}$	0.811 ± 0.014^{ns}
CONV_AG	4710 ± 53^{a}	7.08 ± 0.03^{ns}	$0.998 \pm 1.8E\text{-}04^{ns}$	0.817 ± 0.005^{ns}
CONV_FRES	4530 ± 98^{ab}	7.00 ± 0.04^{ns}	$0.997 \pm 2.1E\text{-}04^{ns}$	0.818 ± 0.010^{ns}
CONV_SLU	4510 ± 289^{ab}	7.01 ± 0.14^{ns}	$0.998 \pm 4.3E\text{-}04^{ns}$	0.814 ± 0.010^{ns}
UNAMEN	4441 ± 47^{ab}	6.95 ± 0.04^{ns}	$0.997 \pm 3.0E\text{-}04^{ns}$	0.805 ± 0.008^{ns}

Means (n = 4) and standard errors. Errors with different letters are significantly different ($p < 0.05$) according to Duncan's multiple range test. ORG_AG, aged manure form organic farm; ORG_FRES, fresh manure from organic farm; ORG_SLU, slurry from organic farm; CONV_AG, aged manure from conventional farm; CONV_FRES, fresh manure from conventional farm; CONV_SLU, slurry from conventional farm; UNAMEN, unamended control. ns, non-significant.

0.21–10.37 for oxytetracycline, 0.61–1.94 for chlortetracycline, 0.22–1.02 for sulfamethoxazole, 0.43–1.76 for norfloxacin and 0.46–4.17 for enrofloxacin (45, 46). On the other hand, once introduced into the soil matrix, antibiotics are susceptible to a variety of processes, such as adsorption, microbial transformation, photodegradation, plant uptake, sequestration, transport (leaching, runoff), etc. (13, 42, 47, 48). In contrast with other studies (49–51), macrolides, sulphonamides, and tetracyclines were not detected in any of the amendments studied here. Actually, in the first analysis, out of the 57 antibiotics analyzed here, only colistin and marbofloxacin were detected. In the second analysis, only colistin (117 $\mu g\ kg^{-1}$) was detected in one of the amendments, i.e., fresh manure from the conventional farm. Nonetheless, we did find genes encoding resistance to those antibiotics in the amendments, which could be due to the fact that: (i) the antibiotics were already completely degraded but the ARGs persisted in the amendments despite the absence of the antibiotics; (ii) antibiotic transformation products, still capable of bioactive effect, are responsible for the induction of the emergence of ARGs in the amendments (42); and/or (iii) although antibiotic concentrations in the amendments were below the detection limit of the technique, sub-inhibitory concentrations result in an enough level of selective pressure to induce AR (52). Furthermore, antibiotic sub-inhibitory concentrations are known to induce horizontal gene transfer (53), which could spread ARGs among different bacterial populations. Interestingly, some studies (40, 54) have reported an increase in AR in soils amended with manure from animals that had not been subjected to any antibiotic treatment.

In our study, the amendment that showed the highest absolute abundances of ARGs and MGE-genes was the slurry from the conventional farm, but this highest resistome risk was then not reflected, as one would expect, in those soils and crops amended with such slurry. Actually, despite the fact that the slurry from both livestock farms presented greater values of absolute abundance for transposase, aminoglycoside, MLSB, tetracycline and multidrug resistance genes (compared to the other amendments), lettuce soils amended with such slurry showed a lower resistome risk than when fertilized with the other amendments. Also, the absolute abundances for aminoglycoside resistance genes were lower in wheat soils amended with slurry vs. fresh and aged manure. Remarkably, within the same treatment, the resistome risk differed between the amendment, the amended soil and, finally, the crop. In other words, according to our data, the resistome risk in manure-amended crops cannot be directly inferred from the analysis of the amendments themselves. Although aging and composting are both effective processes (composting is certainly more effective than aging in this respect) for reducing the concentration of antibiotics and the total abundance of ARGs, the trend in some ARGs is highly gene-specific (55). In our case, the manure was not composted following a controlled procedure, but simply aged for approximately 6 months. In any case, dairy fresh manure from both the conventional and the organic farm presented higher absolute abundances of *intI1*, *sul2,* and 7 tetracycline-resistance than those reported in previous studies (56–58).

On the other hand, slurry samples from both livestock farms showed the lowest metal concentrations, compared to aged and fresh manure. The *co-selection* of antibiotic and metal resistance in bacteria, due to *co-resistance* (when two or more different resistance genes are located on the same genetic element, e.g., a plasmid or a transposon) or *cross-resistance* (when a single mechanism confers resistance to both antibiotics and

metals, e.g., an efflux pump) mechanisms, is widely known (59–62). Moreover, *co-regulatory mechanisms* (when genes that confer resistance to different compounds are controlled by a single regulatory gene) can promote antibiotic-metal co-selection processes. In this respect, Perron et al. (46) reported that the regulatory protein CzcR regulates (i) the expression of the CzcCBA efflux pump, which confers resistance to Zn, Cd and Co; and (ii) the expression of the OprD porin, the route of entry of carbapenems in bacteria. This co-selection phenomenon is of the utmost importance as it can be responsible for the maintenance and dissemination of AR in the absence of antibiotics. In our study, as abovementioned, the values of ARG and MGE-gene absolute abundances were lower in aged and fresh manure than in slurry, but it is possible that the higher metal concentrations detected in aged and fresh manure (vs. slurry) could induce the spread of ARGs once applied to the agricultural soil.

Overall, the values of absolute abundance of ARGs and MGE-genes were higher in soil vs. plant samples, in agreement with previous studies (63, 64). Soils are important reservoir of ARGs (16, 65). In any event, the typology of ARGs found in lettuce and wheat grains was robustly dependent on the typology of ARGs observed in the corresponding soil. Relevantly, much higher absolute abundances of MGE-genes vs. ARGs were detected in both soil and plant samples, pointing out to a high potential risk of dissemination of AR in the studied soils and crops. In addition to the physical contact and interactions among the plants, the soil and the amendments, in some cases, the water used for irrigation is another factor to be considered, as it might be contaminated with ARGs (66). However, in our study, this is not a relevant factor since the same tap water was used to irrigate all the treatments.

Furthermore, we found higher ARG and MGE-gene absolute abundances in wheat vs. lettuce soils. Plants are known to regulate rhizosphere microbial communities through the excretion of root exudates (67). The composition and quantity of root exudates greatly vary depending on the specific plant species and its physiological status (68, 69). The type of crop (lettuce vs. wheat), dose of amendment (here adjusted to 100 vs. 180 kg N ha^{-1} for lettuce and wheat plants, respectively), duration of plant growth until harvest (44 vs. 171 days for lettuce and wheat plants, respectively), type of root system (pivotant vs. fasciculate for lettuce and wheat plants, respectively), and the amount and composition of the rhizodeposition are all factors that can affect the composition of soil microbial communities and the fate and distribution of ARGs and MGE-genes in agricultural soils. No significant differences were observed, in terms of the absolute abundances of ARGs and MGE-genes, between unamended lettuce soils and unamended wheat soils (neither between lettuce and wheat grain samples), which indicates that the amendment application was responsible for the observed differences among treatments.

Although Zhang et al. (64) observed higher ARG abundances in manure-amended lettuce soils (the abundance of ARGs ranged from 4.37×10^9 to 2.02×10^{10} in soils), compared to ours, the transfer of those ARGs from the lettuce soil to the lettuce was approximately between one and two orders of magnitude higher in our study (the abundance of ARGs ranged from 7.45×10^6 to 8.24×10^7 in plant samples). In lettuce soils, the unamended control showed the highest abundance of vancomycin resistance genes. Antibiotic resistance genes have not only been found in antibiotic-free soil (70) but also in environments (e.g., permafrost, isolated caves) that have remained isolated from the impact of anthropic activity much before the beginning of the use of antibiotics for the preventive and curative treatment of bacterial infectious diseases in medicine and veterinary (71, 72).

Regarding the possible links between the presence of certain prokaryotic taxa and AR profiles, the order *Pseudonocardiales* presented a positive correlation with vancomycin resistance genes. Several strains belonging to *Pseudonocardiales* are known to produce biologically active products, such as erythromycin, rifamycin, and vancomycin (73). The unamended control soil showed significantly higher abundance of *Pseudonocardiales* than the other treated soils (and, as already mentioned, the unamended lettuce soil showed the highest abundance of vancomycin resistance genes). In general, the unamended lettuce soil showed lower abundances of those orders negatively correlated with vancomycin resistance genes (*Cytophagales*, *Obscuribacterales,* and *SAR324*), compared to the other treated soils. Many authors (74, 75) have reported that changes in the composition of prokaryotic communities appear to be the key drivers for the magnitude and profile of the antibiotic resistome. The values of bacterial richness and Shannon's diversity detected in the unamended control soil were significantly lower than those observed in the other soils (except for soils amended with aged manure from the organic farm). These data suggest that vancomycin resistance genes most likely did not enter the soil matrix through the application of the amendments, but that they existed previously in such soil. Chaudhry et al. (76) found that the application of amendments to soil could lead to an increase of (i) overall bacterial diversity; and (ii) the dominance of certain bacterial taxa which could then play important roles in a variety of soil processes. Highly diverse soil microbial communities can, for instance, act as a biological barrier against biological invasion (77). The decline in microbial diversity has often been related to a loss of ecosystem multi-functionality (78).

CONCLUSIONS

Despite our initial hypotheses, no single treatment could be identified as the best or worst treatment regarding the risk of antibiotic resistance in soil and plant samples. Interestingly, within the same treatment, the resistome risk differed between the amendment, the amended soil and, finally, the crop. In other words, according to our data, the resistome risk in manure-amended crops cannot be directly inferred from the analysis of the amendments themselves. Then, we concluded that, depending on the specific question under study, the analysis of the resistome risk should specifically focus on the amendment, the amended soil or the crop. In any case, our results confirm the risk of AR dissemination

in agricultural settings where dairy cow manure-derived amendments are used as fertilizers. In this respect, much higher absolute abundances of MGE-genes vs. ARGs were detected in both soil and plant samples, pointing out to a high potential risk of dissemination of AR in the studied soils and crops.

DATA AVAILABILITY STATEMENT

The dataset presented in the study are publicly available. This data can be found here: https://www.ebi.ac.uk/ena, PRJEB41541.

AUTHOR CONTRIBUTIONS

LE, IA, and CG designed the study. LJ and LE performed the analytical work and data treatment. LJ, LE, IA, and CG wrote the manuscript. All authors revised the final version of 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/fvets.2021.633858/full#supplementary-material

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Genomic Insights of Multidrug-Resistant *Escherichia coli* From Wastewater Sources and Their Association With Clinical Pathogens in South Africa

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There is limited information on the comparative genomic diversity of antibiotic-resistant *Escherichia coli* from wastewater. We sought to characterize environmental *E. coli* isolates belonging to various pathotypes obtained from a wastewater treatment plant (WWTP) and its receiving waters using whole-genome sequencing (WGS) and an array of bioinformatics tools to elucidate the resistomes, virulomes, mobilomes, clonality, and phylogenies. Twelve multidrug-resistant (MDR) diarrheagenic *E. coli* isolates were obtained from the final effluent of a WWTP, and the receiving river upstream and downstream of the WWTP were sequenced on an Illumina MiSeq machine. The multilocus sequence typing (MLST) analysis revealed that multiple sequence types (STs), the most common of which was ST69 ($n = 4$) and ST10 ($n = 2$), followed by singletons belonging to ST372, ST101, ST569, ST218, and ST200. One isolate was assigned to a novel ST ST11351. A total of 66.7% isolates were positive for β-lactamase genes with 58.3% harboring the bla_{TEM1B} gene and a single isolate the $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-55}}$ extended-spectrum β-lactamase (ESBL) genes. One isolate was positive for the *mcr*-9 mobilized colistin resistance gene. Most antibiotic resistance genes (ARGs) were associated with mobile genetic support: class 1 integrons (In22, In54, In191, and In369), insertion sequences (ISs), and/or transposons (Tn402 or Tn21). A total of 31 virulence genes were identified across the study isolates, including those responsible for adhesion (*lpfA, iha,* and *aggR*), immunity (*air, gad,* and *iss*), and toxins (*senB, vat, astA,* and *sat*). The virulence genes were mostly associated with IS (IS1, IS3, IS91, IS66, IS630, and IS481) or prophages. Co-resistance to heavy metal/biocide, antibiotics were evident in several isolates. The phylogenomic analysis with South African *E. coli* isolates from different sources (animals, birds, and humans) revealed that isolates from this study mostly clustered with clinical isolates. Phylogenetics linked with metadata revealed that isolates did not cluster according to source but according to ST. The occurrence of pathogenic and MDR isolates in the WWTP effluent and the associated river is a public health concern.

Keywords: whole-genome sequencing, antibiotic resistance, *Escherichia coli*, wastewater treatment plant, sequence types, phylogenomic analysis, public health, river water

INTRODUCTION

The role of the environment in the spread of antibiotic resistance is an evolving issue (1). Wastewater treatment plants (WWTPs) have received a lot of attention because of the central role they play in reducing pollutant loads that include antibiotic-resistant bacteria (ARB), antibiotic resistance genes (ARGs), virulence genes, and their associated mobile genetic elements to acceptable limits before the discharge of treated effluent into receiving water bodies.

With inadequately maintained sanitation infrastructure, low- and middle-income countries (LMICs) and emerging economies like South Africa face challenges with the release of untreated or poorly treated effluent into the environment, which may be a driver for the dissemination of antibiotic resistance in these settings (2). Constant monitoring of WWTPs for the release of multi-drug resistant (MDR) bacteria into receiving waters *via* their effluents is important as it indicates what is disseminated to the environment.

The WWTP investigated in this study is the largest in Pietermaritzburg, the provincial capital of KwaZulu-Natal in South Africa. Runoff from this WWTP is released into the Msunduzi River, a tributary that ultimately discharges into the Umgeni River (3). Upstream of the WWTP, the Msunduzi River receives runoff from rural communities, agricultural areas, urban municipalities (including several hospitals and community health centers), and numerous informal settlements along the river (4). The surface water is a key water source for domestic, agricultural, and recreational purposes to inhabitants of the several informal settlements along its banks. The river water has previously been considered to be polluted with fecal matter and unsuitable for anthropogenic activities (4).

Diarrheagenic *Escherichia coli* pathotypes are a public health concern (5). Pathogenic MDR *E. coli* that affects humans and animals have been reported in the water environment (6–8). However, studies that employ sequencing technologies to investigate environmental *E. coli* or any other bacteria are rare in Africa, including in South Africa. Consequently, there is little information regarding environmental isolates and their association with other isolates from clinical and agricultural sources. We sought to compare the genomics of MDR environmental *E. coli* isolates belonging to various pathotypes obtained from a WWTP and its receiving waters using whole-genome sequencing (WGS) and bioinformatics tools in terms of their lineages, resistomes, virulomes, mobilomes, clonality, and phylogenies to determine associations/correlations with clinical, animal, and environmental isolates.

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 and Sample Description

A longitudinal antibiotic resistance surveillance study was undertaken in the uMgungundlovu District, one of 11 districts in the coastal province of KwaZulu-Natal, South Africa. Water samples were collected fortnightly for 7 months from May to November 2018 at the largest urban WWTP in the district. Manual grab water samples were collected in sterile 500-ml containers according to Kalkhajeh et al. (9), upstream (29°36′10.73″S and 30°25′29.97″E), downstream (29°36′27.54″S 30°27′0.76″E), and from the influent (29°36′3.70″S 30°25′41.71″E) and final effluent (29°35′49.97″S 30°26′19.74″E) of the WWTP.

Bacterial Identification

A total of 580 *E. coli* isolates were putatively identified during enumeration using the Colilert®-18 Quanti-Tray® 2000 system, followed by phenotypic confirmation on eosin methylene blue (EMB) agar. Briefly, before analysis, bottles containing the water samples were thoroughly mixed and then serially diluted using 10-fold dilutions. Samples from upstream and downstream river water as well as final effluent were diluted 1 ml in 100 ml (0.01 dilution) using sterile water. The influent samples were also diluted by 0.05 ml in 100 ml (0.0005 dilution) using sterile water. The 100 ml from each sample was then analyzed using the Colilert®-18 Quanti-Tray® 2000 System (IDEXX Laboratories (Pty) Ltd., Johannesburg, South Africa). *E. coli* was obtained from positive Quanti-Trays, subcultured on EMB (Merck, Darmstadt, Germany) and incubated at 37°C for 18–24 h. At least 10 distinct colonies representing each sampling site were randomly selected from the EMB and further subcultured onto the same medium to obtain pure colonies. Molecular confirmation of the selected *E. coli* isolates was accomplished using real-time PCR targeting the *uidA* (β-D-glucuronidase) gene, as was the delineation of *E. coli* into various diarrheagenic pathotypes [i.e., enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), and enteroinvasive *E. coli* (EIEC)]. All reactions included a no-template control consisting of the reaction mixture. The real-time PCR protocol was done according to Mbanga et al. (10). The primers, virulence genes, and reference strains used to determine pathotypes are shown in **Supplementary Table 1**. The WGS study sample consisted of a subset of 12 MDR diarrhoeagenic isolates obtained from the upstream, downstream, and effluent sites over the study period. The selection of isolates was based on their antibiograms and pathotypes.

Antimicrobial Susceptibility Testing

The resistance of *E. coli* to a panel of 20 antibiotics was determined through the disk diffusion assay, and the results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria (11). The panel consisted of amikacin (AMK, 30 μg), ampicillin (AMP, 10 μg), azithromycin (AZM, 15 μg), amoxicillin–clavulanic acid (AMC, 30 μg), cefepime (FEP, 10 μg), cefotaxime (CTX, 30 μg), cefoxitin (FOX, 30 μg), ceftazidime (CAZ, 30 μg), ceftriaxone (CRO, 30 μg), cephalexin (LEX, 30 μg), ciprofloxacin

(CIP, 5 μg), chloramphenicol (CHL, 30 μg), gentamicin (GEN, 10 μg), imipenem (IPM, 10 μg), meropenem (MEM, 10 μg), nalidixic acid (NAL, 30 μg), piperacillin–tazobactam (TZP, 110 μg), tetracycline (TET, 30 μg), tigecycline (TGC, 15 μg), and trimethoprim–sulfamethoxazole (SXT, 25 μg) (Oxoid Ltd., Basingstoke, UK). Breakpoints for AZM, TET, and NAL were obtained from the Clinical and Laboratory Standards Institute (CLSI) interpretative charts (12). Colistin resistance was undertaken using the broth microdilution assay to determine the colistin minimum inhibitory concentration (MIC). The results were interpreted according to the EUCAST guidelines (11). *E. coli* ATCC 25922 was used as the control.

Whole-Genome Sequencing and Bioinformatic Analysis

The genomic DNA was extracted from the *E. coli* isolates using the GenElute Bacterial Genomic DNA Kit (Sigma Aldrich, St. Louis, MO, USA) following the instructions of the manufacturer before quantification using the 260/280 nm wavelength on a Nanodrop 8000 (Thermo Fisher Scientific Waltham, MA, USA). Library preparation was done using the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA) followed by WGS using an Illumina MiSeq Machine (Illumina, USA). Quality trimming of raw reads was done using Sickle v1.33 (https://github.com/najoshi/sickle). The raw reads were then assembled spontaneously using the SPAdes v3.6.2 assembler (https://cab.spbu.ru/software/spades/). All contiguous sequences were subsequently submitted to GenBank and assigned accession numbers (**Supplementary Table 2**) under BioProject PRJNA609073.

The assembled genomes were analyzed for multilocus sequence typing (MLST) sequence types (STs) on the MLST 1.8 database hosted by the Center for Genomic Epidemiology (CGE) (http://cge.cbs.dtu.dk/services/MLST/). Isolates without STs were submitted to the EnteroBase *Escherichia*/*Shigella* database (https://enterobase.warwick.ac.uk/species/index/ecoli) and assigned novel STs.

Mutations conferring resistance to fluoroquinolones were determined from the assembled genomes using BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch). Briefly, DNA gyrase (*gyrA* and *gyrB*) and DNA topoisomerase IV (*par*C and *parE*) genes and the reference strain *E. coli* ATCC 25922 (Accession number: CP009072) were aligned with the genomes of this study using BLASTN. The mutations in the isolate of the genomes of this study were manually curated and tabulated.

Plasmid replicons types were identified using PlasmidFinder 2.1 on the CGE website (https://cge.cbs.dtu.dk/services/PlasmidFinder/). The assembled genomes were further analyzed for mobile genetic elements (MGEs), including insertion sequences using ISFinder (https://isfinder.biotoul.fr/) and intact prophages using PHASTER (https://phaster.ca/). RAST SEEDVIEWER (https://rast.nmpdr.org/seedviewer.cgi) was also used to annotate and identify the investigated genomes for integrons. Virulence genes were assayed using VirulenceFinder 2.0 on the CGE website (https://cge.cbs.dtu.dk/services/VirulenceFinder/). The synteny and genetic environment of ARGs and associated MGEs was investigated using the general feature format (GFF3) files from GenBank. The genetic environment of virulence genes detected in the study was also determined using a similar approach. The GFF files were imported into Geneious Prime 2020.2 (https://www.geneious.com) for analysis.

Phylogenomic Analyses of the *E. coli* Isolates ($n = 12$) and Isolates From South Africa

Whole-genome sequences of all isolates were uploaded and analyzed on the CSI Phylogeny 1.4 pipeline (https://cge.cbs.dtu.dk/services/CSIPhylogeny/). CSI Phylogeny recognizes, screens, and validates the location of single-nucleotide polymorphisms (SNPs), before deducing a phylogeny founded on the concatenated alignment of the high-quality SNPs. Selection of SNPs was based on default parameters on the CSI Phylogeny, which included: a minimum distance of 10 bp between each SNP, a minimum of 10% of the average depth, mapping quality was above 25, SNP quality above 30, and all insertions and deletions (INDELs) were excluded. The *Morganella morganii* subsp. *morganii* KT genome (Accession number: CP004345.1) served as the outgroup to root the tree enabling the easy configuration of the phylogenetic distance between the isolates on the branches. The phylogeny was visualized with annotations for isolate information and *in-silico* typing (ST) metadata using Phandango (https://jameshadfield.github.io/phandango/#/main) to provide insights into the generated tree.

Additionally, WGS of *E. coli* isolates from South Africa curated at the PATRIC website (https://www.patricbrc.org/) were downloaded and used alongside the isolates of this study for the whole-genome phylogeny analysis to ensure a current epidemiological and evolutionary analysis (Dataset 1). The generated phylogenetic trees were visualized, annotated, and edited using iTOL (https://itol.embl.de/) and Figtree (http://tree.bio.ed.ac.uk/software/figtree/). Isolates of the same host (human, animal, or bird) or from the environment were highlighted with the same color.

RESULTS

Isolate Characteristics

The 12 *E. coli* isolates investigated in this study were obtained from the WWTP and its associated waters. Seven isolates were from the downstream site, four were from the upstream site, and one isolate was obtained from the final effluent. The isolates belonged to the diarrheagenic group of *E. coli*; seven were EAEC, three were EIEC, with one EHEC, and one EPEC (**Supplementary Table 2**).

Antibiotic Susceptibility

The 12 isolates had varying phenotypic resistance patterns, with most being resistant to AMP (83.3%), SXT (75%), and TET (66.7%) (**Table 1**). Some isolates had the same resistance profiles but were isolated at different times (months) from different sampling points. The resistance profiles AMP—TET–NAL–SXT,

TABLE 1 | Source, sequence types (STs), antibiograms, resistance genes, virulence genes, and mobile genetic elements found in the *Escherichia coli* isolates.

Isolate 1D	MLST	Source	Antibiogram	ESBL	ARGs	Integrons	Cassette arrays				Transposons	Virulence genes	Plasmid replicons
							GC1	GC2	GC3	GC4			
D18/5	ST372	Downstream	AMP-TET-NAL-SXT	+	*blaTEM-1B, dfrA7, sul1, sul2, tet(A), mdf(A), aph(3")-Ib, aph(6)-Id*	*In22*	*sul1*	*qacEΔ1dfrA7*	-	-	-	*vat, gad, iss*	IncFIA, IncFIB IncFII (pRSB107), IncQ1
D47/7	ST69	Downstream	AMP-TET-AZM-SXT	+	*blaTEM-1B, dfrA1, dfrA14, dfrA17, sul1, sul2, tet(A), mdf(A), mph(A), aadA1, aadA5, aph (3")-Ib, aph (6)-Id, qnrB19, qnrS*	*In54 In191 In369*	*sul1 dfrA14 - dfrA1*	*qacEΔ1aadA5* - -	*dfrA17* - '-	-	-	*eilA,lpfA,nfaE, air,gad,iss,*	Col156, Col440I, ColpVC, IncFIB, IncFII, IncFII(pCoo)
D64/7	ST101	Downstream	AMP-TET-SXT	-	*tet(A), tet(M), mdf(A)*	*In0*	-	-	-	-	Tn402 (Tn5090)	*lpfA, gad, iss*	IncFII
D69/7	ST218	Downstream	AMP-AMC-SXT	-	*mdf(A)*	-	-	-	-	-	-	*pet, aap, aatA, iha, iss, mchB, mchC, mchF, capU*	IncFII, IncFII(pCoo)
D77/8	ST200	Downstream	TET-NAL-CIP-SXT	+	*blaTEM-1B, dfrA7, sul1, sul2, mdf(A), aph(3")-Ib,aph(6)-Id*	*In22*	*sul1*	*qacEΔ1dfrA7*	-	-	Tn21	*astA, pet, pic, sat, aafA, aafB, aafC, aafD, aap, aar, aatA, aggR, iha, lpfA, gad, mchB, mchC, mchF, ORF3, ORF4, aaiC, capU*	IncFIC(FII), IncQ1
D96/9	ST69	Downstream	FOX-AMP-AMC-TET-LEX	+	*blaTEM-1B, tet(A), mdf(A), mcr-9*	-	-	-	-	-	-	*eilA, lpfA, tsh, iroN, gad, iss, mchB, mchC, mchF, mcmA*	IncFIA, IncFIB IncFIC(FII)
D106/9	ST11351	Downstream	AMP-AMC-LEX-CTX-CAZ -CRO-FEP-SXT	+	*blaCTX-M-14, blaCTX-M-55, dfrA14, sul2, mdf(A), aph(3")-Ib, aph(6)-Id*	-	-	-	-	-	-	*eilA, lpfA, air, gad,*	IncI1, IncY
E13/5	ST569	Effluent	AMP-AMC-SXT	+	*blaTEM-1B, dfrA17, sul1, sul2, mdf(A), mph(A), aadA5, aph(3")-Ib, aph(6)-Id*	*In54*	*sul1*	*qacEΔ1aadA5*	*dfrA17*		Tn21	*senB, vat, gad, iss*	Col156, IncFIB, IncFII(29, IncQ1
U40/6	ST69	Upstream	FOX-AMP-AMC-TET-LEX	+	*blaTEM-1B, dfrA7, sul1, sul2, tet (A), mdf(A), aadA5, aph(3")-Ib, aph(6)-Id*	*In22*	*sul1*	*qacEΔ1dfrA7*	-		Tn402 (Tn5090), Tn21	*astA, eilA, lpfA, air, gad, iss*	Col440I, IncFII(pCoo), IncQ1, IncX4
U69/7	ST10	Upstream	TET-NAL-CIP-SXT	-	*dfrA14, sul2, tet(A), mdf(A)*	*1n191*	*dfrA14*	-	-	-	-	*gad*	IncFIB(pKPHS1), IncFII(K), IncI1, IncR, IncX2, IncX4
U88/8	ST10	Upstream	FOX-AMP-AMC	-	*mdf(A)*	-	-	-	-	-	-	*gad*	No plasmids
U117/10	ST69	Upstream	AMP-TET-NAL-SXT	+	*blaTEM-1B, dfrA14, sul2, tet(B), mdf(A), mph(A), aph(3")-Ib,aph(6)-Id*	*1n191*	*dfrA14*	-	-	-	Tn402 (Tn5090)	*senB, eilA, lpfA, air, gad, iss*	Col156, IncFIA, IncFIB, IncFII(pRSB107)

AMP, ampicillin; AZM, azithromycin; AMC, amoxicillin-clavulanic acid; FOX, cefoxitin; LEX, cephalexin; CIP, ciprofloxacin; NAL, nalidixic acid; TET, tetracycline; SXT, trimethoprim-sulfamethoxazole; bold, Novel type; ARGs, antibiotic resistance genes; ESBL, Extended spectrum β-lactamases; MLST, Multilocus sequence type; + /-, Presence/Absence.

TET–NAL–CIP–SXT, and FOX–AMP–AMC–TET–LEX were common to isolates obtained from the downstream and upstream sites of the WWTP. Three isolates, one from downstream and one from the final effluent, had the resistance profile AMP–AMC–SXT (**Table 1**). The remaining four isolates had unique resistance profiles AMP–TET–AZM–SXT, AMP–TET–SXT, FOX–AMP–AMC, and AMP–AMC–LEX–CTX–CAZ–CRO–FEP–SXT but were all resistant to AMP.

Genome Characteristics

The genomic characteristics of the *E. coli* sequences are presented in **Supplementary Table 2**. The total assembled genome size ranged from 4.7 to 6.1 MB; the GC content ranged from 50.4 to 51.2; and the N50, L50, and the total number of contigs are also shown in **Supplementary Table 2**.

Antibiotic Resistance Genes

All the *E. coli* isolates harbored ARGs, which included the β-lactamases. A total of 8/12 (66.7%) isolates were positive for the β-lactamase genes, with 7 (58.3%) harboring the bla_{TEM1B} gene (**Table 1**). One novel isolate, D106, was ESBL positive for the $bla_{CTX-\text{M}-14}$ and $bla_{CTX-\text{M}-55}$ genes but harbored different genes from other ESBL positive isolates. All ST69 isolates were ESBL positive (bla_{TEM1B} gene), whereas the ST10 isolates were ESBL negative. Additional genes included the *aph*(3″)-Ib, *aph*(6)-Id, *aad*A1, and *aad*A5 (which confer resistance to aminoglycosides); *tet(A), tet(M),* and *tet(B)* (resistance to tetracycline), *sul1* and *sul2* (resistance to sulfonamides), *dfr*A1, *dfr*A14, and *dfr*A17 (resistance to trimethoprim), and *mdf*(A) and *mph*(A) (which confer resistance to macrolides). Notably, one isolate (D96) was positive for the *mcr*-9 gene, which confers resistance to colistin, and another (D47) was positive for the *qnr*B19 gene, which has been implicated in plasmid-mediated quinolone resistance. Tetracycline resistance determinants *tet(M)* and *tet(A)* occurred together in an EPEC isolate (D64), which was phenotypically resistant to tetracycline, with no zone of inhibition (**Table 1**).

The quinolone resistance determinant regions (QRDRs) were investigated for mutations in all isolates. The QRDR consists of DNA gyrase (*gyr*A and *gyr*B) and DNA topoisomerase IV (*par*C and *par*E) genes. The *gyr*A gene (S83L, D678E, L447M, A828S, and D87N), the *gyr*B gene (E703D, A618T, E185D, E58D, and I60V), and the *par*C gene (E62K, D475E, M241I, T718A, and S80I) all had five mutations while the *par*E had four mutations (V136I*, K146T, D475E, and L416F) (**Table 2**). Two isolates, E13 and U69, had mutations in all four QRDR genes. Isolate U69 had known mutations *gyrA* (S83L and D87N) that confers resistance to NAL and CIP, and *parC* (S80I) and *parE* (L416F) that confer resistance to CIP. Isolate E13, which contained unique mutations *gyrA* (D678E* and A828S*), *gyrB* (A618T*), *parC* (E62K*, D475E*, and T718A*), and *parE* (V136I*, K146T*, and D475E*) was susceptible to all investigated quinolones.

TABLE 2 | Point mutation table for gyrA/B and parC/E genes of environmental *E. coli*.

Isolate ID	MLST	*gyr A*	*gyr B*	*parC*	*parE*
D18/5	ST372	S83L	-	E62K*, D475E*	V136I*
D47/7	ST69	D678E*	E703D*	E62K*	-
D64/7	ST101	-	-	E62K*	-
D69/7	ST218	-	-	E62K*, M241I*	-
D77/8	ST200	L447M*	-	E62K*	-
D96/9	ST69	D678E*	E703D*	E62K*	-
D106/9	**ST11351**	D678E*	-	E62K*	-
E13/5	ST569	D678E*, A828S*	A618T*	E62K*, D475E*, T718A*	V136I*, K146T*, D475E*
U40/6	ST69	D678E*,	E703D*	E62K*	-
U69/7	ST10	S83L, D87N	E185D*	S80I, E62K*	L416F
U88/8	ST10	-	E58D*, I60V*	E62K*	-
U117/10	ST69	S83L, D678E*	E703D*	S80I, E62K*	-

**Putatively novel mutations: novel sequence type (bold).*

Mobilome (Plasmids, Insertion Sequences, Intact Prophages, and Integrons)

The IncFII was the most detected plasmid replicon, with 10 (83.3%) isolates harboring it (**Table 1**). The IncFIA (25%), IncFIB (50%), IncQ1 (33.3%), IncI1 (16.7%), Col156 (25%), and Col440I (16.7%) plasmid replicons were also detected in some isolates. Most isolates (83.3%) had more than one plasmid replicon; however, no plasmids were detected in one isolate, U88 (**Table 1**). Of note, there was no unique pattern with respect to the replicon type, ST, and source of isolation.

Class 1 integrons were identified in 8 (66.7%) of the isolates of which 5 had the *qacEΔ1, sul1* genes, which are typically found at the 3'conserved segment in a class 1 integron (**Table 1**). The resistance gene cassettes identified in this study mostly harbored genes encoding resistance to trimethoprim ($n = 8$), streptomycin/spectinomycin ($n = 2$), and aminoglycosides ($n = 2$). The most frequently identified gene cassettes were *dfrA7* and *dfrA1*. Identified integron types included In22, In54, In191, and In369. Isolate D47 had three integron types: In54, In191, and In369 (**Table 1**). Similar integron types with identical gene cassettes were identified in isolates from different clonal types and sampling sites. Isolates from the upstream U69 (ST10), U117 (ST69), and downstream D47 (ST69) had the In191 integron type with identical gene cassette (*dfrA14* gene). Isolates from the upstream U40 (ST69) and downstream D77 (ST200) sites had the In22 with identical gene cassettes sul1, *qacEΔ1,* and *dfrA7*. Cassette arrays did not follow clonal lineages or source (upstream, downstream, and effluent), while isolates belonging to the same STs had different gene cassettes (**Table 1**). Some of the class 1 integrons were bracketed by transposons [e.g., U117 (ST69)]. The integron was flanked by the *TniA* and *TniB* genes associated with the Tn402-like transposons. Two other isolates, D77 (ST200) and E13 (ST569), had class 1 integrons flanked by the Tn21 transposon, which belongs to the Tn3 group of transposons. A transposable element Tn402 (Tn5090) not

linked to an integron was identified in D64 (ST101). The class 1 integron in U40 (ST69) was flanked on one side by Tn21, with the other end harboring the *TniA* and *TniB* genes (**Table 1**). The fluidity and mobility of MGEs was evident from the different permutations and combinations in isolates from different sources (**Table 1**).

The ARGs were mostly co-carried on class 1 integrons or associated with insertion sequences and/or transposons (**Table 3**). The $bla_{\text{TEM-1B}}$ gene was commonly associated with a recombinase, and the IS91 insertion sequence was the most common insertion sequence. The IS91 was also associated with aminoglycoside, trimethoprim, and sulfonamide resistance genes. Insertion sequences, IS5 and IS6, were also found associated with the $bla_{\text{CTX-M55}}$ and *mcr-9* genes, respectively. Tn3 transposons occurred either independently or with class 1 integrons (**Table 3**). The resistance genes and MGEs in the *E. coli* isolates were closely related (98–100% similarity) with target sequences in the GenBank database. Most hits were for plasmids, with the most common being the *E. coli* EcPF40 plasmid p1 (CP054215.1). The rich diversity of ISs and transposons attests to the plasticity of the bacterial genomes and horizontal gene transfer (HGT) of ARGs within and between different isolates.

The co-carriage of heavy metal (mercury and chromate), disinfectant (quaternary ammonium compounds), and ARGs were evident in several isolates. The mercury resistance operon was found associated with a transposase, tetracycline resistance transcriptional repressor *tetR(A)*, and the tetracycline resistance gene *tet(A)* in two isolates from the upstream (U40) and downstream (D16) sites of the WWTP (**Table 3**). The class 1 integrons in isolates D47 (downstream site) and E13 (effluent site) had the *PadR* (a transcriptional regulator) and *chrA* (chromate transport protein) downstream and adjacent to the integrons (**Table 3**). The synteny of heavy metal, disinfectant, and ARGs in these isolates consisted of the *chrA* (chromate resistance), *qacEΔ1* gene (which is a disinfectant resistance gene), and the class 1 integron ARG cassette (**Table 3**).

Phylogenetics linked with metadata revealed that isolates did not cluster according to source but according to ST (**Figure 2**). There was a clear association between the presence of the sulfonamide *sul2* and the aminoglycoside *aph-* genes, and the *sul1* gene and trimethoprim *dfrA* genes. The ESBL positive isolates had more resistance genes than the ESBL negative isolates; the number of resistance genes was not linked to the pathotype or clonality. Isolate D69 (ST218) was the only ESBL-negative EAEC isolate and had only one macrolide resistance gene (*mdfA*), while the other ESBL-positive EAEC isolates had more (**Figure 2**).

A total of 45 IS families were detected across the isolates (**Supplementary Table 3**). There was a great diversity of IS families, with only two occurring more than once.

A total of 19 intact prophages were found across all the investigated isolates (**Supplementary Table 3**). The Entero_mEp460 and Shigel_sfII were the most common prophages occurring in four different isolates each. The Entero_PsP3 ($n = 3$), Salmon_Fels_2 ($n = 3$), and Entero_fAA91_ss ($n = 2$) also occurred in several isolates. None of the prophages carried ARGs; however, some prophages carried virulence genes (**Table 4**). The abundance of ISs and prophages in environmental *E. coli* isolates is evidence of a very flexible genome that is constantly gaining and losing genetic elements through mobilizable regions of the genome.

Virulome and Serotypes

A total of 31 virulence genes were identified across all isolates (**Table 1**). Isolates obtained from downstream of the WWTP had the most virulence genes, including D77 (22 virulence genes), followed by D96 (10) and D69 (9) (**Supplementary Figure 1**). All isolates had at least one virulence gene, with isolates U69 and U88 (from the upstream site) having only one virulence gene each. The most common virulence genes were those encoding immunity *gad* (11 isolates), *iss* (8 isolates) and *air* (four isolates), and adhesion *Ipf*A (seven isolates) and *eil*A (five isolates) (**Supplementary Figure 1**).

The virulence genes were mostly associated with several insertion sequences, including IS1, IS3, IS91, IS66, IS630, and IS481, suggesting that insertion sequences play a prominent role in transferring virulence genes in environmental isolates (**Table 4**). The vacuolating autotransporter protein (*vat*) gene encoding a cytotoxin was mostly found with a transposase and the insertion sequence IS1. The *senB* gene, which encodes an enterotoxin, was mostly associated with IS91. The insertion sequences IS3 (*aafA*, *B, C, D*, *capU*) and IS66 (*nfaE, iha, pet*) were associated with different virulence genes. The increased serum survival (*iss*) gene was bracketed by several prophage genes, including the *RzoD* (outer membrane lipoprotein), *RrrD* (lysozyme), and *EssD* (lysis protein), implying that it is carried on a prophage (**Table 4**). Most of the virulence genes and their associated MGEs were similar (98–100%) to target sequences in GenBank, with the most hits being for chromosomal sequences. This indicates that *E. coli* virulence genes may mostly be carried on chromosomes.

The somatic (O) and flagellar (H) antigens were used for serotyping environmental *E. coli* isolates where nine different O antigens and 11 different H types were identified across all isolates. No O type was detected for isolate D18, which only had the H31 antigen (**Supplementary Table 2**). The complexity and diversity of the virulome coupled with the range of identified capsule types are worrying as they are associated with virulence. Environmental isolates have a rich repertoire of virulence genes mobilized mostly by ISs contributing to the dynamic milieu of resistance, virulence, and MGEs in the water environment.

Sequence Types and Phylogenomic Relationships

The MLST analysis revealed that the *E. coli* isolates belonged to multiple STs. The most common ST was ST69 ($n = 4$), followed by ST10 ($n = 2$), the rest had unique STs ST372, ST101, ST569, ST218, and ST200 (**Table 1**). Isolate D106 was assigned a novel ST, ST 11351. The phylogenetic analysis combined with metadata revealed that isolates of the same MLST clustered together [e.g., isolates from downstream (D47 and D96) and upstream (U40 and U117) sites that all belonged to ST69 (**Figure 1**)]. However, it was interesting to note that the isolates clustered according to the isolation site, with the downstream isolates (D47 and D96) forming their subclade. Some single STs from the downstream and effluent sites also clustered together, including D18 (ST372)

TABLE 3 | Mobile genetic elements associated with antibiotic resistance genes in environmental *E. coli*.

Isolate (MLST)	Contig	Synteny of resistance genes and MGE	Plasmid/chromosomal sequence with closest nucleotide homology (accession number)
D18/5 (ST372)	7	IS91: *bla*$_{TEM-1B}$: recombinase	*E. coli* EcP40 plasmid p1 (CP054215.1)
	21	Transposase:*tetR*(A):*tet(A)*:	*E. coli* AR_0013 chromosome (CP032204.1)
	52	*sul*1: qacEΔ1: *dfrA7*:*IntI1*	*E. coli* SCU_164 chromosome (CP054343.1)
	84	*aph(6)-Id*: *aph(3")-Ib*: *sul2*	*K. pneumoniae* plasmid p19051-FIIK (MN823997.1)
	21*	*merE:merD:mer(II)reductase:merC:merP:merT: merR:transposase:tetR(A):tet(A)*	*E. coli* AR_0013 Chromosome (CP032204.1)
D47/7(ST69)	2	*pspF*: *QnrB19*:	*K. pneumoniae* plasmid pRIVM_C014947_7 (MT560070.1)
	78*	*padR:chrA::sul1*: qacEΔ1:ant*(3")-Ia:dfrA17: IntI1*	*E. coli* strain AH62 plasmid pAH62-3 (CP055262.1)
	95	*bla*$_{TEM-1B}$	*K. quasipneumoniae* strain S174-1 plasmid pS174-1.1(CP063875.1)
	201	*tetR*-FTR (AcrR):: *mph(A)*	*K. pneumoniae* strain S90-2 plasmid pS90-2.3 (CP063884.1)
	390	*aadA1*: *dfrA1*	*E. coli* strain EcPF5 plasmid p1 (CP054237.1)
	401	*dfrA14*	*K. pneumoniae* strain KP20194a plasmid pKP20194a-p3 (CP054783.1)
	430	*tet(A)*: *tetR*(A):relaxase: *aph(6)-Id: aph(3")-Ib:sul2*	*E. coli* strain SCU-103 plasmid pSCU-103-1 (CP054458.1)
D64/7(ST101)	32	*tet(A)*: *tetR*(A):transposase	*E. coli* strain CFS3292 plasmid pCFS3292-1 (CP026936.2)
	36	*tetrLpep: tet(M):*	*E. coli* strain CFS3292 plasmid pCFS3292-1 (CP026936.2)
D77/8(ST200)	2	*sul1*:qacEΔ1: *dfrA7*: *IntI1::recombinase:Tn3(TnAs3)::ISI*	*E. coli* strain RHBSTW-00014 chromosome (CP056902.1)
	62	IS91: *bla*$_{TEM-1B}$:recombinase	*E. coli* EcPF40 plasmid p1 (CP054215.1)
	67	*aph(6)-Id: aph(3")-Ib: sul2:*	*E. coli* F070 plasmid pF070-NDM5 (AP023238.1)
D96/9(ST69)	91	*repA* (incFII):Tn3:recombinase: *bla*$_{TEM-1B}$::recombinase: Tn3 (TnAs1)	*E. coli* strain EC28 plasmid p2 (CP049102.1)
	131	*tet(A)*: *tetR*(A):transposase	*E. coli* strain CFS3273 plasmid pCFS3273-1 (CP026933.2)
	190	*mcr-9*: *WbuC*: IS6 (IS26)	*E. coli* CVM N18EC0432 plasmid pN18EC0432-1 (CP048293.1)
D106/9 (ST11351)	2	*sul2::::IS91: aph(6)-Id: aph(3")-Ib: dfrA14*	*Citrobacter freundii* plasmid pRHB16-C09_5 (CP057750.1)
	32	IS5: *bla*$_{CTX-M-14}$	*K. pneumoniae* plasmid pB16KP0177-4 (CP052528.1)
	42	*bla*$_{CTX-M-55}$*: WbuC::: ParA*	*E. coli* strain RD174 plasmid pHNRD174 (KX246268.1)
E13/5(ST569)	28*	*PadR:chrA::sul1*: *qacEΔ1*:aadA5*:dfrA17: IntI1::*recombinase	*E. coli* strain SCU-482 plasmid pSCU-482-1 (CP040859.1)
	31	*aph(6)-Id: aph(3")-Ib: sul2:*	*E. coli* strain EcPF40 plasmid p1 (CP054215.1)
	34	DDE integrase: *tetR*-FTR (AcrR)::: m*ph(A)*	*K. pneumoniae* strain S183-1 plasmid pS183-1.2 (CP063929.1)
	36	IS91:*bla*$_{TEM-1B}$:recombinase	*E. coli* strain EcPF40 plasmid p1 (CP054215.1)
U40/6(ST69)	39	*transposase:tetR(A):tet(A)*	*E. coli* strain CFS3273 plasmid pCFS3273-1 (CP026933.2)
	40	DDE integrase:TniB::*sul1*: *qacEΔ1*:*dfrA7: IntI1::*recombinase	*E. coli* SCU_164 chromosome (CP054343.1)
	43	*aph(6)-Id: aph(3")-Ib:sul2*	*E. coli* strain UFU_EC98 plasmid pEc98_3 (CP024095.1)
	55	IS91: *bla*$_{TEM-1B}$	*E. coli* strain EcPF5 plasmid p1 (CP054237.1)
	39*	*merE:merD:mer(II)reductase:merC:merP: merT:merR:transposase:tetR(A):tet(A)*	*E. coli strain CFS3273 plasmid pCFS3273-1 (CP026933.2)*
U69/7(ST10)	3	sul2:::IS91(ISVsa3)::recombinase	*K. quasipneumoniae* plasmid pRHBSTW-00138_10 (CP058140.1)
	29	ISKra4(ISKpn19): recombinase::transposase: *tetR*(A):*tet(A)*:::Tn3 (TnAs1):recombinase	*E. coli* strain EC194 plasmid p194 (MH121703.1)
	105	DfrA14:intI1:DDE integrase	*K. pneumoniae* plasmid pF16KP0096-1 (CP052151.1)
U117/10(ST69)	54	*bla*$_{TEM-1B}$:IS91: *aph(6)-Id: aph(3")-Ib:sul2::IS110(IS5075):Tn3*	*K. pneumoniae* strain Xen39 plasmid unnamed1 (CP040859.1)
	59	*tetR(B):tet(B):tet(C)::IS4(ISVa5)*	*Shigella flexneri* strain FDAARGOS_714 chromosome (CP055124.1)
	66	*tetR*-FTR (AcrR):: m*ph(A)*	*K. pneumoniae* plasmid p19110124-1 (CP064175.1)
	73	*mobC:dfrA14:intI1*	*E. coli* strain DH5alpha plasmid pESBL162 (MT230135.1)

**Co-occurrence of a heavy metal resistance gene (HMRG), disinfectant resistance gene (DRG), and antibiotic resistance genes (ARGs).*

and E13 (ST569), and also D64 (ST101) and D77 (ST200) (**Figure 1**).

Compared with South African *E. coli* isolates from different sources (animals, birds, and humans), the isolates from this study mostly clustered with clinical isolates (**Figure 2**). Isolates U40 (ST69), D96 (ST69), U117 (ST69), and DI06 (ST11351) clustered together and were closely related to a clinical isolate (ST648) obtained from a blood sample in Pretoria Hospital.

TABLE 4 | Mobile genetic elements associated with virulence genes in environmental *E. coli*.

Isolate	Contig	Synteny of virulence genes and MGE	Plasmid/chromosomal sequence with closest nucleotide homology (accession number)
D18 (ST372)	6	Prophagetailfibreprot::***iss***:rzoD:lysozyme:::EssD (phagelysisprotein)	*E. coli* CFTO73 chromosome (AE014075.1)
	73	Integrase:IS1:***vat***	*E.coli* strain 144 chromosome (CP041550.1)
D47/7(ST69)	38	IS66transposase:IS66:IS66:***nfaE***	*E. coli* strain 118UI chromosome (CP032515.1)
	144	***lss***:rzoD:RrrD(lysozyme):phageholinprotein	*E. coli* NMBU-W13E19 chromosome (CP043406.1)
D64/7(ST101)	16	Recombinase:tailfibreassemblyprot:tailfbrpro:phageterminase: DNApackagingprot::***iss***:RzoD:lysozyme:phage lysisprot	*E. coli* strain Res13-Lact-PEB01-20 chromosome (CP062868.1)
D69/7 (ST218)	39	***mchI***:***mchB***:::***hlyD***:***mchF***	*E. coli* 2013C chromosome (CP027355.1)
	34	IS30:transposase::virK:***capU***::IS3	*E. coli* strain MEM chromosome (CP012378.1)
	39	Transposase:IS66 (TnpB):IS66:transposase:***iha***	*E. coli* 2013C chromosome (CP027355.1)
	51	DNApakagingprot::***iss***:RzoD:lysozyme(RrrD):phage lysisprot (EssD)	E. coli CFTO73 chromosome (AE014075.1)
	83	***pet***:transposase:::IS66	E. coli strain S50 plasmid (CP010239.1)
D77/8(ST200)	23	IncFII repA:transposase:***ORF3***:***ORF4***::transposase	*E. coli* RHBSTW-00014 Plasmid p RHBSTW-00014_2 (CP056903.1)
	32	***aafA***:***aafD***:IS3:IS3::IS3	*E. coli* 042plasmid pAA(FN554767.1)
	39	***aafC***:***aafB***:IS3:transposase	*E. coli* RHBSTW-00622chromosome (CP055438.1)
	45	***aaiC***:tssC:tssB:IS630:::transposase	E. coli strain 2492 chromosome (CP044021.1)
	49	***mchI***:***mchB***:::***hlyD***:***mchF***	*E. coli* 2013C chromosome (CP027355.1)
	50	IS66::IS66*:IS66:IS66transposase:IS630:***aar***:IS3:IS481:***aap***: IS3:IS3:***aggR***	*E. coli* 042plasmid pAA (FN554767.1)
	59	***pic***:IS256:transposase::iucA:iucB:iucC:iucD:***iutA***::***sat***	*E. coli* strain NRRL B-1109 chromosome (CP039753.1)
	69	***Iha***:transposase:IS66*:IS66:ISEc22	*E. coli* strain NCTC10444 chromosome (LR134092)
D96/9(ST69)	95	***mchI***:***mchB***:***mchC***::***hlyD***:***mchF***	*E. coli* 2013C chromosome (CP027355.1)
	97	IS200.IS605::ISNCY transposase:IS3 transposase::IS3:IS3like:transposase:***iss***:lysisprot:transposase: iroB:iroC:iroD:iroE:iroN	*E. coli* strain CVM N17EC0616 plasmid pN17EC0616-1 (CP043737.1)
	121	EssD:rrrD:RzoD:***iss***	*E. coli* NMBU-W13E19 chromosome (CP043406.1)
	122	***eilA:air***	E. coli WP8-S17-ESBL-12 (AP022222.1)
	150	phageterminaseprot:DNApackprot::***iss***	*E. coli* NMBU-W13E19 chromosome (CP043406.1)
D106/9 (ST11351)	17	**eilA:air**	*E. coli* WP8-S17-ESBL-12 chromosome (AP022222.1)
E13/5(ST569)	1	Integrase:IS1:***vat***	*E. coli* strain 144 chromosome (CP041550.1)
	3	Lysozyme:RzoD:***iss***:DNApackprot:phageterminaseprot	*E. coli* NMBU-W13E19 chromosome (CP043406.1)
	12	phageHolin::RzoD:***iss***:endonuclease:phageterminase	*E. coli* strain SCU-485 chromosome (CP053245.1)
	27	***senB***:Tie:IS91	E. coli strain EcPF7 plasmid p1 (CP054233.1)
U40/6(ST69)	29	***eilA:air***	*E. coli* WP8-S17-ESBL-12 chromosome (AP022222.1)
	53	***lss***:RzoD:RrrD	*E. coli* NMBU-W13E19 chromosome (CP043406.1)
U117/10(ST69)	39	DNApackprot:***lss***:RzoD:RrrD:classIIholin	*E. coli* NMBU-W13E19 chromosome (CP043406.1)
	40	***eilA:air***	*E. coli* strain SCU-313 chromosome (CP051694.1)
	53	***senB***:TieB:IS91	E. coli strain EcPF7 plasmid p1 (CP054233.1)

**Virulence gene(s) in bold.*

Isolates E13 (ST569) and D18 (ST372) clustered together and with other clinical isolates (ST998) obtained from urine samples from hospital patients in Pretoria. D77 (ST200) and U69 (ST10) also clustered with clinical isolates obtained from hospital patients in the Western Cape and Pretoria, respectively, albeit in different clades. An isolate D64 (ST101) was closely related to an isolate from a wild bird obtained from Durban (**Figure 2**). The remaining three isolates, D69 (ST218), U88 (ST10), and D47 (ST69), were more closely related to each other and did not cluster with any isolates from animals, birds, or humans and may be considered a unique aquatic lineage.

DISCUSSION

Genomic insights reported in this study revealed the complexity and diversity of lineages, resistome, mobilome, and virulome of MDR *E. coli* found in wastewater and river water in Kwazulu Natal, South Africa, intimating that the aquatic environment contains a fluid and dynamic milieu of ARB and ARGs. The ARGs were mostly carried on plasmids, transposable elements, and integrons, and fewer were associated with IS. The virulence genes were mostly associated with IS, which are probably central in their rearrangement and transfer. The

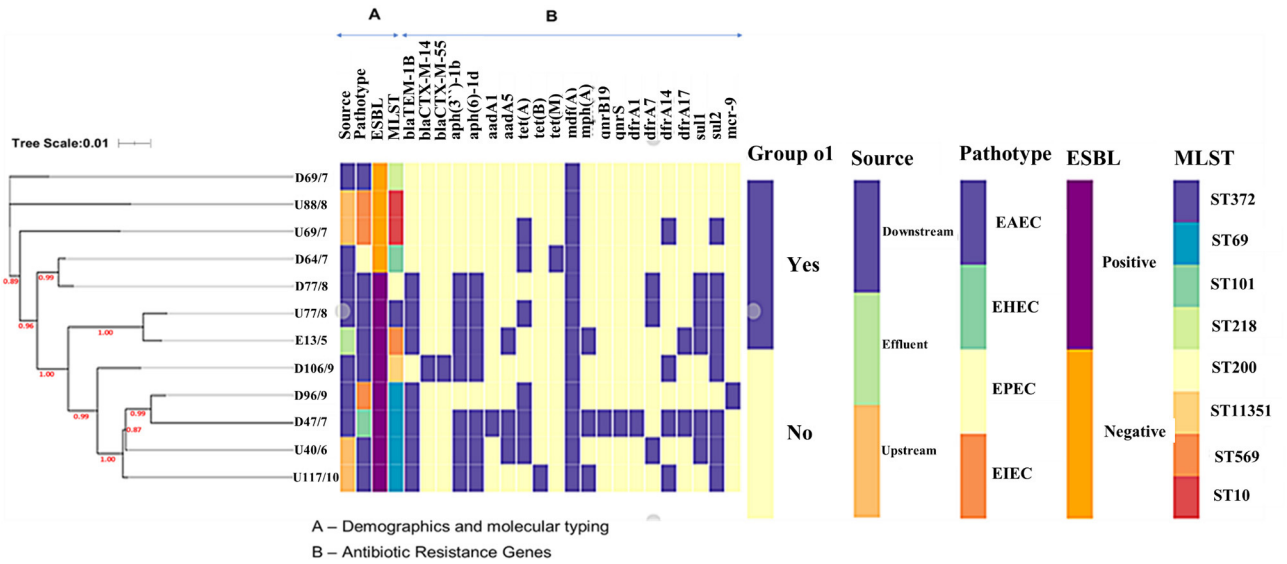

FIGURE 1 | The phylogenetic branch and metadata [demographics, molecular typing, and antibiotic resistance genes (ARGs)] coupled by the use of Phandango (https://github.com/jameshadfield/phandango/wiki) in isolated multidrug resistant *Escherichia coli* strains ($n = 12$) from wastewater sources in South Africa.

occurrence of heavy metal, disinfectant, and ARGs in bacterial isolates is a cause for concern as it may lead to co-selection of ARB.

An assortment of ARGs and MGEs was detected among and within the sampled sites (**Table 1**). The variation in the ARGs and associated MGEs may reflect numerous, distinct horizontal transfer events among environmental isolates. The occurrence of ARGs, most notably the ESBL, tetracycline, sulfonamide, and macrolide genes, was not dependent on the sample source or clonal type. This contrasts with a study done in the USA that studied ESBL and *Klebsiella pneumoniae* carbapenemase (KPC) producing *E. coli* from municipal wastewater, surface water, and a WWTP. WGS of *E. coli* isolates revealed an association between the sample source and the presence of specific ESBL genes (e.g., bla_{TEM} was unique to municipal wastewater isolates, whereas $bla_{\text{CTX-M}}$ was unique to WWTP raw influent isolates (13)). Most ARGs and associated MGEs were carried on plasmids (**Table 3**), signifying that plasmids play a central part in the resistome of environmental *E. coli* isolates. A few ARGs including those encoding tetracycline resistance [*tet(A)*, *tet(B)*, *tet(C)*], sulfonamides (*sul1*), and trimethoprim (*dfrA7*) (carried on a class 1 integron) were found on chromosomes. However, the integrons and transposons were largely associated with ARGs on plasmids, similar to findings in other studies (14). Che et al. (14) used WGS to investigate the ARGs in total DNA extracted from water samples from three WWTPs in Hong Kong and reported that ARGs carried on plasmids were dominant in the resistome of the WWTPs.

In this study, the investigated ARGs were mainly bracketed by transposons, insertion sequences, and class 1 integrons. A novel isolate, D106 (ST11351), had the $bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-55}}$ genes (**Table 1**). Both genes were found on genetic elements IS5: $bla_{CTX-M-14}$ and $bla_{CTX-M-55}$*:WbuC:::ParA* on contigs that had closest nucleotide homology to plasmids from *K. pneumoniae* pB16KP0177-4 (CP052528.1) and *E. coli* pHNRD174 (KX246268.1), respectively. The isolate exhibited phenotypic resistance to tested cephalosporins, including FEP, CTX, CAZ, CRO, and LEX; this was expected since the $bla_{\text{CTX-M-55}}$ is a variant of the $bla_{\text{CTX-M-15}}$, which has heightened cephalosporin-hydrolyzing action (15). The $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-15}}$ remain among the most predominant CTX-M types worldwide (16) and have also been reported in several studies on clinical isolates in South Africa (17–19). However, there are no reports on the occurrence of $bla_{\text{CTX-M-55}}$ in environmental *E. coli* in South Africa. The $bla_{\text{TEM-1B}}$ was found in the same genetic context *IS91:* bla_{TEM-1B}*:recombinase* for isolates from the upstream (U40), effluent (E13), and downstream sites (D18 and D77) (**Table 4**) and had high sequence similarity to *E. coli* EcPF40 plasmid p1 (CP054215.1). The bla_{TEM}genes are often plasmid mediated and are the leading cause of AMP resistance in Gram-negative bacteria (20). The IS91 can mobilize adjacent sequences through a one-ended transposition process, and the association with p1 plasmids points to a plasmid-mediated circulation of these genes in the water environment (21, 22).

An interesting finding in this study was the occurrence of the plasmid-borne *mcr-9* gene in isolate D96 (**Table 1**) that also had ESBL, macrolide, and tetracycline resistance genes. Phenotypic resistance to colistin was then determined using the colistin MIC, and the isolate was found to be susceptible (<4 mg/L). The phenotypic susceptibility to colistin in isolates carrying the *mcr-9* gene was also reported in a study conducted in the USA where 100 *mcr-9* positive *Salmonella enterica* and *E. coli* isolates from the National Antimicrobial Resistance Monitoring System (NARMS), which samples retail meat, reported that all 100 isolates were susceptible to colistin, suggesting that

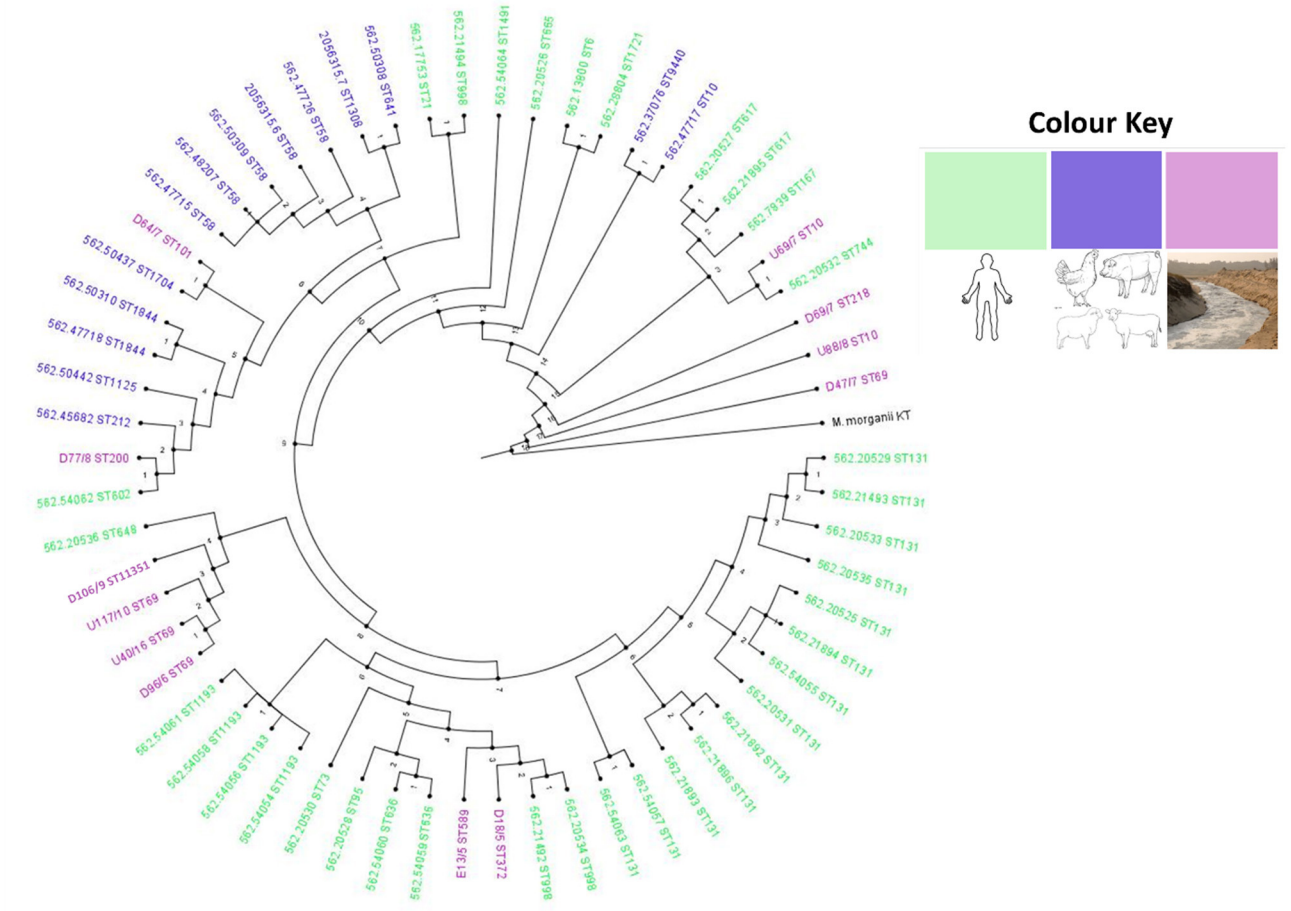

FIGURE 2 | Circular phylogenomic tree with color annotations depicting the relationship between *E. coli* isolates from this study and South African isolates from diverse sources in the one-health continuum. The strains used in this study (from environmental source colored in purple) were basically related to strains from human sources (colored in green).

the *mcr-9* gene may not be associated with colistin resistance (23). In this study, the *mcr-9* gene was found adjacent to an unknown function cupin fold metalloprotein gene (*WbuC*) in genetic context *mcr-9*:*WbuC*:IS6 (IS26). Similar genetic contexts have been reported in *Enterobacter hormaechei,* and *Salmonella* Typhimurium isolates from studies undertaken in the USA and China (24–26). This points to a stable *mcr-9* locus, whose transfer is mediated by insertion sequences. The *mcr-9* gene was first reported in a clinical *Salmonella* Typhimurium isolate in the USA in May 2019 (24). Isolates harboring *mcr-9* were subsequently identified in 21 countries covering six continents, including Europe, Asia, America, Oceania, South America, and Africa (27). An *mcr-9* harboring *Enterobacter hormaechei* isolate obtained from the sputum of a patient in Cairo, Egypt, is to date the only *mcr-9* positive isolate reported in Africa (28). Thus, this is the second report of the *mcr-9* positive isolate in Africa and the first in Southern Africa.

The co-occurrence of aminoglycoside resistance genes [*aph(6)-Id: aph(3")-Ib*] with sulfonamide and trimethoprim or tetracycline genes revealed the presence of resistance islands located on regions with high similarity to plasmids deposited in GenBank. This implies that the transmission of these resistance genes is plasmid mediated (**Table 3**). The genetic context, *aph(6)-Id: aph(3")-Ib:sul2,* has been found complete or incomplete within plasmids, integrative conjugative elements (ICE), and chromosomes in both Gram-negative and Gram-positive organisms (29).

Most TET-resistant isolates harbored the *tet*(A) gene and were phenotypically resistant (**Table 1**). Only one isolate had *tet*(B), and another isolate had *tet(A)* and *tet(M),* and both were phenotypically resistant to TET (**Table 1**). In this study, the *tet(A)* gene was consistently found within a resistance operon adjacent to a transcriptional repressor gene *tetR(A)* and a transposase (**Table 3**). The genetic context *transposase:tetR(A):tet(A)* had high similarity to plasmid sequences deposited in GenBank, especially *E. coli* strain CFS3292 plasmid pCFS3292-1 (CP026936.2). The *tet(A)* family has been constantly associated with conjugative plasmids, which

mediate transfer (30). Isolate U117 had *tet(B)* and *tet(C)* flanked by an insertion sequence IS4 (SVa5), which may be important in mobilizing these resistance genes.

The genes encoding resistance to trimethoprim/sulfonamides, *sul1, sul2*, and *dfrA* gene cassettes, were detected in 8 (66.7%) isolates. The *sul2* genes were consistently co-carried with the aminoglycoside resistance *aph-* genes [*aph(6)-Id: aph(3")-Ib*] with *sul1* being co-carried with the *dfrA* and *qacEΔ1* genes (**Table 1**). There was concordance in the phenotypic and genotypic results in 7 (58.3%) isolates, with one isolate being phenotypically susceptible to SXT but possessing genotypic resistance traits (**Table 1**). A total of 8 (66.7%) isolates harbored class 1 integrons with an array of gene cassettes (**Table 1**). The class 1 integrons are directly linked with the Tn3 transposon family (Tn21 or Tn1697), mainly because of their inability to self-transfer; thus, they rely on conjugative plasmids and transposons for their horizontal or vertical transmission (31). Two isolates (E13 and D77) had class 1 integrons that were carried by the Tn21 transposon (**Table 1**). U40 was, however, unique in that either side of its class 1 integron had different transposons (namely, Tn21 and Tn402). The Tn402 (Tn5090) may carry class 1 integrons or mercury resistance integrons (*MerR*) and are characterized by *TniABQR* genes (32). The TniA codes for a putative transposase, TniB is a nucleoside triphosphate (NTP) binding protein, TniR is a resolvase or integrase, and TniQ is required for transposition (32). The class 1 integrons in isolates D47 and E13 had the *PadR* (a transcriptional regulator) and the *chrA* (chromate transport protein) downstream and adjacent to the integrons (**Table 3**). The *ChrA* gene is a heavy metal resistance gene (HMRG) that encodes resistance to chromate and is usually found on plasmids or chromosomes of bacteria (33). The *qacEΔ1* gene is a disinfectant resistance gene (DRG) that encodes resistance to disinfectants of quaternary ammonium compounds (34). A mercury resistance operon was associated with *tet(A)* resistance genes and transposons in two isolates from upstream (U40) and downstream (D18) of the WWTP (**Table 3**). The co-occurrence of HMRGs, DRGs, and ARGs was recently demonstrated in *E. coli* strains obtained from rivers, streams, and lakes in Brazil (8). The coexistence of HMRGs, DRGs, and ARGs in the studied integrons is important as disinfectants and heavy metals can co-select for ARGs (35). Altogether, these results revealed that ARGs carried on plasmids predominate the investigated water resistome; however, IS, transposable elements, and integrons accentuate the mobility of the plasmid-encoded ARGs and HMRGs.

A huge diversity of virulence genes often associated with pathogenic *E. coli* was found in the genomes of isolates in this study (**Table 1**). Similar virulence factors have been identified in environmental *E. coli* isolates obtained from surface water and WWTPs in previous studies (8, 36). Most virulence genes were associated with the insertion sequences, suggesting that these are important in the mobilization of the bacterial virulome (**Table 4**). The *iss* gene is responsible for increased serum survival and mediates against phagocytosis enabling the evasion of the immune system (37). The genetic environment of the *iss* gene consisted of bacteriophage genes, implying that it is carried on a prophage in *E. coli* isolates (**Table 4**). The *iss* gene is thought to have evolved from a λ phage gene called *bor,* which integrated into the genomes of different *E. coli* pathotypes (37). Virulence genes are frequently clustered together on the bacterial chromosome in pathogenicity islands (PAIs). In Gram-negative bacteria, the PAIs tend to contain insertion sequences that promote reorganizations and transfer of virulence genes (38). Several virulence genes, including *senB* (IS91), *vat* (IS1), and *iha* (IS66), were associated with IS (**Table 4**). The virulome of the environmental isolates investigated in this study revealed a diverse assemblage of virulence genes that are mobilizable and not clone specific.

Phylogenomic analyses revealed that the environmental samples in this study clustered mainly with clinical isolates, mostly from hospital patients (**Figure 2**). Six EAEC and two EIEC isolates were closely related to clinical isolates, implying that they originated from clinical sources. The spread of EIEC and EAEC is frequently associated with food sources or polluted water (8, 39, 40) as was the case in this study. An EPEC isolate (D64) was closely related to an isolate from a wild bird (**Figure 2**). Typical EPEC isolates are rarely isolated from animals as humans are the major natural reservoir; however, atypical EPEC occurs in healthy and sickly animals and humans (5). The EPEC isolate from this study probably originated from an animal host.

This study focused on a small subset of MDR and diarrheagenic *E. coli*; thus, its findings may not be generalized for all *E. coli* pathotypes. Similar studies employing a larger sample size and covering greater geographical area and diversity of *E. coli* should be conducted. However, this study adds to the knowledge that pathogenic *E. coli* can survive and be disseminated in the water environment, which is a public health concern.

CONCLUSIONS

The occurrence of pathogenic and MDR isolates in the WWTP effluent and the associated river is a public health concern. *E. coli* isolates have a wealth of ARGs and virulence genes that have been mobilized on diverse MGEs as evident from the different permutations and combinations of ARGs, virulence genes, and MGEs in *E. coli* STs and pathotypes from the different water sources. The findings of this study may not be typical of all WWTPs and river systems in South Africa or beyond but form a basis of the need for surveillance systems that employ high-throughput technologies like WGS to gain genomic insights into the environmental dimensions of AMR. Surveillance of ARB in wastewater and associated surface waters could serve as a proxy for local antibiotic resistance and how this changes over time.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/genbank/, PRJNA609073.

AUTHOR CONTRIBUTIONS

SYE, ALKA, and JM co-conceptualized the study. JM, ALKA, and DGA performed the experiments. JM, ALKA, DGA, MA, and AI analyzed the data. JM wrote the paper. SYE, ALKA, and DGA supervised. SYE involved in funding acquisition. All the authors undertook critical revision of the manuscript and also reviewed, edited, and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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frontiers in Veterinary Science

BRIEF RESEARCH REPORT
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Occurrence and Quantification of Antimicrobial Resistance Genes in the Gastrointestinal Microbiome of Two Wild Seabird Species With Contrasting Behaviors

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Antimicrobial resistance genes (ARGs) are environmental pollutants and anthropization indicators. We evaluated human interference in the marine ecosystem through the ocurrence and quantification (real-time PCRs) of 21 plasmid-mediated ARGs in enema samples of 25 wild seabirds, upon admission into rehabilitation: kelp gull (*Larus dominicanus*, $n = 14$) and Magellanic penguin (*Spheniscus magellanicus*, $n = 11$). Overall, higher resistance values were observed in kelp gulls (non-migratory coastal synanthropic) in comparison with Magellanic penguins (migratory pelagic non-synanthropic). There were significant differences between species (respectively, kelp gull and Magellanic penguin): ARGs occurrence (bla_{TEM} [$p = 0.032$]; *tet*M [$p = 0.015$]; *tet*A [$p = 0.003$]; and *sul*II [$p = 0.007$]), mean number of ARGs per sample ($p = 0.031$), ARGs mean load percentage (*aad*A [$p = 0.045$], *tet*A [$p = 0.031$], *tet*M [$p = 0.016$], bla_{TEM} [$p = 0.032$], *sul*II [$p = 0.008$]), percentage of genes conferring resistance to an antimicrobial class (betalactams [$p = 0.036$] and sulfonamides [$p = 0.033$]), mean number of genes conferring resistance to one or more antimicrobial classes ($p = 0.024$]), percentage of multiresistant microbiomes ($p = 0.032$), and clustering ($p = 0.006$). These differences are likely due to these species' contrasting biology and ecology - key factors in the epidemiology of ARGs in seabirds. Additionally, this is the first report of *mec*A in seabirds in the Americas. Further studies are necessary to clarify the occurrence and diversity of ARGs in seabirds, and their role as potential sources of infection and dispersal within the One Health chain of ARGs.

Keywords: anthropization, marine pollution, antibiotic resistance, wildlife, gull, penguin, One Health

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INTRODUCTION

Antimicrobial resistance is an issue of serious public health concern with global economic, social and political implications affecting human and animal populations, as well as the environment (1–3). This worldwide phenomenon is compromising our ability to treat infectious diseases, and undermining or preventing advances in health and medicine (4). Microbial resistance is the result of natural bacteria genetic plasticity and interactions between microbial agents, host organisms and the environment (1, 5), enhanced by the selective pressure exerted by antimicrobial usage and over-prescription in human and veterinary medicine treatments, animal and fish production (i.e., growth promoters and prophylaxis), agriculture and food technologies (1, 5, 6). The consequent remodeling of the existing microbiomes (group of all the genomic elements of a specific microbiota), associated with their dissemination capacity, confer antimicrobial resistance genes (ARGs) the role of environmental pollutants (7, 8) and indicators of environmental anthropization (2, 9, 10).

Seabirds are long-lived, wide-ranging, and upper trophic level marine predators present in all marine ecosystems and oceans of the world, from coastline to pelagic and open seas (11). By acting as predators, scavengers and cross-ecosystem nutrient ancillaries, seabirds play important roles in the processes, function and resilience of island and marine ecosystems (12). Essentially, seabirds respond rapidly to environmental changes, and due to their behavior and population dynamics, are excellent sentinels of the marine ecosystem health, reflecting natural and anthropogenic changes to the environment (13), including pollution by ARGs (14–16). In seabirds, most ARGs studies have focused on synanthropic species, due to their proximity to anthropized areas and feeding habits, and relied on classic microbiological techniques (bacterial culture and sensitivity testing) (9, 17, 18). Nevertheless, recent studies have shown that biological and ecological factors (e.g., migration and feeding niche) are also relevant to the issue of ARGs in wild birds (16, 19, 20). Additionally, only a small fraction of bacteria are cultivable (21, 22). Thus, in order to promote a more comprehensive approach, we employed highly sensitive real time polymerase chain reaction (rtPCR) methods (10, 23) to directly detect and quantify 21 selected plasmid-mediated ARGs in the gastrointestinal microbiome of two wild seabirds species (kelp gulls [*Larus dominicanus*] and Magellanic penguins [*Spheniscus magellanicus*]) upon admission to a rehabilitation center. The goals of this study were to (i) assess the presence and load of ARGs in these individuals and (ii) evaluate our findings in light of selected biological and ecological parameters (i.e., dispersal [migratory and non-migratory], feeding niche [coastal and pelagic], and interaction with human-impacted areas [synanthropic and non-synanthropic]). We hypothesized that due to their non-migratory coastal synanthropic behavior (24), kelp gull would present higher occurrence and load of ARGs than the migratory pelagic non-synanthropic Magellanic penguin (25, 26).

METHODS

Sample Collection

Fresh fecal samples were immediately obtained by enema (16) in 25 physically restrained birds (14 kelp gulls and 11 Magellanic penguins) upon admission at the wildlife rehabilitation center (Associação R3 Animal, Florianópolis, Santa Catarina state, southern Brazil), and stored at −20°C until analyses. All birds included in the study came directly from their rescue sites (beach), and did not receive previous veterinary care prior to their arrival at the center. Total DNA extraction was carried out by a pressure filtration technique (QuickGene DNA tissue kit S, Fujifilm, Tokyo, Japan), according with the manufacturer's instructions. The 16S rRNA gene was amplified by real time PCR (rtPCR) in 10-fold dilutions of each extracted sample [(27, 28), **Supplementary Materials**] to verify adequate concentration of bacterial DNA. A sample was considered validated when its 10-fold dilution showed a cycle threshold (Ct) <25 (29). To normalize the study, ct was obtained based on the fluorescence variation value [$(\Delta F/\Delta C) = 0.02$] (30). Once validated, samples were analyzed by rtPCR for 21 selected ARGs encoding resistance to eight antimicrobial classes: tetracyclines (*tet*(A), *tet*(B), *tet*(Y), *tet*(K), *tet*(M), *tet*(Q), *tet*(S), and *tet*(W) (28), aminoglycosides [*aad*A (31) and *str* (32)], sulfonamides (*sul*I, *sul*II), chloramphenicols [*cat*I and *cat*II (28)], macrolides [*erm*(B), *erm*(F) (33)], quinolones [*qnr*B (34) and *qnr*S (35)]; betalactams [bla_{TEM} (31) and *mec*A (36)], and polymyxins [*mcr*-1 (30)] (**Supplementary Materials**). The estimation of the percentage of bacteria harboring ARGs (mean load percentage of each ARG), was based on the formula % gene $X = 10^{[2+0.33(ct16S-ctgeneX)]}$, with ct as the cycle threshold (16S rRNA regarding bacterial determination and X for each evaluated gene), and 0.33 as the mean slope for all the evaluated genes. Results were expressed in $\log_{10}$ scale of the hypothetical percentage of bacteria presenting each gene, ranging from −8 (sample considered negative) to +2 (when 100% of the bacteria in the sample presented the ARG) (30). The same thermal cycle was used for all rtPCR reactions [6′ 95°C, 40x (10″ 95°C, 30″ 60°C)], with alignment and extension in the same step, at constant 60°C. A melting curve step was performed at the end of the rtPCR reaction (30). As per (37), we applied the term "multiresistant microbiome" when a fecal sample presented at least three ARGs encoding resistance to different classes of antimicrobials (10, 29, 30). All samples used in this study were collected as part of the Santos Basin Beach Monitoring Project (Projeto de monitoramento de Praias da Bacia de Santos - PMP-BS), licensed by the Brazilian Institute of the Environment and Renewable Natural Resources (IBAMA) of the Brazilian Ministry of Environment (ABIO N° 640/2015), and in full compliance with the Biodiversity Information and Authorization System (SISBIO 59150-4). All procedures were performed according to the Ethical Committee in Animal Research of the School of Veterinary Medicine and Animal Sciences, University of São Paulo (process number 1753110716).

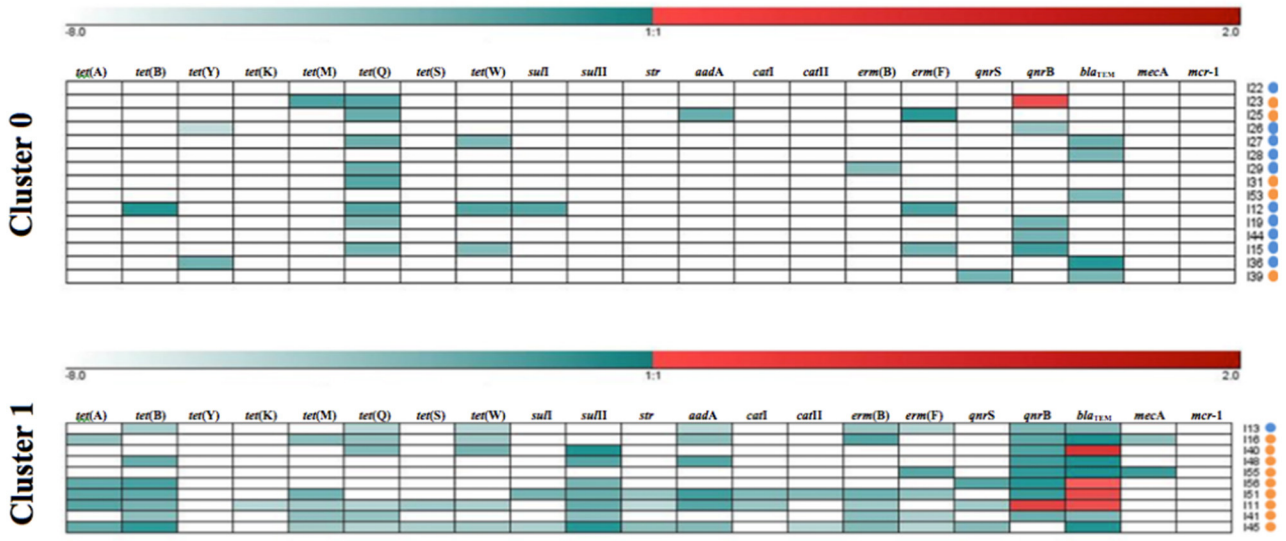

FIGURE 1 | Resistance patterns of kelp gull (*Larus dominicanus*) and Magellanic penguin (*Spheniscus magellanicus*) samples obtained by *k*-means clustering of each antimicrobial resistance gene (ARG). Cluster 1 shows samples with high relative load percentage and Cluster 0 shows samples with low relative load percentage. Relative load percentage is expressed in a color scale (white for negative [−8] and dark red for the maximum value [+2]). The species are indicated on the right side (kelp gull [orange dots] and Magellanic penguin [blue dots]).

Statistical Analysis

The *k*-means clustering method was used to investigate the resistance patterns (GENESIS software v. 1.7.7, Graz University of Technology, Graz, Austria), by assigning each sample to one cluster (**Figure 1**). Two clusters were selected, corresponding to low (value = 0) and high (value = 1) levels of ARGs. The Mann-Whitney *U* non-parametric test was used to establish the differences between species regarding: ARGs occurrence, mean number of ARGs per sample, mean load percentage of each ARG, the mean number of genes conferring resistance to one or more antimicrobial classes in each sample, percentage of multiresistant microbiomes and resistance patterns. Such statistical analyses were performed in R software (R Development Core Team 3.0.1., 2013), with a significance level of $p < 0.05$.

RESULTS

All the tested samples validated for the 16S rRNA gene. All animals, with the exception of one individual (96%, 24/25), were positive to at least one ARG (**Table 1**). ARGs results according with the species are described below.

Kelp Gull

The bla_{TEM} gene presented the highest occurrence (79%, 11/14), followed by *qnr*B (64%, 9/14), *tet*(Q) (57%, 8/14), *sul*II (50%, 7/14), *tet*(B), *tet*(M), and *aad*A (43%, 6/14), *tet*(A), *erm*(B) and *erm*(F) (36%, 5/14), *tet*(W), and *qnr*S (29%, 4/29), *str* (21%, 3/21), *tet*(S), *sul*I, *cat*I, *cat*II, and *mec*A (14%, 2/14), and *tet*(K) (7%, 1/14). The *tet*(Y) and *mcr*-1 genes were not detected in this group. The mean number of ARGs per sample was 6.4 (with min = 1 and max = 15). The bla_{TEM} gene presented the highest mean load percentage (−2.2) (considering ≥-3 as the median value, with −8 [min] and +2 [max]).

When clustered by antimicrobial class, kelp gulls were positive to one or more genes encoding resistance to tetracycline, quinolone and betalactams (79%, 11/14), sulfonamides and macrolides (50%, 7/14), aminoglycosides (43%, 6/14), and phenicols (21%, 3/14). No gulls presented ARGs encoding polymyxin resistance (*mcr-1*). The mean number of genes conferring resistance to one or more antimicrobial classes presented in each gull sample was four. Additionally, 71% (10/14) of the gulls presented multiresistant microbiomes (**Table 1**), of these, five presented two similar patterns: a tetracycline, sulfonamide, quinolone, betalactam, aminoglycoside, phenicol and macrolide combination (30%; 3/10), and a tetracycline, sulfonamide, quinolone and betalactam combination (20%; 2/10).

Magellanic Penguin

The *tet*(Q) gene presented the highest occurrence (55%, 6/11), followed by *qnr*B (45%, 5/11), bla_{TEM} and *tet*(W) (36%, 4/11), *erm*(F) (27%, 3/11), *tet*(B), *tet*(Y), and *erm*(B) (18%, 2/11), *sul*I and *aad*A (9%, 1/11). Genes *tet*(A), *tet*(K), *tet*(M), *tet*(S), *sul*II, *str*, *cat*I, *cat*II, *qnr*S, *mec*A, and *mcr*-1 were not detected. The mean number of ARGs per sample was 2.7 (with a maximum of eight genes per individual). Only one penguin did not present any of the tested ARGs. None of the genes presented mean load percentage ≥−3.

When clustered by antimicrobial class, individuals were positive to one or more genes encoding resistance to tetracyclines (73%, 8/11), quinolone (45%, 5/11), macrolides and betalactams (36%, 4/11), and sulfonamides and aminoglycosides (9%,

TABLE 1 | Microbiome patterns, number of detected genes per sample, and detected genes according with the animal ID and species (kelp gull *Larus dominicanus* and Magellanic penguin *Spheniscus magellanicus*).

ID	Species	Drug class pattern	Number of detected ARGs	Detected ARGs
I11	kelp gull	TET, SUL, AMINO, PHEN, MACR, QUINO, BLACT†	15	*tet*(A), *tet*(B), *tet*(K), *tet*(M), *tet*(Q), *tet*(S), *tet*(W), *sul*II, *str*, *aad*A, *cat*I, *erm*(B), *qnr*S, *qnr*B, bla_{TEM}
I16	kelp gull	TET, AMINO, MACR, QUINO, BLACT†	9	*tet*(A), *tet*(M), *tet*(Q), *tet*(W), *aad*A, *erm*(B), *qnr*B, bla_{TEM}, *mec*A
I23	kelp gull	TET, QUINO	3	*tet*(M), *tet*(Q), *qnr*B
I25	kelp gull	TET, AMINO, MACR†	3	*tet*(Q), *aad*A, *erm*(F)
I56	kelp gull	TET, SUL, QUINO, BLACT†	6	*tet*(A), *tet*(B), *sul*II, *qnr*S, *qnr*B, bla_{TEM}
I31	kelp gull	TET	1	*tet*(Q)
I39	kelp gull	QUINO, BLACT	2	*qnr*S, bla_{TEM}
I40	kelp gull	TET, SUL, QUINO, BLACT†	5	*tet*(Q), *tet*(W), *sul*II, *qnr*B, bla_{TEM}
I41	kelp gull	TET, SUL, MACR, QUINO, BLACT†	8	*tet*(B), *tet*(M), *tet*(Q), *sul*II, *erm*(B), *erm*(F), *qnr*B, bla_{TEM}
I45	kelp gull	TET, SUL, AMINO, PHEN, MACR, QUINO, BLACT†	15	*tet*(A), *tet*(B), *tet*(M), *tet*(Q), *tet*(S), *tet*(W), *sul*I, *sul*II, *str*, *aad*A, *cat*II, *erm*(B), *erm*(F), *qnr*S, bla_{TEM}
I48	kelp gull	TET, SUL, AMINO, QUINO, BLACT†	5	*tet*(B), *sul*II, *aad*A, *qnrB*, bla_{TEM}
I51	kelp gull	TET, SUL, AMINO, PHEN, MACR, QUINO, BLACT†	13	*tet*(A), *tet*(B), *tet*(M), *sul*I, *sul*II, *str*, *aad*A, *cat*I, *cat*II,*erm*(B), *erm*(F), *qnr*B, bla_{TEM}
I53	kelp gull	BLACT	1	bla_{TEM}
I55	kelp gull	MACR, QUINO, BLACT†	4	*erm*(F), *qnr*B, bla_{TEM}, *mec*A
I12	Magellanic penguin	TET, SUL, MACR†	5	*tet*(B), *tet*(Q), *tet*(W), *sul*I, *erm*(F)
I13	Magellanic penguin	TET, AMINO, MACR, QUINO, BLACT†	8	*tet*(B), *tet*(Q), *tet*(W), *aad*A, *erm*(B), *erm*(F), *qnr*B, bla_{TEM}
I15	Magellanic penguin	TET, MACR, QUINO†	4	*tet*(Q), *tet*(W), *erm*(F), *qnr*B
I19	Magellanic penguin	TET, QUINO	2	*tet*(Q), *qnr*B
I22	Magellanic penguin	-	0	-
I26	Magellanic penguin	TET, QUINO	2	*tet*(Y), *qnr*B
I27	Magellanic penguin	TET, BLACT	3	*tet*(Q), *tet*(W), bla_{TEM}
I28	Magellanic penguin	BLACT	1	bla_{TEM}
I29	Magellanic penguin	TET, MACR	2	*tet*(Q), *erm*(B)
I36	Magellanic penguin	TET, BLACT	2	*tet*(Y), bla_{TEM}
I44	Magellanic penguin	QUINO	1	*qnr*B

TET, tetracyclines; SUL, sulfonamides; AMINO, aminoglycosides; PHEN, phenicols; MACR, macrolides; QUINO, quinolone; BLACT, betalactams.
† *Multiresistant microbiomes.*

1/11). None of the individuals presented ARGs encoding chloramphenicol or polimyxin resistance. The mean number of genes conferring resistance to one or more antimicrobial classes presented in each sample was 2.1. Mutiresistant microbiomes were found in 27% (3/11) of the penguins (**Table 1**). Although no common patterns were observed, genes conferring resistance to tetracycline and macrolides were detected in the microbiomes of the three individuals presenting multiresistant profiles.

Qualitative Analysis

There were significant differences between species (respectively, kelp gull and Magellanic penguin) in regards to: ARG occurrence (bla_{TEM} [79 and 36%. $p = 0.032$]; *tet*(M) [43 and 0%. $p = 0.015$]; *tet*(A) [36 and 0%. $p = 0.003$]; and *sul*II [50 and 0%. $p = 0.007$]), mean number of ARGs per sample (6.4 and 2.7. $p = 0.031$), ARG mean load percentage (*aad*A [−5.4 and −7.7. $p = 0.045$], *tet*(A) [−5.8 and −8. $p = 0.031$]; *tet*(M) [−5.8 and −8. $p = 0.016$]; bla_{TEM} [−2.2 and −5.8. $p = 0.032$]; *sul*II [−4.8 and −8. $p = 0.008$]), percentage of genes potentially conferring resistance to an antimicrobial class (betalactams [79 and 36%. $p = 0.036$] and sulfonamides [50 and 9%. $p = 0.033$]), mean number of genes conferring resistance to one or more antimicrobial classes (4 and 2.1. $p = 0.024$]), percentage of multiresistant microbiomes (71 and 27%. $p = 0.032$]), and clustering (0.6 and 0.1. $p = 0.006$]). Statistically significant differences are summarized in **Table 2**.

DISCUSSION

In accordance with our hypothesis, kelp gulls presented higher occurrence and load of ARGs than Magellanic penguins, findings that may potentially be influenced by the contrasting behaviors of these two seabird species in regard to feeding niches, interaction with human-impacted areas and dispersal. The kelp gull is the most widespread and abundant gull species in the Southern Hemisphere (38–40). Like other gull species, kelp gulls are extremely opportunistic and generalist feeders, very adapted to exploiting a wide variety of human-impacted and highly populated areas, and food subsidies (e.g., fishing discards and

TABLE 2 | Statistically significant differences between kelp gull (*Larus dominicanus*) and Magellanic penguin (*Spheniscus magellanicus*): ARG occurrence, mean number of ARGs per sample, mean load percentage of each ARG, the mean number of antimicrobial classes presented in each sample, percentage of multiresistant microbiomes, and resistance patterns.

Parameter	*p*-value	kelp gull ($n = 14$) 95% CI	Magellanic penguin ($n = 11$) 95% CI
Occurrence of *tet*(A)	0.03	36% (7, 64%)	0%
Occurrence of *tet*(M)	0.015	43% (13, 73%)	0%
Occurrence of *sul*II	0.007	50% (20, 80%)	0%
Occurrence of bla_{TEM}	0.036	79% (54, 103%)	36% (2, 70%)
Mean load percentage of *tet*(A)	0.031	−5.8 (−7.6, −4.1)	−8.0
Mean load percentage of *tet*(M)	0.016	−5.8 (−7.4, −4.3)	−8.0
Mean load percentage of *sul*II	0.008	−4.8 (−6.8, −2.9)	−8.0
Mean load percentage of *aad*A	0.045	−5.4 (−7.2, −3.6)	−7.7 (−8.4, −7.0)
Mean load percentage of bla_{TEM}	0.009	−2.2 (−4.1, −0.2)	−5.8 (−7.9, −3.7)
Percentage of resistance to sulfonamides	0.033	50% (20, 80%)	9% (−11, 29%)
Percentage of resistance to betalactams	0.036	79% (54, 103%)	36% (2, 70%)
Mean number of genes	0.031	6.4 (3.6, 9.2)	2.7 (1.2, 4.2)
Mean number of classes	0.024	4.0 (2.8, 5.2)	2.1 (1.2, 3.0)
Percentage of multiresistant microbiomes	0.032	71% (44, 98%)	27% (−4, 59%)
Clustering (0 = low; 1 = high)	0.006	0.6 (0.4, 0.9)	0.1 (−0.1, 0.3)

Mann-Whitney U non-parametric test. Numbers in parenthesis indicate the 95% confidence interval (CI).

refuse disposals) (40–42). Such behaviors have been associated with the presence of ARGs in kelp gulls in Argentina (43), as well as in other gull species worldwide (17, 44, 45). Conversely, the Magellanic penguin is a migratory upper trophic level predator and the most abundant penguin in temperate areas, widely distributed along the southern coast of South America (24). Magellanic penguins remain in their colonies during breeding and molting periods, adopting a pelagic behavior while migrating along the continental shelf off the coast of northern Argentina, Uruguay, and southern Brazil (25, 26). Although scarce, studies on the presence of ARGs in penguins have associated ARGs occurrence with anthropization in remote locations (20, 46).

The *mec*A gene was detected in 14% (2/14) of kelp gulls, but not in penguins. This gene was reported in other wild bird groups in Brazil [passerines (47)] and Europe [corvids (48, 49), storks (50), and vultures (49)]. Nevertheless, to the best of our knowledge, this is the first report of *mec*A in seabirds in the Americas, only previously reported in European herring gulls (*Larus argentatus*) in Lithuania through metagenomics (51). The *mec*A gene is widely disseminated among *Staphylococcus aureus* and other staphylococcal species (52), encoding resistance to methicillin and cross-resistance to other β-lactam antimicrobials (52–54). Methicillin-resistant staphylococci are disseminated worldwide, frequently causing health care- and community-associated infections (52, 55), being considered one of the leading causes of nosocomial infection in Latin America (56), where it was also reported in animals, food products and the environment (57–59).

The bla_{TEM} gene was detected in kelp gulls and Magellanic penguins, being the most prevalent gene in the former species (79%; 11/14). Bla_{TEM} also presented the highest mean load percentage in this study (−2.2, in kelp gull), indicating an increased dissemination potential in comparison with the other ARGs detected here. Furthermore, the Furthermore, the bla_{TEM} gene presented significant differences in kelp gull in comparison with Magellanic penguin in regards to occurrence (79 and 36%. $p = 0.032$) and mean load percentage (−2.2 and −5.8. $p = 0.032$). This gene has been previously described in seabirds in Brazil (16), the United States (14, 60), and Europe Europe (51, 61–64). The TEM betalactamases confer resistance to cephalosporins and penicillins (65), one of the oldest and most widely used antimicrobial classes in humans and veterinary medicine (66, 67), partialy explaining their dissemination in the tested seabirds. Recently, a similar study in Brazil, that evaluated the microbiome of six species of wild seabirds (overall, 304 individuals), found that the bla_{TEM} occurrence and percentage loads ranged from 0 to 25% and −8 to −0.6, respectively, and that the bla_{TEM} prevalence was significantly higher in migratory in comparison with non-migratory species (16). Interestingly, despite the considerable differences regarding species and sampling size, herein we found higher bla_{TEM} occurrence and mean load percentages in kelp gull and Magellanic penguin, and higher bla_{TEM} occurrence in the non-migratory synanthropic species (kelp gull). Epidemiologically, our findings are very concerning, because while the migratory species evaluated by Ewbank et al. (16) were using a pristine habitat (Rocas Atoll), kelp gulls and Magellanic penguins are using anthropized environments. Kelp gulls especially, are using heavily anthropized areas, which likely influence not only the acquisition and potential transmission of ARGs, but also their development and maintenance, once these individuals are continuously more exposed to ARGs sources (e.g., landfills, wastewater), and consequently, to reinfection.

The genes encoding tetracycline resistance (*tet*) were the most prevalent in this study (79%; 11/14 in kelp gull and 73%; 8/11 in Magellanic penguin): *tet*(A), *tet*(M) and *tet*(W) in kelp gull, and *tet*(Q) in Magellanic penguin. Additionally, *tet*(Q) was the most prevalent gene in the penguin group (55%, 6/11). Interestingly, Ewbank et al. (16) found that tetracycline-encoding genes were also the most prevalent antimicrobial class (ranging from 64.5 to 87.9%), significantly greater than the rest of the other ARGs classes (16). Moreover, we observed significant differences between kelp gull and Magellanic penguin in terms of *tet*(M) and *tet*(A) occurrence (43 and 0%. $p = 0.015$, and 36 and 0%. $p = 0.003$, respectively), and mean load percentage (−5.8 and −8. $p = 0.016$, and −5.8 and −8. $p = 0.031$, respectively). The high *tet* occurrence found herein was not surprising, once it had been previously detected in other seabirds in Brazil (16), and its extensive use in human and veterinary medicine, and in agriculture (68, 69). *Tet* genes have been reported in gulls in the Americas (16, 70, 71) and Europe (9, 51, 61–64, 72), and in wild penguins in Antarctica (46, 73) and Brazil (74).

Genes *sul*I and *sul*II were detected in kelp gull (*sul*II: 50% [7/14]) and in a Magellanic penguin (*sul*I: 9% [1/11]). *Sul*I and *sul*II encode resistance to sulfonamides and have been previously reported in wild seabirds in Brazil (16), with the former also reported in gulls in Europe (61–63, 72). *Sul*II presented significant differences in kelp gulls in comparison with Magellanic penguin regarding its occurrence (50% and 0%. p =0.007) and mean load percentage (−4.8 and −8. p = 0.008). Additionally, resistance to sulfonamides was significantly different in kelp gull in comparison with Magellanic penguin (50 and 9%. p = 0.033). Interestingly, the prevalences of sulfonamide and *sul*II gene were statistically significant higher in seabirds from an anthropized in comparison with a pristine environment (16). Sulfonamides are among the oldest synthesized antimicrobials, used in several medical therapies (75). This antimicrobial class is known to persist in the environment (76), and to resist biodegradation in wastewater-treatment processes and in media with elevated microbial activity, such as byproduct sludge (77, 78). Thus, the fact that such antimicrobial class presented more significant findings in the synanthropic coastal species (kelp gull), likely indicates higher ARGs pollution of coastal environments due to anthropogenic impact and environmental contamination (e.g., WWTP effluents and wastewater discharge) (10, 16).

Finally, we also observed significant differences in the *aad*A mean load percentage between kelp gull and Magellanic penguin (respectively, −5.4 and −7.7. p = 0.045). The *aad*A gene encodes resistance to two aminoglycosides: streptomycin and spectinomycin (79). Aminoglycosides are used against several aerobic Gram-negative bacilli, many staphylococci, some streptococci, and mycobacteria. Of note, streptomycin is used in multidrug treatments against multidrug-resistant *M. tuberculosis* infections (80). *Aad*A has been previously reported in gull species (61, 63, 72), and in little penguins (*Eudyptula minor*) (81).

Our findings, especially the detection of the public health relevant *mec*A and bla_{TEM} genes, are very concerning. The present study evaluated samples collected upon the individuals' admission into a rehabilitation center. Thus, the ARGs detected here were acquired in the wild, most likely in the environment (either in anthropized (e.g., landfills, sewage) or natural (e.g., aquatic, continental shelf) epidemiological settings), but potentially from other sources as well, such as infected food items (82) and through intra and/or interspecific interactions (e.g., kleptoparasitism). Wildlife is not naturally exposed to antimicrobial therapy in the wild, but once under treatment in rehabilitation centers, the presence of ARGs in their microbiome may interfere, and even prevent, successful therapy. Similarly to nosocomial settings, due to the intense use of antimicrobials, rehabilitation centers may be highly contaminated by these drugs and their metabolites, as well as by ARGs, and exert intense selective pressure over the local resistome (83, 84). As a consequence, rehabilitation centers may be hot spots for ARGs acquisition, interaction, and development, facilitating resistance exchanges among wildlife, humans (e.g., staff) and the environment, both while in-care and upon release (84). Thus, rehabilitation centers are very important and informative settings for the study of ARGs within the One Health interface.

Magellanic penguins are a migratory species. Bird migrations may cover great distances, through natural bio-barriers such as oceans, thus considered as holders of a potential central epidemiological role in the dissemination of ARGs, even to remote locations (3, 16, 44). Because migratory birds are capable of acquiring ARGs from humans, domestic animals and the environment (15, 17, 20, 44, 45, 85–89), this group has been largely suggested as reservoirs and dispersers of antimicrobial resistance (45, 88, 90). Despite a recent experimental study in captive ring-billed gulls (*Larus delawarensis*) in which the individuals were able to shed and contaminate the artificial environment and infect cospecifics in a controlled setting (91), further studies under natural conditions are necessary to confirm such hypothesis. Herein, migration may have not been a key factor from an epidemiological perspective of ARGs dispersal affecting humans, because despite our significant findings in Magellanic penguin [e.g., detection of ARGs in 10 out of the 11 individuals and of a gene of great public health importance (bla_{TEM})], this is a highly pelagic species that spends a great part of its life cycle in the oceans (26), sustaining limited direct contact with humans. By contrast, kelp gulls are not migratory, only capable of small geographical dislocations (24). Such species presents synanthropic behavior and adaptability to highly anthropized areas, in closer contact with humans and food-producing animals, consequently playing a more relevant role than Magellanic penguins in the epidemiological chain of ARGs within the human-animal-environmental interface. These findings show that all geographical dislocations – from great migrations to small geographical movements, must be considered in the study of ARGs dispersal and epidemiology.

Herein, we showed that the biological and ecological parameters evaluated in this study (i.e., dispersal [migratory and non-migratory], feeding niche [coastal and pelagic], and interaction with human-impacted areas [synanthropic and non-synanthropic]) are key factors in the complex epidemiology of ARGs in wild seabirds. Additionally, we reported the first detection of the *mec*A gene in seabirds in the Americas. Our findings greatly contribute to the current knowledge on ARGs in wild birds both nationally and worldwide, emphasize the importance of ARGs studies in wildlife rehabilitation settings, and reinforce the utility of culture-free highly sensitive molecular diagnostics to assess ARGs in the microbiome of wild birds. Nevertheless, it is important to consider the limitations of our study: (1) our techniques characterize the resistance genotype, not the phenotype, (2) microbiomes were evaluated at the exact point in time of each sample collection, and host-bacteria could eventually lose ARGs-containing plasmids prior to transmission and/or dispersal, and (3) our small sampling size. Admission and pre-release sampling and analysis would allow future assessment of rehabilitation centers as epidemiological settings. Further studies on ARGs in the microbiome of a greater number of seabirds, considering biological and ecological parameters, and the species' natural history (e.g., feeding strategy, habitat, territory), are necessary to broaden our understanding regarding the occurrence and diversity of ARGs in seabirds, and their role as potential sources of infection and dispersal within the One Health chain of ARGs acquisition, interaction, and dissemination.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethical Committee in Animal Research of the School of Veterinary Medicine and Animal Sciences, University of São Paulo (process number 1753110716).

AUTHOR CONTRIBUTIONS

AE, FE, CS, and JC-D: conceptualization. AE, CS, IS, and EN: formal analysis. AE, CS, IS, SC-S, MA, JR, and CK: original draft preparation. All authors: review and editing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.651781/full#supplementary-material

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Polyene-Producing *Streptomyces* spp. From the Fungus-Growing Termite *Macrotermes barneyi* Exhibit High Inhibitory Activity Against the Antagonistic Fungus *Xylaria*

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Fungus-growing termites are engaged in a tripartite mutualism with intestinal microbes and a monocultivar (*Termitomyces* sp.) in the fungus garden. The termites are often plagued by entomopathogen (*Metarhizium anisopliae*) and fungus garden is always threatened by competitors (*Xylaria* spp.). Here, we aim to understand the defensive role of intestinal microbes, the actinomycetes which were isolated from the gut of *Macrotermes barneyi*. We obtained 44 antifungal isolates, which showed moderate to strong inhibition to *Xylaria* sp. HPLC analysis indicated that different types of polyenes (tetraene, pentene, and heptaene) existed in the metabolites of 10 strong antifungal *Streptomyces* strains. Two pentene macrolides (pentamycin and 1′14-dihydroxyisochainin) were firstly purified from *Streptomyces* strain HF10, both exhibiting higher activity against *Xylaria* sp. and *M. anisopliae* than cultivar *Termitomyces*. Subsequently, tetraene and heptaene related gene disruption assay showed that the mutant strains lost the ability to produce corresponding polyenes, and they also had significantly decreased activities against *Xylaria* sp. and *M. anisopliae* compared to that of wild type strains. These results indicate that polyene-producing *Streptomyces* from the guts of *M. barneyi* have strong inhibition to competitor fungus and polyenes contribute to inhibitory effects on *Xylaria* sp.

Keywords: fungus-growing termite, *Macrotermes barneyi*, *Streptomyces*, polyene, *Xylaria*

INTRODUCTION

Many fungus-growing insects (ants, termites, and southern pine beetles) actively cultivate one or two symbiotic fungi (Basidiomycota or Ascomycota) in an obligatory association (Chapela et al., 1994; Aanen et al., 2002; Hofstetter et al., 2006). The fungi serve as food for the insects, and the insects provide nutrients for fungus growth. However, the fungi cultivated by fungus-growing ants (Attini), fungus-growing termites (Macrotermitinae), and fungus-growing beetles

(*Dendroctonus frontalis*) are plagued by specialized pathogens *Escovopsis* spp., *Xylaria* spp., and *Ophiostoma minus*, respectively (Currie et al., 1999a; Hofstetter et al., 2006; Ju and Hsieh, 2007). In addition, entomopathogenic fungi, such as *Beauveria bassiana* and *Metarhizium anisopliae*, are abundant in soil, and can be infectious to fungus-growing insects (Strasser et al., 2000; Mburu et al., 2009).

The multilateral symbioses systems of fungus-growing insects are compared to human agricultural systems. The symbiotic interactions in fungus-growing ant and beetle systems have been documented (Ramadhar et al., 2014). Microbial symbionts of fungus-growing ants and southern pine beetles, mainly *Pseudonocardia* and *Streptomyces* (Currie et al., 1999b; Cafaro et al., 2011), protect their fungal cultivars from competitor fungi via production of antifungal compounds, including dentigerumycin, gerumycins, antimycins, 9-methoxyrebeccamycin, cyphomycin, candicidin, mycangimycin, and macrolactam frontalamides (Scott et al., 2008; Haeder et al., 2009; Oh et al., 2009a,b; Blodgett et al., 2010; Schoenian et al., 2011; Sit et al., 2015; van Arnam et al., 2015; Chevrette et al., 2019b). Leaf-cutting ant-associated *Pseudonocardia* and *Amycolatopsis* isolates with antifungal activities protect hosts from entomopathogenic fungi (Sen et al., 2009; Mattoso et al., 2012).

Fungus-growing termites (Termitidae: Macrotermitinae), a group of higher termites, are abundant in tropical and subtropical regions of Asia and Africa (Liu et al., 2013). They have a significant effect on plant decomposition and element cycling (Li et al., 2017, 2021). The bacteria in termite mound soils could improve the fertility of the soil and suppress soil borne plant pathogens through the production of antibiotics and nutrient fixation, thus they might help reduce the farm use of chemical fertilizers and pesticides (Enagbonma and Babalola, 2019). Actinobacteria from nests of termite species, including *Macrotermes natalensis*, *Microtermes* sp., and *Odontotermes* sp., show higher antifungal activity against cultivar *Termitomyces* than *Xylaria* (Visser et al., 2012), while other Actinobacteria strains, or chemicals such as Actinomycin D and macrotermycins isolated from termite-associated actinomycetes exhibit selective antifungal activity against competitors (*Pseudoxylaria* or *Xylaria*) over *Termitomyces* (Beemelmanns et al., 2017; Yin et al., 2019). The role of actinobacteria in fungus-growing termites remains to be further explored.

Macrotermes barneyi, is a fungus-growing termite in the subfamily of Macrotermitinae, and widely distributed in southern China (Wu et al., 2012). *M. barneyi* lives in obligate symbiosis with a specialized fungal cultivar *Termitomyces* sp. (Basidiomycotina), which is the only visible fungus in active nests. *Xylaria* sp. thrives in abandoned termite nests and is the competitor of *Termitomyces* sp. Here, we assess the antifungal activity of actinomycetes through strain isolation, paired bioassay, HPLC analysis, compound identification, gene disruption and bioactivity assay. The results indicated that *M. barneyi* associated antifungal *Streptomyces* produced different types of polyenes, which contributes to the inhibitory activity against the antagonistic fungi.

MATERIALS AND METHODS

Sample Collection and Tested Fungi

The workers and soldiers of *M. barneyi* were collected in July 2017 and June 2018 from termite nests in Hunan (E 112° 96′, N 26° 58′) and Guangdong (E 113° 60′, N 24° 82′), China. The termites were transferred into sterilized tubes and stored on dry ice. Gut dissection was performed within 48 h. The insect-pathogenic fungi *M. anisopliae* ACCC 30103 and *B. bassiana* ACCC 30730 were purchased from the Agricultural Culture Collection of China, Beijing, China. *Termitomyces* sp. and *Xylaria* sp. were isolated from the fungus gardens of *M. barneyi*. Strain identity was verified by PCR amplification and sequencing of the ITS gene using the primers ITS1 and ITS4 (Heine et al., 2018).

Gut Dissection and DNA Extraction

Termite workers were surface sterilized by successive soaking in sterile water and phosphate buffered saline, and then rinsed with 70% ethanol and sterile water (Ramadhar et al., 2014). 150 workers and 50 soldiers were dissected. The workers guts were aseptically removed with forceps and divided into foregut, midgut, and hindgut, which were immersed in PBS buffer (NaCl 8 g/L, KCl 0.2 g/L, Na_2HPO_4 1.42 g/L, KH_2PO_4 0.27 g/L, pH 7.4) (Schmitt-Wagner et al., 2003). The samples were homogenized and transferred into sterile tubes to be used for actinomycetes isolation.

Strain Isolation and Phylogenetic Analysis

Actinomycetes were isolated by the serial dilution method on Gause's No.1, M2 (Mincer et al., 2002), M4 (Mincer et al., 2002), PY-CMC (Min et al., 1994), HVA (Subramani and Aalbersberg, 2013), and Chitin media (Benndorf et al., 2018). Fifty workers and soldiers were dissected to obtain gut samples. After shaking at 150 rpm for 30 min, gut suspensions of 10^{-1} to 10^{-3}-fold dilutions were plated onto six isolation media with or without inhibitors (50 μg/mL potassium dichromate, 50 μg/mL cycloheximide, and 20 μg/mL nalidixic acid) (Malviya et al., 2014). The plates were incubated at 30°C for 7–15 days. Colonies with distinct morphological characteristics were transferred and purified on yeast extract-malt extract (ISP2) agar plates (Shirling and Gottlieb, 1966). Isolates were kept in 20% glycerol at −80°C for long-term preservation.

For genomic DNA extraction, actinomycetes were grown in nutrient-rich liquid Tryptic Soy Broth (TSB) medium at 30°C for 4 days. Cells were then harvested, and the genomic DNA was extracted using a bacterial DNA Extraction Kit (Omega, BioTek, United States). The 16S rRNA gene was amplified by PCR using general primers 27F and 1492R (Liu et al., 2013). Amplification reactions were standardized in a total volume of 50 μl containing 2× EasyTaq master mix (Takara, Dalian, China), 100 ng genomic DNA of isolated actinomycetes, 10 μM of each primer and sterile water. Cycle parameters for PCR were 10 min at 95°C (initial denaturation); 35 cycles at 95°C for 30 s, 58°C for 30 s and 72°C for 2 min; 7 min at 72°C (final

elongation). The amplified 16S rRNA fragments were cloned into the pMD19-T vector. After sequencing, these complete 16S rRNA gene sequences were compared with available sequences in the GenBank database using the BLAST program in NCBI. The phylogenetic tree was constructed via the neighbor-joining tree algorithm using MEGA version 7.0 (Kumar et al., 2016). The confidence values of nodes in the trees were evaluated by 1000 bootstrap replicates (Felsenstein, 1985).

Actinobacteria–Fungi Paired Challenge Assays

Actinobacteria-fungi paired bioassays were performed according to a previously reported method (Benndorf et al., 2018). Strains were grown at 30°C for 3 days in ISP2 or TSB. Aliquots of 20 μl liquid culture were used to inoculate on the centers of Potato Dextrose Agar (PDA) plates. Then, plates were inoculated at the edges with two agar pieces covered with fungal mycelium (*Xylaria* sp.). All assays were performed in triplicate. Plates were incubated for 7–10 days at 30°C and checked daily until a clear and stable zone of inhibition (ZOI) appeared (normally after 7 days). The ZOI values were given by measuring the distance of two inoculated *Xylaria*. The final ZOI is the average value of three replicates. The categories of strong inhibition (ZOI > 2 cm), moderate inhibition (ZOI 0.5–2 cm), and little or no inhibition (ZOI < 0.5 cm) were defined (Scott et al., 2008).

Antifungal Assay of Crude Extracts

Actinomycetes isolates with strong antifungal activity against *Xylaria* sp. in Actinobacteria-fungi paired bioassays were cultivated in 40 ml TSB, MS (2% D mannitol, 2% soybean meal, pH 7.2) and ISP2 liquid media for 4 days at 30°C with shaking at 150 rpm. The liquid broth of 21 actinomycetes isolates was centrifuged at 12000 × *g* for 10 min. Culture supernatants were dried in vacuum rotary evaporator at 38°C and dissolved in 2 ml methanol, which was used as crude extracts. The assay was conducted using the agar diffusion method (Haeder et al., 2009). First, sterilized stainless-steel Oxford cups (10 × 6 × 8 mm) were placed on PDA plates. Next, PDA agar medium with fungal spores and mycelium was introduced into the upper plates. After solidification, the Oxford cups were removed, the crude extracts (100 μl) were added into each well. The diameters of the inhibition zones around the wells were observed after incubation for 1 days at 30°C. The active crude extracts were subjected to HPLC analysis.

HPLC Analysis of the Active Crude Extracts

The active crude extracts were applied to HPLC (DIONEX Ultimate 3000 instrument) analysis. HPLC was operated at a flow rate of 1 mL/min with a C18 column (YMC-Pack Pro, 250 × 4.6 mm, 5 μm). Water-0.1% formic acid (solvent A) and acetonitrile-0.1% formic acid (solvent B) were used as the mobile phases. The column was eluted for a conventional separation by using elution gradient of 20% B, increased to 35% B in 3 min, 45% B in 10 min, 90% B in 20 min, and 100% B in 22 min (held for 3 min). Afterward, the elution gradient was reduced to 20% B in 27 min and sustained for 3 min.

Genome Sequencing and Analysis

The *Streptomyces* strains were cultivated in 20 ml TSB liquid medium for 2 days at 30°C with shaking at 150 rpm. The culture broth (20 ml) was centrifuged at 12000 × *g* for 10 min, culture supernatant was removed and cell pellets were harvested. Genomic DNA was isolated from cell pellets that were physically ground in liquid N_2 and then extracted using a bacterial DNA Extraction Kit (Omega, BioTek, United States). Whole-genome sequencing was performed using PacBio SMRT sequencing technology by the Novogene sequencing company. Biosynthetic gene clusters (BGCs) of secondary metabolites were predicted by antiSMASH 4.0 (Blin et al., 2017). The annotation of the polyene BGCs was performed using Blastp (non-redundant proteins).

Isolation and Identification of the Active Compounds From *Streptomyces* sp. HF10

To isolate the antifungal compounds from *Streptomyces* sp. HF10, we set out a large-scale culture (20 L ISP7 broth) (Shirling and Gottlieb, 1966) for 4 days at 30°C with shaking at 160 rpm. The culture was centrifuged to obtain the supernatant followed by absorption using macroporous resin D101 overnight. The resin was loaded on a column and washed with water and then eluted with 50% methanol and 100% methanol, respectively. The antifungal activity of each fraction was tested. The active fractions were dried by evaporation to obtain the crude extract.

The crude extract was loaded on a middle-pressure liquid chromatography column (MPLC; 80 g RP-18 silica gel; 20, 40, 60, and 100% acetonitrile containing 0.1% formic acid, 200 ml for each gradient) to yield Fr.1-4. Fr 2 was subjected to Sephadex LH-20 to yield Fr.2a and 2b. Fr.2a and Fr.2b were subjected to MPLC (25%, 30% acetonitrile containing 0.1% formic acid) to yield Fr.2a1 and Fr.2b2. Fr.2a1 and Fr.2b2 were purified by semi-preparative reverse-phase HPLC (DIONEX Ultimate 3000 instrument, YMC-Pack Pro C18, 10 × 250 mm, 5 μm, flow rate 4 mL/min, UV detection at 350 nm) to yield 1 (10 mg, 38% acetonitrile containing 0.1% formic acid, t_R = 9.5 min) and 2 (9 mg, 29% acetonitrile containing 0.1% formic acid, t_R = 8.5 min), respectively. Subsequently, compounds 1 and 2 dissolved in methanol were subjected to high resolution mass spectrometry (HRMS) for determination of molecular mass. Compounds 1 and 2 were dissolved in DMSO-*d* to measure ^{1}H, ^{13}C-NMR and two-dimensional NMR spectra on a 600 MHz spectrometer.

In vitro Antifungal Assay and Determination of Minimal Inhibitory Concentrations

Antifungal activity of the purified compounds was monitored by the paper disc diffusion method (Um et al., 2013). An agar piece covered with fungal mycelium (*Xylaria* sp. or *Termitomyces* sp.) was inoculated at the center of the PDA plates. Plates were incubated for 7 days at 30°C until a clear colony was apparent.

Sterile discs were placed around the colony covered with different amounts (2.5, 5, and 10 μg) of purified pentamycin or 1′14-dihydroxyisochainin. DMSO was used as a negative control. All of the tested discs were observed on a daily basis for 3 days. A liquid antifungal assay was performed to measure minimal inhibitory concentrations (MICs) of compounds. To prepare mycelia or spore suspensions, *Xylaria* sp., *Termitomyces* sp. and two entomopathogens were cultivated on PDA plates 5–7 days, then mycelia or spores were collected and homogenized in potato dextrose broth medium to maintain the absorbance of OD_{600} approximately 0.5. The suspension was transferred into the wells of 96-well microplate followed by adding 2-fold serial dilution of purified compounds or amphotericin with a final concentration of 128, 64, 32, 16, 8, 4, 2, and 1 μg/mL. Subsequently, in order to determine the exact difference in antifungal activity of purified compounds against tested fungi, different concentrations of pentamycin (30, 25, and 20 μg/mL), 1′14-dihydroxyisochainin (150 145, and 140 μg/mL) or amphotericin (60, 55, 50, 45, and 40 μg/mL) were applied against *Termitomyces* sp. The above suspension without adding compound used as negative control was simultaneously cultured. The plates were incubated at 30°C and monitored for inhibition. The MIC value was calculated as the lowest concentration showing complete inhibition of the tested strain. All of the assays were performed in triplicate.

Construction of Natamycin Biosynthetic Gene Disruption Mutant of *Streptomyces* sp. GS7

To inactivate the BGC of natamycin (*pim*) in *Streptomyces* sp. GS7, the polyketide synthase gene (*pimS0*) was replaced with a gene disruption cassette by homologous recombination. The gene disruption cassette containing a selectable apramycin resistance gene *aac(3)IV* and an origin of transfer gene *oriT* were jointly amplified by PCR from plasmid PIJ773 (Gust et al., 2003) with the primers (*aac(3)IV*-P4F/*aac(3)IV*-P4R). Two homologous arms flanking *pim*S0 were amplified from the genomic DNA of *Streptomyces* sp. GS7 with the primers (dP4LF/dP4LR, dP4RF/dP4RR). The above three fragments were assembled into pUC19 by Gibson assembly (Gibson et al., 2009), to yield pUC19-Δ*pim*S0. The constructed plasmid pUC19-Δ*pim*S0 was transformed into *Streptomyces* sp. GS7 by conjugation and the exconjugants were selected on MS agar medium with apramycin (Sangon Biotech, Shanghai, China). After three rounds of non-selective growth, the desired double cross-over mutants were confirmed by PCR. Amplification reactions were performed in a final volume of 25 μl containing 2× Rapid Taq master mix (Vazyme Biotech Co., Nanjing, China), 50 ng genomic DNA of mutants or wide-type strains, 10 μM of each primer (S0vF/S0vR), 5 μl PCR enhancer (Vazyme Biotech Co., Nanjing, China) and sterile water. PCR amplification program involved an initial DNA denaturation at 95°C for 10 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s and elongation at 72°C for 3 min 30s, which followed by a final extension at 72°C for 7 min. All primers were listed in **Supplementary Table 7**.

Construction of the Candicidin Biosynthetic Gene Disruption Mutant of *Streptomyces* sp. GF20

To inactivate the BGC of candicidin (*fsc*) in *Streptomyces* sp. GF20, the polyketide synthase gene (*fsc*A) was replaced with a gene disruption cassette by homologous recombination. The gene disruption cassette *oriT-aac(3)IV* was amplified by PCR from plasmid pSET152 (Bierman et al., 1992) with the primers (*aac(3)IV*-*Xba*I-F/*aac(3)IV*-*Xba*I-F) and digested with *Xba*I. Two homologous arms flanking *fsc*A were amplified from the genomic DNA of *Streptomyces* sp. GF20 with the primers (dP7-*Pst*I-LF/dP7-*Xba*I-LR, dP7-*Xba*I-RF/dP7-*Hin*dIII-RR). Homologous arms were digested with *Pst*I/*Xba*I or *Hin*dIII/*Xba*I, respectively. The three fragments were ligated into pSPRam, which is pOJ260 (Bierman et al., 1992) derivative containing the reporter melanin gene *mel* (Wang et al., 2018) and resistance marker *aadA* (spectinomycin-resistance), to yield pSPRam-Δ*fsc*A. The constructed plasmid pSPRam-Δ*fsc*A was transformed into *Streptomyces* sp. GF20 by conjugation and exconjugants were selected on MS agar medium with apramycin. Then the cells were cultured on MS medium plates containing 5 mg/L copper sulfate and 100 mg/L tyrosine, white colonies as double cross-over mutants were picked (Wang et al., 2018). The double cross-over mutants were confirmed by PCR. The reaction systems were same as described early. Program parameters for PCR were 10 min at 98°C (initial denaturation); 35 cycles at 95°C for 30 s, 58°C for 30 s and 72°C for 6 min; 7 min at 72°C (final elongation). All used primers were listed in **Supplementary Table 7**.

RESULTS

Isolation and Phylogenetic Analysis of Actinomycetes Strains

Actinobacteria strains were isolated from the foregut, midgut and hindgut of workers as well as the gut of soldiers. According to distinct morphological features of actinomycetes and 16S rRNA gene sequence analysis, in total, we obtained 83 strains of actinomycetes belonging to eight genera from two termite samples collected from Hunan and Guangdong (**Figure 1** and **Supplementary Table 1**). Among these, 72 strains were isolated from workers, and 11 from soldiers. Overall, 60 *Streptomyces* strains were isolated from the foregut, midgut and hindgut of workers and the gut of soldiers. 11 *Kitasatospora* strains were isolated from the foregut of workers and the gut of soldiers (**Figure 1**). Defined by a threshold of <98.65% sequence similarity, seven putative new Actinobacteria species (*Streptomyces*, *Kitasatospora*, and *Amycolatopsis*) were obtained from termite workers (**Supplementary Table 2**). Among them, six strains (HF5, HF17, GF5, GF6, GF15, and GF18) were isolated from the foregut and one strain GM8 from the midgut of *M. barneyi*. Phylogenetic analysis of all of the isolates based on 16S rRNA sequences revealed that the isolates formed two clades within the Actinobacteria phylum (**Figure 2A**), *Streptomyces* and

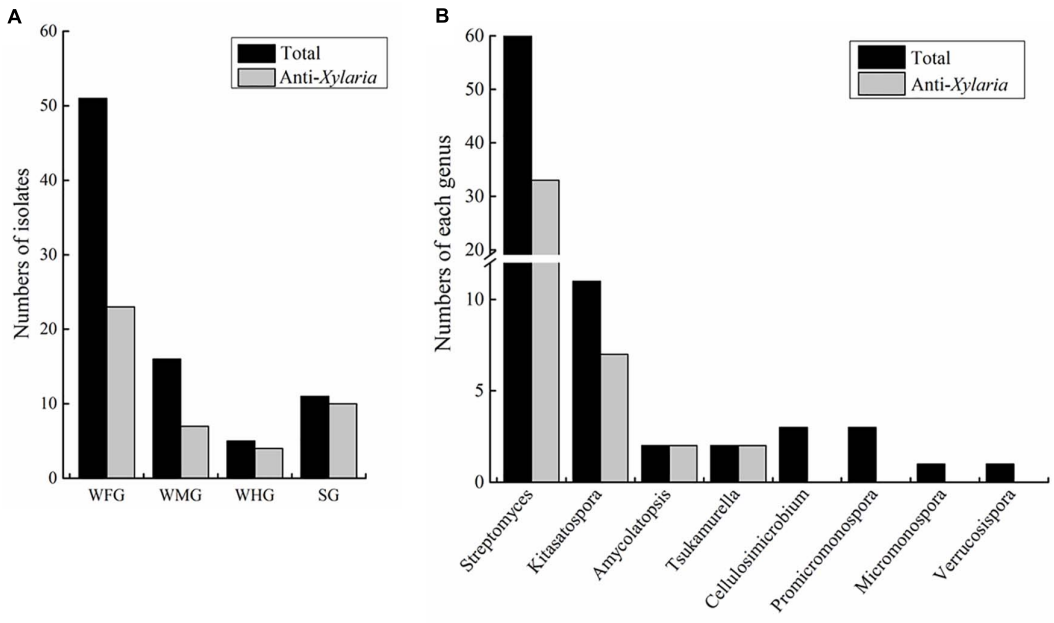

FIGURE 1 | Statistics of Actinobacteria isolated from intestines of *Macrotermes barneyi*. **(A)** Total numbers of Actinobacteria isolates and those with anti-*Xylaria* activity from the worker foregut (WFG), worker midgut (WMG), worker hindgut (WHG), and the soldier gut (SG), respectively. **(B)** Numbers of Actinobacteria isolates and those with anti-*Xylaria* activity in each genus. The anti-*Xylaria* activity was examined by the paired challenge assay.

Kitasatospora isolates clustering into one clade and the remaining 12 isolates clustering into another clade.

The Isolated Actinomycetes Exhibited Antifungal Activity Against *Xylaria* sp.

To explore the antifungal activity of the isolated strains, all the Actinobacteria isolates were challenged against fungal garden competitor *Xylaria* sp. Forty-four actinomycetes isolates, which belongs to four genera (*Streptomyces*, *Kitasatospora*, *Amycolatopsis*, and *Tsukamurella*), exhibited moderate to strong antifungal activity against *Xylaria* sp. (**Figure 2A**). Among these, 23, 7, and 4 strains were isolated from foregut, midgut, and hindgut of workers, respectively, and 10 strains were isolated from the gut of soldiers. Twenty-one isolates with strong antifungal activity belong to two genera (*Streptomyces* and *Kitasatospora*) and cluster into one clade (**Figure 2A**). It was noted that *Streptomyces* strains GF2 and GM11 have the same closest type strain (*Streptomyces drozdowiczii* NRRL B-24297), HF10 and GM9 have the same type strain (*Streptomyces misionensis* JCM 4497), GF26 and HM2 have the same type strain (*Streptomyces sampsonii* ATCC 25495) in the blast search in NCBI (**Supplementary Table 1**), GF2, HF10, and GF26 presented antifungal activity while GM11, GM9, and HM2 had no antifungal activity (**Figure 2A**), which was supported by the study that taxonomic and metabolic incongruence exists in *Streptomyces* (Chevrette et al., 2019a).

Paired challenge assays of five representative strains were shown in **Figure 2B**. As shown in this figure, three *Streptomyces* isolates (HF10, GS7, and GF20) exhibited strong inhibition against *Xylaria* sp. (**Figure 2B**i–iii). *Streptomyces* sp. GM6 exhibited moderate inhibition against *Xylaria* sp. and *Streptomyces* sp. GM1 displayed no inhibition against *Xylaria* sp. (**Figure 2B**iv,v).

Polyene Compounds Were Detected in the Metabolites of Majority of the *Streptomyces* Strains

To identify putative compounds responsible for the antifungal activity, 21 actinomycetes strains with strong antifungal activity against *Xylaria* sp. were selected and cultivated in TSB, MS, and ISP2 liquid media, respectively. Agar diffusion assay showed that the fermentation broth of 15 strains had antifungal activity against *M. anisopliae*. Here *M. anisopliae* was used instead of *Xylaria* sp. owing to the instability of crude extracts. The metabolites of these strains were analyzed by HPLC and the profiles of 10 out of the 15 *Streptomyces* strains, GS7, GS11, GM12, HF10, GF21, GF25, HM3, GF20, GF26, and GH2, showed peaks with typical UV/vis spectra of four type of polyenes (**Table 1** and **Supplementary Figures 1–10**). Among these strains, GS7, GS11, and GM12 showed tetraene peaks (292, 305, and 320 nm) (**Supplementary Figures 1–3**; Mendes et al., 2001); HF10, GF21, and GF25 showed pentene peaks (325, 340, and 358 nm) (**Supplementary Figures 4–6**; Xiong et al., 2012); HM-3 showed linear heptaene peaks (354, 272, and 393 nm) (**Supplementary Figure 7**; Oh et al., 2009b); GF20, GF26, and GH2 showed heptaene peaks (360, 381, and 406 nm) (**Supplementary Figures 8–10**; Haeder et al., 2009). While no obvious polyene peaks were observed in the metabolite profiles of the remaining five strains (*Kitasatospora* sp. HF13, HF15, and GS2, *Streptomyces* sp. HH1 and GS5).

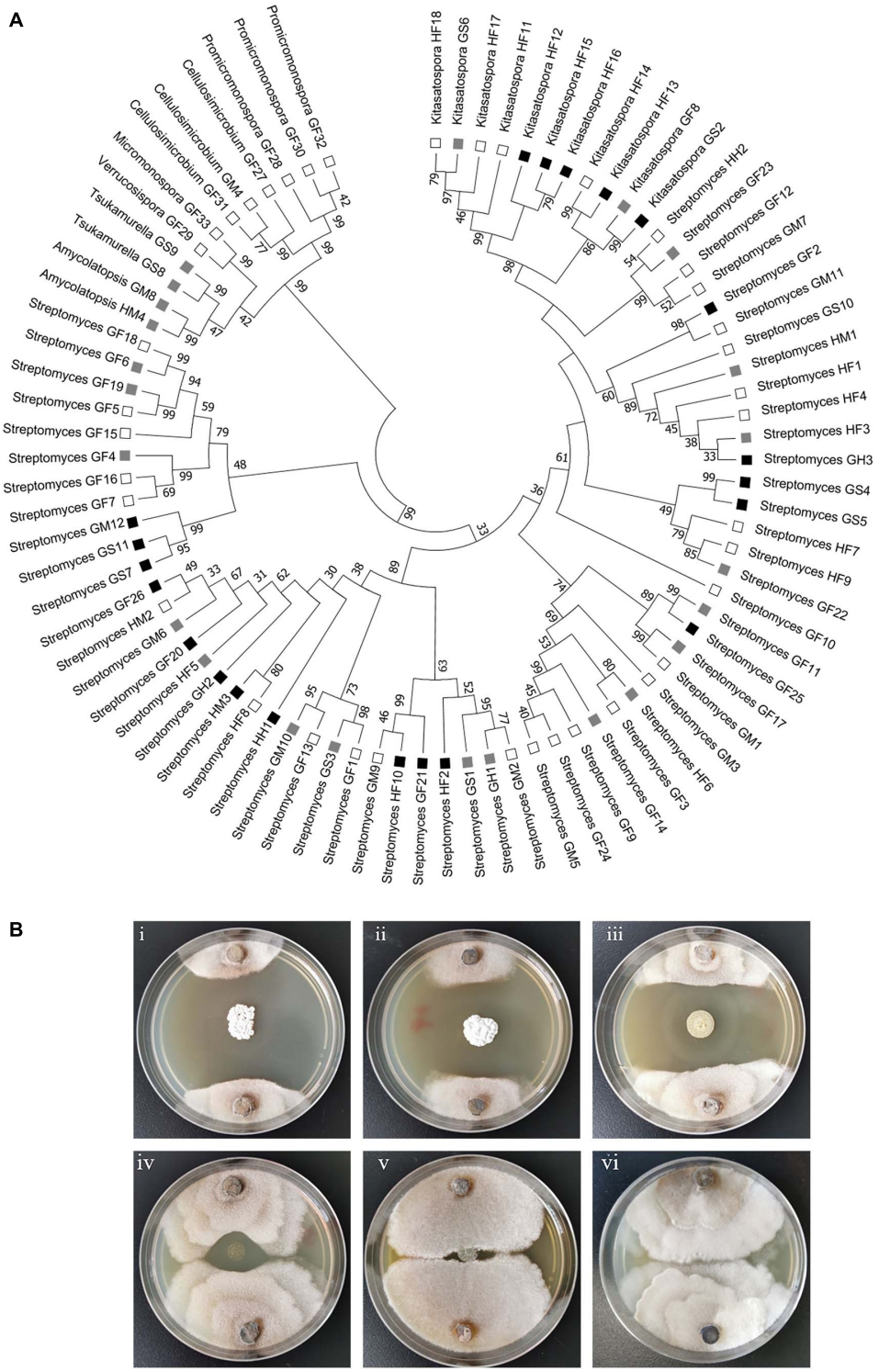

FIGURE 2 | Phylogenetic and anti-*Xylaria* activity analyses of the isolated actinomycetes. **(A)** Phylogenetic tree of all the isolated strains. The tree was constructed with Mega 7.0 by the Neighbor-Joining method. The branch values indicate bootstrap support (>30 are given) of 1000 replicates. The ability to inhibit the growth of *Xylaria* sp. is indicated with black squares (strong inhibition, ZOI > 2 cm), gray squares (moderate inhibition, ZOI = 0.5–2 cm), and open squares (little or no inhibition, ZOI < 0.5 cm) (Scott et al., 2008). HF: Hunan foregut, HM: Hunan midgut, HH: Hunan hindgut, GF: Guangdong foregut, GM: Guangdong midgut, GH: Guangdong hindgut, GS: Guangdong soldier. **(B)** Examination of the anti-*Xylaria* activity of the isolated actinomycetes by paired challenge assay. Inhibition of representative *Streptomyces* against *Xylaria* sp. is shown. (i–v) *Streptomyces* sp. strains against *Xylaria* sp. (i–iii) HF10, GS7, and GF20, strong inhibition; (iv) GM6, moderate inhibition; (v) GM1, no inhibition; (vi) control, *Xylaria* sp.

TABLE 1 | Predicted polyenes from antifungal crude extracts of isolated actinomycetes.

Strains	Isolated resource	Antifungal activity[a]	Absorption peaks (nm)	Polyene type	References
Streptomyces GS7	Solider gut	+++	293,306,320	Cyclic tetraene	Mendes et al., 2001
Streptomyces GS11	Solider gut	+++	293,306,320	Cyclic tetraene	Mendes et al., 2001
Streptomyces GM12	Worker midgut	+++	294,306,321	Cyclic tetraene	Mendes et al., 2001
Streptomyces HF10	Worker foregut	+++	324,340,358	Cyclic pentene	Xiong et al., 2012
Streptomyces GF21	Worker foregut	++	325,340,358	Cyclic pentene	Xiong et al., 2012
Streptomyces GF25	Worker foregut	++	325,340,358	Cyclic pentene	Xiong et al., 2012
Streptomyces HM3	Worker midgut	+++	353,372,393	Linear heptaene	Oh et al., 2009b
Streptomyces GF20	Worker foregut	+++	360,381,400	Cyclic heptaene	Haeder et al., 2009
Streptomyces GF26	Worker foregut	++	360,382,401	Cyclic heptaene	Haeder et al., 2009
Streptomyces GH2	Worker hindgut	+	360,382,401	Cyclic heptaene	Haeder et al., 2009
Kitasatospora HF13	Worker foregut	+++	–	–	–
Kitasatospora HF15	Worker foregut	+++	–	–	–
Streptomyces HH1	Worker hindgut	++	–	–	–
Kitasatospora GS2	Solider gut	++	–	–	–
Streptomyces GS5	Solider gut	+	–	–	–

[a]*The antifungal activity against Metarhizium anisopliae. IZD: Inhibition zone diameter excluding width of Oxford cups. +++: IZD ≥ 1.5 cm. ++: IZD ≥ 1 cm. +: IZD ≥ 0.5 cm.*

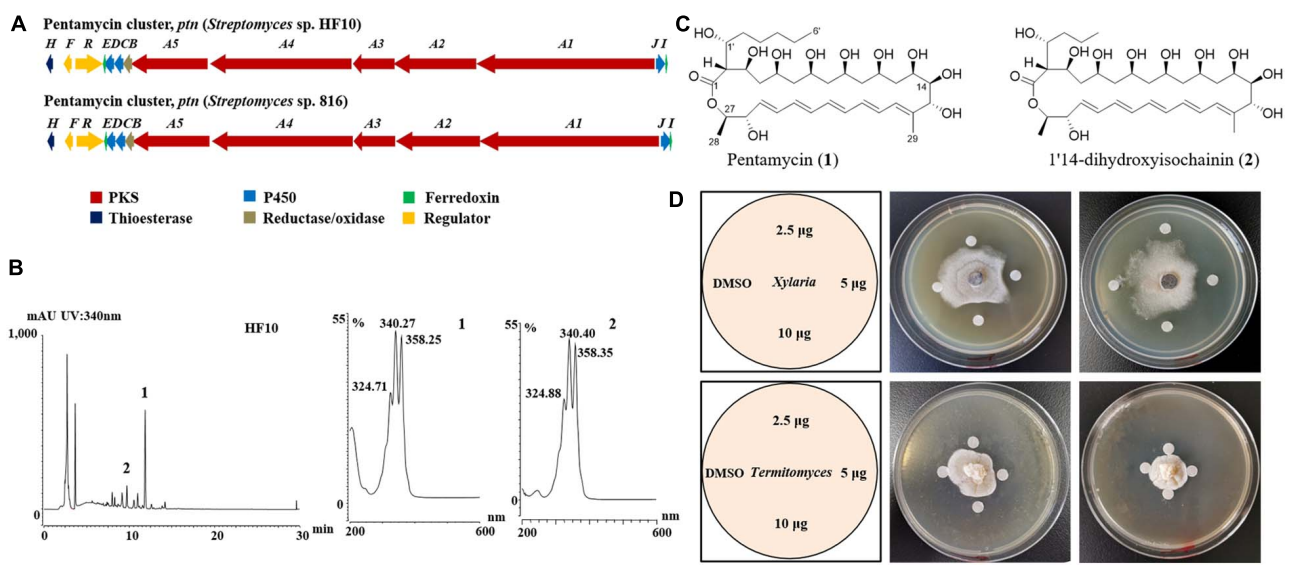

FIGURE 3 | *Streptomyces* sp. strain HF10 produces antibiotics pentamycin and 1′14-dihydroxyisochainin. **(A)** The genetic organization of the pentamycin BGC of strain HF10 compared with the reported one in *Streptomyces* sp. 816. The BGC of pentamycin in strain HF10 was identified by whole genome sequencing and bioinformatic analysis. **(B)** HPLC profiles of metabolites from strain HF10 (left) and UV/vis spectra of compounds 1 and 2 (right). **(C)** The chemical structures of pentamycin (1) and 1′14-dihydroxyisochainin. (2). **(D)** Antifungal activity of pentamycin and 1′14-dihydroxyisochainin by disc diffusion assay. Pentamycin and 1′14-dihydroxyisochainin were purified from strain HF10 as described in section "Materials and Methods." Pentamycin (middle) and 1′14-dihydroxyisochainin (right) against *Xylaria* sp. Pentamycin (middle) and 1′14-dihydroxyisochainin (right) against *Termitomyces* sp. Left, schematic map showing sample arrangement in experiments. A gradient amount (2.5, 5.0, and 10 μg) of pentamycin or 1′14-dihydroxyisochainin was used. An agar piece covered with fungal mycelium (*Xylaria* sp. or *Termitomyces* sp.) was inoculated at the center of the PDA plate for 7 days at 30°C. Sterile discs containing the compounds dissolved in DMSO were placed around the colony. The plates were cultured at 30°C for 3 days and observed on daily basis.

Prediction of BGC and Purification of Antifungal Polyene Compounds From *Streptomyces* sp. HF10 and Bioactivity Assays of Purified Compounds

To identify polyene BGCs, whole genome sequencing of strain HF10 was performed. Comparative analysis revealed that the genetic organization of the target gene cluster in strain HF10 was almost identical to the reported pentamycin gene cluster in *Streptomyces* sp. S816 (**Figure 3A** and **Supplementary Table 3**; Zhou et al., 2019).

To identify the antifungal compounds produced by strain HF10 which had the strongest activity against *Xylaria* sp. as observed in the paired bioassay (**Figure 2B**), we performed

a scale-up fermentation of strain HF10 and obtained 10 mg of pure compound 1 and 9 mg of pure compound 2 after a series of column chromatography purifications (**Figure 3B**). The relative molecular masses of compounds 1 and 2 were detected at m/z 670.3936 (1) and m/z 642.3630 (2) by HRMS, respectively (**Supplementary Figures 11, 12**). The ^{1}H- and ^{13}C-NMR data of 1 and 2 were summarized in **Supplementary Table 4** (**Supplementary Figures 13–20**; Li et al., 1989). Based on these results, the chemical structures of 1 and 2 were determined to be pentamycin and 1′14-dihydroxyisochainin, respectively (**Figure 3C**).

In paper disc diffusion assays, pentamycin (1) exhibited stronger activity against *Xylaria* sp. (**Figure 3D** middle) than *Termitomyces* sp. (**Figure 3D** middle). With the tested concentrations, 1′14-dihydroxyisochainin (2) also selectively suppressed *Xylaria* sp. (**Figure 3D** right) rather than *Termitomyces* sp. (**Figure 3D** right). In the MIC determination assays, the pentamycin (1) exhibited the lowest MIC (4 μg/mL) against *Xylaria* sp., while the MIC of amphotericin B against *Xylaria* sp. was 16 μg/mL (**Table 2**). The pentamycin consistently inhibited the growth of *Xylaria* sp., *M. anisopliae*, and *B. bassiana* more strongly than that of *Termitomyces* sp. While, 1′14-dihydroxyisochainin selectively suppressed the growth of pathogens but not *Termitomyces* sp. Thus, the antifungal activities of the two polyene compounds against *Xylaria* sp. and entomopathogens were stronger than that of the cultivar.

Mutants Completely Abolished the Polyene Production and the Activities of the Mutants Against Antagonistic Fungi Were Greatly Weakened

Two *Streptomyces* strains, GS7 and GF20 (**Figure 2B**), producing tetraene and heptaene compounds (**Supplementary Figures 1, 8**) revealed by HPLC analysis, were subjected to genome sequencing. The sequence alignment showed that the putative tetraene cluster in GS7 was homologous to the natamycin synthesis cluster of *Streptomyces natalensis* ATCC 27448 and showed 77% sequence identity (**Figure 4A**; Mendes et al., 2001). The putative heptaene cluster in GF20 was homologous to the candicidin synthesis cluster of *Streptomyces* sp. FR-008 and showed 100% sequence identity (**Figure 5A**; Chen et al., 2003).

TABLE 2 | Minimal inhibitory concentrations (MIC, μg/mL) of pentamycin and 1′14-dihydroxyisochainin.

Strains	MIC (μg/mL)		
	Pentamycin	1′14-dihydroxyisochainin	Amphotericin B
M. anisopliae ACCC 30103	8	64	16
B. bassiana ACCC 30730	8	64	32
Xylaria sp.	4	32	16
Termitomyces sp.	20	>150	60

Amphotericin B was used as a positive control.

Potential ORFs responsible for natamycin and candicidin biosynthesis were shown in **Supplementary Tables 5,6**.

The 3.2 kb fragment of *pimS0* and 5.7 kb fragment of *fscA*, corresponding to the polyketide synthase gene of polyenes BGCs *pim* (natamycins) and *fsc* (candicidins) in strains GS7 and GF20, respectively, have been replaced with the gene disruption cassette (**Figures 4B**, **5B**). Two mutants GS7Δ*pimS0* and GF20Δ*fsc*A generated by double cross-over were obtained and verified by PCR (**Figures 4C**, **5C**) with primers S0vF/S0vR and AvF/AvR, respectively (**Supplementary Table 7**). HPLC analysis showed that two mutant strains were completely unable to produce compounds 3, 4 (**Figure 6A**), and 5 (**Figure 7A**). *Streptomyces*-fungi paired bioassays showed that both mutants (GS7Δ*pimS0* and GF20Δ*fsc*A) had significantly less inhibitory effects on the growth of *Xylaria* sp. and *M. anisopliae* than the corresponding wild type strains (**Figures 6B,C**, **7B,C**). In addition, both wild type and mutant strains, exhibited stronger inhibition to *Xylaria* sp. than *M. anisopliae*. The results suggested that tetraene and heptaene, produced by *Streptomyces* sp. GS7 and GF20, respectively, are major antifungal compounds against the pathogens (*Xylaria* sp. and *M. anisopliae*), and compared to *M. anisopliae*, *Streptomyces* tends to inhibit *Xylaria* sp. more strongly than *M. anisopliae*.

The relative molecular masses of compounds 3, 4, and 5 were m/z 665.3123, 649.1131, and 1108.5778, respectively (**Figures 6D,E**, **7D**). UV/Vis spectra of compound 3 and 4 were identical to pimaricin and de-epoxypimaricin from *S. natalensis* ATCC 27448, and compound 5 was identical to candicidin D from *Streptomyces* sp. FR-008 (Mendes et al., 2001; Chen et al., 2003). The identity of BGC, UV/Vis spectrum and relative molecular mass indicated that compounds 3, 4, and 5 are probably natamycin/pimaricin, de-epoxynatamycin/de-epoxypimaricin, and candicidin D, respectively (**Figures 6F,G**, **7E**).

DISCUSSION

The success of termite fungiculture depends on multiple factors, such as the control of pathogens within termite fungus farms, termite antimicrobial peptides and gut bacteria with antimicrobial properties (Um et al., 2013; Poulsen, 2015; Bodawatta et al., 2019). The results in this study revealed that fungus-growing termite *M. barneyi*-associated actinomycetes produced different type of polyenes, which greatly inhibited competitor fungus *Xylaria* sp.

Actinobacteria Isolation From the Digestive Tract of *M. barneyi*

The actinomycetes were isolated from *M. barneyi* workers and soldiers. Owing to relative smaller size of soldier guts than that of workers, also a fewer numbers of soldier collected than workers, only worker guts were separated into the different gut sections (foregut, midgut, and hindgut) (**Figure 1A**). By a culture-based approach, we obtained 83 Actinobacteria strains from the guts of *M. barneyi*, which belongs to 8 genera but mainly *Streptomyces* and *Kitasatospora* (**Figure 1B**).

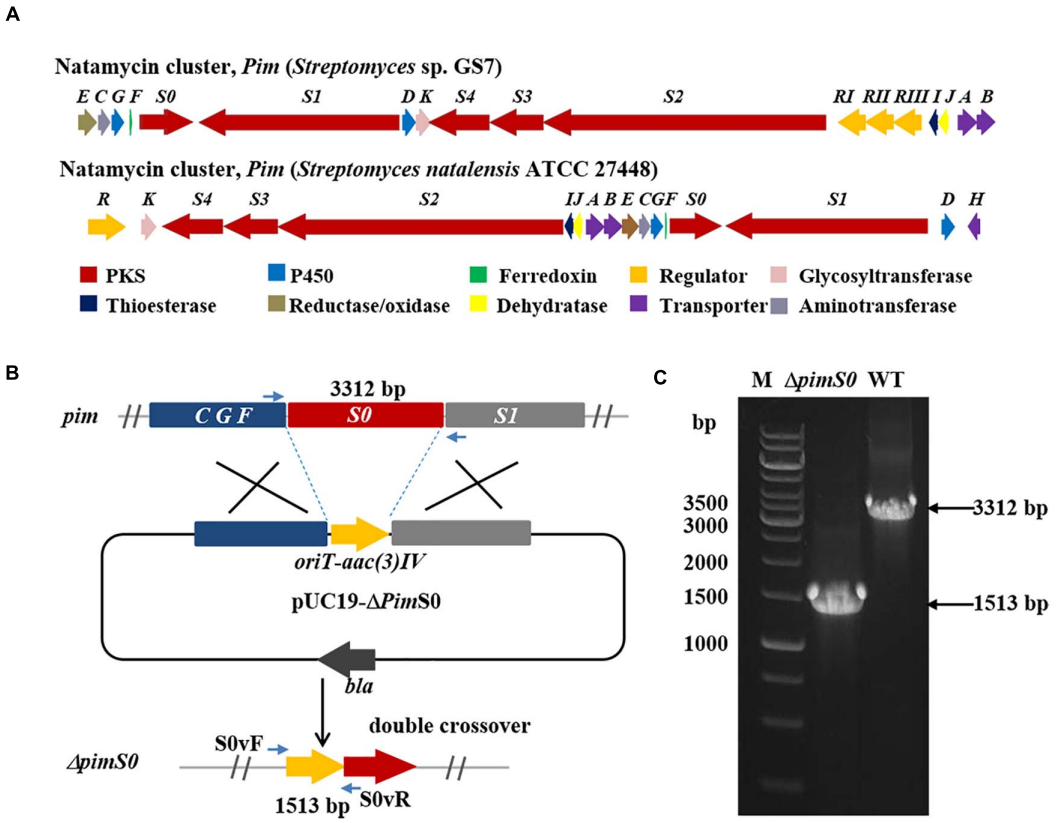

FIGURE 4 | Disruption of a key natamycin biosynthetic gene *pimS0* in *Streptomyces* sp. GS7. **(A)** Comparison of the genetic organization of natamycin BGCs in *Streptomyces* sp. GS7 and *S. natalensis* ATCC 27448. **(B)** Schematic representation for disruption of *pimS0*. **(C)** Verification of the *pimS0*-disrupted mutant by PCR. M, DNA marker. The fragments were amplified using primers of S0vF/S0vR.

Previously, actinomycetes of 4 genera including *Streptomyces*, *Cellulosimicrobium*, *Promicromonospora*, and *Micromonospora* have been isolated from workers intestines of *M. natalensis* and *Odontotermes formosanus* (Benndorf et al., 2018), and genus *Kitasatospora* strains have been isolated from the cuticle of fungus-growing termite (Visser et al., 2012). To our knowledge, this is the first time to obtain actinomycetes isolates of three genera *Amycolatopsis*, *Tsukamurella*, and *Verrucosispora* from fungus-growing termites.

Based on 16S rRNA sequence analysis, seven strains were predicted to be potential novel actinomycetes (**Supplementary Table 1**). Recently, a number of new actinomycetes have been isolated and identified from the worker gut of *M. natalensis* (Benndorf et al., 2020a,b; Schwitalla et al., 2020). The intestinal microflora of fungus-growing termite represents a promising resource of novel actinomycetes.

It was noted there were relatively higher numbers of strains obtained from the foregut of worker than other gut sections. The possible explanation is: the variety and abundance of hindgut microflora are highest among three gut sections (Chew et al., 2018), and actinomycetes are at very low level in termite guts (Otani et al., 2014), thus on the plates spreading with hindgut sample, a large number of bacteria overgrew and slow-growing actinomycetes were difficult to grow. Therefore, there was few actinomycetes obtained from the hindgut. Pre-treatments of hindgut samples by drying heating (Bredholt et al., 2008) or adding inhibitor such as chloramine-T (Hayakawa et al., 1997) may facilitate the selective isolation of actinomycetes from the hindgut. Although majority of actinomycetes were isolated from the foregut, considering the foregut is the first gut section of ingested food, we could not preclude these actinomyces originate from the nest environment, where many actinobacteria strains exist (Visser et al., 2012).

Antifungal Actinobacteria Strains From the Digestive Tract of *M. barneyi* Worker and Soldier

Plate paired assay revealed that 53% of the actinomycetes, mainly *Streptomyces* and *Kitasatospora* genus possessed antifungal activity against antagonistic fungus *Xylaria*. Majority of these antifungal actinomycetes were isolated from worker intestines, which could be explained by the fact that the workers have a greater chance of being exposed to pathogens than that of soldiers, since workers are responsible for transporting and digesting external plant material (Li et al., 2017). Additionally, the defensive microbes (most likely Actinobacteria and *Bacillus*) in the gut is helpful to selectively inhibit the potential antagonists

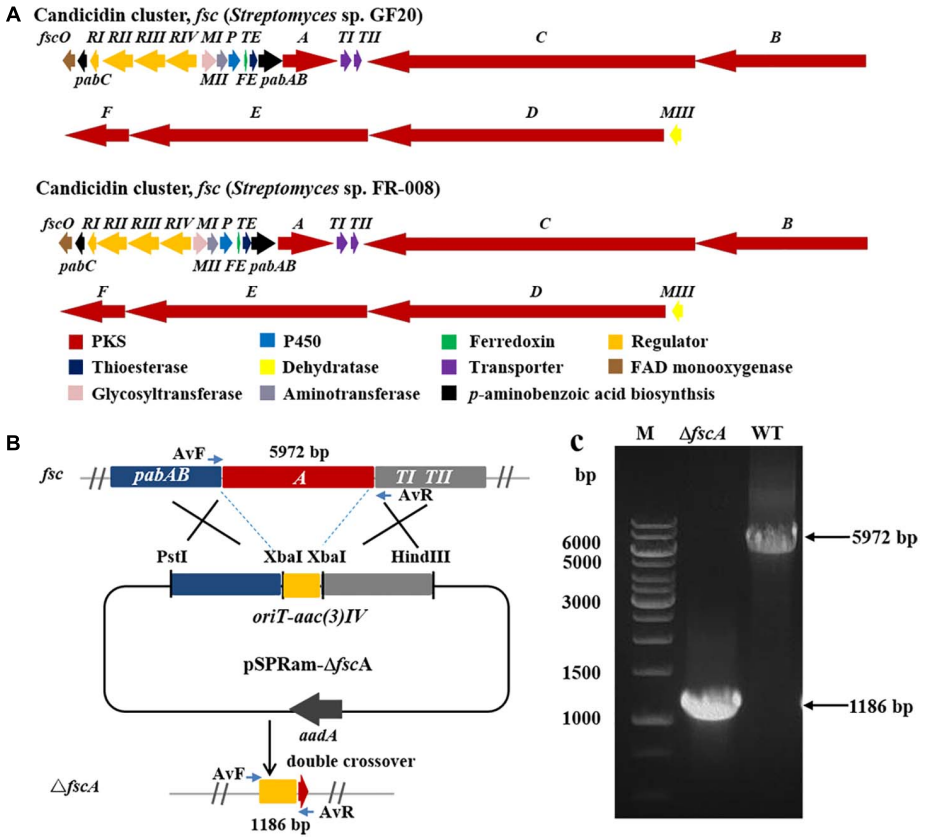

FIGURE 5 | Disruption of candicidin biosynthetic gene *fscA* in *Streptomyces* sp. GF20. **(A)** Comparison of the genetic organization of candicidin BGCs in *Streptomyces* sp. GF20 and *Streptomyces* sp. FR-008. **(B)** Schematic representation for disruption of *fscA* in *Streptomyces* sp. GF20. **(C)** Verification of the *fscA*-disrupted mutant by PCR. M, DNA size marker. The fragments were amplified using primers of AvF/AvR.

when the substrate first passes through the worker gut and avoid the entry of pathogens into fungal comb (Um et al., 2013; Poulsen, 2015).

Soldiers mainly play a defensive role in the colony by using their large and strong mandibles as well as by chemical substances secreted from a frontal gland on the head (He et al., 2018). In the present study, we also obtained *Streptomyces* with antifungal activity against *Xylaria* sp. from soldier guts. Previous study of gut bacterial metagenomic analysis by Poulsen revealed that the soldiers have nearly similar or a bit higher relative abundance of *Streptomyces* than workers in *M. natalensis* (Poulsen et al., 2014). To our knowledge, this is the first time to obtain the *Streptomyces* isolates from the soldier, considering the relatively high percentage of antifungal strains, which suggest the potential role of soldier-associated *Streptomyces* in fungus comb against antagonistic fungus *Xylaria*.

Polyenes-Producing *Streptomyces* Contribution to Inhibition of *Xylaria*

A key strategy of insects coping with environmental threats is the use of molecular defenses from symbiotic microbes (van Arnam et al., 2018), especially from insect-associated *Streptomyces* and *Pseudonocardia* (Scott et al., 2008; Haeder et al., 2009; Oh et al., 2009a,b; Blodgett et al., 2010; Schoenian et al., 2011; Sit et al., 2015; van Arnam et al., 2015; Chevrette et al., 2019b). To identify putative compounds responsible for the antifungal activity, the metabolites of antifungal strains were analyzed by HPLC, and the results revealed that the ten antifungal *Streptomyces* strains produce four type of polyenes with different number of conjugated double bonds. Several polyene compounds, including candicidin (Haeder et al., 2009; Barke et al., 2010), nystatin P1 (Barke et al., 2010), selvamicin (van Arnam et al., 2016), filipins (Gao et al., 2014), and mycangimycin (Oh et al., 2009b) have been reported from the symbiont actinomycetes of fungus-farming ants and southern pine beetles. *Bacillus* sp. from *M. natalensis* produced a polyene polyketide, bacillaene, which selectively inhibits antagonistic fungus of *Termitomyces* (Um et al., 2013), However, as far as we know, polyene compounds have not previously been isolated from fungus-growing termites-associated actinomycetes (Bi et al., 2011; Carr et al., 2012; Zhang et al., 2013, 2020; Kim et al., 2014; Benndorf et al., 2018; Klassen et al., 2019; Guo et al., 2020). Three strains with strong antifungal activities and potential polyene products corresponding to tetraene, pentene and heptaene, respectively, were subjected to bulk culture, probably due

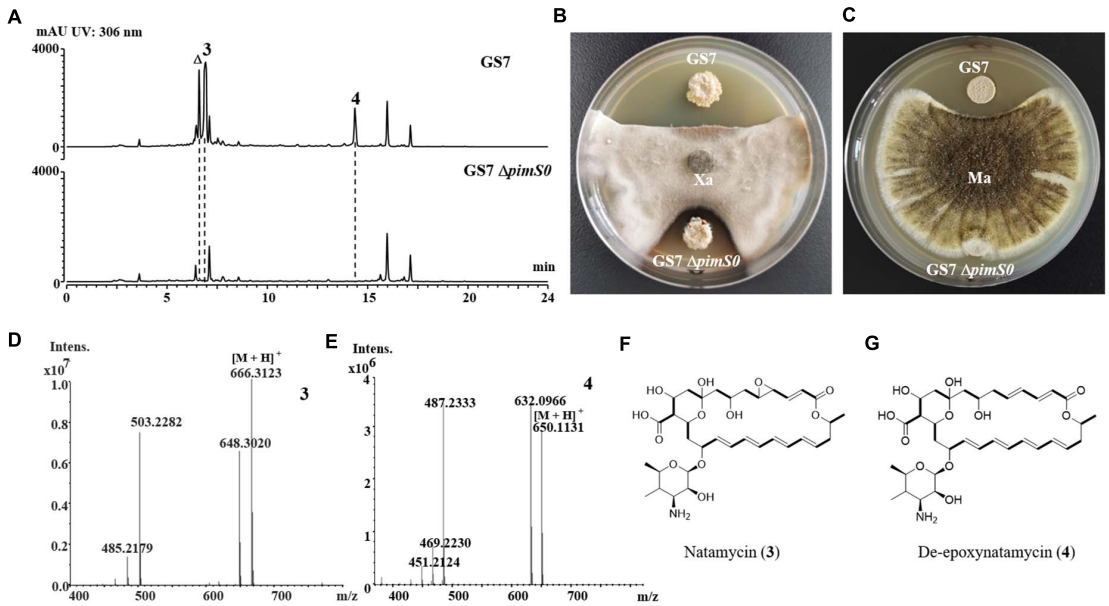

FIGURE 6 | The deletion mutant of natamycins in *Streptomyces* sp. GS7 had weaker antifungal activities against *Xylaria* sp. and *Metarhizium anisopliae*. **(A)** HPLC profiles of the metabolites of *Streptomyces* sp. GS7 wild type and the GS7 Δ*pimS0* mutant. The triangle symbol (Δ) denotes a homolog of natamycin. **(B,C)** Paired challenge assays of GS7 and the deletion mutant (GS7 Δ*pimS0*) against *Xylaria* sp. (Xa, **B**) and *M. anisopliae* (Ma, **C**). **(D,E)** HRMS spectra of compounds 3 and 4 from GS7. **(F,G)** The predicted chemical structures of natamycin/pimaricin (3) and de-epoxynatamycin/de-epoxypimaricin (4).

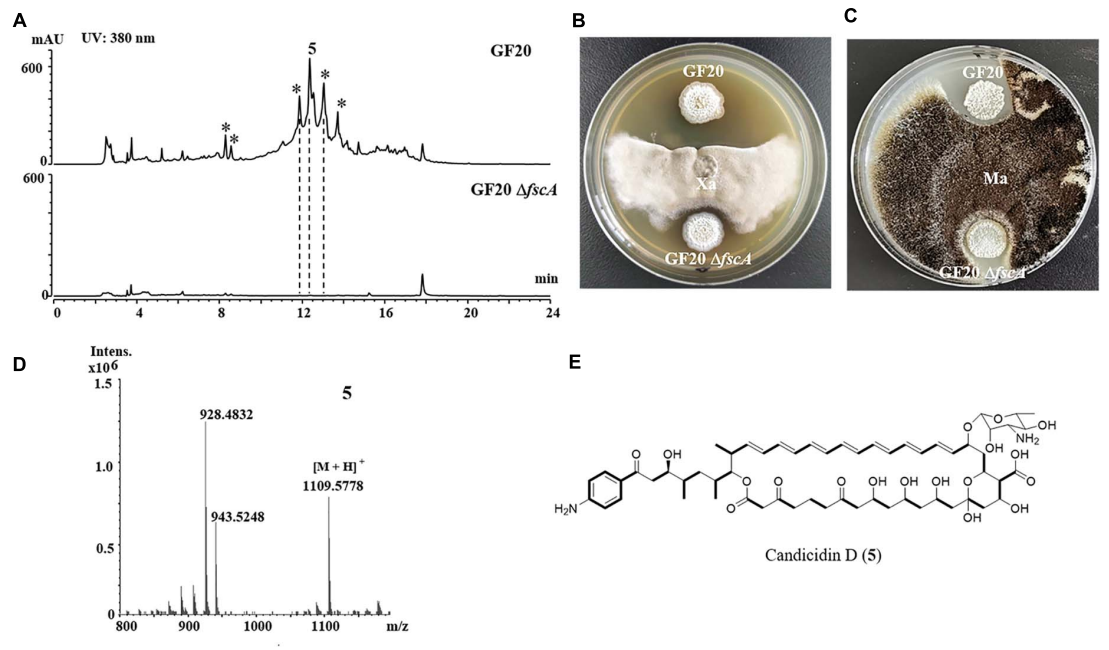

FIGURE 7 | The deletion mutant of candicidins in *Streptomyces* sp. GF20 had weaker antifungal activities against *Xylaria* sp. and *M. anisopliae*. **(A)** HPLC profiles of the metabolites of wide type *Streptomyces* sp. GF20 and mutant GF20 Δ*fscA*. The asterisk symbols (∗) denote homologs of candicidin. **(B,C)**, paired challenge assays of GF20 and the deletion mutant (GF20 Δ*fscA*) against *Xylaria* sp. (Xa, **B**) and *M. anisopliae* (Ma, **C**). **(D)** HRMS spectrum of compound 5 from GF20. **(E)** The predicted structure of candicidin D (5).

to chemical instability of polyenes (Worthen et al., 2001), finally only two pentene compounds (pentamycin and 1′14-dihydroxyisochainin) were purified from *Streptomyces* sp. HF10 (**Figure 3C**). Pentamycin (also called fungichromin) and 1′14-dihydroxyisochainin belong to polyene macrolides, containing the antifungal antibiotics amphotericin B (Sun et al., 2015) and

nystatin (Fjaervik and Zotchev, 2005). Previously, fungichromin have been isolated from endophytic actinomycetes (Human et al., 2016) and lower termite-associated actinomycetes (Mevers et al., 2017). The compound 1′14-dihydroxyisochainin is an analog of chainin, which was first isolated from a soil actinomycete with antifungal activity against phytopathogens (Thirumalachar, 1955; Gopalkrishnan et al., 1968).

Although two purified pentene compounds are not novel products, they represent the first report on polyene compound from fungus-growing termite-associated *Streptomyces*. Bioactivity assays showed that the competitor fungus *Xylaria* sp. was the most susceptible to inhibition of two pentene compounds, compared with entomopathogen (*B. bassiana* and *M. anisopliae*) and fungal cultivar *Termitomyces* sp., which was similar to the studies in leaf-cutting ants and the southern pine beetle (Haeder et al., 2009; Oh et al., 2009b). Candicidin identified from ant-associated *Streptomyces* is highly active against pathogenic fungus *Escovopsis* sp. (Haeder et al., 2009). Our results suggest the potential role of the pentamycin in protecting the fungus comb of *M. barneyi* against competitor fungus *Xylaria* sp.

Since we failed to obtain tetraene and heptaene compounds from cultures of *Streptomyces* sp. GS7 and GF20, disruptive mutants of tetraene and heptaene BGCs were constructed (**Figures 4**, **5**). HPLC analysis revealed that the corresponding polyene peaks disappeared and paired challenge assays showed the mutants had obviously weaker activities against antagonistic fungus *Xylaria* than the wild type strains, suggesting that tetraene and heptaene compounds produced by GS7 and GF20 contribute to the inhibition against the antagonistic fungi. Furthermore, both the wild type and mutant strains exhibited stronger inhibitory effects on antagonistic fungus (*Xylaria* sp.) than entomopathogen (*M. anisopliae*) (**Figures 6B,C**, **7B,C**), suggesting tetraene and heptaene produced by *Streptomyces* firstly selectively inhibited the fungus comb antagonistic fungus *Xylaria*. Interestingly, the recent study by Bodawatta et al. (2019) showed that *M. natalensis* foraging workers significantly avoided the mycopathogen-exposed substrates, and did not show any preference between entomopathogen-exposed and control substrate. Overall, the present study by pentene compound purification, tetraene and heptaene BGCs gene disruption and bioactivity assays suggest that polyenes produced by *M. barneyi*-associated *Streptomyces* greatly contribute to inhibition of antagonistic fungi.

It was noted that mutants retained slight activity against *Xylaria* sp. after disruption of tetraene and heptaene BGCs (**Figures 6B**, **7B**). Thus, except for polyenes, some other active compounds also inhibit the antagonistic fungi. In leaf-cutting ants, different antifungal secondary metabolites exhibited strong synergistic effects against pathogenic fungi (Schoenian et al., 2011). We inferred that in termite guts multiple compounds including polyenes and non-polyenes complement and reinforce the activities against pathogens.

The complex web of interactions involving insects (Poulsen et al., 2014), their fungal crops (Wang et al., 2015; Otani et al., 2019), specialized pathogens (Guo et al., 2016), symbiotic fungus (Xu et al., 2020), and symbiotic bacteria has become both a model system for chemical ecology and a source of naturally occurring small molecules. We are still a long way from identifying additional antifungal compounds in this system and understand thoroughly the chemical basis of symbiotic or antagonistic associations among termites, fungal cultivar, cultivar competitors, entomopathogenic fungi and antibiotic-producing actinomycetes.

CONCLUSION

In conclusion, through actinomycetes isolation, bioactivity assays, active product purification, and BGCs gene disruption analysis, we show that *Streptomyces* isolated from the gut of fungus-growing termite *M. barneyi* are capable of producing a variety of polyenes, which significantly inhibit antagonistic fungus *Xylaria* over entomopathogenic fungi and fungal cultivar *Termitomyces*. The results indicate that potential role of different type of polyenes produced by *Streptomyces* in protection of fungus comb against the antagonistic fungus.

DATA AVAILABILITY STATEMENT

The complete 16S rRNA genes of 83 isolated actinomycetes in this study were deposited in GenBank (MN826234-MN826316). The GenBank accession numbers for the genomes of the three strains are CP047144-CP047145 (*Streptomyces* sp. HF10), CP047146 (*Streptomyces* sp. GS7), and CP047147 (*Streptomyces* sp. GF20).

AUTHOR CONTRIBUTIONS

JL, MS, YJ, and JW performed the experiments. JN, YL, and JL designed the experiments. JL and JN analyzed the data and wrote the manuscript. YL, YS, and QH helped to revision of 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.2021.649962/full#supplementary-material

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Antimicrobial Resistance Glides in the Sky—Free-Living Birds as a Reservoir of Resistant *Escherichia coli* With Zoonotic Potential

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Antimicrobial resistance (AMR) is one of the most important global health concerns; therefore, the identification of AMR reservoirs and vectors is essential. Attention should be paid to the recognition of potential hazards associated with wildlife as this field still seems to be incompletely explored. In this context, the role of free-living birds as AMR carriers is noteworthy. Therefore, we applied methods used in AMR monitoring, supplemented by colistin resistance screening, to investigate the AMR status of *Escherichia coli* from free-living birds coming from natural habitats and rescue centers. Whole-genome sequencing (WGS) of strains enabled to determine resistance mechanisms and investigate their epidemiological relationships and virulence potential. As far as we know, this study is one of the few that applied WGS of that number (n = 71) of strains coming from a wild avian reservoir. The primary concerns arising from our study relate to resistance and its determinants toward antimicrobial classes of the highest priority for the treatment of critical infections in people, e.g., cephalosporins, quinolones, polymyxins, and aminoglycosides, as well as fosfomycin. Among the numerous determinants, $bla_{CTX-M-15}$, bla_{CMY-2}, bla_{SHV-12}, bla_{TEM-1B}, *qnrS1*, *qnrB19*, *mcr-1*, *fosA7*, *aac(3)-IIa*, *ant(3")-Ia*, and *aph(6)-Id* and chromosomal *gyrA*, *parC*, and *parE* mutations were identified. Fifty-two sequence types (STs) noted among 71 *E. coli* included the global lineages ST131, ST10, and ST224 as well as the three novel STs 11104, 11105, and 11194. Numerous virulence factors were noted with the prevailing *terC*, *gad*, *ompT*, *iss*, *traT*, *lpfA*, and *sitA*. Single *E. coli* was Shiga toxin-producing. Our study shows that the clonal spread of *E. coli* lineages of public and animal health relevance is a serious avian-associated hazard.

Keywords: antimicrobial resistance, free-living birds, AMR, wildlife, *E. coli*

INTRODUCTION

The scale of bacterial resistance to antimicrobials is one of the most important global health concerns. Although antimicrobial resistance (AMR) is an ancient and natural phenomenon, the widespread use of antimicrobials in human and veterinary medicine and also in agriculture contributes to its pandemic dissemination (D'Costa et al., 2011; Martínez, 2012; World Health Organization [WHO], 2014). The enormous consequences of AMR cover most of all treatment failures and increased mortality. However, economic losses as a consequence of AMR increase are also significant and they generate extra healthcare costs and a decrease of productivity (European Centre for Disease Prevention and Control, 2009). Nevertheless, losses in agriculture and animal production sector should also be listed here.

The use of antimicrobials (AMU) is considered one of the main drivers of AMR emergence; therefore, the impact of drug residues in municipal waste waters and of organic fertilizers in agriculture is indisputable (Swift et al., 2019). Several other environmental factors may affect AMR dissemination. Chemicals like disinfectants, fungicides, and pesticides widely used in agriculture may effectively co-select AMR (Hansen et al., 2007; European Commission, 2009; Hobman and Crossman, 2015). Similarly, heavy metals (e.g., copper) used in antifungal plant protection agents should also be deliberated (Borkow and Gabbay, 2005; Hobman and Crossman, 2015). All those pollutants contribute to the selection and spread of AMR in the environment affecting wildlife.

Consequently, free-living animals may serve as a reservoir of AMR determinants. The scale of AMR in wildlife, despite several reports on this subject, continually seems to be incompletely known and underestimated (Guenther et al., 2010a; Arnold et al., 2016; Mo et al., 2018; Wasyl et al., 2018). However, in the assessment of wildlife input to the AMR spread, different animal species with diverse habitats and feeding behaviors need to be considered. It should be emphasized here that migratory species seem to pose a significant threat to AMR dissemination. Seasonal migration, often over long distances and between continents, contributes to greater exposure of animals and their intestinal microbiome to various AMR drivers (Arnold et al., 2016). In this context, the role of free-living birds as AMR carriers is noteworthy and has already drawn the attention of several researchers (Pinto et al., 2010; Literak et al., 2012; Poirel et al., 2012; Veldman et al., 2013; Vredenburg et al., 2014; Oh et al., 2016; Ahlstrom et al., 2018; Zurfluh et al., 2019). To illustrate a ubiquitous and diverse avian community, it covers species that avoid human proximity, such as the golden oriole (*Oriolus oriolus*), as well as species living close to human settlements, like the house martin (*Delichon urbicum*). Other examples of the puzzle are herring gulls (*Larus argentatus*) known to prey on landfills and around sewage treatment plants, areas polluted with a variety of AMR determinants and its affecting factors (Bonnedahl and Järhult, 2014), and white stork (*Ciconia ciconia*) popular in Polish spring and summer landscape and wintering far away in Africa.

Recent developments in sequencing techniques provide an invaluable tool to elucidate the background and pathways of AMR transmission. Accurate genotypic characterization of bacteria enables to examine the genotypes circulating among environments and to identify their possible links to clinically relevant resistant pathogens and, thus, human and animal infections. Besides a somewhat unrealistic direct contact with free-living birds, there is a serious possibility of livestock or human contact with, for example, bird droppings (Dolejska and Literak, 2019). A flash point for the current study was multidrug-resistant (MDR) *Escherichia coli* derived from an individual of green woodpecker (*Picus viridis*) found in nature. The strain was isolated in 2013 from fresh feces collected during the delivery of birds to a veterinary clinic due to clinical symptoms.

In this study, we applied microbiological culture methods commonly used in the official monitoring of slaughter animals (European Food Safety Authority, 2019) to investigate the AMR status of *E. coli* isolated from free-living birds. The samples were derived from birds coming from natural habitats and rescue centers. The application of whole-genome sequencing (WGS) aimed to characterize AMR determinants and plasmids associated with AMR horizontal transfer and also to investigate the epidemiological relationships and virulence potential of *E. coli* isolated from free-living birds.

MATERIALS AND METHODS

Sample Collection

A total of 69 samples (20 intestines, 44 feces, 4 goiter swabs, and 1 stomach sample) from 68 free-living birds were collected between 2017 and 2020 within a convenience sampling in the National Reference Laboratory for Antimicrobial Resistance (NRL) at the National Veterinary Research Institute (NVRI) in Puławy, Poland. The most prevalent tested group constituted birds of prey that belonged to Accipitriformes (n = 23) and Strigiformes (n = 3). The other group consisted of migratory species of Pelecaniformes (n = 19) and ubiquitous Passeriformes (n = 15) representing the most abundant avian order. The dataset was completed with orders represented by Anseriformes (n = 5), Gruiformes (n = 1), Charadriiformes (n = 1), and Columbiformes (n = 1).

Twenty-two samples were derived from deceased birds, mostly birds of prey (n = 9) and waterbirds (n = 6), and sent to the NVRI for diagnostics purposes (e.g., defining the cause of death). The second subset of samples was collected from 15 white storks residing in the Center for Rehabilitation of Free-living Birds in Bukwałd due to mechanical injuries. Antimicrobial treatment status of this group remained unknown. Eight fresh feces samples (six from birds of prey and two from white storks) were collected from animals at the Bird of Prey Rehabilitation Center in Dąbrówka on the day of release. The birds were not treated with antimicrobials during their stay in the rescue center. The remaining samples (n = 24) were taken from animals in their natural environment during the ringing activity or nest inspection carried out in 2019 in Lublin region (S-E Poland)

by ornithologists from the Department of Zoology and Nature Protection, UMCS. The study fulfilled the current Polish law and was permitted by the Ministry of the Environment (approval number: DL-III.6713.11.2018.ABR) and the General Directorate for Environmental Protection (approval number: DZP-WG.6401.03.2.2018.jro). The Regional Directorate for Environmental Protection (RDOŚ) in Lublin allowed for the research project through a letter (approval number: WPN. 6401.6.2018.MPR). Ten samples of birds sampled in nature came from juvenile birds of prey—marsh harrier (*Circus aeruginosus*). All tested bird species, their origin, and included sample types are presented in **Supplementary Table 1**.

Isolation and Identification of *E. coli*

Usually, on the day following their collection, the samples were cultured on buffered peptone water for 18 ± 3 h at 37°C and then streaked on MacConkey agar (Oxoid, Hampshire, United Kingdom), MacConkey agar supplemented with cefotaxime (1 mg/L, Oxoid, Hampshire, United Kingdom), chromID^{TM} CARBA, chromID^{TM} OXA-48 agar (bioMérieux, Marcy l'Etoile, France), and MacConkey supplemented with colistin (2 mg/L, Oxoid, Hampshire, United Kingdom) for isolation of commensal, cephalosporin-, carbapenem-, and colistin-resistant *E. coli*, respectively. Suspected colonies were identified with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS, Microflex LT MALDI Biotyper; Bruker Biosciences, Billerica, MA, United States).

Antimicrobial Resistance Testing

All *E. coli* were tested for antimicrobial susceptibility with the microbroth dilution method (Sensititre; TREK Diagnostic Systems, Thermo Fisher Scientific, Waltham, MA, United States). Resistance tests to nine antimicrobial classes—beta-lactams, quinolones, phenicols, aminoglycosides, folate path inhibitors, tetracyclines, polymyxins, macrolides, and glycylcyclines—were performed with EUVSEC plates (as described in Table 1 of the Annex to 2013/652/EC). For all isolates resistant to cephalosporins, the second panel (EUVSEC2 according to Table 4 of the Annex to 2013/652/EC) was applied. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) epidemiological cutoff values (ECOFFs) for minimum inhibitory concentration (MICs) were used as interpretation criteria. *E. coli* strain was regarded as resistant (non-wild type, NWT) when MIC values above the cutoff were obtained. Among the NWT category, strains resistant to at least three antimicrobial classes were referred as multidrug-resistant (MDR) (Magiorakos et al., 2012). *E. coli* with all MIC values below the ECOFF were recognized as susceptible (wild type, WT). The procedure mirrored the official AMR monitoring implemented in the EU according to Directive No. 2013/652/EC.

Whole-Genome Sequencing

Seventy-one *E. coli* strains, including an archival strain from a woodpecker, have been subjected to whole-genome sequencing. Extraction of DNA was prepared with Maxwell® RSC Cultured Cells DNA Kit—Automated DNA Purification from Mammalian and Bacterial Cultured Cells (AS1620 Promega, Madison, Wisconsin, United States) according to the manufacturer's instruction with Maxwell® RSC Instrument (Promega, Madison, Wisconsin, United States). For yield and purity check, all samples were measured with NanoDrop^{TM} One following extraction (Thermo Scientific, Waltham, MA, United States). DNA libraries prepared with Library Preparation Kit (Illumina, Inc., San Diego, CA, United States) according to the manufacturer's instructions were sequenced with the MiSeq platform (Illumina, Inc., San Diego, CA, United States). Paired-end sequencing per flow cell (2 × 300) was applied.

Bioinformatic and Statistical Analyses

FastQC 0.11.5 was used for the raw reads quality check and Trimmomatic 0.36 (Bolger et al., 2014) for the read trimming. Corrected reads were *de novo* assembled by SPAdes 3.9.0 (Bankevich et al., 2012). Resistance and plasmid identification was conducted using abricate 1.0.1 (Seemann, 2019) against ResFinder (Zankari et al., 2012) and PlasmidFinder (Carattoli et al., 2014) databases (2020-07-25) with identity threshold 95% and selected minimum length 60%. PointFinder software 3.1.0 was applied for the identification of chromosomal point mutations (database: 2019-07-02) (Zankari et al., 2012). For the identification of multilocus sequence type (MLST, ST), we used the MLST 2.0 tool (Larsen et al., 2012) with database version 2.0.0 (2020-05-04). Virulence factors were analyzed with VirulenceFinder 2.0 and its database of 2020-05-29 with% ID threshold 90% and minimum length 60% (Joensen et al., 2014). Submission of *E. coli* with unknown ST to EnteroBase v1.1.2[1] allowed to assign new sequence type using the Achtman 7 Gene MLST algorithm (Zhou et al., 2020).

CSI Phylogeny 1.4 (call SNPs and infer phylogeny) CGE with input parameters—minimum depth at single nucleotide polymorphism (SNP) positions: 10, relative depth at SNP positions: 10, minimum distance between SNPs (prune): 10, minimum SNP quality: 30, minimum read mapping quality: 25, minimum *Z*-score: 1.96—was applied for phylogeny tree preparation (Kaas et al., 2014). As reference genome of *E. coli* from one of the two most represented ST types was chosen (14P KOL). The online tool iTOL v5 was applied for phylogeny tree visualization (Letunic and Bork, 2019).

The sequences were deposited at the European Nucleotide Archive (ENA)[2] under accession number PREJB42669[3].

The variability of the noted MLST and virulence genes was measured with Simpson's diversity index (Hunter and Gaston, 1988).

To determine the statistical difference in the occurrence of resistant *E. coli* between groups of birds, a chi-square test with the appropriate correction was applied (**Supplementary Table 2**). Resistance in different groups of birds was assessed with a 95% confidence interval.

[1]https://enterobase.warwick.ac.uk/

[2]http://www.ebi.ac.uk/ena

[3]http://www.ebi.ac.uk/ena/data/view/PRJEB42669

RESULTS

Phenotypic Results of Antimicrobial Resistance

A total of 73 *E. coli* were isolated. Sixty were obtained from MacConkey agar, six from MacConkey supplemented with cefotaxime, and seven from MacConkey with colistin. No carbapenem-resistant *E. coli* were found. To avoid duplicate testing of the same strain, three isolates were excluded, as they were obtained on different culture media from the same sample but showed identical minimum inhibitory concentration (MIC) values. As a result, 70 strains were included in the comparison of resistance in different bird groups. Half of the tested *E. coli* were found resistant (n = 35, 50.0%) and most of them (n = 27, 38.6%) were MDR (resistant to at least three antimicrobial classes). The analyses showed significant differences between the number of resistant strains isolated from birds sampled in nature and all the other groups (p-values 0.0047–0.0003) (**Supplementary Table 2**). Susceptible *E. coli* were derived mostly from birds sampled in nature. Yet, two strains derived from blue tit (*Cyanistes caeruleus*) sampled in nature exhibited non-wild-type MICs with 8 and 11 antimicrobials each.

Of all the antimicrobials assessed, ampicillin and tetracycline resistance dominated (41.4%, each) followed by quinolones (35.7 and 31.4% for ciprofloxacin and nalidixic acid, respectively), as well as folate path inhibitors (28.6% sulfamethoxazole and almost 22.9% trimethoprim). A lower percentage of AMR was observed for chloramphenicol (12.9%) and third-generation cephalosporins (8.6%). Four strains (5.7%) were resistant to gentamicin. **Supplementary Table 3** presents detailed MIC value distribution of the tested *E. coli* on both applied panels. Overall, 20 different AMR profiles were noted (**Figure 1**).

AMR Determinants and Plasmid Replicons

Cephalosporin resistance was defined as extended-spectrum beta-lactamases (ESBLs) and AmpC-type cephalosporinase production. ESBL determinants were identified as $bla_{\mathrm{SHV-12}}$ (archival woodpecker *E. coli*) and $bla_{\mathrm{CTX-M-15}}$ [n = 1, mute swan (*Cygnus olor*)—deceased]. AmpC-type cephalosporinases were determined by $bla_{\mathrm{CMY-2}}$ [n = 5, blue tit—nature, white-tailed eagle (*Haliaeetus albicilla*), buzzard (*Buteo buteo*), crane (*Grus grus*)—deceased, white stork—rescue]. Resistance to ampicillin was linked to $bla_{\mathrm{TEM-1B}}$ most often. In one ampicillin-resistant *E. coli* from buzzard (released), any relevant determinant was noted that could confer resistance for beta-lactams.

Mutations in quinolone resistance determining region (QRDR) dominated ciprofloxacin and nalidixic acid resistance, and *gyrA* substitution S83L was noted in the majority of 26 resistant strains (n = 23). Three quinolone-susceptible strains carried single *parC* mutations [n = 1, marsh harrier (*Circus aeruginosus*)—nature; n = 2, white-tailed eagle —deceased] and one wild-type strain possessed *parE* mutation [marsh harrier—nature].

Plasmid-mediated quinolone resistance (PMQR) genes were identified as *qnrS1* [n = 4; two white storks—rescue, raven (*Corvus corax*), and buzzard—deceased] and *qnrB19* (n = 1; buzzard—deceased). The gene *aac(6′)-Ib-cr* that determines AMR toward quinolones and aminoglycosides was noted in a strain from deceased mute swan carrying $bla_{\mathrm{CTX-M-15}}$ and $bla_{\mathrm{OXA-1}}$.

The genes *aac(3)-IIa* (n = 2, mute swan—deceased) and *aac(3)-IId* (n = 2, blue tit—nature) were associated with gentamicin resistance. Of all determinants noted, genes encoding resistance toward aminoglycosides other than gentamicin (e.g., streptomycin not tested phenotypically) were the most abundant (n = 55), and among them, *ant(3″)-Ia* (n = 18), *aph(3″)-Ib* (n = 13), and *aph(6)-Id* (n = 13) prevailed. Genes *sul1*, *sul2*, *sul3*, and *dfrA1* were the most often noted among strains resistant to folate path inhibitors, while genes *tet*(A) (n = 21) and *tet*(B) (n = 11) dominated in tetracycline-resistant *E. coli*. Resistance toward phenicols was determined by *cmlA1*, *catA1*, *catB3*, and *floR*. Genes *cmlA1* and *catB3* were also found in five strains that were chloramphenicol susceptible.

The *mcr-1* gene conferring colistin resistance was noted in two strains with MIC for this antimicrobial below the cutoff (MIC = 2 mg/L). Both *E. coli* were found in deceased water birds (herring gull and mute swan). WGS also revealed the presence of *fosA7* determining resistance toward fosfomycin (not tested phenotypically) in two *E. coli* (white stork—rescue and white-tailed eagle—released). Three genes that confer resistance for macrolides [*mef*(B), *mph*(A)] and lincosamides [*lnu*(G)] were noted as well (**Figure 1**).

All genotype–phenotype discrepancies in case of *mcr-1* finding in colistin wild-type strain as well as *E. coli* susceptible to chloramphenicol that carried *cmlA1* and *catB3* were confirmed by repeated susceptibility testing. Resistance to ampicillin in *E. coli* without any relevant AMR gene was also verified.

Multiple plasmid replicons (n = 30) were found dispersed in most of the tested *E. coli* (n = 60). The most frequent replicon was IncFIB(AP001918) (n = 47). Few other replicons IncI1_1_Alpha, p0111, IncFIC(FII), IncQ1, and IncFII occurred in a similar number of strains (from 8 up to 12 strains) (**Supplementary Figure 1**). Ten from 11 plasmid replicon-free strains were pan-susceptible and AMR gene-free (**Figure 1**). The exception was one strain from deceased mute swan carrying five resistance genes including genes $bla_{\mathrm{CTX-M-15}}$, $bla_{\mathrm{OXA-1}}$, and *aac(6')-Ib-cr*. Chromosomal location of antimicrobial resistance genes in that strain was confirmed with MinION long-read sequencing (Oxford Nanopore Technologies, data not presented).

Seventeen strains possessed one plasmid replicon and three of them carried resistance genes. Among 34 *E. coli* with two up to four identified plasmid replicons, 25 strains were recognized as non-wild type (NWT), including 17 MDR. Nine *E. coli* carried from five up to eight plasmid replicons and all were MDR (**Figure 1** and **Supplementary Figure 1**).

Virulence Genes

Virulence factors were noted in all tested strains. One *E. coli* possessed a single virulence gene; the remaining carried at least three virulence genes. The majority of *E. coli* (n = 47) contained 10 or more virulence factors. In nine strains, 20 up to 26 virulence genes were found simultaneously. A huge diversity (D = 0.959)

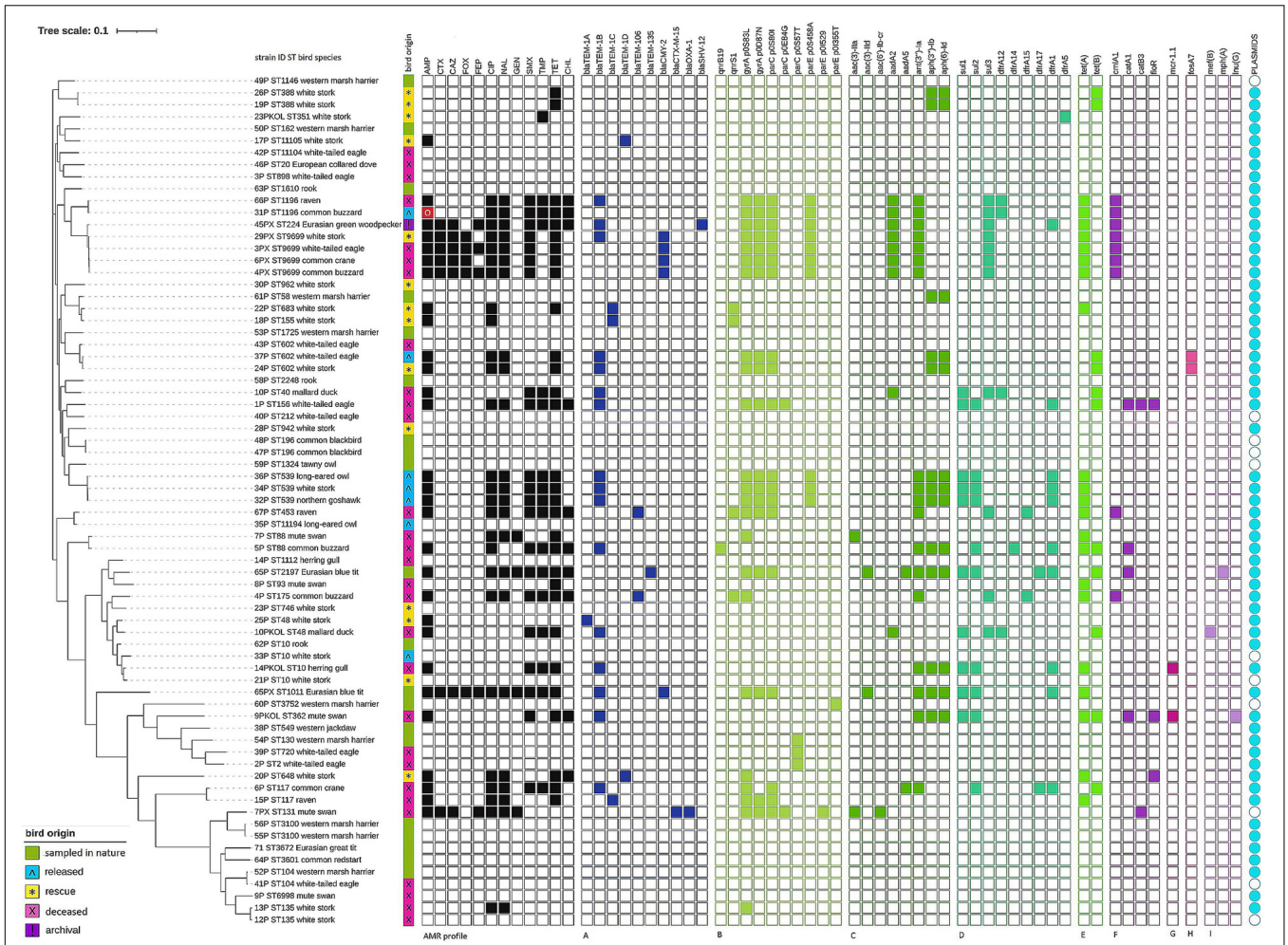

FIGURE 1 | Phylogeny of *Escherichia coli* strains isolated from wild birds. Full and empty squares depict the presence or absence of AMR or AMR determinants. AMR profile in black—phenotypic AMR profiles confirmed by the presence of AMR determinants. Red square with white circle means phenotypical resistance not confirmed by genotypic testing. **(A)** Beta-lactam, **(B)** quinolone, **(C)** aminoglycosides, **(D)** folate path inhibitors, **(E)** tetracyclines, **(F)** phenicols, **(G)** colistin, **(H)** fosfomycin, **(I)** macrolide and lincosamide resistance determinants. Visualization prepared with the online tool iTOL v5.

of virulence factors amounting to 65 different determinants was noted. The most prevalent genes were *terC* ($n = 71$), *gad* ($n = 63$), *ompT* ($n = 51$), *iss* ($n = 49$), *traT* ($n = 48$), *lpfA* ($n = 46$), and *sitA* ($n = 42$) (**Figure 2**). One of the *E. coli* isolated from deceased collared dove (*Streptopelia decaocto*) was recognized as Shiga toxin-producing *E. coli* (STEC). It carried *stx2A* and *stx2B* encoding Shiga toxin and *nleA–C* determining non-LEE encoded effectors.

Phylogenetic Diversity

Multilocus sequence typing (MLST) revealed 52 STs among 71 tested *E. coli* (Simpson diversity index $D = 0.989$). The most abundant were ST10 and ST9699 (four strains each), followed by ST602 and ST539 (three *E. coli* each). Nine ST types (including avian pathogenic ST117) were represented by two strains. One strain belonged to pandemic *E. coli* lineage ST131. The strain was isolated from deceased mute swan, and it was MDR and harbored the CTX-M-15 enzyme. All strains derived from the same sample but different culture media belonged to different STs. The minimum SNP dissimilarities were observed between strains belonging to ST388 (four SNPs). The highest difference of 46,533 SNPs was noted between strain 55P (ST3100) and 54P (ST130). Three novel STs were noted and assigned by EnteroBase as ST11104, ST11105, and ST11194. Those three STs were characterized by new *gyr* alleles (1,037, 1,038, and 1,047, respectively). All *E. coli* belonging to ST9699 were cephalosporin-resistant and carried the $bla_{\mathrm{CMY-2}}$ gene. One ST10 strain from deceased herring gull was MDR and possessed *inter alia mcr-1.1* and $bla_{\mathrm{TEM-1B}}$. The remaining three ST10 strains were susceptible (**Figure 1**).

DISCUSSION

The impact of resistant bacteria on health and environmental issues is unquestionable. Thus, the identification of potential

FIGURE 2 | Virulence of *E. coli* isolated from wild birds. Virulence factor by sequence type and bird's species. Full and empty squares mean the presence or absence of virulence factors. The matrix was visualized with iTOL v5.

hazards associated with wildlife becomes critical to recognize environmental reservoirs of AMR and to take appropriate and prompt actions against possible emerging AMR mechanisms (Davies and Davies, 2010). Our study, although based on a convenience sampling of a limited fraction of free-living bird populations, confirms its role in AMR transmission and reveals the zoonotic potential of *E. coli* coming from the avian reservoir.

AMR in Free-Living Birds—the Possible Impact of Human Activity

The main concerns arising from our study relate to resistance toward antimicrobial classes of the highest priority in human medicine, e.g., cephalosporins, quinolones, polymyxins, and aminoglycosides (World Health Organization [WHO], 2019; European Medicines Agency, 2020), as well as fosfomycin, assigned to substances that should be limited for human prescription in the EU (European Medicines Agency, 2020).

The AMR profiles noted in *E. coli* of avian origin were dominated by resistance toward classes of antimicrobials often used in human and veterinary medicine (Food and Agriculture Organization of the United Nations, 2016; World Health Organization [WHO], 2018; European Medicines Agency and European Surveillance of Veterinary Antimicrobial Consumption, 2019). This finding indicates that ubiquitous birds might be exposed to the anthropogenic impact and acquisition of resistant bacteria through contact with the waste of human or livestock origin. Resistance toward antimicrobial classes that

we found prevailing (beta-lactams, tetracyclines, quinolones, aminoglycosides, folate path inhibitors) was also reported in bird communities from distinct geographical areas worldwide, from the Americas through Africa, Europe, Asia to Australia (Nascimento et al., 2003; Guenther et al., 2010b; Literak et al., 2010; Poirel et al., 2012; Veldman et al., 2013; Mohsin et al., 2016; Guenther et al., 2017; Ahlstrom et al., 2018; Marcelino et al., 2019; Zurfluh et al., 2019; Cao et al., 2020; Fuentes-Castillo et al., 2020; Nabil et al., 2020). Moreover, AMR toward those substances was commonly found in *E. coli* from farm animals and reported from other wildlife species (Navarro-Gonzalez et al., 2013; Wasyl et al., 2013, 2018; Ceccarelli et al., 2020). The AMR status of bacteria isolated from birds living in pristine environments of Antarctica—gentoo penguin (*Pygoscelis papua*)—seems to be contrasting. Some studies showed an almost complete lack of resistance in bacteria from penguins, while other studies revealed resistance but simultaneously indicated that the result might have been influenced by human activity (Bonnedahl et al., 2008; Miller et al., 2009; Rabbia et al., 2016; Marcelino et al., 2019).

The direct anthropogenic impact could have caused resistance noted in *E. coli* from birds originating from rescue centers. Although the animals were not treated with antimicrobials, they were fed by humans and kept in human proximity. The inclusion of that group of birds might be considered as a weakness of the study. Nevertheless, we believe that the results may highlight the immediate risk for people dealing with birds. Furthermore, it reveals the potential "microbial pollution" of the environment after the release of birds into their natural habitats.

Exposure to environmental pollution and anthropogenic factors, e.g., human waste, sewage treatment plant effluents, and manure, seems to be the significant cause inducing resistance in migratory species and waterbirds, e.g., white stork, crane, mute swan, and mallard (*Anas platyrhynchos*), that we found. That scenario has also been postulated by other researchers (Cole et al., 2005; Guenther et al., 2012; Bouaziz et al., 2018; Marcelino et al., 2019).

We assume that limited contact with human-related external factors and the environment explains the lowest number of NWT strains observed among birds sampled in nature as a significant part of that avian category constituted predominantly by young birds. Similar conclusions were derived by researchers from Switzerland that also suggested a higher resistance *E. coli* rate in adult birds and the parental transmission of AMR as the most probable in the case of juveniles (Zurfluh et al., 2019).

Deliberating the possible source of AMR in avian species, the position of birds within the trophic interactions should also be taken into consideration. It cannot be excluded that the multidrug resistance found in our study among *E. coli* from raptorial birds might be the aftermath of transmission and accumulation of resistance determinants from their potential prey (Marrow et al., 2009).

Selected Resistance Mechanisms and Potential Transfer

It should be emphasized that no resistance determinants toward carbapenems were noted among the tested strains, and so far, carbapenem resistance has rarely been reported in Poland in farm animals and other wildlife species (Lalak et al., 2016; Wasyl et al., 2018; Skarżyńska et al., 2020a,b). However, since the first report of carbapenemase-producing *Salmonella* Corvallis from the migratory bird—black kite (*Milvus migrans*) in Germany (Fischer et al., 2013), several new studies that presented carbapenem resistance in bacteria from free-living birds were published (Vittecoq et al., 2017; Bouaziz et al., 2018). One of them described a high prevalence of bla_{IMP}-producing enterobacteria in the silver gull (*Chroicocephalus novaehollandiae*) on Five Islands of Australia (Dolejska et al., 2016). The authors suggested that colonization of birds with resistant bacteria was a result of the feeding habits of the birds at a local waste depot contaminated with clinical material.

Numerous studies reported the occurrence of ESBLs in free-living birds (Guenther et al., 2012, 2017; Mohsin et al., 2017; Zurfluh et al., 2019). It is worth emphasizing that we found CTX-M-15, ESBL of public health concern, in *E. coli* from a deceased mute swan. Although ESBL genes are frequently described on IncF and IncI plasmids (Rozwandowicz et al., 2018) in case of ESBL-producing strains in our study, the resistance genes were not located on the same contigs as plasmid replicons. The gene $bla_{\mathrm{CTX-M-15}}$, as well as the other AMR genes in this strain, was located in the chromosome. It has been suggested that the incorporation of AMR genes into the chromosome favors the maintenance of resistance in the bacterial population of *E. coli* (Rodríguez et al., 2014). The occurrence of chromosomally encoded $bla_{\mathrm{CTX-M}}$ genes was previously reported in studies on avian *E. coli* from Pakistan and Mongolia (Guenther et al., 2017; Mohsin et al., 2017). Moreover, chromosomal integration of $bla_{\mathrm{CTX-M-15}}$ was described in clinical *E. coli* isolates belonging to the clonal group ST131, and our strain represented this lineage (Rodríguez et al., 2014).

The ESBL encoded by $bla_{\mathrm{SHV-12}}$ in archival *E. coli* from woodpecker was one of the most prevalent ESBLs associated with nosocomial infections before the increase of CTX-M enzymes (Coque et al., 2008). The SHV-12 was previously reported in *E. coli* from waterbirds in Poland (Literak et al., 2010), but it was found in free-living birds in Spain and The Netherlands as well (Veldman et al., 2013; Oteo et al., 2018). Moreover, spreading of $bla_{\mathrm{SHV-12}}$ was formerly revealed among indicator *E. coli* isolated from food animals in Poland (Lalak et al., 2016).

It should be emphasized that in our study cephalosporin resistance was mostly linked to AmpC-type cephalosporinase encoded by $bla_{\mathrm{CMY-2}}$. The finding of the gene in *E. coli* from blue tit, often found in close vicinity to human settlements, is a cause for concern. During winter, tits are often fed with pork fat and this raises a question on the direction of the gene transmission. It was formerly revealed that in Poland, $bla_{\mathrm{CMY-2}}$ disseminated among *E. coli* from food-producing animals, e.g., pigs, broilers, and turkeys, as well as from wildlife, e.g., wild boars (Lalak et al., 2016; Wasyl et al., 2018). Among free-living birds, the gene was reported in *E. coli* from species associated with aquatic environments and birds of prey (Poirel et al., 2012; Veldman et al., 2013; Ahlstrom et al., 2018).

AMR mechanisms toward quinolones in tested *E. coli* were dominated with mutations in quinolone resistance determining

region (QRDR), and this result might indicate the presence of quinolone selection pressure in the environment. Naturally, drug residues in the environment are subjected to photo- and biodegradation, but the latter process seems to have lower rates for quinolones as synthetic compounds (Martinez, 2009). It was formerly revealed that even at very low concentrations quinolones might select for resistance (Gullberg et al., 2011; Andersson and Hughes, 2012). Our results show that QRDR mutations seem to spread clonally in certain lineages of *E. coli*. QRDR mutations were reported previously in *E. coli* isolated from gulls and birds of prey. Furthermore, it was demonstrated that the same *E. coli* lineages were present in wastewaters, streams, and gulls (Vredenburg et al., 2014). Former research on *E. coli* derived from free-living birds with septicemia also indicated the role of QRDR mutations but reported only mutation in gyrase A subunit (*gyrA*) leading to the Ser-83Leu amino acid substitution (Jimenez Gomez et al., 2004). Single mutations in *parC* and *parE* noted here did not affect the quinolone susceptibility of the strains (Heisig, 1996; Vila et al., 1996; Komp Lindgren et al., 2003; Ling et al., 2003).

In our study, only a few *qnr* genes were noted conversely to a previous report on *E. coli* from free-living birds sampled on the Polish Baltic sea coast, which revealed *qnrS* gene presence in quinolone-resistant strains accompanied by *gyr* and *par* mutations in some of them (Literak et al., 2010). It should be underlined that the majority of former studies on quinolone resistance in free-living birds from the United States, Europe, and Asia were focused mostly on plasmid-mediated resistance mechanisms and reported the occurrence of *qnrS*, *qnrB*, and *aac(6′)-Ib-cr* (Literak et al., 2010, 2012; Halova et al., 2014; Oh et al., 2016; Mohsin et al., 2017). Interestingly, our study confirmed the presence of *aac(6′)-Ib-cr* that determines resistance toward quinolones and aminoglycosides along with beta-lactamases encoded by $bla_{\mathrm{CTX-M-15}}$ and $bla_{\mathrm{OXA-1}}$ in *E. coli* from a deceased mute swan, supporting the theory on the spread of these resistance gene sets among bird communities (Literak et al., 2010; Veldman et al., 2013; Vredenburg et al., 2014).

The cause for concern was finding plasmid-mediated colistin resistance (*mcr-1*) in deceased mute swan and herring gull. It is worth noting that the first cases of the *mcr-1* occurrence in free-living birds were published in 2016 (Mohsin et al., 2016; Ruzauskas and Vaskeviciute, 2016). Similar to our results, these studies reported *mcr-1* in species associated with aquatic environments: herring gull and coot (*Fulica atra*). A recent study from Egypt revealed over 10% prevalence of *mcr-1* in bacteria from resident birds (e.g., pigeons, crows) and even 20% prevalence of the gene in migratory waterfowls birds (Ahmed et al., 2019). Moreover, the gene was detected in water samples collected in the area of bird trapping. All the above results indicate that birds might be considered an important vector of colistin resistance. Previous studies from Poland showed the wide spread of *mcr-1* among food-producing animals, particularly turkeys (Zając et al., 2019). Corresponding to our study, the gene was revealed *inter alia* on IncX4 plasmid and occurred mostly in isolates with colistin MIC close to ECOFF (2 mg/L). The mentioned Egyptian study also reported *mcr-2* presence, although less frequently (Ahmed et al., 2019). The gene was found neither in this research nor in previous studies from Poland (Literak et al., 2010; Zając et al., 2019).

The finding of *fosA7* gene encoding fosfomycin resistance in *E. coli* derived from birds remaining at rescue centers drew our attention. White-tailed eagle colonized with *E. coli* carrying *fosA7* was released into the natural habitat becoming a source of the resistance in the environment. This perfectly illustrates the feasibility of "microbial pollution" by AMR determinants and resistant bacteria. Recently, the *fosA7*-like gene and other fosfomycin resistance gene, namely *fosA3*, were found in Andean condors (*Vultur gryphus*) (Fuentes-Castillo et al., 2020). Earlier, *fosA7* was described in *Salmonella* isolated from broiler chickens in Canada, as well as from retail meat and clinical incidents in the United States (Rehman et al., 2017; Keefer et al., 2019). The gene was also noted in *E. coli* recovered from soil exposed to anthropogenic activities in North Carolina (Balbin et al., 2020). In Europe, the presence of fosfomycin resistance gene *fosA3* in a *Salmonella* isolated from the migratory bird black kite was reported in Germany (Villa et al., 2015).

Although we noted an infrequent (5.7%) occurrence of resistance to aminoglycosides represented exclusively by gentamicin, we found a spectrum of genes determining resistance toward other compounds of this group, e.g., streptomycin. This result proved that application of phenotypical methods often limited to several antimicrobials, or identification of selected resistance determinants, may lead to underestimation of real AMR status. Similar conclusions were also presented in another study (Rega et al., 2021). That was perfectly illustrated by the *E. coli* recognized as wild type, isolated from a marsh harrier sampled in nature. The analysis revealed that the strain carried aminoglycoside phosphotransferases encoded by *aph(3″)-Ib* and *aph(6)-Id*. On the other hand, identification of the gene (i.e., *mcr-1* discussed earlier) does not always mean resistance. Those findings drive attention to the everlasting discussion of phenotype–genotype congruence and the superiority of the methods applied for testing both aspects.

Phylogeny and Virulence—A Threat to Public and Animal Health

A variety of sequence types was revealed among the tested *E. coli* including some relevant lineages. Furthermore, a wide range and number of virulence factors were observed.

A single isolate from deceased herring gull belonged to ST10, one of the clinically important clones, and carried *mcr-1*. Such an *E. coli* ST10 with *mcr-1* was reported in the Sultanate of Oman from human bloodstream infection (Mohsin et al., 2018). A similar strain was noted in a clinical case in Uruguay (Papa-Ezdra et al., 2020). That *E. coli* variant was also observed in poultry from Poland and China (Yang et al., 2017; Zając et al., 2019) and in agricultural soil of Algeria (Touati et al., 2020).

Another highly virulent lineage—ST131 often associated with extended-spectrum β-lactamase CTX-M-15 spread, was reported as predominant among extraintestinal pathogenic *E. coli* (ExPEC)

(Coque et al., 2008; Nicolas-Chanoine et al., 2014). In Poland, the ST131 clone was a frequent cause of neonatal infections (Chmielarczyk et al., 2013). Indeed, our ST131 from deceased mute swan strain possessed several virulence determinants specific for ExPEC pathotype such as enabling colonization (*pap*) and adherence (*iha*), as well as determining the outer membrane hemin receptor (*chuA*) and secreted autotransporter toxin (*sat*) (Sarowska et al., 2019).

The identification of STEC belonging to ST20 in deceased collared dove captured our attention. Although pigeons were pointed out as a STEC reservoir, most of the researches indicated the presence of Stx2f toxin in the tested strains. A recent study concerning Stx2f-carrying *E. coli* demonstrated that strains responsible for human infections do not directly originate from the pigeon reservoir (van Hoek et al., 2019). However, the *E. coli* tested here possessed two subtypes of toxin Stx2, including Stx2A, which has previously been described as more potent in causing clinical outcomes (Fuller et al., 2011). Moreover, the study on STEC from Switzerland reported ST20 clone carrying *stx2A* from human patients (Fierz et al., 2017).

We also noted *E. coli* ST117 strains from deceased crane and raven harboring *papC* (outer membrane usher P fimbriae), accompanied with *fyuA* (yersiniabactin receptor), *iucC* (aerobactin synthetase), *iroN* (enterobactin siderophore receptor protein), *vat* (vacuolating autotransporter toxin), and *iss* (increased serum survival). It might be perceived as a poultry health risk since the genes were previously reported in avian pathogenic *E. coli* (APEC) ST117 resulting in increased mortality and colibacillosis in broilers in Nordic countries (Ronco et al., 2017).

As a flash point for the study, the archival MDR *E. coli* woodpecker isolate was assigned to the global clone ST224. Noteworthy, our strain possessed high pathogenicity potential carrying multiple virulence genes. MDR *E. coli* belonging to ST224 lineage were previously isolated from patients with urinary tract infections in China (Cao et al., 2014). MDR strains assigned to ST224 were identified among *E. coli* from retail food (chicken carcasses and ground beef) in Egypt (Ramadan et al., 2020). ST224 *E. coli* were also noted among ESBL-producing strains from food-producing animals and wastewater samples in Tunisia (Sghaier et al., 2019). Moreover, *E. coli* ST224 was reported to cause a fatal pneumonia infection in a domestic cat (*Felis catus*) (Silva et al., 2018).

CONCLUSION

All of the above findings indicate free-living bird populations represented by our study group might be considered a source or vector of *E. coli* posing a possible threat to public and animal health. Identification of resistance toward several antimicrobial classes including substances of the highest priority for human medicine, e.g., cephalosporins and quinolones in all tested groups of birds, verified that free-living birds constitute a meaningful AMR reservoir and vector.

Nothing in nature is lost. All pollution of the environment, farmlands, and water might become a possible source of AMR determinants for animals. In consequence, animals affected by resistant bacteria turn into a vector of AMR transmission. Our study shows that the clonal spread of *E. coli* lineages of public and animal health relevance is a serious avian-associated hazard.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: the European Nucleotide Archive (ENA), accession number: PRJEB42669, (http://www.ebi.ac.uk/ena/data/view/PRJEB42669).

ETHICS STATEMENT

The animal study was reviewed and approved by the Ministry of the Environment (approval number: DL-III.6713.11.2018.ABR) General Directorate for Environmental Protection (approval number: DZP-WG.6401.03.2.2018.jro) Regional Directorate for Environmental Protection (RDOŚ) in Lublin (approval number: WPN. 6401.6.2018.MPR).

AUTHOR CONTRIBUTIONS

MS and DW designed the experiments. MP, JW, and WK performed the sampling campaign. MS and MZ performed the experiments. MS and AB performed the NGS analyses. ŁB performed the statistical analyses. MS prepared the manuscript. All authors discussed the results, reviewed and edited the manuscript, and read and approved the final version of 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.2021.656223/full#supplementary-material

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Wide Distribution and Specific Resistance Pattern to Third-Generation Cephalosporins of *Enterobacter cloacae* Complex Members in Humans and in the Environment in Guadeloupe (French West Indies)

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Species belonging to *Enterobacter cloacae* complex have been isolated in numerous environments and samples of various origins. They are also involved in opportunistic infections in plants, animals, and humans. Previous prospection in Guadeloupe (French West Indies) indicated a high frequency of *E. cloacae* complex strains resistant to third-generation cephalosporins (3GCs) in a local lizard population (*Anolis marmoratus*), but knowledge of the distribution and resistance of these strains in humans and the environment is limited. The aim of this study was to compare the distribution and antibiotic susceptibility pattern of *E. cloacae* complex members from different sources in a "one health" approach and to find possible explanations for the high level of resistance in non-human samples. *E. cloacae* complex strains were collected between January 2017 and the end of 2018 from anoles, farm animals, local fresh produce, water, and clinical human samples. Isolates were characterized by the heat-shock protein 60 gene-fragment typing method, and whole-genome sequencing was conducted on the most frequent clusters (i.e., C-VI and C-VIII). The prevalence of resistance to 3GCs was relatively high (56/346, 16.2%) in non-human samples. The associated resistance mechanism was related to an AmpC overproduction; however, in human samples, most of the resistant strains (40/62) produced an extended-spectrum beta-lactamase. No relation was found between resistance in isolates from wild anoles (35/168) and human activities. Specific core-genome phylogenetic analysis highlighted an important diversity in this bacterial population and no wide circulation among the different compartments.

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In our setting, the mutations responsible for resistance to 3GCs, especially in *ampD*, were diverse and not compartment specific. In conclusion, high levels of resistance in non-human *E. cloacae* complex isolates are probably due to environmental factors that favor the selection of these resistant strains, and this will be explored further.

Keywords: *Anolis marmoratus*, cephalosporinase overproduction, *Enterobacter cloacae* complex, ESBL, *hsp60*, one health, phylogeny, Caribbean

INTRODUCTION

Bacteria in the *Enterobacter cloacae* complex (ECC) are widely distributed in numerous terrestrial and aquatic environments, and also in air and space equipment (Singh et al., 2018; Davin-Regli et al., 2019; Uchida et al., 2020). ECC are found in the gut microbiota of animals, including reptiles, mammals, and humans, and some studies have reported that the complex is endophytic (Liu et al., 2012; Singh et al., 2013; Davin-Regli et al., 2019). This bacterial complex also includes phytopathogenic clones (Humann et al., 2011), infection-causing strains in wild fauna and domestic animals (Haenni et al., 2016; Goldberg et al., 2019), and opportunistic pathogens that are involved in a wide variety of human infections, especially those associated with health care (Garinet et al., 2018).

The denomination "complex" refers to different *Enterobacter* species and subspecies, which are difficult to discriminate clearly only with phenotypic approaches. The current classification of ECC members is based on DNA analysis and specifically on partial sequence comparisons of heat-shock protein 60-gene (*hsp60*) fragments (Davin-Regli et al., 2019). The population structure of this complex was initially divided into 12 genetic clusters (C-I to C-XII) and a loosely knit group (C-xiii; Hoffmann and Roggenkamp, 2003). More recently, use of whole-genome sequencing (WGS) revealed a total of 22 phylogenetic clades (A–V), further illustrating the complex taxonomy of this genus (Chavda et al., 2016; Sutton et al., 2018). In addition, some phylogenetic clades are associated with previous *hsp60* clusters, and a novel cluster was reported (Beyrouthy et al., 2018).

The distribution of the ECC clusters was analyzed in studies related to clinical isolates (Kremer and Hoffmann, 2012; Garinet et al., 2018). Strains belonging to C-III, -VI, and -VIII usually predominated and were found to carry various determinants of antibiotic resistance (Stock et al., 2001; Hoffmann et al., 2005; Kremer and Hoffmann, 2012; Peirano et al., 2018). These clusters appeared to be adapted to the hospital environment (Paauw et al., 2008), and geographic specificities were recently reported (Zhou et al., 2018). Few studies, however, have provided information about the distribution of ECC members in animals and other sources, which limits comparisons and impedes understanding of their global epidemiology (Hoffmann and Roggenkamp, 2003; Kämpfer et al., 2008; Haenni et al., 2016).

Enterobacter cloacae complex belongs to the ESKAPE group which referred to *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp., and is recognized as a "priority pathogen" due to its clinical relevance and association with antibiotic resistance and virulence genes (Rice, 2008; World Health Organization, 2017). All members of this complex have similar susceptibility to antibiotics (Davin-Regli et al., 2019), except fosfomycin (Stock et al., 2001). They are intrinsically resistant to aminopenicillin, the combination amoxicillin–clavulanic acid, and the first two generations of cephalosporins because they express an inducible *ampC* cephalosporinase. Under selection pressure, derepressed mutants often emerge, which overproduce cephalosporinase, conferring a high level of resistance to third-generation cephalosporins (3GCs) and increasing the minimum inhibitory concentration required for the fourth generation, such as cefepime (Guérin et al., 2015; Kohlmann et al., 2019; Mizrahi et al., 2020). Since the emergence of extended-spectrum beta-lactamase (ESBL), plasmidic acquisition of ESBL determinants has become an important mechanism in 3GCs resistance (3GC-R), especially among clinical ECC strains, as for most *Enterobacteriaceae.* These bacteria are also capable of acquiring genes that encode for carbapenemases, further restricting therapeutic management (Peirano et al., 2018). ESBL acquisition or cephalosporinase overproduction (CoP) appear to be distributed differently inside *hsp60* clusters, as illustrated by a higher frequency of ESBL production in C-VI and -VIII isolates (Stock et al., 2001; Garinet et al., 2018). As in other high resource countries, community resistance of ECCs to 3GCs is rare in Guadeloupe because of lower selection pressure (Guyomard-Rabenirina, 2016).

Although these bacteria have been described in the environment, the susceptibility of environmental strains is not well understood. There is little information in Guadeloupe, as in other South American countries, on the distribution of ECC members or on the resistance to antibiotics of strains isolated in the environment and in clinical samples. Resistance of *Enterobacteriaceae* to 3GCs was rare in community-acquired urinary tract infections (4.0%), due mainly to ESBL production (Guyomard-Rabenirina, 2016), in contrast to the high frequency of ESBL in clinical ECC (19.7%) from the University Hospital of Guadeloupe (S. Breurec, personal communication). In the local environment, we observed a high prevalence of 3GC-R *Enterobacteriaceae* carriage in feces of *Anolis marmoratus* (89/234, 38.0%), a small endemic lizard, and ECC members

Abbreviations: 3GC, third-generation cephalosporin; 3GC-R, third-generation cephalosporin resistant; ECC, *Enterobacter cloacae* complex; ESBL, extended-spectrum beta-lactamase; ESKAPE, *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp.; CoP, cephalosporinase overproduction; *hsp60*, heat-shock protein 60 gene-fragment; SNP, single nucleotide polymorphism; ST, sequence type; UD, undefined cluster; WGS, whole-genome sequencing; WT, wild-type.

were the most prevalent among isolated strains (57/115, 49.5%; Guyomard-Rabenirina, 2016).

We conducted this study to investigate the distribution of ECC *hsp60* clusters isolated from various sources in Guadeloupe and to characterize their antibiotic susceptibility patterns in a "one health" approach. Genomic analysis was conducted on the main clusters in clinical samples (C-VI and C-VIII) and in different biotopes to compare the strains and to investigate the high level of 3GC-R in non-human samples.

MATERIALS AND METHODS

Collection From Human

Between January and December 2018, clinical ECC isolates obtained during routine bacteriological diagnostics were collected prospectively from patients admitted to the University Hospital of Guadeloupe, a 900-bed teaching hospital in Pointe-à-Pitre/Les Abymes. The date and nature of the sample, and the results of antibiotic susceptibility analysis were recorded anonymously. Isolates were considered to be hospital-acquired if there were collected from patients hospitalized for more than 48 h after admission. The others were notified as to be community-acquired. Human samples were taken in accordance with the requirements of the local ethics committee and did not interfere with laboratory organization (reference A5_19_12_05_TRAMID).

Sampling for Animal and Environmental Isolates

Between January 2017 and December 2018, ECC strains were isolated from water catchment areas, local fruits and vegetables, and fresh fecal samples from *A. marmoratus* and farm animals sampled at different sites in Guadeloupe.

Overall, 168 free-living adult lizards were caught and sampled at 17 sampling sites throughout the island (**Supplementary Table 1**). All the procedures were approved by the regional environment, planning, and housing agency and by the Guadeloupe National Park. The project was also approved by the Committee for Ethics in animal experiments of the French West Indies and Guyana (reference 971-2016-12-20-001). Animals were cared for and used according to French decree No. 2013-118 of 1 February 2013 on the protection of animals, which meets European Union Directive 2010/63 on the protection of animals used for experimental and other scientific purposes (Guyomard-Rabenirina et al., 2020). To study a possible association between ECC carriage and the degree of human activity at the site at which wild individuals were caught, the sampling location and the type of environment (urban, coastline, and mountain forest) were recorded (**Supplementary Table 1**). Urban sites were considered to be associated with moderate to high human activity, and the coastline and mountain forest environments with limited human impact. Fresh fecal samples from 34 pigs and 28 beef cattle were collected at the only slaughterhouse in Guadeloupe, located in Le Moule. The municipality of origin of the sampled animal was recorded (**Supplementary Table 1**). No information was available on antibiotic treatment. A total of 76 samples of fresh, locally produced fruits and vegetables were collected aseptically at four local markets (Bergevin, Convenance, Gourdeliane, and Saint-Jules). The market and the farm of origin were recorded, as well as the type of fertilizer used (organic or chemical) on each identified farm ($n = 27$ in **Supplementary Table 1**). A total of 40 raw water samples, which corresponded to drinking water before treatment, were collected at 24 catchment points, in collaboration with the regional health agency and the hygiene laboratory of the Pasteur Institute of Guadeloupe. Most of these sampling points were located in Basse-Terre. All samples were transported rapidly to the laboratory, stored at $5 \pm 3°C$ and analyzed within 4 h.

ECC Isolation and Antibiotic Susceptibility Analysis

All non-human samples were enriched. Animal stools, fruits, and vegetables were mixed with buffered peptone water. For water samples, 100 mL of serially diluted samples were filtered through a 0.45-μm membrane filter (Millipore, Guyancourt, France), and the membranes were placed in 9 mL of buffered peptone water solution. The non-selective enrichment broth for all samples was incubated for 16–20 h at 37°C. Then, 100 μL were inoculated onto chromogenic agar (CCA, CHROMagar, Paris, France). In addition, to increase the chances of detection of 3GC-R strains in the bacterial population, the same medium supplemented with ceftriaxone at 4 mg/L was also inoculated. Plates were incubated for 16–20 h at 37°C. This antibiotic was selected as it is considered to be a weak inducer of CoP as other 3GCs (Mizrahi et al., 2020). A maximum of five presumptive ECC colonies from each plate were isolated and identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Shimadzu Biotech, Kyoto, Japan) and associated software.

Susceptibility to ampicillin (10 μg), amikacin (30 μg), amoxicillin–clavulanic acid (20–10 μg), aztreonam (30 μg), cefepim (30 μg), cefotaxime (5 μg), cefoxitin (30 μg), ceftazidime (10 μg), ciprofloxacin (5 μg), ertapenem (10 μg), gentamicin (10 μg), nalidixic acid (30 μg), temocillin (30 μg), ticarcillin (75 μg), tigecycline (15 μg), and trimethoprim–sulfamethoxazole (1.25–23.75 μg) was determined for all ECC strains by the disk diffusion method on Mueller-Hinton agar (Bio-Rad, Marnes-la-Coquette, France). Isolates were classified as resistant, intermediate, or susceptible according to the 2018 guidelines of CA-SFM/EUCAST[1]. Isolates of intermediate susceptibility were grouped with resistant isolates for data analysis (**Supplementary Table 2**). Production of ESBL was detected with the double-disk synergy test, according to CA-SFM/EUCAST recommendations.

DNA Extraction

Total bacterial DNA was initially extracted from pure cultures with the Qiagen QIAamp DNA minikit (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

ESBL Resistance Gene Screening

Extended-spectrum beta-lactamase-encoding genes were screened by polymerase chain reaction (PCR). On the basis of

[1]http://www.sfm-microbiologie.org

previous epidemiological evidence for ECC in the community, PCR was performed only for bla_{CTX-M} group 1 (Dallenne et al., 2010; Guyomard-Rabenirina, 2016). Amplicons were sequenced at Eurofins (Eurofins Genomic SAS, Les Ullis, France). Resistance genes were identified from the ResFinder database (Zankari et al., 2012).

ECC *hsp60* Typing

As ECC members cannot be differentiated reliably with classic identification methods, we conducted sequence analysis of the partial *hsp60* gene, as described previously on 313 local strains (Hoffmann and Roggenkamp, 2003). PCR products were sequenced at Eurofins, and DNA sequences and chromatograms were analyzed with ApE software[2]. Maximum likelihood phylogenetic reconstruction was performed with RAxML in 100 replications (Stamatakis, 2014). The tree was drawn with iTOL (Letunic and Bork, 2019) and rooted with *Klebsiella aerogenes hsp60* partial sequence (AB008141.1; Hoffmann and Roggenkamp, 2003). Accession numbers for previously identified *hps60* cluster sequences are listed in **Supplementary Figure 1**. To complete the analysis, *hsp60* partial sequences were extracted from the assembled genomes of ECC ST873 strains, characterized, and added as a new ECC cluster (C-XIV – Clade S; Beyrouthy et al., 2018). Similar initial bioinformatic analysis was conducted on a selection of different ECC strains used and identified by Chavda et al. (2016), and Sutton et al. (2018; **Supplementary Figure 1**). We also included *hsp60* partial sequences of recently named strains: *E. wuhouensis*, *E. quasihormaechei* (Wang et al., 2020), *E. huaxiensis*, *E. chuandaensis* (Wu et al., 2019), and *E. oligotrophicus* (formerly *E. oligotrophica*) (Akita et al., 2019). The collected strains were assigned to a cluster on the basis of the reference data set used and bootstrap values (**Supplementary Figure 1**). When multiple strains were found in the same sample, only one in each cluster or resistance phenotype profile against beta-lactam antibiotics was conserved for the analysis, to avoid duplicates.

Core-Genome Phylogenetic Analyses

To better describe possible circulation of ECC lineages collected from animals, fresh food, raw water, and humans and to gain understanding of the emergence of 3GC-R ECC from non-human samples, WGS was conducted on randomly selected strains in the two most prevalent *hsp60* clusters: C-VI (n = 45) and C-VIII (n = 86). Their origins were: 47 from *Anolis*, 5 from domestic animals, 14 from fresh produce, 10 from raw water samples, and 55 from human isolates. WGS was performed at the "Plateforme de microbiologie mutualisée" of the Pasteur International Bioresources network (Institut Pasteur, Paris, France). The method and software used for sequencing, quality checking and core-genome extraction were described previously (Guyomard-Rabenirina et al., 2020). Raw reads were trimmed and filtered with AlienTrimmer (Criscuolo and Brisse, 2014). Genomes were assembled with SPAdes software (Bankevich et al., 2012), and final quality was appreciated with QUAST and BUSCO score (Gurevich et al., 2013; Simão et al., 2015).

Total core single nucleotide polymorphisms (SNPs) were detected with Snippy software version 4.4.5, with GENC200 strain as reference for C-VI isolates and GENC071 strain for C-VIII[3]. Recombination elements were removed from the global core genome alignment with ClonalFrameML software (Didelot and Wilson, 2015), The two maximum likelihood phylogenetic reconstructions were performed with RAxML software in the GTR-CAT model and 1000 bootstrap replicates, and the trees were drawn with iTOL. Multilocus sequence typing was performed *in silico* with mlst software[4] against the PubMLST database (Jolley et al., 2018), and virulence gene factors were identified with Abricate software[5] associated with the Virulence Factor Database with a threshold of 95% coverage and 75% nucleotide identity (Chen et al., 2016). Abricate was used with these parameters to assess plasmid replicon and antibiotic resistance gene content associated with PlasmidFinder and ResFinder databases, respectively (**Supplementary Table 3**; Zankari et al., 2012; Carattoli et al., 2014).

Focus on Cephalosporinase Genes and Mutations

In the ECC collection, 11 wild-type (WT)/CoP pairs from the same sample were analyzed for mutations. Each strain pair differed by fewer than 45 SNPs (**Supplementary Table 4**). Nine pairs belonging to ECC C-VIII and two to C-VI were selected. Most of the strains (14/22) were isolated from reptiles. Mutation analyses were performed with Snippy software, and the corresponding GenBank flat file format for each gene was retrieved from the NCBI website[6] (Benson et al., 2018). The accession numbers of the sequences used as references were: NZ_CP012165.1 (*E. hormaechei* subsp. *oharae* strain 34978, complete genome) and NC_014121.1 (*E. cloacae* subsp. *cloacae* ATCC 13047 chromosome, complete genome). All genes which could be involved in CoP and 3GC-R phenotype were analyzed and listed in **Supplementary Table 4** (see **Reference.gbk**). An in-house Perl script was used to extract results from files generated by Snippy and to create a table of the numbers of non-synonymous and synonymous gene mutations in the ECC sequences retrieved from the same host but with different antibiotic resistance profiles.

Statistical Analyses

The analyses and data collection were performed with Microsoft Access 2003. Pearson's χ^2 or Fisher's exact test was used. P values < 0.05 were considered significant.

[2]https://jorgensen.biology.utah.edu/wayned/ape/

[3]https://github.com/tseemann/snippy

[4]https://github.com/tseemann/mlst

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

[6]https://www.ncbi.nlm.nih.gov/genbank/

RESULTS

Between January 2017 and December 2018, 313 unique ECC strains were isolated from various sample types, after removal of duplicates (**Table 1**). In non-human isolates, ECC strains (WT and 3GC-R) were isolated from 57.9% (44/76) of fresh produce and 42.9% (72/168) of anole samples; half of the raw water samples (20/40) were positive, while ECC strains were isolated from only 29.0% of livestock samples (18/62). Overall, resistance to 3GCs was found in 16.2% of non-human samples (56/346) and was due only to CoP. Most of these 3GC-R strains exhibited a 6 mm inhibition diameter on Mueller-Hinton agar plate for cefotaxime and ceftazidime antibiotics (data not shown). The prevalence of CoP was higher in anole samples (35/168, 20.8%) than in vegetable (13/76, 17.1%), livestock (6/62, 9.7%), or water isolates (2/40, 5.0%). Most of the anoles were trapped in areas impacted by human activities (n = 112, 66.7%); however, no significant difference was found in the rates of CoP by degree of human activity (**Table 2**). A positive association was found between the prevalence of positive for ECC and use of organic fertilizer (11/11) rather than chemical fertilizer (4/12; P = 0.001) from the 27 identified farms, but a similar association was not found for the CoP rate (P = 0.15, i.e., 4/11 and 1/12; **Supplementary Table 1**).

Most of human isolates were hospital-acquired (95/107) and 3GC-R were mainly associated to ESBL production (40/62; **Table 1** and **Supplementary Table 2**). Genes encoding for ESBL were identified in all clinical strains positive in the double-disk synergy test (40/107, 37.4%). Amplicon sequencing revealed a $bla_{\mathrm{CTX-M-15}}$ gene in 38 isolates (95.0%). In addition, WGS allowed us to identify a $bla_{\mathrm{GES-7}}$ gene on the two last ESBL producers (GENC084, GENC220). One strain of human origin carried a $bla_{\mathrm{OXA-48}}$ gene (GENC133).

Co-resistance against beta-lactams (including 3GCs) and other antibiotic families was observed mainly in human associated strains and was usually to fluoroquinolones (41/62, 66.2%; **Table 1**). Co-resistance to gentamicin and trimethoprim–sulfamethoxazole was also found frequently, in 29 3GC-R clinical strains (46.8%) but not in samples from other sources. Only one strain from a raw water sample was resistant to nalidixic acid (ECC403), and one strain from livestock was notified resistant to trimethoprim–sulfamethoxazole and tigecycline (ECC408).

Distribution of *hsp60* Clusters

Strain diversity was first investigated with *hsp60* typing. A neighbor-joining tree was constructed with 129 alignment patterns of 313 partial *hsp60* sequences from the different isolates, comprising 131 strains from the animal collection (110 from anole, 9 from pig, and 12 from beef cattle), 23 from water, 52 from fresh produce, and 107 from clinical samples (**Table 3** and **Supplementary Figure 1**). All *Enterobacter* Hoffman clusters except C-VII (*E. hormaechei* subsp. *hormaechei*) were represented in our study, including C-XIV (n = 2) in human and domestic animal samples

TABLE 1 | Prevalence of resistance to each tested antibiotic of *E. cloacae* complex (ECC) isolates.

Antibiotic	Human (N = 107)				*Anolis* (N = 110)		Fresh produce (N = 52)		Water (N = 23)		Livestock (N = 21)	
	WT (N = 45)	CoP (N = 21)	ESBL (N = 40)	CP (N = 1)	WT (N = 70)	CoP (N = 40)	WT (N = 38)	CoP (N = 14)	WT (N = 21)	CoP (N = 2)	WT (N = 15)	CoP (N = 6)
Ticarcillin	3 (6.7)	21 (100.0)	40 (100.0)	1 (100.0)	4 (5.7)	36 (90.0)	4 (10.5)	14 (100.0)	–	2 (100.0)	1 (6.7)	6 (100.0)
Temocillin	–	17 (81.0)	20 (50.0)	1 (100.0)	1 (1.4)	17 (42.5)	–	9 (64.3)	–	–	–	2 (33.3)
Cefotaxime	–	21 (100.0)	40 (100.0)	1 (100.0)	–	33 (82.5)	–	14 (100.0)	–	2 (100.0)	–	6 (100.0)
Ceftazidime	–	19 (90.5)	40 (100.0)	1 (100.0)	–	40 (100.0)	–	14 (100.0)	–	2 (100.0)	–	6 (100.0)
Aztreonam	–	14 (66.7)	39 (97.5)	1 (100.0)	–	34 (85.0)	–	13 (92.9)	–	1 (50.0)	–	6 (100.0)
Cefepim	–	–	40 (100.0)	1 (100.0)	–	–	–	–	–	–	–	–
Ertapenem	–	1 (4.8)	1 (2.5)	1 (100.0)	–	20 (50.0)	–	9 (64.3)	–	1 (50.0)	–	4 (66.7)
Nalidixic acid	4 (8.9)	4 (19.0)	38 (95.0)	1 (100.0)	–	–	–	–	1 (4.8)	–	–	–
Ciprofloxacin	3 (6.7)	4 (19.0)	36 (90.0)	1 (100.0)	–	–	–	–	–	–	–	–
Gentamicin	–	2 (9.5)	31 (77.5)	–	–	–	–	–	–	–	–	–
Amikacin	–	–	1 (2.5)	–	–	–	–	–	–	–	–	–
Tigecycline	3 (6.7)	1 (4.8)	5 (12.5)	–	–	–	–	–	–	–	1 (6.7)	–
Trimethoprim – sulfamethoxazole	3 (6.7)	2 (9.5)	35 (87.5)	1 (100.0)	–	–	–	–	–	–	1 (6.7)	–

*ECC strains (n = 313) were grouped according to their origin and resistance to beta-lactam antibiotics: wild-type (WT), cephalosporinase overproduction (CoP), extended-spectrum beta-lactamase (ESBL), or carbapenemase production (CP). All strains were resistant to ampicillin, amoxicillin–clavulanic acid, and cefoxitin (i.e., "R" **Supplementary Table 2**).*

TABLE 2 | Carriage of *E. cloacae* complex and third-generation cephalosporin-resistant (3GC-R) strains in the *Anolis* population, according to degree of human activity.

Sampling site			*Anolis marmoratus* sampled	*E. cloacae* complex isolation			
Type	**(*N* = 17)[a]**	**Degree of human activity**	**(*N* = 168)**	**Total of positive sample *N* (%)**	***p-value***	**3GC-R *N* (%)**	***p-value***
Urban	11	Moderate-high	112	53 (47.3)	0.1	25 (22.3)	0.5
Coastline or mountain forest	6	Limited	56	19 (33.9)		10 (17.9)	

[a]*GPS coordinates are provided in* ***Supplementary Table 1****.*

(Hoffmann and Roggenkamp, 2003; Beyrouthy et al., 2018); 28 strains, mainly human isolates (14/28), did not correspond to any of the 14 previously defined *hsp60* clusters. They were grouped into six undefined *hsp60* clusters (recorded as UD1–6), which included ECC clades recently identified by WGS (K, L, P, N, T) and *E. oligotrophica* (**Table 3**, **Supplementary Table 2**, and **Supplementary Figure 1**; Chavda et al., 2016; Sutton et al., 2018; citealpBR1).

Cluster VIII was best represented, except in livestock and fresh produce. C-VI was the second most frequent in clinical strains, whereas C-xiii was over-represented among anole isolates (28.2%), and *E. asburiae* was more frequent in water samples (C-I, 17.4%). In livestock, C-XI, -VI, and -IX predominated (**Table 3** and **Supplementary Figure 1**). CoP strains were found in nearly all clusters except C-II, -V, and UD1, 3 and 5. The highest rate of CoP strains was found in cluster VIII (**Table 3**).

Genetic Analysis of ECC Clusters VI and VIII Populations

The WGS generated a mean of 149.87 bp paired-end reads, with an estimate coverage of 77.851-fold (AlienTrimmer; Criscuolo and Brisse, 2014). Quality of the assembly indicated a mean N50 of 309532 (minimum 43293, maximum 782933), and a mean single-copy BUSCO score of 98.6% completeness (Gurevich et al., 2013; Simão et al., 2015). Of the 45 sequenced isolates assigned to C-VI, 42 belonged to *E. hormaechei* subsp. *xiangfangensis* (clade A), and only three were identified as *E. hormaechei* subsp. *oharae* (clade C; **Table 3**; Sutton et al., 2018). These clade C strains were isolated from fresh produce and human samples (ECC 312, ECC336, and GENC003) and were not conserved for further analysis. Maximum likelihood phylogenetic analysis of *E. hormaechei* subsp. *xiangfangensis* strains indicated wide diversity among isolates (mean SNP of all isolates n = 17973, minimum n = 4, and maximum n = 22821). Fifteen strains were not assigned to a well-known sequence type (ST) or identified as new, whereas the others belonged to nine clearly identified sequence types (**Figure 1**). The largest cluster contained 11 strains in the international clone ST114 (mean SNP between isolates n = 935, minimum n = 4, and maximum n = 2122), which were isolated only from humans. Most of the strains (8/11) were ESBL producers. Some genetically related clusters with isolates of different origins were observed, comprising a cluster with three ST98 strains (two from humans and one from a cucumber; SNP mean = 484, minimum = 68) and a second with three vegetables strains belonging to ST344. The samples, taken at the same time at Bergevin market on 12 January 2018 (**Supplementary Table 1**), were from two farms, had the same antibiotic resistance profile (3GC-R), and differed by a mean of 45 SNPs (minimum = 36). One strain from *Anolis* clustered with an isolate from fresh produce, with only 65 SNPs difference (**Figure 1**).

Phylogenetic analysis of C-VIII revealed greater diversity, especially among anole strains (mean SNP for all isolates n = 23125, minimum n = 8, and maximum n = 27680). Various lineages were observed in the same sample (samples a, e, and f in **Figure 2** and **Supplementary Table 4**). *In silico* analyses revealed the presence of two main groups in this second tree. The first (C-VIII-A, n = 32) consisted mainly of clinical isolates (20/32; mean SNP among isolates n = 26166, minimum n = 8, and maximum n = 31449), while reptile strains predominated in the second one (C-VIII-B, 31/49). ESBL producers were found only in C-VIII-A and, as expected, only in human isolates. Like ST114 in ECC C-VI, ST113 was well represented in human samples for C-VIII (n = 9), and four were ESBL producers. This ST was not found in other biotopes (**Supplementary Table 3**).

As observed in the C-VI phylogenetic tree, some strains of different origins were genetically related. ST90 was recovered from three different *A. marmoratus*, one water sample, and one human (two strains from the same patient, l in **Figure 2** and **Supplementary Table 4**; mean SNP among isolates n = 1283, minimum n = 8, and maximum n = 2221). Most of these strains presented a CoP (5/6). Two other STs found in wild fauna and human samples clustered together: ST50, (ECC239–GENC185, 225 SNPs) and ST304 (ECC273–GENC100, 7698 SNPs). The human strain GENC117 shared the same target sequence of seven housekeeping genes with ECC386 isolated from water and a difference of 123 SNPs (i.e., new ST1493). One strain from *Anolis* clustered with an isolate from water, with a difference of only 101 SNPs (ECC443–ECC426). Two strains isolated from two *Anolis* 28 km apart clustered, with a difference of only 81 SNPs (ECC140–ECC300).

Few different resistance gene types were shared by ECC isolates from the 5 origins (**Supplementary Table 3**). The genes belonged to bla_{ACT}, and one gene conferred resistance to fosfomycin (*fosA*). Genes encoding for efflux pumps (*oqxA–oqxB* and *mdfA*) were observed in human and non-human isolates. Genes encoding for cephalosporinase were specific for each cluster. *E. hormaechei* subsp. *xiangfangensis* was related to the $bla_{\mathrm{ACT-16}}$ gene type, while $bla_{\mathrm{ACT-15}}$ and $bla_{\mathrm{ACT-7}}$ were associated with *E. hormaechei* subsp. *steigerwaltii*. Human strains belonging to C-VI and C-VIII expressed more resistance genes (mean, nine) than those from other compartments (mean, three; **Supplementary Table 3**). All sequenced and analyzed

TABLE 3 | Distribution of *E. cloacae* complex (ECC) members in samples of different origin.

***Enterobacter* species or subspecies names**	***hsp60* cluster[a]**	**WGS clade[a]**	**Number of collected strains**	**Origin N (%)**					**3GC-R *N* (%)**		**ESBL[c] *N* (%)**
				Human	***Anolis***	**Fresh produce**	**Water**	**Livestock**	**Human**	**Non-human[d]**	
			(*N* = 313)	**(*N* = 107)**	**(*N* = 110)**	**(*N* = 52)**	**(*N* = 23)**	**(*N* = 21)**	**(*N* = 62)**	**(*N* = 62)**	**(*N* = 40)**
E. asburiae	I	J	13	6 (46.1)	–	2 (15.4)	4 (30.8)	1 (7.7)	4 (30.8)	–	3 (23.1)
E. kobei	II	Q	3	2 (66.7)	–	1 (33.3)	–	–	–	–	–
E. hormaechei subsp. *hoffmannii*	III	D	1	1 (100.0)	–	–	–	–	1 (100.0)	–	–
E. roggenkampii	IV	M	18	4 (22.2)	6 (33.3)	6 (33.3)	1 (5.6)	1 (5.6)	3 (16.7)	3 (16.7)	–
E. ludwigii	V	I	1	1 (100.0)	–	–	–	–	–	–	–
E. hormaechei subsp. *xiangfangensis*	VI	A	48	28 (58.3)	6 (12.5)	9 (18.8)	–	5 (10.4)	22 (45,8)	10 (20.8)	16 (33.3)
E. hormaechei subsp. *oharae*	VI	C	3	1 (33.3)	–	2 (66.7)	–	–	–	1 (33.3)	–
E. hormaechei subsp. *hormaechei*	VII	E	–	–	–	–	–	–	–	–	–
E. hormaechei subsp. *steigerwaltii*	VIII	B	87	31 (35.6)	43 (49.4)	3 (3.5)	10 (11.5)	–	13 (14,9)	18 (20,7)	7 (8.0)
E. bugandensis	IX	R	32	10 (31.2)	7 (21.9)	9 (28.1)	2 (6.3)	4 (12.5)	4 (12.5)	6 (18.6)	1 (3.1)
E. cloacae subsp. *cloacae*	XI	G	19	5 (26.3)	1 (5.3)	5 (26.3)	1 (5.3)	7 (36.8)	2 (10.5)	1 (5.3)	1 (5.3)
E. cloacae subsp. *dissolvens*	XII	H	21	2 (9.5)	8 (38.1)	8 (38.1)	1 (4.8)	2 (9.5)	1 (4,8)	4 (19,0)	–
E. cloacae complex	xiii	na	37	1 (2.7)	31 (83.8)	3 (8.1)	2 (5.4)	–	–	16 (43.2)	–
E. quasihormaechei	XIV	S	2	1 (50.0)	–	–	–	1 (50.0)	1 (50.0)	–	1 (50.0)
Undefined clusters[b]	UD1	P	1	1 (100.0)	–	–	–	–	–	–	–
	UD2	N	2	–	1 (50.0)	1 (50.0)	–	–	–	1 (50.0)	–
	UD3	K	2	–	1 (50.0)	1 (50.0)	–	–	–	–	–
	UD4	L	14	13 (92.9)	–	–	1 (7.1)	–	11 (78.6)	1 (7.1)	11 (78.6)
	UD5	na	1	–	1 (100.0)	–	–	–	–	–	–
	UD6	T	8	–	5 (62.5)	2 (25.0)	1 (12.5)	–	–	1 (12.5)	–

*[a] Details of the strains and sequences used as reference are provided in **Supplementary Figure 1** and in **Supplementary Table 2**. [b] Undefined cluster numbers proposed in this article. [c] Extended-spectrum beta-lactamase (ESBL) producers were only recovered from human samples. [d] Most of the third-generation cephalosporin-resistant (3GC-R) strains isolated from non-human samples were identified from CCA medium supplemented with ceftriaxone 4 mg/L (56/62; see **Supplementary Table 2**); na, not attributed.*

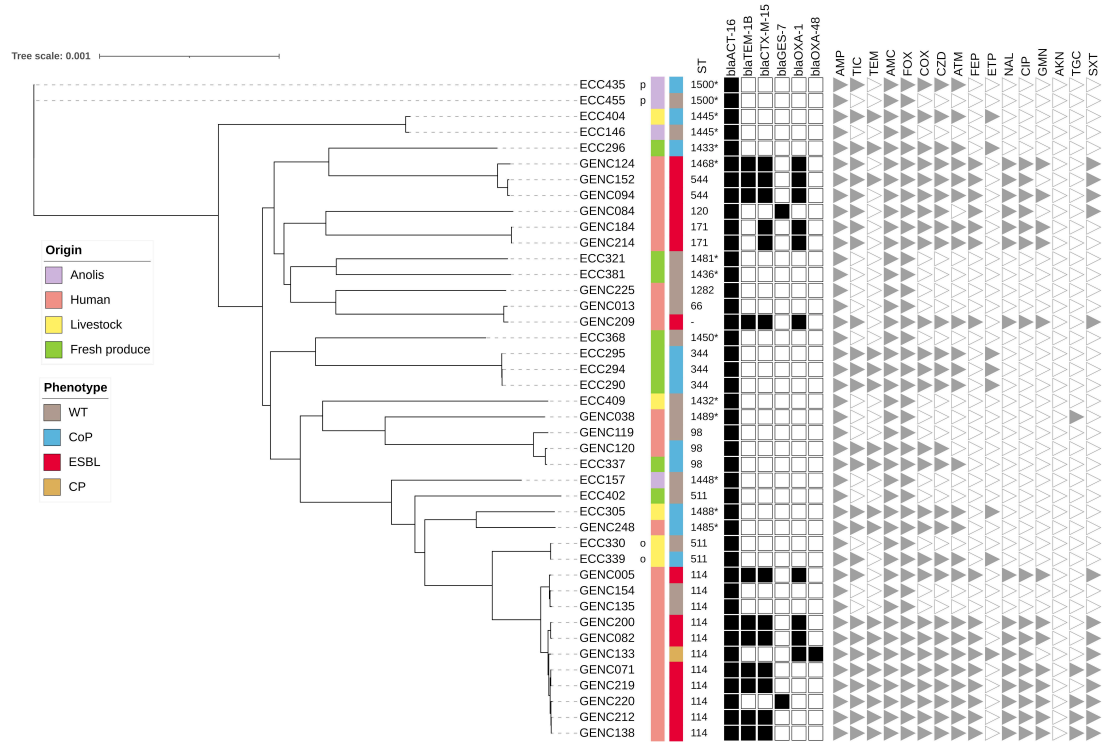

FIGURE 1 | Maximum likelihood phylogenetic tree of *E. cloacae* complex C-VI – clade A isolates recovered in Guadeloupe (n = 42). Maximum likelihood phylogenetic reconstructions were performed with RAxML software (1000 bootstrap replicates), and the tree was drawn with iTOL. Hosts and phenotypes are indicated by vertical colored strips. The letters indicate specific wild-type and cephalosporinase overproduction pairs in the same sample. New sequence types (STs) identified in this study are indicated by a star, while unknown ST is denoted by a dash. Only genes that confer resistance to beta-lactam antibiotics were included. They were characterized by ResFinder and are indicated by black squares; all genetic details are provided in **Supplementary Table 3**. Antibiotic resistance profiles are indicated by gray triangles; AKN, amikacin; AMC, amoxicillin–clavulanic acid; AMP, ampicillin; ATM, aztreonam; CIP, ciprofloxacin; COX, cefotaxime; CZD, ceftazidime; ETP, ertapenem; FEP, cefepim; FOX, cefoxitin; GMN, gentamicin; NAL, nalidixic acid; TEM, temocillin; TGC, tigecycline; TIC, ticarcillin; and SXT, trimethoprim–sulfamethoxazole.

strains resistant to fluoroquinolones (28/128; except GENC200) harbored quinolone resistance genes (*qnrB1*, *qnrB19* or *qnrS2*). Regarding the plasmid distribution, 50.0% (64/128) of the sequenced C-VI and C-VIII carried at least one identifiable replicon type. Among them Col (48/64), IncFII (n = 24), IncHI2 (n = 21), and IncFIB (n = 20) were the most often identified. Only 24.3% (18/74) of non-human strains presented at least one replicon. This prevalence was higher in the human collection (46/54, 85.2%), and whole-genome sequenced ESBL-producing ECC were especially associated with an IncHI2 signature (19/22, 86.4%; **Supplementary Table 3**). We also identified genes associated with virulence in C-VI and C-VIII populations in both human and non-human strains, which are involved in bacterial adherence, iron uptake, motility, or toxin production (**Supplementary Table 3**). Globally, human and non-human isolates expressed a similar number of virulence genes (mean twenty-one). All sequenced C-VIII strains (n = 86) harbored genes involved in the salmochelin siderophore system (*iroB*, *iroC*, *iroD*, *iroE*, and *iroN*), while this system was not found in the C-VI population. The yersiniabactin system (*fyuA*, *irp1*, *irp2*, *ybtA*, *ybtE*, *ybtP*, *ybtQ*, *ybtS*, *ybtT*, *ybtU*, and *ybtX*) was identified in two non-human strains belonging to ST90 (ECC169 and ECC403).

Cephalosporinase Genes Mutation Profile Analysis

In isolates analyzed for cephalosporinase mutation, alignment of the AmpD protein sequences belonging to the WT ECC showed relatively good conservation (with an overall identity of 94.65%). Few mismatches were found (**Supplementary Table 4**). Comparison of 3GC-R with WT isolates from the same sample indicated that differences in mutations were found mainly in the *ampD* gene. Significant mutations are listed in the last column of **Supplementary Table 4**. Furthermore, other genes such as *dacB* and *ramR* could play a non-negligible role in the differentiation of resistant and susceptible strains. Our analysis indicates non-synonymous mutations different from those was observed previously, although Ala60Val substitution in *ampD* was also detected in ECC033 and ECC037 (Flury et al., 2016).

DISCUSSION

This study of ECC diversity in samples from different compartments in Guadeloupe showed by *hsp60* typing analysis that six clusters (C-IV, -VI, -VIII, -IX, -XI, and -XII) are identified in more than two thirds of the strains (228/313, 72.8%), and most

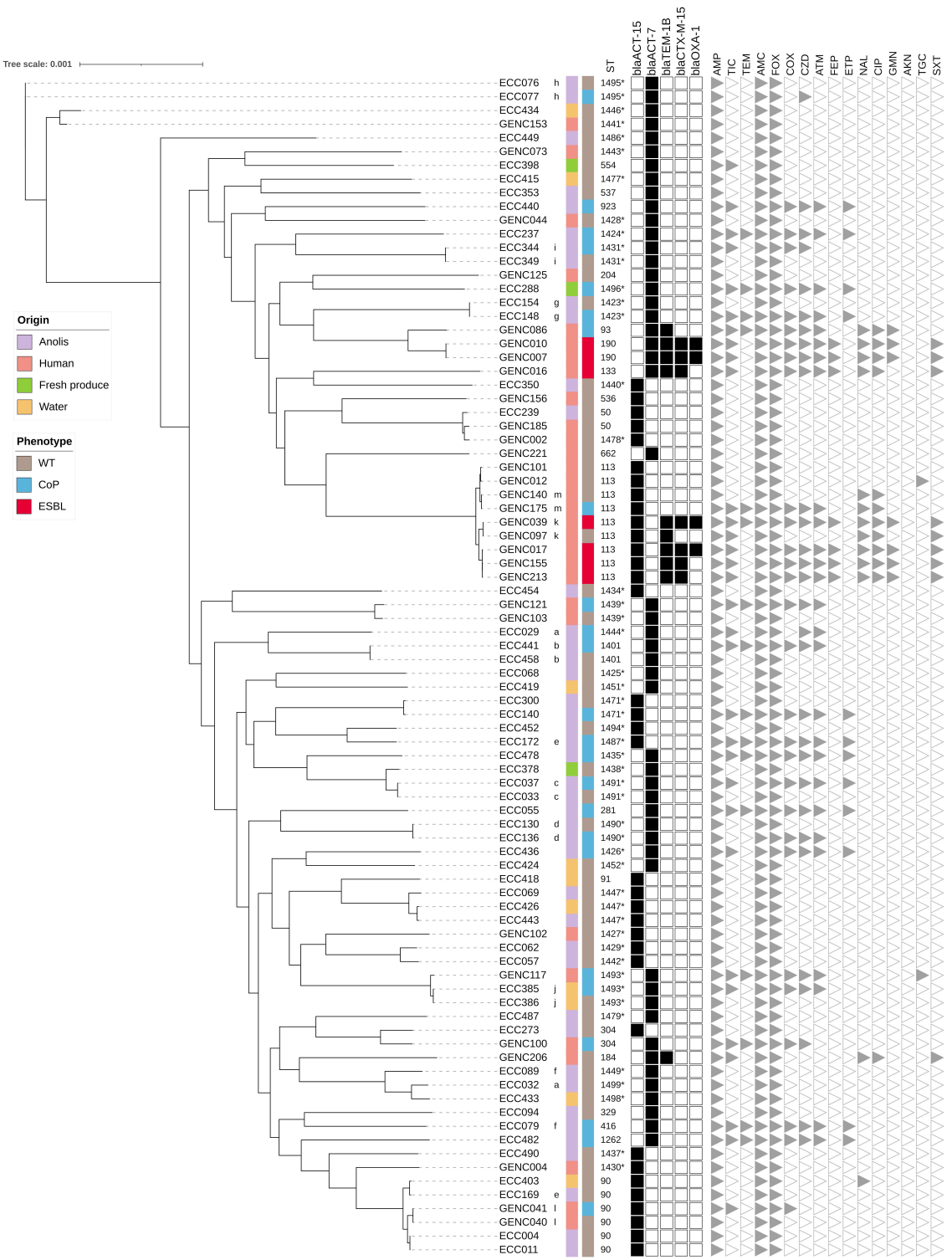

FIGURE 2 | Maximum likelihood phylogenetic tree of *E. cloacae* complex C-VIII isolates recovered in Guadeloupe (n = 86). Maximum likelihood phylogenetic reconstructions were performed with RAxML software (1000 bootstrap replicates), and the tree was drawn with iTOL. Hosts and phenotypes are indicated by vertical colored strips. The letters indicate specific wild-type and cephalosporinase overproduction pairs in the same sample. New sequence types (STs) identified in this study are indicated by a star. Only genes that confer resistance to beta-lactam antibiotics were included. They were characterized by ResFinder and are indicated by black squares; all genetic details are provided in **Supplementary Table 3**. Antibiotic resistance profiles are indicated by gray triangles; AKN, amikacin; AMC, amoxicillin–clavulanic acid; AMP, ampicillin; ATM, aztreonam; CIP, ciprofloxacin; COX, cefotaxime; CZD, ceftazidime; ETP, ertapenem; FEP, cefepim; FOX, cefoxitin; GMN, gentamicin; NAL, nalidixic acid; TEM, temocillin; TGC, tigecycline; TIC, ticarcillin; and SXT, trimethoprim–sulfamethoxazole.

are present in all sample types. Although this typing method is limited and focus on only one gene (Hoffmann and Roggenkamp, 2003; Wu et al., 2020), it indicates a high degree of diversity of this bacterial complex which is present in a wide variety of compartments.

The *E. hormaechei* metacluster predominated especially with C-VI (51/313, 16.3%) and C-VIII (87/313, 27.8%), which were also the most frequent clusters in human infections (27.1 and 29.0%, respectively). Surprisingly, C-III was rare in Guadeloupe, with only one strain in a human sample, whereas this *E. hormaechei* subspecies is one of the most frequently reported in clinical studies (Hoffmann and Roggenkamp, 2003; Kremer and Hoffmann, 2012; Garinet et al., 2018). In contrast, we notified the high prevalence of the *hsp60* UD4 cluster in our set of clinical isolates which should referred to ECC clade L (Sutton et al., 2018), and a possible new successful ESBL-producing lineage.

Among other important clusters, *E. bugandensis* (C-IX) accounted for 10 of the 107 clinical strains, and 22 isolates were recovered from non-clinical samples. This species was found mainly in fresh produce (9/22) and reptile feces (7/22). It has been reported in wild fauna, livestock, and the environment in only a few studies (Khanna et al., 2013; Singh et al., 2018; Matteoli et al., 2019; Wu et al., 2020). *E. bugandensis* has been described as the most virulent and pathogenic of all ECC strains (Doijad et al., 2016; Pati et al., 2018), which raises concern, as fresh produce may be the origin of dissemination of virulent pathogens in the community, as for some *Escherichia coli* lineages (Luna-Guevara et al., 2019).

As C-VI and -VIII were the most prevalent *hsp60* clusters in this collection, the WGS analysis was conducted to investigate their potential clone diffusion among human, animal, and environmental samples. Among C-VI, *E. hormaechei* subsp. *xiangfangensis* was overrepresented in comparison to the other related subspecies *E. hormaechei* subsp. *oharae* (3/45) as reported in previous studies (Peirano et al., 2018; Sutton et al., 2018). Core-genome analysis showed that clonal spread at hospital level was limited to a few genetic backgrounds which were previously found in human infections (i.e., ST113 and ST114; Peirano et al., 2018; Siebor et al., 2019). In accordance with our observations, they have rarely been observed in samples of other origins, except from companion and wild animals (Haenni et al., 2016; Harada et al., 2017; Goldberg et al., 2019). Different sample types shared a few lineages, one being ST90 (n = 5), which is also involved in human and animals infections (Peirano et al., 2018; Zhu et al., 2020). Moreover, two ST90 strains isolated from an anole and a raw water sample harbored genes encoding for the yersiniabactin system. The presence of this highly pathogenic island has previously been described in other *Enterobacteriaceae* species and was associated with a specific *E. hormaechei* clone that caused a hospital outbreak in Netherlands (Paauw et al., 2009). Another ST previously found in a clinical sample (ST98) was present in both a human collection and fresh produce (Izdebski et al., 2015). ST344 clones were found in three fresh products but from two different market stands, suggesting manual strain circulation.

This study also showed a high frequency of 3GC-R ECC members in non-human samples such as livestock and fresh local produce, and confirmed previous observations on the local anole population (Guyomard-Rabenirina, 2016). Three hypotheses have been proposed to explain this high prevalence of CoP ECC strains in these compartments. The first one was a human origin of these resistant strains, with the spread of successful lineages among compartments; however, *hsp*60 clusters distribution within each sample type and the WGS analysis of C-VI and -VIII, indicated a wide diversity, which was not in favor of exchanges. Although some whole-genomes sequenced strains isolated from humans, lizards, and other origins were genetically close, most of them were grouped separately.

The second hypothesis was an impact of human activities, which could exert selective pressure for resistant ECC strains, as few international ST were found in non-clinical isolates (ST90, ST98; Izdebski et al., 2015; Peirano et al., 2018). No significant difference was found between *Anolis* individuals sampled near or far from areas of human activity, which is congruent with the results of a study on 3GC-R *E. coli* carriage in lizard in Guadeloupe (Guyomard-Rabenirina et al., 2020). To go further in a recent survey, *Anolis* (n = 20) and other wild animals (n = 67; rat, bird, toad, and cockroach) living near the hospital sewers and at the associated wastewater treatment plant were sampled. Among them, 21.8% (19/87) carried 3GC-R ECC including one *Anolis*. These strains were exclusively ESBL-producers, and WGS analysis indicated the presence of a ST114 lineage closely related to human samples and the dissemination of an IncHI2/$bla_{\mathrm{CTX-M-15}}$ plasmid. Taken together, these results suggested that only specific polluted environments associated with an important selective pressure are in favor of a large dissemination and maintenance of human related resistant strains in the wild fauna compartment (Pot et al., 2021). This second hypothesis is less well supported for fruits and vegetables, as the origin of the resistant clones in such samples could be multifactorial (Hölzel et al., 2018). Overall, the prevalence of 3GC-R clones in fresh produce was higher than in a previous larger collection, but only for CoP clones (van Hoek et al., 2015), in contrast to other published reports of ESBL producers (van Hoek et al., 2015; Hölzel et al., 2018). Our results suggest that organic fertilization is associated with a higher load of ECC strains in fresh produce but is not correlated with higher counts of CoP ECC, as described previously (Marti et al., 2013).

As it has been suggested that ECC members have higher mutation rates due to derepression than other genera with constitutive *ampC* (Kohlmann et al., 2018), we explored a third hypothesis, that non-human strains have a greater ability to acquire specific mutations in genes encoding for AmpC. This hypothesis was rapidly excluded by analysis of 11 WT/CoP pairs, in which most mutations were shared by strains from the different compartments and especially mutations in *ampD*, which has been suggested to be the leading mechanism in ECC with CoP in human samples (Guérin et al., 2015).

Our results increase the understanding of reservoirs definition and sources of ECC infections in a tropical setting. Several limitations should be pointed in this study. First, human

isolates were recovered from only infectious sites and were mostly specified to be hospital-acquired. Nevertheless, since ECC members are opportunistic pathogens and belong to *Enterobacteriaceae*, most of infections came from endogenous colonizing strains (Gorrie et al., 2018). As we used selective medium to facilitate resistance detection, it could led to the overrepresentation of 3GC-R ECC in non-human isolates. This selection bias was limited by using ceftriaxone as selective antibiotic, described as a weak CoP inducer (Mizrahi et al., 2020). Moreover, previous authors indicated a relatively low rate of derepressed mutant in ECC population (3×10^{-8}; Kohlmann et al., 2018), although we did not estimate the mutation rate in our non-human samples due to the presence of various cultivable genus and species in agar plates.

CONCLUSION

Our findings highlight the widely diverse distribution of ECC members in non-human and human samples. We found a high prevalence of 3GC-R ECC in non-human samples, due exclusively to CoP. None of our hypotheses could explain this prevalence, and higher mutation rate is not excluded. These results suggest that this characteristic confers a selective advantage for these strains. Unknown persistent environmental factors, which should be further explored, may favor such overproduction.

DATA AVAILABILITY STATEMENT

The 313 partial *hsp60* sequences are available in **Supplementary Table 2** with strain details, while the whole-genome sequences of C-VI, clade A (n = 42) and C-VIII (n = 86) are deposited in GenBank under BioSample accession numbers SAMN15680734 to SAMN15680861 (**Supplementary Table 3**).

ETHICS STATEMENT

The parts of this project involving human participants were reviewed and approved by Commission de recherche éthique, Direction de la recherche et de l'innovation, CHU de la Guadeloupe (A5_19_12_05_TRAMID). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements. The animal study was reviewed and approved by the Committee for Ethics in animal experiments of the French West Indies and Guyana (reference 971-2016-12-20-001). Written informed consent for participation was not obtained from the owners because feces were collected from livestock animals at the slaughterhouse, just before slaughter. No data concerning farm was avalaible. Only the municipality origin of screened animals batchs was recorded.

AUTHOR CONTRIBUTIONS

MP, SG-R, and AT conceived and designed the study. MP, SB, CD, SF, GG, SG-R, EM, and AT collected biological samples, isolates, and epidemiological data. MP, DC, FGu, SG-R, FGr, AT, and YR analyzed the data. MP, SB, DC, SG-R, and AT wrote the manuscript. All the authors critically revised and approved the final version of 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.2021.628058/full#supplementary-material

Supplementary Figure 1 | Maximum likelihood phylogenetic tree based on the partial sequence *hsp60* gene of *E. cloacae* complex (ECC) isolates recovered in Guadeloupe (n = 313). Maximum likelihood phylogenetic reconstructions were performed with RAxML software (100 bootstrap replicates), and the tree rooted with *Klebsiella aerogenes hsp60* sequence (AB008141.1) was drawn with iTOL. All the sequences used as reference in the tree have a corresponding GenBank accession number, and only sequences extracted from whole-genome of ECC strains were labeled with current names. Each *hsp60* cluster is identified by a specific color, and bootstrap values $\geq$ 60 are indicated. *Hsp60* sequences from C-X, which correspond to *Lelliottia nimipressuralis* (formerly *Enterobacter nimipressuralis*) are inserted (AJ567887.1, AJ567900.1). For better readability, C-IV nodes are grouped. The hosts of each local strain are specified by a circle (human), a square (livestock), a star (fresh produce), a right-pointing triangle (water), or a left-pointing triangle (*Anolis*). Phenotypes are indicated by a white form for wild-type against beta-lactam antibiotics, and a black form for third-generation cephalosporin-resistant strains. The global distribution is provided in **Table 3**, and all *hsp60* partial sequences are available in **Supplementary Table 2**. List of ECC partial *hps60* sequences included in this figure (associated cluster: C or UD, and accession number): **C-I**: AJ417140.1, AJ567846.1, AJ567893.1, FXLQ00000000; **C-II**: AJ567849.1, AJ567862.1, AJ567886.1, AJ567888.1, AJ567899.1, CP017181.1, **C-III**: AJ543781.1, AJ543789.1, AJ567871.1, AJ567872.1, AJ567877.1, AJ567880.1, CP017186.1; **C-IV**: AJ543784.1, AJ543806.1, AJ543807.1, AJ543867.1, AJ543877.1, AJ543889.1, AJ543893.1, CP017184.1; **C-V**: AJ417114.1, AJ862859.1, AJ862861.1, AJ862862.1, AJ862863.1, CP017279.1; **C-VI**: AJ543778.1, AJ543782.1, AJ567878.1, AJ866507.1, CP017180.1, CP017183.1; **C-VII**: AJ417108.1,

AJ862866.1, AJ862867.1, AJ866491.1, CAI28773.1, MKEQ00000000.1; **C-VIII**: AJ543821.1, AJ543849.1, AJ543857.1, AJ543908.1, AJ567884.1, AJ567889.1, AJ567890.1, AJ567892.1, CAD66305, CAI28810.1, CP017179.1; **C-IX**: AJ543819.1, AJ543820.1, AJ543878.1, AJ543881.1, CAD66281.1, CAD66282.1, LT992502; **C-XI**: AJ417139.1, AJ417142.1, AJ543768.1, AJ543855.1, NC014121.1; **C-XII**: AJ417143.1, AJ543817.1, AJ543847.1, AJ862872.1, CP003678.1; **C-xiii**: AJ417128.1, AJ543837.1, AJ543870.1, AJ543872.1, CAD66299.1, CAD66332.1, CAD66334.1, CAD99100.1, LVUF00000000, FYBB00000000, SJOO00000000, AEXB00000000, QZCT01000000, FYBA00000000; **C-XIV**: ERS2281247, ERS2281248, LXPT00000000.1, LZEN00000000, NSIZ00000000.1, SJON00000000.1; **UD1**: JDWG00000000.1, JDWH00000000.1, JUZJ00000000.1, JUZQ00000000; **UD2**: BBUQ00000000.1, CP021851.1, JZYX00000000.1, NPNR00000000; **UD3**: AZXZ00000000.1, FCNO00000000.1, JACW00000000.1, JWAU00000000; **UD4**: CP043318.1, JVIL00000000.1, JZKT00000000.1, LEDN00000000; **UD5**: AP019007.1; and **UD6**: MTKD00000000, QZCS00000000.

Supplementary Table 1 | Details of non-clinical samples: sampling date, location, type of environment, farm characteristics.

Supplementary Table 2 | Details of strains: *hsp60* partial sequence and results for antibiotic resistance. Resistance profiles were obtained against 16 antibiotics. Intermediate or resistant results are labeled "R"; "s" corresponds to a susceptible phenotype.

Supplementary Table 3 | Details of genetic content of sequenced *E. hormaechei* subsp. *xiangfangensis* (C-VI – clade A; $n = 42$) and *E. hormaechei* subsp. *steigerwaltii* (C-VIII – clade B; $n = 86$).

Supplementary Table 4 | Cephalosporinase gene complex mutation. *Clone numbers refer to letters used in **Figures 1**, **2** and correspond to two strains from the same sample with different resistance profile against beta-lactam antibiotics: wild-type or cephalosporinase overproduction. Each strain is considered to be a clone that differs by < 45 single nucleotide polymorphisms (SNPs). na: not attributed. The abbreviation "N/S" after the gene names indicates the number of non-synonymous mutations (N) versus the number of synonymous mutations (S) in comparison to the reference data. Please note that only the different non-synonymous mutations between strains from the same sample have been conserved in the observation column.

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Isolation of Ciprofloxacin and Ceftazidime-Resistant Enterobacterales From Vegetables and River Water Is Strongly Associated With the Season and the Sample Type

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The dissemination of antibiotic-resistant bacteria (ARB) from water used for crop irrigation to vegetables is poorly studied. During a year, five farmer markets in a city in Central Chile were visited, and 478 vegetable samples (parsleys, corianders, celeries, lettuces, chards, and beets) were collected. Simultaneously, 32 water samples were collected from two rivers which are used to irrigate the vegetables produced in the area. Resistant Enterobacterales were isolated and identified. Colistin resistance gene *mcr-1* and extended spectrum β-lactamases (ESBL) were molecularly detected. The association of environmental factors was evaluated, with the outcomes being the presence of Enterobacterales resistant to four antibiotic families and the presence of multidrug resistance (MDR) phenotypes. Parsley, coriander, and celery showed the highest prevalence of resistant Enterobacterales (41.9% for ciprofloxacin and 18.5% for ceftazidime). A total of 155 isolates were obtained, including *Escherichia coli* ($n = 109$), *Citrobacter* sp. ($n = 20$), *Enterobacter cloacae* complex ($n = 8$), *Klebsiella pneumoniae* ($n = 8$), and *Klebsiella aerogenes* ($n = 1$). Resistance to ampicillin (63.2%) and ciprofloxacin (74.2%) was most frequently found; 34.5% of the isolates showed resistance to third-generation cephalosporins, and the MDR phenotype represented 51.6% of the isolates. In two *E. coli* isolates (1.29%), the gene *mcr-1* was found and ESBL genes were found in 23/62 isolates (37%), with $bla_{\text{CTX-M}}$ being the most frequently found in 20 isolates (32%). Resistant Enterobacterales isolated during the rainy

season were less likely to be MDR as compared to the dry season. Understanding environmental associations represent the first step toward an improved understanding of the public health impact of ARB in vegetables and water.

Keywords: multidrug resistance, vegetable, river water, Chile, environmental risk factors, Enterobacterales

INTRODUCTION

Antimicrobial resistance (AMR) is one of the most pressing global public health concerns and has been prioritized by the WHO, World Health Organization (2019). The AMR crisis is directly related to the overuse and misuse of antibiotics in multiple activities, including human health, animal production, and agriculture (Manage and Liyanage, 2019). Factors like environmental pollution and livestock production practices likely accelerate the dissemination of antibiotic-resistant bacteria (ARB) and antimicrobial resistance genes (ARGs) in the environment (e.g., water and soil), facilitating food contamination (Hernando-Amado et al., 2019). Understanding the specific role of each of these factors in the dispersion of ARB and/or ARGs is critical to tackle the fast increase of AMR (Hernando-Amado et al., 2019).

Extended spectrum β-lactamase (ESBL)-producing Enterobacterales, such as *Escherichia coli* and *Klebsiella pneumoniae*, are among the top AMR threats (Rajendran et al., 2019). Indeed, the WHO ranked ESBL-producing and carbapenem-resistant Enterobacterales within the critically important pathogens, against which novel strategies are urgently needed (WHO, World Health Organization, 2019). Infections due to ESBL-producing Enterobacterales result in 197,400 hospitalizations and 9,100 deaths per year only in the United States (CDC, Centers for Disease Control and Prevention, 2019). In addition, these pathogens have also been reported in numerous animals, including livestock (poultry and cattle) and companion animals (cats and dogs) (Saliu et al., 2017; Melo et al., 2018; Dantas Palmeira and Ferreira, 2020). In the same way, ESBL-producing Enterobacterales have been found in food (Ye et al., 2018) and water sources (Tanner et al., 2019), highlighting their dissemination into the environment.

In terms of food safety, most of the interest in AMR has focused on recognized foodborne pathogens (e.g., *Salmonella* spp. and *Campylobacter* spp.). Not only have these shown a considerable rise in the overall levels of AMR, but also there are increasing reports of emerging resistance to third-generation cephalosporins (3GC) and other critical antibiotics (e.g., colistin and carbapenems) (Lima et al., 2019). Less attention has been given to the presence of clinically relevant ARB in food, whereas current evidence suggests that their presence could be widespread in food products (Hudson et al., 2017). For instance, studies conducted on leafy greens and vegetables have described the presence of multidrug resistance (MDR) Enterobacterales (Zekar et al., 2017; Hölzel et al., 2018; Sapkota et al., 2019; Oh et al., 2020). Similarly, Saksena et al. (2020) described the presence of resistant Enterobacterales, including *Klebsiella* spp., *Escherichia coli*, *Citrobacter* spp., and *Enterobacter* spp., in a wide range of fruits and vegetables (e.g., apples, tomatoes, and cucumbers, among others) (Saksena et al., 2020). Importantly, previous studies have shown that most of the ARB recovered from vegetables presented resistance to 3GC. In addition, another study reported the presence of fluoroquinolone-resistant *E. coli* in cabbage, lettuce, and spinach in South Africa (Jongman and Korsten, 2016).

Contaminated water used to irrigate crops is one of the main contributors to vegetable contamination with ARB and ARGs (Iwu and Okoh, 2019). This phenomenon has been attributed not only to surface water but also to water wells and groundwater, which have been shown to be capable of transmitting ARB to vegetables and animals (Wu et al., 2016; Guo et al., 2019). Antimicrobial-resistant Enterobacterales have been reported in surface water, with MDR *E. coli* being the most common bacteria detected in a previous study conducted on the Caribbean island of Guadeloupe, where areas with a low population density showed lower numbers of resistant *E. coli* in comparison with highly populated urban areas (Guyomard-Rabenirina et al., 2017). While several factors could influence water contamination with ARB, including climate, land usage, and urbanization (Yuan et al., 2019; Weller et al., 2020), in general, the main sources of contamination are feces from humans and animals, including run-off from farms (feed-lots or dairy), manure used as fertilizer, and waste from water treatment plants (Liu et al., 2013; Luna-Guevara et al., 2019). Importantly, in numerous developing countries, rural areas lack drinking water and proper sewage disposal. Consequently, human waste material accumulates in surface water (rivers) (Ferronato and Torretta, 2019). In these circumstances, the use of surface water to irrigate crops is particularly relevant as a potential source of ARB dissemination, particularly in vegetables that are consumed raw or without proper cooking.

While previous studies have highlighted the potential risk to humans of water and vegetables contaminated with ARB, little is currently known about the environmental factors associated with the presence of ARB in water and vegetable sources. Hence, this study aims to determine the presence of clinically relevant antibiotic-resistant Enterobacterales in vegetables and surface water in an agricultural town in central Chile, and their association with environmental factors like season: dry and rainy; source: vegetable type and river water; ambient temperature; produce state (fresh, partly rotten, or completely rotten); presence of insects in farmer market sampled; produce stored at ground level; rain event 5 days before sample collection; pest control present in farmer market; seller wearing gloves when manipulating vegetables; vegetables stored separately by type; and pets present in farmer market.

MATERIALS AND METHODS

Study Location

This study was carried out in Molina, a city of approximately 45,000 inhabitants in central Chile (**Figure 1**). Molina is located in a region that comprises 17.2% of the national surface for agricultural and livestock production, from which the main activities are the production of crops, such as cereals, fruit trees, and vineyards (ODEPA, Oficina de Estudios y Políticas Agrarias, 2018). There are 761,981.2 hectares of land used for crop activities (ODEPA, Oficina de Estudios y Políticas Agrarias, 2018), mainly apples, cherries, and kiwis (ODEPA, Oficina de Estudios y Políticas Agrarias, 2018). Also, there are numerous small and medium-sized farms with backyard flocks and other animals, and small- and medium-scale production of vegetables (e.g., lettuce and spinach). Irrigation mainly derives from surface water obtained from two rivers (Lontue and Claro rivers) running parallel to the northern and southern borders of Molina (**Figure 1A**). The most frequently used irrigation system by small and medium producers is primary and secondary open furrows, using untreated river water distributed by different canal systems. The study area contains one main wastewater treatment plant (WWTP) that discharges the treated effluent into the Carretones Creek which discharges into the river beyond the sampling sites and area evaluated; therefore, this creek was not sampled. The region has a Mediterranean climate, with four seasons, characterized by rainy winters and falls, and dry springs and summers.

Vegetable Samplings

To determine the prevalence of ARB in vegetables harvested and commercialized in the city of Molina, we selected the following vegetables: parsleys, lettuces, beets, chards, celeries, and corianders. This selection fulfilled the following criteria: (i) year-round cultivation in the region, (ii) raw consumption, and (iii) cultivated at ground level. These vegetables were locally grown by small, not industrialized farmers, and had a variety of distribution channels, from collection in larger markets and resale to direct sale in small farmer market. In this location, traceability of sold vegetables is either minimal or absent. Five farmer market selling locally harvested vegetables were selected (**Figure 1B** and **Supplementary Figure S1**). Four sampling efforts were performed between May 2019 and January 2020. Samplings were collected in May (fall), July (winter), both represented the rainy season, November (spring) and January (summer), both represented the dry season. A total of 478 vegetable samples were collected, including parsleys ($n = 103$),

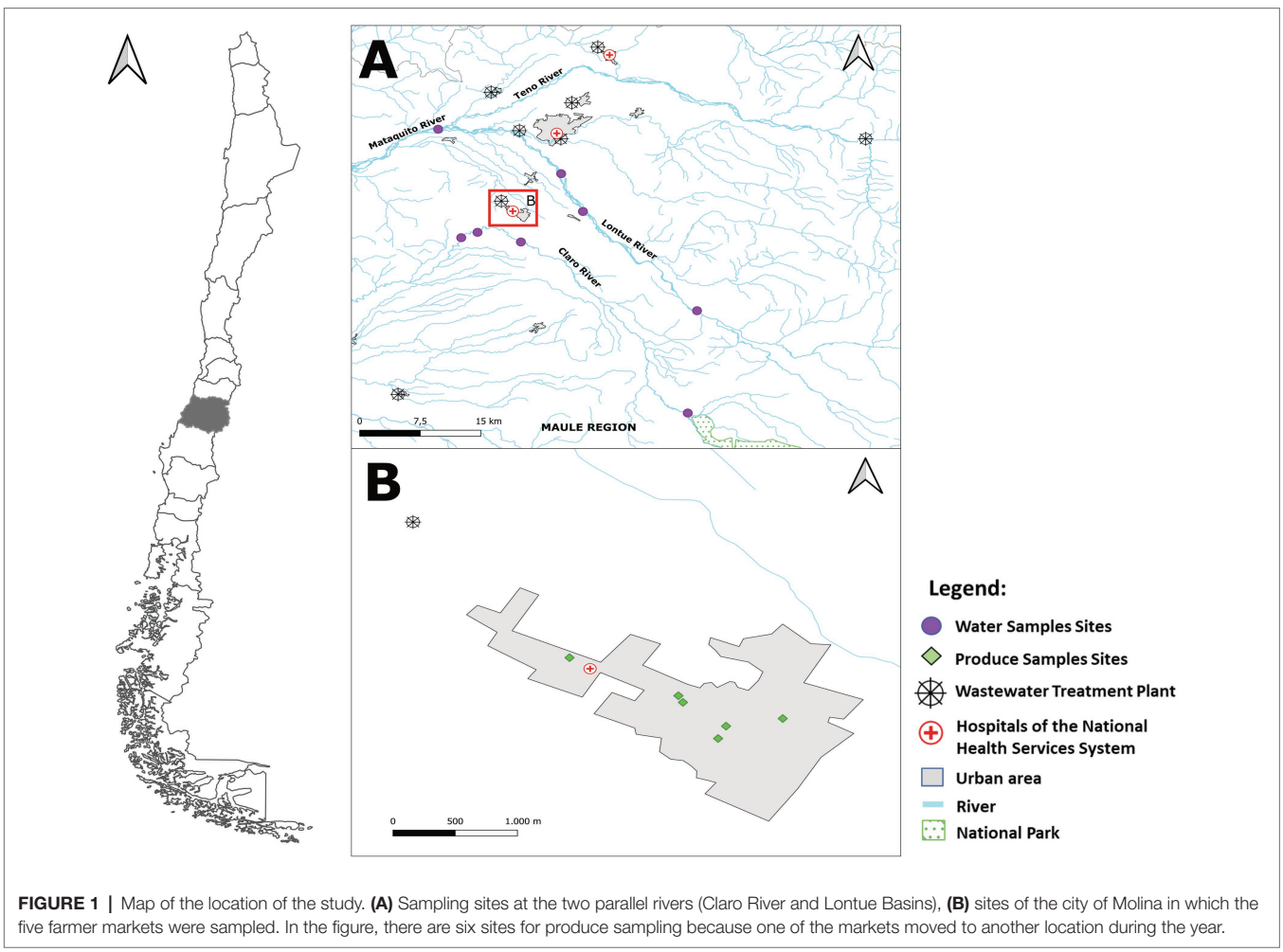

FIGURE 1 | Map of the location of the study. **(A)** Sampling sites at the two parallel rivers (Claro River and Lontue Basins), **(B)** sites of the city of Molina in which the five farmer markets were sampled. In the figure, there are six sites for produce sampling because one of the markets moved to another location during the year.

lettuces ($n = 132$), beets ($n = 114$), chards ($n = 108$), celeries ($n = 6$), and corianders ($n = 15$). In the absence of a given vegetable, these were replaced by a similar one (e.g., parsley was replaced by coriander). Vegetables were collected on the same market in each sampling; samples were maintained in sterile zip-locked plastic bags and transported aseptically to the Laboratory at Universidad Andres Bello. They were kept at 4°C and processed within 48 h.

The following environmental factors were registered by samplers during each sampling effort: (i) ambient temperature (°C), (ii) produce state (fresh, partly rotten, or completely rotten), (iii) presence of insects in farmer market sampled (yes/no), (iv) produce stored at ground level (yes/no), (v) rain event 5 days before sample collection (yes/no), (vi) pest control presented in farmer market, (vii) seller wearing gloves when manipulating vegetables, (viii) vegetables stored separately by type (yes/no), and (ix) pets present in farmer market (yes/no).

Water Sampling and Processing

Water sampling was conducted in the course of the Claro and Lontue rivers, both of which originate in the Andes Mountains. The Lontue basin is composed of three river branches (Lontue, Mataquito, and Teno) and the Claro river flows, but flow into the Maule river. All of them finally flow into the Pacific Ocean (**Figure 1A**).

The Lontue and Claro rivers fulfilled the following characteristics: (i) run close to the location where vegetables were collected and produced; (ii) used as irrigation for agricultural purposes; (iii) have perennial flow; and (iv) provide secure access for researchers on foot and/or motor vehicle to collect samples. We sampled these rivers as their water is used to irrigate the area where the vegetables are produced. Sampling points corresponded to areas where each river crossed communes with population densities between 30.28 and 111.88 habs/km^2 (INE, Instituto Nacional de Estadísticas, 2017), including small populated areas with a few households and a middle-sized city. All sampling sites were georeferenced (**Figure 1A**).

A total of 32 water samples were collected from four sampling sites per river every 3 months between May 2019 and January 2020, likewise vegetable samples, two visits represented the dry season, and two visits represented the rainy season. The first sampling site was located in the highest level at which water samples could be collected in the Andes Mountains to determine the original state of the river. The second site was located before the city where vegetables were collected for this study, while the third and fourth sampling sites were located after the city. As the farms of origin of the vegetables collected in the farmers market are scattered along the entire area, we selected different sites along the river's course to evaluate the water quality used for irrigation. Experimentally, 10 liters of river water were collected using the Modified Moore Swab water filtration method (Sbodio et al., 2013). Briefly, this filtration system incorporates a filter gauze into a cassette to retain bacteria from the water sample as it is passed through using a portable peristaltic pump. All cassettes were kept in individual sterile zip-locked plastic bags, stored at 4°C, and transported aseptically to the Laboratory at Universidad Andres Bello, in Santiago, for processing.

The physical parameters of the river water—pH, water temperature (°C), conductivity (μs), salinity (ppm), and total dissolved solvents (ppm)—were measured and recorded *in situ* at each site using the Yalitech AM 006 Waterproof Multiparameter Meter Combo 6. Also, environmental factors from each visit were registered as: weather conditions (sunny, rainy, partly cloudy, and cloudy), rain event 5 days prior to sample collection (yes/no), ambient temperature (continuous variable °C), presence of visible feces (yes/no), visible presence of domestic animals (yes/no), presence of garbage in sampling site (yes/no), observation of crops nearby sampling site (yes/no), and presence of aquatic plants in sampling sites (yes/no). These variables were further analyzed as described below.

Bacterial Isolation and Identification

A total of 25 *g* of each vegetable were obtained with a sterile scalpel and placed in bags containing 225 ml of buffered peptone water (BD, Franklin Lakes, NJ, United States). Samples were homogenized in a stomacher (IUL Instruments, Spain) for 1 minute and then incubated at 37°C for 18–24 h. The same microbiological procedure was followed for water samples after removing the full gauzes from the cassettes and placed them in peptone water. After incubation, two plates with MacConkey agar medium (BD) were inoculated with 100 μl from each stomacher bag; one plate was supplemented with 2 μg/ml of ceftazidime (CAZ; Sigma, Germany) and the other with 2 μg/ml of ciprofloxacin (CIP; Sigma, Germany). In all experiments, *K. pneumoniae* SCL 2346 (CAZ MIC >16 μg/ml and CIP MIC >2 μg/ml) and *E. coli* ATCC 25922 were used as resistant and susceptible controls, respectively. All plates were incubated at 37°C for 18–24 h. Colonies were selected according to morphology, using a magnifying glass. This selection was made by classifying the colonies according to standard patterns: colony shape, color (pigmentation), texture, and edge shape, as described previously (Higuera-Llantén et al., 2018). Distinct morphotypes phenotypically consistent with Enterobacterales were further identified by MALDI-TOF (Bruker Daltonics, Germany).

Antimicrobial Susceptibility Testing

All isolates confirmed as Enterobacterales were tested against a panel of 15 antibiotics using the disk diffusion method following CLSI guidelines (CLSI, Clinical and Laboratory Standards Institute, 2018). Antibiotic tested included as ampicillin (AMP, 10 μg); cefazolin (CFZ, 30 μg); ceftazidime (CAZ, 30 μg); ceftriaxone (CRO, 30 μg); cefepime (FEP, 30 μg); ertapenem (ETP, 10 μg); imipenem (IPM, 10 μg); meropenem (MEM, 10 μg); ampicillin/sulbactam (SAM, 10/10 μg); piperacillin/tazobactam (TZP, 100/10 μg); ciprofloxacin (CIP, 5 μg); amikacin (AMK, 30 μg); gentamicin (GEN, 10 μg); fosfomycin/trometamol (FOF, 200 μg); and trimethoprim/sulfamethoxazole (SXT, 1.25/23.75 μg), all of which were

supplied by OXOID (Hampshire, England). Bacterial isolates resistant to three or more antimicrobial classes were cataloged as MDR following previously standardized criteria (Magiorakos et al., 2012). Intrinsic resistance was not considered for susceptibility analysis in *Klebsiella aerogenes* (CFZ and SAM), *Citrobacter freundii* (AMP, CFZ, and SAM), and *Enterobacter cloacae* (CFZ). Importantly, all *Citrobacter* spp. were analyzed as members of the *C. freundii* complex due to the impossibility of identification down to the species level using the MALDI-TOF technique (Kolínská et al., 2015). Isolates classified as intermediate were considered resistant bacteria for the purpose of this study.

Molecular Detection of Extended Spectrum β-lactamases and *mcr-1*

Isolates exhibiting resistance to third- or fourth-generation cephalosporins (CAZ, CRO, and FEP) or carbapenems (ETP and IMP) were tested to detect the presence of genes encoding ESBLs (bla_{TEM}, bla_{SHV}, and bla_{CTX-M}) by a multiplex PCR scheme as previously reported (Salgado-Caxito et al., 2021).

To test a possible resistance to colistin in all isolates, a PCR to detect the *mcr-1* gene was performed. Primers used were previously reported (Rebelo et al., 2018). Briefly, running conditions were as: 1 cycle of denaturation at 95°C for 15 min, followed by 10 cycles of extension: 30 s at 95°C, 30 s at 58°C, and 1 min at 72°C. The final cycle of elongation was performed for 5 min at 72°C. The amplification was visualized by electrophoresis using 2% agarose. *E. coli* isolate SCL1290 (mcr-1 positive) was used as the positive control.

Statistical Analysis

Antimicrobial resistance profiles were compared among sources by a clustering and a heat map. These represented each isolate resistance/no resistance to previously described antibiotics. Analysis was conducted using Heat-map.plus in R (version 4.0.0). The heat map also included the antibiotic used for isolation, season of isolation collection, bacterial species, source of isolation, and MDR profile (yes/no).

We used generalized linear models (GLM) to explore possible associations between the environmental variables registered during each sampling effort and isolation of MDR Enterobacterales, evaluating each matrix separately. The multivariate statistical model for vegetable samples included all explanatory variables and was selected as the best model based on the Akaike information criterion (AIC = 142.11) compared to models including only one variable (AIC > 216.49). The statistical significance ($p < 0.05$) of each variable in the full model was assessed using Wald's test. As an insufficient number of Enterobacterales was isolated from water samples, GLM could not be conducted. Instead, boxplot (R) or histograms (Excel) were constructed to display the distribution of the data based on isolation of MDR Enterobacterales considering different environmental factors. Some environmental factors were not recorded during the first vegetable sampling (produce condition, wearing gloves while manipulating vegetables, presence of pets, and presence of pest control devices), these were not included in the statistical analysis. Drinking water was available and used in all the farmer markets studied, as well as produce separation by type, therefore, these variables were also not included in the GLM analysis.

It was evaluated the association between each Enterobacterales isolate presenting MDR as defined above (yes/no), with two independent categorical variables (source of the sample and season) that were present in both the vegetable and river dataset: (i) source in which the sample was collected, including "river," "beet," "celery," chard," and "parsley", except for "coriander" that had to be removed from the analysis for having only one sample in which MDR Enterobacterales was isolated, which meant the analysis could not be run; (ii) season in which the sample was collected, with "rainy" including winter and fall and "dry" including spring and summer; and (iii) the variable "CIP/CAZ selection" corresponding to the antibiotic used as a supplement in the MacConkey agar to isolate clinically relevant Enterobacterales (CIP/CAZ). A similar analysis was conducted categorizing each Enterobacterales isolate resistant to antibiotics of different families: (i) resistant to 3GC (CAZ and/or CRO); (ii) resistant to penicillin with β-lactamase inhibitor (RPIB) antibiotics (SAM and/or TZP); (iii) resistant to the quinolone CIP (RCIP); and (iv) resistant to β-lactam (RBET) antibiotics (AMP, ETP, IPM, MEM, CAZ, CRO, CFZ, FEP, SAM, and/or TZP). However, in these models, the variable "CIP/CAZ selection" was not included. Resistance (yes/no) was characterized as described above based on the CLSI standards.

The binary nature of the response variable (yes/no) required the use of generalized linear models (GLM) with binomial errors (such as logistic regression), including the two variables (source and season) listed above. Analyses of the generalized linear model were performed using the R software[1] (version 4.0.0) The multivariate statistical model included all explanatory variables and was selected as the best model based on the Akaike information criterion (AIC = 189.16 for the MDR outcome, AIC = 214.51 for the 3GC outcome, AIC = 177.77 for the RPIB outcome, AIC = 171 for the RCIP outcome, and AIC = 190.07 for the RBET outcome) compared to models including only one variable (AIC > 216.49). The statistical significance ($p < 0.05$) of each variable in the full model was assessed using Wald's test.

GLM could not be performed for the models to evaluate the association between each Enterobacterales isolates selected using CIP and CAZ resistant to: (i) at least one antibiotic, regardless of their family, as all the isolates were resistant to at least one of the antibiotics evaluated in the study; (ii) carbapenems (ETP, IPM, and/or MEM); and (iii) aminoglycosides (AMK and/or GEN). The GLM for the carbapenem and aminoglycoside outcomes could not be performed because all the Enterobacterales isolates recovered from beets ($n = 9$) were susceptible to carbapenems and aminoglycoside antibiotics as per the CLSI standards.

[1]http://www.R-project.org

RESULTS

Prevalence of Resistant Enterobacterales in Vegetables

CAZ- and CIP-resistant Enterobacterales were obtained from vegetables during the four samplings. Isolates showed an overall prevalence of 8.8% (42/478) and 17.5% (84/478) for those selected with CAZ or CIP, respectively. For CAZ resistance, the highest prevalence of 35.7% (10/28) and 30.6% (11/36) was found for samples from the *Apiaceae* family (i.e., parsley, coriander, and celery) in the spring and summer, respectively. This was followed by chard samples, which presented a prevalence of 27.6% (8/29) in the summer and 12.5% (3/24) in the spring. Beet and lettuce, also during the summer, showed the higher prevalence (**Table 1**). Interestingly, all 120 vegetable samples obtained during the winter were negative for CAZ-resistant Enterobacterales.

The highest prevalence of CIP-resistance was found in samples from *Apiaceae* during the fall [73.3% (22/30)], spring [50% (14/28)], and summer [44.4% (16/36)]. In chard samples, we observed a prevalence of 30% (9/30) for the fall, 8.3% (2/24) for the spring, and 27.6% (8/29) for the summer. Lettuce samples showed a prevalence of 10% (3/30), 8.3% (3/36), and 6.5% (2/31) for the fall, spring, and summer, respectively. Finally, beet samples were positive only during the fall [13.3% (4/30)] and the summer [4.2% (1/24)] (**Table 1**). Similar to CAZ-resistant Enterobacterales, none of the 120 vegetable samples obtained during the winter season were positive.

Prevalence of Resistant Enterobacterales Isolated From Water

For the prevalence of isolates obtained in CAZ contained plates, the highest prevalence of 37.5% (3/8) was observed in water samples during the fall (**Table 1**); however, in the water samples, none of the tested samples were positive for CAZ-resistant Enterobacterales during the other seasons. On the other hand, CIP-resistant Enterobacterales were observed in three seasons, with a prevalence of 37.5% (3/8) for the fall and summer and a prevalence of 25% (2/8) in the winter (**Table 1**).

Identification and Distribution of Antibiotic-Resistant Enterobacterales Species in Vegetables and Water

A total of 155 ARB was confirmed as Enterobacterales. The predominant species in vegetables and river samples was *E. coli*, with 70.3% (109/155) of the isolates, which were obtained from the *Apiaceae* family ($n=57$ strains), chard ($n=18$), water samples ($n=17$), lettuces ($n=10$), and beets ($n=7$; **Table 2**). Moreover, *Citrobacter* spp. was the second most common (18.7%, 29/155) and was found in the *Apiaceae* family ($n=17$), chards ($n=7$), beets ($n=2$), and lettuces ($n=2$); one isolate was recovered from a water sample. Nine isolates belonging to the genus *Klebsiella* spp. were found (5.8%, 9/155), corresponding to only two species: *K. pneumoniae* and *K. aerogenes*; while the first one was found in the *Apiaceae* family, and in chards and water, *K. aerogenes* was found only once in a parsley. Finally, *E. cloacae* complex (2.5%, 8/155) was detected in parsleys, a lettuce, and a chard (**Table 2**). In addition to the Enterobacterales order, other isolates were identified, including *Aeromonas* sp., which was recovered from vegetables collected in both rainy and dry seasons (data not shown).

Antimicrobial Resistance Profile in Isolates Recovered From Vegetables and Water

Considering the 155 bacterial isolates from vegetables and water, the susceptibility test showed that the highest resistance was to AMP and CIP, with 63.2% and 74.2%, respectively. Resistance to cephalosporins was observed in CFZ, which presented 38.5% of resistance, CAZ 34.8%, CRO 34.2%, and FEP 16.1%. The carbapenems ETP and IPM showed 13.5% and 1.9% of resistance, respectively, while no MEM resistant isolates were obtained during the study. For β-lactams + β-lactamase inhibitor, SAM presented 24.8% of resistance and TZP 8.4%. For aminoglycoside

TABLE 1 | Prevalence of Enterobacterales detected with ceftazidime and ciprofloxacin added plates on vegetable and river samples.

Season[1]	Percentage of positive sa ıples[2] (Number of positive amples/total samples) in a given matrix obtained on p' tes with CAZ and CIP									
	Parsley, Coriander, and Celery[3]		Lettuce		Beet		Chard		Water[4]	
	CAZ	CIP	CAZ	CIP	CAZ	CIP	CAZ	CIP	CAZ	CIP
Fall	6.7% (2/30)	73.3% (22/30)	0% (0/30)	10% (3/30)	0% (0/30)	13.3% (4/30)	0% (0/30)	30% (9/30)	37.5% (3/8)	37.5% (3/8)
Winter	0% (0/30)	0% (0/30)	0% (0/35)	0% (0/35)	0% (0/30)	0% (0/30)	0% (0/25)	0% (0/25)	0% (0/8)	25% (2/8)
Spring	35.7% (10/28)	50% (14/28)	2.8% (1/36)	8.3% (3/36)	3.3% (1/30)	0% (0/30)	12.5% (3/24)	8.3% (2/24)	0% (0/8)	0% (0/8)
Summer	30.6% (11/36)	44.4% (16/36)	9.7% (3/31)	6.5% (2/31)	12.5% (3/24)	4.2% (1/24)	27.6% (8/29)	27.6% (8/29)	0% (0/8)	37.5% (3/8)
Total	18.5% (23/124)	41.9% (52/124)	3% (4/132)	6.1% (8/132)	3.5% (4/114)	4.4% (5/114)	10.2% (11/108)	17.6% (19/108)	9.4% (3/32)	25% (8/32)

[1]*For all seasons, five farmer markets were sampled.*

[2]*Included samples with at least one isolate recovered per vegetable and water samples. Total of vegetable samples was 478 and total of river samples was 32.*

[3]*These three vegetables were grouped together since they belong to the same family of Apiaceae.*

[4]*Water samples included samples of Lontue and Claro rivers.*

CIP, ciprofloxacin; CAZ, ceftazidime.

TABLE 2 | Identification and distribution of Enterobacterales strains isolated from vegetable and river samples collected during four seasons during 2019–2020.

	Number of isolates obtained					
Species	**Parsley, Coriander, and Celery[1]**	**Lettuce**	**Beet**	**Chard**	**Water[3]**	**Total**
Escherichia coli	57[2]	10	7	18	17[b]	109
Citrobacter sp	17[2]	2	2	7	1	29
Klebsiella pneumoniae	2	0	0	5[2]	1	8
Klebsiella aerogenes	1	0	0	0	0	1
Enterobacter cloacae complex	6[2]	1	0	1	0	8
Total	83	13	9	31	19	155

[1]*These three vegetables were grouped together since they belong to the same family of Apiaceae.*
[2]*More than one isolate was obtained from the same sample.*
[3]*Water samples included samples of Lontue and Claro rivers.*

resistance, AMK and GEN showed 1.9% and 3.2%, respectively. For FOF, 2.6% of the strains presented resistance. Finally, for SXT, 41.9% showed resistance (**Supplementary Table S1**).

To analyze similarity among antibiotic resistance patterns and sources, a clustering was performed (**Figure 2**). Five main clusters were identified as: Cluster 1 grouped 21 CAZ-selected isolates [*Citrobacter* spp. ($n=20$) from parsley, chard, lettuce, beet, and celery and *K. aerogenes* ($n=1$) from parsley] (**Figure 2**). Nine isolates in cluster 1 presented an MDR phenotype. Cluster 2 grouped 18 isolates (three *E. coli* from water, six *E. coli* from parsley, beet, and lettuce, two *Citrobacter* spp. from parsley and water, and seven *K. pneumoniae* from parsley, chard, celery, and water), all selected in CAZ from vegetables collected in the fall, spring, and summer and in water samples collected during the fall. All isolates except two *K. pneumoniae* from chards presented an MDR profile. Cluster 3 grouped 26 MDR isolates (five *E. coli* from water, 13 *E. coli* from parsley, chard, and lettuce, and eight *E. cloacae* complex from parsley, chard, and lettuce) selected in CAZ and CIP from vegetables collected in three seasons (fall, spring, and summer) and from water collected in the fall and summer. Cluster 4 grouped 40 isolates (39 *E. coli* isolated from parsley, lettuce, chard, beet, celery, coriander, and water and one *K. pneumoniae* isolated from a chard), all selected in CIP and included isolates from vegetables collected in the fall, spring, and summer, along with isolates from water collected in the fall and summer. A total of 29 *E. coli* presented MDR. Finally, cluster 5 grouped 50 isolates (43 *E. coli* isolated from parsley, chard, beet, lettuce, celery, and water and seven *Citrobacter* spp. isolated from parsley and chard) that were selected and resistant only to CIP; these isolates were obtained from vegetables collected in the fall, spring, and summer, and from samples from water collected in the fall and winter. None of the isolates in cluster 5 presented MDR.

Presence of *mcr-1* and bla_{ESBL} Genes in Isolates From Vegetables and Water

The gene *mcr-1* was found in 2/155 isolates; both isolates corresponded to *E. coli* that were isolated during the summer season, from two distinct beet samples from the same market. These two *E. coli* isolates presented identical antibiotic resistance patterns (**Table 3**). Detection of ESBL genes was performed in 62/155 isolates that presented resistance to third-generation cephalosporins and/or carbapenems. ESBL genes were found in 23/62 isolates tested, representing seven isolates of *K. pneumoniae* (11.3%) and 16 isolates of *E. coli* (25.8%; **Table 3**). The most common ESBL gene detected was $bla_{CTX\text{-}M}$ found in *E. coli* ($n=15$) and *K. pneumoniae* ($n=5$), followed by bla_{SHV} found in *K. pneumoniae* ($n=5$). Finally, bla_{TEM} was detected in *E. coli* ($n=3$) and *K. pneumoniae* ($n=1$). Combinations of $bla_{CTX\text{-}M}$, bla_{SHV} and $bla_{CTX\text{-}M}$, bla_{TEM} were also found. Summer was the season with more isolates containing ESBL genes, with 10/23 isolates; no ESBL genes were detected in the isolates collected during the winter.

Descriptive Evaluation of Environmental Factors and MDR Enterobacterales Isolation in River Water Samples

The results of the descriptive statistics of isolation of MDR Enterobacterales in water samples based on continuous environmental factors registered when samples were collected are shown in **Supplementary Figure S2**. In water samples, 50% of the MDR Enterobacterales isolated in river water were recovered in water with the following characteristics: pH ranging between 6.1 and 7.7, temperature between 5°C and 13°C, conductivity between 100 µs and 250 µs, total dissolved solids between 20 and 160 ppm; salinity between 20 ppm and 120 ppm; and an ambient temperature between 1°C and 14°C (**Supplementary Figure S2**).

The descriptive statistics conducted for the categorical environmental factor (**Supplementary Figure S3**) show that MDR Enterobacterales were (i) more frequently isolated from the Claro river in the rainy season and (ii) slightly more frequent using CAZ instead of CIP as a supplement in the MacConkey agar, (iii) in sunny weather conditions, (iv) in sampling sites in which there was no observation of crops nearby, (v) in sites where a rain event occurred 5 days prior to the water sample collection, (vi) in sites where at least one animal was present when the sample was collected, and (vii) in sites where aquatic plants were present in the sampling point.

Association Between Environmental Factors and MDR Enterobacterales Isolation in Vegetable Samples

The descriptive statistics conducted for the categorical and continuous environmental factors (**Supplementary Figure S4**)

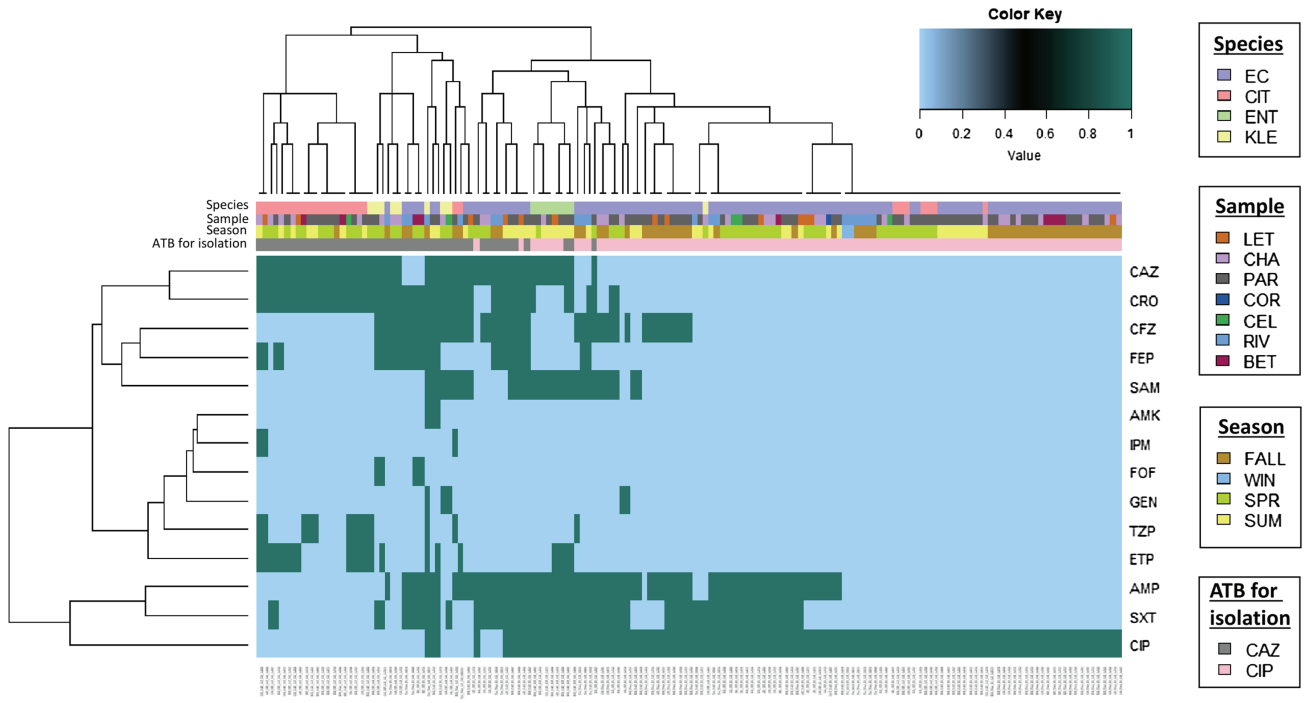

FIGURE 2 | Heat map representation of antimicrobial resistance (AMR) profiles. Cluster analysis of AMR profiles, in the vertical axis the abbreviations for the antimicrobial tested and in the horizontal axis are the isolates obtained in this study. Light blue represents sensitivity and dark green represents resistance. Five clusters were found and are labeled from 1 to 5 in the dendrogram.

show that MDR Enterobacterales detected in vegetables were (i) more frequently isolated from parsley and chard, (ii) during the dry season, (iii) in sites where a rain event did not occurred 5 days prior to the vegetable sample collection, (iv) in partly cloudy weather conditions, (v) slightly more frequent using CAZ instead of CIP as a supplement in the MacConkey agar, and (iv) in ambient temperature ranging from 18°C to 27°C approximately.

The generalized linear model presented in **Table 4** shows that Enterobacterales that grew in MacConkey agar supplemented with CAZ were statistically significantly associated with the isolation of MDR Enterobacterales in vegetable samples, with CAZ used as supplement having a 3.1 times higher likelihood of isolating MDR Enterobacterales compared to those that were isolated in plates supplemented with CIP (OR = 0,073; 95% CI: [1.217, 8.2623]; $p < 0.021$). All the other environmental factors (produce type, season, produce state, insect in farmers market, and produce at ground level) presented a non-significant association with MDR Enterobacterales in the GLM model (value of $p > 0.05$).

Association Between the Environmental Factors Season and Source of the Sample, and AMR Enterobacterales Isolation in Both Vegetable and Water Samples

The seasonality analyses shown in **Table 5** indicate that Enterobacterales isolates recovered from vegetable samples collected during the rainy season were significantly less likely to be resistant to at least one 3GC (OR = 0.08; 95% CI: [0.023–0.227]; $p < 0.00001$) compared to those recovered in samples collected in the dry season (**Table 5**). Similarly, Enterobacterales isolates recovered from samples collected during the rainy season were significantly less likely to be resistant to at least one penicillin combined with a β-lactamase inhibitor (OR = 0.18; 95% CI: [0.05–0.47]; $p < 0.0015$) and to at least one β-lactam antibiotic (OR = 0.22; 95% CI: [0.098–0.482]; $p < 0.0001$) compared to Enterobacterales isolates recovered from samples collected during the dry season (**Table 5**). In contrast, Enterobacterales previously isolated from samples collected during the rainy season were 5.12 times more likely to be resistant to CIP in the disk diffusion method than isolates recovered in samples collected during the rainy season (OR = 5.109; 95% CI: [1.069, 19.806]; $p = 0.0034$).

For the variable matrix, the statistical analyses showed that Enterobacterales isolated from chard and parsley were 0.2 times less likely to be resistant to at least one 3GC compared to the isolates obtained from river (OR = 0.203 [95% CI: 0.038, 0.930]; $p = 0.046$ and OR = 0.202 [95% CI: 0.043, 0.814]; $p < 0.029$, respectively; **Table 5**). Similarly, Enterobacterales isolated from parsley were 4.51 more likely to be resistant to CIP compared to those isolated from the river ([95% CI: 0.5945633, 26.576365]; $p = 0.039$).

The generalized linear model showed that Enterobacterales that grew in MacConkey agar supplemented with CAZ were the only statistically significant variable associated with the isolation of MDR Enterobacterales (**Table 5**); this indicates

TABLE 3 | Resistance profile and presence of *mcr-1* and *bla*ESBL genes on *E. coli* and *K. pneumoniae* isolates.

Species	Source	Season	Resistance Profile[2]	*bla* genes or *mcr*
E. coli	Parsley	Spring	AMP-CFZ-CAZ-CRO-FEP-ETP-SAM-CIP-AMK-SXT	bla_{CTX-M}
E. coli	Parsley	Spring	AMP-CFZ-CAZ-CRO-FEP-SAM-CIP-AMK-SXT	bla_{CTX-M}
E. coli	Lettuce	Spring	AMP-CFZ-CAZ-CRO-FEP-SAM-CIP-SXT	bla_{CTX-M}
E. coli	Parsley	Spring	AMP-CFZ-CAZ-CRO-FEP-SAM-CIP-SXT	bla_{CTX-M}
E. coli	Parsley	Spring	AMP-CFZ-CAZ-CRO-FEP-SAM-CIP-SXT	bla_{CTX-M}
E. coli	Parsley	Spring	AMP-CFZ-CAZ-CRO-FEP-SAM-CIP-SXT	bla_{CTX-M}
E. coli	Lettuce	Spring	AMP-CFZ-CAZ-CRO-FEP-CIP-SXT	bla_{CTX-M}
E. coli	River	Summer	AMP-CFZ-CRO-FEP-SAM-CIP-SXT	bla_{CTX-M}
E. coli	Beet	Summer	AMP-CFZ-CRO-FEP-FOF-SXT	bla_{CTX-M}, bla_{TEM}, *mcr-1*
E. coli	Beet	Summer	AMP-CFZ-CRO-FEP-FOF-SXT	bla_{CTX-M}, bla_{TEM}, *mcr-1*
E. coli	Parsley	Summer	AMP-CAZ-CIP-SXT	bla_{TEM}
E. coli	River	Fall	AMP-CFZ-CAZ-FEP-CRO-SXT	bla_{CTX-M}
E. coli	River	Fall	AMP-CFZ-CAZ-FEP-CRO-SXT	bla_{CTX-M}
E. coli	River	Fall	AMP-CFZ -CAZ-FEP-CRO	bla_{CTX-M}
E. coli	River	Fall	AMP-CFZ-FEP-CRO-SXT	bla_{CTX-M}
E. coli	River	Fall	AMP-CFZ-FEP-CRO-SXT	bla_{CTX-M}
K. pneumoniae	Chard	Summer	CFZ-CAZ-CRO-FEP-ETP-FOF-SXT	bla_{CTX-M}, bla_{SHV}
K. pneumoniae	Celery	Summer	CFZ-CAZ-CRO-SAM-GEN-SXT	bla_{SHV}
K. pneumoniae	Parsley	Summer	CFZ-CAZ-CRO-FEP-FOF-SXT	bla_{CTX-M}, bla_{SHV}
K. pneumoniae	Chard	Summer	CFZ-CAZ-CRO-SAM-GEN	SHV
K. pneumoniae[1]	Chard	Summer	CFZ-CAZ-CRO-FEP	bla_{CTX-M}, bla_{SHV}
K. pneumoniae[1]	Chard	Summer	CFZ-CAZ-CRO-FEP	bla_{CTX-M}, bla_{SHV}
K. pneumoniae	River	Fall	AMP-CFZ -CAZ-CRO-FEP-ETP-SAM-CIP-GEN-SXT-TZP-AMK	bla_{CTX-M}, bla_{TEM}

[1]*Isolates resistant only to cephalosporins.*
[2]*AMP, ampicillin; CFZ, cefazolin; CAZ, ceftazidime; CRO, ceftriaxone; FEP, cefepime; ETP, ertapenem; IPM, imipenem; MEM, meropenem; SAM, ampicillin/sulbactam; TZP, piperacillin/tazobactam; CIP, ciprofloxacin; AMK, amikacin; GEN, gentamicin; FOF, fosfomycin/trometamol; and SXT, trimethoprim/sulfamethoxazole.*

that Enterobacterales that grew in CAZ supplemented plates were 3.28 more likely to be MDR Enterobacterales compared to those that grew in CIP supplemented plates (OR = 3.28 [95% CI: 1.519, 7.421] value of p: 0.003). All the other variables included in the model were not significantly associated with the isolation of MDR Enterobacterales (value of p >0.05).

DISCUSSION

In this study, antibiotic-resistant Enterobacterales were recovered and characterized from vegetables and water samples in an agricultural city in central Chile; variation in seasons and source composition were analyzed to search for associations with the presence of resistant and MDR Enterobacterales. The main findings of this work are as: (i) wide distribution and significant prevalence of resistant and clinically relevant Enterobacterales in both vegetables and surface water; (ii) colistin resistance gene *mcr-1* and ESBL coding genes found in isolates obtained from vegetables and water; and (iii) association of seasons of the year with the isolation of MDR and third-generation cephalosporin resistant Enterobacterales in vegetables and water samples.

Wide Distribution and Significant Prevalence of Resistant and Clinically Relevant Enterobacterales in Vegetables and Water

Currently, there is scarce information about AMR in vegetables and the risk of ARB acquisition by consumption of contaminated vegetables; in this work, we found a high prevalence of resistant and MDR Enterobacterales collected from vegetables, mostly in sampled parsleys, corianders, and celeries. While many food safety research has focused on well-known foodborne pathogens, such as *Salmonella*, *Listeria monocytogenes*, and *E. coli* O157-H7 in vegetables (Rajabzadeh et al., 2018), our study screened for antibiotic-resistant Enterobacterales. In other studies, distinct prevalence of resistant Enterobacterales has been reported in food, for instance, Richter et al., 2019 found that spinaches and tomatoes, obtained at different points of sale (grocery

TABLE 4 | Multivariable generalized linear model showing risk factor associations between environmental factors and isolation of MDR Enterobacterales strains isolated from vegetables collected in November 2019 and January 2020.

Variable	Categories or levels[1]	Odds ratio estimates[2]	SD Error	95% CI limits	*P*
Produce	Parsley[3]				
	Beet	0.802	1.0731	(0.096, 7.718)	0.837
	Celery	3.596	1.2200	(0.415, 78.524)	0.294
	Chard	0.698	0.5888	(0.215, 2.205)	0.541
	Lettuce	1.183	0.8666	(0.220, 7.200)	0.846
Season[4]	Summer[3]				
	Spring	1.913	0.9730	(0.298, 16.139)	0.505
Produce State	Fresh[3]				
	Partly Rotten	2.927	0.7116	(0.764, 13.104)	0.131
	Completely Rotten	0.437	1.1446	(0.039, 3.981)	0.470
Insects in farmers market	Yes[3]				
	No	0.3969	1.0318	(0.0495, 2.965)	0.371
Produce at ground level	Yes[3]				
	No	0.498	0.7339	(0.112, 2.123)	0.342
Enterobacterales isolation with CAZ or CIP	CIP[3]				
	CAZ	3.073	0.4854	(1.217, 8.2623)	0.021[5]

[1]*Vegetable samples with Enterobacterales strains isolated in MacConkey supplemented with 2 μg/ml of ciprofloxacin (CIP) and 2 μg/ml of ceftazidime (CAZ).*
[2]*AIC = 142.11; R-sqr = 0.1091288.*
[3]*Used as the reference category for statistical comparisons.*
[4]*Spring: September 21st to Dec 20th 2021; Summer: Dec 21st to March 20th.*
[5]*Risk factors with statistically significant results.*

stores, vendors, and farmer markets), were the most frequently contaminated, with a prevalence of 17.4%, which was similar to our study. However, a much lower prevalence (2.7%) was reported for 3GC resistance bacteria in supermarket vegetables in Netherlands (Blaak et al., 2014). This highlights the relevance of understanding local level of contamination and the public health implications of the consumption of ready-to-eat vegetables contaminated with resistant Enterobacterales.

In this study, we also investigated resistant Enterobacterales in water samples; here, we found an important prevalence of resistance and MDR Enterobacterales. Likewise vegetable samples, different prevalence results have been reported, as a similar study that reported highly contaminated water samples (15/22 samples) collected in estuaries in the Lebanon (Diab et al., 2018), where 45% of these isolates presented an MDR profile (*E. coli, K. pneumoniae*, and *Citrobacter* spp). These results suggest that rivers used to irrigate the farms, carry antimicrobial-resistant Enterobacterales, and could be a potential source of contamination for the vegetables that were locally produced and consumed throughout the year (Jongman and Korsten, 2016; Hudson et al., 2017). While traceability of sampled vegetables was not possible with our approach (sampling at farmer markets), rather than at the harvesting sites, further whole genome sequencing could facilitate to elucidate closeness of resistant Enterobacterales obtained from water and vegetable samples.

In this work, we not only found high prevalence, but also presence of clinically relevant Enterobacterales (3GC resistant *E. cloacae* complex and *E. coli,* and *K. pneumoniae*). *K. pneumoniae* and *E. cloacae* complex are considered opportunistic pathogens, and several reports indicated that they are associated with important hospital-acquired infections (De Oliveira et al., 2020). Other similar studies have also reported *Enterobacter* and *Klebsiella* genera in fresh vegetables; more concerning, they found a higher concentration of *E. cloacae* in ready-to-eat food products (Falomir et al., 2013). Our findings along with previous studies are extremely interesting because it creates the possibility of knowing the potential risk of acquiring clinically relevant bacteria from food. These bacteria could colonize the gut microbiota of people or could even cause a higher risk of developing an infection in immunocompromised people. Although the evidence is strong, more studies are needed to trace these bacteria and their fate after people ingest them.

Colistin Resistance Gene *mcr-1* and ESBL Were Found in Isolates Obtained From Vegetables and Water

In this publication, two *E. coli* isolates that carry the *mcr-1* gene were found. This result represents the first report of colistin-resistant bacteria in vegetables in Latin America and the first time that the *mcr-1* gene has been described in non-clinical environments in Chile. Nowadays, colistin is considered "last-line therapy" to treat infections caused by MDR-resistant Gram-negative bacteria, such as *E. coli* and *K. pneumoniae* (Jones et al., 2013) and there is an increasing worldwide interest in bacteria isolated from vegetables carrying the *mcr-1* gene due to the potential risk of acquiring this gene through food consumption. Colistin-resistant bacteria isolated from vegetables and carrying some alleles of the *mcr-1* gene have already been described in diverse other countries (Zurfuh et al., 2016; Liu et al., 2020; Manageiro et al., 2020), which constitutes a major public health problem. Importantly, the two isolates that carried *mcr-1* genes found in this study, also carried the β-lactamases bla_{CTX-M} and bla_{TEM}. Although this work did not study the genetic context of these genes, it would be interesting to evaluate it in a future study since it has been described that the *mcr-1* gene with different β-lactamases can coexist in the same plasmid that are highly transmissible (Migura-Garcia et al., 2020).

TABLE 5 | Multivariate generalized linear model testing the influence of Matrix and season on the likelihood of each Enterobacterales isolate's resistance based on Kirby-Bauer and CLSI standards: **(A)** Multidrug resistance (MDR)[1] and resistance to at least one antibiotic of the following families: **(B)** third-generation cephalosporin (3GC)[2]; **(C)** Penicillin with β-lactamase inhibitor (RPIB)[3]; **(D)** Ciprofloxacin (RCIP)[4]; and **(E)** β-lactam (RBET)[5].

Model	Variable	Level	OR	OR 95%CI	Std. error	*z* value	Pr (>|z|)
A. MDR[1]	Matrix[6,7]	Beet	0.243	(0.035, 1.421)	0.93	–1.521	0.1283
		Celery	1.960	(0.194, 46.447)	1.29	0.522	0.6017
		Chard	0.450	(0.119, 1.636)	0.66	–1.205	0.2281
		Lettuce	1.399	(0.280, 7.550)	0.82	0.406	0.6849
		Parsley	0.601	(0.185, 1.901)	0.58	–0.866	0.3865
	Season[8,9]	Rainy	0.555	(0.245, 1.224)	0.40	–1.444	0.1487
	CIP/CAZ Selection[10]		3.282	(1.519, 7.420)	0.40	2.953	0.0032[11]
B. 3GC[2]	Matrix[6,7]	Beet	0.411	(0.053, 2.862)	1.00	–0.883	0.3774
		Celery	0.129	(0.011, 1.235)	1.17	–1.752	0.0797
		Chard	0.203	(0.039, 0.930)	0.80	–1.992	0.0464[11]
		Lettuce	0.256	(0.039, 1.508)	0.92	–1.476	0.1399
		Parsley	0.201	(0.043, 0.814)	0.73	–2.178	0.0294[11]
	Season[8,9]	Rainy	0.082	(0.023, 0.226)	1.00	–4.386	<0.0001[11]
C. RPIB[3]	Matrix[6,7]	Beet	0.189	(0.008, 1.822)	1.28	–1.305	0.1919
		Celery	0.604	(0.054, 5.955)	1.16	–0.433	0.6642
		Chard	0.371	(0.072, 1.910)	0.81	–1.208	0.2270
		Lettuce	0.536	(0.082, 3.338)	0.93	–0.669	0.5033
		Parsley	0.616	(0.146, 2.722)	0.73	–0.667	0.5046
	Season[8,9]	Rainy	0.174	(0.053, 0.472)	0.55	–3.179	0.0014[11]
D. RCIP[4]	Matrix[6,7]	Beet	0.896	(0.170, 2.86)	0.94	–0.117	0.9066
		Celery	2.115	(0.141, 5.958)	1.15	0.651	0.5153
		Chard	1.761	(0.222, 23.604)	0.76	0.745	0.4560
		Lettuce	3.647	(0.394, 8.173)	0.95	1.359	0.1740
		Parsley	4.513	(0.595, 26.576)	0.73	2.067	0.0387[11]
	Season[8,9]	Rainy	5.109	(1.069, 19.806)	0.56	2.929	0.0034[11]
E. RBET[5]	Matrix[6,7]	Beet	0.431	(0.072, 2.523)	0.89	–0.945	0.3448
		Celery	0.689	(0.070, 16.050)	1.28	–0.291	0.7707
		Chard	0.509	(0.131, 1.889)	0.68	–0.999	0.3180
		Lettuce	3.557	(0.462, 75.096)	1.19	1.070	0.2847
		Parsley	0.475	(0.140, 1.502)	0.60	–1.243	0.2140
	Season[8,9]	Rainy	0.221	(0.098, 0.482)	0.40	–3.726	0.0002[11]

[1]AIC = 189.16; r2 = 0.07131851.

[2]AIC = 214.51; r2 = 0.1678267.

[3]AIC = 177.77; r2 = 0.0984252.

[4]AIC = 171; r2 = 0.107578.

[5]AIC = 190.07; r2 = 0.1302794.

[6]For the variable Matrix, the level "coriander" had to be removed from the model because this food had only one Enterobacterales isolate, which did not present MDR, therefore the GLM presented error.

[7]Water sample was used as the reference for the variable Matrix to estimate the effects of each variable category.

[8]Dry was used as the reference for the variable Matrix to estimate the effects of each variable category.

[9]Season in which the sample was collected with "Rainy" representing March 21st to September 20th, and "Dry" from September 21st to March 20th for a Mediterranean climate in the Southern Hemisphere of the American continent.

[10]CIP/CAZ selection corresponds the antibiotic used to select Enterobacterales AMR isolates. CIP was used as the reference for the variable CIP/CAZ selection to estimate the effects of each variable category.

[11]Variables with statistical significance ($p < 0.05$).

Our work found that ESBL had a prevalence of 11.3% in *K. pneumoniae* and 25.8% in *E. coli*. Similar results were presented in a previous study that collected 109 vegetable samples from 18 farms in Tunisia and detected ESBL genes in isolates of *E. coli*, *Klebsiella*, and *Citrobacter*; likewise our study, $bla_{\text{CTX-M}}$ was the most common ESBL detected, and bla_{TEM} and bla_{SHV} were also found (Said et al., 2015). Additionally, ESBL genes have also been reported from different water sources, Caltagirone et al., 2017 conducted a study in Italy and reported the presence of $bla_{\text{CTX-M}}$ along with $bla_{\text{CTX–M}}$ in combination with bla_{SHV} in isolates obtained from wells, streams, and water treatment plants. In Chile, a recent study reported the prevalence of ESBL in *E. coli* in domestic and wild animals, indicating a 30% prevalence in livestock, 24% in dogs, and 0.5% in wild animals; meanwhile, CTX-M enzymes were the most common enzymes in this study, coinciding with the results reported in the present work (Benavides et al., 2021). Other studies in the country have reported $bla_{\text{CTX-M}}$ in foxes, Andean condors, and wild felids (Cevidanes et al., 2020; Sacristán et al., 2020). These results highlight a high incidence of $bla_{\text{CTX-M}}$ in different environments in Chile and the fact that run-off from domestic animals could be a source of contamination for water and food. Significantly, AMR should be studied with a focus on one health because $bla_{\text{CTX-M}}$ was found to present a high prevalence in an intensive care unit

in the country (Pavez et al., 2019). The present study, along with previously reported studies, highlights the relevance of improving our understanding of the environmental dissemination of ESBL in water sources and vegetables. Further studies are necessary, including whole genome sequencing of the collected isolated harboring ESBL, to understand at the genomic level the isolates and mobile genetic elements that are involved in the dispersion and transmission of these genes.

Seasons Is Associated With the Isolation of MDR and Third-Generation Cephalosporins Enterobacterales in Vegetables and Water

Statistical analysis performed in this work indicated that the rainy season (fall and winter) has a lower likelihood of isolating Enterobacterales that displayed an MDR phenotype and resistance to at least one 3GC compared to the dry season. Conversely, a previous study carried out in North Africa characterized 3GC resistant Gram-negative bacteria in which a higher frequency of contamination of fruits and vegetables was identified during the rainy season (winter and fall for this region; Zekar et al., 2017). Other, previous research also found a higher load of microorganisms in vegetables during rainy seasons, due mainly to an increase in rainfall that carries garbage along with lower solar irradiation (Allende et al., 2017). The difference between our results could be due in Chile, the dry season is during the summer and spring, in which temperature of water could increase, river water flow could decrease, and rains are scarce, but if there is a rain event, this could drag accumulated material on the banks of the rivers, but this hypothesis should be further investigated. Moreover, none of the investigations mentioned before evaluated AMR. Very few studies have focused on AMR and environmental factors, one previous work has related the increase of bacteria (*E. coli* and *K. pneumoniae*) that carry ESBL in the human population during the summer, concluding that seasonality could play a fundamental role in the dissemination of ESBL (Wielders et al., 2020). These results agreed with those obtained in our work, as it is precisely in the summer that the highest number of ESBLs was found. While this study presented data for only 1 year of sampling, longitudinal data for longer than 1 year are necessary. Overall, understanding seasons with higher contamination with resistant Enterobacterales contribute to a better understanding of the transmission dynamics of AMR through food and to further develop interventions.

Our study indicates that performing screening on plates supplemented with ceftazidime increases the probability of finding MDR Enterobacterales in vegetable samples. Therefore, this antibiotic could be used as a marker for multidrug resistance in Enterobacterales in our region, agreeing with data from other regions, such as Europe, where cefotaxime is used as a marker of resistance (Shaw et al., 2021). Before this study, it was thought that supplementing with ciprofloxacin could have a similar effect on the detection of MDR Enterobacterales, as in this region a high prevalence of resistance to this antibiotic is reported (ISP, Instituto de Salud Publica, 2015; Durán, 2018). In addition, ciprofloxacin is usually found in aquatic environments, such as river water that is used to water vegetables due to its low biodegradation in aquatic environments (Girardi et al., 2011).

CONCLUSION

This study emphasizes the importance of improving our understanding of environmental and food contamination with antimicrobial-resistant bacteria. The presence of MDR Enterobacterales isolates in vegetables that are mostly consumed without further cooking could represent a public health concern. MDR isolates, ESBL producers, and *mcr-1* were found in vegetables and river water that may irrigate those vegetables, these findings highlight the potential wide spread of MDR Enterobacterales and ESBL genes in the studied region. Because AMR is a global concern, reports from underrepresented regions in which environmental surveillance of AMR is not conducted could help to develop local awareness of AMR, especially for food-producing countries that may have underestimated the importance of environmental AMR.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

CD-G, AM-S, AA, JO-P, and CB designed the study, conducted the experiments, and wrote the manuscript. AA ran the statistical analysis while CD-G constructed the heat map. LR, PG, and FA conducted experiments. GG-R, SC, AO-C, JM, and RA designed experiments and critically 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.2021.604567/full#supplementary-material

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