EXPOSURE, RISKS, AND DRIVERS OF THE MOBILE ANTIMICROBIAL RESISTANCE GENES IN THE ENVIRONMENT – A GLOBAL PERSPECTIVE

EDITED BY: Abasiofiok Mark Ibekwe, Lisa M. Durso and John P. Brooks

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EXPOSURE, RISKS, AND DRIVERS OF THE MOBILE ANTIMICROBIAL RESISTANCE GENES IN THE ENVIRONMENT – A GLOBAL PERSPECTIVE

Topic Editors:
Abasiofiok Mark Ibekwe, United States Department of Agriculture (USDA), United States
Lisa M. Durso, United States Department of Agriculture, United States
John P. Brooks, United States Department of Agriculture, United States

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EDITORIAL
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Editorial: Exposure, Risks, and Drivers of the Mobile Antimicrobial Resistance Genes in the Environment—a Global Perspective

John P. Brooks[*†], *Lisa M. Durso*[†] *and Abasiofiok Mark Ibekwe*[†]

Agricultural Research Service, United States Department of Agriculture, Washington, DC, United States

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Editorial on the Research Topic

Exposure, Risks, and Drivers of the Mobile Antimicrobial Resistance Genes in the Environment—a Global Perspective

The life and work of Heather K. Allen
October 24, 1980 – March 7th, 2020

In 2010, Dr. Heather K. Allen published a foundational review paper on the emerging field of environmental antibiotic resistance. "Call of the wild: antibiotic resistance genes in natural environments" (Allen et al., 2010) introduced a conceptual framework for understanding and assessing antibiotic resistance in water, soil, air, and wildlife, at a time when the focus of antibiotic resistance work was primarily on human clinical settings. The paper provided a framework that built on Dr. Allen's Ph.D. work with Dr. Jo Handelsman, integrating microbial ecology with the then-new metagenomic sequencing tools to probe antibiotic resistance in natural and agricultural settings. The paper defined priority Research Topics that have guided a generation of researchers in the field and has received over 1,900 citations to date. Dr. Allen's "Call of the Wild" highlighted the important role of naturally occurring antibiotic resistance; the diversity and role of antibiotic resistance genes in bacterial communities from both naturally occurring and human-impacted sites; and the need to better understand factors contributing to the spread of antibiotic resistant bacteria and their genes in the environment. These are the topics covered by this current Frontiers Research Topic "*Exposure, Risks, and Drivers of the Mobile Antimicrobial Resistance Genes in the Environment—a Global Perspective.*"

After leaving the Handelsman laboratory, Dr. Allen took a position at the USDA-Agricultural Research Service in Ames Iowa. While the focus of her antibiotic resistance research shifted to the gut microbiome of swine, she remained a thought leader in the field of environmental antibiotic resistance. During her short career she received numerous awards for her work on antibiotic resistance, including the prestigious Arthur S. Flemming award for scientific achievements and the Presidential Early Career Award for Scientists and Engineers.

Heather had a sharply analytical mind, visionary creativity, and a talent for writing that changed the conversation around environmental antibiotic resistance. She leaves a lasting and positive influence on the field, both through her innovative research, and through her gracious collegiality.

The potential sources and movement of antimicrobial resistant (AMR) bacteria and antibiotic resistance determinants (ARD) through the soil and water can lead to a seemingly endless web of interconnected public health consequences and the environment. While we, generally, are aware of the many sources of AMR, we still don't understand the environmental fate and ultimately the

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Edited and reviewed by:
Giovanna Batoni,
University of Pisa, Italy

***Correspondence:**
John P. Brooks
john.brooks@usda.gov

[†]These authors have contributed equally to this work

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public health consequences of AMR bacteria. The goal of this Research Topic is to present current research on the source of AMR from both anthropogenic and animal sources, elaborate on the potential movement through the environment, and present new approaches to surveying for AMR and ARD.

ANIMAL AND HUMAN SOURCES OF AMR

The human and animal sources of AMR have long been tied to spread to the environment. Long considered a major source for environmental pollution, these respective sources are key control points in the reduction of AMR in the environment. Animal feeding operations and municipal wastewater treatment plants are considered "ideal" milieu for promoting the proliferation of AMR and selection and exchange of ARD. In the current special section, we have gathered international contributions from teams working on the presence of AMR in the environment, with those focusing on farm and wastewater treatment plants. While foodborne bacterial pathogens are typically investigated, and rightly so, we have papers focused on commensal bacteria as well. We have gathered papers covering the diversity of gene cassettes in commensal *E. coli* at swine farms (Zhang et al.) to whole genome sequencing of common foodborne pathogen indicator species, such as vancomycin resistant *Enterococcus* spp. (Foka et al.). Poultry production in Brazil is covered by original research demonstrating extended spectrum beta lactamase producing *E. coli* in samples through production and the immediate food production environment (Gazal et al.). On farm anaerobic treatment of manure and effects of wastewater treatment plants on ARD and their surveillance were investigated by Agga et al.; Majeed et al., respectively. Surveillance of ARD and AMR bacteria utilizing metagenomics is a highly sought-after topic, particularly while trying to understand the context of baseline levels of these pollutants in the environment. Finally, we have near retail meat products destined for human consumption and potential for AMR exposure (Wang et al.), and review of the current literature summarizing antibiotic resistance genes in animal manure and potential fate following land application.

AMR IN WATER

One primary interface between the public and AMR bacteria is in watersheds and surface freshwater. Manure and wastewater treatment by products are often disposed of in pasture and row crop fields which are buffered by surface water bodies. One process that drives AMR bacteria and ARGs from manure is via rain associated runoff. In the current special section, we have an article covering the effect of long-term pasture management on ARD in runoff (Yang et al.). We have also gathered similar research in an urban watershed impacted by wastewater treatment plant recharge (Mukherjee et al.). These two studies focused on the targeting of ARD in the environment. A method to control surface runoff is irrigation return flows, however return flows are known to harbor high levels of contaminants and bacteria. A study by Dungan and Bjorneberg investigated *E. coli* and enterococcal AMR profiles in return flows.

NEW TOOLS FOR UNDERSTANDING DISSEMINATION OF ANTIBIOTICS AND ANTIBIOTIC RESISTANCE FROM THE BROAD ENVIRONMENT TO CROPS

More advanced knowledge in chemistry, molecular microbiology, metagenomics, and bioinformatics are needed to understand the ecology of transmission of antibiotics and ARGs from the environment to crops and potentially to human. Matrices such as manures, biosolids, and wastewater are highly significant sources of antibiotics and ARDs. In addition to these, direct gene transfers between microbes present in the waste material and the soil can also develop resistance owing to the selective pressure applied by the presence of antibiotic compounds in the waste material (Duan et al., 2017). The occurrence of antibiotics in such systems may lead to genetic changes in sensitive bacteria (Martinez, 2009). MGEs would further enhance the dissemination and promotion of genetic recombination of ARGs via horizontal gene transfer (HGT) (Vikesland et al., 2017). In a minireview, Bartkova et al. outlined the current technologies used to characterize microplastics based ecosystems termed "plastisphere" and their AMR promoting elements and highlighted emerging technologies that could be useful for systems-level investigations of AMR in the plastisphere. When analyzing these ecosystems scientists can integrate metagenomics and metatranscriptomics with machine-learning tools such as DeepARG or any other artificial intelligent tools (AI) to find the existing and novel ARGs and MGEs for future AMR work (Arango-Argoty et al., 2016; Cuadrat et al., 2020).

In the broad environment, livestock manure application to cropland for soil fertility presents a concern that ARG and bacteria may proliferate and be transported in the environment (Miller et al.). For instance, swine manure application to agricultural soil can introduce diverse set of *tet* resistances genes into low *tet* resistance agricultural soil. One of the major concerns after manure or wastewater application to agricultural soil is the dissemination of chemicals of emerging concern (CEC) through the food chain. This was observed when 11 fosfomycin-resistant *Citrobacter freundii* strains from 270 samples were identified from flowers and the retail environment (Cheng et al.). The authors noted that these isolates were multidrug-resistant, and most were simultaneously resistant to fosfomycin, cefotaxime, ciprofloxacin, and amikacin, therefore potentially posing a public health threat to workers. It is interesting to note that there is a high prevalence of widespread acquired resistance genes among bacteria such as *Enterococcus* strains exposed to anthropogenic antibiotic pressure in the environment (Aun et al.). However, they noted that *E. faecium* strains in their dataset were found within the same host species or environmental origin, confirming the previous findings that *E. faecalis* from the same ST can be found in human as well as in other animal species, while *E. faecium* strains tend to be host specific (Hammerum, 2012). The biggest concern of AMR is their transferability from the environment; one of the routes may be through fresh produce that are eaten raw or with minimally processed. Due to high

water demands especially in the southwestern part of the United States, there is a high demand for new water reuse programs, including treated municipal wastewater usage. The use of wastewater to grow leafy greens could pose potential health risks due to the high probability of AMR in such wastewater (Summerlin et al.). These authors suggested that successful reuse of wastewater in agriculture will depend on appropriate mitigation and management strategies to guarantee safe water supply. In an integrated study to understand factors influencing the occurrence, distribution, and fate of antibiotic resistance genes in vegetable production systems, Wind et al., suggested that pre-harvest and potentially post-harvest interventions may be warranted to minimize risk of propagating antibiotic resistance in the food chain. They demonstrated a holistic approach as the best options to identifying key control points for the propagation of ARGs in vegetable production systems, identifying potential ARG-MGE combinations that could inform future surveillance. They suggested that agroecosystems are a key reservoir of antibiotic resistance and appropriate mitigation strategies are needed to limit potential for ARGs to spread and negatively influence human and animal health.

All told, this special section covers a wide swath of research from AMR and ARD in wastes and products derived from both human and animal sources, and their potential movement through the environment, ultimately culminating in potential public health consequences.

AUTHOR CONTRIBUTIONS

JB, LD, and AI were involved in the conception, execution, and editing. All authors contributed to the article and approved the submitted version.

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REFERENCES

Allen, H. K., Donato, J., Wang, H. H., Cloud-Hansen, K. A., Davies, J., and Handelsman, J. (2010). Call of the wild: antibiotic resistance genes in natural environments. *Nat. Rev. Microbiol.* 8, 251–259. doi: 10.1038/nrmicro2312

Arango-Argoty, G., Singh, G., Heath, L. S., Pruden, A., Ziao, W., and Zhang, L. (2016). MetaStorm: a public resource for customizable metagenomics annotation. *PLoS ONE* 11:e0162442. doi: 10.1371/journal.pone.0162442

Cuadrat, R. R. C., Sorokina, M., and Andrade, B. G. (2020). Supporting data for Global ocean resistome revealed: exploring antibiotic resistance gene abundance and distribution in TARA Oceans samples. *GigaScience Database* 2020:12. doi: 10. 5524/100739

Duan, M., Li, H., Gu, J., Tuo, X., and Wang, X. (2017). Effects of biochar on reducing the abundance of oxytetracycline, antibiotic resistance genes, and human pathogenic bacteria in soil and lettuce. *Environ. Pollut*. 224, 787–795. doi: 10.1016/j.envpol.2017.01.021

Hammerum, A. M. (2012). Enterococci of animal origin and their significance for public health. *Clin. Microbiol. Infect*. 18, 619–625. doi: 10.1111/j.1469-0691. 2012.03829.x

Martinez, J. L. (2009). Environmental pollution by antibiotics and by antibiotic resistance determinants. *Environ. Pollut*. 157, 2893–2902. doi: 10.1016/j.envpol.2009.05.051

Vikesland, P. J., Pruden, A., Alvarez, P. J. J., Aga, D., Bürgmann, H., Li, X. D., et al. (2017). Toward a comprehensive strategy to mitigate dissemination of environmental sources of antibiotic resistance. *Environ. Sci. Technol*. 51, 13061–13069. doi: 10.1021/acs.est.7b03623

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ORIGINAL RESEARCH
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Diverse Gene Cassette Arrays Prevail in Commensal *Escherichia coli* From Intensive Farming Swine in Four Provinces of China

Xiuping Zhang[1,2†], Xinxin Li[1†], Weihua Wang[3†], Jiali Qi[1], Dong Wang[1], Lei Xu[4], Yong Liu[1], Yanming Zhang[1] and Kangkang Guo[1]*

[1]College of Veterinary Medicine, Northwest A&F University, Yangling, China, [2]College of Animal Science, Tarim University, Alar, China, [3]Weinan Vocational and Technical College, Weinan, China, [4]College of Life Science, Northwest A&F University, Yangling, China

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Edited by:
Lisa Durso, United States Department of Agriculture, United States

Reviewed by:
Dingqiang Chen, Southern Medical University, China
Bing Gu, Affiliated Hospital of Xuzhou Medical University, China

***Correspondence:**
Kangkang Guo
guokk2007@nwsuaf.edu.cn

†These authors have contributed equally to this work

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Multiple-drug resistance bacteria containing antimicrobial resistance genes (ARGs) are a concern for public health. Integrons are bacterial genetic elements that can capture, rearrange, and express mobile gene cassettes responsible for the spread of ARGs. Few studies link genotype and phenotype of swine-related ARGs in the context of mobile gene cassette arrays among commensal *Escherichia coli* (*E. coli*) in nonclinical livestock isolates from intensive farms. In the present study, a total of 264 isolates were obtained from 330 rectal swabs to determine the prevalence and characteristics of antibiotic-resistant gene being carried by commensal *E. coli* in the healthy swine from four intensive farms at Anhui, Hebei, Shanxi, and Shaanxi, in China. Antimicrobial resistance phenotypes of the recovered isolates were determined for 19 antimicrobials. The *E. coli* isolates were commonly nonsusceptible to doxycycline (75.8%), tetracycline (73.5%), sulfamethoxazole-trimethoprim (71.6%), amoxicillin (68.2%), sulfasalazine (67.1%), ampicillin (58.0%), florfenicol (56.1%), and streptomycin (53.0%), but all isolates were susceptible to imipenem (100%). Isolates [184 (69.7%)] exhibited multiple drug resistance with 11 patterns. Moreover, 197 isolates (74.6%) were detected carrying the integron-integrase gene (*intI*1) of class 1 integrons. A higher incidence of antimicrobial resistance was observed in the *intI*1-positive *E. coli* isolates than in the *intI*1-negative *E. coli* isolates. Furthermore, there were 17 kinds of gene cassette arrays in the 70 integrons as detected by sequencing amplicons of variable regions, with 66 isolates (94.3%) expressing their gene cassettes encoding for multiple drug resistance phenotypes for streptomycin, neomycin, gentamicin, kanamycin, amikacin, sulfamethoxazole-trimethoprim, sulfasalazine, and florfenicol. Notably, due to harboring multiple, hybrid, and recombination cassettes, complex cassette arrays were attributed to multiple drug resistance patterns than simple arrays. In conclusion, we demonstrated that the prevalence of multiple drug resistance and the incidence of class 1 integrons were 69.7 and 74.6% in commensal *E. coli* isolated from healthy swine, which were lower in frequency than that previously reported in China.

Keywords: antimicrobial resistance, multiple-drug resistance, class 1 integrons, gene cassette, commensal *Escherichia coli*, antibiotic-resistant bacteria, antibiotic-resistant genes

INTRODUCTION

Antimicrobial-resistant bacteria, especially multiple drug-resistant (MDR) strains have caused many outbreaks of food-borne diseases and infectious diseases worldwide, threatening human and animal health (Paitan, 2018). Integrons located on either chromosome or mobile genetic elements (MGEs), such as plasmids and transposons, are considered responsible for the horizontal gene transfer of antimicrobial resistance (AMR; Stalder et al., 2012). Integrons are natural recombination and expression systems with the ability to acquire gene cassettes (Partridge et al., 2018). Gene cassettes are a major source of the resistance genes found in clinical, commensal, and environmental isolates of bacteria. A gene cassette is a small mobile element (0.5–1 kb) consisting of a single gene (occasionally two) and a recombination site (*attC*; Partridge et al., 2009). Gene cassettes encode proteins that facilitate interactions with their extracellular environment (Timothy et al., 2019). Several cassettes may be inserted into the same integron forming a tandem array. Class 1 integrons in bacteria ubiquitously reside in gastrointestinal tracts of animals and humans, and their abundance and genetic diversity can readily change in response to environmental pressures (Alonso et al., 2017). The class 1 integrons are diverse and significant players in the spread of AMR. *E. coli* has been shown to be a significant reservoir of genes encoding for AMR and has been suggested as a useful indicator for resistance in bacterial communities (Ge et al., 2020; Holcomb and Stewart, 2020; Zhang et al., 2020b). Highly dynamic and diverse of *E. coli* populations exist in the swine intestinal microbiota and in the farm environment throughout the full production cycle, suggesting the potential for carriage of antimicrobial resistance and the presence of clinical integrons (Marchant and Morenoa, 2013; Van den Meersche et al., 2020).

Development of MDR in intestinal flora is closely associated with integrons and their gene cassettes (Olivier et al., 2018). Integrons are genetic platforms for captured gene cassettes, which are regarded as adaptation and evolution of bacteria (José, 2018). They have traditional genetic structure of two conserved segments (5'-CS and 3'-CS) and a variable region (Barraud and Ploy, 2015). At least 130 different (<98% identical) cassettes that carry known or predicted antibiotic resistance genes have been identified, along with many cassettes of unknown function (Partridge et al., 2009). Class 1 integrons play a crucial role in the propagation of ARGs, and have been surveyed in numerous ecosystems and animals. Zhang et al. found the average content of class I integrons as 1.31×10^4 copies/100 ml in drinking water from 71 cities in China (Zhang et al., 2020a). However, the data are still lacking on the epidemiology of class 1 integrons and integron-borne gene cassettes in commensal *E. coli* among livestock herds, in particular at the source of pork production.

To promote effective antimicrobial stewardship, the government of China issued the National Action Plan to Contain Animal Original Antimicrobial Resistance (2017–2020) on June 22, 2017. This action is in line with the strategic objectives of WHO's Global Action Plan on antimicrobial resistance. Tracking and surveillance of antimicrobial resistant bacteria in livestock is a critical step toward protecting humans and animals from infections. Anhui, Hebei, Shanxi, and Shaanxi provinces are mainly livestock-raising areas in China, with the amount of fattening swine for market (unit: million heads) of 283.74, 370.96, 81.46, 115.08, with the number of stocks at year-end (unit: million heads) of 135.63, 182.08, 54.95, 83.9, respectively, in 2019 (NBSC, 2019). However, data of antimicrobial resistance in livestock are inadequate in these areas.

The aim of the present study was to obtain the phenotypic and genotypic characterization of selected antimicrobial resistance determinants found in commensal *E. coli* isolated from intensively farmed swine. We detected the MDR phenotypic profiles, the abundance of class 1 integrons, and gene cassettes of class 1 integrons through antimicrobial susceptibility testing, PCR assay, and DNA sequencing.

MATERIALS AND METHODS

Sampling and Isolation

From March to May, 2019, a total of 330 rectal sterile swabs were collected from healthy growing-finishing swine of four intensive farms in Anhui, Hebei, Shanxi, and Shaanxi province, China (**Table 1**). The annual production of the intensive farms was 1,000–1,200 heads (i.e., the breeding stock was 500–600 heads), with growing-finishing swine (mixed sex, same aged from 70 to 185 days) raised in the mode of all-in and all-out. Random sampling was conducted on 90- to 180-days growing-finishing swine in 10% of the breeding stock herds, with one swab per swine. Healthy swines were determined by observation indicators of their good physical and mental state, with normal body temperature, appearance, and behavior, feed intake and drinking, and excretion. The Animal Welfare and Research Ethics Committee of Northwest A&F University (Yangling, China) approved the protocol of the experiment (protocol number: NWAFUSM2018005). All the rectal sterile swabs were placed into an ice box and transferred to the laboratory within 6 h for further bacteriological analysis.

Isolation and identification of *E. coli* were performed with rectal sterile swabs and transferred to sterile culture tubes containing 5 ml of Luria-Bertani (LB) broth and mixed vigorously (200 r/min) at 37°C for 8 h. After enrichment, a loop of LB broth culture was streaked onto eosin-methylene blue medium (EMB) agar and incubated at 37°C for 24 h. Colonies showing

TABLE 1 | Samples from Anhui, Hebei, Shanxi, and Shaanxi.

Time[a]	Anhui	Hebei	Shanxi	Shaanxi
March	30	30	30	30
April	30	30	30	30
May	20[b]	20[b]	20[b]	30
Total	80	80	80	90

[a]*Sampling was originally planned on the 1st, 15th, 30th of March, April, and May in each farm, when the ages of the growing-finishing swine varied from 90 to 180 days. In total, 30 samples were to be collected in each farm every month.*
[b]*Sampling plan was not conducted on the 30th of May in Anhui, Hebei, and Shanxi, so only 20 samples were obtained from their farms, respectively.*

a metallic sheen were considered presumptive *E. coli* isolates, and positive colonies were chosen for further biochemical identification using Gram-negative identification cards of an automated VITEK2 microbial identification system (BioMerieux, France), according to the manufacturers.

Antimicrobial Susceptibility Testing

All *E. coli* isolates were tested susceptible to seven classes of antimicrobials by the Kirby-Bauer disk diffusion method on Mueller-Hinton (MH) agar plates following Clinical and Laboratory Standards Institute (CLSI) procedures (CLSI, 2017). A panel of 19 antimicrobial agents (**Table 2**) are commonly used against clinical infections. In animal husbandry, discs containing streptomycin (STR, 10 μg), neomycin (NEO, 30 μg), gentamicin (GEN, 10 μg), kanamycin (K, 30 μg), amikacin (AK, 30 μg), amoxicillin (AMC, 20 μg), ampicillin (AMP, 10 μg), cephalexin (CL, 30 μg), cefotaxime (CTX, 30 μg), imipenem (IMP, 10 μg), ciprofloxacin (CIP, 5 μg), enrofloxacin (ENR, 10 μg), norfloxacin (NOR, 10 μg), tetracycline (TET, 30 μg), doxycycline (DX, 30 μg), sulfamethoxazole-trimethoprim (SMZ/TMP, 25 μg), sulfasalazine (SIZ, 300 μg), erythromycin (ERY, 15 μg), and florfenicol (FF, 30 μg) were used. The reference strain *E. coli* ATCC 25922 was used for quality control. Susceptibility decision was described as resistant (R), intermediate (I), or susceptible (S) as the CLSI and the sensitivity criteria for *Enterobacteriaceae* (Hangzhou Microbiology Co., Ltd., China, 2019). Isolates were considered as nonsusceptible when reported as either intermediate susceptible (I) or resistant (R; Jarlier et al., 2019). *E. coli* isolates exhibiting MDR was defined as acquired nonsusceptible to ≥1 agent in ≥3 antimicrobial categories (Magiorakos et al., 2012).

PCR Detection of *intI*1 Gene in *E. coli* Isolates

All *E. coli* isolates were screened by PCR for the class 1 integron-integrase (*intI*1) gene to confirm the presence of class 1 integrons. The primers were *intI*1 F 5'-ACGAGCGCAAGGTTTCGGT-3' and *intI1* R 5'-GAAAGGTCTGGTCATACATG-3' (Bass et al., 1999). The amplification protocol was performed as follows: initial denaturation (94°C for 5 min), followed by 30 cycles of denaturation (94°C for 30 s), annealing (56°C for 30 s), extension (72°C for 2 min), then a final extension (72°C for 10 min). Amplicons were separated by electrophoresis in a 1.5% agarose gel and sequenced (Beijing Qingke Biotech Co., Ltd. China).

Arrangement of Resistance Gene Cassettes in Class 1 Integrons

The variable regions of the integron-positive isolates were further amplified for gene cassettes by PCR using primers of *intI1*-V FP 5'-TCATGGCTTGTTATGACTGT-3' and *intI*-V RP 5'-GTAG GGCTTATTATGCACGC-3' (White et al., 2000). PCR conditions were the same as above except for the annealing temperatures for the fragments. After separated by electrophoresis in 1.5% agarose gel, the gel-recovered product was ligated with pMD19-T vector (TaKaRa Bio Group, Japan) at 16°C for 4 h and then transferred into *E. coli* DH5α cells (QiaGen Biotech CO., LTD, China). The *E. coli* DH5α cultures were inoculated on the LB agar plates supplemented with 100 μg/ml ampicillin. The amplified fragments

TABLE 2 | Antimicrobial agents were used to define the susceptibility of *Escherichia coli* isolates.

Antimicrobials[a]		Content (μg/disc)	Breakpoint (mm)		
			R[b]	I[b]	S[b]
Aminoglycosides	Streptomycin (STR)	10	≤12	13–14	≥15
	Neomycin (NEO)	30	≤12	13–16	≥17
	Gentamicin (GEN)	10	≤13	14–17	≥18
	Kanamycin (K)	30	≤13	14–17	≥18
	Amikacin (AK)	30	≤14	15–16	≥17
β-Lactams	Amoxicillin (AMC)	20	≤13	14–17	≥18
	Ampicillin (AMP)	10	≤13	14–16	≥17
	Cephalexin (CL)	30	≤14	15–17	≥18
	Cefotaxime (CTX)	30	≤14	15–22	≥23
	Imipenem (IPM)	10	≤13	14 15	≥16
Quinolones	Ciprofloxacin (CIP)	5	≤15	16–20	≥21
	Enrofloxacin (ENR)	10	≤21	22–28	≥29
	Norfloxacin (NOR)	10	≤12	13–16	≥17
Tetracyclines	Tetracycline (TET)	30	≤14	15–18	≥19
	Doxycycline (DX)	30	≤12	13–15	≥16
Sulfonamides	Sulfamethaxazole/ trimethoprim (SMZ/TMP)	25	≤10	11–15	≥16
	Sulfasalazine (SIZ)	300	≤12	13–16	≥17
Macrolides	Erythromycin (ERY)	15	≤13	14–22	≥23
Chloramphenicol	Florfenicol (FF)	30	≤12	13–17	≥18

[a]*Veterinary antibiotics were chosen for testing including aminoglycoside (streptomycin STR, neomycin NEO, gentamicin GEN, kanamycin K, amikacin AK), β-lactam (amoxicillin AMC and ampicillin AMP, cephalexin CL, cefotaxime CTX, mipenem IPM), quinolone (ciprofloxacin CIP, for enrofloxacin ENR and norfloxacin NOR), tetracycline (tetracycline TET, doxycycline DX), sulfonamide (sulfamethoxazole-trimethoprim SMZ/TMP, and sulfasalazine SIZ), macrolides (erythromycin ERY), and chloramphenicol (florfenicol FF).*
[b]*R stands for resistant, I for intermediate resistant, S for susceptible.*

of different sizes were sequenced. The nucleotide sequences were analyzed by using Nucleotide BLAST on NCBI website[1] and the integron database INTEGRALL.[2] Standard Nucleotide BLAST was set as Search database Nucleotide collection (nr/nt) using Megablast (optimized for highly similar sequences). The general parameters were set by selecting the maximum number of aligned sequences to display (100) and expected threshold (10). Once the nucleotide BLAST results were available, pairwise and CDS features were chosen for alignment view.

Statistical Analysis

Data were analyzed using the Statistical Product and Service Solutions software (SPSS, version 20.0). Chi square test and Fisher's exact test of analysis of variance (ANOVA) were used to determine the statistical significance of data. Values were considered as having a statistically significant difference if $p < 0.05$ and as an extremely distinct difference if $p < 0.01$.

RESULT

Antimicrobial Resistance Profiles of *E. coli* in Four Provinces

In our study, we investigated the prevalence of antimicrobial resistance in 264 *E. coli* isolates recovered from rectal swabs. They were resistant to at least one antimicrobial agent (**Table 3**, left). A high proportion of *E. coli* isolates were nonsusceptible to doxycycline (75.8%), tetracycline (73.5%), sulfamethoxazole-trimethoprim (71.6%), amoxicillin (68.2%), sulfasalazine (67.1%), ampicillin (58.0%), florfenicol (56.1%), and streptomycin (53.0%). However, all isolates were susceptible to imipenem. A total of 184 (69.7%) *E. coli* isolates exhibited MDR, with 11 MDR patterns (**Figure 1**). Importantly, 16 *E. coli* isolates (6.0%) displayed resistance against 13 antimicrobial agents. The prevalence of *E. coli* MDR in Anhui, Hebei, Shanxi, and Shaanxi were 78.6, 80, 74.4, and 58.0%, respectively (**Table 4**).

The Incidence of Class 1 Integrons and Their Association With Antimicrobial Resistance in *E. coli* Isolates

The *IntI*1 gene was detected in 197 (74.6%) *E. coli* isolates, including 155 (78.7%) MDR isolates. I*ntI*1-positive *E. coli* isolates were most commonly nonsusceptible to doxycycline (70.1%), tetracycline (69.0%), sulfamethoxazole-trimethoprim (67.0%), amoxicillin (66.5%), sulfasalazine (64.5%), florfenicol (54.3%), and ampicillin (53.3%). There were significant differences in AMR between *intI*1-positive *E. coli* isolates and *intI*1-negative *E. coli* isolates, not only in single antimicrobial resistance but also in multiple drugs resistance ($p < 0.01$), except sulfasalazine ($p = 0.313$), kanamycin ($p = 0.055$), and amikacin ($p = 0.128$).

After sequencing the variable regions of class 1 integrons, we found 17 gene cassette arrays and 10 gene cassettes (*aadA1*, *aadA2*, *aadA5*, *aadA16*; *dfrA1*, *dfrA7*, *dfrA12*, *dfrA17*, *cmlA1*, and *orfF*) in 70 *E. coli* isolates (**Table 5**). Among them, 66 (85.7%) isolates had gene cassettes that corresponded with respective phenotypic MDR patterns, encoding resistance to streptomycin, neomycin, gentamicin, kanamycin, amikacin, sulfamethoxazole-trimethoprim, sulfasalazine, and florfenicol. However, there were three *E. coli* isolates that did not express their cassette arrays for MDR patterns at all, including *dfrA12-orfF-aadA2/1*, *aadA1-aadA2*, *aadA2*, and *aadA1* (**Table 5**, marked with lower case c). Different gene cassette arrays, even from the same array showed different phenotypic patterns of MDR. In addition, prevailing arrays were different in the four farms: five arrays in Anhui ($n = 19$), five arrays in Hebei ($n = 11$), eight arrays in Shanxi ($n = 10$), and six arrays in Shaanxi ($n = 30$).

Cassette arrays showed the following characteristics. First, the top three cassette arrays were *dfrA17–aadA5* (9.6%), *dfrA12–orfF–aadA2* (6.0%), and *dfrA12–orfF-aadA2–adA1–cmlA1* (4.0%). They were confirmed by the same or similar sizes of amplicons, but they exhibited different patterns of phenotypical MDR. This phenomenon also occurred in *dfrA1–aadA2/1* (2.5%), *aadA2* (1.5%), and *dfrA12–orfF–aadA2/1* (1.0%). Second, in six arrays, *E. coli* isolates with *aadA2/1* and *aadA2/aadA2* hybrid gene cassettes exhibited more complex MDR, such as *dfrA1–aadA2/1*, *dfrA7–aadA1-2–aadA1-1, dfrA12–orfF–aadA2/aadA2–aadA1–cmlA1*, *dfrA12-orfF–aadA2/aadA1–cmlA1*, *dfrA12–orfF–aadA2/aadA1–cmlA1*, and *dfrA12–orfF–aadA2/1*. Third, *E. coli* isolates with simple cassette arrays performed poorly on MDR, including *dfrA12–orfF*, *dfrA1–aadA2*, *dfrA1–aadA1*, *aadA1–dfrA1*, *aadA1–aadA2*, *aadA2*, and *aadA1*. In addition, one isolate yielded two amplicons, 2,026 and 1,122 bp, and they formed into two arrays of *dfrA12–orfF–aadA2/aadA1–cmlA1* and *aadA2*. Another special array, *aadA16–aadA5–dfrA17* was found for the first time.

DISCUSSION

A Lower and Still Challenging Prevalence of Antimicrobial Resistance *E. coli* in Anhui, Hebei, Shanxi, and Shaanxi

In the present study, we reported the frequency of MDR and the incidence of class 1 integrons from nonclinical swine and were 69.7 and 74.6% in commensal *E. coli*, which was lower than those that had been reported among swine origin nonclinical *E. coli* in several provinces of China, such as Sichuan (99.23, 87.69%; Lin et al., 2014), Xiamen (100, 92.2%; Liu et al., 2015), Harbin (100, 91.67%; Zhang et al., 2013), Hulunbeir (77.8, 95.59%; Qiu, 2015), Liaoning (92.31, 71.43%; Zhao et al., 2017), and Yunnan (96.15, 84.62%; Li et al., 2019) between 2010 and 2019. This indicates that there is a clear difference in prevalence between the previous investigated districts and the four provinces. Antimicrobial application is responsible for emergence and spread of MDR intestinal bacteria in animal husbandry (Matjuda and Aiyegoro, 2016). Veterinary antimicrobials always have the most priority treatment, and some of them are often taken as growth promoters for swine fattening (Ochoa et al., 2016). Increasing diversity and abundance of the antimicrobial resistance in *E. coli* clinical isolates witnessed the extensive and excessive usage of veterinary antimicrobials in swine herds of 18 provinces in China

[1]http://www.ncbi.nlm.nih.gov/BLAST
[2]http://integrall.bio.ua.pt

TABLE 3 | Frequency of antimicrobial susceptibility and *intI1* gene in 264 *E. coli* isolates.

Antimicrobials	Numbers of *E. coli* isolates (%)				
	Nonsusceptible	Susceptible	*intI1+*[a] (*n* = 197)	*intI1–*[a] (*n* = 67)	*P*[b]
Doxycycline	200 (75.8)	64 (24.2)	138 (70.1)	28 (14.2)	0.000
Tetracycline	194 (73.5)	70 (26.5)	136 (69.0)	32 (16.2)	0.000
Sulfamethaxazole/ trimethoprim	189 (71.6)	75 (28.4)	132 (67.0)	20 (10.2)	0.005
Amoxicillin	180 (68.2)	84 (31.8)	131 (66.5)	14 (7.1)	0.000
Sulfasalazine	177 (67.1)	87 (32.9)	127 (64.5)	30 (15.2)	0.313
Ampicillin	153 (58.0)	111 (42.0)	105 (53.3)	7 (3.6)	0.000
Florfenicol	148 (56.1)	116 (43.9)	107 (54.3)	21 (10.7)	0.000
Streptomycin	140 (53.0)	124 (47.0)	75 (38.1)	10 (5.1)	0.000
Erythromycin	128 (48.5)	136 (51.5)	47 (23.9)	12 (6.1)	0.001
Enrofloxacin	113 (46.6)	141 (53.4)	74 (37.6)	12 (6.1)	0.003
Neomycin	89 (33.7)	175 (66.3)	55 (27.9)	4 (2.0)	0.000
Kanamycin	87 (33.0)	177 (67.0)	59 (29.9)	12 (6.1)	0.055
Cephalexin	86 (32.6)	178 (67.4)	50 (25.4)	0 (0.0)	0.000
Ciprofloxacin	63 (23.9)	201 (76.1)	34 (17.3)	1 (0.5)	0.001
Gentamicin	57 (21.6)	207 (78.4)	25 (12.7)	0 (0.0)	0.002
Norfloxacin	53 (21.1)	211 (79.9)	27 (13.7)	3 (1.5)	0.040
Amikacin	46 (17.4)	218 (82.6)	21 (10.7)	3 (1.5)	0.128
Cefotaxime	44 (16.7)	220 (83.3)	24 (12.2)	0 (0.0)	0.003
Imipenem	0 (0)	264 (100.0)	-[d]	-	-
Multiple Drugs[c]			155 (78.7)	29 (43.3)	0.000

[a]*intI1+ stands for intI1 gene-positive isolate and intI1– for intI1 gene-negative isolate.*
[b]*p means the difference between intI1-positive isolate and intI1-negative isolate. Chi square test and Fisher's exact test were used.*
[c]*Multiple drugs meant at least more than three antimicrobial agents.*
[d]*intI1 was not detected in E. coli isolates susceptible to imipenem.*

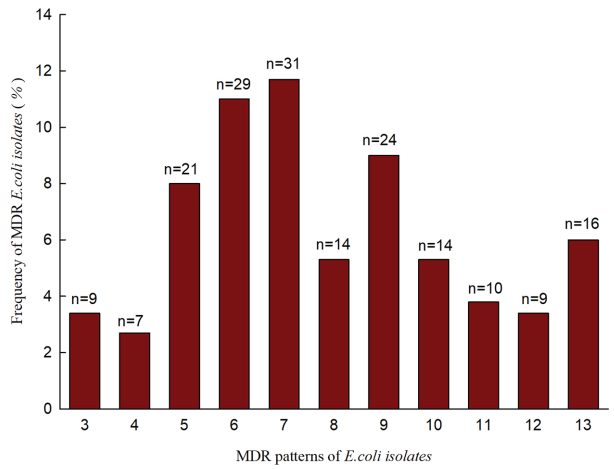

FIGURE 1 | Antimicrobial resistance pattern and frequency of 184 MDR *E. coli* isolates.

between 2010 and 2019 (Zhang et al., 2017, 2019a; Yang et al., 2019). Swine farming could lead to enhanced concentration levels of various veterinary antimicrobials and ARGs in groundwater and soils (Qiao et al., 2018). The increasing prevalence of MDR *E. coli* and other antimicrobial-resistant microorganisms causes unpredictable consequences to the environment and humans. In China, alternative strategies have been effectively explored to combat veterinary antimicrobial resistance, such as vaccination, chicken egg yolk antibodies (Zhang et al., 2011), lactic acid bacteria (Yang et al., 2015), antimicrobial peptides (Kang et al., 2017), bacteriocins (Qian et al., 2020), antibiotic adjuvants (Liu et al., 2019), phytocompounds (Chang et al., 2019), and metal-based nanoparticles (Ali et al., 2020). In addition, the implementation of management strategies has been done to reduce antimicrobial usage in animal husbandry, including good hygiene practice and biosecurity measures (Hu and Cowling, 2020). Therefore, it is possible that the frequency of MDR and the incidence of class 1 integrons in the present intensive farms are in the decline process due to good veterinary hygiene managements and reduced antimicrobial usage.

Phenotypic susceptibility testing showed that doxycycline, tetracycline, sulfamethaxazole/trimethoprim, amoxicillin, sulfasalazine, florfenicol, and ampicillin resistance are still challenging intensive farms in Anhui, Hebei, Shanxi, and Shaanxi. Many studies pointed out that *E. coli* strains from swine farms had formed high resistance to the first- or the second-generation antibiotics, and some of the original drugs had lost their antibacterial effect, such as streptomycin, tetracycline, cephalothin, ampicillin, ofloxacin, and sulfamethoxazole (Tang, 2014; Zhang et al., 2017). The evolution of *E. coli* antimicrobial resistance is closely correlated with the emergence and dissemination of specific ARGs (Jiang et al., 2017a; Divya and Hatha, 2019; Wang et al., 2019) and virulence genes (VGs; Wang et al., 2010; Cheng et al., 2020). This indicates that commensal *E. coli* in healthy swine, with resistance against doxycycline, tetracycline, sulfamethaxazole/ trimethoprim, amoxicillin, sulfasalazine, florfenicol, and ampicillin, may easily obtain antibiotic resistance genes and virulence genes that jeopardize swine health.

TABLE 4 | Distribution of multiple drug-resistant (MDR), class 1 integrons, and gene cassettes among *E. coli* isolates in Anhui, Hebei, Shanxi, and Shaanxi.

Characteristic	Isolate No. (%)				
	Anhui (*n* = 56)	Hebei (*n* = 30)	Shanxi (*n* = 78)	Shaanxi (*n* = 100)	Total (*n* = 264)
MDR	44 (78.6)	24 (80.0)	58 (74.4)	58 (58.0)	184 (69.7)
*Intl*1	50 (89.2)	25 (83.3)	58 (74.4)	64 (64.0)	197 (74.6)
MDR-*intl*1	40 (71.4)	19 (63.3)	52 (66.7)	44 (44.0)	155 (58.7)
*intl*1-gene cassette-MDR	19 (33.9)	11 (36.7)	10 (12.8)	30 (30.0)	70 (26.5)

Notably, 11 isolates were highly resistant to florfenicol for carrying the *cmlA* gene (in arrays of *dfrA12–orfF–aadA2–adA1–cmlA1*, *dfrA12–orfF–aadA2/aadA2–aadA1–cmlA1*, and *dfrA12–orfF–aadA2/aadA1–cmlA1*), which encodes a specific chloramphenicol transporter. Florfenicol, a fluorinated chloramphenicol derivative, has been widely used against both Gram-positive and Gram-negative bacteria (Belaynehe et al., 2018). Another notable antimicrobial agent is imipenem, belonging to the carbapenems antibiotics, which has a broader range of activity against Gram-negative and Gram-positive bacteria. The carbapenem-resistant genes (*blaOXA-48*, *blaGES-1*, *blaKPC-2*, and *blaNDM-1*) were considered to be the most harmful to human health (Yang et al., 2016). In China, KPC-2, NDM, and OXA-48-like carbapenemases were predominant among the carbapenem-resistant *Enterobacteriaceae* (CRE) isolates from adult and children patients (Han et al., 2020). However, in the present study, all *E. coli* isolates were susceptible to imipenem, and carbapenem-resistant genes were not detected by PCR. It demonstrates that the carbapenem-resistant genes did not exist or was extremely low in the investigated farms, which is consistent with the carbapenem-resistant pollution that is not severe in the drinking water of China (Xin et al., 2019).

Diverse Gene Cassette Arrays Are Common Among Intensive Farms

In the present study, the prevalence of MDR *E. coli* is closely associated with the presence of class 1 integrons. Our findings are in agreement with the previous studies that class 1 integrons are the most ubiquitous classes of integrons in the enteric bacteria (Stokes et al., 2001). According to the study of Yohann Lacotte, in the stress-free environmental settings of *E. coli*, class 1 integrons live a relaxed life at low-cost structure, which can favor their maintenance and prevalence in cassette networks (Lacotte et al., 2017). In the present study, the healthy swine gut meant a safe environment for the low-cost class 1 integrons, thus 74.6 % (*n* = 197) integrons were highly prevalent in common *E. coli* isolates. They can offer a wider platform for the acquisition, rearrangement, and expression of gene cassettes. In addition, isolates (*n* = 29) without integrons also showed MDR, suggesting that other determinants might contribute to their resistance. Farm animals and manure are a source of food-borne and water-borne human pathogens (Bailey et al., 2011). Being a DNA pollutant (Gillings, 2018), the abundance of *intI*1 ranged from 3.83×10^{-4} to 4.26×10^{0} *intI*1/cell in eight ecosystems, even in giant pandas (47%) and remote rural area animals (6.7%) in China (Ma et al., 2017; Rehman et al., 2017; Zou et al., 2018). Xia et al. reviewed that *E. coli* had the highest positive rates (65.4%) of integrons from human patients with Gram-negative bacteria isolates in China during 2000–2014 (Xia et al., 2016). It suggests that MDR *E. coli* with a high number of class 1 integrons were widely distributed in swine farms, also indicating a higher incidence of lateral gene-transfer events.

In the present study, 17 gene cassette arrays in the commensal *E. coli* isolates were more diverse than those that had been reported in different animals and their products during the past 10 years in China, Spain, and Australia. There are nine arrays that are summarized from swine farming settings and pork products (*dfrA1–aadA1*, *aadA22*, *dfr17–aadA5*, *dfrA12–orfF–aadA2*, *dfrXII–orfF–aadA2*, *aadA2*, *dfrA1–catB3–aacA4*, *aadB–aadA2*, and *dfrA12–aadA2–cmlA1–aadA1*; Chen, 2013; Wei, 2014; Zhang et al., 2017). Nine arrays were present in *E. coli* isolates from beef carcasses, including *linF–aadA2*, *dfrA17–aadA5*, *aadB–blaOXA-10*, *dfr12–orfF–aadA2*, *dfrA1–aadA1*, *dfrA12–aadA2*, *aadA2*, *dfr12*, and *aadB–aadA2* (Chen et al., 2017). Five arrays were in *E. coli* isolates from waterfowls (*dfrA1–orfC*, *aadA2*, *aadA1*, *dfrA1–aadA1*, and *dfrA1–orfC–aadA1*; Zhang et al., 2019b). The different arrangements of gene cassettes of *E. coli* isolates from healthy swine were not only reported in China but also in other countries. For instance, nine different gene cassette arrays were described in 393 intestinal *E. coli* isolates in Spain, with *dfrA1–aadA1* in a dominant position (Marchant et al., 2013). Ten gene cassette arrays were observed in 103 class 1 integron-positive *E. coli* from two commercial production facilities in New South Wales, Australia (Reid et al., 2017). These results highlighted the role of pork, beef, and poultry products as a potential source for MDR *E. coli* strains and the necessity for controlling animal product safety.

In this study, the common gene cassette arrays were still prevalent in MDR *E. coli*. High frequency of 11 simple gene arrays were found, including *dfrA17–aadA5*, *dfrA12–orfF–aadA2*, *dfrA1–aadA1*, *dfrA12–orfF*, *dfrA1–aadA2*, *aadA1–dfrA1–aadA2*, *aadA1–dfrA1*, *aadA1–aadA2*, and *aadA2*. Then, the complex gene cassette arrays exhibited more MDR patterns through multiple cassettes, hybrid cassettes, and recombination cassettes. Isolates could contain two, three, four, and five cassettes in a single array. The complexity of the cassette array is strongly correlated with MDR patterns and phenotype.

In addition, six isolates were identified carrying hybrid gene cassettes (gene cassettes arrays marked in lowercase letter b, in **Table 3**). Our results were in agreement with the reported array *dfrA12–orfF–aadA2* cassette, in which *aadA2* often was replaced by the corresponding part of the *aadA1* cassette (Alicia et al., 2005). Moreover, *aadA16–aadA5–dfrA17* was a novel array. To the best of our knowledge, this was the first time to report a relative higher incidence of complex cassettes prevailing in intensive-farming swine in China.

TABLE 5 | Characterization of cassette arrays and integron-associated antibiotic resistance in 70 isolates of *E. coli*.

	Gene cassette array	Resistance phenotype[a]	No. of antimicrobials	Amplicon size (bp)	Isolate number	sites	Frequency (*n* = 197,%)
1	*dfrA17–aadA5*	TET + AMC + **NEO** + AMP + DX + **SMZ/TMP**	6	1,778	5	Anhui	19 (9.6)
		ERY + TET + AMC + **NEO** + AMP + DX + **SMZ/TMP**[b]	7	1,780	11	Shaanxi	
		TET + **NEO** + ENR + DX + **STR** + NOR + **SMZ/TMP**	7	1,787	1	Shanxi	
		ERY + TET + AMC + **NEO** + **GEN** + DX + **SMZ/TMP**	7	1,794	1		
		ERY + TET + **STR**	3	1,794	1		
		ERY + TET + AMC + FF + CL + **STR**	6	2,035	1		
		ERY + TET + AMC + ENR + **GEN** + AMP + DX + **SMZ/TMP**	8	2,035	1		
2	*dfrA12–orfF–aadA2*	ERY + TET + AMC + ENR + DX + AMP + **SMZ/TMP**	7	2,036	4	Shaanxi	12 (6.0)
		ERY + TET + AMC + **NEO** + AMP + DX + **SMZ/TMP**[b]	7	2,037	5		
		STR + **NEO** + **AK** + AMC + AMP + CL + CIP + ENR + TET + DX + **SIZ** + **SMZ/TMP**	12	2,070	1	Hebei	
3	*dfrA12–orfF–aadA2–adA1–cmlA1*	**STR** + **NEO** + **AK** + AMC + AMP + CL + CIP + ENR + TET + **SMZ/TMP** + **SIZ** + ERY + **FF**	13	2,084	8	Anhui	8 (4.0)
4	*dfrA12–orfF–aadA2/aadA2–aadA1–cmlA1*[c]	TET + AMC + **FF** + **GEN** + AMP + DX + **SMZ/TMP**	7	2,054	2	Hebei	2 (1.0)
5	*dfrA12–orfF–aadA2/aadA1–cmlA1*[c]	ERY + TET + AMC + AMP + DX + **STR** + **SMZ/TMP**	7	2,036	1	Shaanxi	2 (1.0)
		TET + AMC + AMP + DX[d]	4	2,026[e]	1	Shanxi	
6	*dfrA12–orfF–aadA2/1*[c]	ERY + TET + AMC + **FF** + **GEN** + AMP + CL + DX + **STR** + CTX + **SMZ/TMP**	11	2,026	1		1 (0.5)
7	*dfrA12–orf F*	CL + **K** + **SMZ/TMP**	3	1,197	3	Hebei	3 (1.5)
8	*dfrA7–aadA1-2–aadA1-1*[c]	ERY + TET + AMC + **NEO** + **GEN** + AMP + ENR + DX + **STR** + **SMZ/TMP**	10	1,175	2	Shaanxi	2 (1.0)
9	*dfrA1–aadA2/1*[c]	TET + AMC + FF + DX + **SMZ/TMP**	5	1,735	4	Shanxi	5 (2.5)
		STR + **AK** + AMC + AMP + NOR + CL + ENR + TET + DX + SIZ + ERY + FF + **SMZ/TMP**	13	1,699	1		
10	*dfrA1–aadA2*	ERY + TET + **NEO** + DX + **STR**	5	1,685	4	Hebei	4 (2.0)
11	*dfrA1–aadA1*	TET + AMC + **NEO** + FF + **SMZ/TMP**	5	1,706	1	Anhui	1 (0.5)
12	*aadA1–dfrA1–aadA2*	ERY + TET + AMC + **GEN** + AMP + DX + **SMZ/TMP**	7	1,709	4		4 (2.0)
13	*aadA1–dfrA1*	ERY + TET + AMC + **NEO** + DX + NOR + **STR** + **SMZ/TMP**	8	1,699	2	Shanxi	2 (1.0)
14	*aadA2*	ERY + TET + AMC + **NEO** + **FF** + **GEN** + AMP + **K** + **STR** + **SMZ/TMP**	10	1,174	2	Shaanxi	2 (1.0)
		TET + AMC + AMP + DX[d]	4	1,122[e]	-	Shanxi	
15	*aadA16–aadA5–dfrA17*	ERY + TET + AMC + ENR + AMP + DX + **SMZ/TMP** + NOR	8	1,181	1		1 (0.5)
16	*aadA1–aadA2*	CL + CTX[d]	2	1,155	1	Hebei	1 (0.5)
17	*aadA1*	ERY + TET[d]	2	1,124	1	Anhui	1 (0.5)

[a]*Italic bold means phenotypic resistance could track their gene cassettes.*

[b]*Two different gene cassette arrays have the same phenotypic MDR patterns.*

[c]*aadA2/1 or A2/aadA1 hybrid harbored in these arrays.*

[d]*Gene array was not exhibited in their corresponding phenotype(s) of antimicrobial agents.*

[e]*Two amplicons were in the same isolate.*

Future Work Directions Complex Structure Arrays

We noticed that 12 isolates had a larger variable region containing four or five different gene cassettes, *dfrA12–orfF–aadA2–adA1–cmlA1* ($n = 8$), *dfrA12–orfF-aadA2/aadA2–aadA1–cmlA1* ($n = 2$), *dfrA12–orfF–aadA2/aadA1–cmlA1* ($n = 1$), and *dfrA12–orfF–aadA2/1* ($n = 1$). They were highly consistent with nonclassic integrons that Partridge (Partridge et al., 2009) summarized as the 5'-CS but not the typical 3'-CS or incomplete transposition (tni) region. Similar arrays have been reported in one *E. coli* isolate from retail meat (pork; Jiang et al., 2017b), two commensal *E. coli* strains from feces (Moran et al., 2016; Oliva et al., 2018), and three clinical *E. coli* strains from different sources (samples from patients, dogs, swine, food products, and environment; Antunes et al., 2007; Sa'enz et al., 2010; María et al., 2011; Siqueira et al., 2016). In addition, four gene arrays did not exhibit their corresponding MDR phenotypes. This may due to the fact that many would affect the expression of gene cassettes, such as the strict regulation of integrase expression, the strength of the Pc promoter, the gene cassette arrangement, and the antibiotic concentrations (Vinué et al., 2011). Therefore, our further work will focus on these special arrays and their roles in conferring antibiotic resistance.

In conclusion, this study reported that the prevalence of antimicrobial resistance and the presence of class 1 integrons of commensal *E. coli* in the present four provinces were lower than previously reported in other regions. A high number of commensal *E. coli* with class 1 integrons were widely distributed in investigated farms, indicating a higher incidence of lateral resistant gene-transfer events. What is more, a portion of commensal *E. coli* harboring diverse cassette arrays contributed to their MDR phenotypes. Our finding indicates that the nonclinical swine raised in intensive farm would be a reservoir of MDR *E. coli*, which is a potential health risk.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

KG and YZ conceived and designed the study. XZ performed the data analyses and wrote the manuscript. XL and JQ performed antimicrobial susceptibility testing and molecular experiments. WW, DW, and YL collected the samples and isolated *Escherichia coli*. LX reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

Ali, A., Ovais, M., Cui, X., Rui, Y., and Chen, C. (2020). Safety assessment of nanomaterials for antimicrobial applications. *Chem. Res. Toxicol.* 33, 1082–1109. doi: 10.1021/acs.chemrestox.9b00519

Alicia, M., Gestal, H., Stokes, W., Partridge, S. R., and Hall, R. M. (2005). Recombination between the dfrA12-orfF-aadA2 cassette array and an aadA1 gene cassette creates a hybrid cassette, aadA8b. *Appl. Environ. Microbiol.* 49, 4771–4774. doi: 10.1128/AAC.49.11.4771-4774.2005

Alonso, C., Zarazaga, M., Ben, S. R., Jouini, A., Ben, S. K., and Torres, C. (2017). Antibiotic resistance in *Escherichia coli* in husbandry animals: the African perspective. *Lett. Appl. Microbiol.* 64, 318–334. doi: 10.1111/lam.12724

Antunes, P., Machado, J., and Peixe, L. (2007). Dissemination of sul3-containing elements linked to class 1 integrons with an unusual 3' conserved sequence region among Salmonella isolates. *Antimicrob. Agents Chemother.* 51, 1545–1548. doi: 10.1128/AAC.01275-06

Bailey, K. G. B., Gaze, W. H., Zhang, L., Kay, P., Boxall, A., Hawkey, P. M., et al. (2011). Integron prevalence and diversity in manured soil. *Appl. Environ. Microbiol.* 77, 684–687. doi: 10.1128/AEM.01425-10

Barraud, O., and Ploy, M. C. (2015). Diversity of class 1 integron gene cassette rearrangements selected under antibiotic pressure. *J. Bacteriol.* 197, 2171–2178. doi: 10.1128/JB.02455-14

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

Belaynehe, K. M., Shin, S. W., and Yoo, H. S. (2018). Interrelationship between tetracycline resistance determinants, phylogenetic group affiliation and carriage of class 1 integrons in commensal *Escherichia coli* isolates from cattle farms. *BMC Vet. Res.* 14:340. doi: 10.1186/s12917-018-1661-3

Chang, F., Shen, S., Shi, P., Zhang, H., Ye, L., Zhou, Q., et al. (2019). Antimicrobial resins with quaternary ammonium salts as a supplement to combat the antibiotic resistome in drinking water treatment plants. *Chemosphere* 221, 132–140. doi: 10.1016/j.chemosphere.2019.01.047

Chen, C. (2013). Multi-antibiotics resistance and integron gene cassette system analysis of *E. coli* isolated from pork production chain. Master's degree dissertation (in Chinese), Sichuan Agricultural University

Chen, C., Ke, S., Li, C., Wu, Y., Chen, T., Lai, C., et al. (2017). High diversity of antimicrobial resistance genes, class 1 integrons, and genotypes of multidrug-resistant *Escherichia coli* in beef carcasses. *Microb. Drug Resist.* 23, 915–924. doi: 10.1089/mdr.2016.0223

Cheng, P., Yang, Y., Zhang, J., Li, F., Li, X., Liu, H., et al. (2020). Antimicrobial resistance and virulence profiles of mcr-1-positive *Escherichia coli* isolated from swine farms in Heilongjiang Province of China. *J. Food Prot.* doi: 10.4315/jfp-20-190 [Epub ahead of print]

CLSI (2017). Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing: Twentieth-third Informational Supplement M100-S23. Wayne, PA, USA: Clinical and Laboratory Standards Institute.

Divya, S., and Hatha, A. (2019). Screening of tropical estuarine water in south-west coast of India reveals emergence of ARGs-harboring hypervirulent *Escherichia coli* of global significance. *Int. J. Hyg. Environ. Health* 222, 235–248. doi: 10.1016/j.ijheh.2018.11.002

Ge, B., Domesle, K., Gaines, S., Lam, C., Bodeis Jones, S., Yang, Q., et al. (2020). Prevalence and antimicrobial susceptibility of Indicator organisms Escherichia coli and Enterococcus spp. isolated from U.S. animal food, 2005-2011. *Microorganisms* 8:1048. doi: 10.3390/microorganisms8071048

Gillings, M. R. (2018). DNA as a pollutant: the clinical class 1 integron. *Curr. Pollut. Rep.* 4, 49–55. doi: 10.1007/s40726-018-0076-x

Han, R., Shi, Q., Wu, S., Yin, D., Peng, M., Dong, D., et al. (2020). Dissemination of Carbapenemases (KPC, NDM, OXA-48, IMP, and VIM) among

carbapenem-resistant *Enterobacteriaceae* isolated from adult and children patients in China. *Front. Cell. Infect. Microbiol.* 10:314. doi: 10.3389/fcimb.2020.00314

Holcomb, D., and Stewart, J. (2020). Microbial indicators of fecal pollution: recent progress and challenges in assessing water quality. *Current Environ. Health Rep.* 7, 311–324. doi: 10.1007/s40572-020-00278-1

Hu, Y., and Cowling, B. (2020). Reducing antibiotic use in livestock, China. *Bull. World Health Organ.* 98, 360–361. doi: 10.2471/blt.19.243501

Jarlier, V., Högberg, L. D., Heuer, O. E., Campos, J., and Simonsen, G. S. (2019). Strong correlation between the rates of intrinsically antibiotic-resistant species and the rates of acquired resistance in gram-negative species causing bacteraemia, EU/EEA, 2016. *Euro Surveill.* 24:1800538. doi: 10.2807/1560-7917.ES.2019.24.33.1800538

Jiang, X., Ellabaan, M., Charusanti, P., Munck, C., Blin, K., Tong, Y., et al. (2017a). Dissemination of antibiotic resistance genes from antibiotic producers to pathogens. *Nat. Commun.* 8:15784. doi: 10.1038/ncomms15784

Jiang, X., Xu, Y., Li, Y., Zhang, K., Liu, L., Wang, H., et al. (2017b). Characterization and horizontal transfer of qacH-associated class 1 integrons in *Escherichia coli* isolated from retail meats. *Int. J. Food Microbiol.* 258, 12–17. doi: 10.1016/j.ijfoodmicro.2017.07.009

José, A. E., Loot, C., and Mazel, D. (2018). "Integrons as adaptive devices" in *Molecular mechanisms of microbial evolution. Grand challenges in biology and biotechnology*. ed. P. H. Rampelotto (Cham: Springer).

Kang, W., Liu, H., Ma, L., Wang, M., Wei, S., Sun, P., et al. (2017). Effective antimicrobial activity of a peptide mutant Cbf-14-2 against penicillin-resistant bacteria based on its unnatural amino acids. *Eur. J. Pharm. Sci.* 105, 169–177. doi: 10.1016/j.ejps.2017.05.030

Lacotte, Y., Cécile, P. M., and Sophie, R. (2017). Class 1 integrons are low-cost structures in *Escherichia coli*. *ISME J.* 11, 1535–1544. doi: 10.1038/ismej.2017.38

Li, F., Liao, D., Li, N., Miao, H., Song, J., Zhao, W., et al. (2019). Correlations between integron gene cassttes and antimicrobial ressitancen of pathogenic *Escherichia coli* isolated from pigs. *China J. Vet. Sci.* 39, 228–234. doi: 10.16303/j.cnki.1005-4545.2019.02.07

Lin, J., Shu, G., Zhang, H., Cao, S., and Wen, X. (2014). Detection of resistance and characterization of integron-cassettes in *Escherichia coli* from intestinal tract of healthy food-animals, enteric strains. *Chinese J. Vet. Sci.* 34, 55–60. doi: 10.16303/j.cnki.1005-4545.2014.01.011

Liu, Y., Li, R., Xiao, X., and Wang, Z. (2019). Antibiotic adjuvants: an alternative approach to overcome multi-drug resistant gram-negative bacteria. *Crit. Rev. Microbiol.* 45, 301–314. doi: 10.1080/1040841x.2019.1599813

Liu, Z., Zhang, Z., Yan, H., Li, J., and Shi, L. (2015). Isolation and molecular characterization of multidrug-resistant Enterobacteriaceae strains from pork and environmental samples in Xiamen, China. *J. Food Prot.* 78, 78–88. doi: 10.4315/0362-028X.JFP-14-172

Ma, L., Li, A. D., Yin, X. L., and Zhang, T. (2017). The prevalence of integrons as the carrier of antibiotic resistance genes in natural and man-made environments. *Environ. Sci. Technol.* 51, 5721–5728. doi: 10.1021/acs.est.6b05887

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

Marchant, M., and Morenoa, M. A. (2013). Dynamics and diversity of *Escherichia coli* in animals and system management of the manure on a commercial farrow-to-finish pig farm. *Appl. Environ. Microbiol.* 79, 853–859. doi: 10.1128/AEM.02866-12

Marchant, M., Vinué, L., Torres, C., and Moreno, M. (2013). Change of integrons over time in *Escherichia coli* isolates recovered from healthy pigs and chickens. *Vet. Microbiol.* 163, 124–132. doi: 10.1016/j.vetmic.2012.12.011

María de, T. H., Yolanda, D., Mansilla, E. C., Bezares, B. R., and Marta, G. C. (2011). Genetic characterization of the mechanisms of resistance to amoxicillin/clavulanate and third-generation cephalosporins in *Salmonella enterica* from three Spanish hospitals. *Int. Microbiol.* 14, 173–181. doi: 10.2436/20.1501.01.146

Matjuda, D. S., and Aiyegoro, O. A. (2016). Soil bacteriological pollution in pig farm vicinity: assessment of bacterial dynamics and detection of antimicrobial resistance gene. *Afr. J. Microbiol. Res.* 10, 1625–1636. doi: 10.5897/AJMR2016.8138

Moran, R. A., Holt, K. E., and Hall, R. M. (2016). pCERC3 from a commensal ST95 *Escherichia coli*: a ColV virulence-multiresistance plasmid carrying a sul3-associated class 1 integron. *Plasmid* 84, 11–19. doi: 10.1016/j.plasmid.2016.02.002

NBSC (2019). China Statistical Yearbook [Online]. Available: http://www.stats.gov.cn/tjsj/ndsj/2019/indexch.htm (Accessed September 22, 2020).

Ochoa, T. J., Duarte, G., and Oscar, G. (2016). "Antibiotic resistance in *Escherichia coli*" in *Escherichia coli in the Americas*. ed. A. G. Torres (Cham: Springer International Publishing).

Oliva, M., Monno, R., Addabbo, P., Pesole, G., Scrascia, M., Calia, C., et al. (2018). IS26 mediated antimicrobial resistance gene shuffling from the chromosome to a mosaic conjugative FII plasmid. *Plasmid* 100, 22–30. doi: 10.1016/j.plasmid.2018.10.001

Olivier, B., Marianne, P., Elodie, C. D., Delphine, C., Claire, B., Vincent, G., et al. (2018). Antibiotic resistance acquisition in the first week of life. *Front. Microbiol.* 9:1467. doi: 10.3389/fmicb.2018.01467

Paitan, Y. (2018). Current trends in antimicrobial resistance of *Escherichia coli*. *Curr. Top. Microbiol. Immunol.* 416, 181–211. doi: 10.1007/82_2018_110

Partridge, S., Guy, T., Enrico, C., and Jonathan, R. I. (2009). Gene cassettes and cassette arrays in mobile resistance integrons. *FEMS Microbiol. Rev.* 33, 757–784. doi: 10.1111/j.1574-6976.2009.00175.x

Partridge, S. R., Kwong, S. M., Firth, N., and Jensen, S. O. (2018). Mobile genetic elements associated with antimicrobial resistance. *Clin. Microbiol. Rev.* 31, e00088–e00017. doi: 10.1128/CMR.00088-17

Qian, Z., Zhao, D., Yin, Y., Zhu, H., and Chen, D. (2020). Antibacterial activity of lactobacillus strains isolated from Mongolian yogurt against *Gardnerella vaginalis*. *Biomed. Res. Int.* 2020:3548618. doi: 10.1155/2020/3548618

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

Qiu, J. (2015). "Incidence investigation and prevention of swine colibacillosis in Hulunbeir" in *Dissertation for the veterinary doctoral degree (in Chinese)*. Northeast Agricultural University.

Rehman, M. U., Zhang, H., Huang, S., Iqbal, M. K., Mehmood, K., Luo, H., et al. (2017). Characteristics of integrons and associated gene cassettes in antibiotic-resistant *Escherichia coli* isolated from free-ranging food animals in China. *J. Food Sci.* 82, 1902–1907. doi: 10.1111/1750-3841.13795

Reid, C., Wyrsch, E., Roy, C., Zingali, T., Liu, M., Darling, A., et al. (2017). Porcine commensal *Escherichia coli*: a reservoir for class 1 integrons associated with IS26. *Microb. Genom.* 3:e000143. doi: 10.1099/mgen.0.000143

Sa'enz, Y., Vinue´, L., Ruiz, E., Somalo, S., Martı´nez, S., Rojo-Bezares, B., et al. (2010). Class 1 integrons lacking qacEΔ1 and sul1 genes in *Escherichia coli* isolates of food, animal and human origins. *Vet. Microbiol.* 144, 493–497. doi: 10.1016/j.vetmic.2010.01.026

Siqueira, A. K., Michael, G. B., Domingos, D. F., Ferraz, M. M., Ribeiro, M. G., Schwarz, S., et al. (2016). Diversity of class 1 and 2 integrons detected in *Escherichia coli* isolates from diseased and apparently healthy dogs. *Vet. Microbiol.* 194, 79–83. doi: 10.1016/j.vetmic.2016.05.005

Stalder, T., Barraud, O., Casellas, M., Dagot, C., and Ploy, M. (2012). Integron involvement in environmental spread of antibiotic resistance. *Front. Microbiol.* 3:119. doi: 10.3389/fmicb.2012.00119

Stokes, H. W., Holmes, A. J., Nield, B. S., Holley, M. P., Nevalainen, H. K. M., Mabbutt, B. C., et al. (2001). Gene cassette PCR: sequence-independent recovery of entire genes from environmental DNA. *Appl. Eniviron. Microbiol.* 67, 5240–5246. doi: 10.1128/AEM.67.11.5240-5246.2001

Tang, Q. (2014). Study on the correlation between the drug-resistance of Escherichia coli and drug use in pig farms. Doctoral dissertation. Hunan Agricultural University.

Timothy, M. G., Jemma, L. G., John, A., and Gillings, M. (2019). High diversity and rapid spatial turnover of integron gene cassettes in soil. *Environ. Microbiol.* 21, 1567–1574. doi: 10.1111/1462-2920.14551

Van den Meersche, T., Rasschaert, G., Vanden Nest, T., Haesebrouck, F., Herman, L., Van Coillie, E., et al. (2020). Longitudinal screening of antibiotic residues, antibiotic resistance genes and zoonotic bacteria in soils fertilized with pig manure. *Environ. Sci. Pollut. Res. Int.* 27, 28016–28029. doi: 10.1007/s11356-020-09119-y

Vinué, L., Jové, T., Torres, C., and Ploy, M. C. (2011). Diversity of class 1 integron gene cassette pc promoter variants in clinical *Escherichia coli* strains and description of a new P2 promoter variant. *Int. J. Antimicrob. Agents* 38, 526–529. doi: 10.1016/j.ijantimicag.2011.07.007

Wang, X., Jiang, H., Liao, X., Liu, J., Zhang, W., Zhang, H., et al. (2010). Antimicrobial resistance, virulence genes, and phylogenetic background in *Escherichia coli* isolates from diseased pigs. *FEMS Microbiol. Lett.* 306, 15–21. doi: 10.1111/j.1574-6968.2010.01917.x

Wang, L., Wang, J., Wang, J., Zhu, L., Yang, L., and Yang, R. (2019). Distribution characteristics of antibiotic resistant bacteria and genes in fresh and composted manures of livestock farms. *Sci. Total Environ.* 695:133781. doi: 10.1016/j.scitotenv.2019.133781

Wei, W. (2014). The establishment of multiplex PCR for porcine Enterotoxingenitc *Escherichia coli* and analysis of integron which mediated it multiresistance. Master's doctoral dissertation. Jiangxi Agricultural University.

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

Xia, W., Xu, T., Qin, T., Liu, P., Liu, Y., Kang, H., et al. (2016). Characterization of integrons and novel cassette arrays in bacteria from clinical isloates in China, 2000-2014. *J. Biomed. Res.* 30, 292–303. doi: 10.7555/jbr.30.20150153

Xin, R., Zhang, K., Wu, N., Zhang, Y., and Niu, Z. (2019). The pollution level of the Bla carbapenemase gene in coastal water and its host bacteria characteristics. *Environ. Pollut.* 244, 66–71. doi: 10.1016/j.envpol.2018.10.023

Yang, F., Hou, C., Zeng, X., and Qiao, S. (2015). The use of lactic acid bacteria as a probiotic in swine diets. *Pathogens* 4, 34–45. doi: 10.3390/pathogens4010034

Yang, F., Mao, D., Zhou, H., and Luo, Y. (2016). Prevalence and fate of carbapenemase genes in a wastewater treatment plant in northern China. *PLoS One* 11:e0156383. doi: 10.1371/journal.pone.0156383

Yang, F., Zhang, K., Zhi, S., Li, J., Tian, X., Gu, Y., et al. (2019). High prevalence and dissemination of β-lactamase genes in swine farms in northern China. *Sci. Total Environ.* 651, 2507–2513. doi: 10.1016/j.scitotenv.2018.10.144

Zhang, S., Abbas, M., Rehman, M., Huang, Y., Zhou, R., Gong, S., et al. (2020b). Dissemination of antibiotic resistance genes (ARGs) via integrons in *Escherichia coli*: a risk to human health. *Environ. Pollut.* 266:115260. doi: 10.1016/j.envpol.2020.115260

Zhang, B., Ku, X., Yu, X., Sun, Q., Wu, H., Chen, F., et al. (2019a). Prevalence and antimicrobial susceptibilities of bacterial pathogens in Chinese pig farms from 2013 to 2017. *Sci. Rep.* 9, 1–11. doi: 10.1038/s41598-019-45482-8

Zhang, P., Shen, Z., Zhang, C., Song, L., Wang, B., Shang, J., et al. (2017). Surveillance of antimicrobial resistance among *Escherichia coli* from chicken and swine, China, 2008–2015. *Vet. Microbiol.* 203, 49–55. doi: 10.1016/j.vetmic.2017.02.008

Zhang, S., Wang, S., Li, H., and Li, L. (2011). Vitellogenin, a multivalent sensor and an antimicrobial effector. *Int. J. Biochem. Cell Biol.* 43, 303–305. doi: 10.1016/j.biocel.2010.11.003

Zhang, X., Wang, L., Xu, G., Yue, J., Su, H., Zhang, L., et al. (2013). Inspection of multidrug resistant *Escherichia coli* conjuagal plasmid and derived from swine in Harbin. *J. Northeast Agric. Univ.* 44, 40–45.

Zhang, K., Xin, R., Zhao, Z., Ma, Y., Zhang, Y., and Niu, Z. (2020a). Antibiotic resistance genes in drinking water of China: occurrence, distribution and influencing factors. *Ecotoxicol. Environ. Saf.* 188:109837. doi: 10.1016/j.ecoenv.2019.109837

Zhang, S., Yang, H., Rehman, M., Yang, K., Dong, M., Yang, J., et al. (2019b). Class 1 integrons as predominant carriers in *Escherichia coli* isolates from waterfowls in Hainan, China. *Ecotoxicol. Environ. Saf.* 183:109514. doi: 10.1016/j.ecoenv.2019.109514

Zhao, F., Zhu, Q., Guan, M., ChunJing, Wang, Z. Cao, D., et al. (2017b). Investigation and analysis of whole carrying integrons of *Escherichia coli* from swine in Liaoning area. Progress in Veterinary Medicine (Chinese with English abstract).

Zou, W., Li, C., Yang, X., Wang, Y., Cheng, G., Zeng, J., et al. (2018). Frequency of antimicrobial resistance and integron gene cassettes in *Escherichia coli* isolated from giant pandas (*Ailuropoda melanoleuca*) in China. *Microb. Pathog.* 116, 173–179. doi: 10.1016/j.micpath.2018.01.034

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Complete Genomic Analysis of VRE From a Cattle Feedlot: Focus on 2 Antibiotic Resistance

*Frank Eric Tatsing Foka[1], Charlotte Mienie[2], Cornelius Carlos Bezuidenhout[2] and Collins Njie Ateba[1]**

[1] Food Security and Safety Niche Area, Faculty of Natural and Agricultural Sciences, North-West University, Mmabatho, South Africa, [2] Unit for Environmental Sciences and Management, North-West University, Potchefstroom, South Africa

Practices in intensive animal farming such as the extensive use of antimicrobials have significant impacts on the genetic make-up of bacterial communities, especially on that of human/animal commensals. In this report, whole genome sequencing of two vancomycin-resistant enterococci (VRE) isolates from a cattle feedlot in the North West Province, South Africa, was used to highlight the threats that extensive antimicrobial usage in intensive animal rearing represents for environmental microbiomes and the food chain. The genomic DNA of the studied strains was extracted using a DNA extraction kit. Whole-genome sequencing was performed through next-generation sequencing. The genomes of *Enterococcus durans* strain NWUTAL1 and *Enterococcus gallinarum* strain S52016 consisted of 3,279,618 and 2,374,946 bp, respectively with G + C contents of 40.76 and 43.13%, respectively. Antibiotic resistance genes (ARG), plasmids and virulence factors (involved in biofilm formation, colonization and copper/silver efflux system), were detected in the genomes of both strains. The presence of these genetic determinants in the studied strains is a cause for concern as they may disseminate and find their way into the food chain via horizontal gene transfer amongst bacteria of the different ecological niches. Issues of this nature cannot be undermined and are relevant as far as food safety is concerned.

Keywords: vancomycin-resistant enterococci, *E. durans* strain NWUTAL1, *E. gallinarum* strain S52016, whole-genome sequencing, food safety

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***Correspondence:**
Collins Njie Ateba
collins.ateba@nwu.ac.za

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INTRODUCTION

The discovery of antibiotics was a significant hallmark in the evolution of mankind as they became important life-saving compounds both for animals and humans (Gonzalez-Zorn and Escudero, 2012). In fact, antimicrobials have impacted significantly on society and the health of humans and animals mainly because life expectancy could be ameliorated as common infections have become curable, thus promoting rapid growth of the population (Gonzalez-Zorn and Escudero, 2012). Unfortunately, as the therapeutic effects of antibiotics were discovered, their growth-promoting attributes became apparent, resulting in the extensive use of these agents as growth promoters in intensive animal rearing (Acar et al., 2012; Economou and Gousia, 2015). According to Marshall and Levy (2011), subtherapeutic doses of certain antibiotics that are used as growth promoters improve feed conversion, animal growth and diminish mortality and morbidity rates that arise

from clinical and subclinical diseases. However, the mechanism through which this is achieved is unclear (Marshall and Levy, 2011). Consequently, multidrug resistant isolates have emerged not only because of the abusive use of antibiotics/antimicrobials in communities and clinics, but mostly because of widespread use of antimicrobials in industrial animal farming (Boxall et al., 2002; Acar et al., 2012). However, there are studies that highlight that resistant bacteria and resistance mechanisms were present long before antibiotics were produced or used in clinical practise (Boxall et al., 2002; Acar et al., 2012).

A significant consequence of the widespread use of antibiotics in industrial animal farming is the presence of genetic resistance determinants in the environment and its ecological niches (Ding et al., 2014). This also results from the fact that antibiotics are not totally degraded into inactive compounds in the body of treated animals and excreted with feces in manure where they regain their initial molecular structure after some time (Forsberg et al., 2014). The manure becomes a hotspot for resistance determinants, which when mixed with soil, genetic material is transferred to other bacteria of the soil (Forsberg et al., 2014; Thanner et al., 2016). Moreover, as a result of agricultural lands runoffs, water bodies become contaminated with resistant strains that exchange genetic material with other commensals present in the water bodies which may eventually find their way into the food chain (Economou and Gousia, 2015).

Enterococci are commensals of the gastrointestinal tract of warm-blooded animals. Enterococci have the ability to cause illnesses both in animals and immunocompromized individuals. In fact, they can cause endocarditis, septicemia, urinary tract infections, burn wound and deep tissue infections in humans meanwhile they are responsible for intramammary infections and clinical mastitis in cattle (Myllys and Rautala, 1995; Bager et al., 1999; Aarestrup et al., 2000). Vancomycin-resistant enterococci (VRE) emerged four decades ago due to the misuse of avoparcin (a glycopeptide analog of vancomycin) as a growth promoter in intensive animal rearing and the abuse of vancomycin in clinics for the therapeutic management of community-acquired enterococcal infections (Myllys and Rautala, 1995; Bager et al., 1999). Since then, avoparcin has been banned in intensive animal farming (Bager et al., 1999). However, the constant detection of VRE worldwide (Arthur et al., 1996; Depardieu et al., 2004; Courvalin, 2006; Sundermann et al., 2019; Tatsing and Ateba, 2019) is indicative of the fact that factors other than avoparcin may be the source of the dissemination of VRE in the environment. Resistance to vancomycin can be either intrinsic or acquired. Eight types of vancomycin resistance gene clusters have been characterized so far (*vanA*, *vanB*, *vanC*, *vanD*, *vanE*, *vanG*, *vanL*, *vanM*, and *vanN*) (Depardieu et al., 2004).

Although there are several studies on the detection of antibiotic resistant strains such as VRE worldwide (Myllys and Rautala, 1995; Arthur et al., 1996; Depardieu et al., 2004; Courvalin, 2006; Tatsing and Ateba, 2019), there is a need to investigate the possible effects that practices such as the misuse of antimicrobials/antibiotics in industrial animal farming facilities, have on the genetic constitution of environmental bacteria and, consequently, on the different ecological niches of the environment and the food chain. A decade ago, whole genome sequencing (WGS) technologies were introduced in epidemiological studies, thus generating huge amounts of relevant data. WGS has been used since then to decode the genetic constitution of a considerable number of enterococcal species from various sources, thereby putting in the spotlight, genetic determinants involved in antibiotic resistance as well as those involved in pathogenesis processes which were previously less studied (Rangel et al., 2019; Sundermann et al., 2019). As WGS tools were gradually used in epidemiological investigations, *Enterococcus faecium* and *Enterococcus faecalis* have become the most studied enterococci, disregarding other supposedly harmless species such as *Enterococcus durans* and *Enterococcus gallinarum*, which have evolved into highly resistant strains with time (Rogers et al., 1992; Taucer-Kapteijin et al., 2016; Tatsing and Ateba, 2019). Since *E. durans* and *E. gallinarum* are mostly associated with environmental samples, less focus has been given to these species, due to which their whole genomic data are insufficient as compared to those of *E. faecalis* and *E. faecium* strains (Rogers et al., 1992; Jenney et al., 2000).

The aim of the study was to analyze the whole genomes of two vancomycin-resistant enterococcal strains, specifically *E. durans* NWUTAL1 and *E. gallinarum* S52016 isolated from a feedlot (cattle feces and soil, respectively) and further, demonstrate the impact of antimicrobial usage in animal farming on the genetic constitution of these strains (by evaluating their genomic diversity as well as their resistome) and the risk that such strains represent for food safety.

MATERIALS AND METHODS

Bacterial Strains

Two vancomycin-resistant strains, *E. durans* NWUTAL1 was recovered from fecal samples obtained from cattle while *E. gallinarum* S52016 was recovered from samples obtained from feedlot soil in Rooigrond, North-West Province, South Africa (Tatsing and Ateba, 2019), and stored at −80°C in Luria-Bertani broth supplemented with 50% (v/v) glycerol. These isolates were resistant to tetracycline (TET–30 μg), ampicillin (AMP–10 μg), amoxicillin (AMX–10 μg), vancomycin (VAN–30 μg), penicillin (PEN–10 μg), linezolid (LIN–30 μg), and erythromycin (ERY–15 μg) thus the multi-drug phenotypes were TET^R-AMP^R-AMX^R-VAN^R-PEN^R-LIN^R-ERY^R. They also harbored resistant determinants *vanA*, *vanB*, *vanC*, *tetK*, *tetL*, *msrA/B*, and *mefA* as well as the virulence genes *cylA*, *hyl*, *esp*, *gelE*, and *asa1*. The identities of the *E. durans* strain NWUTAL1 and *E. gallinarum* strain S52016 were confirmed in a previous study (Tatsing and Ateba, 2019; Tatsing, 2020) and their 16S rRNA gene sequences were deposited in GeneBank with accession numbers MK086097 and MK086099, respectively.

Genomic DNA Extraction and Detection of Vancomycin-Resistant Enterococci (VREs)

Pure *E. durans* strain NWUTAL1 and *E. gallinarum* strain S52016 colonies were revived by sub-culturing on nutrient agar.

Pure colonies were inoculated in 20 ml brain heart infusion broth (BHI, Merck, South Africa) and incubated overnight at 37°C. Bacteria cells were harvested through centrifugation. Genomic DNA was extracted with a DNA extraction kit (Zymo Research Genomic DNATM–Tissue MiniPrep Kit, ZR Corp. Irvine, United States) and quantified using a NanoDrop TM 1000 spectrophotometer (Thermo Fischer Scientific, United States).

Sequencing and Library Preparation of Whole Genome

The draft genomes were obtained through WGS using an Illumina Miseq platform. 1 ng of the genomic DNA was tagmented with the Nextera XT DNA library prep kit according to the manufacturer's protocol. The kit reagents fragment the DNA with simultaneous addition of adapter sequences. The libraries were amplified with a limited-cycle PCR program (12 cycles) to add the index 1 (i7) and index 2 (i5) adapters, containing sequences required for cluster generation of the Illumina flow cell. The library was purified using 0.6× Agencourt AMPure XP beads (Beckman Coulter). The quality and sizes of the resulting DNA fragments were evaluated on a 1.5% (w/v) agarose gel. The libraries were quantified with a fluorometric method (Qubit, Life Technologies) and normalized to 4 nM using a standard dilution method. The libraries were pooled, denatured with 0.1 N NaOH and diluted to the final loading concentration of 12 pmol. An identically treated PhiX Control v3 adapter-ligated library at low-concentration spike-in of 1% was added as an in-lane positive control for alignment calculations and quantification efficiency. Paired-end sequencing was done on an Illumina MiSeq platform using a MiSeq Reagent Kit V3 600 cycles.

Sequence Quality Checking, Trimming and Assembly

Sequence data from Illumina platform were extracted and uploaded on Kbase. The quality of the raw sequences reads were assessed with FastQC (v0.11.5) (Wingett and Andrews, 2018). Low quality sequences and adapters were removed with Trimmomatic (v0.36) (Bolger et al., 2014). The sequences reads were *de novo* assembled using SPAdes (v3.13.0) (Bankevich et al., 2012).

Genome Annotation and Comparative Analysis

The genomes of our strains of interest were annotated using Prokka (v1.12) (Seemann, 2014), RAST (v0.11) (Overbeek et al., 2014) and the NCBI prokaryotic genome annotation pipeline (Tatusova et al., 2015). Algorithms of the Pathosystems Resource Integration Center (PATRIC 3.5.41) (Wattam et al., 2017), ResFinder (v3.1.0) (Zankari et al., 2012) and PlasmidFinder (v2.0) (Carattoli et al., 2014) were used to assess the resistome, plasmids and virulence factors in the draft genomes. The Genome Annotation Service in PATRIC uses k-mer-based Antibiotic resistance genes (ARG) detection method, which utilizes PATRIC's curated collection of representative ARG sequence variants and assigns to each ARG, functional annotation, broad mechanism of antibiotic resistance, drug class and, in some cases, specific antibiotic it confers resistance to. CGView server was used to generate a circular map of the genomes (Grant and Stothard, 2008). The phylogenetic relationships with other strains of the respective species of interest were also assessed with PATRIC (v3.5.41) (Wattam et al., 2017). Finally, the presence of clustered regularly interspaced short palindromic repeats (CRISPR) and bacteriophages in the draft genomes of interest were assessed with CRISPRFinder (Grissa et al., 2007) and PHASTER (Arndt et al., 2016).

Reference genomes from NCBI were used by PATRIC algorithms to generate a phylogenetic tree. The closest reference and representative genomes to our strains of interest were identified by Mash/MinHash (Ondov et al., 2016). PGfams were selected from these genomes to determine the phylogenetic placement of our genomes of interest. The protein sequences from these families were aligned with MUSCLE (Edgar, 2004), and the nucleotides for each of these sequences were mapped to the protein alignment. The joint set of amino acid and nucleotide alignments were concatenated into a data matrix, and RaxML (Stamatakis, 2014) was used to analyze this matrix with fast bootstrapping (Stamatakis et al., 2008) in order to generate the support values in the phylogenetic tree.

Data Analysis

Statistica 13 (StatSoft, TIBCO software Inc., United States) was utilized to organize and interpret the data generated in this study.

RESULTS

Genomic Assembly Features of *E. durans* NWUTAL1 and *E. gallinarum* S52016

VR *E. durans* Strain NWUTAL1 was recovered from fecal samples obtained from cattle while VR *E. gallinarum* strain S52016 was recovered from samples obtained from feedlot soil. Both strains possessed *vanC* resistance gene and their genome sequences were submitted to NCBI GenBank. Data derived from the assembly and the annotation of the genomes studied are summarized in **Table 1**. The genomes have 3,517 versus 2,351 protein coding sequences, respectively, 59 versus 30 transfer RNA sequences, respectively and 4 versus 5 ribosomal RNA sequences, respectively for strains NWUTAL1 and S52016.

Moreover, no miscellaneous RNA sequences were detected in these genomes (**Table 1**).

Genomic Annotation of Strains NWUTAL1 and S52016

Protein Features of Strains NWUTAL1 and S52016

Annotation generated data that included hypothetical proteins and proteins with functional assignments are shown in **Table 2**. Proteins with functional assignment included proteins with enzyme commission (EC) numbers, those with gene ontology (GO) assignments and those mapping on KEGG pathways. Annotation with PATRIC included two types of

TABLE 1 | Assembly reports of *E. durans* NWUTAL1 and *E. gallinarum* S52016 genomes.

Features	*E. durans* NWUTAL1	*E. gallinarum* S52016
Genome size (bp)	3,279,618	2,374,946
DNA G + C content	40.76%	43.13%
Number of contigs	747	18
Contig N50	7,961	288,028
Contig L50	92	4
CDS	3,517	2,351
tRNA	59	30
rRNA	4	5
Partial CDS	0	0
Miscellaneous RNA	0	0
Chromosomes	Present	Present

TABLE 2 | Protein features of *E. durans* NWUTAL1 and *E. gallinarum* S52016.

Protein features	*E. durans* NWUTAL1	*E. gallinarum* S52016
Hypothetical proteins	934	507
Proteins with functional assignments	2,583	1,844
Proteins with EC number assignments	833	619
Proteins with GO assignments	684	487
Proteins with pathway assignments	554	429
Proteins with PLfam assignments	3,082	2,168
Proteins with PGfam assignments	3,246	2,262

protein families: those of the genus-specific protein families (PLfams) and those belonging to the cross-genus protein family (PGfams). The protein features of the studied strains are presented in **Table 2**.

Subsystem Analysis of Strains NWUTAL1 and S52016 Genomes

A subsystem refers to a set of proteins that, altogether, implement a specific biological process or structural complex (Overbeek et al., 2005). PATRIC generated an overview of the subsystems inherent to each of the studied genomes (**Figure 1**). Genes involved in the different cellular processes were summed up and assigned to their respective subsystems.

VR *E. durans* NWUTAL1 strain displayed 122 genes belonging to 33 subsystems, which play a role in stress response, defense and virulence mechanisms, compared to *E. gallinarum* strain S52016, which displayed 76 genes belonging to 25 subsystems involved in the same mechanisms. Moreover, miscellaneous genes and subsystems were not detected in strain S52016 compared to strain NWUTAL1 (**Figure 1**). A circular graphic display of the distribution of the genomes annotations was generated (**Figure 2**).

Genes Involved in Virulence and Antimicrobial Resistance

Analysis of the genomes revealed the presence of ARG as well as virulence genes. Both strains possessed glycopeptide resistance genes, aminoglycoside resistance genes, β-lactam resistance genes, macrolide resistance genes, tetracycline resistance genes and peptide antibiotics resistance genes, among others. ARG were identified either as genes involved in antibiotic resistance processes (such as *tet* and *bla* genes), or as genes encoding targets that may play a role in resistance mechanisms (such as *gyrA* and *gyrB* which are housekeeping genes) (**Table 3**).

Some genes reported to be associated with virulence in pathogenic bacteria were noticed as follows: *pgaA* and *bopD* (biofilm formation); *cspE* (cold shock protein); *purB* (colonization factor); *ompA* (outer membrane porin); *ecbA* (cell wall surface anchor protein); and *perR* (peroxide stress regulator) for strain NWUTAL1; while *purB* (colonization factor), *ebpC* and *pgaA* (biofilm formation), *cspE* (a cold shock protein) and *ompA* as well as *ompF* (outer membrane porins) were detected in strain S52016. Moreover, a Copper/silver efflux RND transporter, outer membrane protein (*cusC*) was detected in both strains.

Assessment of CRISPR, Phages and Plasmids

No phages were detected in both strains. However, plasmids were detected in both strains (*E. durans* NWUTAL1 and *E. gallinarum* S52016) and aligned with reference plasmid sequences of the Enterobacteriaceae plasmids database. The plasmids detected in *E. durans* NWUTAL1 showed an identity of 99.3, 99.23, 96.54, 95.04, and 93.84% to plasmids *Incl1* (accession number: AP005147), *IncFII* (accession number: AY458016), *rep1* (accession number: NC011140), *IncFll*(pCoo) (accession number: CR942285), and *IncFIB*(AP001918) (accession number: AP001918), respectively. Comparatively, four plasmid sequences were detected in *E. gallinarum* S52016 and these demonstrated an identity of 97.32, 100, 99.38, and 95.04% to plasmids *IncFII* (accession number: AY458016), *IncFIA* (accession number: AP001918) *IncFIB* (pB171) (accession number: AB024946), and *IncFII*(pCoo) (accession number: CR942285), respectively. CRISPRFinder predicted three CRISPR on nodes 5, 307, and 729 in the genome of strain NWUTAL1. Three CRISPR were also detected on nodes 6, 1029, and 1030 in the genome of strain S52016.

Comparatively, three plasmid sequences were detected in *E. gallinarum* S52016 and these included IncFII, Incl1 and rep1. CRISPRFinder predicted three CRISPR on nodes 5, 307, and 729 in the genome of strain NWUTAL1. Three CRISPR were also detected on nodes 6, 1029, and 1030 in the genome of strain S52016.

Phylogenetic Assessment of Nucleotide Sequences of Strains NWUTAL1 and S52016

Based on the alignment of the 16S rDNA sequences, a high similarity was detected between strain NWUTAL1 and other strains of the same species from different sources. Similarly, strain S52016 was compared with other *E. gallinarum* strains and a high similarity was detected between them as well (**Figure 3**).

Moreover, sequences of the studied strains *vanC* genes were compared with that of a reference strain (accession number AF162694) that also possessed *vanC* gene. Identity scores of the *vanC* genes were 99.88 and 100 for strain NWUTAL1 and strain S52016, respectively.

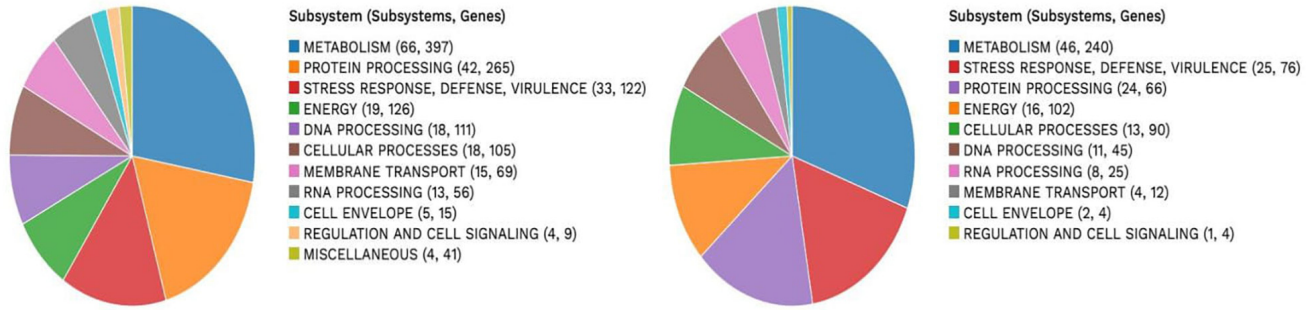

FIGURE 1 | Subsystem analysis of strain NWUTAL1 **(left)** and strain S52016 **(right)**.

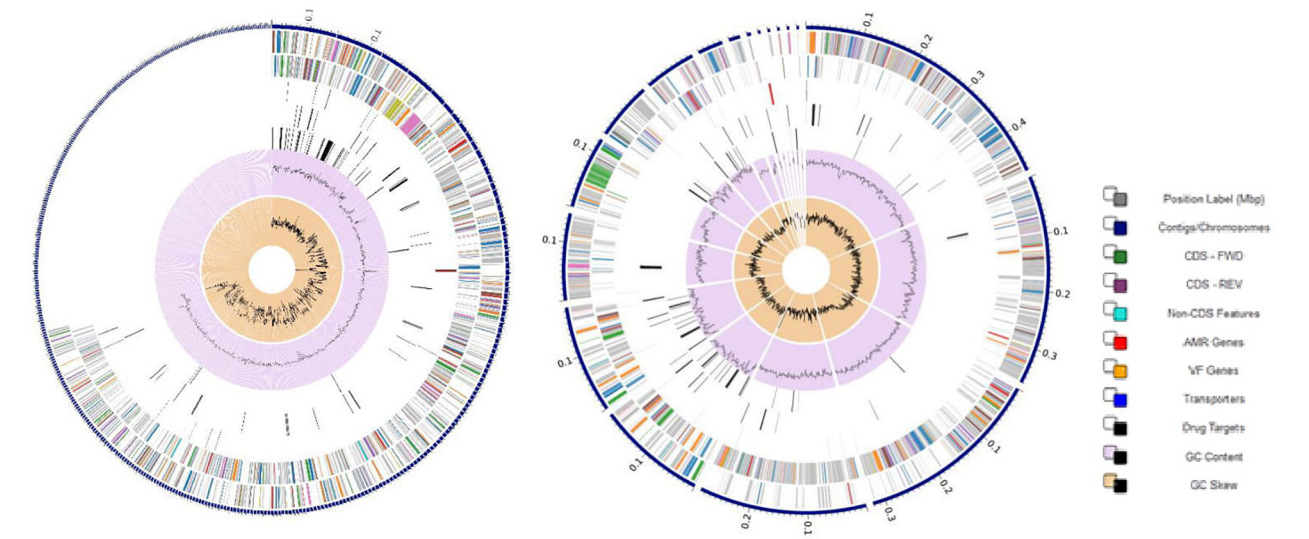

FIGURE 2 | Circular graphical display of the distribution of the annotated genomes of strain NWUTAL1 **(left)** and strain S52016 **(right)**. This includes, from outer to inner rings, the contigs, CDS on the forward strand, CDS on the reverse strand, RNA genes, CDS with homology to known ARG, CDS with homology to know virulence factors, GC content and GC skew. The colors of the CDS on the forward and reverse strand indicate the subsystem that these genes belong to (see legend).

DISCUSSION

Systematic monitoring of antibiotic usage and prevalence of antibiotic resistance among humans and animals as well as their pattern of spread in the environment, is of utmost importance as far as the management of bacterial infectious diseases and food safety are concerned (World Health Organization, 2015). The unavailability of infrastructure and resources in certain low income countries, such as insufficient financial means and underequipped hospitals with poor healthcare systems, has created gaps in the data generated worldwide, causing inefficient surveillance systems (World Health Organization, 2018). This study is in accordance with the "One Health Perspective" which was, therefore, designed to close these gaps in the antibiotic resistance surveillance data while emphasizing on the interconnections between the health and well-being of animals, humans, plants and their environment (World Health Organization, 2018).

The present investigation reveals features that are inherent to the genomes of two enterococcal isolates, namely: *E. durans* strain NWUTAL1 isolated from cattle feces of feedlots and *E. gallinarum* strain S52016 isolated from the soil of the same cattle feedlot. Data from whole genome sequence was used in the present study to assess their resistome and some virulence factors of importance. An explanation of the multidrug resistant nature of these isolates may be the ability of enterococci to adapt to their environment by incorporating, in their genomes, genetic determinants such as plasmids that harbor multiple genes, which altogether, code for resistance to either a single drug or multiple drugs (Clewell et al., 2014). Additionally, another explanation of these observations is the increased expression by enterococci of genes that code for multiple-drug efflux pumps, thus conferring to them, the ability to flush out of their cells, a wide range of antimicrobials (Miller et al., 2014). Moreover, antibiotic resistance in some cases is an inherent feature located in the chromosome, which is transmitted to progenies.

TABLE 3 | ARGs detected in strains NWUTAL1 and S52016.

NWUTAL1	S52016	Resistance genes	Antibiotic to which resistance is conferred	Antibiotic group	Function
	✓	*vanC1*	vancomycin	Glycopeptides	D-alanine–D-serine ligase
✓		*vanC2/C3*	vancomycin		D-alanine–D-serine ligase
✓	✓	*vanXY-C*	vancomycin		D-Ala-D-Ala dipeptidase/carboxypeptidase
✓	✓	*vanC/E/L/N*-type	vancomycin		vancomycin (or other glycopeptides) response regulator VanR
✓	✓	*macA*, *macB*	macrolides	Macrolides	macrolide-specific efflux protein *macA*, Macrolide export ATP-binding/permease protein *macB*
✓	✓	*rlmA*(II)	tylosin		23S rRNA (guanine(748)-N(1))-methyltransferase
	✓	*erm(A)*	erythromycin	macrolides, streptogramins	23SrRNA(adenine(2058)-N(6))-dimethyltransferase
✓	✓	*aac(6′)-Ia*	–	aminoglycosides	aminoglycoside N(6′)-acetyltransferase
✓	✓	*blaEC*	–	β-lactams	class C β-lactamase
✓		*tet(A)*	tetracycline	Tetracyclines	tetracycline resistance, MFS efflux pump
	✓	*tet(L)*	tetracycline	tetracyclines	tetracycline resistance, MFS efflux pump
✓	✓	*S10p*	tetracycline	tetracyclines	SSU ribosomal protein S10p
✓		*gyrA*	ciprofloxacine	Quinolones	DNA gyrase subunit A
✓	✓	*gyrB*	ciprofloxacine	Quinolones	DNA gyrase subunit B
	✓	*msbA*	–	Quinolones	efflux pump conferring antibiotic resistance
✓	✓	*S12p*	streptomycin	aminoglycosides	SSU ribosomal protein S12p
✓	✓	*rpoB*, *rpoC*	myxopiremine	Peptides	DNA-directed RNA polymerase β-subunit
✓		*mdfA/cmr*	multidrug efflux pump, quaternary ammonium compounds resistance		multidrug efflux pump *mdfA/cmr* (of MFS type), broad spectrum
✓	✓	*liaF, liaR, liaS*	daptomycin	peptide	membrane protein *liaF(VraT)*, specific inhibitor of *liaRS(VraRS)* signaling pathway, cell envelope stress response system *liaFSR*, response regulator *liaR(VraR)*, cell envelope stress response system *liaFSR*, sensor histidine kinase *liaS*
✓		*bcrC*	bacitracin	Polypetide	undecaprenyl-diphosphatase *BcrC* (EC 3.6.1.27), conveys bacitracin resistance
✓		*mprF*	moenomycin	phosphoglycolipid	L-O-lysylphosphatidylglycerol synthase
✓		*pgsA*	Daptomycin	peptide	CDP-diacylglycerol–glycerol-3-phosphate 3-phosphatidyltransferase
✓	✓	*ef-G*	fusidic acid	Fusidane	translation elongation factor G
✓	✓	*ef-TU*	Elfamycins		translation elongation factor Tu
✓	✓	*ddl, alr*	cycloserines		D-alanine–D-alanine ligase and Alanine racemase
✓	✓	*kasA*	isoniazid, triclosan		3-oxoacyl-[acyl-carrier-protein] synthase, KASII
✓	✓	*isotRNA*	mupirocin	carboxylic acid	isoleucyl-tRNA synthetase
✓	✓	*inhA, fabI*	isoniazid, triclosan		Enoyl-[acyl-carrier-protein] reductase [NADH]
✓	✓	*murA*	fosfomycin	fosfonic antibiotics	UDP-N-acetylglucosamine 1-carboxyvinyltransferase
✓	✓	*folA, Dfr*	trimethoprim		Dihydrofolate reductase

In this study, plasmids (Incl1, IncFII, rep1 and IncFIB) and most importantly, vancomycin (glycopeptide) resistance genes as well as genes of resistance to peptides, macrolides, tetracyclines, aminoglycosides, streptogramins, quinolones and β-lactams were detected in the genomes of the studied strains, with many other resistance genes to antibiotics such as bacitracin, fosfomycin, trimethoprim and fusidic acid, among others (**Table 3**). Vancomycin resistance can be either intrinsic or acquired. Intrinsic resistance or low-level resistance refers to the ineffectiveness of a drug due to the possession of certain genetic features, which are inherent to a species. This type of resistance is common in *Enterococcus casseliflavus*, *E. durans* and *E. gallinarum*, and *vanC* (*vanC1*, *vanC2/C3*) resistance gene confers such type of resistance (Ahmed and Baptiste, 2017). Comparatively, acquired resistance arises due to the uptake of genetic determinants either from the environment or from another bacterium. This type of resistance is common in *E. faecalis*, *E. faecium*, *E. durans* and less often, *Enterococcus avium* and *Enterococcus raffinosus*, and *vanA*, *vanB*, *vanD*, *vanE*, *vanG*, *vanL*, *vanM*, and *vanN* code for this type of resistance (Arthur et al., 1996; Ahmed and Baptiste, 2017). The vancomycin resistance genes detected in this study are involved in the intrinsic type of resistance mechanism and the same findings were reported elsewhere (Reid et al., 2001). Broadly, intrinsic glycopeptide resistance in enterococci arises when the peptidoglycan layer synthesis pathway is altered in

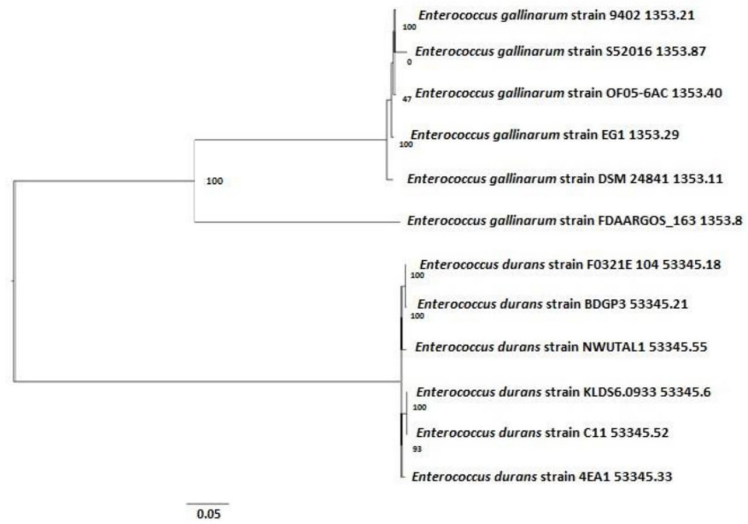

FIGURE 3 | Phylogenetic tree determining the relationship between strains NWUTAL1, S52016 and other enterococci of the same species.

such a way that D-alanine-D-Alanine (D-Ala-D-Ala) is replaced by D-Alanine-D-Serine (D-Ala-D-Ser). This is mediated by chromosomal attributes that render glycopeptides inactive on such strains and their offspring. Although avoparcin, a growth promoter, which was initially incriminated for the emergence of VREs, has been banned three decades ago, VREs are continuously detected worldwide as it is the case in this study. It has been proved that the emergence of VREs is due to the usage of alternative growth promoters and antimicrobials, which continue to co-select vancomycin resistance due to selective pressure (Aarestrup, 2000). As a matter of fact, the use of the macrolide tylosin in Danish pig farms was found to co-select for vancomycin resistance among enterococci (Aarestrup, 2000). Moreover, some studies have revealed that usage of erythromycin and tetracyclines in animal rearing settings accounts for the co-selection of vancomycin resistance (Aarestrup, 2000). An exhaustive list of antibiotics currently used in animal farming settings in South Africa is provided in **Supplementary Table S1**. This list of antimicrobials conforms to our findings as far as vancomycin resistance and the other types of ARGs detected in this study are concerned (**Table 3** and **Supplementary Table S1**). However, there is a need to further elucidate the mechanisms through which some of these antimicrobials co-select vancomycin resistance and this is a limitation of this study.

Administration of antimicrobials to animals, either as therapeutics or as growth promoters, causes drastic changes in the gut microbiota of animals, enhancing the proliferation of drug-resistant strains such as VREs. As demonstrated by a wide range of studies, enterococci, which were initially resistant to vancomycin or any other drug may acquire more antibiotic resistance genetic determinants and additional virulence factors with plasmids upon interaction with other bacteria of the gut, giving rise to multidrug resistant isolates, which may become pathogenic and subsequently, be shed with fecal matter (Doucet-Populaire et al., 1991; Rizzotti et al., 2009; Toomey et al., 2009). This assertion may be an additional explanation of our findings. Most of the virulence factors and the ARGs detected in this study have been previously screened in other enteric isolates (Ahmed and Baptiste, 2017). The antibiotic susceptibility profiles of strains NWUTAL1 and S52016 were previously assessed against nine antibiotics (vancomycin 30 μg, tetracycline 30 μg, erythromycin 15 μg, ampicillin 10 μg, amoxicillin 10 μg, chloramphenicol 30 μg, linezolid 30 μg, ciprofloxacin 5 μg, and penicillin 10 μg) (Tatsing and Ateba, 2019). The measurement and interpretation of the zones of inhibition revealed they were both intermediate for ciprofloxacin according to the CLSI guideline (CLSI, 2017). The antibiotic resistance profile for both strains was TET^R-AMP^R-AMX^R-VAN^R-CHL^R-PEN^R-LIN^R-ERY^R. From these findings, it is suggested that presence of multidrug resistant VREs in the environment may play a significant role in the transmission and acquisition of multidrug-resistant determinants such as *vanA*, *vanB*, *vanC*, *tetK*, *tetL*, *msrA/B*, and *mefA*. With the alarming increase in antibiotic resistance globally, these new and highly sensitive techniques such as WGS may be required to mitigate the role that environment plays in the transmission of antimicrobial resistant isolates.

When soil is mixed with manure in agricultural processes, resistance genes can be transferred either vertically or horizontally to soil microbiota. Through this process, commensals and human pathogens pick up genetic determinants such as resistance genes and virulence factors with plasmids in the already polluted soil environment (Boxall et al., 2002; Ding et al., 2014; Forsberg et al., 2014; Thanner et al., 2016; Wei et al., 2019; Zhang et al., 2019). This assertion may additionally justify the detection of ARGs in the studied strains.

The effects of usage of antimicrobials in intensive rearing cannot be undermined as it has a significant impact on the environment and, consequently, on the safety of food items. Wastes from such farms may find their way into water bodies

used either in irrigation processes or for recreational purposes (Economou and Gousia, 2015). Consequently, these water bodies may be contaminated with ARG that may be incorporated into the genetic make-up of their microbiota; and whenever water from such sources is used in irrigation processes, ARG and multidrug resistant isolates are propagated unto crops, which will later on be eaten by humans and animals. This will consequently lead to a never ending cycle of transmission of ARGs to commensals and other potentially pathogenic bacteria, through the food chain and various microbiomes of the environment (Acar et al., 2012; Gonzalez-Zorn and Escudero, 2012; Wei et al., 2019; Zhang et al., 2019). Moreover, even if waste from such farms were treated before being released into the environment, the problem will not be resolved since antibiotics are not completely deactivated in the process of waste treatment and after a while in the environment, they always revert to their initial active form (Ding et al., 2014). Reports of food products contaminated by multidrug resistant isolates as a consequence of extensive usage of antimicrobials in intensive animal farming are numerous (Petersen et al., 2002; Rizzotti et al., 2009; Toomey et al., 2009; Shah et al., 2012; Drissner and Zürcher, 2014; Forsberg et al., 2014; Thanner et al., 2016; Wei et al., 2019; Zhang et al., 2019). Thus, such issues that could seriously impact food safety, should be addressed promptly.

CONCLUSION

The well-being of living beings depends undoubtedly on the quality of food ingested and the quality of the environment in which they thrive. Ever since antimicrobials were discovered and introduced in therapeutic regimens and intensive animal farming, the world has spawned into what many scientists call the "post-antibiotic era," with its huge consequences on the environment and food safety. One of such consequences is the emergence of multidrug resistant strains of bacteria and the probable availability of ARG into the environment that will later on contaminate food items through previously described mechanisms. This report highlights, on a microbiological perspective, the impact of intensive animal rearing on food safety. Two multidrug resistant enterococcal strains (namely; *E. durans* strain NWUTAL1 and *E. gallinarum* strain S52016), isolated from a cattle feedlot in the North West Province, South Africa, were assessed through genomics. The detection of ARGs that code for vancomycin, tylosin, tetracycline, erythromycin, β-lactam antibiotics, quinolones, fusidic acid, bacitracin and fosfomycin, among others, in their genomes, highlights the role that intensive farming practices, such as the abusive usage of antimicrobials has on the spread and the dissemination of resistant strains such as VREs in the environment, but most importantly the risk that such strains present as far as food safety is concerned. Environment has become a pool where genetic determinants are exchanged horizontally and vertically between organisms of different ecological niches. The consequences of industrial animal rearing on food safety and subsequently on human and animal health cannot be overemphasized, thus there is an urgent need to consider alternatives to antibiotics and adopt lifestyles that are healthier and more environment-friendly.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Ethical clearance was issued by the Faculty of Natural and Agricultural Sciences (FNAS) Ethics Committee. The ethics certificate number is NWU-01221-19-S9. Moreover, authorization was granted by owners of the feedlots before collection of samples.

AUTHOR CONTRIBUTIONS

CA: conceptualization, resources, and funding acquisition. FF, CM, CB, and CA: methodology, software, validation, and investigation. FF and CM: formal analysis. FF: writing – original draft preparation. CM, CB, and CA: writing – review and editing, supervision, and project administration. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.571958/full#supplementary-material

Supplementary Table S1 | Antibiotics currently used in animal farming in South Africa.

REFERENCES

Aarestrup, F. M. (2000). Characterization of glycopeptide-resistant *Enterococcus faecium* (GRE) from broilers and pigs in Denmark: genetic evidence that persistence of GRE in pig herds is associated with co-selection by resistance to macrolides. *J. Clin. Microbiol.* 38, 2774–2777. doi: 10.1128/JCM.38.7.2774-2777.2000

Aarestrup, F. M., Kruse, H., Tast, E., Hammerum, A. M., and Jensen, L. B. (2000). Associations between the use of antimicrobial agents for growth promotion and the occurrence of resistance among *Enterococcus faecium* from broilers and pigs in Denmark. *Finland, and Norway. Microb. Drug Resist.* 6, 63–70. doi: 10.1089/mdr.2000.6.63

Acar, J. F., Moulin, G., Page, S. W., and Pastoret, P. P. (2012). Antimicrobial resistance in animal and public health: introduction and classification of antimicrobial agents. *Rev. Sci. Tech.* 31, 15–21. doi: 10.20506/rst.31.1.2093

Ahmed, M. O., and Baptiste, K. E. (2017). Vancomycin-resistant enterococci: a review of antimicrobial resistant mechanisms and perspectives of human and animal health. *Microb. Drug Resist.* 24, 590–606. doi: 10.1089/mdr.2017.0147

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

Arthur, M., Reynolds, P., and Courvalin, P. (1996). Glycopeptide resistance in enterococci. *Trends Microbiol.* 4, 401–407. doi: 10.1016/0966-842X(96)10063-9

Bager, F., Madsen, M., Christensen, J., and Aarestrup, F. M. (1999). Avoparcin used as a growth promoter is associated with the occurrence of vancomycin-resistant *Enterococcus faecium* on Danish poultry and pig farms. *Prev. Vet. Med.* 31, 95–112. doi: 10.1016/s0167-5877(96)01119-1

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., et al. (2012). SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol.* 19, 455–477. doi: 10.1089/cmb.2012.0021

Bolger, A. M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30, 2114–2120. doi: 10.1093/bioinformatics/btu170

Boxall, A. B., Blackwell, P., Cavallo, R., Kay, P., and Tolls, J. (2002). The sorption and transport of a sulphonamide antibiotic in soil systems. *Toxicol. Lett.* 131, 19–28. doi: 10.1016/S0378-4274(02)00063-2

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

Clewell, D. B., Weaver, K. E., Dunny, G. M., Coque, T. M., Francia, M. V., and Hayes, F. (2014). "Extrachromosomal and mobile elements in enterococci: transmission, maintenance, and epidemiology," in . *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection*, eds M. S. Gilmore, D. B. Clewell, Y. Ike, and N. Shankar (Boston, MA: Eye and Ear Infirmary).

CLSI (2017). *Performance Standards for Antimicrobial Susceptibility Testing*, 27th Edn. Wayne, PA: Clinical and Laboratory Standards Institute.

Courvalin, P. (2006). Vancomycin resistance in gram-positive cocci. *Clin. Infect. Dis.* 42, 25–34. doi: 10.1086/491711

Depardieu, F., Perichon, B., and Courvalin, P. (2004). Detection of the van alphabet and identification of enterococci and staphylococci at the species level by multiplex PCR. *J. Clin. Microbiol.* 42, 5857–5860. doi: 10.1128/JCM.42.12.5857-5860.2004

Ding, G. C., Radl, V., Schloter-Hai, B., Jechalke, S., Heuer, H., Smalla, K., et al. (2014). Dynamics of soil bacterial communities in response to repeated application of manure containing sulfadiazine. *PLoS One* 9:e92958. doi: 10.1371/journal.pone.0092958

Doucet-Populaire, F., Trieu-Cuot, P., Dosbaa, I., Andremont, A., and Courvalin, P. (1991). Inducible transfer of conjugative transposon Tn1545 from *Enterococcus faecalis* to *Listeria monocytogenes* in the digestive tracts of gnotobiotic mice. *Antimicrob. Agents Chemother.* 35, 185–187. doi: 10.1128/aac.35.1.185

Drissner, D., and Zürcher, U. (2014). "Microbial safety of fresh fruits and vegetables," in *Encyclopedia of Food Safety*, Vol. 3, ed. Y. Motarjemi (Oxford: Elsevier).

Economou, V., and Gousia, P. (2015). Agriculture and food animals as a source of antimicrobial-resistant bacteria. *Infect. Drug Resist.* 8, 49–61. doi: 10.2147/IDR.S55778

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 32, 1792–1797. doi: 10.1093/nar/gkh340

Forsberg, K. J., Patel, S., Gibson, M. K., Lauber, C. L., Knight, R., Fierer, N., et al. (2014). Bacterial phylogeny structures soil resistomes across habitats. *Nature* 509, 612–616. doi: 10.1038/nature13377

Gonzalez-Zorn, B., and Escudero, J. A. (2012). Ecology of antimicrobial resistance: humans, animals, food and environment. *Int. Microbiol.* 15, 101–109. doi: 10.2436/20.1501.01.163

Grant, J. R., and Stothard, P. (2008). The CGView server: a comparative genomics tool for circular genomes. *Nucleic Acids Res.* 36, W181–W184. doi: 10.1093/nar/gkn179

Grissa, I., Vergnaud, G., and Pourcel, C. (2007). CRISPRFinder: a web tool to identify clustered regularly interspaced short palindromic repeats. *Nucleic Acids Res.* 35, W52–W57. doi: 10.1093/nar/gkm360

Jenney, A., Franklin, C., Liolos, L., and Spelman, D. (2000). *Enterococcus durans* vanB. *J. Antimicrob. Chemother.* 46, 513–519. doi: 10.1093/jac/46.3.515

Marshall, B. M., and Levy, S. B. (2011). Food animals and antimicrobials: impacts on human health. *Clin. Microbiol. Rev.* 24, 718–733. doi: 10.1128/CMR.00002-11

Miller, W. R., Munita, J. M., and Arias, C. A. (2014). Mechanisms of antibiotic resistance in enterococci. *Expert Rev. Anti-Infect. Ther.* 12, 1221–1236. doi: 10.1586/14787210.2014.956092

Myllys, V., and Rautala, H. (1995). Characterization of clinical mastitis in primiparous heifers. *J. Dairy Sci.* 78, 538–545. doi: 10.3168/jds.s0022-0302(95)76664-4

Ondov, B. D. T., Reangen, T. J., Melsted, P., Mallonee, A. B., Bergman, N. H., Koren, S., et al. (2016). Mash: fast genome and metagenome distance estimation using MinHash. *Genome Biol.* 17:132. doi: 10.1186/s13059-016-0997-x

Overbeek, R., Begley, T., Butler, R. M., Choudhuri, J. V., Chuang, H. Y., Cohoon, M., et al. (2005). The subsystems approach to genome annotation and its use in the project to annotate 1000 genomes. *Nucleic Acids Res.* 33, 5691–5702. doi: 10.1093/nar/gki866

Overbeek, R., Olson, R., Pusch, G. D., Olsen, G. J., Davis, J. J., Disz, T., et al. (2014). The SEED and the rapid annotation of microbial genomes using subsystems technology (RAST). *Nucleic Acids Res.* 42, D206–D214. doi: 10.1093/nar/gkt1226

Petersen, A., Andersen, J. S., Kaewmack, T., Somsiri, T., and Dalsgaard, A. (2002). Impact of integrated fish farming on antimicrobial resistance in a pond environment. *Appl. Environ. Microbiol.* 68, 6036–6042. doi: 10.1128/AEM.68.12.6036-6042.2002

Rangel, F. A. A., Rodrigues, S. S. S., Faria, A. R., Planet, P. J., Carreira, M. V. L., and Teixeira, M. L. (2019). Draft genome sequence of multidrug-resistant *Enterococcus faecium* Strain E1298, with a Sequence Type 1274 profile, recovered from the cloacal microbiome of a tropical screech Owl (*Megascops choliba*) in Rio de Janeiro, Brazil. *Microbiol. Resour. Announc.* 8:e00168-19. doi: 10.1128/MRA.00168-19

Reid, K. C., Cockerill, F. R. III, and Patel, R. (2001). Clinical and epidemiological features of Enterococcus casseliflavus/flavescens and Enterococcus gallinarum bacteremia: a report of 20 cases. *Clin. Infect. Dis.* 32, 1540–1546. doi: 10.1186/s12879-019-4136-7

Rizzotti, L., La Gioia, F., Dellaglio, F., and Torriani, S. (2009). Molecular diversity and transferability of the tetracycline resistance gene *tet(M)*, carried on Tn916-1545 family transposons, in enterococci from a total food chain. *Antimicrob. Agents Chemother.* 96, 43–52. doi: 10.1007/s10482-009-9334-7

Rogers, D. G., Zeman, D. H., and Erikson, E. D. (1992). Diarrhea associated with *Enterococcus durans* in calves. *J. Vet. Diagn. Invest.* 4, 471–472. doi: 10.1177/104063879200400423

Seemann, T. (2014). Prokka: rapid prokaryotic genome annotation. *Bioinformatics* 30, 2068–2069. doi: 10.1093/bioinformatics/btu153

Shah, S. Q. A., Colquhoun, D. J., Nikuli, H. L., and Sørum, H. (2012). Prevalence of antibiotic resistance genes in the bacterial flora of integrated fish farming environments of Pakistan and Tanzania. *Environ. Sci. Technol.* 46, 8672–8679. doi: 10.1021/es3018607

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

Stamatakis, A., Hoover, P., and Rougemont, J. (2008). A rapid bootstrap algorithm for the RAxML web servers. *Syst. Biol.* 57, 758–771. doi: 10.1080/10635150802429642

Sundermann, A. J., Babiker, A., Marsh, J. W., Shutt, K. A., Mustapha, M. M., Pasculle, A. W., et al. (2019). Outbreak of vancomycin-resistant *Enterococcus faecium* in interventional radiology: detection through whole genome sequencing-based surveillance. *Clin. Infect. Dis.* 70, 2336–2343. doi: 10.1093/cid/ciz666

Tatsing, F. F. E. (2020). *Assessment of Feedlots Cattle in the Development and Spread of Vancomycin Resistant Enterococci*. Ph.D. thesis, North West University, Vanderbijlpark.

Tatsing, F. F. E., and Ateba, C. N. (2019). Detection of virulence genes in multidrug resistant enterococci isolated from feedlot dairy and beef cattle: implications for human health and food safety. *Biomed. Res. Int.* 2019:5921840. doi: 10.1155/2019/5921840

Tatusova, T., DiCuccio, M., Badretdin, A., Chetvernin, V., Nawrocki, E. P., Zaslavsky, L., et al. (2015). NCBI prokaryotic genome annotation pipeline. *Nucleic Acids Res.* 43, D599–D605. doi: 10.1093/nar/gkw569

Taucer-Kapteijin, M., Hoogenboezem, W., Heiliegers, L., Danny de Bolster, H., and Medema, G. (2016). Screening municipal wastewater effluent and surface water used for drinking water production for the presence of ampicillin and vancomycin resistant enterococci. *Int. J. Hyg. Environ. Health* 16, 1–7. doi: 10.1016/j.ijheh.2016.04.007

Thanner, S., Drissner, D., and Walsh, F. (2016). Antimicrobial resistance in agriculture. *mBio* 7:e02227-15. doi: 10.1128/mBio.02227-15

Toomey, N., Monaghan, A., Fanning, S., and Bolton, D. J. (2009). Assessment of antimicrobial resistance transfer between lactic acid bacteria and potential foodborne pathogens using in vitro methods and mating in a food matrix. *Foodborne Pathog. Dis.* 6, 925–933. doi: 10.1089/fpd.2009.0278

Wattam, A. R., Davis, J. J., Assaf, R., Boisvert, S., Brettin, T., Bun, C., et al. (2017). Improvements to PATRIC, the all-bacterial bioinformatics database and analysis resource center. *Nucleic Acids Res.* 45, D535–D542. doi: 10.1093/nar/gkw1017

Wei, R., He, T., Zhang, S., Zhu, L., Shang, B., Li, Z., et al. (2019). Occurrence of seventeen veterinary antibiotics and resistant bacteria in manure-fertilized vegetable farm soil in four provinces of China. *Chemosphere* 215, 234–240. doi: 10.1016/j.chemosphere.2018.09.152

Wingett, S. W., and Andrews, S. (2018). FastQ screen: a tool for multi-genome mapping and quality control. *F1000 Res.* 7:1338. doi: 10.12688/f1000research.15931.2

World Health Organization (2015). *Global Action Plan on Antimicrobial Resistance*. Geneva: WHO.

World Health Organization (2018). *WHO Report on Surveillance of Antibiotic Consumption: 2016–2018 Early Implementation*. Geneva: WHO.

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

Zhang, Y.-J., Hu, H.-W., Chen, Q.-L., Singh, B. K., Yan, H., Chen, D., et al. (2019). Transfer of antibiotic resistance from manure-amended soils to vegetable microbiomes. *Environ. Int.* 130:104912. doi: 10.1016/j.envint.2019.104912

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Simulated Winter Incubation of Soil With Swine Manure Differentially Affects Multiple Antimicrobial Resistance Elements

Daniel N. Miller[1], Madison E. Jurgens[2], Lisa M. Durso[1] and Amy M. Schmidt[3]*

[1] USDA-ARS, Agroecosystem Management Research Unit, Lincoln, NE, United States, [2] University of Nebraska, Lincoln, NE, United States, [3] Department of Biological Systems Engineering, University of Nebraska, Lincoln, NE, United States

Gastrointestinal bacteria that harbor antibiotic resistance genes (ARG) become enriched with antibiotic use. Livestock manure application to cropland for soil fertility presents a concern that ARG and bacteria may proliferate and be transported in the environment. In the United States, manure applications typically occur during autumn with slow mineralization until spring planting season. A laboratory soil incubation study was conducted mimicking autumn swine manure application to soils with concentrations of selected ARG monitored during simulated 120-day winter incubation with multiple freeze-thaw events. Additionally, the effects of two soil moistures [10 and 30% water holding capacity (WHC)] and two manure treatments [raw versus hydrated lime alkaline stabilization (HLAS)] were assessed. Fourteen tetracycline resistance genes were evaluated; *tet*(D), *tet*(G), and *tet*(L) were detected in background soil while swine manure contained *tet*(A), *tet*(B), *tet*(C), *tet*(G), *tet*(M), *tet*(O), *tet*(Q), and *tet*(X). By day 120, the manure-borne *tet*(M) and *tet*(O) were still detected while *tet*(C), *tet*(D), *tet*(L), and *tet*(X) genes were detected less frequently. Other *tet* resistance genes were detected rarely, if at all. The sum of unique *tet* resistance genes among all treatments decreased during the incubation from an average of 8.9 to 3.8 unique *tet* resistance genes. Four resistance elements, *int*I1, $bla_{ctx-m-32}$, *sul*(I), *erm*(B), and 16s rRNA genes were measured using quantitative PCR. ARG abundances relative to 16S abundance were initially greater in the raw manure compared to background soil (−1.53 to −3.92 log abundance in manure; −4.02 to <−6.7 log abundance in soil). In the mixed manure/soil, relative abundance of the four resistance elements decreased (0.87 to 1.94 log abundance) during the incubation largely because 16S rRNA genes increased by 1.21 log abundance. Throughout the incubation, the abundance of *int*I1, $bla_{ctx-m-32}$, *sul*(I), and *erm*(B) per gram in soil amended with HLAS-treated manure was lower than in soil amended with raw manure. Under low initial soil moisture conditions, HLAS treatment reduced the abundance of *int*I1 and resulted in loss of $bla_{ctx-m-32}$,

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Christophe Merlin, Université de Lorraine, France

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***Correspondence:**
Daniel N. Miller
dan.miller@usda.gov

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sul(I), and *erm*(B)] compared to other treatment-moisture combinations. Although one might expect antibiotic resistance to be relatively unchanged after simulated winter manure application to soil, a variety of changes in diversity and relative abundance can be expected.

Keywords: antibiotic resistance, freeze-thaw, manure application, manure treatment, soil moisture, swine

INTRODUCTION

Manure from animal production is a valuable source of fertilizer nutrients and organic matter for crop production offsetting the need for chemical fertilizer inputs and enhancing many soil attributes (Edmeades, 2003). However, manure management presents potential challenges, including odors, runoff, greenhouse gas emissions, and pathogens when best management practices are not used. Impairment of surface waters with zoonotic pathogens that may be associated with manure application have lent more focus toward pathogen control/inactivation prior to and during manure application to cropland. Contributions from livestock manure to antibiotic resistance in environmental matrices presents a growing concern about the fate of resistant microorganisms or the dissemination of antibiotic resistance genes (ARG) during manure treatment or following manure application to soil (Durso and Cook, 2014; Topp et al., 2018). The use of manure as a soil and crop fertility input could represent an important contribution of antibiotic resistant bacteria (ARB) and ARG to the soil environment (Franklin et al., 2016).

Croplands receiving manure application contain indigenous soil microorganisms that normally harbor a low, yet diverse, abundance of background of ARG (Durso et al., 2016). Once in the soil environment, ARGs originating from animal manures or human biosolids could potentially move into indigenous soil microbial populations though horizontal gene transfer of mobile genetic elements (von Wintersdorff et al., 2016), which are often found on particularly stable plasmids that increase the chance of inheritance between species (Heuer et al., 2009). Once acquired, the low fitness cost of antibiotic resistance enables soil bacteria harboring ARGs to persist in the environment even though antibiotic pressures are no longer present (Cook et al., 2014). Although this chain of events seems exceedingly rare, Forsberg et al. (2012) identified non-pathogenic soil microorganisms containing several long resistance genes having 100% nucleotide identity to those in human pathogens indicating a possible recent transfer event.

The abundance of specific ARGs may increase, decrease, or remain unchanged overtime with continuous/long term manure inputs (Chee-Sanford et al., 2009; Mantz et al., 2013; Peng et al., 2015; Tang et al., 2015; Durso et al., 2018; McKinney et al., 2018), indicating the fate of ARB and ARG in soils is likely dependent upon repeated manure inputs and a multitude environmental factors. Proliferation of indigenous ARB with the influx of manure nutrients and substrates can enhance ARB persistence (Heuer et al., 2011; Jechalke et al., 2013; Udikovic-Kolic et al., 2014; Fang et al., 2015) but may not be as important as the manure microbes themselves (Peng et al., 2016). Manure source (cattle, swine, or poultry), environmental factors (soil type, moisture and temperature), and management decisions (tillage practices, application timing, and method, and location of manure application) also likely influence persistence of ARG in soils.

In confined swine production systems, manure is typically collected and stored (with or without treatment) for a period of 6 months to a year to reduce the occurrence of pathogens (Hill, 2003; McLaughlin et al., 2012). During manure storage/treatment, biological, chemical, and physical processes reduce pathogen load and provide some capacity to reduce, but not eliminate, ARGs (Joy et al., 2014). Untreated human biosolids have many of the same characteristics as swine manure, and a preferred method to reduce pathogen loads in human biosolids is through alkaline stabilization using hydrated lime to raise the pH of the biosolids for a sustained period of time (US Environmental Protection Agency, 2000).

Recently, a hydrated lime alkaline stabilization (HLAS) manure treatment, modeled after the alkaline stabilization used on human biosolids, has been shown to reduce porcine epidemic diarrhea virus (PEDV), an important coronavirus disease in swine (Stevens et al., 2018). Research investigating the impact of HLAS manure treatment on ARG abundance is limited; however, a survey of three resistance genes [*tet(*W*), tet(*O*),* and *sul(*I*)*] in manure, soil, human biosolids, and lime-stabilized human biosolids found lower abundances of these ARG in lime-stabilized biosolids (Munir and Xagoraraki, 2011). The long-term impact of manure or biosolids application on soil microbial communities in this study was less clear, with considerable farm-to-farm variability. It should be noted that soil pH is an important driver of microbial community structure (Lauber et al., 2009; Tan et al., 2020). Indeed, agricultural lime addition to soils, a practice used to raise the pH of acidic soils, also affects soil community structure (Barth et al., 2018; Lin et al., 2018) and may impact diversity and abundance of soil ARG.

In swine production areas across the midwestern United States, stored manure slurry is land applied in the fall because manure nutrients are more easily retained in the cold soil over winter and available for new crop growth in the spring. Winter application of manure is discouraged due to contaminant runoff concerns resulting from frozen, snow-covered or saturated soil. Two manure applications methods are commonly used depending upon the solids content of the manure: surface and sub-surface application. Surface application via irrigation is common where manure is stored in treatment lagoons, as the very low solids content in lagoon effluent does not interfere with spray nozzle performance. An alternative storage method used in many swine production systems to minimize manure volume and retain valuable nitrogen for crop fertilization is collection

in deep pits below the production area. This "slurry" manure has higher solids content and greater concentration of nutrients and is typically injected in narrow bands into crop soils to minimize nitrogen losses via volatilization of ammonia. Manure injection produces a unique microenvironment where manure microbes and nutrients are in close contact with soil microbes. During application of liquid or slurry manure application, soil moisture also increases based upon manure water content, soil characteristics and hydrologic conditions. Application of manure to saturated soils is both difficult and discouraged as it contributes to manure runoff. Following fall manure application, low temperatures and soil freeze-thaw during winter months may impact bacterial competition, survival, and persistence of organisms and ARG.

This study examines how ARGs indigenous to either agricultural soil or swine manure change after simulated manure application and 120-day winter soil incubation with multiple freeze-thaw events, with and without manure HLAS treatment. We hypothesized that (1) HLAS treatment would have a strong immediate effect on resistance by directly limiting the survival of microorganisms; however, (2) few changes in diversity or persistence of ARG would occur over time under "winter" incubation conditions. Additionally, the effects of two initial soil moisture contents were assessed. Fourteen different tetracycline resistance genes were evaluated, and the abundance of four resistance elements both in bulk soil and relative to 16s rRNA gene abundance were measured using qPCR molecular techniques.

MATERIALS AND METHODS

Soil and Manure Treatments and Incubations

A silty clay loam soil was collected from a crop field in eastern Nebraska, sieved, and air dried (soil properties: 5.45 $\mu g\ g^{-1}$ NO_3-N, 2.43 $\mu g\ g^{-1}$ NH_4-N, 0.23 dS m^{-1} EC, 7.00 pH, 63.03 $\mu g\ P\ g^{-1}$ Mehlich P). The WHC of the soil was determined gravimetrically (Klute, 1986) and two stock soil mixtures were prepared by thoroughly incorporating deionized water to be achieve 10 or 30% of the maximum WHC of the soil (i.e., "dry" and "moist" soil conditions, respectively). Expressed on a moisture content basis, the 10 and 30% WHC soils contained 82.6 and 247.7 g H_2O kg^{-1} dry soil, respectively. From each soil mixture, 30 g (oven dry equivalent) soil was added to multiple 50 mL tubes. A soil cavity (simulated manure furrow) was made in the center of each soil tube by pressing a 10 mL pipet tip into the soil and tubes were immediately capped to prevent moisture loss.

Manure slurry was collected from the deep pit of a commercial swine production site in south central Nebraska and refrigerated at 4°C for less than 48 h prior to setting up the soil incubation. The day prior to setting up the incubations, manure slurry was divided into two stocks and equilibrated to room temperature (20°C). One manure slurry stock was amended with hydrated quicklime (10 g L^{-1} of manure slurry) yielding a final, stable pH of 11.5 and designated HLAS-treated. Although a higher pH of 12 for 2 h is recommended for biosolids treatment (US Code of Federal Regulations, 2018), an earlier study demonstrated that pH 10 for 12 h effectively controlled PEDV (Stevens et al., 2018). The other manure slurry stock (Raw) received no quicklime treatment. Both treatments were incubated at room temperature overnight since effective HLAS treatment recommends at least a 6-h exposure time at high pH. On day 0, 80 soil incubations were prepared by adding 10 g of manure slurry to the soil cavity in each vial yielding 20 replicates of each moisture (10 and 30% WHC) and manure (HLAS and raw manure) combination. Four samples of each treatment combination were immediately stored at −80°C as Day 0 samples. All other samples were capped loosely, secured in tube racks, and randomly placed in a refrigerated incubator (Fisherbrand Isotemp BOD Refrigerated Incubator, Fisher Scientific, Waltham, MA, United States). The incubator temperature was adjusted daily to simulate mean winter soil temperatures (5.1 cm depth) at the University of Minnesota Southern Research and Outreach Center in Waseca, Minnesota (**Figure 1**). The Waseca location was selected based upon the extensive soil temperature (daily high and low) records, Waseca's location in an area of dense swine production, and because seasonal temperature changes spanned the range where soils froze for extended periods of time. Twice weekly during the first 10 weeks of incubation, the racks of tubes were removed from the incubator and either allowed to briefly (over 20 min) freeze in the −80°C freezer (if the current incubation temperature was above 0°C) or thaw at room temperature (if the current incubation temperature was below 0°C). The number of freeze/thaw events was selected based upon the number of freeze/thaw events observed within November, December, and March at 5 cm soil depth in soils at Waseca, MN in 2009, 2011, 2012, 2013, and 2014, and ranged from six in 2013 to 22 in 2011. At Days 30, 60, 90, and 120, four samples of each treatment combination were retrieved and stored at −80°C for subsequent DNA extraction.

DNA Extraction, Multiplex PCR, and Quantitative PCR Amplification

Immediately prior to DNA extraction, deionized water was thoroughly mixed into each manure/soil sample to yield a uniform sample moisture content (25% moisture content wet basis) since incubation tubes lost moisture over the 120-day incubation (determined gravimetrically). DNA was extracted from 250 mg of slurry using the PowerSoil DNA Isolation Kit (MoBio Laboratories, Solana Beach, CA, United States) according to the manufacturer's protocol. DNA extracts were stored at −80°C until polymerase chain reaction (PCR) analysis.

A variety of target genes were assessed to gage how manure treatments and simulated winter incubation conditions affected resistance (**Table 1**). These target genes included "highly" and "critically important" resistance genes conferring resistance to antibiotics used in animal production. Four multiplex PCR amplification reactions for the presence/absence of fourteen tetracycline resistance genes (**Table 2**) were performed on each DNA extract according to the method of Ng et al. (2001), with each 25-μL reaction containing 12.5 μL Jumpstart RedTaq Master Mix and 0.5 μL of each primer. Extractions were processed in a StepOnePlus Real-Time PCR System (Applied

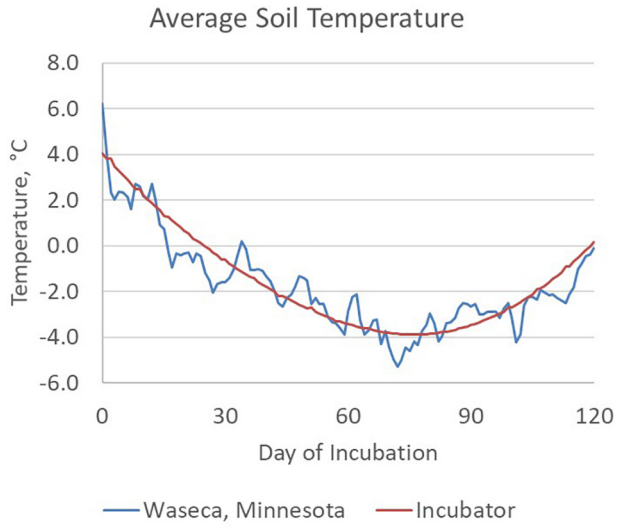

FIGURE 1 | Average soil temperature between November 11th and March 11th calculated for a 6-year period (2009 – 2014) of field data at the Waseca, MN experimental station. Two freeze/thaw cycles were conducted each week during the first 5 weeks of the incubation, typical for surface soils at this location.

Biosystems, Waltham, MA, United States) at settings of 5 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 55°C, 90 s at 72°C; and 5 min at 72°C. Positive controls and blanks were included in each PCR run, and PCR product was separated using agarose gel, stained (ethidium bromide), and photographed (UVP GelDoc-It TS3 Imager, Upland, CA, United States) to visualize PCR amplification products and confirm PCR product sizes.

Quantitative PCR reaction amplifications of 16s rRNA, *intI1, sul*(I), *erm*(B), and $bla_{ctx-m-32}$ were performed according to **Table 2**. Standards were prepared from serial dilutions of 4-gene multiplex with 16s rRNA gene (Integrated DNA Technologies, Coralville, IA, United States). Each 5-μL volume of standard contained from 10^1 to 10^8 gene copies. Standards and reagent blanks were included in each run. All samples were quantified in triplicate and averaged. Standard curve R^2 ranged from 0.960 to 0.998 while efficiency ranged from 80 to 117%.

Statistics

Equality of proportions tests and ANOVA were performed using SAS statistical software (SAS Institute, Cary, NC, United States). The two-sample test of equality of proportions was chosen to generate probabilities related to the significance of initial soil moisture and manure treatment on the percent of each tetracycline resistance gene. Analysis of variance was used to evaluate the significance of antecedent soil moisture and manure treatment on (i) the diversity of tetracycline resistance genes present over time (ii) the log abundance of *intI1, sul*(I), $bla_{CTX-M-32}$, and *erm*(B) in soil, and (iii) the log abundance of of *intI1, sul*(I), $bla_{CTX-M-32}$, and *erm*(B) normalized to 16s rRNA gene abundance. A *P*-value of <0.05 was considered significant.

RESULTS

Initial Resistances in Manure and Soil

Eight *tet* resistance genes were detected in the raw manure while background soil revealed three *tet* resistance genes (**Table 3**). There was no difference in the detection of *tet* resistance genes between HLAS-treated and raw manure. The *tet*(G) gene was detected in both soil and manure samples, while *tet*(E), *tet*(K), *tet*(S), and *tet*(A/P) genes were not initially detected in either soil or manure. Detections between replicate soil or manure samples did not differ; *tet* genes were either detected in 100% of samples or not detected at all.

From a quantitative perspective (**Table 3**), microbial abundance (based upon 16S rRNA genes per gram) in initial sources of raw manure and soil were similar (8.43 and 8.42 log abundance, respectively) with 16S rRNA genes in HLAS-treated manure slightly higher (8.68 log abundance) than the raw manure and soil. Comparing soil to manure, soil contained much lower (10- to 100-fold) abundances of *intI1*, $bla_{ctx-m-32}$, and *sul*(I) compared to manure (raw and HLAS-treated). Furthermore, *erm*(B) was not detected (50 targets per gram detection limit) in soil but was detected in the manure at 6.65 and 7.15 log abundance in raw and HLAS-treated manure, respectively. Comparing manures, the abundance of *sul*(I) and *erm*(B) were slightly higher while $bla_{ctx-m-32}$ was slightly lower in HLAS-treated manure. Normalizing to the number of 16S rRNA genes, the differences between soil and manures remained consistent. Likewise, the significant differences observed between manures for *sul*(I) and *erm*(B), when compared on a mass basis, were insignificant when normalized to 16S rRNA gene abundance.

Temporal Trends in Soil/Manure Slurry Tetracycline Resistance

The multiplex PCR results revealed gene-specific persistence patterns (**Table 4**). Some genes were unaffected and consistently present over time. For instance, *tet*(M) and *tet*(O) genes originating in the manure were present with 100% frequency in all samples, regardless of treatment or time. Other *tet* genes [i.e., *tet*(E), *tet*(L), and *tet*(S)] were detected very infrequently or only sporadically with no obvious differences attributed to soil moisture, manure treatment, or temporal trend. Other genes [i.e., *tet*(G), *tet*(K), *tet*(A/P), and *tet*(Q)] detected initially or during the incubation were absent by Day 120 of the incubations. The frequency of *tet*(X) detection in soils receiving HLAS-treated manure was significantly lower than in samples receiving raw manure. Specifically, 75 to 100% of the soil reps receiving HLAS-treated manure were positive for *tet*(X) on Day 0, but just one of 24 reps (3.5%) were positive for *tet*(X) after Day 30. In comparison, the proportion of tests positive for *tet*(X) in soil receiving raw manure slurry after Day 30 (20 detections out of 24 tests, or 83%) was much greater ($P < 0.0001$). Similarly, the low-moisture soil (10% WHC) receiving raw manure retained *tet*(A), *tet*(B), and *tet*(C) at a greater frequency ($P < 0.001$) during the incubation period (69, 87, and 100%, respectively, after Day 0) when compared to higher moisture soil receiving raw manure (6, 19, and 31%, respectively, after Day 0).

TABLE 1 | List of target genes.

Gene	Function*
*int*I1	Encodes for an integron-integrase gene that helps antibiotic resistance genes spread from cell to cell. It has been proposed as a gene that will help identify resistance that is associated with human activities (Gillings et al., 2015), and as a marker for "pollutants" including antibiotic resistance, heavy metals, and disinfectants.
sul(I)	Encodes for sulfonamide-resistance. One of the most studied resistance genes in environmental samples. Sulfonamides are classified as "Highly Important" (the second category) by the World Health Organization (WHO, 2018). Three drugs in this class are used in food animals and administered to groups of animals via food and water.
$bla_{CTX-M-32}$	Encodes for third-generation cephalosporin resistance, one type of β-lactamase resistant drug. These drugs are classified as "Critically Important" (the top category) by World Health Organization. Most individual drugs in the class are limited to use on humans, pets (dog/cat), and horses, however, two (cefquinome and ceftiofur) are indicated for use in food animals, though they are not administered to groups of animals via food or water.
erm(B)	Encodes for resistance to macrolide drugs, such as erythromycin. These drugs are classified as "Critically Important" (the top category) by the World Health Organization. Erythromycin is used in large and small animals, is FDA approved for use in cattle, swine, and poultry, and is administered to food animals via food and water. A related macrolide, azithromycin, is also approved for use in sick food animals, and is individually listed on the CDC Antibiotic Resistance Threat list as a concern for some foodborne pathogens (CDC, 2019).
tet	Encodes for resistance to tetracycline drugs, including tetracycline, chlortetracycline, oxytetracycline, and doxycycline. The tetracyclines are the most widely used drugs in food animal production both globally, and in the United States. The tetracyclines account for 36% of all drugs sold in the United States in 2018 for veterinary purposes, with 44% of those attributed to use in cattle and 48% in swine (US Food and Drug Administration, 2019). Although these drugs are classified as "Highly Important" (the second category) by the World Health Organization, they are not listed on the CDC Antibiotic Resistance Threats in the United States 2019 publication.

TABLE 2 | Quantitative PCR primers and thermocycler conditions utilized in this study.

PCR Target	Primer name	Primer sequence	Thermocycler program	Source
16s rRNA	TB 331F-16srRNA	5′-TCCTACGGGAGGCAGCAGT-3′	95°C (15 min)	Denman and McSweeney (2006)
	TB 518R-16srRNA	5′-ATTACCGCGGCTGCTGG-3′	35 cycles of 95°C (15 s), 55°C (20 s), 72°C (10 s)	
erm(B)	ermB F TB	5′-GGTTGCTCTTGCACACTCAAG-3′	94°C (4 min) 5 cycles* of 94°C (30 s), 63–58°C (30 s), 72°C (60 s)	Koike et al. (2010)
	ermB R TB	5′-CAGTTGACGATATTCTCGATTG-3′	30 cycles of 94°C (30 s), 58°C (30 s), 72°C (45 s) 72°C (7 min)	
intI1	TB intI1LC5 FW	5′-GATCGGTCGAATGCGTGT-3′	95°C (15 min)	Barraud et al. (2010)
	TB intI1LC1 RV	5′-GCCTTGATGTTACCCGAGAG-3′	40 cycles of 95°C (15 s), 55°C (30 s), 72°C (10 s)	
sul(I)	SμLI FW	5′-CGCACCGGAAACATCGCTGCAC-3′	95°C (15 min)	Pei et al. (2006)
	SμLI RVS	5′-TGAAGTTCCGCCGCAAGGCTCG-3′	50 cycles of 95°C (15 s), 65°C (30 s), 72°C (30 s)	
$bla_{ctx-m-32}$	TB ctx-m-32FWD	5′-CGTCACGCTGTTGTTAGGAA-3′	95°C (15 min)	Szczepanowski et al. (2009)
	TB ctx-m-32RVS	5′-CGCTCATCAGCACGATAAAG-3′	35 cycles of 95°C (15 s), 63°C (30 s), 72°C (10 s)	

**Touchdown cycle decrease annealing temperature by 1°C/cycle.*
Standard curve R^2 ranged from 0.960 to 0.998. Efficiency ranged from 80 to 117%.

Comparing *tet* resistance gene prevalence on the final day of the incubation, the occurrence of *tet(*M*)* and *tet(*O*)* were unaffected by manure treatment or soil moisture (100% of samples were positive). Manure treatment affected the frequency of detection of two *tet* resistance genes; *tet(*X*)* gene was less prevalent (0% in soil receiving HLAS-treated manure vs. 75% and 100% in the low and high moisture content soils, respectively, receiving raw manure; $P \leq 0.028$), but *tet(*D*)* was more prevalent in soil receiving HLAS-treated manure (75% detection frequency) than in soil receiving raw manure (0% detection frequency); ($P = 0.028$) on Day 120. Soil moisture content alone did not yield a consistent effect on the frequency of *tet* resistance gene detection; however, the interaction of low soil moisture (10% WHC) and application of raw manure yielded greater frequency of *tet(*A*), tet(*B*)*, and *tet(*C*)* retention (detection frequencies of 75, 50, and 100%, respectively), in samples on Day 120. In comparison, *tet(A)*, *tet(*B*)*, and *tet(*C*)* were found at a frequency of 25% or less in other treatment combinations. Statistically significant differences between any two treatment combinations/sampling dates depended upon the difference in percentage positive. Differences between 25 or 50% detection frequencies were not significant ($P = 0.285$ and 0.102, respectively), while differences between 75 or 100% detection frequencies were significant ($P = 0.028$ and 0.005, respectively) based upon four replicates tested per treatment combination.

Finally, the overall diversity of *tet* resistance genes among all treatment combinations declined during the simulated incubation period (**Figure 2**). The average number of different *tet* resistance genes detected in any treatment combination ranged from 7.5 to 9.75 on Day 0 but declined to a range of 3 to 5.25 by Day 120 ($P < 0.001$). Comparisons made on individual sampling days revealed that soil at initial 10% WHC receiving raw manure

TABLE 3 | Initial resistances in soil, manure slurry, and hydrated lime alkaline stabilized (HLAS) manure slurry assessed by multiplex PCR of tetracycline resistance genes and quantitative PCR of select resistance elements.

Mulitplex PCR, % positive†	Soil	Raw manure	HLAS-treated manure	Resistance mechanism/action
tet(A)	0	100	100	Efflux pump
tet(B)	0	100	100	Efflux pump
tet(C)	0	100	100	Efflux pump
tet(D)	100	0	0	Efflux pump
tet(E)	0	0	0	Efflux pump
tet(G)	100	100	100	Efflux pump
tet(K)	0	0	0	Efflux pump
tet(L)	100	0	0	Efflux pump
tet(A/P)	0	0	0	Efflux pump
tet(M)	0	100	100	Ribosome protection
tet(O)	0	100	100	Ribosome protection
tet(Q)	0	100	100	Ribosome protection
tet(S)	0	0	0	Ribosome protection
tet(X)	0	100	100	Antibiotic metabolism
Quantitative PCR‡				
Per gram dry matte2r				
16S rRNA gene	8.42 (0.08) A	8.43 (0.02) A	8.68 (0.04) B	Protein synthesis
*int*I1	3.48 (0.10) A	6.11 (0.02) B	6.15 (0.01) B	Integrase gene
$bla_{CTX-M-32}$	3.80 (0.27) A	5.03 (0.03) B	4.76 (0.01) C	Antibiotic metabolism/ring cleavage
sul(I)	4.39 (0.07) A	6.70 (0.03) B	6.84 (0.03) C	Alternative dihydropteroate synthase
erm(B)	<1.7 A	6.65 (0.04) B	7.15 (0.02) C	Ribosome protection/dimethylation
Log abundance normalized per 16S rRNA gene				
*int*I1	−4.94 (0.05) A	−2.31 (0.04) B	−2.53 (0.05) B	
$bla_{CTX-M-32}$	−4.78 (0.29) A	−3.40 (0.04) B	−3.92 (0.10) C	
sul(I)	−4.02 (0.05) A	−1.73 (0.05) B	−1.84 (0.07) B	
erm(B)	<−6.70 A	−1.78 (0.06) B	−1.53 (0.05) B	
Abundance normalized per10^6 16S rRNA gene				
*int*I1	12 A	4900 B	2980 B	
$bla_{CTX-M-32}$	17 A	400 B	120 C	
sul(I)	96 A	18900 B	14700 B	
erm(B)	<0.2 A	16800 B	29800 B	

†*Results of multiplex PCR expressed as percentage of PCR-positive soil or manure samples (n = 3).*
‡*For quantitative PCR, each sample was amplified three times and averaged. The average log abundance (±SE) for three samples is reported. "ABC" denotes a difference ($P < 0.05$) between sample types (i.e., soil, manure, and HLAS-treated manure).*

had greater diversity of *tet* resistance genes ($P < 0.05$) on Day 90 and 120 compared to all other treatments ($P = 0.021$). On Day 60, the diversity of *tet* resistance genes in soil receiving raw manure (at initial 10 and 30% WHC) were greater ($P < 0.03$) than in the soil receiving HLAS-treated manure.

Temporal Trends in Resistance Elements and 16S rRNA Genes

The abundances of *int*I1, $bla_{CTX-M-32}$, *sul*(I), *erm*(B), and 16S rRNA genes over the 120-day incubation period, when expressed on a per gram of dry slurry basis, varied among genes (**Figure 3**, left hand panels). Depending on the treatment combination, abundances could be (i) relatively stable [sul(I)], (ii) increasing over the incubation period (16S rRNA genes and intI1 in soil at 10% WHC receiving raw manure), (iii) decreasing slowly over the incubation period [erm(B)], or (iv) quite dynamic (blaCTX-M-32). Of the genes assessed, the abundance of the16S rRNA gene demonstrated the most consistent pattern (CV = 2.26 to 5.61%) regardless of treatments, increased through time, ranging from 7.86 to 8.11 log abundance copies per gram of dried manure/soil on Day 0 to 8.93 to 9.37 log abundance on Day 120.

Comparing among the four treatment combinations, soil at 10% WHC receiving raw manure yielded roughly 10-fold greater abundances of *int*I1, $bla_{CTX-M-32}$, and *sul*(I) on Day 120 compared to all other treatment combinations. The result for *erm*(B) was consistent with other resistance genes and was 0.5 log abundance greater for soil at 10% WHC receiving raw manure than the other treatments. The abundance of resistance elements in soil receiving HLAS-treated manure was consistently lower throughout the incubation period compared to soils receiving raw manure; initial soil moisture did not have a large effect on abundance of resistance elements in soil receiving HLAS-treated manure. However, initial soil moisture did impact the abundance of resistance elements in soil receiving raw manure, with lower resistance gene abundances ($P = 0.002$) noted at

TABLE 4 | Percent positive detection of *tet* genes in soil at 10 or 30% water holding capacity (WHC) with either raw manure or hydrated lime alkaline stabilized (HLAS) manure during simulated winter incubation using multiplex PCR*.

Manure	Moisture,%WHC	Day	*tet* gene (Source)†													
			A (M)	B (M)	C (M)	D (S)	E (-)	G (SM)	K (-)	L (S)	M (M)	O (M)	A(P) (-)	Q (M)	S (-)	X (M)
Raw	10	0	100	100	100	100	0	25	100	0	100	100	0	100	0	100
		30	0	100	100	0	0	0	100	0	100	100	0	0	0	100
		60	100	100	100	0	0	100	0	0	100	100	0	0	0	100
		90	100	100	100	0	0	0	100	0	100	100	0	0	0	100
		120	75	50	100	0	0	0	0	0	100	100	0	0	0	100
	30	0	100	100	100	100	0	75	100	0	100	100	0	100	0	100
		30	0	25	0	100	0	0	0	0	100	100	100	100	0	100
		60	25	50	75	100	0	100	0	0	100	100	50	75	0	75
		90	0	0	25	0	0	0	0	0	100	100	50	50	0	50
		120	0	0	25	0	25	0	0	0	100	100	0	0	0	75
HLAS	10	0	100	100	100	0	0	50	25	0	100	100	0	75	0	100
		30	50	0	75	100	0	50	0	0	100	100	75	50	0	100
		60	0	100	25	0	0	0	0	100	100	100	50	25	0	0
		90	0	100	0	0	0	100	0	0	100	100	100	0	0	25
		120	0	0	25	75	0	0	0	0	100	100	0	0	0	0
	30	0	100	0	100	100	0	100	100	0	100	100	50	75	0	75
		30	0	0	0	100	0	100	0	25	100	100	100	25	0	0
		60	0	100	0	0	0	0	0	25	100	100	75	50	25	0
		90	0	100	0	0	0	50	0	0	100	100	50	25	0	0
		120	0	25	0	75	0	25	0	0	100	100	0	0	25	0

*Four replicates were analyzed for each manure/soil mixture. Statistical comparisons using the equality of proportions test (SAS) between cells can be calculated by the difference in percentage positive detections between two cells: 100% P = 0.005; 75% P = 0.028; 50% P = 0.102; 25% P = 0.285.

†Source indicated by detection in soil (S), manure (M), both soil and manure (SM), or undetermined (–) as described in **Table 3**.

Shading indicates the percent positive detection representing 0% (white), 25% (light gray), 50% (medium gray), 75% (dark gray), and 100% (black) detection.

the 30% WHC level compared to the 10% WHC level (5.54 versus 6.05 log abundance, respectively). There was no evidence of a similar synergistic effect for higher moisture soil receiving HLAS-treated manure.

Normalizing resistance elements to 16s rRNA genes helps to account for unequal initial microbial abundances and changes in the microbial population throughout the incubation. In this study, a very consistent pattern of decreasing resistance element abundance throughout the incubation period is evident (**Figure 3**, right hand panels). Although the abundance of an individual resistance element may have increased slightly during incubation (i.e., *int*I1 for soil at 10% WHC receiving raw manure), the proportion of the resistance element normalized to 16s rRNA genes in the microbial population decreased 10-fold (1 log). Interestingly, *int*I1 abundance in soil receiving HLAS-treated manure was only slightly reduced (roughly 0.5 log) relative to 16S rRNA. For resistance elements that showed decreasing abundance per gram of dried slurry [i.e., *erm(*B*)* and some $bla_{CTX-M-32}$ treatments], the relative reduction was even greater (100-fold or 2 logs).

DISCUSSION

Manure injection is a common swine manure slurry application practice across the United States. When examined at a fine scale (<1–2 cm), this likely has a profound effect on the immediate soil environment and microbial processes. Considering the issue of antibiotic resistance, the narrow band of injected manure introduces a suite of very diverse *tet* resistance genes and resistance elements into the soil at abundances 10- to 100-fold greater than background soil concentrations. One might expect at very low winter-time temperatures that there would be limited microbial growth and very little microbial activity, having little impact on antibiotic resistance. However, studies on wheat straw decomposition at low temperatures indicate that although the rates of CO_2 evolution are decreased, they are still substantial (Stott et al., 1986). In a more recent study, respiration rates measured at 0°C in agricultural and a humus forest soils from southern Sweden were positive and not predicted to reach zero respiration until −6°C (Pietikäinen et al., 2005). Supporting evidence from Arctic and Antarctic soils demonstrates that microorganisms mediate nutrient and carbon cycles at these very low temperatures (Eriksson et al., 2001; Kotsyurbenko, 2005). Stott et al. (1986) speculate that salts in soils may lower the freezing point of water enabling limited microbial decomposition. At the fine scale of manure injection bands in crop fields, this may be particularly important since swine manure slurries usually have high salinity and could depress the freezing point well below 0°C. The results of the simulated winter incubation presented herein, which included multiple freeze/thaw events, indicate that resistance in

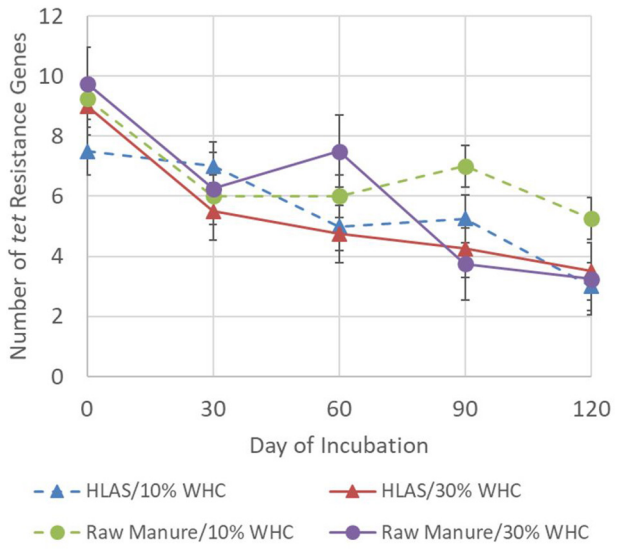

FIGURE 2 | Number of different tetracycline resistance genes detected using the multiplex PCR method for the four treatment combinations, hydrated lime alkaline stabilized (HLAS) swine manure slurry (triangles) or raw swine manure slurry (circles) in soil originally at 10% (dashed line) or 30% (solid line) water holding capacity (WHC). Error bars = standard error.

soil can be substantially altered even during the winter climate experienced in southwestern Minnesota, a region of extensive swine production.

Assessing a suite of tetracycline resistance genes through multiplex PCR reactions showed that both their occurrence and diversity were impacted by the simulated winter incubation. Ultimately, the presence of *tet* resistance genes on Day 120 may be the most important indicator of how soil moisture and manure treatment affect tetracycline resistance after a simulated winter incubation. From this perspective, the results are more easily interpreted. Particular *tet* resistance genes in the swine manure [*tet*(M) and *tet*(O)] showed no change after 120 days, yet others [*tet*(K), *tet*(L), *tet*(A/P), and *tet*(Q)] present in the initial samples or occasionally detected during the incubation were no longer detected after 120 days. Furthermore, the persistence of other *tet* resistance genes [*tet*(A), *tet*(B), *tet*(C), *tet*(D), and *tet*(X)] were affected by application of HLAS-treated manure and/or initial soil moisture and were quite dynamic, disappearing entirely from some of the treatments. Finally, assessed *tet* resistance gene diversity illustrated a consistent decline in all the treatments during the simulated winter incubation, but less of a decline in the drier soil receiving raw manure. It is possible that the slightly lower moisture content provided conditions where manure microbes harboring *tet*(A), *tet*(B), and *tet*(C) were able to persist within the soil microbial community. An alternative hypothesis is that the increased water content enhanced ice crystal formation during freezing, which increased the likelihood that manure bacteria were ruptured during multiple freeze/thaw events. Clearly additional research needs to be done to explore these hypotheses.

Converting the initial log abundances of resistance elements *int*I1, $bla_{CTX-M-32}$, *sul*(I), and *erm*(B) to numbers of targets observed per million copies of the 16S rRNA gene revealed that resistance elements in the soil were much less abundant (<100 copies) compared to those in manures, which contained hundreds to tens of thousands more resistance elements per million 16S rRNA genes (**Table 3**). Although, *int*I1, $bla_{CTX-M-32}$, *sul*(I), and *erm*(B) normalized to 16S rRNA abundance demonstrated a 10- to 100-fold reduction over the simulated winter incubation (**Figure 3**). A substantial portion of resistance element decrease could be attributed to the 10-fold increase in 16S rRNA abundance during the incubation. Although one would expect greatest increases in microbial abundance (i.e., 16S rRNA gene abundance) during the warmest periods (Day 0 to 30), the largest increases were observed from Day 30 to 60 and Day 90 to 120 when temperatures were slightly less than 0°C. One hypothesis that may explain these observations is that the series of freeze/thaw events (Day 0 to 30) lysed some microorganisms and/or released useful microbial substrates from manure solids. Cold tolerant species were then able to proliferate as substrates became available. A change in microbial community reflected in 16S rRNA sequence diversity would support this hypothesis but needs to be conducted.

The application of raw manure to soil at the lower WHC (10 vs. 30%) produced somewhat greater microbial abundance, which effectively diluted the relative abundance of most manure resistance elements for this treatment to a lower concentration. Somewhat to our surprise, even HLAS-treated manure also stimulated microbial growth. Although we presumed that the highly alkaline conditions in the manure would have reduced the capacity for microbial activity and survival, the soil environment was able to moderate the high pH in the treated swine slurry, and soil microbes were able to utilize the carbon and nutrient resources in the manure. For the resistance elements assessed using quantitative PCR in this study, nutrient addition did not preferentially stimulate proliferation of particular ARB over the simulated winter incubation, as was found by Udikovic-Kolic et al. (2014).

Specific *tet* resistance mechanisms in soil after the winter incubation were affected by swine manure application. Initially, only efflux pump types of resistance [*tet*(D), *tet*(G), and *tet*(L)] were detected in background soil, but application of swine manure introduced ribosome protection [*tet*(M), *tet* (O), and *tet*(Q)] and antibiotic metabolism [*tet*(X)] types of resistance to the soil environment. Although tet(Q) was no longer detectable by Day 120, the ribosomal protection resistance mechanism of *tet*(M) and *tet*(O) was still easily detected. Manure treatment also seemed to influence antibiotic metabolism resistance [*tet*(X)]. While *tet*(X) was still detected on Day 120 in soil receiving raw manure, *tet*(X) was no longer detectable in the soil receiving HLAS-treated manure. It is likely that the type of bacterial species harboring these *tet* resistances may be particularly sensitive to high pH and easily disrupted during HLAS.

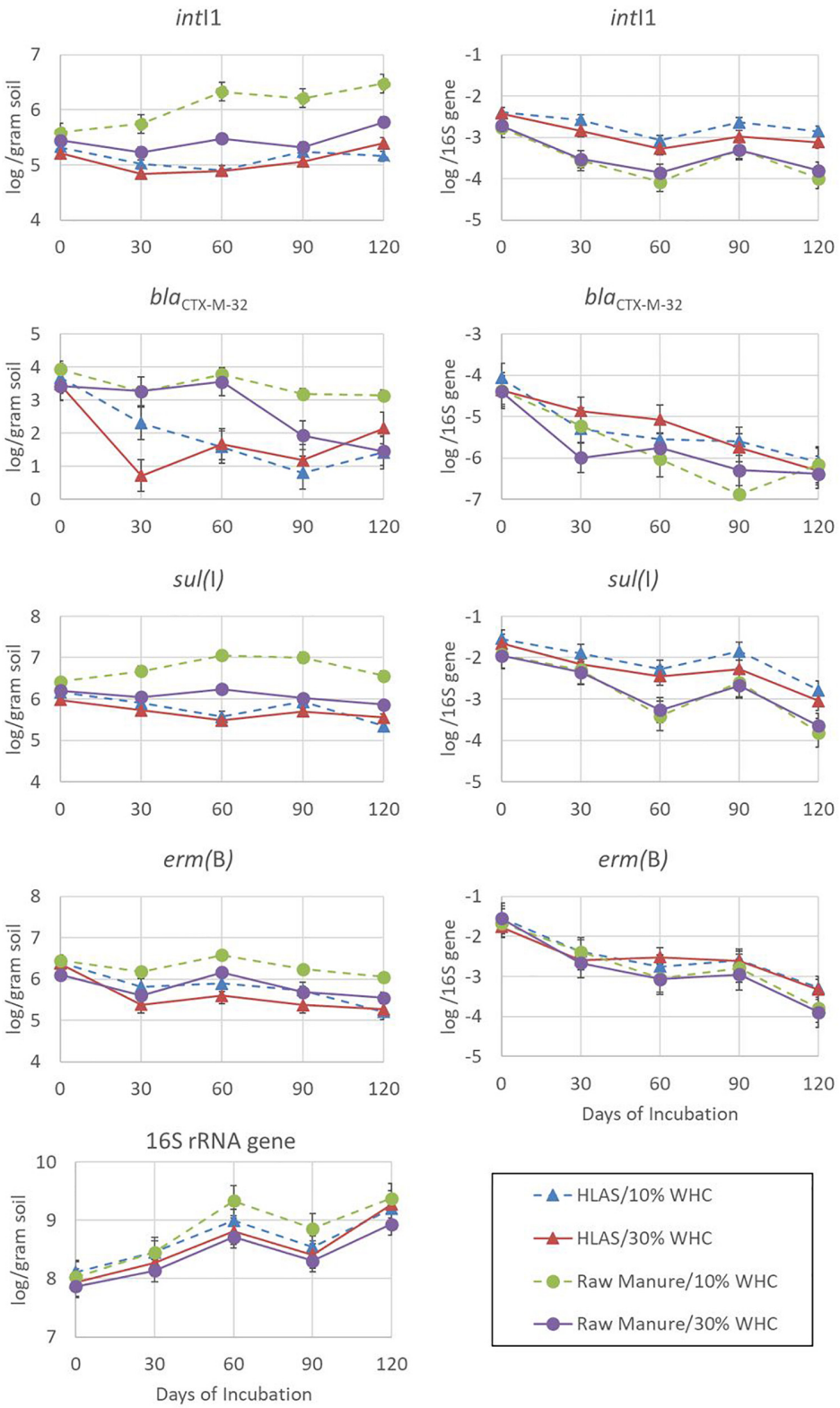

FIGURE 3 | Abundance determined using quantitative polymerase chain reaction of resistance elements *int*I1, $bla_{CTX-M-32}$, *sul*(I), and *erm*(B) in addition to 16S rRNA genes over the 120-day simulated winter incubation. The four treatment combinations were hydrated lime alkaline stabilized (HLAS) swine manure slurry (triangles) or raw swine manure slurry (circles) in soil originally at 10% (dashed line) or 30% (solid line) water holding capacity (WHC) and are expressed in terms of log abundance per gram of dry soil (left-hand column of figures) or log abundance per 16S rRNA gene (right-hand column of figures). Error bars = standard error.

The findings of these laboratory incubations present a unique look at changes in resistance at very low temperatures. However, these results are generally consistent with earlier studies investigating antibiotic resistance in swine manure slurry applied to soil and do not challenge consensus that the abundances of manure resistant genes may temporarily increase in the soil but decrease with time at seasonal scales. Even prior to swine manure application to soil, abundances of *tet* and *erm* genes in swine manure storage pits generally decrease by orders of magnitude as the fresh manure undergoes decomposition (Joy et al., 2014). A field study conducted by Garder et al. (2014) in Iowa examined swine manure injected into soil and may offer the closest comparison to the research presented here. The Iowa study collected data seasonally and found that the absolute abundance (per gram soil) of *erm(*B*)* and *erm*(F) decreased by 5 – 6 log abundance over a year. At the shortest time step, fall to spring, *erm(*F*)* absolute abundance decreased by 2 – 3 log per gram soil. Although over a short time period (65 days) and at higher temperature (25°C), another study conducted in a greenhouse found *tet(*C*)* and *tet(*Z*)* decreased from <0.5 to 2 log abundance after swine manure was initially added to soil (Kang et al., 2017). Finally, a recent study conducted using dairy manure investigated how resistance elements respond to freeze/thaw and various WHC (25 to 75%) at low (5°C) temperature over a 56-day incubation (McKinney and Dungan, 2020). Examining *int*I1, *sul(*I*)*, *tet(*M*)*, *erm(*B*)*, and 16S rRNA genes, they were unable to detect erm(B) but found decreasing absolute abundance (per gram soil) of all other resistance elements at lowest temperature and for all resistance elements except for *tet(*M*)* which did not change at the lowest WHC. Freeze/thaw had little effect on resistance element absolute abundance except for *sul(*I*)* which declined slightly. One subtle difference with our findings was that 16S rRNA gene abundance did not change during the incubation at lowest temperatures (5°C) and WHC (25%). It is interesting to note that 16S rRNA abundance was already above 9 log abundance per gram soil while the abundance in this study started at 8 log abundance and ended near 9 log abundance. Would the 16S rRNA abundance in our study have continued to increase with a longer incubation time at 0°C or would it have plateaued?

Initially, we hypothesized that HLAS treatment would have a strong effect on resistance by directly affecting the viability of microorganisms, but that incubation time would yield minimal changes in resistance after the imposition of "winter" incubation conditions due to low microbial and enzymatic activity. However, our results demonstrate that the soil community even at low temperatures is quite responsive to manure nutrient input. Furthermore, resistance elements were surprisingly dynamic during a presumed inactive period.

CONCLUSION

Swine manure application introduced a diverse set of *tet* resistance genes into low *tet* resistance diversity agricultural soil, increasing the diversity from three of fourteen assessed genes to ten different *tet* genes in the mixed manure/soil. During a simulated winter incubation, the diversity of tetracycline resistance genes declined. However, two swine manure associated resistance genes [*tet(*M*)* and *tet(*O*)*] persisted. The persistence of other *tet* resistance genes [*tet(*A*)*, *tet(*B*)*, *tet(*C*)*, *tet(*D*)*, and *tet(*X*)*] were affected by HLAS treatment and initial soil moisture. Other resistance elements [*int*I1, $bla_{CTX-M-32}$, *sul(*I*)*, and *erm(*B*)*] normalized to 16S rRNA abundance demonstrated a 10- to 100-fold reduction over the simulated winter incubation, due in part to the 10-fold increase in 16S rRNA abundance, and to a limited reduction of most resistance element abundances.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

DM was the principal scientist responsible for conducting the analysis of soil and manure samples, reviewing statistical analyses, developing figures and tables, and finishing the final drafts of the manuscript. MJ was an undergraduate student and responsible for conducting lab analyses and preparing initial statistics, figures, and drafts of the manuscript. LD contributed to laboratory analysis and manuscript preparation. AS was responsible for conducting the incubations and revising manuscripts. All authors contributed to the article and approved the submitted version.

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REFERENCES

Barraud, O., Baclet, M. C., Denis, F., and Ploy, M. C. (2010). Quantitative multiplex real-time PCR for detecting class 1, 2, and 3 integrons. *J. Antimicrob. Chemother.* 65, 1642–1645. doi: 10.1093/jac/dkq167

Barth, V. P., Reardon, C. R., Coffey, T., Klein, A. M., McFarland, C. L., Huggins, D. R., et al. (2018). Stratification of soil chemical and microbial properties under no-till management after lime amendment. *Appl. Soil Ecol.* 130, 169–177. doi: 10.1016/j.apsoil.2018.06.001

CDC, (2019). *Antibiotic Resistance Threats in the United States, 2019*. Atlanta, GA: U.S. Department of Health and Human Services, CDC.

Chee-Sanford, J. C., Mackie, R. I., Koike, S., Krapac, I. G., Lin, Y., Yannarell, A. C., et al. (2009). Fate and transport of antibiotic residues and antibiotic resistance

genes following land application of manure waste. *J. Env. Qual.* 38, 1086–1108. doi: 10.2134/jeq2008.0128

Cook, K. L., Netthisinghe, A. M. P., and Gilfillen, R. A. (2014). Detection of pathogens, indicators, and antibiotic resistance genes after land application of poultry litter. *J. Env. Qual.* 43, 1546–1558. doi: 10.2134/jeq2013.10.0432

Denman, S., and McSweeney, C. (2006). Development of a real-time PCR assay for monitoring anaerobic fungal and cellulolytic bacterial populations within the rumen. *FEMS Microbiol. Ecol.* 58, 572–582. doi: 10.1111/j.1574-6941.2006.00190.x

Durso, L. M., and Cook, K. L. (2014). Impacts of antibiotic use in agriculture: what are the benefits and risks? *Curr. Opin. Microbiol.* 19, 37–44. doi: 10.1016/j.mib.2014.05.019

Durso, L. M., Miller, D. N., and Henry, C. G. (2018). Impact of vegetative treatment systems on multiple measures of antibiotic resistance in agricultural wastewater. *Int. J. Environ. Res. Public Health* 15:1295.

Durso, L. M., Wedin, D. A., Gilley, J. E., Miller, D. N., and Marx, D. B. (2016). Assessment of selected antibiotic resistance in ungrazed native Nebraska prairie soils. *J. Env. Qual.* 45, 454–462. doi: 10.2134/jeq2015.06.0280

Edmeades, D. C. (2003). The long-term effects of manures and fertilisers on soil productivity and quality: a review. *Nutr. Cycl. Agroecosys.* 66, 165–180.

Eriksson, M., Ka, J. O., and Mohn, W. W. (2001). Effects of low temperature and freeze-thaw cycles on hydrocarbon biodegradation in Arctic tundra soil. *Appl. Environ. Microb.* 67, 5107–5112. doi: 10.1128/aem.67.11.5107-5112.2001

Fang, H., Wang, H., Cai, L., and Yu, Y. (2015). Prevalence of antibiotic resistance genes and bacterial pathogens in long-term manured greenhouse soils as revealed by metagenomics survey. *Env. Sci. Tech.* 49, 1095–1104. doi: 10.1021/es504157v

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

Franklin, A. M., Aga, D. S., Cytryn, E., Durso, L. M., McLain, J. E., Pruden, A., et al. (2016). Antibiotics in agroecosystems: introduction to the special section. *J. Env. Qual.* 45, 377–393. doi: 10.2134/jeq2016.01.0023

Garder, J. L., Moorman, T. B., and Soupir, M. L. (2014). Transport and persistence of tylosin-resistant Enterococci, erm genes, and tylosin in soil and drainage water from fields receiving swine manure. *J. Env. Qual.* 43, 1484–1493. doi: 10.2134/jeq2013.09.0379

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

Heuer, H., Kopmann, C., Binh, C. T. T., Topp, E., and Smalla, K. (2009). Spreading antibiotic resistance through spread manure: characteristics of a novel plasmid type with low %G+C content. *Environ. Microb.* 11, 937–949. doi: 10.1111/j.1462-2920.2008.01819.x

Heuer, H., Solehati, Q., Zimmerling, U., Kleineidam, K., Schloter, M., Muller, T., et al. (2011). Accumulation of sulfonamide resistance genes in arable soils due to repeated application of manure containing sulfadiazine. *Appl. Environ. Microb.* 77, 2527–2530. doi: 10.1128/aem.02577-10

Hill, V. R. (2003). Prospects for pathogen reductions in livestock wastewaters: a review. *Crit. Rev. Env. Sci. Tech.* 33, 187–235. doi: 10.1080/10643380390814532

Jechalke, S., Kopmann, C., Rosendahl, I., Groeneweg, J., Weichelt, V., Krogerrecklenfort, E., et al. (2013). Increased abundance and transferability of resistance genes after field application of manure from sulfadiazine-treated pigs. *Appl. Environ. Microb.* 79, 1704–1711. doi: 10.1128/aem.03172-12

Joy, S. R., Li, X., Snow, D. D., Gilley, J. E., Woodbury, B., and Bartelt-Hunt, S. L. (2014). Fate of antimicrobials and antimicrobial resistance genes in simulated swine manure storage. *Sci. Total Env.* 481, 69–74. doi: 10.1016/j.scitotenv.2014.02.027

Kang, Y., Hao, Y., Xia, D., Shen, M., Li, Q., and Hu, J. (2017). The effects of pig manure application on the spread of tetracycline resistance in bulk and cucumber rhizosphere soils: a greenhouse experiment. *Can. J. Microbiol.* 63, 563–572. doi: 10.1139/cjm-2016-0767

Klute, A. (1986). "Water retention: laboratory methods," in *Methods of Soil Analysis. I. Physical and Mineralogical Methods*, ed. C. A. Black, (Madison, WI: ASA; SSSA), 635–662. doi: 10.2136/sssabookser5.1.2ed.c26

Koike, S., Aminov, R., Yannarell, A., Grans, H., Krapac, I., Chee-Sanford, J., et al. (2010). Molecular ecology of macrolide–lincosamide–streptogramin B methylases in water lagoons and subsurface waters associated with swine production. *Microb. Ecol.* 59, 487–498. doi: 10.1007/s00248-009-9610-0

Kotsyurbenko, O. R. (2005). Trophic interactions in the methanogenic microbial community of low-temperature terrestrial ecosystems. *FEMS Microb. Ecol.* 53, 3–13. doi: 10.1016/j.femsec.2004.12.009

Lauber, C. L., Hamady, M., Knight, R., and Fierer, N. (2009). Pyrosequencing-based assessment of soil pH as a predictor of community structure at the continental scale. *Appl. Environ. Microb.* 75, 5111–5120. doi: 10.1128/aem.00335-09

Lin, Y., Ye, G., Liu, D., Ledgard, S., Luo, J., Fan, J., et al. (2018). Long-term application of lime or pig manure rather than plant residues suppressed diazotroph abundance and diversity and altered community structure in an acidic Ultisol. *Soil Biol. Biochem.* 123, 218–228. doi: 10.1016/j.soilbio.2018.05.018

Mantz, A. R., Miller, D. N., Spiehs, M. J., Woodbury, B. L., and Durso, L. M. (2013). Persistence of erythromycin resistance genes erm(B) in cattle feedlot pens over time. *Agric. Food Anal. Bacteriol.* 3, 312–320.

McKinney, C. W., and Dungan, R. S. (2020). Influence of environmental conditions on extracellular and intracellular antibiotic resistance genes in manure-amended soil: a microcosm study. *Soil Sci. Soc. Am. J.* 84, 747–759. doi: 10.1002/saj2.20049

McKinney, C. W., Dungan, R. S., Moore, A., and Leytem, A. B. (2018). Occurrence and abundance of antibiotic resistance genes in agricultural soil receiving dairy manure. *FEMS Microb. Ecol.* 94:fiy010.

McLaughlin, M. R., Brooks, J. P., and Adeli, A. (2012). Temporal flux and spatial dynamics of nutrients, fecal indicators, and zoonotic pathogens in anaerobic swine manure lagoon water. *Water Res.* 46, 4949–4960. doi: 10.1016/j.watres.2012.06.023

Munir, M., and Xagoraraki, I. (2011). Levels of antibiotic resistance genes in manure, biosolids, and fertilized soil. *J. Env. Qual.* 40, 248–255. doi: 10.2134/jeq2010.0209

Ng, L. K., Martin, I., Alfa, M., and Mulvey, M. (2001). Multiplex PCR for the detection og tetracycline resistant genes. *Mol. Cell. Probe.* 15, 209–215. doi: 10.1006/mcpr.2001.0363

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

Peng, S., Wang, Y., Zhou, B., and Lin, X. (2015). Long-term application of fresh and composted manure increase tetracycline resistance in the arable soil of eastern China. *Sci. Total Environ.* 506, 279–286. doi: 10.1016/j.scitotenv.2014.11.010

Peng, S., Zhou, B., Wang, Y., Lin, X., Wang, H., and Qiu, C. (2016). Bacteria play a more important role than nutrients in the accumulation of tetracycline resistance in manure-treated soil. *Biol. Fert. Soils* 52, 655–663. doi: 10.1007/s00374-016-1105-9

Pietikäinen, J., Pettersson, M., and Bååth, E. (2005). Comparison of temperature effects on soil respiration and bacterial and fungal growth rates. *FEMS Microbiol. Ecol.* 52, 49–58. doi: 10.1016/j.femsec.2004.10.002

Stevens, E. E., Miller, D. N., Brittenham, B. A., Vitosh-Sillman, S. J., Brodersen, B. W., Jin, V. L., et al. (2018). Alkaline stabilization of manure slurry inactivates porcine epidemic diarrhea virus. *J. Swine Health Prod.* 26, 95–100.

Stott, D. E., Elliott, L. F., Papendick, R. I., and Campbell, G. S. (1986). Low temperature or low water potential effects on the microbial decomposition of wheat residue. *Soil Biol. Biochem.* 18, 577–582.

Szczepanowski, R., Linke, B., Krahn, I., Gartemann, K., Gutzkow, T., Eicher, W., et al. (2009). Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing reduced susceptibility to selected antibiotics. *Microbiology* 155, 2306–2319. doi: 10.1099/mic.0.028233-0

Tan, W., Wang, W., Bai, W., Qi, J., and Chen, W. (2020). Soil bacterial diversity correlates with precipitation and soil pH in long-term maize cropping systems. *Sci. Rep.* 10:6012.

Tang, X., Lou, C., Wang, S., Lu, Y., Liu, M., Hashmi, M. Z., et al. (2015). Effects of long-term manure applications on the occurrence of antibiotics and antibiotic resistance genes (ARGs) in paddy soils: evidence from four field experiments in south of China. *Soil Biol. Biochem.* 90, 179–187. doi: 10.1016/j.soilbio.2015.07.027

Topp, E., Larsson, D. G. J., Miller, D. N., Van den Eede, C., and Virta, M. P. J. (2018). Antimicrobial resistance and the environment: assessment of advances,

gaps and recommendations for agriculture, aquaculture and pharmaceutical manufacturing. *FEMS Microb. Ecol.* 94:fix185.

Udikovic-Kolic, N., Wichmann, F., Broderick, N. A., and Handelsman, J. (2014). Bloom of resident antibiotic-resistant bacteria in soil following manure fertilization. *PNAS* 111, 15202–15207. doi: 10.1073/pnas.1409836111

US Code of Federal Regulations (2018). *Standards for the Use or Disposal of Sewage Sludge, 40 C.F.R. §503 Appendix B*. Available online at: https://www.govinfo.gov/content/pkg/CFR-2018-title40-vol32/xml/CFR-2018-title40-vol32-part503.xml#seqnum503.25 (accessed 1 July, 2018).

US Environmental Protection Agency (2000). *Biosolids Technology Fact Sheet. Alkaline stabilization of biosolids. EPA 832-F-00-052*. Available online at: https://www.epa.gov/biosolids/fact-sheet-alkaline-stabilization-biosolids (accessed September, 2000)

US Food and Drug Administration, (2019). *Summary Report on Antimicrobials Sold or Distributed fo Use in Food-Producing Animals*. Silver Spring, MD: US Food & Drug Administration.

von Wintersdorff, C. J. H., Penders, J., van Niekerk, J. M., Mills, N. D., Majumder, S., van Alphen, L. B., et al. (2016). Dissemination of antimicrobial resistance in microbial ecosystems through horizontal gene transfer. *Front. Microbiol.* 7:173. doi: 10.3389/fmicb.2016.00173

WHO, (2018). *Critically Important Antimicrobials for Human Medicine*. Geneva: World Health Organization.

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Anaerobic Digestion of Tetracycline Spiked Livestock Manure and Poultry Litter Increased the Abundances of Antibiotic and Heavy Metal Resistance Genes

Getahun E. Agga[1], John Kasumba[2], John H. Loughrin[1] and Eric D. Conte[2]*

[1] USDA, Agricultural Research Service, Food Animal Environmental Systems Research Unit, Bowling Green, KY, United States, [2] Department of Chemistry, Western Kentucky University, Bowling Green, KY, United States

Anaerobic digestion is used for the treatment of animal manure by generating biogas. Heavy metals cause environmental pollutions and co-select for antimicrobial resistance. We evaluated the impact of mesophilic anaerobic digestion of cattle manure (CM), swine manure (SM) and poultry litter (PL) on the concentrations of seven tetracycline [*tet*(A), *tet*(B), *tet*(G), *tet*(M), *tet*(O), *tet*(Q), and *tet*(W)], macrolide [*erm*(B)], methicillin (*mec*A and *mec*C), copper (*cop*B, *pco*A, *pco*D, and *tcr*B) and zinc (*czr*C) resistance genes, and three bacterial species (*E. coli*, *Enterococcus* spp. and *Staphylococcus aureus*). The total bacterial population and total abundance of the seven *tet* genes significantly increased in the three manure types after digestion. Concentration of *tet*(M) was strongly correlated with that of *erm*(B) and enterococci. As concentration of tetracyclines declined during anaerobic digestion, that of four *tet* genes (A, B, Q, and W) and 16S rRNA increased, that of *tet*(M) decreased, and that of *tet*(G) and *tet*(O) did not change. Concentrations of *cop*B and *pco*A did not change; while that of *pco*D did not change in the PL, it increased in the SM and CM. While the concentration of enterococci remained unchanged in CM, it significantly increased in the PL and SM. Concentrations of *tcr*B significantly increased in the three manure types. While concentrations of *S. aureus* significantly increased in the CM and PL, that of SM was not affected. Concentrations of *mec*C significantly increased in all manure types after digestion; while *mec*A concentrations did not change in the SM, they significantly increased in CM and PL. While concentration of *czr*C remained low in the CM, it increased in the PL but declined in the SM. In conclusion, while mesophilic anaerobic digestion of animal manure decreased concentration of tetracyclines, it increased the concentrations of total bacteria, *tet* genes, *E. coli*, enterococci and *S. aureus* and methicillin resistance genes. It did not have any effect on concentrations of heavy metals; concentrations of heavy metal resistance genes either increased or remained unaffected depending on the animal species. This study showed the need for post-digestion treatments of animal manure to remove bacteria, antibiotic resistance genes, heavy metals and their resistance genes.

Keywords: antimicrobial resistance, antimicrobial resistance genes, anaerobic digestion, tetracycline resistance, heavy metal resistance, animal manure, poultry litter, bacteria

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Abasiofiok Mark Ibekwe, United States Department of Agriculture (USDA), United States

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****Correspondence:***
Getahun E. Agga
Getahun.agga@usda.gov

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INTRODUCTION

Antibiotics play a significant role in food animals to treat, prevent and control bacterial infections. Although the use of medically important antibiotics for growth promotion are banned in many countries, they are still used in other parts of the world (OIE, 2020). Tetracyclines are the most widely used antibiotics in food animals in the United States (FDA, 2019) and worldwide (OIE, 2020) making it a good choice to evaluate mitigation strategies to reduce antibiotic resistance determinants. Antibiotics are released into the environment through feces and urine mostly unchanged or as transformation products along with antibiotic resistant bacteria (ARB) and associated antibiotic resistance genes (ARGs) (Williams-Nguyen et al., 2016; Oliver et al., 2020). Once in the environment, antibiotics can exert selection pressure on bacteria (Pruden et al., 2006) resulting in the propagation and spread of ARB via hydrologic processes beyond the point of use, consequently resulting in environmental and public health concerns (Peak et al., 2007). Antibiotic resistant bacteria can cause severe, difficult to treat, and sometimes fatal infections, with groundwater serving as a potential source of antimicrobial resistant pathogens in the human food chain (Chee-Sanford et al., 2001; Campagnolo et al., 2002) or when animal manure is land applied as soil amendment (Miller et al., 2019). Several studies reported multiple ARGs in various environments including water, sludge, farm soils, sediment, animal manure, and municipal wastewater (Chen et al., 2010; Knapp et al., 2010; Munir and Xagoraraki, 2011; Jiang et al., 2013; Agga et al., 2015a, 2019; Rodriguez-Mozaz et al., 2015; Sui et al., 2016). Manure from livestock and poultry farming plays an important role in the dissemination of ARB and ARGs in the environment when applied as fertilizer on agricultural farms. Manure is a reservoir of ARB and antibiotic compounds, and its application on agricultural soils can significantly increase ARGs and selects for ARB populations in the farm soils and other environmental compartments (Heuer et al., 2011; Udikovic-Kolic et al., 2014; Miller et al., 2019; Meyers et al., 2020). Cattle and swine manure storage lagoons are known to carry ARGs including various tetracycline resistance (*tet*) genes (Koike et al., 2007; Peak et al., 2007).

Heavy metals such as copper (Cu) and zinc (Zn) are widely used in animal agriculture as normal nutrient requirements in the form of feed supplements. Copper and Zn are also added to animal feed in higher concentrations than required for growth promotion, disease prevention and therapy (Rensing et al., 2018). They are particularly considered as a potential alternative to antibiotics in food animals due to increased pressure to avoid the use of medically important antibiotics for growth promotion or their routine use for disease prevention. However, concerns are growing with the excessive use of heavy metals since studies have shown the use of Cu and Zn are associated with antibiotic resistance and heavy metal resistance co-selects for antibiotic resistance genes (Yazdankhah et al., 2014). Moreover, environmental pollution is a concern. They are excreted in feces and contaminate water sources and plants from animal manure runoff or from manure land application as a soil amendment and persist in the environment leading to environmental toxicity (Jensen et al., 2016; Rensing et al., 2018).

Animal manure management technologies such as composting, anaerobic digestion (AD), aerobic digestion, chemical stabilization, and others can be employed to treat animal manure to reduce the concentrations of antibiotic residues, bacteria and ARGs before disposal and land application (Zhang et al., 2015). Anaerobic digestion is a widely used manure treatment technology because of its ability to reduce the volume of the manure, remove pathogens and ARGs, and simultaneously produce useful biogas (Sahlström, 2003; Novak et al., 2007; Ma et al., 2011; Kwietniewska and Tys, 2014). Anaerobic digestion of swine lagoon (Sui et al., 2016), and municipal wastewater solids (Diehl and LaPara, 2010) resulted up to 1.34 logs reductions in the concentrations of tetracycline, sulfonamide and macrolide resistance genes. However, our recent study found that depending on the amount and frequency of addition of feed to the digesters, AD of swine manure either reduced, increased, or had no significant effect on the abundances of the *tet* genes quantified (Couch et al., 2019). Similarly, Chen et al. reported that AD and lagoon storage did not reduce the abundances of macrolide (*erm*) and *tet* genes in swine manure (Chen et al., 2010).

Because of the discrepancies in the previous studies regarding the effect of AD on ARGs in animal manure, more research on AD as an on-farm manure treatment technology is still desired. In a study (Kasumba et al., 2019) that evaluated the effect of mesophilic AD of cattle and swine manure, and poultry litter on the concentrations of tetracyclines, we observed differences in the concentrations of Cu and Zn by animal species. The objectives of this study were to evaluate the effect of AD of livestock and poultry manure on the abundances of seven *tet* genes *tet*(A), *tet*(B), *tet*(G), *tet*(M), *tet*(O), *tet*(Q), and *tet*(W), heavy metal (Cu and Zn) resistance genes, and macrolide resistance gene *erm*(B) and three bacterial pathogens *E. coli*, *Enterococcus* spp., and *Staphylococcus aureus* reported to be associated with heavy metal resistance. While *tet*(A), *tet*(B), and *tet*(G) encode for efflux proteins, *tet*(M), *tet*(O), *tet*(Q), and *tet*(W) encode for ribosomal protection proteins (Roberts and Schwarz, 2016). These genes were commonly reported from swine feces (Agga et al., 2015b) and swine waste lagoons (Koike et al., 2007; Agga et al., 2015a).

MATERIALS AND METHODS

Anaerobic Digestion Experiments

The experimental setup of the AD is previously described (Kasumba et al., 2019). Briefly, cattle manure (CM), swine manure (SM) slurry, and poultry litter (PL) were obtained from independently owned commercial farms in central Kentucky. Cattle manure was obtained from an animal kept in a pen of animals with no antibiotics at Western Kentucky University's feedlot cattle operation. Attempts were not successful to obtain antibiotic use information from the swine and poultry farms. However, we previously reported that the corn used at the swine farm was antibiotics free which may suggest raised without antibiotics production system (Couch et al., 2019). Two 100 mL

samples of SM (∼103 g each) were weighed into two separate beakers, each diluted five times with deionized (DI) water to 500 mL to approximately 5% total solids. Because CM and PL had lower moisture contents than SM, ∼50 g of CM and PL were weighed into beakers and diluted with DI water to 500 mL. Each slurry sample was spiked with a mixture of TC, CTC, and OTC adjusted to a final concentration of 1 μg/mL each in the samples. The digestion experiment was conducted in duplicates. Diluted samples were transferred to 3 L airtight polyvinyl chloride (PVC) batch reactors where the AD experiments were conducted for 64 days. On day one, 5 mL of a 50 μg/mL glucose solution (in water) was added to each reactor as an additional energy source for the microorganisms, thereafter 1 mL of the glucose solution was added every week until the end of the experiment. The headspace of each PVC reactor was blown with nitrogen gas to remove air before the AD experiment was initiated. After reactors were agitated to ensure homogeneity, 20–25 mL liquid samples were collected from each reactor every 8 days.

DNA Extraction and Gene Quantification

Total community DNA was extracted from 500 μL of the liquid samples using the FastDNA Spin kit for soils (MP Biomedical, Santa Ana, CA, United States) following the manufacturer's instructions. Real time quantitative PCR (qPCR) was used to quantify the concentrations of genes encoding for all bacteria (through 16S rRNA), and seven *tet* genes *tet(*A), *tet*(B), *tet*(G), *tet*(M), *tet*(O), *tet*(Q), and *tet*(W) using published primers, probes and protocols (**Supplementary Table 1**). The primers were obtained from Sigma-Genosys (The Woodlands, TX, United States), and the dual-labeled black hole quencher probes for the 16S rRNA TaqMan assay were from Biosearch Technologies, Inc., (Petaluma, CA, United States). The qPCR assays were performed in QuantiTect SYBR green master mix (Qiagen, Valencia, CA, United States) in a total reaction volume of 25 μL. The assay consisted of 12.5 μL, 1.5 μL of 10 μm each of the forward and reverse primers, 200 nm of probe (for 16S rRNA), and 5 μL of 10 ng of sample DNA or the standard (ranging from 10^2 to 10^8 copies), and 4.5 μL of water. Sample DNA was diluted to 1:500 ratio to reduce the effect of PCR inhibitors in the samples. Typical qPCR reaction consisted of initial activation at 95°C for 15 min followed by 40 cycles of denaturation at 95°C for 15 s and annealing at specific temperatures (see **Supplementary Table 1**) for 20 s, followed by a final extension at 72°C for 30 s. Melt curve analysis was conducted between 65°C and 95°C with an increment of 0.2°C for 1 s. qPCR reactions were run on the CFX 96 real-time PCR detection system (Bio Rad Laboratories Inc., Hercules, CA, United States). From day 0 (undigested raw manure) and day 64 (digested manure) samples for which Cu and Zn concentrations were measured (Kasumba et al., 2019) bacteria previously reported to carry Cu and Zn resistance genes, and *erm*(B) and methicillin resistance genes (*mec*A and *mec*C) were quantified using published primers and protocols (**Supplementary Table 1**) using QX200 droplet digital PCR (ddPCR) system (Bio Rad Laboratories Inc.). We used TaqMan probes for *E. coli* and *Enterococcus* spp., and Eva Green assays for *S. aureus*, *erm*(B), *mec*A, and *mec*C and heavy metal resistance genes.

Data Analysis

Gene copies of total bacteria (16S rRNA), antibiotic- and heavy metal- resistance genes were analyzed as count outcomes. The effects of sampling day (i.e., AD effect) and manure type on these outcomes were analyzed by negative binomial regression using mean as a dispersion parameter. Day 0 sampling was used as a baseline to evaluate the effect of AD. Since the proportions of non-detects (i.e., zero counts) in the PL were high for *tet*(B), *tet*(G) and *tet*(Q), only CM and SM were compared. Mean gene counts were converted to $\log_{10}$/mL and plotted over sampling days. The association between total tetracycline concentration, and 16S rRNA and the *tet* genes was evaluated using a negative binomial model, and $\log_{10}$ predicted gene copies were plotted against tetracycline concentration over time. Linear regression was used to compare the concentrations of Cu and Zn (as a continuous variable) among the manure types and between the sampling days. Manure type and sampling day were first evaluated in a univariate analysis. Full models with the sampling day and manure type, and their interaction terms were first modeled, and each model was subsequently evaluated after removing a statistically non-significant term. When univariate analyses were significant, sampling day was included in the model. Associations between the heavy metals, heavy metal resistance genes and selected bacterial concentrations were analyzed by a pairwise Pearson correlation coefficient. All statistical analyses were done in STATA 16 (StataCorp LLC, College Station, TX, United States).

RESULTS

Effect of Anaerobic Digestion of Animal Manure on the Abundances of Bacteria

Mean concentrations of total bacteria, *E. coli*, enterococci, and *S. aureus* are shown in **Figure 1** as a function of manure type and effect of AD. The mean concentration of the total bacteria (16S rRNA) was higher in the CM (9 logs) than SM (8.2 logs) or PL (8.5 logs) on day 0. Overall, the abundances of the total bacterial genes increased during AD in all manure types; day 64 concentrations were 4.7 (CM), 4.0 (SM), and 5.5 (PL) times higher than their respective day 0 concentrations. The 16S rRNA gene concentrations steadily increased from their baseline levels for the three manure types during the first 24 days of AD and remained unchanged thereafter. Poultry litter showed the greatest increase in the mean abundances of the 16S rRNA genes and its concentrations were higher than those of CM or SM starting on day 4 of AD. Cattle manure had the highest baseline gene copies of *E. coli* and SM had the least. While concentrations of *E. coli* significantly increased in CM and PL following AD, in SM it did not change from day 0 level. On day 64, CM and PL had similar levels of *E. coli* concentrations which was higher than that of SM. On day 0, the highest and lowest gene copies of enterococci were observed in the CM and SM, respectively. While enterococci concentrations significantly increased in both the PL and SM following AD, that of CM did not change from baseline level. On day 64, the highest and lowest concentration of enterococci

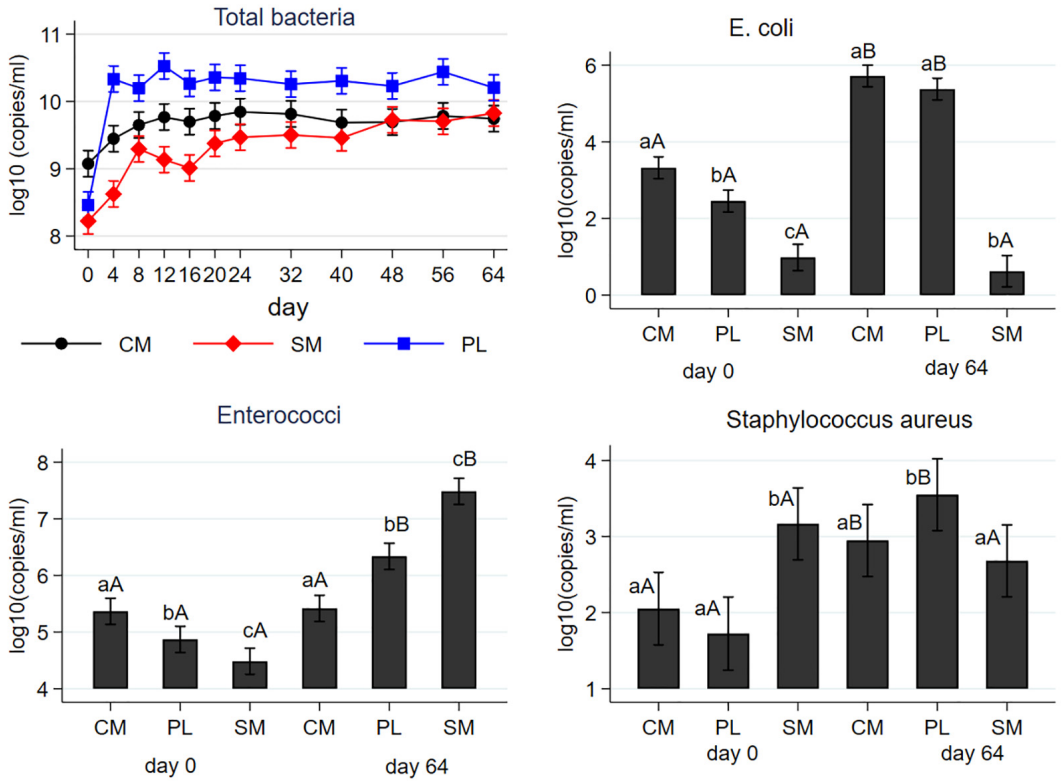

FIGURE 1 | Effect of mesophilic anaerobic digestion of cattle manure (CM), swine manure (SM) and poultry litter (PL) on the mean concentrations of total bacteria (16S rRNA), *E. coli (uidA)*, enterococci (23S rRNA) and *Staphylococcus aureus* (*nuc*). Data were analyzed by negative binomial regression considering gene copy numbers as count outcomes. Plots are shown as the mean concentrations along with their 95% confidence intervals. Different lower-case letters within the same sampling day indicate significant differences between the manure types; different upper-case letters within the same manure type indicate significant differences by sampling day (i.e., digestion effect). Significance was assessed at $P < 0.05$.

were observed in the SM and CM, respectively. Concentration of *S. aureus* significantly differed both by the manure type and AD. In the pre-digested samples, the highest concentration was observed in SM with no difference between CM and PL. In the digested samples, PL had the highest concentration with no significant difference between CM and SM. While the levels of *S. aureus* significantly increased in CM and PL due to AD, that of SM did not change from its pre-digested level.

Effect of Anaerobic Digestion of Animal Manure on the Abundances of Antibiotic Resistance Genes

Combined and individual concentrations of seven tetracycline resistance genes are shown in **Figure 2** as a function of manure type and sampling day. Combined total *tet* gene concentration varied at the baseline among the manure types (CM > SM > PL). Total *tet* gene concentrations increased from their pre-digestion levels in the three manure types during digestion; and total concentrations were significantly higher in the SM starting on day 24 during AD compared to CM or PL. The percentages of PL samples (n = 24) with non-detects (observations with zero gene copies i.e., with no PCR amplification) were 8.3% for *tet*(A), 83.3% for *tet*(B), 20.8% for *tet*(G) and 45.8% for *tet*(Q). Consequently, only *tet*(A) could be compared among the three manure types, while *tet*(B), *tet*(G), and *tet*(Q) were compared only between CM and SM under negative binomial models. Concentrations of *tet*(A), *tet*(B), *tet*(G), *tet*(M), *tet*(O), and *tet*(Q) increased from their baseline levels during AD in CM and SM. Concentration of *tet*(A) did not change from its baseline level in PL during AD. However, concentrations of *tet*(W) increased during AD of PL with no changes in the CM and SM. Comparing by manure type, CM had higher baseline concentrations of *tet*(A), *tet*(B), *tet*(G), *tet*(M), *tet*(O), and *tet*(W) than those of SM or PL. Concentrations of *tet*(A), *tet*(B), and *tet*(Q) in CM remained higher than that of SM or PL on most of the sampling days during AD. Concentrations of *tet*(G) in CM were higher than that of SM during the first 20 days of AD. Cattle manure had the lowest concentrations of *tet*(M) during AD compared to SM or PL.

Relative abundances (calculated as a ratio of *tet* gene copies per 16S rRNA gene copy) and distribution of the *tet* genes also varied considerably among the manure types (**Figure 3**). The abundances of the *tet* genes relative to 16S rRNA gene were considerably higher in the SM compared to CM or PL. The *tet*(M) gene had the highest relative abundances in SM and PL, while *tet*(A) and *tet*(Q) had the highest relative abundances in CM. Total tetracycline concentration generally decreased during AD regardless of the manure type (**Figure 4**).

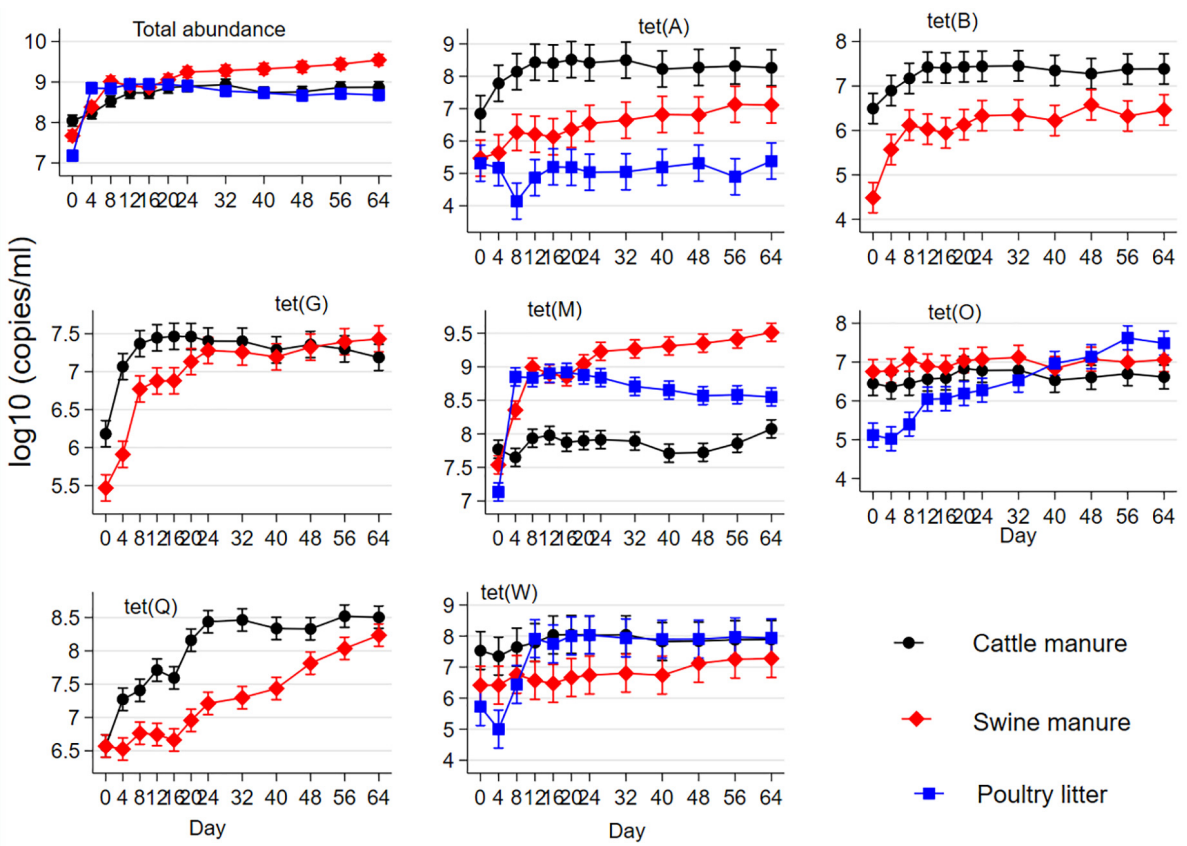

FIGURE 2 | Effect of mesophilic anaerobic digestion of cattle and swine manure and poultry litter on the mean concentrations of total and individual abundances of seven tetracycline resistance genes [*tet*(A), *tet*(B), *tet*(G), *tet*(M), *tet*(O), *tet*(Q), and *tet*(W)]. Data were analyzed by negative binomial regression considering gene copy numbers as count outcomes and displayed as the mean concentrations and their 95% confidence intervals.

Total bacterial population increased as the total tetracycline concentrations decreased over time during AD in the three manure types. A similar negative association was observed for *tet*(A), *tet*(B), *tet*(Q) and *tet*(W). Interestingly, however, a positive association was observed between *tet*(M) and tetracycline concentration in which concentrations of *tet*(M) dropped as the tetracycline concentrations decreased over time regardless of manure type. *tet*(G) and *tet*(O) were not significantly associated with tetracycline concentration.

Macrolide resistance [*erm*(B)] and methicillin resistance (*mec*A and *mec*C) genes were measured at baseline (day 0) and post digestion (day 64) and results are shown in **Figure 5**. Concentrations of *erm*(B) significantly differed by the manure type both at the baseline and after digestion with the highest and lowest concentrations observed in the SM and CM, respectively. While concentrations in the CM remained unchanged, concentrations in the PL decreased, and concentrations in the SM significantly increased from the pre-digestion level at the end of digestion (**Figure 5**). Concentrations of *mec*A gene was significantly higher in the SM before digestion compared to CM or PL. However, it was significantly higher in PL than either CM or SM after digestion (**Figure 5**). Levels of *mec*A genes significantly increased in CM and PL with no change in SM following AD. The mean concentrations of *mec*C gene did not differ by manure type both in the pre- and post- digested samples (**Figure 5**). However, its concentrations significantly increased from predigested levels due to digestion in all manure types.

Effect of Anaerobic Digestion of Animal Manure on the Concentrations of Heavy Metals, and Heavy Metal Resistance Genes

Sampling day (i.e., AD) and its interaction with manure type did not have significant ($P > 0.05$) effects on Cu and Zn concentrations. Manure type had a significant impact on the concentrations of both heavy metals; SM had the highest concentrations of both metals and CM had the least concentrations (**Table 1**). Gram negative Cu resistance gene *cop*B was not detected from the PL. Its mean gene copies were significantly higher in the SM compared to CM (**Table 1**). Anaerobic digestion did not have any significant ($P = 0.472$) effect on the mean *cop*B copies. Mean *pco*A gene copies were not significantly ($P = 0.608$) affected by AD (**Figure 6**); PL had significantly higher mean gene copies than CM or SM (**Table 1**). The *pco*D gene differed both by AD and manure type (**Table 1**); undigested PL had the highest mean copies and remained unaffected by AD while mean copies in the CM and

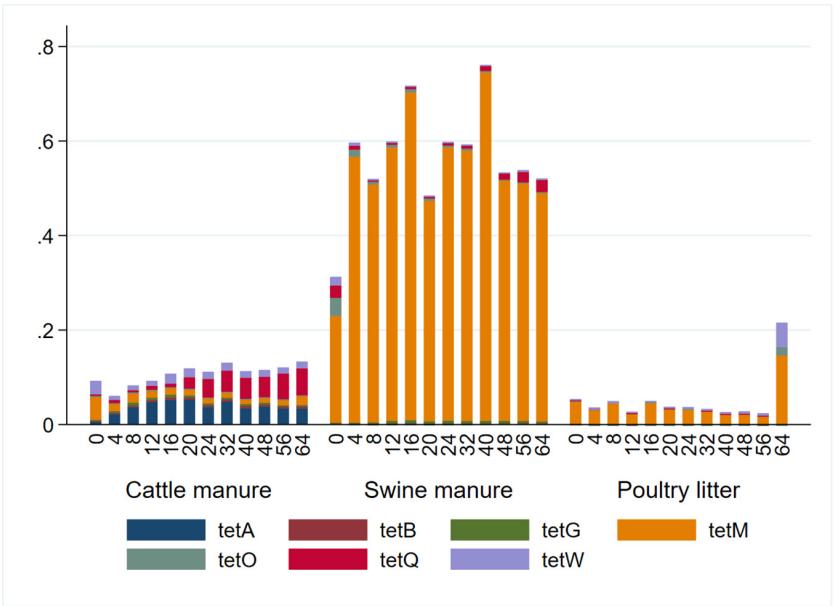

FIGURE 3 | Relative abundance of seven tetracycline resistance (*tet*) genes by sampling day and manure type. Relative abundances were calculated as a ratio of the *tet* genes to 16S rRNA concentrations.

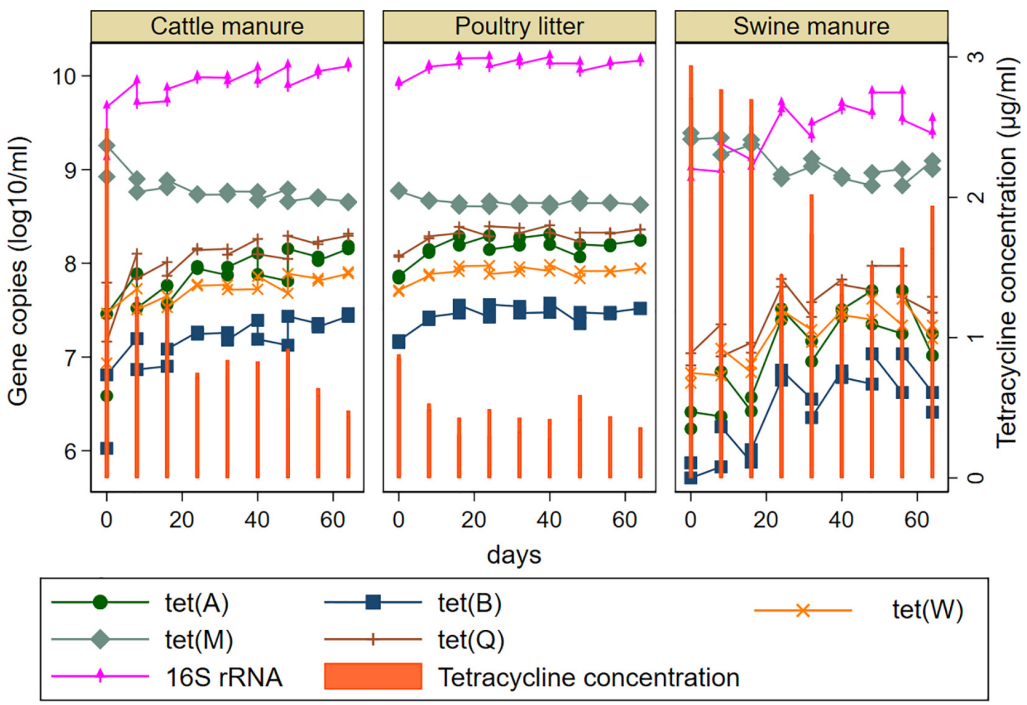

FIGURE 4 | Effect of tetracycline concentrations on the model predicted abundances of total bacteria and five tetracycline resistance genes [*tet*(A), *tet*(B), *tet*(M), *tet*(Q), and *tet*(W) during mesophilic anaerobic digestion of cattle and swine manure, and poultry litter. *tet*(G) and *tet*(O) were not shown since they were not significantly associated with the concentration of tetracyclines. Predicted values for the *tet* genes was obtained after linear regression using tetracycline concentrations as a continuous variable.

SM significantly increased during AD (**Figure 6**). The commonly reported transferable copper resistance *tcr*B gene in enterococci and other gram-positive bacteria was significantly higher in the PL (**Table 1**) both before- and after- digestion when compared to CM or SM. Mean concentrations of *tcr*B significantly increased from its pre-digestion level following digestion in all manure

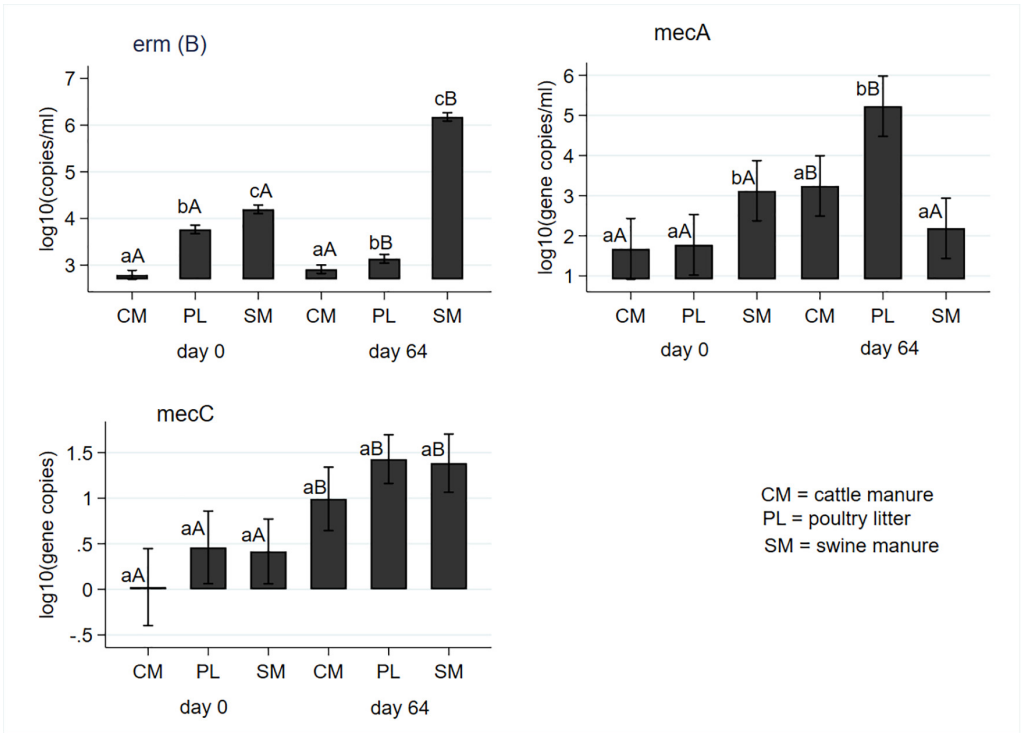

FIGURE 5 | Effect of mesophilic anaerobic digestion of cattle and swine manure, and poultry litter on concentrations of macrolide *erm*(B) and methicillin (*mec*A and *mec*C) resistance genes. Data were analyzed with negative binomial regression and results were plotted as mean values with 95% confidence intervals. Different lower-case letters within the same sampling day indicate significant differences between the manure types; different upper-case letters within the same manure type indicate significant differences by sampling day (i.e., digestion effect). Significance was assessed at $P < 0.05$.

types (**Figure 6**). Zinc resistance gene *czr*C significantly differed both by the manure type and AD. The highest concentration was observed in the pre-digested SM samples; PL had the highest concentrations in the post-digestion samples. Mean *czr*C concentration significantly increased in PL, decreased in SM, and did not change in the CM (**Figure 6**). In general, its concentration in the CM was the lowest (**Table 1**).

We also examined pairwise correlations between concentrations of heavy metals, heavy metal resistance genes, and three bacterial genera (*E. coli*, *Enterococcus* spp. and *S. aureus*) as shown in **Table 2**. Strong positive correlations were observed between *tet*(M), *erm*(B) and enterococci; between the heavy metals; between gram positive (*tcr*B) and gram-negative (*pco*A) copper resistance genes; and between methicillin- and copper-resistance genes.

DISCUSSION

The main goal of this study was to evaluate the impact of mesophilic AD of tetracycline-containing animal manure, on tetracycline resistance genes and the total bacterial population. We also evaluated its impact on heavy metal resistance genes, and bacterial species and ARGs associated with heavy metal resistance. The increase in the total bacterial population (16S rRNA), which could be explained by decrease in tetracycline concentration, indicates a functioning AD system perhaps predominated by strictly anaerobic bacteria (Couch et al., 2019). While anaerobic bacteria play a significant role in hydrolysis, acidogenesis and acetogenesis steps, the methanogens play a significant role in the final methanogenesis of forming methane, with hydrolysis being a rate limiting step in AD process (Appels et al., 2008). Concentrations of five of the seven *tet* genes were increased from their pre-digested levels in the manures of the three animal species; *tet*(A) increased in the CM and SM but was not affected in the PL; *tet*(W) was increased in the PL while remaining unaffected in the CM and SM (**Supplementary Table 2**). The dynamics (increase or not) of the *tet* genes detected could be related to specific groups of bacteria carrying them and their ability to grow under the conditions tested. Also, it could be related to the ability of the genetic elements carrying the *tet* genes to be transferred among a wide range of bacterial genera or be more restricted within some groups. Mesophilic AD of wastewater sludge under lab-scale setup, increased the concentrations of both the 16S rRNA and *tet*(G) gene and the authors concluded that mesophilic AD of municipal waste enhances the survival of ARB and horizontal gene transfer (Miller et al., 2016). Similarly, another study (Ghosh et al., 2009) reported an increase in the concentrations of tetracycline resistance genes and *int*I1, a mobilizable genetic element commonly used as an indicator of horizontal gene transfer, under mesophilic AD of municipal wastewater. In another study (Ma et al., 2011) however,

TABLE 1 | Comparisons of heavy metal concentrations and heavy metal resistance genes by manure type.

Heavy metal	Manure type	Mean (95% CI)			Pairwise comparisons				
		Mean	95% CI		Paired comparisons	Contrast	95% CI		*P*-value
Copper (μg/ml)	Cattle manure	0.6	0.4	0.8	PL vs. CM	12.2	6.7	17.7	**0.001**
	Poultry litter	12.8	9.0	16.7	SM vs. CM	27.3	21.8	32.8	**<0.001**
	Swine manure	27.9	24.0	31.8	SM vs. PL	15.0	9.5	20.5	**<0.001**
Zinc (μg/ml)	Cattle manure	1.8	1.3	2.3	PL vs. CM	9.4	1.8	17.1	**0.021**
	Poultry litter	11.3	5.9	16.6	SM vs. CM	33.3	25.7	41.0	**<0.001**
	Swine manure	35.2	29.8	40.5	SM vs. PL	23.9	16.3	31.5	**<0.001**
*cop*B (log_{10} copies/ml)	Cattle manure	−0.2	−0.8	0.4	PL vs. CM	N/A			
	Poultry litter	N/A			SM vs. CM	0.8	0.1	1.5	**0.033**
	Swine manure	0.6	0.2	0.9	SM vs. PL	N/A			
*pco*A (log_{10} copies/ml)	Cattle manure	0.3	−0.6	1.2	PL vs. CM	2.4	1.2	3.7	**<0.001**
	Poultry litter	2.7	1.9	3.6	SM vs. CM	1.0	−0.2	2.2	0.101
	Swine manure	1.3	0.5	2.2	SM vs. PL	−1.4	−2.6	−0.2	**0.019**
*pco*D (log_{10} copies/ml)	Cattle manure	3.4	2.9	3.9	PL vs. CM	0.2	−0.5	0.9	0.584
	Poultry litter	3.6	3.1	4.1	SM vs. CM	−0.6	−1.3	0.1	0.077
	Swine manure	2.7	2.2	3.2	SM vs. PL	−0.8	−1.5	−0.1	**0.021**
*tcr*B (log_{10} copies/ml)	Cattle manure	2.4	1.9	2.9	PL vs. CM	2.0	1.3	2.7	**<0.001**
	Poultry litter	4.4	3.9	4.9	SM vs. CM	−0.3	−1.0	0.4	0.389
	Swine manure	2.1	1.6	2.6	SM vs. PL	−2.3	−3.0	−1.6	**<0.001**
*czr*C (log_{10} copies/ml)	Cattle manure	0.2	−0.6	1.0	PL vs. CM	3.5	2.4	4.6	**<0.001**
	Poultry litter	3.8	3.0	4.5	SM vs. CM	2.4	1.3	3.5	**<0.001**
	Swine manure	2.6	1.9	3.4	SM vs. PL	−1.1	−2.2	−0.1	**<0.035**

CM = cattle manure; PL = poultry litter; SM = swine manure; CI = confidence interval.
Copper and zinc concentrations were analyzed with linear regression while gene copies of the copper resistance genes shown here were analyzed by negative binomial regression. Bonferroni adjusted pairwise comparisons of the contrast were obtained. P-values with bold face indicate significant difference.

mesophilic AD of municipal wastewater led to mixed results in which the concentration of *tet*(G) declined while that of *tet*(W) increased. Another study (Wallace et al., 2018) also reported that mesophilic AD of dairy manure did not have any effect on the levels of *tet*(O) and *tet*(W). The effect of mesophilic AD on the concentration of macrolide resistance gene *erm*(B) also varied by the manure type: not affected in CM, increased in SM and decreased in PL. In the mesophilic AD of wastewater study, macrolide resistance genes *erm*(B) and *erm*(F) were increased (Ma et al., 2011).

Strong negative correlations between the concentrations of tetracyclines and the 16S rRNA genes suggest that the microbial communities continued to increase during the AD process, while the tetracyclines were degraded. Similarly, the negative correlations between the tetracycline concentrations and most of the *tet* genes may in part suggest that the decrease in tetracycline concentrations potentially led to propagation of the resistant bacterial strains that increased the abundance of the *tet* genes in the manure during AD. The increase in the concentrations of four *tet* genes *tet*(A), *tet*(B), *tet*(Q), and *tet*(W) and maintenance in the other two genes *tet*(G) and *tet*(O) may indicate the persistence and propagation of tetracycline resistant bacterial population carrying these genes in the AD independent of tetracycline concentrations. While *tet*(A), *tet*(B) and *tet*(G) are exclusively detected from gram negative bacteria, *tet*(O), *tet*(Q) and *tet*(W) are found in both gram positive and gram negative bacteria (Roberts and Schwarz, 2016). Interestingly, the concentration of *tet*(M) declined as the tetracycline concentrations declined. The *tet*(M) is the most widespread gene being detected in 75 genera equally distributed between gram positive and gram-negative bacteria. This widespread distribution can be due to its association with chromosomally linked conjugative transposons with broad host range for direct transfer or linked with plasmids (Rice, 1998; Roberts and Schwarz, 2016). Drop in the concentration of *tet*(M) as the tetracycline concentration decreased could be related to its association with a variety of conjugative transposons; and conjugation modules were reported to be induced in the presence of tetracycline (Rice, 1998).

In general, variations in the concentrations of the tetracycline resistance genes in the current and previous studies suggest that microbial communities in anaerobic digesters and operating conditions that influence the development and maintenance of that community play an important role in determining the fate of ARGs (Miller et al., 2013). Furthermore, studies showed the sorption of antibiotics and antibiotic resistance genes to the biosolid following AD, which clearly indicates that mesophilic AD alone is not efficient to remove these substances and secondary treatments such as composting are required before the sludge is used as soil amendment (Wallace et al., 2018; Couch et al., 2019). On the other hand, methanogens are resistant to many of the commonly used antibiotics due to their lack of specific targets (Whitman et al., 2006).

We also quantified the effect of mesophilic AD on the concentrations of bacterial pathogens. While the concentrations

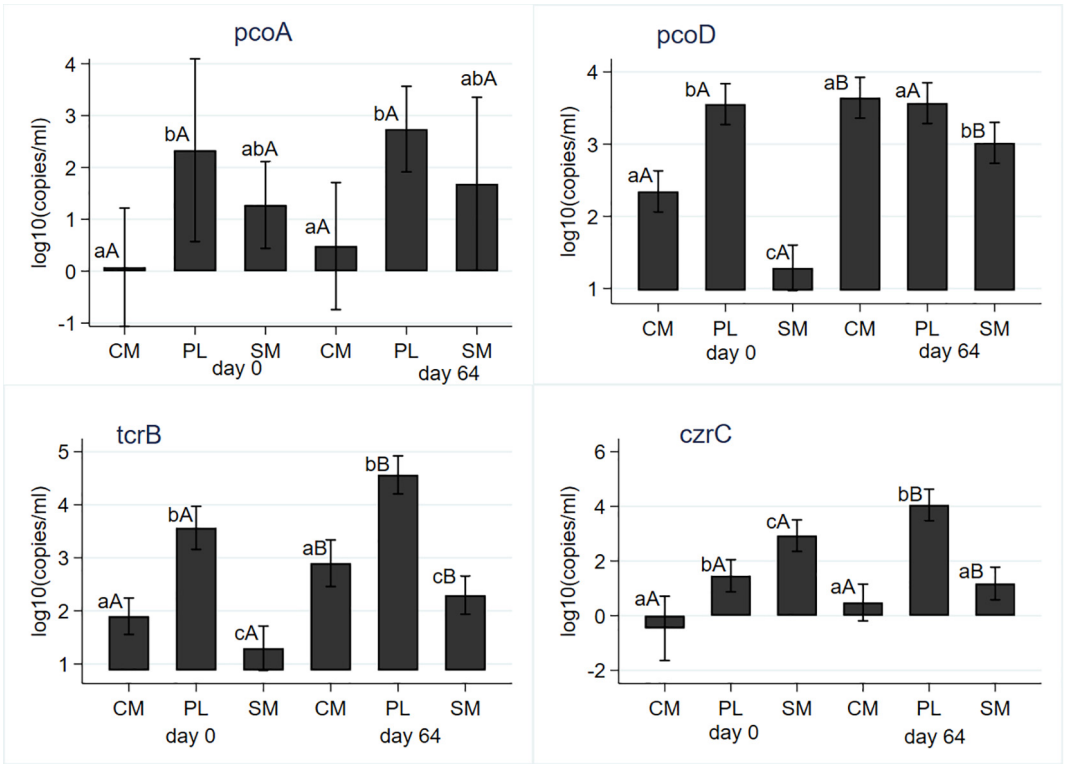

FIGURE 6 | Effect of mesophilic anaerobic digestion of cattle manure (CM), swine manure (SM), and poultry litter (PL) on concentrations of copper (*pco*A, *pco*D, and *tcr*B) and zinc (*czr*C) resistance genes. Data were analyzed with negative binomial regression and results were plotted as mean values with 95% confidence intervals. Different lower-case letters within the same sampling day indicate significant differences between the manure types; different upper-case letters within the same manure type indicate significant differences by sampling day (i.e., digestion effect). Significance was assessed at $P < 0.05$.

of *E. coli* and *S. aureus* in the CM and PL increased following AD, they were not affected in the SM. Concentrations of *Enterococcus* spp. significantly increased in the SM and PL with no effect in the CM. Interestingly, the concentrations of *S. aureus* significantly increased in the three manure types during digestion. An increase or no effect in the concentrations of these bacterial pathogens following mesophilic AD of manure from food animals clearly indicates the need for secondary treatment of the digestate before land application. A previous study (Ziemba and Peccia, 2011) indicated that the rate of inactivation of *E. coli* and *Enterococcus faecalis* increased as the temperature of the AD increased suggesting that mesophilic digestion is not effective in removing pathogens. *S. aureus* was detected using qPCR in mesophilic and thermophilic anaerobically digested wastewater samples but not in composted mesophilic digested samples suggesting the need for post-treatment of mesophilic anaerobically digested animal manure (Viau and Peccia, 2009). A study (Börjesson et al., 2009) that was conducted in a municipal wastewater treatment plant detected *mec*A, *S. aureus* and MRSA in anaerobically digested wastewater effluent samples. Based on the detection of *S. aureus* and *mec*A in human wastewater effluent samples in these previous studies and in animal manure in the current study, it is possible to conclude that MRSA can persist in mesophilic anaerobically digested materials and that secondary treatments are required.

A strong positive correlation between copper and zinc concentrations (**Table 2**) can simply be due to the fact that both of them are used as feed supplements to provide essential cellular functions in both swine and poultry (Rensing et al., 2018). The effect of mesophilic AD on Cu and Zn resistance genes also varied by the manure type, much like that of the bacterial pathogens and some of the ARGs. Heavy metal resistance genes were either not affected (*cop*B and *pco*A) or increased (*tcr*B) in the three manure types (**Supplementary Table 2**), indicating the ineffectiveness of mesophilic AD in removing heavy metal resistance genes. Almost perfect positive correlation between concentration of enterococci, and *erm*(B) and *tet*(M) is in line with culture-based studies that reported association between macrolide resistance [*erm*(B)] and tetracycline resistance [*tet*(M)] in enterococci (Amachawadi et al., 2015). Unlike that finding, *tcr*B was not correlated with *erm*(B) and *tet*(M) in the present ecological study although these three genes have been reported to be co-located on the same genetic elements thus enabling their transfer through selection pressures from heavy metals or the antibiotics (Rensing et al., 2018). Although the positive and strong correlation between *tcr*B and *pco*A can be explained by ecological association since they were tested from same samples, it requires further studies. Both of these genes are plasmid borne and confer Cu resistance in gram positive *Enterococcus* spp. and *Enterobacteria* such as *E. coli*,

TABLE 2 | Correlation of heavy metal concentrations, heavy metal resistance genes and antibiotic resistance genes across all samples.

Variable	*erm*(B)	*tet(*M)	*mec*A	*mec*C	*cop*B	*pco*A	*pco*D	*tcr*B	*czr*C	Enterococci	*E. coli*	*Staphylococcus spp.*	Copper	Zinc
erm(B)	1													
tet(M)	**0.99***	1												
	<0.000													
*mec*A	−0.14	−0.06	1											
	0.67	0.86												
*mec*C	0.22	0.31	0.57	1										
	0.50	0.33	0.0535											
*cop*B	0.35	0.33	−0.19	−0.26	1									
	0.26	0.30	0.56	0.41										
*pco*A	−0.15	−0.06	**0.996***	**0.63**	−0.20	1								
	0.64	0.84	**<0.0001**	**0.0289**	0.54									
*pco*D	−0.23	−0.21	−0.06	0.31	**−0.63**	−0.03	1							
	0.46	0.52	0.84	0.32	**0.0295**	0.93								
*tcr*B	−0.15	−0.07	**0.999***	**0.58**	−0.21	**0.996***	−0.04	1						
	0.65	0.84	**<0.0001**	**0.0492**	0.52	**<0.0001**	0.91							
*czr*C	−0.15	−0.11	−0.05	**0.64**	−0.19	0.04	0. 50	−0.03	1					
	0.63	0.74	0.88	**0.0257**	0.55	0.90	0.10	0.93						
Enterococci	**0.996***	**0.998***	−0.05	0.28	0.32	−0.06	−0.23	−0.06	−0.15	1				
	<0.0001	**<0.0001**	0.88	0.37	0.32	0.85	0.48	0.86	0.64					
E. coli	−0.27	−0.22	0.43	0.23	−0.37	0.41	0. 46	0.42	−0.10	−0.23	1			
	0.39	0.50	0.17	0.48	0.23	0.19	0.14	0.18	0.75	0.48				
Staphylococcus spp.	−0.18	−0.10	**0.84***	**0.67***	−0.21	**0.86***	0. 01	**0.83***	0. 20	−0.10	0. 42	1		
	0.58	0.76	**0.0007**	**0.0178**	0.51	**0.0003**	0.99	**0.0007**	0.53	0.75	0.18			
Copper	0.52	0.51	−0.05	0.25	0.45	−0.03	−0.33	−0.06	0.14	0.51	−0.49	0.20	1	
	0.08	0.09	0.87	0.44	0.15	0.93	0.29	0.86	0.67	0.09	0.10	0.53		
Zinc	0.46	0.43	−0.06	0.07	0.52	−0.06	−0.49	−0.08	−0.07	0.44	−0.44	0.18	**0.96***	1
	0.13	0.16	0.84	0.83	0.09	0.86	0.10	0.81	0.84	0.15	0.15	0.57	**<0.000**	

**Shows statistically significant Pearson's correlation coefficients. For each measured outcome, the number in the first row is a correlation coefficient and the number in the second row is the associated P-value. Correlation coefficients and P-values with statistical significance are in bold face.*

respectively (Rensing et al., 2018). Another new finding in this study is the association of these two plasmid-borne Cu resistance genes and *S. aureus*, *mec*C and *mec*A. Although the use of Zn and zinc resistance gene (*czr*C) were shown to be associated with MRSA and the responsible gene (*mec*A) (Cavaco et al., 2011), an association between Cu resistance genes and MRSA has never been reported. These ecological associations generate hypotheses that need to be tested under field studies. Mesophilic AD of livestock and poultry manure did not result in significant reductions of the heavy metals Cu and Zn although these metals are known to be precipitated at pH values typical of AD (Ayres et al., 1994). The presence of heavy metal resistance genes and bacteria known to carry these genes indicates environmental and antibiotic resistance risks associated with the use of these metals in food animal production. Lack of significant effect of AD on the concentration of heavy metals can be attributed to lack of biodegradable properties of metals as supported by the detection of heavy metals after AD of municipal solid waste (Xie et al., 2015). Variations in the concentrations of the heavy metals by animal species may indicate differences in the amount of the heavy metals fed (Hejna et al., 2019), in addition to basal requirements such as for disease prevention, control and growth promotion. To mitigate these risks, a recommendation is made that concentrations of these heavy metals should be reduced and adjusted to the essential requirements in animal production (Rensing et al., 2018).

The limitation of this study was a lack of a control manure not spiked with tetracyclines. However, the effect of AD was evaluated based on before/after design comparing all outcomes to their baseline values. This method was similarly used in another study (Couch et al., 2019) by this group that evaluated the impact of AD of swine manure on the same seven tetracycline resistance genes targeted in the current study. The use of baseline values was suggested to evaluate interventions related to AMR in the absence of background values or untreated controls (Rothrock et al., 2016). In a study (Miller et al., 2013) that compared AD of sludges spiked with sulfamethoxazole and control sludge under mesophilic conditions, reductions in the concentrations of ARGs remained constant despite selection pressure. This led the authors to conclude that digester operating conditions strongly influence the bacterial community composition and ARGs compared to selective agents. Also, we do not know if the three tetracycline drugs combined and spiked into the manure samples as a mixture had any interaction effects.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

GA, JK, JL, and EC contributed to the conception and design of the study. GA and JK performed the laboratory analysis and wrote the first draft of the manuscript. GA performed the statistical analysis. All authors contributed to manuscript revision, read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.614424/full#supplementary-material

Supplementary Table 1 | Primer sequences used for quantification of antibiotic and heavy metal resistance and bacterial marker genes from livestock manure and poultry litter under anaerobic digestion system.

Supplementary Table 2 | Summary of the effects of anaerobic digestion of cattle and swine manure, and poultry litter.

REFERENCES

Agga, G. E., Arthur, T. M., Durso, L. M., Harhay, D. M., and Schmidt, J. W. (2015a). Antimicrobial-resistant bacterial populations and antimicrobial resistance genes obtained from environments impacted by livestock and municipal waste. *PLoS One* 10:e0132586. doi: 10.1371/journal.pone.0132586

Agga, G. E., Scott, H. M., Vinasco, J., Nagaraja, T. G., Amachawadi, R. G., Bai, J., et al. (2015b). Effects of chlortetracycline and copper supplementation on the prevalence, distribution, and quantity of antimicrobial resistance genes in the fecal metagenome of weaned pigs. *Prev. Vet. Med.* 119, 179–189. doi: 10.1016/j.prevetmed.2015.02.008

Agga, G. E., Cook, K. L., Netthisinghe, A. M. P., Gilfillen, R. A., Woosley, P. B., and Sistani, K. R. (2019). Persistence of antibiotic resistance genes in beef cattle backgrounding environment over two years after cessation of operation. *PLoS One* 14:e0212510. doi: 10.1371/journal.pone.0212510

Amachawadi, R. G., Scott, H. M., Vinasco, J., Tokach, M. D., Dritz, S. S., Nelssen, J. L., et al. (2015). Effects of in-feed copper, chlortetracycline, and tylosin on the prevalence of transferable copper resistance gene, *tcr*B, among fecal enterococci of weaned piglets. *Foodborne Pathog. Dis.* 12, 670–678.

Appels, L., Baeyens, J., Degrève, J., and Dewil, R. (2008). Principles and potential of the anaerobic digestion of waste-activated sludge. *Prog. Energy Combust. Sci.* 34, 755–781.

Ayres, D. M., Davis, A. P., and Gietka, P. M. (1994). *Removing Heavy Metals From Wastewater. University of maryland engineering research centre report*. Available Online at: www.watertechusa.com/userdata/userfiles/file/Heavy%20Metals%20Removal.pdf (accessed 9/25/2020).

Börjesson, S., Melin, S., Matussek, A., and Lindgren, P.-E. (2009). A seasonal study of the mecA gene and *Staphylococcus aureus* including methicillin-resistant *S. aureus* in a municipal wastewater treatment plant. *Water Res.* 43, 925–932. doi: 10.1016/j.watres.2008.11.036

Campagnolo, E. R., Johnson, K. R., Karpati, A., Rubin, C. S., Kolpin, D. W., Meyer, M. T., et al. (2002). Antimicrobial residues in animal waste and water resources proximal to large-scale swine and poultry feeding operations. *Sci. Total Environ.* 299, 89–95. doi: 10.1016/s0048-9697(02)00233-4

Cavaco, L. M., Hasman, H., Aarestrup, F. M., Wagenaar, J. A., Graveland, H., Veldman, K., et al. (2011). Zinc resistance of *Staphylococcus aureus* of animal origin is strongly associated with methicillin resistance. *Vet. Microbiol.* 150, 344–348.

Chee-Sanford, J. C., Aminov, R. I., Krapac, I. J., Garrigues-Jeanjean, N., and Mackie, R. I. (2001). Occurrence and diversity of tetracycline resistance genes in lagoons and groundwater underlying two swine production facilities. *Appl. Environ. Microbiol.* 67, 1494–1502. doi: 10.1128/AEM.67.4.1494-1502.2001

Chen, J., Michel, F. C. Jr., Sreevatsan, S., Morrison, M., and Yu, Z. (2010). Occurrence and persistence of erythromycin resistance genes (*erm*) and tetracycline resistance genes (tet) in waste treatment systems on swine farms. *Microb. Ecol.* 60, 479–486. doi: 10.1007/s00248-010-9634-5

Couch, M., Agga, G. E., Kasumba, J., Parekh, R. R., Loughrin, J. H., and Conte, E. D. (2019). Abundances of tetracycline resistance genes and tetracycline antibiotics during anaerobic digestion of swine waste. *J. Environ. Qual.* 48, 171–178. doi: 10.2134/jeq2018.09.0331

Diehl, D. L., and LaPara, T. M. (2010). Effect of temperature on the fate of genes encoding tetracycline resistance and the integrase of class 1 integrons within anaerobic and aerobic digesters treating municipal wastewater solids. *Environ. Sci. Technol.* 44, 9128–9133.

FDA (2019). *2018 Summary report on antimicrobials sold or distributed for use in food-producing animals*. Silver Spring: FDA.

Ghosh, S., Ramsden, S. J., and LaPara, T. M. (2009). The role of anaerobic digestion in controlling the release of tetracycline resistance genes and class 1 integrons from municipal wastewater treatment plants. *Appl. Microbiol. Biotechnol.* 84, 791–796. doi: 10.1007/s00253-009-2125-2

Hejna, M., Moscatelli, A., Onelli, E., Baldi, A., Pilu, S., and Rossi, L. (2019). Evaluation of concentration of heavy metals in animal rearing system. *Italian J. Anim. Sci.* 18, 1372–1384. doi: 10.1080/1828051X.2019.1642806

Heuer, H., Schmitt, H., and Smalla, K. (2011). Antibiotic resistance gene spread due to manure application on agricultural fields. *Curr. Opinion Microbiol.* 14, 236–243. doi: 10.1016/j.mib.2011.04.009

Jensen, J., Larsen, M. M., and Bak, J. (2016). National monitoring study in Denmark finds increased and critical levels of copper and zinc in arable soils fertilized with pig slurry. *Environ. Pollut.* 214, 334–340. doi: 10.1016/j.envpol.2016.03.034

Jiang, L., Hu, X., Xu, T., Zhang, H., Sheng, D., and Yin, D. (2013). Prevalence of antibiotic resistance genes and their relationship with antibiotics in the Huangpu River and the drinking water sources, Shanghai, China. *Sci. Total Environ.* 458-460, 267–272. doi: 10.1016/j.scitotenv.2013.04.038

Kasumba, J., Appala, K., Agga, G. E., Loughrin, J. H., and Conte, E. D. (2019). Anaerobic digestion of livestock and poultry manures spiked with tetracycline antibiotics. *J. Environ. Sci. Health B* 55, 135–147. doi: 10.1080/03601234.2019.1667190

Knapp, C. W., Dolfing, J., Ehlert, P. A., and Graham, D. W. (2010). Evidence of increasing antibiotic resistance gene abundances in archived soils since 1940. *Environ. Sci. Technol.* 44, 580–587. doi: 10.1021/es901221x

Koike, S., Krapac, I. G., Oliver, H. D., Yannarell, A. C., Chee-Sanford, J. C., Aminov, R. I., et al. (2007). Monitoring and source tracking of tetracycline resistance genes in lagoons and groundwater adjacent to swine production facilities over a 3-year period. *Appl. Environ. Microbiol.* 73, 4813–4823. doi: 10.1128/AEM.00665-07

Kwietniewska, E., and Tys, J. (2014). Process characteristics, inhibition factors and methane yields of anaerobic digestion process, with particular focus on microalgal biomass fermentation. *Renewable Sustainable Energy Rev.* 34, 491–500.

Ma, Y., Wilson, C. A., Novak, J. T., Riffat, R., Aynur, S., Murthy, S., et al. (2011). Effect of various sludge digestion conditions on sulfonamide, macrolide, and tetracycline resistance genes and class I integrons. *Environ. Sci. Technol.* 45, 7855–7861. doi: 10.1021/es200827t

Meyers, M. A., Durso, L. M., Gilley, J. E., Waldrip, H. M., Castleberry, L., and Millmier-Schmidt, A. (2020). Antibiotic resistance gene profile changes in cropland soil after manure application and rainfall. *J. Environ. Qual.* 49, 754–761. doi: 10.1002/jeq2.20060

Miller, E., Spiehs, M., Arthur, T. M., Woodbury, B., Cortus, E., Chatterjee, A., et al. (2019). Cropland amendment with beef cattle manure minimally affects antimicrobial resistance. *J. Environ. Qual.* 48, 1683–1693. doi: 10.2134/jeq2019.02.0042

Miller, J. H., Novak, J. T., Knocke, W. R., and Pruden, A. (2016). Survival of antibiotic resistant bacteria and horizontal gene transfer control antibiotic resistance gene content in anaerobic digesters. *Front. Microbiol.* 7:263. doi: 10.3389/fmicb.2016.00263

Miller, J. H., Novak, J. T., Knocke, W. R., Young, K., Hong, Y., Vikesland, P. J., et al. (2013). Effect of silver nanoparticles and antibiotics on antibiotic resistance genes in anaerobic digestion. *Water Environ. Res.* 85, 411–421. doi: 10.2175/106143012x13373575831394

Munir, M., and Xagoraraki, I. (2011). Levels of antibiotic resistance genes in manure, biosolids, and fertilized soil. *J. Environ. Qual.* 40, 248–255. doi: 10.2134/jeq2010.0209

Novak, J. T., Chon, D. H., Curtis, B.-A., and Doyle, M. (2007). Biological solids reduction using the cannibal process. *Water Environ. Res.* 79, 2380–2386. doi: 10.2175/106143007x183862

OIE (2020). *World Organisation For Animal Health (Oie) Annual Report On Antimicrobial Agents Intended For Use In Animals (Fourth Report)*. Available online at: https://www.oie.int/fileadmin/Home/eng/Our_scientific_expertise/docs/pdf/AMR/A_Fourth_Annual_Report_AMR.pdf (accessed August 10, 2020).

Oliver, J. P., Gooch, C. A., Lansing, S., Schueler, J., Hurst, J. J., Sassoubre, L., et al. (2020). Invited review: fate of antibiotic residues, antibiotic-resistant bacteria, and antibiotic resistance genes in US dairy manure management systems. *J. Dairy Sci.* 103, 1051–1071. doi: 10.3168/jds.2019-16778

Peak, N., Knapp, C. W., Yang, R. K., Hanfelt, M. M., Smith, M. S., Aga, D. S., et al. (2007). Abundance of six tetracycline resistance genes in wastewater lagoons at cattle feedlots with different antibiotic use strategies. *Environ. Microbiol.* 9, 143–151. doi: 10.1111/j.1462-2920.2006.01123.x

Pruden, A., Pei, R., Storteboom, H., and Carlson, K. H. (2006). Antibiotic resistance genes as emerging contaminants: studies in northern Colorado. *Environ. Sci. Technol.* 40, 7445–7450.

Rensing, C., Moodley, A., Cavaco, L. M., and McDevitt, S. F. (2018). Resistance to metals used in agricultural production. *Microbiol. Spectr.* 6:29676247. doi: 10.1128/microbiolspec.ARBA-0025-2017

Rice, L. B. (1998). Tn916 family conjugative transposons and dissemination of antimicrobial resistance determinants. *Antimicrob. Agents Chemother.* 42, 1871–1877. doi: 10.1128/aac.42.8.1871

Roberts, M. C., and Schwarz, S. (2016). Tetracycline and phenicol resistance genes and mechanisms: Importance for agriculture, the environment, and humans. *J. Environ. Qual.* 45, 576–592. doi: 10.2134/jeq2015.04.0207

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

Rothrock, M. J., Keen, P. L., Cook, K. L., Durso, L. M., Franklin, A. M., and Dungan, R. S. (2016). How should we be determining background and baseline antibiotic resistance levels in agroecosystem research? *J. Environ. Qual.* 45, 420–431. doi: 10.2134/jeq2015.06.0327

Sahlström, L. (2003). A review of survival of pathogenic bacteria in organic waste used in biogas plants. *Biores. Technol.* 87, 161–166. doi: 10.1016/S0960-8524(02)00168-2

Sui, Q., Zhang, J., Chen, M., Tong, J., Wang, R., and Wei, Y. (2016). Distribution of antibiotic resistance genes (ARGs) in anaerobic digestion and land application of swine wastewater. *Environ. Pollut.* 213, 751–759. doi: 10.1016/j.envpol.2016.03.038

Udikovic-Kolic, N., Wichmann, F., Broderick, N. A., and Handelsman, J. (2014). Bloom of resident antibiotic-resistant bacteria in soil following manure fertilization. *Proc. Natl. Acad. Sci. U S A.* 111, 15202–15207. doi: 10.1073/pnas.1409836111

Viau, E., and Peccia, J. (2009). Survey of wastewater indicators and human pathogen genomes in biosolids produced by class A and class B stabilization treatments. *Appl. Environ. Microbiol.* 75:164. doi: 10.1128/AEM.01331-08

Wallace, J. S., Garner, E., Pruden, A., and Aga, D. S. (2018). Occurrence and transformation of veterinary antibiotics and antibiotic resistance genes in dairy manure treated by advanced anaerobic digestion and conventional treatment methods. *Environ. Pollut.* 236, 764–772. doi: 10.1016/j.envpol.2018.02.024

Whitman, W. B., Bowen, T. L., and Boone, D. R. (2006). The methanogenic bacteria. *Prokaryotes* 3, 165–207.

Williams-Nguyen, J., Sallach, J. B., Bartelt-Hunt, S., Boxall, A. B., Durso, L. M., McLain, J. E., et al. (2016). Antibiotics and antibiotic resistance in agroecosystems: state of the science. *J. Environ. Qual.* 45, 394–406. doi: 10.2134/jeq2015.07.0336

Xie, S., Ma, Y., Strong, P. J., and Clarke, W. P. (2015). Fluctuation of dissolved heavy metal concentrations in the leachate from anaerobic digestion of municipal solid waste in commercial scale landfill bioreactors: the effect of pH and associated mechanisms. *J. Hazard. Mater.* 299, 577–583. doi: 10.1016/j.jhazmat.2015.07.065

Yazdankhah, S., Rudi, K., and Bernhoft, A. (2014). Zinc and copper in animal feed – development of resistance and co-resistance to antimicrobial agents in bacteria of animal origin. *Microbial. Ecol. Health Dis.* 25:25862. doi: 10.3402/mehd.v25.25862

Zhang, T., Yang, Y., and Pruden, A. (2015). Effect of temperature on removal of antibiotic resistance genes by anaerobic digestion of activated sludge revealed by metagenomic approach. *Appl. Microbiol. Biotechnol.* 99, 7771–7779. doi: 10.1007/s00253-015-6688-9

Ziemba, C., and Peccia, J. (2011). Net energy production associated with pathogen inactivation during mesophilic and thermophilic anaerobic digestion of sewage sludge. *Water Res.* 45, 4758–4768. doi: 10.1016/j.watres.2011.06.014

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Detection of ESBL/AmpC-Producing and Fosfomycin-Resistant *Escherichia coli* From Different Sources in Poultry Production in Southern Brazil

*Luís Eduardo de Souza Gazal[1], Leonardo Pinto Medeiros[1], Miriam Dibo[1], Erick Kenji Nishio[1], Vanessa Lumi Koga[1], Bruna Carolina Gonçalves[1], Tiela Trapp Grassotti[2], Taiara Carolaine Leal de Camargo[2], João Juliano Pinheiro[2], Eliana Carolina Vespero[3], Kelly Cristina Tagliari de Brito[2], Benito Guimarães de Brito[2], Gerson Nakazato[1] and Renata Katsuko Takayama Kobayashi[1]**

[1] Department of Microbiology, Biological Sciences Center, State University of Londrina, Londrina, Brazil, [2] Postgraduate Program in Animal Health, Avian Health Laboratory, Veterinary Research Institute Desidério Finamor, Agricultural Diagnosis and Research Department, Secretariat of Agriculture Livestock Rural and Development, Eldorado do Sul, Brazil, [3] Department of Pathology, Clinical Analysis and Toxicology, Health Sciences Center, State University of Londrina, Londrina, Brazil

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***Correspondence:**
Renata Katsuko Takayama Kobayashi
kobayashirkt@uel.br

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This study discussed the use of antimicrobials in the commercial chicken production system and the possible factors influencing the presence of Extended-spectrum β-lactamase (ESBL)/AmpC producers strains in the broiler production chain. The aim of this study was to perform longitudinal monitoring of ESBL-producing and fosfomycin-resistant *Escherichia coli* from poultry farms in southern Brazil (Paraná and Rio Grande do Sul states) and determine the possible critical points that may be reservoirs for these strains. Samples of poultry litter, cloacal swabs, poultry feed, water, and beetles (*Alphitobius* sp.) were collected during three distinct samplings. Phenotypic and genotypic tests were performed for characterization of antimicrobial resistant strains. A total of 117 strains were isolated and 78 (66%) were positive for ESBL production. The poultry litter presented ESBL positive strains in all three sampled periods, whereas the cloacal swab presented positive strains only from the second period. The poultry litter represents a significant risk factor mainly at the beginning poultry production (odds ratio 6.43, 95% confidence interval 1–41.21, $p < 0.05$). All beetles presented ESBL positive strains. The predominant gene was bla_{CTX-M} group 2, which occurred in approximately 55% of the ESBL-producing *E. coli*. The *cit* gene was found in approximately 13% of the ESBL-producing *E. coli* as AmpC type determinants. A total of 19 out of 26 fosfomycin-resistant strains showed the *fos*A3 gene, all of which produced ESBL. The correlation between *fos*A3 and bla_{CTX-M} group 1 ($bla_{CTX-M55}$) genes was significant among ESBL-producing *E. coli* isolated from Paraná (OR 3.66, 95% CI 1.9–9.68) and these genetic determinants can be transmitted by conjugation to broiler chicken microbiota strains. Our data revealed that poultry litter and beetles were critical points during poultry

production and the presence of fosfomycin-resistant strains indicate the possibility of risks associated with the use of this antimicrobial during production. Furthermore, the genetic determinants encoding CTX-M and fosA3 enzymes can be transferred to *E. coli* strains from broiler chicken microbiota, thereby creating a risk to public health.

Keywords: Avian, multidrug resistance (MDR), Enterobacteriaceae, fosfomycin, poultry litter, public health

INTRODUCTION

Antimicrobial resistance is one of the most alarming public health problems in recent years. According to O'Neill (2014), by 2050, bacterial resistance could cause the deaths of approximately 10 million people each year. The widespread use of antimicrobial drugs, both in humans and animals (including livestock animals), has favored the selection and dissemination of bacterial resistance worldwide (World Health Organization, 2014).

Extended-spectrum β-lactamase (ESBL) and AmpC-like enzymes are among the best-known mechanisms of bacterial resistance, which are both mediated by plasmid genes (Ceccarelli et al., 2019) and can be achieved through the horizontal transfer of mobile genetic elements, in both intestinal and extra-intestinal environments (Lazarus et al., 2014). ESBL and AmpC enzymes are capable of hydrolyzing various β-lactam antimicrobials such as cephalosporins and monobactams, increasing the difficulty of treating these infections (Paterson and Bonomo, 2005; Bush and Fisher, 2011).

Initially, the detection of ESBL/AmpC-producing bacteria was related to cases of infection in humans (Smet et al., 2009; Ewers et al., 2012). However, several studies have reported the presence of these resistant strains in animals, whether domestic (Wieler et al., 2011; Bortolami et al., 2019) or livestock (Pitout and Laupland, 2008; Smet et al., 2009; Dierikx et al., 2013; Laube et al., 2013; Dominguez et al., 2018). ESBL/AmpC enzymes are found in Enterobacteriaceae family members, such as *Escherichia coli*, which have often been isolated in livestock, especially during poultry production (Blanc et al., 2006; Carattoli, 2008; Li et al., 2015). Among the most relevant β-lactamases, CTX-M is one of the main enzymes present in *E. coli* that colonize and infect poultry (Olsen et al., 2014).

Worldwide, ESBL-producing Enterobacteriaceae have emerged in farm animals in recent decades (Carattoli, 2008). The pressure exerted by antimicrobial use, particularly in broiler chickens, led to sensitive strains elimination and selection of resistant ones (Saliu et al., 2017). Although antimicrobial resistance is a natural phenomenon, the prevalence of ESBL-producing strains in broilers has increased due to the antimicrobial use in production (Dierikx et al., 2013). Another relevant finding regarding the use of antimicrobials in production is the presence of fosfomycin-resistant strains in poultry carcasses (Cyoia et al., 2019). Fosfomycin is approved in several countries for urinary tract infections treatment in humans (Keating, 2013; Falagas et al., 2019). Thus, the presence of fosfomycin-resistant strains in poultry raises public health safety concerns.

The possibility that bacterial strains, especially ESBL/AmpC-producing and fosfomycin-resistant *E. coli*, may reach the human population via chicken meat consumption is a public health concern because, compared to other types of meat (e.g., pork and beef), chicken meat has been found to become highly contaminated with ESBL-producing bacteria (Friese et al., 2013). Koga et al. (2015) reported the presence of ESBL/AmpC-producing *E. coli* strains isolated from poultry carcasses in southern Brazil. Cyoia et al. (2019) demonstrated that ESBL-producing *E. coli* strains were capable of transferring genes encoding CTX-M enzymes to a human *E. coli* strain. Plasmid incompatibility groups (Inc groups) are categorized by the propagation inability, in the same cell, of two plasmids belonging to the same group. These plasmids may carry resistance genes and can be found in *E. coli* (Datta and Hedges, 1971; Carattoli, 2011). The *bla*CTX-M (e.g., CTX-M15 and CTX-M55) and *bla*TEM genes are associated with plasmids belonging to the IncF and IncI groups (Cantón and Coque, 2006; Carattoli, 2013).

The presence of ESBL/AmpC-producing and fosfomycin-resistant *E. coli* in poultry carcasses reveals that there are possibly critical points during industrial broiler production where these strains can be found and selected by antimicrobials. Thus, the present study aimed to monitor ESBL/AmpC-producing and fosfomycin-resistant *E. coli* in poultry production and determine the possible critical points that may be reservoirs of these strains.

MATERIALS AND METHODS

Farm Characterization

Monitoring was undertaken in the biggest broiler producers states from Brazil. Five farms in the Rio Grande do Sul (RS) state were sampled between February and May 2016, and three farms in the Paraná (PR) state were sampled between January and March 2018. All farms sampled employed an all-in all-out system. In this system, the barns are emptied for slaughter; the poultry litter is turned and covered with a plastic canvas, remaining inside the barn to be reused; the feeders and drinking fountains are disinfected; and the place remains closed for 15 days (depopulation period), until the arrival of new chicks. Sampling was performed in one barn per farm and monitored at three different times: (1) first day (one-day-old restocking chicks), (2) between 20th and 25th days, and (3) between 36th and 38th days of fattening period (**Supplementary Table 1**). It is important to note that in the first sampling time, chicks samples were collected, but they did not come into contact with water, feed or poultry litter of barn, so that this would not interfere with the analysis of the results. Besides, a questionnaire was given to the producers to obtain

information regarding property characteristics, management, and biosecurity.

In the RS state, the production system was characterized as manual. On average, the farmers restocked approximately 15,000 one-day-old chicks in the poultry houses, one or two poultry houses per farm, and the slaughtering period ranged from 38 to 45 days. The water came from artesian wells and was chlorinated in a reservoir present in each poultry house. In general, the poultry litter was composed of rice husk and reused in up to five subsequent flocks without undergoing treatment. The antimicrobials used in the RS farms were enrofloxacin (ENR), halquinol, and virginiamycin.

In the PR state, the production system used was automated (dark house system). The average restocking was approximately 25,000 one-day-old chicks, one or two poultry houses per farm, and the slaughtering period ranged from 38 to 42 days. Similar to the RS farms, the water used in the bird drinkers was chlorinated. The poultry litter was made of wood shavings and reused without undergoing treatment. The antimicrobials used for treatment in the PR farms were norfloxacin (NOR), ciprofloxacin (CIP), and fosfomycin. All barns investigated were disinfected between a previous and a subsequent flock, followed by a depopulation period to approximately 15 days.

Ethics Statement

The present study was approved by the Animal Ethics Committee of State University of Londrina (CEUA/UEL) (processing number – 22867.2015.23).

Sampling

The sampling methodology was based on the procedure described by Laube et al. (2013), with some modifications. During each fattening period, samples of poultry litter (boot swab), cloacal swab (20 randomly selected broiler), poultry feed (500 g), and water (500 mL) were collected. In the PR state, samples of *Alphitobius* sp. (approximately 100 beetles), popularly known as "darkling beetle," were also collected from the farms. The poultry litter was collected using a sterile boot swab by walking the entire house length in a "zigzag" pattern. The poultry feed was obtained directly from the reservoir and water was collected from the farm reservoir taps. Therefore, a total of 45 samples from PR, and 60 samples in RS were collected.

The collected samples were refrigerated (4°C) and sent to the laboratory for processing on the same day. A total of 25 g of feed was weighed and diluted in 225 mL of buffered peptone water, followed by manual homogenization (approximately 10 min). The boot swab was soaked with buffered peptone water, followed by manual homogenization. The water was processed using the multi-tube method (most probable number method), using Lauryl Triptose broth, Brilliant Green broth, and *E. coli* broth (Himedia Laboratories Pvt. Ltd., Mumbai, India). The beetles were processed following the protocol described by Segabinazi et al. (2005). After preprocessing, all samples were plated on MacConkey agar (MC) (Neogen Corporation, Lansing, Michigan, United States) and cefotaxime-supplemented MacConkey agar (MC/CTX) at a concentration of 8 μg/mL to select positive ESBL/AmpC strains (Jarlier et al., 1988).

E. coli Isolation

MacConkey agar and MC/CTX plates were analyzed for the growth of characteristic colonies of *E. coli*. The grew colonies in MC/CTX (possible ESBL/AmpC-producing *E. coli*) were prioritized, with a collection of one to five colonies. In MC/CTX plates that presented no growth, one to five colonies from the MC plates were collected. The colonies were submitted for biochemical identification using media Escola Paulista de Medicina (Probac, Brazil) (Toledo et al., 1982a; Edwards and Ewing, 1986), Motility, Indole and Lysine (Probac, Brazil) (Toledo et al., 1982b; Edwards and Ewing, 1986), and Simmons citrate (Merck, Darmstadt, Germany). *E. coli* positive colonies were stored in Brain Heart Infusion broth (Himedia Laboratories Pvt. Ltd., Mumbai, India) supplemented with 30% glycerol at −20 and −80°C for subsequent phenotypic and genotypic characterization.

Antimicrobial Susceptibility Test

The antimicrobial susceptibility test was performed using the standard disk diffusion method recommended by the Clinical and Laboratory Standards Institute (CLSI, 2018). The antimicrobials used included several classes, such as β-lactams: cefotaxime (CTX, 30 μg), ceftazidime (CAZ, 30 μg), cefepime (FEP, 30 μg), aztreonam (ATM, 30 μg), cefoxitin (FOX, 30 μg), imipenem (IPM, 30 μg) and amoxicillin-clavulanic acid (AMC, 20/10 μg); quinolones: CIP (5 μg), NOR (10 μg), enrofloxacin (ENR, 10 μg), and nalidixic acid (NAL, 30 μg); sulfonamides: trimethoprim-sulfamethoxazole (SXT, 1.25/23.75 μg); tetracycline (TET, 30 μg); aminoglycosides: gentamicin (GEN, 10 μg), phenicols: chloramphenicol (CHL, 30 μg); nitrofurans: nitrofurantoin (NIT, 300 μg); and fosfomycins: fosfomycin-trometamol (FOT, 200 μg) (Oxoid Ltd., Basingstoke, Hants, United Kingdom). All strains were confirmed for ESBL production using the double-disk approximation test, described by Jarlier et al. (1988). *E. coli* strain ATCC 25922 was used as quality control and results were interpreted based on the CLSI (2018) criteria.

DNA Template

DNA samples used for polymerase chain reaction (PCR) assays and sequencing, were extracted using Pure Link® Genomic DNA Mini Kit (Invitrogen®).

Detection of ESBL/AmpC Genes in *E. coli* Strains

A previously described PCR method was used for the detection of the following antimicrobial resistance genes: ESBL producer (bla_{CTX-M} groups – 1, 2, 8, 9, and 25) (Arlet and Philippon, 1991; Woodford et al., 2005); and AmpC-type producer (*mox*, *fox*, *ebc*, *acc*, *dha,* and *cit*) (Pérez-Pérez and Hanson, 2002). The PCR products positive to bla_{CTX-M} group 1 were characterized for bidirectional Sanger sequencing on ABI-PRISM 3500 XL (Applied Biosystems), following the manufacturer's recommendations.

Detection of Other Antimicrobial Resistance Genes

The presence of fosfomycin (*fos*A3) and colistin (*mcr*-1) resistance was examined as described previously by Sato et al. (2013) and Liu et al. (2016), respectively.

Phylogenetic Analysis

All *E. coli* isolates were assigned to phylogenetic groups A, B1, B2, or D using PCR according to the methodology described by Clermont et al. (2000). Isolates were grouped into the following groups: group A (*chu*A$^-$, *yja*A$^-$, and TspE4.C2$^-$); group B1 (*chu*A$^-$, *yja*A$^+$, TspE4.C2$^-$); group B2 (*chu*A$^+$, *yja*A$^+$, TspE4.C2- or *chu*A$^+$, *yja*A$^+$, TspE4.C2$^+$); and group D (*chu*A$^+$, *yja*A$^-$, TspE4.C2$^+$).

Conjugation Experiments

Horizontal transmission of bla_{CTX-M} and *fos*A3 genes was investigated using conjugation assays. ESBL-producing *E. coli* from poultry litter, harboring the resistance genes, were chosen as donor strains. Non-ESBL-producing *E. coli* from chicks microbiota were selected as possible recipients, based on the antibiogram test and phylogenetic profile. The selected colonies were strains were grown overnight in Luria Bertani (LB) broth (Difco, Sparks, MD, United States) under agitation at 36°C. Then, 1 mL of each strain was centrifuged at 12,000 × *g* for 2 min at 25°C, the supernatants were discarded and resuspended in new LB broth. A total of 100 μL of a recipient strain and 50 μL of a donor strain were added in 3 mL of new LB broth and incubated overnight without shaking at 36°C. Then, 100 μL of the conjugated samples were seeded on LB agar supplemented with GEN (10 μg/mL) and CTX (4 μg/mL), and the plates were incubated at 36°C for growth. The transconjugants selected were used for phylogenetic analysis and tested for the presence of bla_{CTX-M} and *fos*A3 genes.

Plasmid-Based Replicon Typing

All isolates were characterized by the Inc group using plasmid-based replicon typing (PBRT; Carattoli et al., 2005). Simplex-PCR was used to recognize the eight incompatibility plasmids: FIA, FIB, FIC, FII, I1, HI1, HI2, and N (Carattoli et al., 2005).

Enterobacterial Repetitive Intergenic Consensus Sequence–PCR Analysis

A template DNA was used to amplify the repetitive elements from bacterial isolates using the PCR technique to generate DNA fingerprint patterns. The isolates' clonality was determined by homology among fragments amplified using enterobacterial repetitive intergenic consensus sequence (ERIC)–PCR (Versalovic et al., 1991). Gel analysis was performed using BioNumerics software, version 7.6 (Applied Maths, Sint-Martens-Laten, Belgium). Similarities in the amplicon profile were compared using a DICE coefficient at 1% tolerance and 0.5% optimization. A dendrogram was constructed with the unweighted-pair group method using the arithmetic mean clustering method with a cut-off of 80% similarity (McLellan et al., 2003).

Statistical Analysis

The data obtained were analyzed using a logistic regression model to calculate the odds ratio (OR) and 95% confidence interval (CI), with a significance level set at $p < 0.05$, and the statistical software R version 3.5.1. In general, the analysis was performed to verify whether there was any significant trend between the isolates producing ESBL and their origin.

RESULTS

Poultry Farm Samples

A total of 117 *E. coli* strains were isolated from eight poultry farms in two states, 58 strains from three PR farms, and 59 strains from five RS farms. *E. coli* samples grown in MC/CTX were obtained from poultry litter in all periods in both states, whereas it was only present in cloacal samples isolated from the second period (**Table 1**). ESBL-producing strains were detected in poultry feed and beetle samples from PR state. In both states, the water samples showed no *E. coli* growth in MC/CTX (**Table 1**).

ESBL-producing *E. coli* were confirmed, respectively, in 49/58 (84%) and 29/59 (49%) isolates from PR and RS. The distribution of ESBL-producing and non-ESBL-producing *E. coli* strains over the sampling periods are shown in **Figure 1**. Among the 117 samples, ESBL-producing *E. coli* strains were detected in poultry litter in all sampling periods: first period (OR 6.42, 95% CI 1–41.21, $p < 0.05$), second period (OR 3.75, 95% CI 0.4–35.54, $p < 0.05$), and third period (OR 1.05, 95% CI 0.28–3.78, $p < 0.05$). The profile of cloacal poultry strains revealed that ESBL-producing *E. coli* was also present in the second sampling period (OR 2.61, 95% CI 0.27–24.94, $p < 0.05$) and third sampling period (OR 15, 95% CI 1.78–126.59, $p < 0.05$). All the strains isolated from beetles were characterized as ESBL producers from all poultry farms in the PR state (OR > 100, 95% CI 0–inf., $p < 0.05$). No ESBL-producing strains were found in water samples. Moreover, low frequency was found in poultry feed only in the second sampling (OR 0.65, 95% CI 0.06–7.01 $p < 0.05$).

E. coli Antimicrobial Resistance

The antimicrobial susceptibility test indicated that the strains isolated from the poultry farms presented a high frequency of antimicrobial resistance, with 90 and 73% of strains considered as multidrug-resistant (MDR), from PR and RS, respectively (**Supplementary Tables 2,3**).

In the PR state, most isolates were resistant to CTX (85%), NAL (85%), FEP (81%), TET (79%), SXT (78%), ATM (74%), ENR (66%), CIP, and NOR (55%). Most isolates from the RS state were resistant to GEN (70%), TET (63%), CTX (58%), ATM (56%), NAL (54%), and FEP (51%), presenting a different resistance profile from PR state.

The frequency of resistance observed for fosfomycin in PR and RS strains was 40 and 5%, respectively. In both states, no IPM-resistant *E. coli* strain was found. ESBL-producing *E. coli* were resistant to a higher number of antimicrobials (except FOX, NIT, and IPM) compared to non-ESBL-producing *E. coli* ($p < 0.05$) (**Figure 2**).

TABLE 1 | Detection of ESBL-producing *E. coli* isolated in MC/CTX agar from farms in PR and RS states.

***E. coli* source**	**PR farms**									**RS farms**														
	Farm 1			**Farm 2**			**Farm 3**			**Farm 4**			**Farm 5**			**Farm 6**			**Farm 7**			**Farm 8**		
	1°	**2°**	**3°**	**1°**	**2°**	**3°**	**1°**	**2°**	**3°**	**1°**	**2°**	**3°**	**1°**	**2°**	**3°**	**1°**	**2°**	**3°**	**1°**	**2°**	**3°**	**1°**	**2°**	**3°**
Poultry Litter	+	+	+	+	+	+	−	+	+	−	−	+	+	−	+	+	+	−	+	+	+	−	−	+
Poultry	−	+	+	−	+	+	−	+	+	−	+	+	−	+	+	−	+	−	−	+	+	−	+	+
Poultry Feed	−	+	−	−	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
Water	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Beetle	+	−	+	−	+	+	−	+	+	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

(+) Presence; (–) Absence; (NS) Non Sampling.

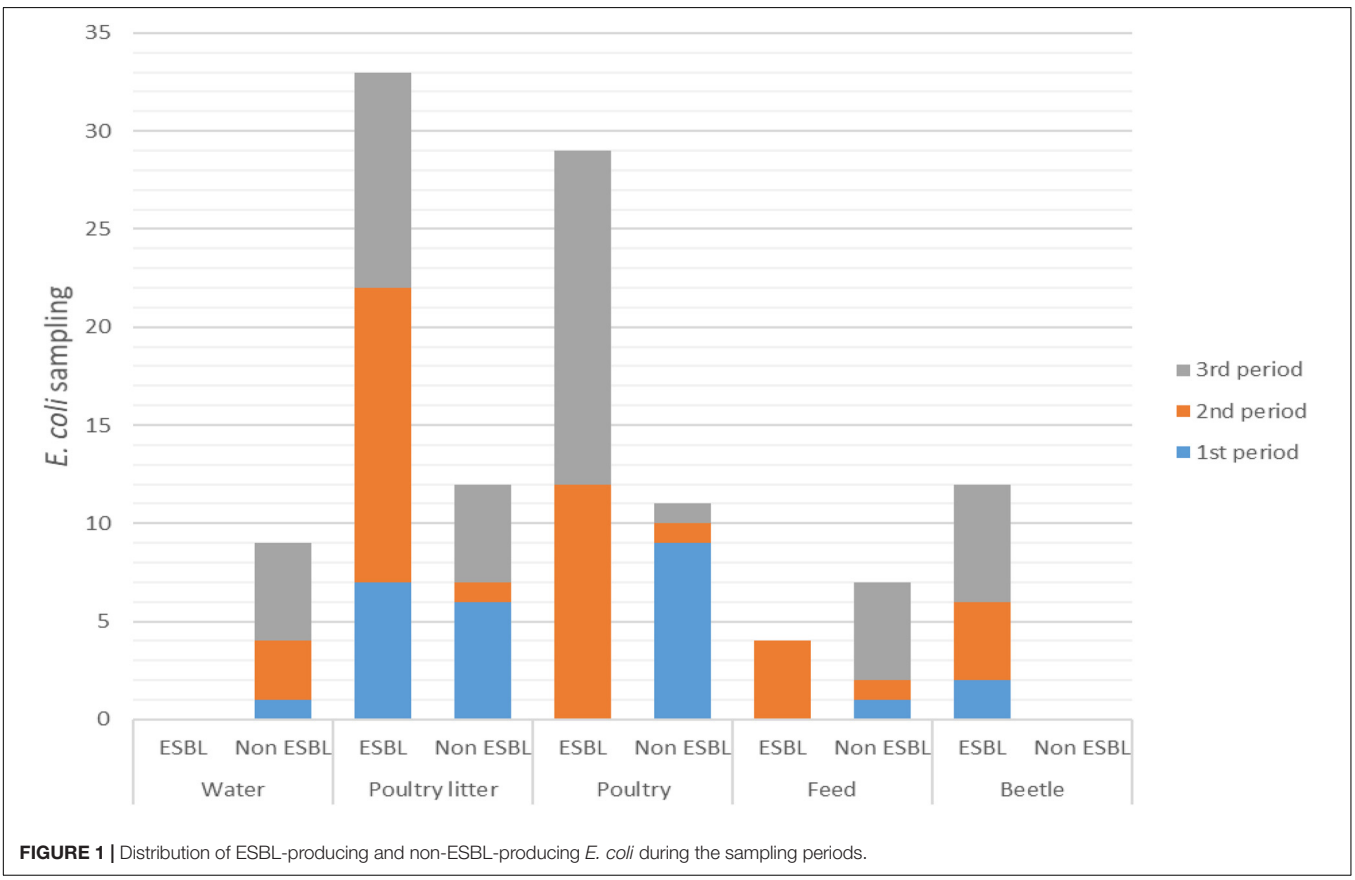

FIGURE 1 | Distribution of ESBL-producing and non-ESBL-producing *E. coli* during the sampling periods.

Detection of ESBL/AmpC, *fos*A3, and *mcr*-1 Genes

Polymerase chain reaction analysis identified ESBL genes in 92/117 (77%) strains isolated from poultry farms (**Supplementary Tables 2,3**). Approximately 77/78 (99%) of the ESBL-producing *E. coli* strains were positive for the ESBL genes investigated. In these strains, the genes bla_{CTX-M} group 2 (55%), bla_{CTX-M} group 1 (38%), and bla_{CTX-M} group 8 (10%) were detected. The only AmpC gene detected was *cit*, found in 14/117 isolates, which was associated with bla_{CTX-M} genes in nine strains. The bla_{CTX-M} group 9 and bla_{CTX-M} group 25 genes were not detected and one ESBL-producing strain carried none of the investigated genes. By sequencing, we found that 100% of bla_{CTX-M} group 1 positive isolates were identified as CTX-M55.

Among the 26 fosfomycin-resistant *E. coli* strains, 19 (73%) harbored the *fos*A3 gene, all had ESBL phenotypes features and 17 of these also harbored the $bla_{CTX-M55}$ gene (OR 3.66, 95% CI 1.39–9.68, $p < 0.05$). All of these *fos*A3 positive strains were from PR farms that used fosfomycin in broiler production. Three fosfomycin-resistant strains were isolated from the RS state and none of them presented the *fos*A3 gene. The *mcr*-1 gene was detected in only one isolate (EcRS60) from RS. Among the ESBL-producing strains, 14 combinations between the resistance genes detected were observed and most of them are distributed among poultry and poultry litter isolates (**Table 2**).

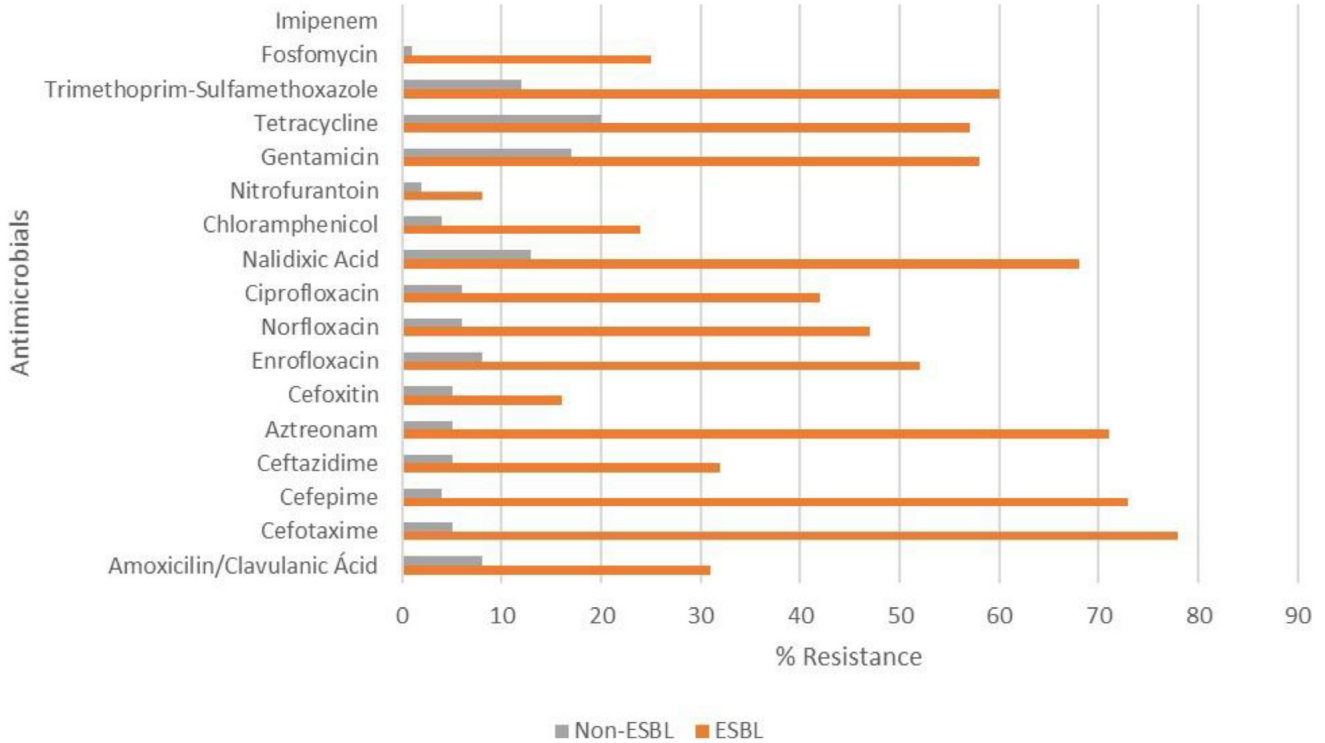

FIGURE 2 | Resistance of ESBL-producing and non-ESBL-producing *E. coli* strains isolated from poultry farms in southern Brazil. There were significant differences between ESBL-producing and non-producing strains ($p < 0.05$), except for cefoxitin, nitrofurantoin and imipenem (all isolates were sensitive).

Inc Group Plasmid

Tables 2, **3** show the results obtained from the strains submitted to the PBRT technique. Replicons were detected in 97/117 (83%) isolates and the Inc typing showed the presence of FIB (n = 89; 76%), I1 (n = 37; 32%), HI2 (n = 7; 6%), FIA (n = 5; 4%), FIC (n = 5; 4%), and N (n = 4; 3%). The presence of HI1 and FII groups was not detected and 19 strains were negative for all tested replicons. The most frequent replicons among the ESBL-producing samples were FIB (71%) and I1 (36%).

TABLE 2 | Distribution of ESBL-producing *E. coli* strains based on a combination of genes (or CTX-M group genes) and sources in PR and RS states.

	Poultry litter		Poultry		Feed		Beetle	
	PR	RS	PR	RS	PR	RS	PR	RS
CTX-M1	1	–	3	1	1	–	5	NS
CTX-M1 + *fos*A3	6	–	4	–	2	–	3	NS
CTX-M1 + *cit*	–	–	–	1	–	–	–	NS
CTX-M1 + CTX-M2 + CTX-M8	1	–	–	–	–	–	–	NS
CTX-M1 + CTX-M8 + *fos*A3	1	–	–	–	–	–	–	NS
CTX-M1 + *cit* + *fos*A3	–	–	1	–	–	–	–	NS
CTX-M2	7	9	6	10	–	–	4	NS
CTX-M2 + *mcr*-1	–	1	–	–	–	–	–	NS
CTX-M2 + *cit*	–	1	–	–	–	–	–	NS
CTX-M2 + CTX-M8	–	–	–	1	–	–	–	NS
CTX-M2 + CTX-M8 + *cit*	–	2	–	1	–	–	–	NS
CTX-M8 + *cit*	–	–	2	–	–	–	–	NS
*fos*A3	–	–	1	–	1	–	–	NS
cit	–	–	–	2–	–	–	–	NS

(–) Absence; (NS) Non Sampling; Water not presented ESBL-producing E. coli positive.

Conjugation Experiments

Two transconjugant strains were obtained in the conjugation assay (T1 and T4), which presented *bla*CTX-M1 and *fos*A3 genes and ESBL phenotype (**Table 3**).

TABLE 3 | Conjugation experiment between ESBL-producing *E. coli* from poultry litter and non-ESBL-producing *E. coli* from the microbiota of chicks.

	Strain	Phylogenetic group	bla_{CTX-M} gene	*fos*A3 gene
Donors	EcPR1	B1	$bla_{CTX-M55}$	+
	EcPR2	B1	$bla_{CTX-M55}$	+
Recipients	EcRS1	D	bla_{CTX-M8}	–
	EcRS2	D	bla_{CTX-M8}	–
Transconjugants	T1 (EcPR1+EcRS1)	D	$bla_{CTX-M8/}$ $bla_{CTX-M55}$	+
	T4 (EcPR1+EcRS2)	D	$bla_{CTX-M8/}$ $bla_{CTX-M55}$	+

(+) Presence; (–) Absence.

Phylogenetic Group and ERIC-PCR Analysis

All strains were assigned to four main phylogenetic groups (Clermont et al., 2000). Most strains belonged to the phylogenetic group D (n = 54; 46%), followed by group B1 (n = 28; 24%), group A (n = 27; 23%), and group B2 (n = 8; 7%) (**Table 4**). The ERIC-PCR analysis showed 16 sub-clusters in the PR state and 15 clusters in the RS state samples, with 19 and 17 singletons remaining, respectively (**Supplementary Figures 1,2**).

DISCUSSION

The present study revealed a high occurrence of ESBL/AmpC-producing and fosfomycin-resistant *E. coli* in poultry farms from southern Brazil. ESBL phenotype positive strains were found in poultry litter in all sampled periods, and similar was observed by Laube et al. (2013) in Germany. We showed in our study that the chance of ESBL-producing *E. coli* occurrence in poultry litter was significant in all sampling periods, mainly at the beginning of poultry production (OR 6.42). Furthermore, ESBL-producing *E. coli* strains were detected in poultry since the second period, with an increased occurrence in the third period. These data indicate that poultry litter could be a risk factor for ESBL-producing *E. coli* dissemination in poultry houses, including the colonization of one-day-old chicks at the beginning of production. Besides, poultry litter may present a potential risk in the formation of bioaerosols containing antimicrobial-resistant bacteria. Brooks et al. (2010) observed a high concentration of these aerosolized bacteria in poultry houses. The results showed an increase in bacterial concentrations between pre-flock (29%) and late-flock (66%). Studies must be carried out to verify the potential risk of bioaerosols on the health of producers that handle poultry daily. Our results proved that water (OR 0) and poultry feed (OR 0.65) were not sources of ESBL-producing *E. coli* strains.

Besides poultry litter, all *E. coli* strains isolated from beetles were positive for ESBL production (OR > 100). These beetles were collected only in the PR state due their presence and abundance in the local farms. These insects are omnivorous scavengers that feed on fecal matter and debris; therefore, they are commonly found in poultry litter (Axtell, 1999). Many insects that inhabit poultry houses can be carriers or reservoirs of MDR bacteria. Studies performed by Blaak et al. (2014) and Solà-Ginés et al. (2015) showed the presence of ESBL-producing *E. coli* in flies isolated from poultry farms.

Daehre et al. (2018) demonstrated the influence of previous fattening flocks on ESBL-producing strains of the following broiler flock. Through the whole-genome analysis, the authors showed the transmission of ESBL-producing strains between the contaminated environment and poultry, suggesting that cleaning and disinfection practices should be applied. These practices are essential for decreasing the risk of ESBL-producing *E. coli* spreading to the next flocks, according to Mo et al. (2016). The authors analyzed the risk factors for the presence of cephalosporin-resistant *E. coli* during broiler production, which is 0.1 times after a rigorous disinfection process and 9 times after people entering the sheds. These authors suggest that good cleaning practices and the control of people's access to the barn can reduce the occurrence of these resistant strains in the barn environment. In line with these studies, our work reaffirms the importance of applying disinfection techniques to reduce MDR strains in the breeding broiler environment.

Another biosecurity measure in poultry production is the poultry litter processing by composting, a widely used method to ensure that organic waste is safe before use (Wilkinson et al., 2011). Composting implies a reduction in organic waste volume and considerably decreases pathogenic microorganisms (Bernal et al., 2009). Gazal et al. (2015) analyzed poultry litter after the composting process, observing that few isolates (6.3%) contained virulence genes from extra-intestinal pathogenic *E. coli* and were susceptible to a large number of antimicrobials. Siller et al. (2020) demonstrated that short-term storage reduces the amount of ESBL-producing *E. coli* in poultry litter. Therefore, the practice of composting or storage is a good alternative for eliminating possible pathogens and multiresistant strains.

Escherichia coli strains found in water samples were negative for ESBL production. All water from the poultry houses was chlorinated, which excludes the possibility of being a source of ESBL-producing *E. coli*.

The MDR strain frequency in the present study is concerning, and many strains were detected as ESBL-producing. A similar resistance profile has been detected in *E. coli* strains from poultry farms in Italy (Ghodousi et al., 2015) and Germany (Laube et al., 2013). Cyoia et al. (2019) detected MDR in 80% of commercialized chicken carcasses in both PR and RS states. Moreover, 30% of these strains were characterized as ESBL producers. Poultry-derived products are considered the main ESBL-producing bacteria sources among all animal products (Saliu et al., 2017). In Brazil, many antimicrobial agents that have been previously used as growth promoters are now prohibited

TABLE 4 | Phylogenetic distribution of 78 ESBL-producing *E. coli* strains and 39 non-ESBL-producing *E. coli* strains based on the isolate source.

	ESBL producer – number of strains (%)					Non-ESBL producer – number of strains (%)				
	A	B1	B2	D	Total	A	B1	B2	D	Total
Poultry Litter	12 (15.4%)	9 (11.5%)	0 (0%)	8 (10.3%)	29	2 (5.1%)	2 (5.1%)	3 (7.7%)	5 (12.8%)	12
Poultry	6 (7.7%)	3 (3.8%)	1 (1.3%)	23 (29.5%)	33	1 (2.6%)	5 (12.8%)	1 (2.6%)	4 (10.3%)	11
Poultry Feed	0 (0%)	1 (1.3%)	0 (0%)	3 (3.8%)	4	4 (10.3%)	0 (0%)	2 (5.1%)	1 (2.6%)	7
Water	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0	2 (5.1%)	4 (10.3%)	0 (0%)	3 (7.7%)	9
Beetle	0 (0%)	4 (5.1%)	1 (1.3%)	7 (9%)	12	NS (0%)	NS (0%)	NS (0%)	NS (0%)	NS

(NS) Non Sampling.

(MAPA, 2003, 2009); however, some antimicrobials are still used for treatment or as a prophylactic measure. In both states, the investigated farms reported the use of quinolones during poultry production. Moreover, we found high resistance to quinolones, especially in ESBL strains (**Figure 2**), which also showed high resistance to other antimicrobials, whose use in poultry production was not mentioned, such as GEN, SXT, and TET. Cyoia et al. (2019) also found that ESBL-producing *E. coli* were more resistant to a higher number of antimicrobials than non-ESBL strains. Besides, Zeng and Lin (2017) reported reported the association between the resistance of ESBL and other antimicrobial classes, such as aminoglycosides and fluoroquinolones.

Currently, resistance to third-generation cephalosporins induced by ESBL production represents a major public health problem (Blaak et al., 2015; Centers for Disease Control and Prevention, 2015). These enzymes are very relevant as they confer resistance to antimicrobials used in veterinary medicine, such as penicillin, aminopenicillin, and cephalosporins (e.g., ceftiofur) (Poirel et al., 2018). In the present study, 92/117 (77%) *E. coli* isolates showed *bla* genes (CTX-M1, 2 and 8 groups). Approximately 66% were confirmed as ESBL-producing, and the majority presented the bla_{CTX-M} group 2 (55%) gene, followed by bla_{CTX-M} group 1 (38%) and bla_{CTX-M} group 8 (10%). These data corroborate the description made by Silva and Lincopan (2012) regarding the epidemiology of ESBL genes in Brazilian territory, especially in animal production. Four strains were negative for ESBL phenotype, but harbored both ESBL and AmpC genes (**Supplementary Table 2**). According to Nishimura et al. (2018), the presence of AmpC determinants interfere with ESBL phenotype and increases false negative detection.

The selective pressure of antimicrobials in poultry farming leads to the death of sensitive strains and also selects the resistant ones (Poole, 2012; Saliu et al., 2017). Moreover, resistance to determined antimicrobials can lead to cross-resistance and co-selection, with stronger promoters increasing ESBL genes' expression (Gniadkowski, 2008).

Approximately 73% of strains harbored the *fos*A3 gene among the 26 phenotypical fosfomycin-resistant strains. Fosfomycin is a bactericidal antimicrobial that inhibits peptidoglycan synthesis and is widely used in human medicine to treat urinary tract infections (Falagas et al., 2016, 2019). Notably, all the *fos*A3 positive strains were also characterized as ESBL-producing. The relation between *fos*A3 and $bla_{CTX-M55}$ (bla_{CTX-M} group 1) genes was significant among ESBL-producing *E. coli* isolated from PR (OR 3.66) and is more evident on strains isolated from RS (OR 6.44, CI 95% 2.89–14.34, $p < 0.05$) since only two strains presented $bla_{CTX-M55}$ and none strains harboring the *fos*A3 gene were isolated. The *fos*A3 gene was found in isolates from properties that used fosfomycin during poultry production in the PR state. Sato et al. (2013) suggested that ESBL-producing strains that harbor the bla_{CTX-M} gene (especially the CTX-M55 enzyme) associated with the *fos*A3 gene naturally reside in the intestinal microbiota of individuals in clinical and veterinary environments. Thus, the use of fosfomycin in Brazil's poultry production may lead to the co-selection of ESBL-producing strains. This is the first study reporting positive strains for the *fos*A3 gene isolated from poultry production in Brazil.

Another important finding was the high heterogeneity among *E. coli* strains analyzed by ERIC-PCR between PR and RS strains that showed several sub-clusters in both states. These results demonstrated that ESBL-producing *E. coli* strains isolated from poultry production in southern Brazil were not caused by clonal spreading. Ghodousi et al. (2015) presented similar data regarding the analysis of ESBL-producing *E. coli* strains isolated from poultry farms in Italy. The spread of ESBL genes among strains isolated from animals occurs by horizontal gene transference (Poirel et al., 2018). Thus, the large number of clusters associated with the high genetic diversity showed in our study suggests that the dissemination of resistance genes is due to horizontal transfer by conjugative plasmids.

IncFIB (n = 89; 76%) followed by IncI1 (N = 37, 32%) were the dominant replicon types in our samples. Several plasmid families carry ESBL/AmpC genes in ESBL/AmpC-producing Enterobacteriaceae (Carattoli, 2009), of which IncF, IncI, and IncK types are frequently found (Liebana et al., 2012). Our results corroborate with other studies that investigated the presence of ESBL/AmpC-producing *E. coli* strains in poultry (Leverstein-van Hall et al., 2011; Mnif et al., 2012).

The present study also demonstrated the conjugation between ESBL-producing *E. coli* from poultry litter and non-ESBL-producing *E. coli* from chicken microbiota, in which the recipient strains became positive to $bla_{CTX-M55}$ and *fos*A3 genes (T1 and T4). Cyoia et al. (2019) showed that the genetic determinants encoding CTX-M enzymes were transferred to the J53 strain from a human source. Thus, the conjugation results indicated two relevant points. First, it reinforces the association between $bla_{CTX-M55}$ and *fos*A3 genes because both were found in the transconjugants. Second, poultry litter could be a starting point for the spreading of resistance genes to poultry and, consequently, to humans via the food chain. Future research intends to clarify the relation between ESBL-producing strains and the production chain in Brazil. In the Netherlands, Apostolakos et al. (2019) showed that ESBL/AmpC producing *E. coli* was prevalent in the broiler production chain, with significant transference to subsequent production levels, indicating that all production levels need to be investigated.

These results show the need for monitoring systems to investigate and understand antimicrobial resistance spreading in animal production, and thus encourage the promotion of antimicrobial rational use. Brazil is the second-largest producer of poultry meat and the leader of exports worldwide (Associação Brasileira de Proteína Animal, 2018). Therefore, Brazil's production has great relevance in the world poultry scenario and, consequently, influences public health through the food chain.

Currently, the One Health concept has been presented as a way to raise awareness regarding the relation among human, animal, and environmental health (Kahn, 2011). This approach is considered by international organizations, such as the World Health Organization, as an important element in disease control and prevention (Lerner and Berg, 2015). However, this awareness should not only be limited to the control and prevention of pathogens, but also address aspects of antimicrobial resistance.

Lammie and Hughes (2016) suggest that due to the challenge presented by antimicrobial resistance and its relation to human, animal, and environmental health, it is essential to include this issue in the One Health approach.

CONCLUSION

The antimicrobial use in poultry production has a great influence on MDR bacteria selection and severely influences public health, as well as the national productive sector. A longitudinal monitoring program in poultry production should be implemented to provide data regarding antimicrobial use. The improvement in management techniques, such as poultry litter treatment and good clean practices, may decrease MDR strain frequency in poultry farms. These measures can optimize poultry production and increase animal and human health preservation.

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 Animal Ethics Committee of State University of Londrina (CEUA/UEL).

AUTHOR CONTRIBUTIONS

LG contributed to the development of experimental research, data analysis, and wrote the manuscript. LM, MD, VK, BG, TG, TC, and JP contributed to the development of experimental research. EN contributed to the statistical analysis. RK, GN, KB, BB, and EV contributed to and assisted in the design of the work and in preparation of the article, and critically reviewed the manuscript. All authors have participated in this study and commented on the manuscript.

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SUPPLEMENTARY MATERIAL

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REFERENCES

Apostolakos, I., Mughini-Gras, L., Fasolato, L., and Piccirillo, A. (2019). Assessing the occurrence and transfer dynamics of ESBL/pAmpC-producing *Escherichia coli* across the broiler production pyramid. *PLoS One* 14:e0217174. doi: 10.1159/000500311

Arlet, G., and Philippon, A. (1991). Construction by polymerase chain reaction and intragenic DNA probes for three main types of transferable β-lactamases (TEM, SHV, CARB). *FEMS Microbiol. Lett.* 82, 19–25. doi: 10.1111/j.1574-6968.1991.tb04833.x

Associação Brasileira de Proteína Animal (2018). *Annual Report of Brazilian Poultry Association*. São Paulo: Brazilian Poultry Union.

Axtell, R. C. (1999). Poultry integrated pest management: status and future. *Integr. Pest Manag. Rev.* 4, 53–73. doi: 10.1023/A:1009637116897

Bernal, M. P., Alburquerque, J. A., and Moral, R. (2009). Composting of animal manures and chemical criteria for compost maturity assessment. A review. *Bioresour. Technol.* 100, 5444–5453. doi: 10.1016/j.biortech.2008.11.027

Blaak, H., Hamidjaja, R. A., van Hoek, A. H., de Heer, L., de Roda Husman, A. M., and Schets, F. M. (2014). Detection of extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* on flies at poultry farms. *Appl. Environ. Microbiol.* 80, 239–246. doi: 10.1128/AEM.02616-13

Blaak, H., van Hoek, A. H., Hamidjaja, R. A., van der Plaats, R. Q., Kerkhof-de Heer, L., de Roda Husman, A. M., et al. (2015). Distribution, numbers, and diversity of ESBL-producing E. coli in the poultry farm environment. *PLoS One* 10:e0135402. doi: 10.1371/journal.pone.0135402

Blanc, V., Mesa, R., Saco, M., Lavilla, S., Prats, G., Miró, E., et al. (2006). ESBL-and plasmidic class C β-lactamase-producing E. coli strains isolated from poultry, pig and rabbit farms. *Vet. Microbiol.* 118, 299–304. doi: 10.1016/j.vetmic.2006.08.002

Bortolami, A., Zendri, F., Maciuca, E. I, Wattret, A., Ellis, C., Schmidt, V., et al. (2019). Diversity, virulence, and clinical significance of extended-spectrum β-Lactamase-and pAmpC-producing *Escherichia coli* from companion animals. *Front. Microbiol.* 10:1260. doi: 10.3389/fmicb.2019.01260

Brooks, J. P., McLaughlin, M. R., Scheffler, B., and Miles, D. M. (2010). Microbial and antibiotic resistant constituents associated with biological aerosols and poultry litter within a commercial poultry house. *Sci. Total Env.* 408, 4770–4777. doi: 10.1016/j.scitotenv.2010.06.038

Bush, K., and Fisher, J. F. (2011). Epidemiological expansion, structural studies, and clinical challenges of new β-lactamases from gram-negative bacteria. *Annu. Rev. Microbiol.* 65, 455–478. doi: 10.1146/annurev-micro-090110-102911

Cantón, R., and Coque, T. M. (2006). The CTX-M β-lactamase pandemic. *Curr. Opin. Microbiol.* 9, 466–475. doi: 10.1016/j.mib.2006.08.011

Carattoli, A. (2008). Animal reservoirs for extended spectrum β-lactamase producers. *Clin. Microbiol. Infect.* 14, 117–123. doi: 10.1111/j.1469-0691.2007.01851.x

Carattoli, A. (2009). Resistance plasmid families in *Enterobacteriaceae*. *Antimicrob. Agents Chemother.* 53, 2227–2238. doi: 10.1128/AAC.01707-08

Carattoli, A. (2011). Plasmids in Gram negatives: molecular typing of resistance plasmids. *Int. J. Med. Microbiol.* 301, 654–658. doi: 10.1016/j.ijmm.2011.09.003

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

Carattoli, A., Bertini, A., Villa, L., Falbo, V., Hopkins, K. L., and Threlfall, E. J. (2005). Identification of plasmids by PCR-based replicon typing. *J. Microbiol. Methods.* 63, 219–228. doi: 10.1016/j.mimet.2005.03.018

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

Centers for Disease Control and Prevention (2015). *Antibiotic Resistance Threats in the United States, 2013*. Atlanta: CDC.

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

CLSI (2018). *Performance Standards for Antimicrobial Susceptibility Testing. Twenty-Fifth Informational Supplement CLSI Document M100*. Wayne, PA: Clinical and Laboratory Standards Institute.

Cyoia, P. S., Koga, V. L., Nishio, E. K., Houle, S., Dozois, C. M., de Brito, K. C. T., et al. (2019). Distribution of ExPEC virulence factors, blaCTX-M, fosA3, and mcr-1 in *Escherichia coli* isolated from commercialized chicken carcasses. *Front. Microbiol.* 9:3254. doi: 10.3389/fmicb.2018.03254

Daehre, K., Projahn, M., Semmler, T., Roesler, U., and Friese, A. (2018). Extended-spectrum beta-lactamase-/AmpC beta-lactamase-producing *enterobacteriaceae* in broiler farms: transmission dynamics at farm level. *Microb. Drug Resist.* 24, 511–518. doi: 10.1089/mdr.2017.0150

Datta, N., and Hedges, R. W. (1971). Compatibility groups among fi–R factors. *Nature* 234, 222. doi: 10.1038/234222a0

Dierikx, C. M., van der Goot, J. A., Smith, H. E., Kant, A., and Mevius, D. J. (2013). Presence of ESBL/AmpC-producing *Escherichia coli* in the broiler production pyramid: a descriptive study. *PLoS One* 8:e79005. doi: 10.1371/journal.pone.0079005

Dominguez, J. E., Redondo, L. M., Figueroa Espinosa, R. A., Cejas, D., Gutkind, G. O., Chacana, P. A., et al. (2018). Simultaneous carriage of mcr-1 and other antimicrobial resistance determinants in *Escherichia coli* from poultry. *Front. Microbiol.* 9:1679. doi: 10.3389/fmicb.2018.01679

Edwards, P. R., and Ewing, W. H. (1986). *Edwards and Ewing's Identification of Enterobacteriaceae*. Amsterdam: Elsevier Publishing Company.

Ewers, C., Bethe, A., Semmler, T., Guenther, S., and Wieler, L. H. (2012). Extended-spectrum β-lactamase-producing and AmpC-producing *Escherichia coli* from livestock and companion animals, and their putative impact on public health: a global perspective. *Clin. Microbiol. Infect.* 18, 646–655. doi: 10.1111/j.1469-0691.2012.03850.x

Falagas, M. E., Athanasaki, F., Voulgaris, G. L., Triarides, N. A., and Vardakas, K. Z. (2019). Resistance to fosfomycin: mechanisms, frequency and clinical consequences. *Int. J. Antimicrob. Agents.* 53, 22–28. doi: 10.1016/j.ijantimicag.2018.09.013

Falagas, M. E., Vouloumanou, E. K., Samonis, G., and Vardakas, K. Z. (2016). Fosfomycin. *Clin. Microbiol. Rev.* 29, 321–347. doi: 10.1128/CMR.00068-15

Friese, A., Schulz, J., Laube, H., Hartung, J., and Roesler, U. (2013). Faecal occurrence and emissions of livestock-associated methicillin-resistant Staphylococcus aureus (laMRSA) and ESBL/AmpC-producing E. coli from animal farms in Germany. *Berl. Munch. Tierarztl. Wochenschr.* 126, 175–180. doi: 10.1037/e411862008-001

Gazal, L. E. S., Puño-Sarmiento, J. J., Medeiros, L. P., Cyoia, P. S., da Silveira, W. D., Kobayashi, R. K., et al. (2015). Presence of pathogenicity islands and virulence genes of extraintestinal pathogenic *Escherichia coli* (ExPEC) in isolates from avian organic fertilizer. *Poult. Sci.* 94, 3025–3033. doi: 10.3382/ps/pev278

Ghodousi, A., Bonura, C., Di Noto, A. M., and Mammina, C. (2015). Extended-spectrum ß-lactamase, AmpC-producing, and fluoroquinolone-resistant *Escherichia coli* in retail broiler chicken meat, Italy. *Foodborne Pathog. Dis.* 12, 619–625. doi: 10.1089/fpd.2015.1936

Gniadkowski, M. (2008). Evolution of extended-spectrum β-lactamases by mutation. *Clin. Microbiol. Infect.* 14, 11–32. doi: 10.1111/j.1469-0691.2007.01854.x

Jarlier, V., Nicolas, M. H., Fournier, G., and Philippon, A. (1988). Extended broad-spectrum β-lactamases conferring transferable resistance to newer β-lactam agents in *Enterobacteriaceae*: hospital prevalence and susceptibility patterns. *Clin. Infect. Dis.* 10, 867–878. doi: 10.1093/clinids/10.4.867

Kahn, L. H. (2011). The need for one health degree programs. *Infect. Ecol. Epidemiol.* 1, 7919. doi: 10.3402/iee.v1i0.7919

Keating, G. M. (2013). Fosfomycin trometamol: a review of its use as a single-dose oral treatment for patients with acute lower urinary tract infections and pregnant women with asymptomatic bacteriuria. *Drugs* 73, 1951–1966. doi: 10.1007/s40265-013-0143-y

Koga, V. L., Rodrigues, G. R., Scandorieiro, S., Vespero, E. C., Oba, A., de Brito, B. G., et al. (2015). Evaluation of the antibiotic resistance and virulence of *Escherichia coli* strains isolated from chicken carcasses in 2007 and 2013 from Paraná, Brazil. *Foodborne Pathog. Dis.* 12, 479–485. doi: 10.1089/fpd.2014.1888

Lammie, S. L., and Hughes, J. M. (2016). Antimicrobial resistance, food safety, and one health: the need for convergence. *Annu. Ver. Food Sci. T.* 7, 287–312. doi: 10.1146/annurev-food-041715-033251

Laube, H., Friese, A., Von Salviati, C., Guerra, B., Käsbohrer, A., Kreienbrock, L., et al. (2013). Longitudinal monitoring of extended-spectrum-beta-lactamase/AmpC-producing *Escherichia coli* at German broiler chicken fattening farms. *Appl. Environ. Microbiol.* 79, 4815–4820. doi: 10.1128/AEM.00856-13

Lazarus, B., Paterson, D. L., Mollinger, J. L., and Rogers, B. A. (2014). Do human extraintestinal *Escherichia coli* infections resistant to expanded-spectrum cephalosporins originate from food-producing animals? A systematic review. *Clin. Infect. Dis.* 60, 439–452. doi: 10.1093/cid/ciu785

Lerner, H., and Berg, C. (2015). The concept of health in One Health and some practical implications for research and education: what is One Health? *Infect. Ecol. Epidemiol.* 5, 25300. doi: 10.3402/iee.v5.2530

Leverstein-van Hall, M. A., Dierikx, C. M., Cohen Stuart, J., Voets, G. M., Van Den Munckhof, M. P., van Essen-Zandbergen, A., et al. (2011). Dutch patients, retail chicken meat and poultry share the same ESBL genes, plasmids and strains. *Clin. Microbiol. Infect.* 17, 873–880. doi: 10.1111/j.1469-0691.2011.03497.x

Li, Y., Chen, L., Wu, X., and Huo, S. (2015). Molecular characterization of multidrug-resistant avian pathogenic *Escherichia coli* isolated from septicemic broilers. *Poult. Sci.* 94, 601–611. doi: 10.3382/ps/pev008

Liebana, E., Carattoli, A., Coque, T. M., Hasman, H., Magiorakos, A. P., Mevius, D., et al. (2012). Public health risks of enterobacterial isolates producing extended-spectrum β-lactamases or AmpC β-lactamases in food and food-producing animals: an EU perspective of epidemiology, analytical methods, risk factors, and control options. *Clin. Infect. Dis.* 56, 1030–1037. doi: 10.1093/cid/cis1043

Liu, Y. Y., Wang, Y., Walsh, T. R., Yi, L. X., Zhang, R., Spencer, J., et al. (2016). Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infect. Dis.* 16, 161–168. doi: 10.1016/S1473-3099(15)00424-7

MAPA (2003). "Pecuária e Abastecimento," in 27 de junho de 2003. Proíbe a Fabricação, a Manipulação, o Fracionamento, a Comercialização, a Importação e o Uso dos Princípios Ativos Cloranfenicol e Nitrofuranos e os Produtos que contenham estes Princípios Ativos, para uso Veterinário e Susceptível de Emprego na Alimentação de Todos os Animais e Insetos. Instrução Normativa (Portuguese). Available online at: https://www.gov.br/agricultura/pt-br/assuntos/insumos-agropecuarios/insumos-pecuarios/alimentacao-animal/arquivos-alimentacao-animal/legislacao/instrucao-normativa-no-9-de-27-de-junho-de-2003.pdf

MAPA (2009). "Pecuária e Abastecimento," in *Regulamento Técnico Para a Fabricação, o Controle de Qualidade, a Comercialização e o Emprego de Produtos Antimicrobianos de Uso Veterinário. Instrução Normativa 26, Ministério da Agricultura, Pecuária e Abastecimento Brasília (Portuguese)*. Available online at: https://www.gov.br/agricultura/pt-br/assuntos/insumos-agropecuarios/insumos-pecuarios/alimentacao-animal/arquivos-alimentacao-animal/legislacao/instrucao-normativa-no-26-de-9-de-julho-de-2009.pdf

McLellan, S. L., Daniels, A. D., and Salmore, A. K. (2003). Genetic characterization of *Escherichia coli* populations from host sources of fecal pollution by using DNA fingerprinting. *Appl. Environ. Microbiol.* 69, 2587–2594. doi: 10.1128/AEM.69.5.2587-2594.2003

Mnif, B., Ktari, S., Rhimi, F. M., and Hammami, A. (2012). Extensive dissemination of CTX-M-1-and CMY-2-producing *Escherichia coli* in poultry farms in Tunisia. *Lett. Appl. Microbiol.* 55, 407–413. doi: 10.1111/j.1472-765X.2012.03309.x

Mo, S. S., Kristoffersen, A. B., Sunde, M., Nødtvedt, A., and Norström, M. (2016). Risk factors for occurrence of cephalosporin-resistant *Escherichia coli* in Norwegian broiler flocks. *Prev. Vet. Med.* 130, 112–118. doi: 10.1016/j.prevetmed.2016.06.011

Nishimura, F., Morinaga, Y., Akamatsu, N., Matsuda, J., Kaku, N., Takeda, K., et al. (2018). Plasmid-mediated AmpC β-lactamase and underestimation of extended-spectrum β-lactamase in cefepime-susceptible, elevated-ceftazidime-MIC *Enterobacteriaceae* isolates. *Jpn. J. Infect. Dis.* 71, 281–285. doi: 10.7883/yoken.jjid.2017.469

Olsen, R. H., Bisgaard, M., Löhren, U., Robineau, B., and Christensen, H. (2014). Extended-spectrum β-lactamase-producing *Escherichia coli* isolated from poultry: a review of current problems, illustrated with some laboratory findings. *Avian Pathol.* 43, 199–208. doi: 10.1080/03079457.2014.907866

O'Neill, J. (2014). *The Review on Antimicrobial Resistance. Antimicrobial Resistance: Tackling a Crisis for the Health and Wealth of Nations*. Available online at: https://amr-review.org/sites/default/files/AMR%20Review%20Paper%20-%20Tackling%20a%20crisis%20for%20the%20health%20and%20wealth%20of%20nations_1.pdf (accessed June 20, 2019).

Paterson, D. L., and Bonomo, R. A. (2005). Extended-spectrum β-lactamases: a clinical update. *Clin. Microbiol. Rev.* 18, 657–686. doi: 10.1128/CMR.18.4.657-686.2005

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

Pitout, J. D., and Laupland, K. B. (2008). Extended-spectrum β-lactamase-producing *Enterobacteriaceae*: an emerging public-health concern. *Lancet Infect. Dis.* 8, 159–166. doi: 10.1016/S1473-3099(08)70041-0

Poirel, L., Madec, J. Y., Lupo, A., Schink, A. K., Kieffer, N., Nordmann, P., et al. (2018). Antimicrobial resistance in *Escherichia coli*. *Microbiol. Spectr.* 6, 289–316. doi: 10.1128/9781555819804.ch13

Poole, K. (2012). Bacterial stress responses as determinants of antimicrobial resistance. *J. Antimicrob. Chemother.* 67, 2069–2089. doi: 10.1093/jac/dks196

Saliu, E. M., Vahjen, W., and Zentek, J. (2017). Types and prevalence of extended–spectrum beta–lactamase producing *Enterobacteriaceae* in poultry. *Anim. Health Res. Rev.* 18, 46–57. doi: 10.1017/S1466252317000020

Sato, N., Kawamura, K., Nakane, K., Wachino, J. I., and Arakawa, Y. (2013). First detection of fosfomycin resistance gene fosA3 in CTX-M-producing *Escherichia coli* isolates from healthy individuals in Japan. *Microb. Drug Resis.* 19, 477–482. doi: 10.1089/mdr.2013.0061

Segabinazi, S. D., Flôres, M. L., da Silva Barcelos, A., Jacobsen, G., and Eltz, R. D. (2005). Bactérias da família *Enterobacteriaceae* em Alphitobius diaperinus oriundos de granjas avícolas dos Estados do Rio Grande do Sul e Santa Catarina, Brasil. *Acta Sci. Vet.* 33, 51–55. doi: 10.22456/1679-9216.14449

Siller, P., Daehre, K., Thiel, N., Nübel, U., and Roesler, U. (2020). Impact of short-term storage on the quantity of extended-spectrum beta-lactamase–producing *Escherichia coli* in broiler litter under practical conditions. *Poultry Sci.* 99, 2125–2135. doi: 10.1016/j.psj.2019.11.043

Silva, K. C. D., and Lincopan, N. (2012). Epidemiologia das betalactamases de espectro estendido no Brasil: impacto clínico e implicações para o agronegócio. *J. Bras. Patol. Med. Lab.* 48, 91–99. doi: 10.1590/S1676-24442012000200004

Smet, A., Martel, A., Persoons, D., Dewulf, J., Heyndrickx, M., Herman, L., et al. (2009). Broad-spectrum β-lactamases among *Enterobacteriaceae* of animal origin: molecular aspects, mobility and impact on public health. *FEMS Microbiol. Rev.* 34, 295–316. doi: 10.1111/j.1574-6976.2009.00198.x

Solà-Ginés, M., González-López, J. J., Cameron-Veas, K., Piedra-Carrasco, N., Cerdà-Cuéllar, M., and Migura-Garcia, L. (2015). Houseflies (Musca domestica) as vectors for extended-spectrum β-lactamase-producing *Escherichia coli* on Spanish broiler farms. *Appl. Environ. Microbiol.* 81, 3604–3611. doi: 10.1128/AEM.04252-14

Toledo, M. R. F., Fontes, C. F., and Trabulsi, L. R. (1982a). EPM-A modification of Rugai and Araujo medium for simultaneous test of gas production from glucose, H2S, urease and tryptophan deaminase. *Rev. Microbiol.* 13, 230–235.

Toledo, M. R. F., Fontes, C. F., and Trabulsi, L. R. (1982b). MILi-a medium for detection of motility, indole and lysine decarboxylase. *Rev. Microbiol.* 13, 230–235.

Versalovic, J., Koeuth, T., and Lupski, R. (1991). Distribution of repetitive DNA sequences in eubacteria and application to finerpriting of bacterial enomes. *Nucleic Acids Res.* 19, 6823–6831. doi: 10.1093/nar/19.24.6823

Wieler, L. H., Ewers, C., Guenther, S., Walther, B., and Lübke-Becker, A. (2011). Methicillin-resistant staphylococci (MRS) and extended-spectrum beta-lactamases (ESBL)-producing *Enterobacteriaceae* in companion animals: nosocomial infections as one reason for the rising prevalence of these potential zoonotic pathogens in clinical samples. *Int. J. Med. Microbiol.* 301, 635–641. doi: 10.1016/j.ijmm.2011.09.009

Wilkinson, K. G., Tee, E., Tomkins, R. B., Hepworth, G., and Premier, R. (2011). Effect of heating and aging of poultry litter on the persistence of enteric bacteria. *Poul. Sci.* 90, 10–18. doi: 10.3382/ps.2010-01023

Woodford, N., Fagan, E. J., and Ellington, M. J. (2005). Multiplex PCR for rapid detection of genes encoding CTX-M extended-spectrum β-lactamases. *J. Antimicrob. Chemother.* 57, 154–155. doi: 10.1093/jac/dki412

World Health Organization (2014). *Antimicrobial resistance: global report on surveillance*. Avaliable online at: https://apps.who.int/iris/bitstream/handle/10665/112642/9789241564748_eng.pdf;jsessionid=9BD9CFC92083023155E1028C22D10F1F?sequence=1 (accessed June 20, 2019).

Zeng, X., and Lin, J. (2017). Factors influencing horizontal gene transfer in the intestine. *Anim. Health Res. Rev.* 18, 153–159. doi: 10.1017/S1466252317000159

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Emergence of *fosA3* and $bla_{CTX-M-14}$ in Multidrug-Resistant *Citrobacter freundii* Isolates From Flowers and the Retail Environment in China

Ke Cheng[1†], Liang-Xing Fang[1,2†], Qian-Wen Ge[1], Dong Wang[1], Bing He[1], Jia-Qi Lu[1], Zi-Xing Zhong[1], Xi-Ran Wang[1], Yang Yu[1,2], Xin-Lei Lian[1,2], Xiao-Ping Liao[1,2,3], Jian Sun[1,2,3] and Ya-Hong Liu[1,2,3,4]**

[1] National Risk Assessment Laboratory for Antimicrobial Resistance of Animal Original Bacteria, South China Agricultural University, Guangzhou, China, [2] Guangdong Provincial Key Laboratory of Veterinary Pharmaceutics Development and Safety Evaluation, South China Agricultural University, Guangzhou, China, [3] Guangdong Laboratory for Lingnan Modern Agriculture, Guangzhou, China, [4] Jiangsu Co-Innovation Center for the Prevention and Control of Important Animal Infectious Disease and Zoonoses, Yangzhou University, Yangzhou, China

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Oak Ridge Institute for Science and Education (ORISE), United States

***Correspondence:**
Jian Sun
jiansun@scau.edu.cn
Ya-Hong Liu
lyh@scau.edu.cn

† These authors have contributed equally to this work

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We examined the prevalence and transmission of the *fosA3* gene among *Citrobacter freundii* isolates from flowers and the retail environments. We identified 11 fosfomycin-resistant *C. freundii* strains (>256 μg/mL) from 270 samples that included petals (n = 7), leaves (n = 2), dust (n = 1) and water (n = 1). These 11 isolates were multidrug-resistant and most were simultaneously resistant to fosfomycin, cefotaxime, ciprofloxacin and amikacin. Consistently, all 11 isolates also possessed $bla_{CTX-M-14}$, $bla_{CMY-65/122}$, *aac(6')-Ib-cr*, *qnrS1*, *qnrB13/6/38* and *rmtB*. These *fosA3*-positive isolates were assigned to two distinct PFGE patterns and one (n = 9) predominated indicating clonal expansion of *fosA3*-positive isolates across flower markets and shops. Correspondingly, *fosA3* was co-transferred with $bla_{CTX-M-14}$ *via* two plasmid types by conjugation possessing sizes of 110 kb (n = 9) and 260 kb (n = 2). Two representatives were fully sequenced and p12-1 and pS39-1 possessed one and two unclassified replicons, respectively. These plasmids shared a distinctive and conserved backbone in common with *fosA3*-carrying *C. freundii* and other *Enterobacteriaceae* from human and food animals. However, the *fosA3*-$bla_{CTX-M-14}$-containing multidrug resistance regions on these untypable plasmids were highly heterogeneous. To the best of our knowledge, this is the first report of *fosA3* and $bla_{CTX-M-14}$ that were present in bacterial contaminants from flower shops and markets. These findings underscore a public health threat posed by untypable and transferable p12-1-like and pS39-1-like plasmids bearing *fosA3*-$bla_{CTX-M-14}$ that could circulate among *Enterobacteriaceae* species and in particular *C. freundi* in environmental isolates.

Keywords: flower, fosfomycin-resistance, *Citrobacter freundii*, *fosA3*, $bla_{CTX-M-14}$

INTRODUCTION

There are two major problems in the treatment of bacterial infections: the spread of multidrug-resistant (MDR) or extensively drug-resistant (XDR) pathogens and lack of development of new antibiotics active against these bacteria (Falagas et al., 2016). This situation has renewed interest in older antibiotics such as fosfomycin as alternatives or "last resort" therapies (Falagas et al., 2016; Sastry and Doi, 2016). Fosfomycin is viewed as a suitable empirical drug that has retained activity against resistant strains (Popovic et al., 2010). The World Health Organization has reclassified fosfomycin as a "critically important antimicrobial" based on its broad-spectrum bactericidal reactivity and good pharmacological properties (World Health Organization [WHO], 2011).

Clinical cases of fosfomycin resistance have increased in the last decade especially due to inactivation of the drug *via* plasmid-mediated fosfomycin-modification (*fos*) genes in *Enterobacteriaceae* (Diez-Aguilar et al., 2013). Fosfomycin covalently binds to a cysteine thiol in the active site of MurA and interferes with peptidoglycan synthesis at an earlier step than the action of β-lactams or glycopeptides. FosA is a glutathione S-transferase that covalently modifies fosfomycin for inactivation. There are currently more than 10 *fos* types, and *fosA*, its subtypes, and *fosC2* are primarily found in the *Enterobacteriaceae* (Yang et al., 2017). In China, plasmid-encoded *fosA3* in *Escherichia coli* isolates from food and pets have been reported with high detection rates (Hou et al., 2012; Yang et al., 2014; Yao et al., 2016; Wang et al., 2017) although fosfomycin has not been approved for veterinary use. Interestingly, *fosA3* is often found co-localized with bla_{CTX-M} on epidemic plasmids and this most likely promotes the transfer and dissemination of *fosA3* in humans and animals (Yang et al., 2014; Feng et al., 2015). Co-spread of *fosA3* with other important antibiotic resistance genes (ARG) is concerning due to the potential to rapidly develop into MDR *Enterobacteriaceae* strains.

Additionally, *E. coli* strains carrying *fosA3* and $bla_{CTX-M-14}$ have been isolated from vegetables in Netherlands (Freitag et al., 2018), even more *mcr-1* gene in *E. coli* was identified in fresh vegetables in Guangzhou, China (Luo et al., 2017). Literature also have claimed that plants could be contaminated by manure and wastewater from animal farming, contributing to the widely spread of AMR (Zeng et al., 2019; Sun et al., 2020). As a kind of plant, flowers are closely related to human life and possible vectors for AMR genes transformation. However, few reports have focused on the significance of flowers as a pathway for AMR spread.

Therefore, we investigated drug-resistant bacteria/drug-resistant genes from flowers and retail environment including water and dust in florists in Guangzhou, China. There are relatively few reports of plasmid-borne *fosA3* in *Citrobacter. freundii*, a bacterium associated with opportunistic nosocomial infections of the respiratory and urinary tracts and blood (Feng et al., 2015; Li M. et al., 2018). Herein, we present the first report of the emergence of *fosA3* in *C. freundii* isolates from flowers and the retail environments. We further investigated the molecular epidemiology of *fosA3*-carrying *C. freundii* isolates and characterized the *fosA3*-bearing plasmids.

MATERIALS AND METHODS

Bacterial Strains and Detection of fos Genes Materials and Methods

A total of 270 samples were randomly collected from 3 flower markets and 6 flower shops in Guangzhou, China during March 2017. The samples included lily petals (n = 90), lily leaves (n = 90), dust (n = 45), and water (n = 45). Flowers and leaves were separately collected in sterile sealed bags. Water for watering flowers was collected in 50 mL centrifuge tubes. Dust samples were wiped with sterile cotton swabs in the surface dust from tables or floors (each 10-cm × 10-cm area) in retail shops, and then were rinsed in 2 mL sterile physiological saline solution. One petal and one leaf of each lily flowers were picked and washed with 10 mL of sterile saline solution. Then 100 μL of dust-resuspension, flower washed fluid and water samples were incubated in 4 mL drug-free LB broth for 12–16 h at 37°C and plated on MacConkey agar plates containing 256 μg/mL fosfomycin plus 25 μg/mL glucose-6-phosphate. After 18 h incubation at 37°C, 1–2 red colonies of different morphologies from each plate were selected. The bacterial species identification was performed using the MALDI-TOF MS (Shimadzu-Biotech, Japan) and 16S rRNA gene sequencing (16srRNA-F: AGAGTTTGATCATGGCTC; 16srRNA-R: GGTTACCTTGTTACGACTT). Fosfomycin-resistant *C. freundii* isolates (≥256 mg/L) were screened for the presence of the plasmid-mediated fosfomycin resistance genes *fosA1*, *fosA2*, *fosA3*, *fosA4*, *fosA5*, *fosA6*, *fosA7*, and *fosC2* by PCR amplification and sequencing using primers as previously described (Huang et al., 2020).

Antimicrobial Susceptibility Testing and Resistance Genes Detecting

All *fos*-positive isolates were screened for the minimum inhibitory concentration (MIC) using the Mueller Hinton (MH) agar dilution method (MH agar was purchased from Huankai Co., Ltd., Guangzhou, China) and the results were interpreted according to CLSI (Clinical and Laboratory Standards Institute [CLSI], 2018) and veterinary CLSI (Clinical and Laboratory Standards Institute [CLSI], 2019) guidelines. The following antimicrobials were tested: fosfomycin (FOS), cefotaxime (CTX), amoxicillin-clavulanate (AMC), meropenem (MEM), tetracycline (TET), chlortetracycline (CTET), doxycycline (DOX), ciprofloxacin (CIP), gentamicin (GEN), amikacin (AMK), sulfamethoxazole/trimethoprim (SXT), florfenicol (FFC), chloramphenicol (CHL), and rifampicin (RIF) (above drugs were purchased from Sigma Chemical Co., St Louis, MO, United States). MICs of tigecycline and colistin were determined by MH broth microdilution (MH broth was purchased from Huankai Co., Ltd., Guangzhou, China) and the resistance breakpoint was interpreted according to EUCAST criteria

(>2 μg/mL) and FDA criteria (≥8 μg/mL). *E. coli* ATCC 25922 was used as a quality control strain.

All *fos*-positive isolates were further screened for the presence of the extended spectrum β-lactamase (ESBL) gene $bla_{CTX-M-9G/1G}$, the plasmid-mediated AmpC β-lactamase gene bla_{CMY}, the plasmid-mediated quinolone resistance (PMQR) genes *qnrA*, *qnrB*, *qnrS*, *aac-(6′)-Ib-cr*, *qepA*, *oqxA* and *oqxB*), *floR* as well as 16S-RMTase genes (*armA*/*rmtB*) using PCR amplification as previously described (Phuc Nguyen et al., 2009; Fang et al., 2016; Liu et al., 2016). Notably, $bla_{CTX-M-9G}$-positive isolates were further tested by PCR amplification using previous primers of ESBL-encoding genes (IS*Ecp1*-F: CTATCCGTACAAGGGAGTGT; IS*903*-R: TTTCCACTCGCCTTCACC) and protocols to confirm the subtypes of ESBL-encoding genes and other genotypes. All PCR products were sent for sequencing and the DNA sequences were blasted with GenBank database[1].

Molecular Typing

Chromosomal DNA digested with *Xba*I restriction enzyme was used for PFGE (Gautom, 1997) to analyze the genetic relatedness of all isolates containing *fos*. PFGE patterns were analyzed with the Dice coefficient and the unweighted pair group method with average linkages (UPGMA) clustering method using BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium). PFGE types were defined with >90% similarity between clusters.

Transfer of fos Genes, Gene Location and Plasmid Replicon Typing

To determine the transferability of *fosA3* genes, isolates positive for *fosA3* were selected for conjugation experiments using the broth-mating method and streptomycin-resistant *E. coli* strain C600 (MIC > 2000 μg/mL) as the recipient. The donor and recipient strains were inoculated into 4 mL LB broth (Huankai Co., Ltd., Guangzhou, China) and shaken at 37°C for 4 h, then the donors and recipients were mixed in a 1:4 (100 and 400 μL) ratio in a 2 mL EP tubes and incubated at 37°C for 20 h. Transconjugants were selected on MacConkey agar plates supplemented with 2000 μg/mL streptomycin and 256 μg/mL fosfomycin (Yang et al., 2014). Antimicrobial susceptibility testing of the transconjugants and co-transfer of other resistance genes were determined as mentioned above. Incompatibility (Inc) groups were determined using PCR-based replicon typing (PBRT) (Carattoli et al., 2005).

The bacterial cell of the transconjugants were lysed with the ESP buffer (0.5 M EDTA, pH 9.0; 1% sodium lauroyl sarcosinate; 1 mg of proteinase K per mL) and then the bacterial DNA was embedded in the gel block. The S1-PFGE protocol is detailed in previous reports (Barton et al., 1995). The *fosA3* gene genomic locations were identified by linearization of plasmids from transconjugants using S1 nuclease followed by PFGE (Yang et al., 2014). Southern blotting was carried out from S1-PFGE gels using a digoxigenin-labelled probe specific for *fosA3*.

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

WGS Sequencing and Characterization of fosA3-Bearing Plasmids

Based on the results of plasmid analysis and PFGE typing, the total genomic DNA was extracted from two *C. freundii* strains S39 and H12-3-2 using a TIANamp Bacteria DNA Kit (Tiangen) and DNA libraries were constructed with 250-bp paired-end whole-genome sequencing using the Illumina HiSeq system (Illumina, San Diego, CA, United States) (Ma et al., 2020). The obtained paired-end Illumina reads were assembled *de novo* using SPAdes v3.6.2 (default parameters except –careful and –k 21,33,55,77,99,127) (Bankevich et al., 2012). In addition, to obtain long reads sequence, selected strains were further sequenced using Oxford Nanopore MinION flowcell R9.4 (Li R. et al., 2018). *De novo* hybrid assembly was performed using a combined Illumina HiSeq and Nanopore sequencing approach (Nextomics). Genome assembly was performed with Unicycler version 0.4.1 (Wick et al., 2017) using a combination of short and long reads, followed by error correction with Pilon version 1.12 (Walker et al., 2014). Gene prediction and annotation were performed using RAST (Overbeek et al., 2014)[2] and BLAST[3] and rechecked manually. Alignments with highly homologous sequences (with >90% coverage and >90% nucleotide identity) from the NCBI database and generation of plasmid maps were performed with BRIG (Alikhan et al., 2011) and Easyfig (Sullivan et al., 2011).

Nucleotide Sequence Accession Numbers

The representative *fosA3*-bearing genome sequences S39 and H12-1-2 were submitted to NCBI with the accession numbers CP045555 and CP045837, respectively, and *fosA3*-bearing plasmids sequences pS39-1 and pH12-1 with the accession numbers CP045556 and CP045838, respectively.

RESULTS

Antimicrobial Susceptibility and Prevalence of fosA3 Genes

We isolated 17 fosfomycin-resistant Enterobacteriaceae from flower retail environments including 11 *C. freundii* and 6 *E. coli* from our group of 270 samples. The 11 *C. freundii* isolates were from 2 flowers markets and 3 flowers shops and samples included petals (n = 7), leaves (n = 2), dust (n = 1), and water (n = 1). All 11 isolates possessed the *fosA3* gene and no other *fos* gene was detected by PCR. These 11 were all were concurrently resistant to fosfomycin, cefotaxim, amoxicillin-clavulanate, tetracycline, chlortetracycline, doxycycline, ciprofloxacin, gentamicin, sulfamethoxazole/trimethoprim, florfenicol, chloramphenicol, and rifampin (**Table 1**). In addition, most of these isolates were also resistant to amikacin with MICs > 256 μg/mL (n = 9). However, all *fosA3*-positive isolates were susceptible to meropenem and tigecycline.

[2]https://rast.nmpdr.org/

[3]https://blast.ncbi.nlm.nih.gov/Blast.cgi

TABLE 1 | Background information and characteristics of *fosA3*-positive *Citrobacter freundii*.

Strains[1]	Locations	Source	PFGE Typing	Resistance genes[2]	Resistance profiles[3]	Replicon type[4]	Plasmids size (kb)
H12-3-2	Flower market 1	Lily petals	I	*fosA3*, *$bla_{CTX-M-14}$*, *$bla_{CMY-122}$*, *floR*, *aac(6')-Ib-cr*, *rmtB*, *qnrB13*, *qnrS1*	FOS, CTX, AMC, TET, CETE, DOX, CIP, GEN, AMK, SXT, FFC, CHL, RFP	UT	~110
H41-7-2	Flower market 2	Lily petals	I	*fosA3*, *$bla_{CTX-M-14}$*, *bla_{CMY}*, *floR*, *aac(6')-Ib-cr*, *rmtB*, *qnrB*, *qnrS*	FOS, CTX, AMC, TET, CETE, DOX, CIP, GEN, AMK, SXT, FFC, CHL, RFP	UT	~110
HR5	Flower market1	Dust	I	*fosA3*, *$bla_{CTX-M-14}$*, *bla_{CMY}*, *floR*, *aac(6')-Ib-cr*, *rmtB*, *qnrB*, *qnrS*	FOS, CTX, AMC, TET, CETE, DOX, CIP, GEN, AMK, SXT, FFC, CHL, RFP	UT	~110
N21-1	Flower shop 1	Lily petals	I	*fosA3*, *$bla_{CTX-M-14}$*, *bla_{CMY}*, *floR*, *aac(6')-Ib-cr*, *rmtB*, *qnrB*, *qnrS*	FOS, CTX, AMC, TET, CETE, DOX, CIP, GEN, AMK, SXT, FFC, CHL, RFP	UT	~110
N52-2	Flower shop 2	Lily petals	I	*fosA3*, *$bla_{CTX-M-14}$*, *bla_{CMY}*, *floR*, *aac(6')-Ib-cr*, *rmtB*, *qnrB*, *qnrS*	FOS, CTX, AMC, TET, CETE, DOX, CIP, GEN, AMK, SXT, FFC, CHL, RFP	UT	~110
N89-1-1	Flower shop 3	Lily petals	I	*fosA3*, *$bla_{CTX-M-14}$*, *bla_{CMY}*, *floR*, *aac(6')-Ib-cr*, *rmtB*, *qnrB*, *qnrS*	FOS, CTX, AMC, TET, CETE, DOX, CIP, GEN, AMK, SXT, FFC, CHL, RFP	UT	~110
NW2-2	Flower shop 1	Water	I	*fosA3*, *$bla_{CTX-M-14}$*, *bla_{CMY}*, *floR*, *aac(6')-Ib-cr*, *rmtB*, *qnrB*, *qnrS*	FOS, CTX, AMC, TET, CETE, DOX, CIP, GEN, AMK, SXT, FFC, CHL, RFP	UT	~110
S66-1-1	Flower shop 3	Lily petals	I	*fosA3*, *$bla_{CTX-M-14}$*, *bla_{CMY}*, *floR*, *aac(6')-Ib-cr*, *rmtB*, *qnrB*, *qnrS*	FOS, CTX, AMC, TET, CETE, DOX, CIP, GEN, AMK, SXT, FFC, CHL, RFP	UT	~110
S40-1-2	Flower shop 2	Lily petals	I	*fosA3*, *$bla_{CTX-M-14}$*, *bla_{CMY}*, *floR*, *aac(6')-Ib-cr*, *rmtB*, *qnrB*, *qnrS*	FOS, CTX, AMC, TET, CETE, DOX, CIP, GEN, AMK, SXT, FFC, CHL, RFP	UT	~110
S39	Flower shop 2	Lily leaf	II	*fosA3*, *$bla_{CTX-M-14}$*, *bla_{CMY}*, *floR*, *aac(6')-Ib-cr*, *qnrB38*, *qnrB6*, *qnrS1*	FOS, CTX, AMC, TET, CETE, DOX, CIP, GEN, SXT, FFC, CHL, RFP	UT	~261
S45-1	Flower shop 3	Lily leaf	II	*fosA3*, *$bla_{CTX-M-14}$*, *bla_{CMY}*, *floR*, *aac(6')-Ib-cr*, *qnrB*, *qnrB*, *qnrS*	FOS, CTX, AMC, TET, CETE, DOX, CIP, GEN, SXT, FFC, CHL, RFP	UT	~261

[1]WGS sequence are underlined. [2]Resistance phenotypes transferred to the recipient by conjugation are underlined. FOS, fosfomycin; CTX, cefotaxime; AMC, amoxicillin-clavulanate; MEM, meropenem; TIG, tigecycline; TET, tetracycline; CTET, chlortetracycline; DOX, doxycycline; CIP, ciprofloxacin; GEN, gentamicin; AMK, amikacin; SXT, sulfamethoxazole/trimethoprim; FFC, florfenicol; CHL, chloramphenicol; RIF, rifampicin. [3]Transfer of fosA3 and co-transfer of this gene with other genotypes and phenotypes are underlined. [4]UT, Untypeable.

We further screened these 11 *fosA3*-positive *C. freundii* isolates for the presence of other important ARGs and identified numerous gene combinations. These included $bla_{CTX-M-14}$-bla_{CMY}-*rmtB-aac(6')-Ib-cr-qnrB-qnrS-floR* (n = 9) and $bla_{CTX-M-14}$-bla_{CMY}-*aac(6')-Ib-cr-qnrB-qnrB-qnrS-floR* (n = 2) (**Table 1**).

Pulsed-Field Gel Electrophoresis (PFGE) Typing

We successfully performed PFGE typing for all 11 *fosA3*-positive *C. freundii* isolates. We found two different PFGE patterns that retained >90% similarity and were designated types I and II. Type I predominated and contained nine *C. freundii* isolates recovered from Lily petals, dust and water from two markets and three flower shops (**Figure 1**).

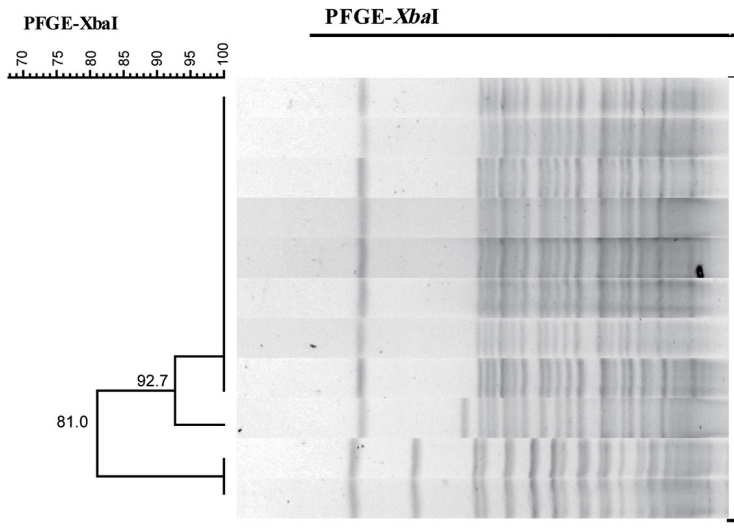
Location of *fosA3* Gene and Transferability of Plasmids Carrying *fosA3*

All 11 *fosA3*-positive isolates were able to mobilize and transfer *fosA3* to recipient strain *E. coli* C600. These transconjugants were highly resistant to FOS (MIC > 512 μg/mL) with high-level resistance to CTX (MICs ≥ 64 μg/mL). In addition, two transconjugants also showed high-level resistance to TET, CETE, GEN, SXT, CHL, and RFP (MICs ≥ 64 μg/mL) and their MICs to DOX and CIP were increased 16- and 32-fold, respectively, when compared with the recipient strain (**Table 1** and **Supplementary Table S1**). Correspondingly, $bla_{CTX-M-14}$ (n = 11), *aac(6')-Ib-cr* (2) and *qnrB* (n = 2) were co-transferred with *fosA3* in 11 donors (**Table 1**). Gene location and plasmid replicon typing demonstrated that the transferable *fosA3* gene coexisted with $bla_{CTX-M-14}$ (n = 9) and $bla_{CTX-M-14}$-*aac(6')-Ib-cr-qnrS* (n = 2) that were present on the untypable plasmids with sizes of 110 kb (n = 9) and 260 kb (n = 2), respectively (**Table 1** and **Supplementary Figure S1**).

fosA3-Carrying Plasmid Analysis

Based on the results of PFGE typing and plasmid analysis, we selected two representative *C. freundii* strains S39 and H12-3-2 for complete sequencing. Strain S39 contained one chromosome (4,806,164 bp) and 4 plasmids including one *fosA3*-carrying plasmid pS39-1. Strain H12-3-2 contained one chromosome (4,948,530 bp) and harbored 2 plasmids including a *fosA3*-carrying plasmid pH12-1. BWA (Li and Durbin, 2009) and Samtools (Li et al., 2009) software were used to obtain the sequence depth of each base, and the average sequencing depths of each nucleotide were calculated. The chromosome sequence of strain H12-1-2 was covered 96 × on average, and the chromosome sequence of strain S39 was covered 159 × on average. The plasmid sequence of pH12-1-1 was covered 106 × on average, and the plasmid sequence of pS39-1 was covered 90 × on average. In addition to the presence of *fosA3*, $bla_{CTX-M-14}$, bla_{CMY-65}, *aac(6')-Ib-cr, qnrB6, qnrS1* and *qnrB38*, strain S39 also harbored bla_{TEM-1B}, *catA2, floR, aac(3)-IIa, aadA16, aph(3")-Ib, aph(6)-Id, sul1, sul2, dfrA27, tet(A), tet(D)*, and *arr-3*. In addition to *fosA3*, $bla_{CTX-M-14}$, $bla_{CMY-122}$, *aac(6')-Ib-cr, qnrS1, qnrB13*, and *rmtB*, strain H12-3-2 also harbored bla_{OXA-1}, bla_{TEM-1B}, *catB3, floR, aadA2b, aph(3")-Ib, aph(6)-Id, sul1, sul2, dfrA12*, and *arr-3*. These ARGs were all located on plasmids except for the chromosomal *qnrB38* and bla_{CMY-65} in strain S39 and chromosomal *qnrB13 and* $bla_{CMY-122}$ in strain H12-3-2. Furthermore, ARG carriage was consistent with the resistance phenotypes for these strains (**Table 1**).

Plasmid pS39-1 was 261, 631 bp in length and included 332 coding sequences (CDS), an overall GC content of 49% and was untypable. The backbone sequence of pS39-1 was almost identical to the *fosA3*-bearing plasmids pMH17-012N_4 (Acc. No. AP018570) and pTEM-2262 (Acc. No. MG387191) (**Figure 2A**). These three plasmids contained 203 kb conserved backbone regions and the primary differences contained between



PFGE-*Xba*I

Strains	Locations	Source	PFGE Typing
H12-3-2	Flower market 1	Lily petals	I
H41-7-2	Flower market 2	Lily petals	I
HR5	Flower market1	Dust	I
N21-1	Flower shop 1	Lily petals	I
N52-2	Flower shop 2	Lily petals	I
N89-1-1	Flower shop 3	Lily petals	I
NW2-2	Flower shop 1	Water	I
S66-1-1	Flower shop 3	Lily petals	I
S40-1-2	Flower shop 2	Lily petals	I
S39	Flower shop 2	Lily leaf	II
S45-1	Flower shop 3	Lily leaf	II

FIGURE 1 | PFGE dendrogram showing the genetic relatedness of 13 *Citrobacter freundii* isolates including 2 *fosA3*-positive isolates.

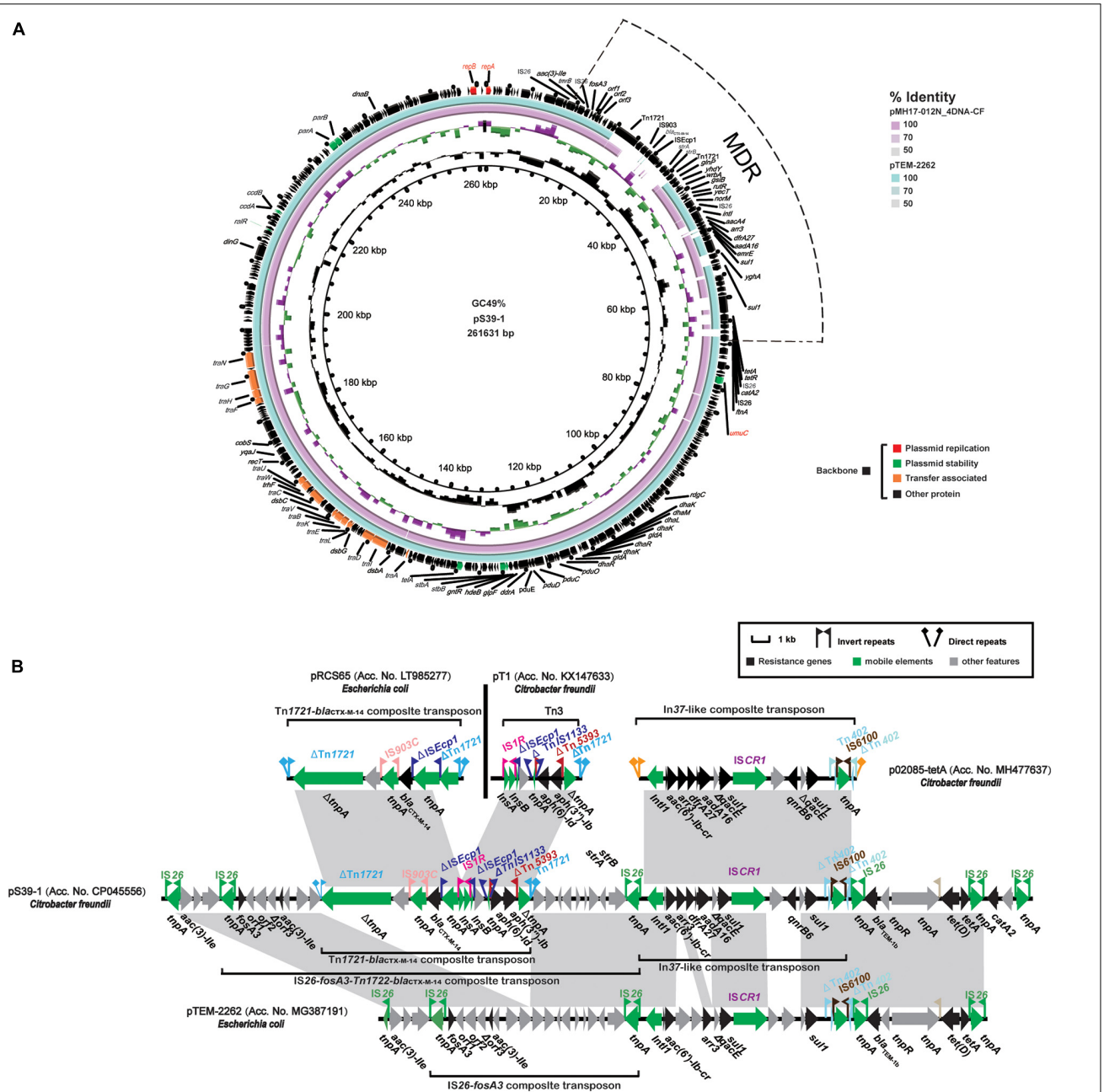


FIGURE 2 | Characteristics of the complete nucleotide sequences of the *fosA3* plasmid pS39-1 identified in this study. **(A)** A comparison of pS39-1 with two untypable *fosA3* plasmids pTEM-2262 (Acc. No. MG387191) and pMH17-012N_4DNA-CF (Acc. No. AP018570). **(B)** Comparison of the *fosA3*-containing MRR with the corresponding regions from pRCS65 (Acc. No. LT985277), pT1 (Acc. No. KX147633), p02085-tetA (Acc. No. MH477637), and pTEM-2262 (Acc. No. MG387191). Genes are denoted by arrows. Genes, mobile elements, and other features are colored based on functional classification. Shaded regions denote shared DNA homology (>95% nucleotide identity).

repA (plasmid replication) and *umuC* (plasmid stability) that included large multidrug-resistance region (MRR). The conserved backbone regions encoding functions for plasmid replication (*repA* and *repB*), stability (*parAB*, *stbAB*, *ccdAB*, *ralR*, *telA*, *umuC*, and *higA*) and transfer (15 *tra/trh* genes). Of note, *repA* (837 bp) and *repB* (1119 bp) located on plasmid pS39-1 belonged to unclassified replicons and were nearly identical to the corresponding *repA* and *repB* genes of plasmids pMH17-012N_4 and pTEM-2262 with 0 and 1 SNP differences for *repA* and *repB*, respectively.

In addition to the plasmid backbone region, plasmid pS39-1 possessed a 53.4 kb MDR containing 15 ARGs (*fosA3*, $bla_{CTX-M-14}$, bla_{TEM-1B}, *catA2*, *aac(3)-IIa*, *aadA16*, *aac(6')-Ib-cr*, *aph(3")-Ib*, *aph(6)-Id*, *sul1*, *qnrB6*, *dfrA27*, *tet(A)*, *tet(D)*,

and *arr-3*) interspersed with different complete or truncated insertion sequences and transposons (IS*26*, ΔTn*1721*, IS*903C*, ΔIS*Ecp1*, IS*1R*, IS*CR1*, ΔTn*402*, and IS*6100*) (**Figure 2A**). This 53.4 kb MDR was highly similar to that in *fosA3*-bearing plasmid pTEM-2262. The primary difference between the two were full and partial deletions of the Tn*1721-bla*$_{CTX-M-14}$-composite transposon (13,196 bp) and the In*37-like* composite transposon (12,959 bp), respectively. Of note, the Tn*1721-bla*$_{CTX-M-14}$-composite transposon on pS39-1 was composed of two parts that were almost identical to the Tn*1721-bla*$_{CTX-M-14}$-composite transposon (10,459 bp) on pRCS65 (Acc. No. LT985277) and the Tn*3* transposon (4569 bp) on pT1 (Acc. No. KX147633), respectively. Furthermore, the In*37-like* composite transposon of plasmid pS39-1 was highly similar to that in plasmid p02085-tetA (Acc. No. MH477637) (**Figure 2B**).

Plasmid pH12-1 was 110, 138 bp in length and contained 117 CDSs and an overall GC content of 51%. The plasmid was untypable although its replication gene *repA*(867 bp) was similar to orthologs of 261 bp on the IncFII plasmids pAMA1167-NDM-5 (Acc. No. CP024805) and pRSB107 (Acc. No. AJ851089) with a 69.7% coverage and 73.6% identity. Sequence comparisons demonstrated that the backbone region of plasmid p12-1 was highly similar to corresponding regions on plasmids pQnrS1-1502262 (Acc. No. CP031572) and p5-20710 (Acc. No. CP030079) and p112298-KPC (Acc. No. KP987215) except that pH12-1 possessed a 32.7 kb insertion between the *parB* (plasmid partition) and *umuD* (DNA polymerase V subunit) genes (**Figure 3A**). The conserved backbone regions in plasmid pH12-1 (91.4 kb) contained *repA*, *parABM*, *umuCD*, *stbD*, *staB*, and 16 *tra*/*trh* transfer gene modules. The 32.7 kb insertion region was primarily composed of hypothetical proteins except for the structure Δ*tnpA-pinE-acrR-yurZ-dsbA-frsA*-IS*4321R* (11.3 kb) and the resistance region containing *bla*$_{CTX-M-14}$ and *fosA3* (7.1 kb). The former was highly similar to that in pEC-IMP (Acc. No. EU855787) and the latter was highly similar to *fosA3-bla*$_{CTX-M-14}$-carrying plasmids pECM13 (Acc. No. KY865323) and pCTXM-2248 (Acc. No. MG836696) except for partial gene deletions (**Figure 3B**).

DISCUSSION

Fosfomycin plays a critical role against MDR and XDR Gram-negative pathogens and in particular, it commonly plays a synergistic role paired with β-lactams and aminoglycosides in treating urinary tract infections (Sastry and Doi, 2016). However, the occurrence and dissemination of plasmid-borne fosfomycin resistance genes, especially *fosA3*, has cast a shadow over the use of fosfomycin in clinics. In this study, we identified *fosA3*-carrying *C. freundi* isolates with a detection rate of 4.1% among 270 samples from flowers and the retail environments in Guangzhou, China. The prevalence of *fosA3* among *C. freundii* strains from flowers was relatively low when compared with *E. coli* isolated from food animals (8.8%, during 2009–2011; 10.5%, during 2015–2016) in China (Yang et al., 2014; Wang et al., 2017). However, our *fosA3* detection rate was consistent with the recent report that *fosA3* sporadically occurs in *C. freundii* and other *Enterobacteriaceae* including *Salmonella spp.*, *Proteus mirabilis,* and *Enterobacter fergusonii* from humans, food animals, pets, retail meat as well as wild birds (Lin and Chen, 2015; Villa et al., 2015; Wong et al., 2016; Yao et al., 2016; Fang et al., 2019). Perhaps of great concern is the emergence of the *fosA3* gene in different *Enterobacteriaceae* species from these diverse origins.

Plants including vegetables and flowers can be contaminated with ARGs *via* wastewater irrigation or manure application. Interestingly, an MDR *C. freundii* strain (WCHCF65) in sewage from a Chinese hospital carried multiple clinically significant ARGs including *bla*$_{CTX-M-12}$, *bla*$_{CTX-M-14}$, *bla*$_{SHV-12}$, *bla*$_{NDM-1}$ and *bla*$_{KPC-2}$ as well as *fosA3* (Wu et al., 2016). Additionally, *mcr-1* was present with *fosA3* and *bla*$_{CTX-M-14}$ in *Raoultella ornithinolytica* and *E. coli* isolates from retail vegetables in Guangzhou, China (Luo et al., 2017). MDR *E. coli* strains co-harboring *fosA3* and *bla*$_{CTX-M-14}$ also occurred in fresh vegetables in Netherlands (Freitag et al., 2018). These observations indicate that plants are potential ARG reservoirs including *fosA3*.

In the present study, we identified the presence of *fosA3* on flowers and the retail environments. These *fosA3*-positive *C. freundi* isolates exhibited resistance to most of the tested antibiotics including cefotaxime, ciprofloxacin, and amikacin in addition to fosfomycin. Consistently we found that *fosA3* coexisted with the ESBL *bla*$_{CTX-M-14}$, the pAmpCs *bla*$_{CMY-65}$ and *bla*$_{CMY-122}$, the PMQR genes *aac(6')-Ib-cr*, *qnrS1*, *qnrB13/qnrB6/qnrB38* as well as *rmtB*. Additionally, WGS analysis demonstrated that *bla*$_{CMY-65}$/*bla*$_{CMY-122}$ and *qnrB13/qnrB38* were located in the chromosome. This was consistent with the origin of plasmid-mediated *qnrB* and *bla*$_{CMY-2}$ -like genes from the chromosome of *Citrobacter spp.* (Verdet et al., 2009; Jacoby et al., 2011; Liao et al., 2015). Importantly, flowers contaminated with MDR bacteria will most likely come into direct contact with humans complicating the treatment and management of disease.

In this study, the *fosA3*-carrying *C. freundii* isolates were genetically related as judged by their PFGE profiles. In particular, we found an epidemic PFGE type that was composed of isolates from diverse origins across flower markets and shops. This indicated possible clonal dissemination of MDR *fosA3*-positive *C. freundii* isolates from flower markets and shops in a local region.

The spread of bacterial plasmids is an increasing global problem contributing to widespread ARG dissemination (San Millan, 2018). Several plasmid types are associated with the spread of *fosA3* and in particular, the epidemic IncF33:A-:B- and ST3-IncHI2 plasmids in *Enterobacteriaceae* from pets and food animals (Hou et al., 2012; Yang et al., 2014; Fang et al., 2019). However, we found that all our 11 *fosA3*-carrying plasmids including p12-1 and pS39-1 in *C. freundi* could not be assigned to any known *Enterobacteriaceae* incompatibility group. The single replicon gene *repA* possessed in plasmid p12-1 was highly similar to that in plasmid p112298-KPC where the *repA* was assigned to the IncFII RepA superfamily (Feng et al., 2015). Plasmid p12-1 showed a similar backbone region to the untypable

fosA3-bearing plasmids in *E. hormaechei* and *C. freundii* isolates from humans in China and the United States (Wang et al., 2018; **Figure 3**).

Interestingly, a similar scenario was also observed for the other untypable *fosA3*-bearing plasmid pS39-1. Linear genomic comparisons revealed that a conserved backbone, including the two unclassified replicons *repA* and *repB*, were identified between pS39-1 and another two untypable *fosA3*-bearing plasmids pTEM-2262 and pMH17-012N_4 in *C. freundii* isolates of pig and human origin from China (Li M. et al., 2018; Zhang et al., 2019; **Figure 2**). Noticeably, pTEM-2262 also shared a conserved backbone with another four untypable non-*fosA3*-bearing plasmids from different species including *C. freundii*, *Kosakonia radicincitans*, and *Citrobacter werkmanii* with origins including the environment, vegetables and humans (Zhou et al., 2017; Zurfluh et al., 2017; Becker et al., 2018; Barry et al., 2019). These indicated that the untypable and conjugal p12-1-like and pS39-1-like plasmids could act as vectors for *fosA3*

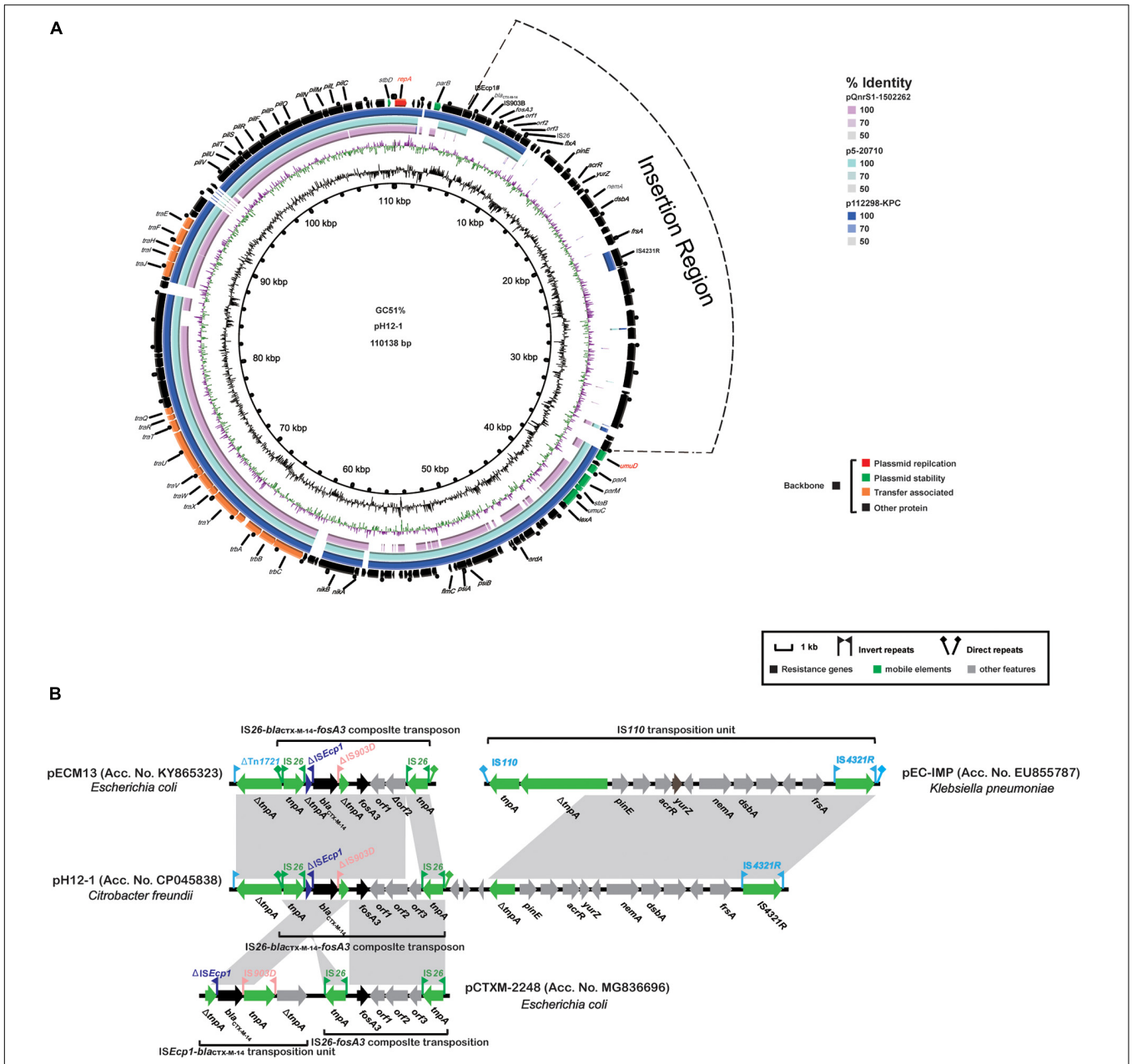


FIGURE 3 | Characteristics of the complete nucleotide sequences of *fosA3*-carrying pH12-1 identified in this study. **(A)** A comparison with two *fosA3*-carrying untypeable plasmids p112298-KPC (Acc. No. KP987215), pQnrS1-1502262 (Acc. No. CP031572), and p5-20710 (Acc. No. CP030079). **(B)** Comparison of the *fosA3*-containing MRR in pH12-1 with the corresponding regions of pECM13 (Acc. No. KY865323), pEC-IMP (Acc. No. EU855787), and pCTXM-2248 (Acc. No. MG836696). See **Figure 2** for annotation features.

transmission between different *Enterobacteriaceae* from different ecological niches.

In contrast to the conserved backbones, the *fosA3*-$bla_{CTX-M-14}$-containing MDR of these untypable plasmids from the GenBank were highly heterogeneous. This was primarily due to acquisition or deletion of resistance determinants mediated by mobile genetic elements and recombination. Plasmid pS39-1 possessed a large *fosA3*-containing MRR composed of 15 ARGs and diverse insertion sequences and transposons. Furthermore, the large *fosA3*-containing MRRs in plasmid pS39-1 partially resembled analogous regions from different plasmids indicating the MRR likely originated from the recombination of genetic contents from different plasmids as previously described (Hammerum et al., 2016; Li M. et al., 2018; Jing et al., 2019; Maherault et al., 2019). Unlike pS39-1, an additional insertion region mainly composed of hypothetical proteins was also integrated into the variable region of plasmid p12-1 in addition to the *fosA3*-$bla_{CTX-M-14}$-containing resistance region. Interestingly, heterogeneous *fosA3*-containing multidrug resistance regions have been also identified on the epidemic ST3-IncHI2 and F33:A-:B- plasmids with a conserved backbone in *Salmonella* (Yang et al., 2014). These data indicated that diverse and flexible transmission of *fosA3* was associated with heterogeneous MRRs and conserved backbones of a specific group of plasmids including ST3-IncHI2 and F33:A-:B- as well as untypable replicons in *Enterobacteriaceae*.

In conclusion, this study revealed the presence of *fosA3* that co-existed with $bla_{CTX-M-14}$ in MDR *C. freundii* isolates from flowers and the retail environments. Clonal expansion and horizontal transmission of untypable plasmids were involved in the spread of *fosA3* and $bla_{CTX-M-14}$ among these *C. freundi* isolates. To the best of our knowledge, this is the first report of the identification of *fosA3* in bacteria isolated from flower shops and markets. The *fosA3*- and $bla_{CTX-M-14}$-bearing untypable and transferable p12-1-like and pS39-1-like plasmids could circulate among diverse *Enterobacteriaceae* species from diverse origins, including plants and humans. Future studies are necessary to monitor the prevalence and transmission of these plasmids in *Enterobacteriaceae* species especially *C. freundi*, to better understand the potential threat to public health.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

Y-HL, JS, and X-PL designed the study. KC, L-XF, DW, BH, Q-WG, J-QL, and Z-XZ performed the experiments and collected the data. KC, L-XF, DW, BH, Q-WG, J-QL, Z-XZ, X-LL, YY, and X-RW analyzed and interpreted the data. KC wrote the draft of the manuscript. L-XF, Y-HL, JS, X-PL, and X-LL edited and revised the manuscript. Y-HL and JS coordinated the whole project. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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REFERENCES

Alikhan, N. F., Petty, N. K., Ben Zakour, N. L., and Beatson, S. A. (2011). BLAST ring image generator (BRIG): simple prokaryote genome comparisons. *BMC Genomics* 12:402. doi: 10.1186/1471-2164-12-402

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A., Dvorkin, M., Kulikov, A., et al. (2012). SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol.* 19, 455–477. doi: 10.1089/cmb.2012.0021

Barry, K. E., Wailan, A. M., Sheppard, A. E., Crook, D., Vegesana, K., Stoesser, N., et al. (2019). Don't overlook the little guy: an evaluation of the frequency of small plasmids co-conjugating with larger carbapenemase gene containing plasmids. *Plasmid* 103, 1–8. doi: 10.1016/j.plasmid.2019.03.005

Barton, B. M., Harding, G. P., and Zuccarelli, A. J. (1995). A general method for detecting and sizing large plasmids. *Anal. Biochem.* 226, 235–240. doi: 10.1006/abio.1995.1220

Becker, M., Patz, S., Becker, Y., Berger, B., Drungowski, M., Bunk, B., et al. (2018). Comparative genomics reveal a flagellar system, a type VI secretion system and plant growth-promoting gene clusters unique to the endophytic bacterium *Kosakonia radicincitans*. *Front. Microbiol.* 9:1997. doi: 10.3389/fmicb.2018.01997

Carattoli, A., Bertini, A., Villa, L., Falbo, V., Hopkins, K. L., and Threlfall, E. J. (2005). Identification of plasmids by PCR-based replicon typing. *J. Microbiol. Methods* 63, 219–228. doi: 10.1016/j.mimet.2005.03.018

Clinical and Laboratory Standards Institute [CLSI] (2018). *Performance Standards for Antimicrobial Susceptibility Testing*, 28th Edn. Wayne, PA: Clinical and Laboratory Standards Institute. ClSI Suppl. M100.

Clinical and Laboratory Standards Institute [CLSI] (2019). *Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacterial Isolated from Animals*, 5th Edn. Wayne, PA: Clinical and Laboratory Standards Institute. VET01Ed5E.

Diez-Aguilar, M., Morosini, M. I., Del Campo, R., Garcia-Castillo, M., Zamora, J., and Canton, R. (2013). In vitro activity of fosfomycin against a collection of clinical *Pseudomonas aeruginosa* isolates from 16 Spanish hospitals: establishing the validity of standard broth microdilution as susceptibility testing method. *Antimicrob. Agents Chemother.* 57, 5701–5703. doi: 10.1128/aac.00589-13

Falagas, M. E., Vouloumanou, E. K., Samonis, G., and Vardakas, K. Z. (2016). Fosfomycin. *Clin. Microbiol. Rev.* 29, 321–347.

Fang, L., Li, X., Li, L., Li, S., Liao, X., Sun, J., et al. (2016). Co-spread of metal and antibiotic resistance within ST3-IncHI2 plasmids from *E. coli* isolates of food-producing animals. *Sci. Rep.* 6:25312.

Fang, L. X., Deng, G. H., Jiang, Q., Cen, D. J., Yang, R. S., Feng, Y. Y., et al. (2019). Clonal expansion and horizontal transmission of epidemic F2:A1:B1 plasmids involved in co-spread of rmtB with qepA and bla$_{CTX-M-27}$ in extensively drug-resistant *Salmonella enterica* serovar indiana isolates. *J. Antimicrob. Chemother.* 74, 334–341. doi: 10.1093/jac/dky441

Feng, J., Qiu, Y., Yin, Z., Chen, W., Yang, H., Yang, W., et al. (2015). Coexistence of a novel KPC-2-encoding MDR plasmid and an NDM-1-encoding pNDM-HN380-like plasmid in a clinical isolate of *Citrobacter freundii*. *J. Antimicrob. Chemother.* 70, 2987–2991. doi: 10.1093/jac/dkv232

Freitag, C., Michael, G. B., Li, J., Kadlec, K., Wang, Y., Hassel, M., et al. (2018). Occurrence and characterisation of ESBL-encoding plasmids among *Escherichia coli* isolates from fresh vegetables. *Vet. Microbiol.* 219, 63–69. doi: 10.1016/j.vetmic.2018.03.028

Gautom, R. K. (1997). Rapid pulsed-field gel electrophoresis protocol for typing of *Escherichia coli* O157:H7 and other gram-negative organisms in 1 day. *J. Clin. Microbiol.* 35, 2977–2980. doi: 10.1128/jcm.35.11.2977-2980.1997

Hammerum, A. M., Hansen, F., Nielsen, H. L., Jakobsen, L., Stegger, M., Andersen, P. S., et al. (2016). Use of WGS data for investigation of a long-term NDM-1-producing *Citrobacter freundii* outbreak and secondary in vivo spread of bla$_{NDM-1}$ to *Escherichia coli*. *Klebsiella pneumoniae* and *Klebsiella oxytoca*. *J. Antimicrob. Chemother.* 71, 3117–3124.

Hou, J., Huang, X., Deng, Y., He, L., Yang, T., Zeng, Z., et al. (2012). Dissemination of the fosfomycin resistance gene fosA3 with CTX-M beta-lactamase genes and rmtB carried on IncFII plasmids among *Escherichia coli* isolates from pets in China. *Antimicrob. Agents Chemother.* 56, 2135–2138. doi: 10.1128/aac.05104-11

Huang, Y., Lin, Q., Zhou, Q., Lv, L., Wan, M., Gao, X., et al. (2020). Identification of fosA10, a novel plasmid-mediated fosfomycin resistance gene of *Klebsiella pneumoniae* origin, in *Escherichia coli*. *Infect. Drug Resist.* 13, 1273–1279. doi: 10.2147/idr.s251360

Jacoby, G. A., Griffin, C. M., and Hooper, D. C. (2011). *Citrobacter* spp. as a source of qnrB Alleles. *Antimicrob. Agents Chemother.* 55, 4979–4984. doi: 10.1128/aac.05187-11

Jing, Y., Jiang, X., Yin, Z., Hu, L., Zhang, Y., Yang, W., et al. (2019). Genomic diversification of IncR plasmids from China. *J. Glob. Antimicrob. Resist.* 19, 358–364. doi: 10.1016/j.jgar.2019.06.007

Li, H., and Durbin, R. (2009). Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* 25, 1754–1760. doi: 10.1093/bioinformatics/btp324

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

Li, M., Li, F., Mi, Z., Zhao, Y., Zhang, X., Jiang, Z., et al. (2018). Comparative genomics analysis of pTEM-2262, an MDR plasmid from *Citrobacter freundii*, harboring two unclassified replicons. *Future Microbiol.* 13, 1657–1668. doi: 10.2217/fmb-2018-0243

Li, R., Xie, M., Dong, N., Lin, D., Yang, X., Wong, M. H. Y., et al. (2018). Efficient generation of complete sequences of MDR-encoding plasmids by rapid assembly of MinION barcoding sequencing data. *Gigascience* 7, 1–9.

Liao, X., Fang, L., Li, L., Sun, J., Li, X., Chen, M., et al. (2015). Characterization of chromosomal qnrB and ampC alleles in *Citrobacter freundii* isolates from different origins. *Infect. Genet. Evol.* 35, 214–220. doi: 10.1016/j.meegid.2015.07.011

Lin, D., and Chen, S. (2015). First detection of conjugative plasmid-borne fosfomycin resistance gene fosA3 in *Salmonella* isolates of food origin. *Antimicrob. Agents Chemother.* 59, 1381–1383. doi: 10.1128/aac.04750-14

Liu, Y. Y., Wang, Y., Walsh, T. R., Yi, L. X., Zhang, R., Spencer, J., et al. (2016). Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infect. Dis.* 16, 161–168. doi: 10.1016/s1473-3099(15)00424-7

Luo, J., Yao, X., Lv, L., Doi, Y., Huang, X., Huang, S., et al. (2017). Emergence of mcr-1 in *Raoultella ornithinolytica* and *Escherichia coli* isolates from retail vegetables in China. *Antimicrob. Agents Chemother.* 61:e1139-17.

Ma, K., Feng, Y., Liu, L., Yao, Z., and Zong, Z. (2020). A Cluster of colistin- and carbapenem-resistant *Klebsiella pneumoniae* carrying bla$_{NDM-1}$ and mcr-8.2. *J. Infect. Dis.* 221, S237–S242.

Maherault, A. C., Kemble, H., Magnan, M., Gachet, B., Roche, D., Le Nagard, H., et al. (2019). Advantage of the F2:A1:B- IncF pandemic plasmid over IncC plasmids in in vitro acquisition and evolution of bla$_{CTX-M}$ gene-bearing plasmids in *Escherichia coli*. *Antimicrob. Agents Chemother.* 63, e1130-19.

Overbeek, R., Olson, R., Pusch, G. D., Olsen, G. J., Davis, J. J., Disz, T., et al. (2014). The SEED and the rapid annotation of microbial genomes using subsystems technology (RAST). *Nucleic Acids Res.* 42, D206–D214.

Phuc Nguyen, M. C., Woerther, P. L., Bouvet, M., Andremont, A., Leclercq, R., and Canu, A. (2009). *Escherichia coli* as reservoir for macrolide resistance genes. *Emerg. Infect. Dis.* 15, 1648–1650. doi: 10.3201/eid1510.090696

Popovic, M., Steinort, D., Pillai, S., and Joukhadar, C. (2010). Fosfomycin: an old, new friend? *Eur. J. Clin. Microbiol. Infect. Dis.* 29, 127–142. doi: 10.1007/s10096-009-0833-2

San Millan, A. (2018). Evolution of plasmid-mediated antibiotic resistance in the clinical context. *Trends Microbiol.* 26, 978–985. doi: 10.1016/j.tim.2018.06.007

Sastry, S., and Doi, Y. (2016). Fosfomycin: resurgence of an old companion. *J. Infect. Chemother.* 22, 273–280. doi: 10.1016/j.jiac.2016.01.010

Sullivan, M. J., Petty, N. K., and Beatson, S. A. (2011). Easyfig: a genome comparison visualizer. *Bioinformatics* 27, 1009–1010. doi: 10.1093/bioinformatics/btr039

Sun, J., Liao, X. P., D'souza, A. W., Boolchandani, M., Li, S. H., Cheng, K., et al. (2020). Environmental remodeling of human gut microbiota and antibiotic resistome in livestock farms. *Nat. Commun.* 11:1427.

Verdet, C., Gautier, V., Chachaty, E., Ronco, E., Hidri, N., Decre, D., et al. (2009). Genetic context of plasmid-carried bla$_{CMY-2}$-like genes in Enterobacteriaceae. *Antimicrob. Agents Chemother.* 53, 4002–4006. doi: 10.1128/aac.00753-08

Villa, L., Guerra, B., Schmoger, S., Fischer, J., Helmuth, R., Zong, Z., et al. (2015). IncA/C Plasmid Carrying bla$_{(NDM-1)}$, bla$_{(CMY-16)}$, and fosA3 in a *Salmonella enterica* serovar Corvallis strain isolated from a migratory wild bird in Germany. *Antimicrob. Agents Chemother.* 59, 6597–6600. doi: 10.1128/aac.00944-15

Walker, B. J., Abeel, T., Shea, T., Priest, M., Abouelliel, A., Sakthikumar, S., et al. (2014). Pilon: an integrated tool for comprehensive microbial variant detection and genome assembly improvement. *PLoS One* 9:e112963. doi: 10.1371/journal.pone.0112963

Wang, J., Yao, X., Luo, J., Lv, L., Zeng, Z., and Liu, J. H. (2018). Emergence of *Escherichia coli* co-producing *NDM-1* and *KPC-2* carbapenemases from a retail vegetable, China. *J. Antimicrob. Chemother.* 73, 252–254. doi: 10.1093/jac/dkx335

Wang, X. M., Dong, Z., Schwarz, S., Zhu, Y., Hua, X., Zhang, Y., et al. (2017). Plasmids of diverse inc groups disseminate the fosfomycin resistance gene fosa3 among *Escherichia coli* isolates from pigs, chickens, and dairy cows in Northeast China. *Antimicrob. Agents Chemother.* 61, e859-17.

World Health Organization [WHO] (2011). *Critically Important Antimicrobials for Human Medicine, 3rd Revision 2011. WHO Advisory Group on Integrated Surveillance of Antimicrobial Resistance (AGISAR)*. Geneva: WHO.

Wick, R. R., Judd, L. M., Gorrie, C. L., and Holt, K. E. (2017). Unicycler: resolving bacterial genome assemblies from short and long sequencing reads. *PLoS Comput. Biol.* 13:e1005595. doi: 10.1371/journal.pcbi.1005595

Wong, M. H., Chan, E. W., Xie, L., Li, R., and Chen, S. (2016). IncHI2 plasmids are the key vectors responsible for oqxab transmission among *Salmonella Species*. *Antimicrob. Agents Chemother.* 60, 6911–6915. doi: 10.1128/aac.01555-16

Wu, W., Espedido, B., Feng, Y., and Zong, Z. (2016). *Citrobacter freundii* carrying bla$_{KPC-2}$ and bla$_{NDM-1}$: characterization by whole genome sequencing. *Sci. Rep.* 6:30670.

Yang, T. Y., Lu, P. L., and Tseng, S. P. (2017). Update on fosfomycin-modified genes in Enterobacteriaceae. *J. Microbiol. Immunol. Infect.* 52, 9–21. doi: 10.1016/j.jmii.2017.10.006

Yang, X., Liu, W., Liu, Y., Wang, J., Lv, L., Chen, X., et al. (2014). F33: A-: B-, IncHI2/ST3, and IncI1/ST71 plasmids drive the dissemination of fosA3 and bla$_{CTX-M-55/-14/-65}$ in *Escherichia coli* from chickens in China. *Front. Microbiol.* 5:688. doi: 10.3389/fmicb.2014.00688

Yao, H., Wu, D., Lei, L., Shen, Z., Wang, Y., and Liao, K. (2016). The detection of fosfomycin resistance genes in Enterobacteriaceae from pets and their owners. *Vet. Microbiol.* 193, 67–71. doi: 10.1016/j.vetmic.2016.07.019

Zeng, J., Pan, Y., Yang, J., Hou, M., Zeng, Z., and Xiong, W. (2019). Metagenomic insights into the distribution of antibiotic resistome between the gut-associated environments and the pristine environments. *Environ. Int.* 126, 346–354. doi: 10.1016/j.envint.2019.02.052

Zhang, Y., Lei, C.-W., and Wang, H.-N. (2019). Identification of a novel conjugative plasmid carrying the multiresistance gene cfr in *Proteus vulgaris* isolated from swine origin in China. *Plasmid* 105:102440. doi: 10.1016/j.plasmid.2019.102440

Zhou, G., Peng, H., Wang, Y. S., Huang, X. M., Xie, X. B., and Shi, Q. S. (2017). Complete genome sequence of *Citrobacter werkmanii* strain BF-6 isolated from industrial putrefaction. *BMC Genomics* 18:765. doi: 10.1186/s12864-017-4157-9

Zurfluh, K., Stephan, R., Klumpp, J., Nuesch-Inderbinen, M., Hummerjohann, J., Bagutti, C., et al. (2017). Complete genome sequence of *Citrobacter freundii* 705SK3, an OXA-48-encoding wastewater isolate. *Genome Announc.* 5:e842-17.

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ORIGINAL RESEARCH
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Molecular Characterization of *Enterococcus* Isolates From Different Sources in Estonia Reveals Potential Transmission of Resistance Genes Among Different Reservoirs

Erki Aun[1], Veljo Kisand[2], Mailis Laht[2], Kaidi Telling[3], Piret Kalmus[4], Ülo Väli[5], Age Brauer[1], Maido Remm[1] and Tanel Tenson[2]*

[1] Department of Bioinformatics, Institute of Molecular and Cell Biology, University of Tartu, Tartu, Estonia, [2] Institute of Technology, University of Tartu, Tartu, Estonia, [3] Department of Microbiology, Institute of Biomedicine and Translational Medicine, University of Tartu, Tartu, Estonia, [4] Department of Clinical Veterinary Medicine, Institute of Veterinary Medicine and Animal Sciences, Estonian University of Life Sciences, Tartu, Estonia, [5] Institute of Agricultural and Environmental Sciences, Estonian University of Life Sciences, Tartu, Estonia

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***Correspondence:**
Erki Aun
erki.aun@ut.ee

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In this study, we aimed to characterize the population structure, drug resistance mechanisms, and virulence genes of *Enterococcus* isolates in Estonia. Sixty-one *Enterococcus faecalis* and 34 *Enterococcus faecium* isolates were collected between 2012 and 2014 across the country from various sites and sources, including farm animals and poultry (n = 53), humans (n = 12), environment (n = 24), and wild birds (n = 44). Clonal relationships of the strains were determined by whole-genome sequencing and analyzed by multi-locus sequence typing. We determined the presence of acquired antimicrobial resistance genes and 23S rRNA mutations, virulence genes, and also the plasmid or chromosomal origin of the genes using dedicated DNA sequence analysis tools available and/or homology search against an *ad hoc* compiled database of relevant sequences. Two *E. faecalis* isolates from human with *vanB* genes were highly resistant to vancomycin. Closely related *E. faecalis* strains were isolated from different host species. This indicates interspecies spread of strains and potential transfer of antibiotic resistance. Genomic context analysis of the resistance genes indicated frequent association with plasmids and mobile genetic elements. Resistance genes are often present in the identical genetic context in strains with diverse origins, suggesting the occurrence of transfer events.

Keywords: *E. faecium*, *E. faecalis*, antibiotic resistance, virulence factors, *van* genes, whole-genome sequencing, multi-locus sequence typing, phylogenetic analysis

Abbreviations: AMR, antimicrobial resistance; MLST, multi-locus sequence type; ST, sequence type; VRE, vancomycin-resistant *Enterococcus*.

INTRODUCTION

Enterococcus is a genus of Gram-positive bacteria, with 67 species, belonging to the lactic acid bacteria from the phylum Firmicutes (Arias and Murray, 2012; Parte, 2014). *Enterococcus* species are non-spore-forming facultative anaerobes tolerant to a wide range of environmental conditions (Bondi et al., 2020), which has enabled them to become widespread in nature especially as a part of the commensal flora of nearly all land animals, including mammals, birds, reptiles, and insects; but, they also occur in soil, plants, and aquatic ecosystems (Sadowy and Luczkiewicz, 2014; Guzman Prieto et al., 2016; Dubin and Pamer, 2017).

In humans, *Enterococcus faecium* and *Enterococcus faecalis* are the two most abundant *Enterococcus* commensals of the gastrointestinal and genitourinary tracts, the oral cavity, the vagina, and the skin. In addition to their commensal role, these two *Enterococcus* species have recently emerged as important human pathogens causing infectious diseases, including endocarditis and bacteremia. Their ability to withstand harsh environmental conditions and their high intrinsic resistance or tolerance to many antimicrobials accompanied by their ability to easily acquire high-level resistance to new antimicrobial agents *via* horizontal gene transfer have enabled them to survive and spread within hospital settings and become one of the leading causes of nosocomial infections (Kayser, 2003; Ørstavik, 2004; Heikens et al., 2007).

Human pathogenic *Enterococcus* poses a threat especially to immunocompromised patients (Kommineni et al., 2016). Due to a variety of intrinsic resistance mechanisms, the therapeutic options for *Enterococcus* infections are limited, and the described ease of acquisition of resistance genes from other bacteria makes the treatment even more challenging. The first-line choices for the treatment of *Enterococcus* infections are β-lactam (for example, ampicillin) and aminoglycoside antibiotics (for example, gentamicin, streptomycin), either separately or in synergetic bactericidal combination (Cetinkaya et al., 2000; Gagetti et al., 2019). Glycopeptide antibiotics like vancomycin (available in United States and Europe) and teicoplanin (available in Europe) are used as the second-line drugs for the treatment of infections caused by β-lactam-resistant *Enterococcus* or in the case of patients with serious β-lactam allergies (Levine, 2006; Arias and Murray, 2012; Richey et al., 2015). However, the vancomycin resistance in *Enterococci* also spreads rapidly, and at present, vancomycin-resistant *Enterococcus* (VRE) can be found all over the world, posing a serious threat to global health (McDonald et al., 1997; WHO, 2017). The last-resort antibiotics against enterococcal infections that cannot be treated with β-lactams or glycopeptides include, for example, oxazolidinones like tedizolid and linezolid, daptomycin, tigecycline, and a synergistic combination of streptogramin A and streptogramin B (Cetinkaya et al., 2000; Arias et al., 2010; Rybak and Roberts, 2015; Ahmed and Baptiste, 2018; Bender et al., 2018; Abbo et al., 2019).

In farm animals, *Enterococcus* infections are uncommon (Aphis., 2014), and they are rarely specifically targeted with antibiotics in these settings. However, as a normal part of their commensal intestinal microbiota, *Enterococcus* spp. are exposed to antibiotics administered to animals to treat or prevent infections caused by other bacteria or given in sub-therapeutic doses to achieve the growth-promoting effects (banned in the EU in 2006 and in the US in 2017 and currently allowed in Brazil and China) (Daniel et al., 2015; Roth et al., 2019; Ibrahim et al., 2020). Therefore, the use of antimicrobials in food animal production has been associated with the development of antimicrobial resistance (AMR) in *Enterococci* (Hayes et al., 2004; Gadde et al., 2018). The antimicrobial-resistant bacteria that have emerged and live in the animal production environment are observed to spread to humans *via* direct or indirect human–animal contact or *via* the consumption of or contact with animal products (Marshall and Levy, 2011; Daniel et al., 2015; Fan and Archbold, 2015). While the *E. faecium* isolates from human samples tend to be of different types than the *E. faecium* isolates from animal samples, the same types of *E. faecalis* isolates have been found in both humans and other animal species. This suggests that the antimicrobial-resistant *Enterococcus* strains may be capable of transmission from animals to humans (Hammerum, 2012). In addition to the possible risk of inter-host transmission, these bacteria harbor a pool of mobile genetic elements and may serve as a reservoir for acquisition of antibiotic resistance genes. Thereby, they could also contribute to the spread of resistance genes by distributing them among Gram-positive bacteria, including the possible transfer of resistance genes from animal-associated *Enterococcus* to human bacteria (Marshall and Levy, 2011; Radhouani et al., 2011).

The resistance genes developed in food animal commensal *Enterococcus* strains can also make their way over time into human pathogenic bacteria after entering into environment and ecosystems *via* manure application as fertilizer or through discharges from the wastewater treatment process (Marshall and Levy, 2011; Daniel et al., 2015). In the environment, the bacterial resistance may be transferred to wild animals living in close association with humans (Blanco et al., 2009; Jardine et al., 2012; Shobrak and Abo-Amer, 2014). Birds of prey, especially migratory raptors, travel long distances through different ecological niches and prey on synanthropic rodents and small birds in urban and rural environments. They potentially become host reservoirs of bacteria of the variety of animals on which they feed and may serve as important indicators of environmental contamination with antimicrobial resistance bacteria of different origins, including those from animal husbandry (Marrow et al., 2009; Radhouani et al., 2011; Stępień-Pyśniak et al., 2018).

In the current work, we isolate *Enterococci* from different host species and environments to characterize the circulating strains in terms of their antimicrobial and virulence profile and map the potential interspecies spread by performing multi-locus sequence typing (MLST) and phylogenetic analysis.

MATERIALS AND METHODS

Collection of Study Materials

The collection of samples was carried out between the years 2012 and 2014. The samples were collected from the environment, wild

birds, farm animals, and humans over the territory of Estonia (**Supplementary Table S1**).

The environmental samples ($n = 66$) were collected during 16 sampling campaigns that covered different seasons. Agriculture-related environmental habitat samples were taken at three farms as follows: (i) slurry and manure, (ii) soil from the fields receiving manure, and (iii) surface water from streams and rivers connected to the fields. City-related environmental samples were collected from a city of 100,000 inhabitants as follows: (i) wastewater treatment plant effluent, (ii) an effluent receiving stream, and (iii) the city environment, including a river inside the city and an artificial outdoor bathing lake connected to the river.

The animal samples were collected from farm animals including poultry, swine, and cattle as well as from wild-living birds, mostly raptors. The farm animal samples included samples from healthy swine and cattle and fecal samples from healthy poultry. The fecal samples from swine and cattle were collected in the course of the annual national *Salmonella* surveillance program carried out in Estonia in 2012–2014. The fecal samples from poultry were collected *post-mortem* in slaughterhouses during the national *Salmonella* surveillance program. All samples were sent to the National Veterinary and Food Laboratory for isolation and identification of *Enterococcus*. The samples from the raptors were collected from the nestlings using cloacal swabbing. Three raptor species foraging in an agricultural landscape were selected for our study: the goshawk *Accipiter gentilis* (feeds mainly on birds in the study area), lesser spotted eagle *Clanga pomarina* (a generalist hunting mostly small mammals), and common buzzard *Buteo* (a generalist with a wide spectrum of diet).

The human samples were collected from two major sources in 2012–2013. Firstly, isolates from the clinical samples of the patients of the largest Estonian hospitals were included. A second source of strains was the fecal samples of healthy volunteers (including pig farmers and dog owners; $n = 207$).

Isolation Procedures, Vancomycin Resistance Selection, and Testing

The selective cultivation of *Enterococcus* from the environmental samples was carried out according to standardized environmental monitoring methods for the detection and enumeration of major intestinal enterococci (International Organization for Standardization, 1998). The samples were cultured on selective 4-methylumbelliferyl-β-D-glucoside (MUD) microplates (Bio Rad MUD/SF Microplates for *Enterococcus* Test) at 44 ± 0.5°C for 36–72 h. Three positive wells from lowest or second lowest dilution on MUD were selected, and 100 μl per well was used as bacterial suspension for 10-fold dilutions (1:10–1:10–5) with NaCl (0.9%) solution. Then, 50 μl of dilution 1:10 was cultured on the selective VRE agar plates (Oxoid Brilliance VRE Agar) at 37°C for 24–48 h. For a control and total numbering of the *Enterococcus* in the selected well, 50 μl of dilutions 1:10–4 and 1:10–5 was plated on selective Slanetz and Bartley (SB) agar plates for 48 h at 42°C. Three typical colonies from each morphological subset on the VRE plates and one typical colony from SB agar were selected and plated on Luria–Bertani (LB) agar.

The selective cultivation of *Enterococcus* from the fecal samples of farm animals and poultry was carried out by incubating 1 g of feces at 37°C overnight in enrichment broth agar (6.5% NaCl brain heart infusion), and 10 μl of enrichment suspension was spread on Slanetz–Bartley agar and incubated for 48 h at 42°C. Up to four colonies with morphology typical of *Enterococcus* were sub-cultivated on blood agar. Colonies were identified by the following criteria: hemolysis on blood agar, aesculin hydrolysis on Edward's medium, growth in the presence of tellurite, and the ability to ferment mannitol, sorbitol, arabinose, and raffinose.

The selective cultivation of *Enterococcus* from the cloacal and fecal swab samples of wild birds was carried out by first shacking the swabs on room temperature in 100 ml QSR for 30 min. The solution was filtrated through 45 μm filters, which were then incubated on Slanetz and Bartley agar for 48 h at 42°C. The colonies grown on SB agar were plated with needle on VRE agar plates and incubated at 37°C for 24–48 h. Three typical colonies from each morphological subset on the VRE plates and one typical colony from SB agar were selected and plated on LB agar.

The isolation of *Enterococcus* from human clinical specimens was conducted using standard clinical laboratory methods, which were in accordance with the guidelines of the American Society of Microbiology. The selection of the *Enterococcus* isolates from the fecal samples of human volunteers was carried out by plating the samples on selective medium (BrillianceTM VRE Agar, Oxoid, Basingstoke, United Kingdom). The plates were incubated at 37°C for 24 h, and the negative plates were re-incubated for an additional 24 h. Two colonies per plate with morphology suggestive of *Enterococcus* were selected and confirmed at species level using matrix-assisted laser desorption ionization–time of flight mass spectrometry (Bruker Daltonics, Bremen, Germany).

The minimal inhibitory concentrations of vancomycin for all strains were detected using epsilometer test (Etest, bioMérieux, Marcy l'Etoile, France), and The European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints were used for the interpretation of the results (EUCAST, 2018).

Isolate stocks were made from a single colony (LB plates) of the overnight cultures (LB liquid media). For long-term storage, 15% glycerol stocks of the isolates were made after incubation and stored at −80°C. For DNA extraction and PCR analysis, bacterial pellets were made and stored at—20°C.

DNA Extraction, Genome Sequencing, and Assembly

DNA was extracted from single bacterial colonies grown on blood agar plates (human isolates) or isolate pellets (grown on LB; all other isolates) using the GuSCN-silica protocol (Boom et al., 1990) modified with bead beating (Telling et al., 2020).

Bacterial genomic DNA was quantified using Qubit® 2.0 Fluorometer (Invitrogen, Grand Island, United States) and 2200 TapeStation (Agilent Technologies, Santa Clara, United States). One nanogram of sample DNA was processed for the sequencing libraries using the Illumina Nextera XT sample preparation kit (Illumina, San Diego, United States) following the manufacturer's protocols. Libraries were validated by qPCR with Kapa Library

Quantification Kit (Kapa Biosystems, Woburn, United States) in order to optimize cluster generation. Ninety-six ssDNA Nextera XT libraries originating from 96 isolates were pooled and sequenced on one high-output lane of HiSeq2500 (Illumina, San Diego, United States), with paired-end, 150 bp reads. Demultiplexing was conducted using CASAVA 1.8.2. (Illumina, San Diego, United States), allowing one mismatch in the index reads. Thereafter, all Illumina reads were assembled *de novo* with the SPAdes genome assembler (ver 3.5.0) using MismatchCorrector (Bankevich et al., 2012).

Collection of the Reference Genome Set

Sixteen *E. faecalis* and 15 *E. faecium* reference genomes were obtained from RefSeq database (release 90) and included in the analysis for comparison. In selecting the reference genomes for comparison, we prioritized the genomes of strains isolated from nearby countries and/or wild birds, but a set of *Enterococcus* strains from other various sources and distant countries was included as well (for a more detailed description of the included reference strains, see **Supplementary Table S2**).

Species Identification, Multi-Locus Sequence Typing, and Population Structure Analysis

The final species identification was conducted from raw sequencing reads of our isolates using StrainSeeker software (Roosaare et al., 2017); this was followed by MLST and phylogenetic analysis to examine the relatedness of our isolates. The MLST type was determined *in silico* using the software MLST[1] which scans the contig files against traditional PubMLST typing schemes based on the sequence of seven house-keeping genes *gdh*, *gyd*, *pstS*, *gki*, *aroE*, *xpt*, and *yqiL* for *E. faecalis* and *atpA*, *ddl*, *gdh*, *purK*, *gyd*, *pstS*, and *adk* for *E. faecium* (Jolley et al., 2018).

The core-genome alignments of the *E. faecalis* and *E. faecium* isolates were constructed using the ParSNP tool (version 1.2) from the Harvest Suite software for fast multiple alignment of genomic sequences (Treangen et al., 2014). Thereafter, recombinant regions in the core genomes were identified using BRATNextGen software (Marttinen et al., 2012) and masked to create alignments, free of the potential confounding influence of homologous recombination, for phylogenetic analysis. These alignments were used as an input for RaxML-NG software (version 0.7.0 BETA) to reconstruct a maximum likelihood phylogenetic tree using GTR-GAMMA model with four rate categories (Kozlov et al., 2019).

Determining the Presence of AMR and Virulence Genes, Their Plasmid, or Chromosomal Location and Genomic Context

The presence of acquired and intrinsic resistance genes was determined using ResFinder 3.2 software and ResFinder database as of October 1, 2019 (Zankari et al., 2012). The search was conducted against all AMR classes in the database, with the ResFinder's minimum coverage cutoff raised from a default of 0.6 to 0.8 and the minimum identity percent cutoff raised from a default of 0.9 to 0.95.

As the *Enterococcus* resistance to last-resort antibiotic linezolid often results from point mutations in polyclonal chromosomal 23S rRNA gene, we used LRE-Finder software tool (Hasman et al., 2019), which is dedicated to detect these mutations and other linezolid resistance-associated genes [*optrA*, *cfr*, *cfr(B)*, and *poxtA*] on the sequencing raw reads of our isolates.

We searched the genomes of our isolates for the presence of the *E. faecalis* and *E. faecium* virulence factors associated in the literature with human infections. The *E. faecalis*-specific virulence genes included in our search were *ace*, *asa1*, *cylA*, $efaA_{fs}$, *espfs*, *gelE*, hyl*A*, and *hylB* (Singh et al., 1998, 2010; Vankerckhoven et al., 2004; Stępień-Pyśniak et al., 2019; Kiruthiga et al., 2020), and the *E. faecium*-specific virulence factors included in our search were *acm*, $efaA_{fm}$, *ecbA*, *espfm*, *hylEfm*, *ptsD*, *scm*, *sgrA*, *orf1481*, and IS*16* and four hospital variants of complete pili gene clusters (Freitas et al., 2018; Stępień-Pyśniak et al., 2019). The search was conducted by the alignment of the gene sequences against the genome assemblies using BLASTn (Altschul et al., 1997), with an identity threshold of 95% and gene coverage threshold per hit of 80% (for a more detailed description of the searched virulence genes, see **Supplementary Table S3**).

The plasmid or chromosomal origin of the detected virulence and antimicrobial resistance factors was determined by combining the results of the PlasmidFinder software tool with default parameters (Carattoli et al., 2014) and BLASTn homology search of the corresponding contigs against the *ad hoc* compiled database of plasmid sequences derived from NCBI RefSeq database as described in Roosaare et al. (2018). The BLAST search was conducted using identity threshold of 70% and plasmid coverage threshold per hit of 10%. The PlasmidFinder results were ignored if the hit for plasmid replicon was found in the complete chromosomal sequence.

The genomic regions containing multiple resistance genes were studied in more detail, and the organization of the genes in these regions was reconstructed by the prediction of gene and corresponding protein sequences in these regions using Prodigal software (Hyatt et al., 2010). The predicted genes were annotated by the comparison of the corresponding protein sequences to available annotated sequences in public databases using BLASTp (Altschul et al., 1997).

Analysis and Visualization

All analytic scripts were written in Bash[2] or Python3 ([3]RRID:SCR_008394) programming languages.

The constructed phylogenetic trees were visualized using Python's ETE 3 (Environment for Tree Exploration) toolkit (Huerta-Cepas et al., 2016). All the plots were created

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

[2]https://www.gnu.org/software/bash/

[3]https://www.python.org/

using Python's Pandas (McKinney, 2010) and Matplotlib (RRID:SCR_008624) (Hunter, 2007) libraries.

RESULTS

Recovery of Enterococcus Isolates

In total, 61 *E. faecalis* isolates were recovered from the collected samples and involved in our study. These included eight isolates from environmental sources (river = 3, manure = 5), six isolates from farm animals (*Bos taurus* = 4, *Sus scrofa* = 2), 17 isolates from poultry, 21 isolates from wild raptors (*Buteo buteo* = 5, *A. gentilis* = 7, *C. pomarina* = 9), and nine isolates from human samples (clinical = 6, healthy = 4). The distribution of the isolation sources is plotted in **Figure 1**.

For *E. faecium*, in total, 34 isolates were recovered from the collected samples, and these included four isolates from environmental sources (river = 3, manure = 1), three isolates from farm animals (*B. taurus* = 1, *S. scrofa* = 2), 23 isolates from poultry, one isolate from free living pigeon *Columba livia*, and three isolates from human samples (clinical = 2, healthy = 1). The distribution of the isolation sources is plotted in **Figure 1**.

The MLST Analysis of Enterococcus Isolates

The 61 *E.* faecalis isolates were resolved into 30 sequence types (STs), of which 18 were represented by a single isolate. The most abundant sequence type was ST49, with eight isolates from poultry (*Gallus gallus*), followed by ST936 (six isolates), ST287 (five isolates), and ST4 (four isolates) from wild raptors *A. gentilis*, *Buteo*, and *C. pomarina*, respectively. The *E. faecalis* isolates from hospitalized patients were of sequence types 16, 40, 49, and 774, while three of four colonizing isolates from healthy volunteers were of previously undescribed STs, and one was of sequence type 133. Novel *E. faecalis* sequence types (ST933–ST941 and ST943) were submitted to the PubMLST database (Jolley et al., 2018).

The 34 *E. faecium* isolates were resolved into 24 STs, of which 19 were represented by a single isolate. The most abundant ST was ST258, with six isolates from poultry (*Gallus gallus*). The only human-colonizing *E. faecium* isolate from healthy volunteers was of ST822, and two isolates from hospitalized patients were of ST117. We also discovered six novel *E. faecium* STs, which were submitted to the PubMLST database and assigned to the sequence types ST1634–ST1639.

For *E. faecalis*, ST287 was found in *A. gentilis* and *C. pomarina*, ST4 was found in *G. gallus*, *C. pomarina*, and *B. buteo*, ST49 was found in *G. gallus* and *H. sapiens*, and ST16 was found in *H. sapiens* and *B. taurus* and also in manure. In contrast, all *E. faecium* isolates in our dataset were found within the same host species or environmental origin.

Detection of Virulence Genes

The virulence genes *ace*, *asa1*, *cylA*, *efaA*, *gelE*, *hylA*, and *hylB* were detected in *E. faecalis* isolates, with the adhesin-like antigen encoding gene *efaA* found to be present in the chromosome of all isolates of this species. It was followed by gelatinase coccolysine gene *gelE*, which was present in 53 (87%), and hyaluronidase gene *hylB* present in 48 (79%) *E. faecalis* isolates. As expected, the aggregation substance encoding gene *asa1* was exclusively found to be located on a plasmid. A plasmid- or chromosome-located cytolysin encoding gene *cylA* was carried by a plasmid in 10 out of 12 (83%) of our samples harboring that gene. Most virulence genes (*asa1* or *ace* and *gelE*, *cylA*, *efaA*, *hylA*, and *hylB*) were detected in six *E. faecalis* strains isolated from wild raptors *A. gentilis* and *C. pomarina*. None of these genes was detected in *E. faecium* isolates.

In *E. faecium* isolates, we found virulence genes IS*16*, *ptsD*, *orf1481*, *efaAfm*, *acm*, *ecbA*, and *scm* and all four pili gene clusters.

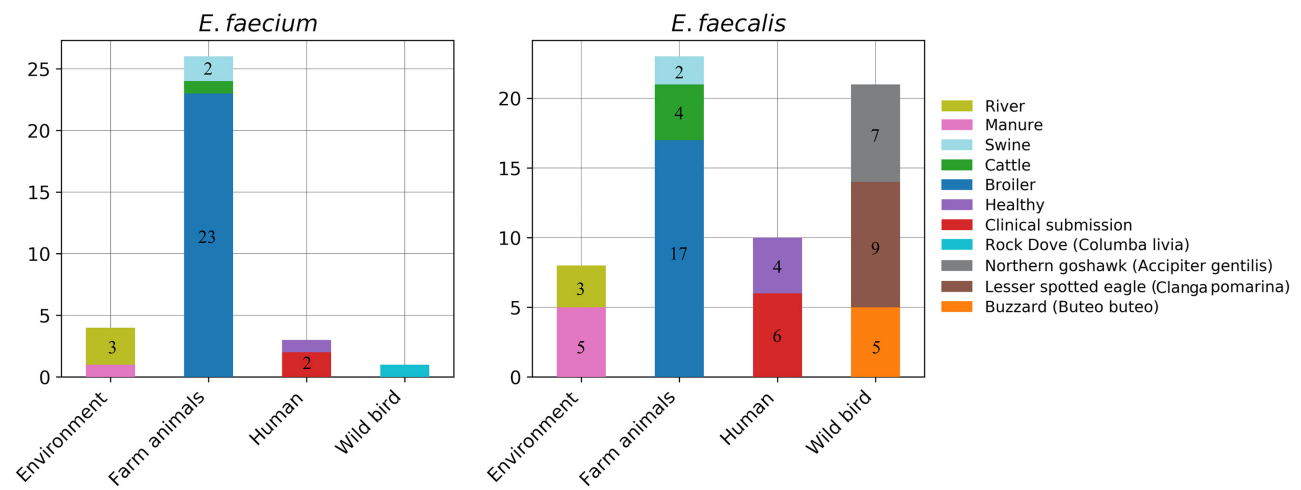

FIGURE 1 | Isolation sources and hosts of the collected *Enterococcus* strains.

None of these virulence factors were present in all strains, but the most frequent was collagen adhesin *acm*, which was present in 25 (74%) of our isolates. The pili gene cluster 1 was found in 18 (53%) of our isolates, and it was exclusively located on a plasmid, which is in agreement with previous reports by other investigators. Most virulence genes, IS*16*, *ptsD*, *orf1481*, *acm*, and *ecbA*, and all pili gene clusters were found in the two clinical *E. faecium* isolates HUM-574 and HUM-575. In our strains, the endocarditis-specific antigen *efaAfm* was found only in the plasmid of one environmental isolate ENV-120. The two *efaAfm*-harboring reference strains were also not of human origin.

The surface protein encoding genes *espfs* and *espfm* as well as *E. faecium* virulence genes *hylEfm* and *sgrA* were not found in any of our isolates.

Antibiotic Susceptibility of Enterococcus Isolates

Two of the *E. faecalis* and none of the E. *faecium* isolates were vancomycin resistant according to EUCAST breakpoints (MIC $\leq$ 4—sensitive, MIC > 4—resistant) (EUCAST, 2018). These two *E. faecalis* were highly resistant human isolates from sequence type 774. They carried *vanB* gene clusters and showed vancomycin MICs of 256 and 32 mg/L (**Figure 2**).

Detection of Antimicrobial Resistance Genes

Genes encoding proteins conferring resistance to aminoglycoside (*aac*, *aph*, *aac-aph*, *ant*, and *str*), phenicol (*cat*), trimethoprim (*dfrG*), macrolide (*erm* and *msr*), lincosamide (*erm*, *lnu*, and *lsa*), tetracyclin (*tet*), streptogramin A (*lsa*), streptogramin B (*erm* and *msr*), pleuromutilins (*lsa*), and vancomycin (*van*) antibiotics were found in our isolates. Macrolide and streptogramin B resistance-encoding *msr* genes were found exclusively in *E. faecium* isolates, and vancomycin resistance-encoding *van* genes were found exclusively in *E. faecalis* isolates of human origin, while other major AMR gene classes were detected in isolates of both species and different origins. In our *Enterococcus* isolates, the tetracycline resistance gene variants *tet(L)_2_M29725* and *tet(M)_10_EU182585* (ResFinder database IDs; Zankari et al., 2012) were found together exclusively in the isolates of animal husbandry origin. In contrast, all reference *E. faecium* strains, which harbored these two genes, were strains of human origin. None of the tetracycline resistance genes was detected in the isolates of wild birds. The resistance genes found in this study, together with the antibiotic classes they confer resistance to, and a description of the function of their products are listed in **Supplementary Table S4**.

Resistance Genes in *E. faecalis*

All of our *E. faecalis* isolates showed the presence of species-specific chromosomal gene *lsa(A)*, which is responsible for the intrinsic resistance to lincosamide (clindamycin and lincomycin), pleuromutilin, and streptogramin A (dalfopristin, pristinamycin II, and virginiamycin) antibiotics. Five out of 61 (8%) *E. faecalis* isolates also showed the presence of additional acquired *lsa(E)* gene, probably increasing these isolates' resistance to the mentioned antibiotics even further. Thirty-one (51%) *E. faecalis* isolates showed the presence of resistance genes against tetracycline antibiotics *tetL*, *tetM*, or *tetO*. Tetracycline resistance genes were often found together with erythromycin-resistant methylase encoding *erm(B)* genes (17 isolates, 28%), aminoglycoside resistance genes (15 isolates, 24%), dihydrofolate reductase encoding *dfrG* genes (nine isolates, 15%), chloramphenicol acetyltransferase encoding *cat* genes (five isolates, 8%), and lincosamide nucleotidyl transferase encoding *lnu* genes (five isolates, 8%). Two human isolates showed the presence of *van* gene cluster conferring resistance to vancomycin, which is one of the most important antibiotics against *Enterococcus*.

In total, acquired resistance genes were found from 32 (53%) of our *E. faecalis* isolates. One isolate had a single *erm(B)* gene, and 11 isolates had acquired resistance genes only for tetracycline. Other 20 isolates possessed resistance genes for more than one class of antibiotics, of which 11 isolates harbored resistance genes from four or more AMR gene classes, potentially conferring them resistance for up to seven different antibiotic classes. These highly multi-resistant isolates of *E. faecalis* originated from humans (five), manure (four), and livestock (three). Although we detected two VRE isolates, we did not detect the resistance genes against ampicillin or linezolid, which are other clinically important antibiotics used to treat *Enterococcus* infections. The distribution of resistance genes in our *E. faecalis* isolates is shown in **Figure 2**.

Resistance Genes in *E. faecium*

All of our *E. faecium* isolates showed the presence of chromosomal *msrC* gene associated with macrolide–streptogramin B resistance and *aac(6')-Ii_1_L12710* gene associated with the intrinsic low-level resistance to aminoglycosides of this species. Thirteen out of 34 (38%) *E. faecium* isolates also showed the presence of additional acquired aminoglycoside resistance genes, possibly contributing to the higher resistance to aminoglycosides of these strains. The acquired aminoglycoside resistance genes were often found together with erythromycin-resistant methylase encoding *erm* genes (nine isolates, 27%), lincosamide nucleotidyl transferase encoding *lnu* genes (nine isolates, 27%), lincosamide, pleuromutilins, and streptogramin A resistance-associated *lsa* gene (six isolates, 18%), and trimethoprim-resistant dihydrofolate reductase encoding *dfrG* gene (six isolates, 18%). None of the *dfrG*, *erm*, *lnu*, or *lsa* genes was found in isolates without acquired aminoglycoside resistance genes. In addition, four (12%) isolates showed the presence of chloramphenicol acetyltransferase-encoding *cat* genes, and 10 isolates (29%) showed the presence of tetracycline resistance genes *tetL* or *tetM*.

In total, acquired resistance genes were found in 17 (50%) of our *E. faecium* isolates. Fifteen of our isolates possessed acquired resistance genes for more than one class of antibiotics, with nine isolates possessing acquired resistance genes from four or more AMR gene classes, potentially conferring them resistance for up to seven different antibiotic classes. These highly multi-resistant isolates of *E. faecium* originated from poultry (five), livestock (one), manure (one), and humans (two). However, we did not detect the resistance genes or mutations against vancomycin,

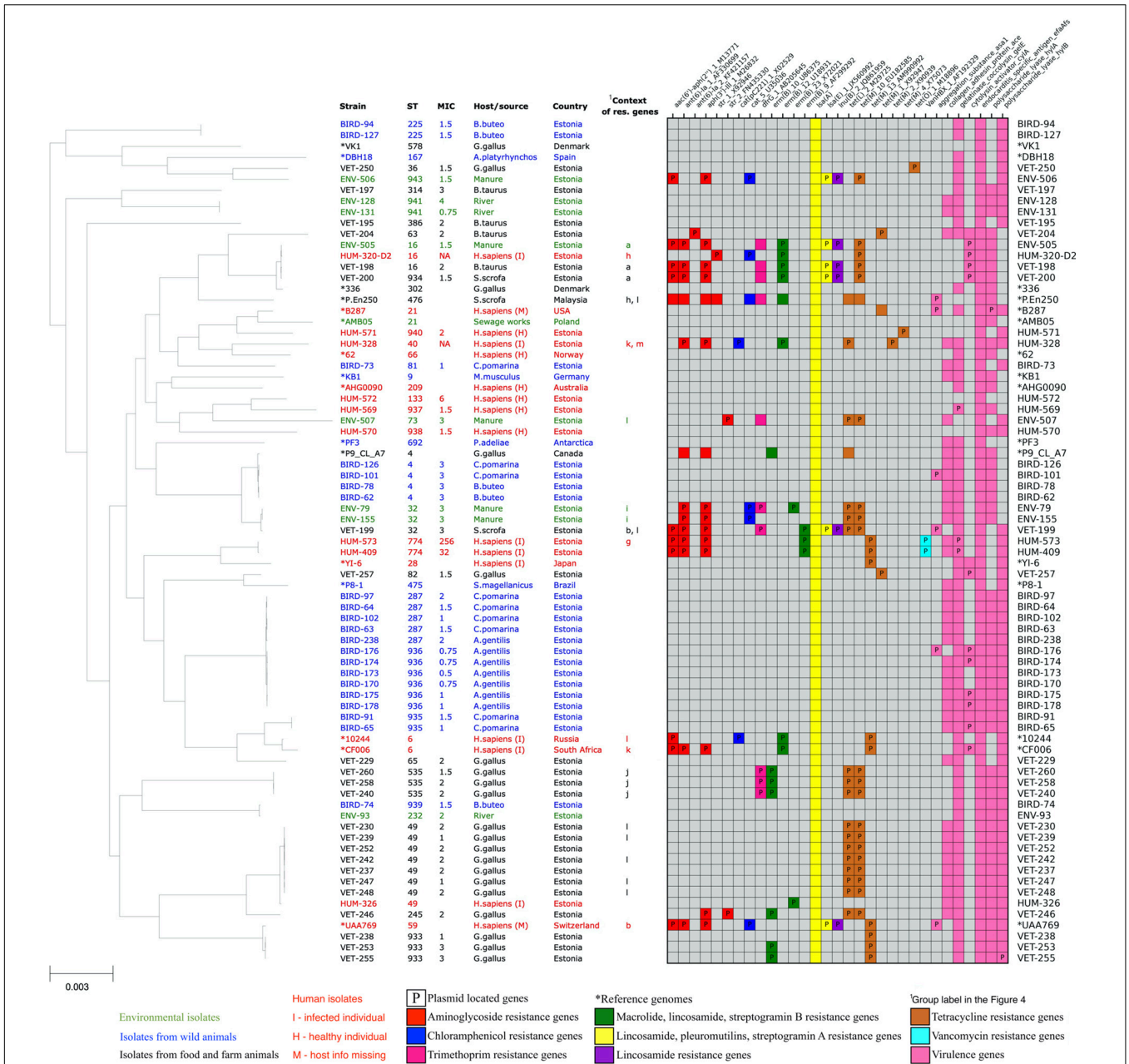

FIGURE 2 | Core-genome phylogenetic tree of our collected 61 *Enterococcus faecium* isolates and 16 reference genomes with the distribution matrix of the antimicrobial resistance and virulence genes detected in their whole-genome sequences.

ampicillin, or linezolid, which are the clinically most important antibiotics against *Enterococcus* infections. The distribution of resistance genes in our *E. faecium* isolates is shown in **Figure 3**.

Genomic Context of the Resistance Genes

The genomic contexts of the resistance genes in the antibiotic resistance islands were reconstructed and schematically illustrated in **Figure 4**. This analysis confirmed the plasmid origin of these genes, as many of them were flanked by plasmid-related genes, for example, plasmid recombination enzyme encoding genes next to tetracycline resistance genes (regions e, f, and l in **Figure 4**) and between chloramphenicol and streptomycin resistance genes (region h in **Figure 4**). The studied resistance genes were also often flanked by transposon-specific genes. We found the transposase genes in regions a, b, d, g, h, and j and the transposon protein TcpC-encoding genes in regions i and l in **Figure 4**. The resistance genes were found in the identical genetic context in the strains of different isolation sources or geographical origins (comparison strains). Furthermore, some

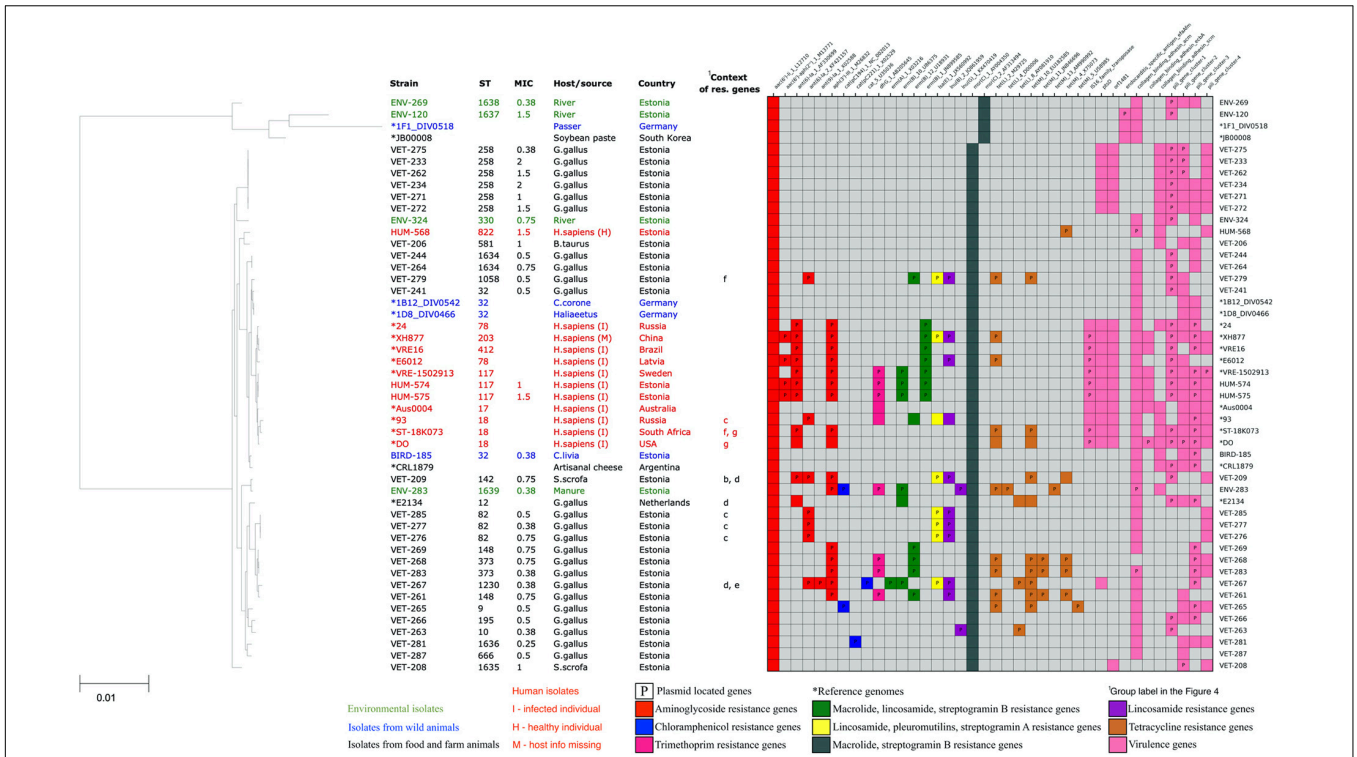

FIGURE 3 | Core-genome phylogenetic tree of our collected 34 *Enterococcus faecium* isolates and 16 reference genomes with the distribution matrix of the antimicrobial resistance and virulence genes detected in their whole-genome sequences.

resistance genes were found in the same genetic context in *E. faecium* and *E. faecalis* isolates. These findings suggest that plasmids and transposons play an important role in the dissemination of resistance genes from and to *Enterococcus*. This genetic context analysis also reveals that the tetracycline resistance genes *tet(L)_2_M29725* and *tet(M)_10_EU182585*, which in our isolated strains are exclusively associated with animal husbandry origin, are adjacent genes included into a leader peptide-controlled tetracycline resistance cluster.

The genomic context analysis also revealed that the aminoglycoside resistance genes *ant(6)-Ia_1_AF330699*, *aph(3')-III_1_M26832*, lincosamide resistance gene *lnu(B)_2_JQ861959*, and lincosamide, pleuromutilins, and streptogramin A resistance gene *lsa(E)_1_JX560992* are located in close proximity in a region named *lsa(E)*-carrying multiresistance gene cluster (Si et al., 2015). This multiresistance gene cluster was present in three strains, which were isolated from different animal husbandry-related sources (manure, *B. taurus*, *S. scrofa*) but were phylogenetically similar and clustered into one branch in the phylogenetic tree. The fourth strain from this branch was isolated from a tracheal aspirate of a person with pneumonia, and this strain was missing this multiresistance gene cluster.

DISCUSSION

Both *E. faecalis* and *E. faecium* are known to colonize different animal species. In our dataset, we have *E. faecalis* sequence types colonizing different species. ST287 was shared between different raptor species. ST4 was shared between broiler and wild bird samples (*C. pomarina* and *B. buteo*). ST16 was shared by humans and cattle, and ST49 was isolated from human and broiler samples, which has been also described by other researchers (Braga et al., 2018). In contrast, all *E. faecium* strains in our dataset were found within the same host species or environmental origin. This is in accordance with previous findings that *E. faecalis* strains of the same sequence type can be found in humans as well as in other animal species, while *E. faecium* strains tend to be more host specific (Hammerum, 2012).

Two of our *E. faecium* strains isolated from hospitalized patients had the multi-resistance sequence type 117 associated with nosocomial outbreaks in multiple European countries (Tedim et al., 2017). Although multi-resistant, we did not find our ST117 strains to possess vancomycin resistance genes as confirmed by vancomycin susceptibility testing, which is in contrast to many other reports (Papagiannitsis et al., 2017; Falgenhauer et al., 2019; Olearo et al., 2021). Our only human-colonizing *E.* faecium strain isolated from healthy patients was of sequence type 822, and it did not show the presence of any acquired resistance genes, although strains of the same sequence type are described as multi-resistant, including resistance to vancomycin, by other researchers (Castro-Nallar et al., 2017; Zangue, 2017).

For *E. faecalis*, we had two vancomycin-resistant isolates obtained in 2013 from the wound discharges of hospitalized

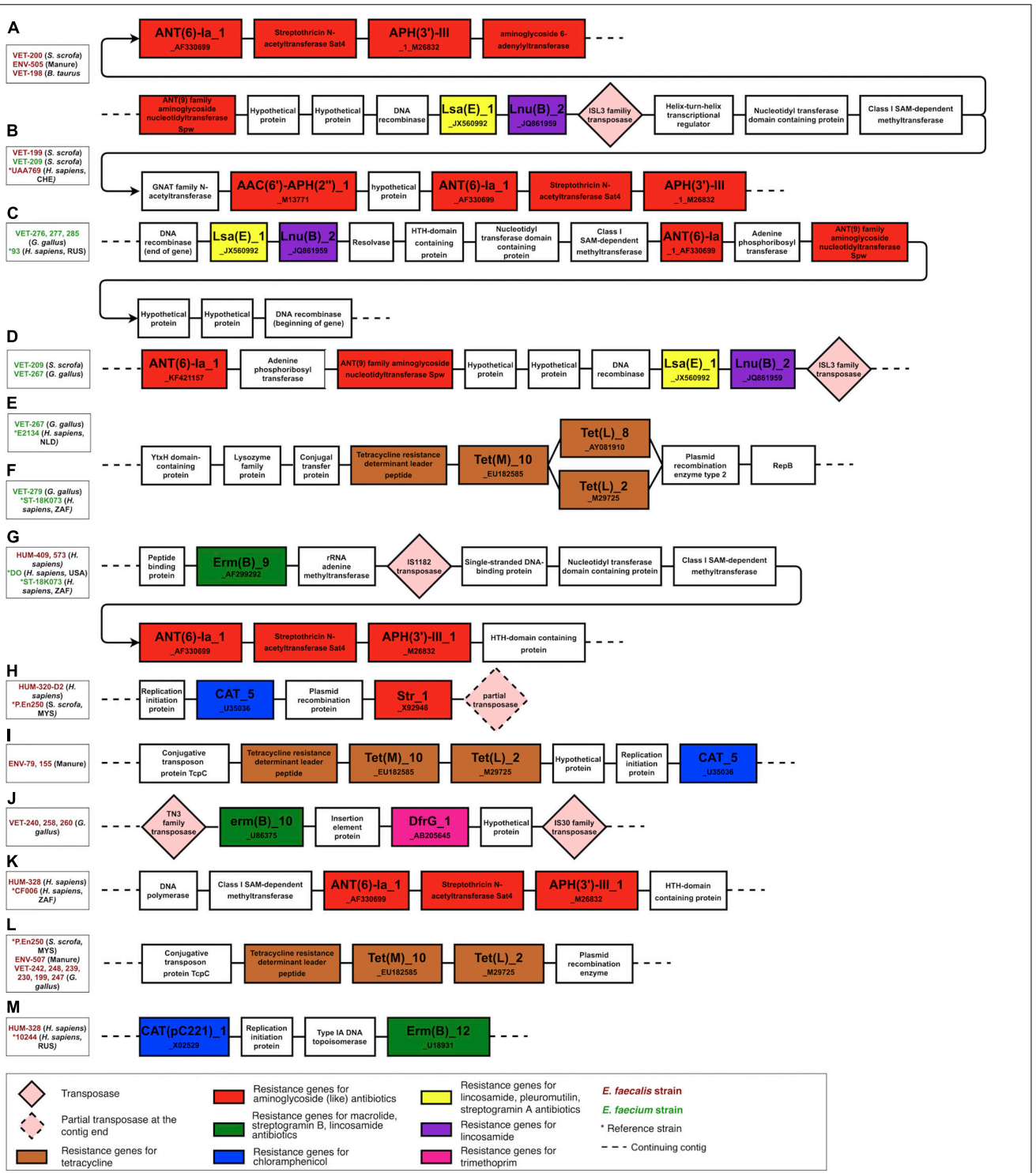

FIGURE 4 | The genomic context of the resistance genes in the antibiotic resistance islands. Most of the clustered resistance genes were found in the same genetic context in strains of different origins and were flanked by mobile genetic elements and plasmid-related genes. The figure illustrates only the context and is not scaled to the actual length of the genes and intergenic sequences. **(A–D)** a *lsa(E)*, *lnu(B)* and aminoglycoside resistance genes harboring genomic islands found in husbandry and human related strains, **(E,F,L)** a tetracycline resistance gene cluster variants found in human and husbandry related strains, **(G)** a *erm(B)* and aminoglycoside resistance genes harboring genomic in human related strains, **(H)** a *cat_5* and *str_1* genes harboring genomic island in human and husbandry related strains, **(I)** a tetracycline resistance cluster and *cat_5* gene harboring genomic island found in husbandry related strains, **(J)** a *erm(B)* and *dfr(G)* genes harboring genomic island found in poultry related strains, **(K)** an aminoglycoside resistance genes harboring genomic island found in human related strains, **(M)** *cat(pc221)_1* and *erm(B)* genes harbouring genomic island found in human related strains.

patients. These isolates belonged to ST774, which is assigned to uropathogenic strains by other researchers (Elena Aleksandrovna et al., 2019; Strateva et al., 2019). Strateva et al. describe in their article the *vanA* gene cluster carrying vancomycin-resistant *E. faecalis* strains of ST774 in Bulgaria in 2015. In contrast, our strains of ST774 were vancomycin resistant due to the presence of *vanB* genes. Other *E. faecalis* strains from hospitalized patients belonged to the sequence types ST16, ST40, and ST49, which are described as widespread STs obtained from different sources and conditions, including human clinical infections, healthy volunteers as well as animals and environment (Ruiz-Garbajosa et al., 2006; Zischka et al., 2015; Braga et al., 2018). ST133 was the only previously described ST of our *E. faecalis* isolates from healthy volunteers. In this isolate (HUM-572), we detected no acquired resistance genes and only one of the searched virulence gene. The strain belonging to ST133 has also been shown as a gut colonizer of healthy individuals in another study (Moles et al., 2015).

It has been described that the virulence of *Enterococcus* is associated with the presence of certain virulence genes. We have analyzed the genomes for eight virulence genes described mainly in *E. faecalis* and 14 virulence genes or gene clusters specific to *E. faecium*. In *E. faecalis*, we did not observe a clear enrichment of the virulence genes in human-derived strains. We instead found the highest number of virulence genes in strains isolated from wild raptors *A. gentilis* and *C. pomarina.* In contrast, in *E. faecium*, we observed evident enrichment of virulence genes in human clinical isolates as compared to human commensal or non-human isolates. However, all of our isolates carried at least one virulence gene, with *E. faecalis asa1* carried only by two raptor- and one swine-derived strain and *E. faecium efaAfm* carried only by one environmental isolate. With that in mind, we can only speculate if these non-human virulence gene-carrying strains can contribute to the virulence of human pathogenic strains when coming into contact with them or if they are able to cause diseases themselves when transferred to humans, making wild and domesticated birds potential reservoirs for zoonotic outbreaks.

Several antibiotic resistance genes were detected. As described previously, *lsa(A)*, causing resistance to lincosamides, pleuromutilines, and streptogramin A, was present in all strains of *E. faecalis*. All *E. faecium* isolates had a chromosomal *msrC* gene associated with macrolide–streptogramin B resistance and *aac(6')-Ii_1_L12710* gene associated with the intrinsic low-level resistance to aminoglycosides. These genes contribute to the intrinsic antibiotic resistance of *Enterococcus*. Moreover, 53% of *E. faecalis* strains and 50% of *E. faecium* strains have genes for acquired antibiotic resistance. Most strains with acquired resistance genes contain multiple genes predicted to give resistance to several antibiotics. As the acquired resistance genes are often located on transposons and/or plasmids, it is expected that the resistance can be transferred between different strains. This is expected to contribute to the constantly increasing resistance levels in *Enterococcus* (Manson et al., 2010; Palmer et al., 2010).

The expected transmission of resistance genes by mobile genetic elements is in accordance with our finding that the resistance genes are often present in the identical genetic context in strains with diverse origins. Furthermore, most of the detected resistance genes were found in strains isolated from different hosts or environmental sources, with the exception of the tetracycline resistance gene cluster with *tet(M)* and *tet(L)* genes, which was found exclusively in our strains of animal husbandry origin. Nevertheless, the presence of this tetracycline gene cluster in human comparison strains from other countries indicates a potential for transmission of this gene cluster to human strains, where it could pose additional health risks related to antimicrobial resistance. The absence of *lsa(E)*-carrying multi-resistance gene cluster (**Figure 4**) in the strain of human origin but its presence in other phylogenetically similar strains of animal husbandry origin suggests a possible cluster loss event in this human-related strain isolated from the tracheal aspirate of a person with pneumonia. None of the strains isolated from wild birds harbored acquired resistance genes, which indicates that the environmental contamination with antibiotics and AMR genes is low in Estonia and that neither wild raptors nor their prey is coming into contact with antimicrobials or bacteria capable of transferring resistance genes.

Vancomycin is one of the critical antibiotics against infections caused by multi-resistant *Enterococcus*. This resistance is caused by *van* genes. Complete *vanB* gene clusters were found from two (3%) of *E. faecalis* strains. As expected, these two strains containing the *vanB* gene cluster were resistant to vancomycin. During the sample collection period, the prevalence of VRE in Estonia was low. As the resistance levels are increasing globally, continuous monitoring and research is needed for mapping the spread and potentially designing containment measures.

CONCLUSION

Our study has shown the widespread prevalence of acquired resistance genes among *Enterococcus* strains exposed to anthropogenic antibiotic pressure. Additionally, the study has also shown that *E. faecalis* strains colonizing different farm animal species and humans could be closely related and contain many potentially mobile antibiotic resistance genes that can contribute to the spread of resistance among different reservoirs. However, no mobile resistance genes were found in *Enterococcus* from free-living birds, which suggests that areas with lower contamination with antibiotics or AMR genes still exist and that the spread of antibiotic resistance to wildlife can be prevented or postponed. The restricted use of antibiotics in animal husbandry and the elimination of potential resistance transmission routes are essential to maintain the currently low prevalence of resistance genes in wildlife in Estonia.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA630475, PRJNA630475.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Research Ethics Committee of the University of Tartu. Written informed consent to participate in this study was provided by the participants and/or their legal guardian/next of kin for minors. For the animal studies, neither written informed consents from the owners nor the ethical review and approval were obtained or required, as collection of fecal samples is not considered an animal experiment according to Estonian law. Also, the analyzed animal fecal samples were collected earlier in the course of the national *Salmonella* surveillance programme and were already present in laboratory.

AUTHOR CONTRIBUTIONS

EA contributed to formal analysis, manuscript writing, and preparation. VK contributed to data curation, resources, manuscript writing, and review. ML and PK contributed to resources, manuscript writing, and review. KT contributed to resources and manuscript review. ÜV contributed to resources and manuscript review. AB contributed to supervision and manuscript review. MR contributed to funding acquisition, project administration, supervision, and manuscript review. TT contributed to funding acquisition, project administration, data curation, resources, manuscript writing, and review. 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.2021.601490/full#supplementary-material

REFERENCES

Abbo, L., Shukla, B. S., Giles, A., Aragon, L., Jimenez, A., Camargo, J. F., et al. (2019). Linezolid-and vancomycin-resistant enterococcus faecium in solid organ transplant recipients: infection control and antimicrobial stewardship using whole genome sequencing. *Clin. Infect. Dis.* 69, 259–265. doi: 10.1093/cid/ciy903

Ahmed, M. O., and Baptiste, K. E. (2018). Vancomycin-resistant enterococci: a review of antimicrobial resistance mechanisms and perspectives of human and animal health. *Microb. Drug Resist.* 24, 590–606. doi: 10.1089/mdr.2017.0147

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., et al. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25, 3389–3402. doi: 10.1093/nar/25.17.3389

Aphis. (2014). *Enterococcus on U . S . Sheep and Lamb Operations*. Riverdale Park, MD: APHIS.

Arias, C. A., and Murray, B. E. (2012). The rise of the enterococcus: beyond vancomycin resistance. *Nat. Rev. Microbiol.* 10, 266–278. doi: 10.1038/nrmicro2761

Arias, C. A., Contreras, G. A., and Murray, B. E. (2010). Management of multi-drug resistant enterococcal infections. *Clin. Microbiol. Infect.* 16, 552–562.

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., et al. (2012). SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol.* 19, 455–477. doi: 10.1089/cmb.2012.0021

Bender, J. K., Cattoir, V., Hegstad, K., Sadowy, E., Coque, T. M., Westh, H., et al. (2018). Update on prevalence and mechanisms of resistance to linezolid, tigecycline and daptomycin in enterococci in Europe: towards a common nomenclature. *Drug Resist. Updat.* 40, 25–39. doi: 10.1016/j.drup.2018.10.002

Blanco, G., Lemus, J. A., and Grande, J. (2009). Microbial pollution in wildlife: linking agricultural manuring and bacterial antibiotic resistance in red-billed choughs. *Environ. Res.* 109, 405–412. doi: 10.1016/j.envres.2009.01.007

Bondi, M., Laukova, A., de Niederhausern, S., Messi, P., Papadopoulou, C., and Economou, V. (2020). Controversial aspects displayed by enterococci: probiotics or pathogens? *Biomed. Res. Int.* 2020:9816185.

Boom, R., Sol, C. J. A., Salimans, M. M. M., Jansen, C. L., Wertheim-Van Dillen, P. M. E., and Van Der Noordaa, J. (1990). Rapid and simple method for purification of nucleic acids. *J. Clin. Microbiol.* 28, 495–503.

Braga, J. F. V., Leal, C. A. G., Silva, C. C., Fernandes, A. A., Martins, N. R. S., and Ecco, R. (2018). Genetic diversity and antimicrobial resistance profile of *Enterococcus faecalis* isolated from broilers with vertebral osteomyelitis in Southeast Brazil. *Avian. Pathol.* 47, 14–22. doi: 10.1080/03079457.2017.1359403

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

Castro-Nallar, E., Valenzuela, S. L., Baquedano, S., Sánchez, C., Fernández, F., and Trombert, A. N. (2017). Draft genome sequences of five Enterococcus species isolated from the gut of patients with suspected clostridium difficile infection. *Genome Announc.* 5, e379–e317.

Cetinkaya, Y., Falk, P., and Mayhall, C. G. (2000). Vancomycin-resistant enterococci. *Clin. Microbiol. Rev.* 13, 686–707.

Daniel, D. S., Lee, S. M., Dykes, G. A., and Rahman, S. (2015). Public health risks of multiple-drug-resistant Enterococcus spp. in Southeast Asia. *Appl. Environ. Microbiol.* 81, 6090–6097. doi: 10.1128/aem.01741-15

Dubin, K., and Pamer, E. G. (2017). "Enterococci and their interactions with the intestinal microbiome," in *Bugs as Drugs* (American Society of Microbiology), 309–330. doi: 10.1128/microbiolspec.bad-0014-2016

Elena Aleksandrovna, Z., Tatiana Sergeevna, K., Elena Aleksandrovna, M., Andrey Mikhailovich, S., and Valentina Nikolaevna, L. (2019). "Phenotypic and genetic diversity of uropathogenic *Enterococcus faecalis* strains isolated in the primorsky region of russia," in *Microbiology of Urinary Tract Infections - Microbial Agents and Predisposing Factors (IntechOpen)*, ed. P. Behzadi (Germany: BoD – Books on Demand).

EUCAST (2018). *The European Committee on Antimicrobial Susceptibility Testing. Breakpoint Tables for Interpretation of MICs and Zone Version 9.0, 2019*.

Falgenhauer, L., Fritzenwanker, M., Imirzalioglu, C., Steul, K., Scherer, M., Albert-Braun, S., et al. (2019). Near-ubiquitous presence of a vancomycin-resistant Enterococcus faecium ST117/CT71/vanB -clone in the Rhine-main metropolitan area of Germany. *Antimicrob. Resist. Infect. Control* 8:128.

Fan, M. Z., and Archbold, T. (2015). Novel and disruptive biological strategies for resolving gut health challenges in monogastric food animal production. *Anim. Nutr.* 1, 138–143. doi: 10.1016/j.aninu.2015.10.002

Freitas, A. R., Tedim, A. P., Novais, C., Coque, T. M., and Peixe, L. (2018). Distribution of putative virulence markers in *Enterococcus faecium*: towards a safety profile review. *J. Antimicrob. Chemother.* 73, 306–319. doi: 10.1093/jac/dkx387

Gadde, U. D., Oh, S., Lillehoj, H. S., and Lillehoj, E. P. (2018). Antibiotic growth promoters virginiamycin and bacitracin methylene disalicylate alter the chicken intestinal metabolome. *Sci. Rep.* 8, 2–9.

Gagetti, P., Bonofiglio, L., García Gabarrot, G., Kaufman, S., Mollerach, M., Vigliarolo, L., et al. (2019). Resistance to β-lactams in enterococci. *Rev. Argent. Microbiol.* 51, 179–183.

Guzman Prieto, A. M., van Schaik, W., Rogers, M. R. C., Coque, T. M., Baquero, F., Corander, J., et al. (2016). Global emergence and dissemination of enterococci as nosocomial pathogens: attack of the clones? *Front. Microbiol.* 7:788.

Hammerum, A. M. (2012). Enterococci of animal origin and their significance for public health. *Clin. Microbiol. Infect.* 18, 619–625. doi: 10.1111/j.1469-0691.2012.03829.x

Hasman, H., Clausen, P. T. L. C., Kaya, H., Hansen, F., Knudsen, J. D., Wang, M., et al. (2019). LRE-Finder, a Web tool for detection of the 23S rRNA mutations and the optrA, cfr, cfr(B) and poxtA genes encoding linezolid resistance in enterococci from whole-genome sequences. *J. Antimicrob. Chemother.* 74, 1473–1476. doi: 10.1093/jac/dkz092

Hayes, J. R., English, L. L., Carr, L. E., Wagner, D. D., and Joseph, S. W. (2004). Multiple-antibiotic resistance of Enterococcus spp. isolated from commercial poultry production environments. *Appl. Environ. Microbiol.* 70, 6005–6011. doi: 10.1128/aem.70.10.6005-6011.2004

Heikens, E., Bonten, M. J. M., and Willems, R. J. L. (2007). Enterococcal surface protein esp is important for biofilm formation of *Enterococcus faecium* E1162. *J. Bacteriol.* 189, 8233–8240. doi: 10.1128/jb.01205-07

Huerta-Cepas, J., Serra, F., and Bork, P. (2016). ETE 3: reconstruction, analysis, and visualization of phylogenomic data. *Mol. Biol. Evol.* 33, 1635–1638. doi: 10.1093/molbev/msw046

Hunter, J. D. (2007). Matplotlib: a 2D graphics environment. *Comput. Sci. Eng.* 9, 99–104.

Hyatt, D., Chen, G. L., LoCascio, P. F., Land, M. L., Larimer, F. W., and Hauser, L. J. (2010). Prodigal: prokaryotic gene recognition and translation initiation site identification. *BMC Bioinformatics* 11:119.

Ibrahim, M., Ahmad, F., Yaqub, B., Ramzan, A., Imran, A., Afzaal, M., et al. (2020). "Current trends of antimicrobials used in food animals and aquaculture," in *Antibiotics and Antimicrobial Resistance Genes in the Environment*, ed. M. Z. Hashmi (Amsterdam: Elsevier), 39–69. doi: 10.1016/b978-0-12-818882-8.00004-8

International Organization for Standardization (1998). *ISO 7899-1:1998(en), Water Quality — Detection and Enumeration of Intestinal Enterococci — Part 1: Miniaturized Method (Most Probable Number) for Surface and Waste Water*. Available online at: https://www.iso.org/obp/ui/#iso:std:iso:7899:-1:ed-2:v1:en [Accessed May 23, 2020]

Jardine, C. M., Janecko, N., Allan, M., Boerlin, P., Chalmers, G., Kozak, G., et al. (2012). Antimicrobial resistance in escherichia coli isolates from raccoons (*procyon lotor*) in southern ontario, Canada. *Appl. Environ. Microbiol.* 78, 3873–3879. doi: 10.1128/aem.00705-12

Jolley, K. A., Bray, J. E., and Maiden, M. C. J. (2018). Open-access bacterial population genomics: BIGSdb software, the PubMLST.org website and their applications. *Wellcome Open Res.* 3:124. doi: 10.12688/wellcomeopenres.14826.1

Kayser, F. H. (2003). Safety aspects of enterococci from the medical point of view. *Int. J. Food Microbiol.* 88, 255–262. doi: 10.1016/s0168-1605(03)00188-0

Kiruthiga, A., Padmavathy, K., Shabana, P., Naveenkumar, V., Gnanadesikan, S., and Malaiyan, J. (2020). Improved detection of esp, hyl, asa1, gelE, cylA virulence genes among clinical isolates of Enterococci. *BMC Res. Notes* 13:170.

Kommineni, S., Kristich, C. J., and Salzman, N. H. (2016). Harnessing bacteriocin biology as targeted therapy in the GI tract. *Gut Microbes* 7, 512–517. doi: 10.1080/19490976.2016.1233089

Kozlov, A. M., Darriba, D., Flouri, T., Morel, B., Stamatakis, A., and Wren, J. (2019). RAxML-NG: a fast, scalable and user-friendly tool for maximum likelihood phylogenetic inference. *Bioinformatics* 35, 4453–4455. doi: 10.1093/bioinformatics/btz305

Levine, D. P. (2006). Vancomycin?: a history. *Clin. Infect. Dis.* 42, 5–12.

Manson, J. M., Hancock, L. E., and Gilmore, M. S. (2010). Mechanism of chromosomal transfer of *Enterococcus faecalis* pathogenicity island, capsule, antimicrobial resistance, and other traits. *Proc. Natl. Acad. Sci. U. S. A.* 107, 12269–12274. doi: 10.1073/pnas.1000139107

Marrow, J., Whittington, J. K., Mitchell, M., Hoyer, L. L., and Maddox, C. (2009). Prevalence and antibiotic-resistance characteristics of Enterococcus spp. isolated from free-living and captive raptors in central illinois. *J. Wildl. Dis.* 45, 302–313. doi: 10.7589/0090-3558-45.2.302

Marshall, B. M., and Levy, S. B. (2011). Food animals and antimicrobials: impacts on human health. *Clin. Microbiol. Rev.* 24, 718–733. doi: 10.1128/cmr.00002-11

Marttinen, P., Hanage, W. P., Croucher, N. J., Connor, T. R., Harris, S. R., Bentley, S. D., et al. (2012). Detection of recombination events in bacterial genomes from large population samples. *Nucleic Acids Res.* 40:e6. doi: 10.1093/nar/gkr928

McDonald, L. C., Kuehnert, M. J., Tenover, F. C., and Jarvis, W. R. (1997). Vancomycin-resistant enterococci outside the health-care setting: prevalence, sources, and public health implications. *Emerg. Infect. Dis.* 3, 311–317. doi: 10.3201/eid0303.970307

McKinney, W. (2010). "Data structures for statistical computing in python," in *Proceedings of the 9th Python in Science Conference*. Greenwich, CT: AQR Capital Management.

Moles, L., Gómez, M., Jiménez, E., Fernández, L., Bustos, G., Chaves, F., et al. (2015). Preterm infant gut colonization in the neonatal ICU and complete restoration 2 years later. *Clin. Microbiol. Infect.* 21:936.e1-10.

Olearo, F., Both, A., Belmar Campos, C., Hilgarth, H., Klupp, E. M., Hansen, J. L., et al. (2021). Emergence of linezolid-resistance in vancomycin-resistant Enterococcus faecium ST117 associated with increased linezolid-consumption. *Int. J. Med. Microbiol.* 311:151477. doi: 10.1016/j.ijmm.2021.151477

Ørstavik, D. (2004). V Irulence F Actors of E nterococcus faecalis?. *Open Microbiol. J.* 15, 308–320.

Palmer, K. L., Kos, V. N., and Gilmore, M. S. (2010). Horizontal gene transfer and the genomics of enterococcal antibiotic resistance. *Curr. Opin. Microbiol.* 13, 632–639. doi: 10.1016/j.mib.2010.08.004

Papagiannitsis, C. C., Malli, E., Florou, Z., Medvecky, M., Sarrou, S., Hrabak, J., et al. (2017). First description in Europe of the emergence of *Enterococcus faecium* ST117 carrying both vanA and vanB genes, isolated in Greece. *J. Glob. Antimicrob. Resist.* 11, 68–70. doi: 10.1016/j.jgar.2017.07.010

Parte, A. C. (2014). LPSN - List of prokaryotic names with standing in nomenclature. *Nucleic Acids Res.* 42, 613–616.

Radhouani, H., Igrejas, G., Pinto, L., Gonalves, A., Coelho, C., Rodrigues, J., et al. (2011). Molecular characterization of antibiotic resistance in enterococci recovered from seagulls (*Larus cachinnans*) representing an environmental health problem. *J. Environ. Monit.* 13, 2227–2233. doi: 10.1039/c0em00682c

Richey, E. M., Waters, P. W., Jovic, M., and Rakhman, C. (2015). Treatment of ampicillin-resistant *Enterococcus faecium* urinary tract infections. *Fed. Pract.* 32, 20–23.

Roosaare, M., Puustusmaa, M., Möls, M., Vaher, M., and Remm, M. (2018). PlasmidSeeker: identification of known plasmids from bacterial whole genome sequencing reads. *PeerJ* 2018:e4588. doi: 10.7717/peerj.4588

Roosaare, M., Vaher, M., Kaplinski, L., Möls, M., Andreson, R., Lepamets, M., et al. (2017). StrainSeeker: fast identification of bacterial strains from raw sequencing reads using user-provided guide trees. *PeerJ* 2017:e3353. doi: 10.7717/peerj.3353

Roth, N., Käsbohrer, A., Mayrhofer, S., Zitz, U., Hofacre, C., and Domig, K. J. (2019). The application of antibiotics in broiler production and the resulting antibiotic resistance in *Escherichia coli*: a global overview. *Poult. Sci.* 98, 1791–1804. doi: 10.3382/ps/pey539

Ruiz-Garbajosa, P., Bonten, M. J. M., Robinson, D. A., Top, J., Nallapareddy, S. R., Torres, C., et al. (2006). Multilocus sequence typing scheme for *Enterococcus faecalis* reveals hospital-adapted genetic complexes in a background of high

rates of recombination. *J. Clin. Microbiol.* 44, 2220–2228. doi: 10.1128/jcm.02596-05

Rybak, J. M., and Roberts, K. (2015). Tedizolid phosphate: a next-generation oxazolidinone. *Infect. Dis. Ther.* 4, 1–14. doi: 10.1007/s40121-015-0060-3

Sadowy, E., and Luczkiewicz, A. (2014). Drug-resistant and hospital-associated *Enterococcus faecium* from wastewater, riverine estuary and anthropogenically impacted marine catchment basin. *BMC Microbiol.* 14:66. doi: 10.1186/1471-2180-14-66

Shobrak, M. Y., and Abo-Amer, A. E. (2014). Role of wild birds as carriers of multi-drug resistant *Escherichia coli* and *Escherichia* vulneris. *Braz. J. Microbiol.* 45, 1199–1209. doi: 10.1590/s1517-83822014000400010

Si, H., Zhang, W. J., Chu, S., Wang, X. M., Dai, L., Hua, X., et al. (2015). Novel plasmid-borne multidrug resistance gene cluster including lsa(E) from a Linezolid-resistant enterococcus faecium isolate of swine origin. *Antimicrob. Agents Chemother.* 59, 7113–7116. doi: 10.1128/aac.01394-15

Singh, K. V., Coque, T. M., Weinstock, G. M., and Murray, B. E. (1998). In vivo testing of an *Enterococcus faecalis* efaA mutant and use of efaA homologs for species identification. *FEMS Immunol. Med. Microbiol.* 21, 323–331. doi: 10.1016/s0928-8244(98)00087-x

Singh, K. V., Nallapareddy, S. R., Sillanpää, J., and Murray, B. E. (2010). Importance of the collagen adhesin ace in pathogenesis and protection against *Enterococcus faecalis* experimental endocarditis. *PLoS Pathog.* 6:e1000716. doi: 10.1371/journal.ppat.1000716

Stępień-Pyśniak, D., Hauschild, T., Kosikowska, U., Dec, M., and Urban-Chmiel, R. (2019). Biofilm formation capacity and presence of virulence factors among commensal Enterococcus spp. from wild birds. *Sci. Rep.* 9:11204.

Stępień-Pyśniak, D., Hauschild, T., Nowaczek, A., Marek, A., and Dec, M. (2018). Wild birds as a potential source of known and novel multilocus sequence types of antibiotic-resistant *Enterococcus faecalis*. *J. Wildl. Dis.* 54, 219–228. doi: 10.7589/2017-05-118

Strateva, T., Peykov, S., Sirakov, I., Savov, E., Dimov, S., and Mitov, I. (2019). First detection and characterisation of a VanA-type *Enterococcus faecalis* clinical isolate from Bulgaria. *J. Glob. Antimicrob. Resist.* 18, 260–262. doi: 10.1016/j.jgar.2019.07.030

Tedim, A. P., Lanza, V. F., Manrique, M., Parej, E., Ruiz-Garbajosa, P., Cantón, R., et al. (2017). Complete genome sequences of isolates of Enterococcus faecium sequence type 117, a globally disseminated multidrug-resistant clone. *Genome Announc.* 5, e1553–e1516.

Telling, K., Brauer, A., Laht, M., Kalmus, P., Toompere, K., Kisand, V., et al. (2020). Characteristics of extended-spectrum beta-lactamase-producing *enterobacteriaceae* and contact to animals in Estonia. *Microorganisms* 8:1130. doi: 10.3390/microorganisms8081130

Treangen, T. J., Ondov, B. D., Koren, S., and Phillippy, A. M. (2014). The harvest suite for rapid core-genome alignment and visualization of thousands of intraspecific microbial genomes. *Genome Biol.* 15:524.

Vankerckhoven, V., Van Autgaerden, T., Vael, C., Lammens, C., Chapelle, S., Rossi, R., et al. (2004). Development of a multiplex PCR for the detection of asaI, gelE, cylA, esp, and hyl genes in enterococci and survey for virulence determinants among european hospital isolates of Enterococcus faecium. *J. Clin. Microbiol.* 42, 4473–4479. doi: 10.1128/jcm.42.10.4473-4479.2004

WHO (2017). *WHO Publishes List of Bacteria for Which New Antibiotics are Urgently Needed*. Geneva: WHO.

Zangue, R. C. F. (2017). Molecular epidemiology of antibiotic resistant eskape pathogens isolated from public sector hospitals in umgungundlovu district, kwazulu-natal, South Africa. *Antibiotics* 9:850.

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

Zischka, M., Künne, C. T., Blom, J., Wobser, D., Sakinç, T., and Schmidt-Hohagen, K. (2015). Comprehensive molecular, genomic and phenotypic analysis of a major clone of *Enterococcus faecalis* MLST ST40. *BMC Genomics* 16:175.

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Techniques Used for Analyzing Microplastics, Antimicrobial Resistance and Microbial Community Composition: A Mini-Review

Simona Bartkova[1], Anne Kahru[2,3], Margit Heinlaan[2]*† and Ott Scheler[1]†*

[1] Department of Chemistry and Biotechnology, Tallinn University of Technology, Tallinn, Estonia, [2] Laboratory of Environmental Toxicology, National Institute of Chemical Physics and Biophysics, Tallinn, Estonia, [3] Estonian Academy of Sciences, Tallinn, Estonia

Antimicrobial resistance (AMR) is a global health threat. Antibiotics, heavy metals, and microplastics are environmental pollutants that together potentially have a positive synergetic effect on the development, persistence, transport, and ecology of antibiotic resistant bacteria in the environment. To evaluate this, a wide array of experimental methods would be needed to quantify the occurrence of antibiotics, heavy metals, and microplastics as well as associated microbial communities in the natural environment. In this mini-review, we outline the current technologies used to characterize microplastics based ecosystems termed "plastisphere" and their AMR promoting elements (antibiotics, heavy metals, and microbial inhabitants) and highlight emerging technologies that could be useful for systems-level investigations of AMR in the plastisphere.

Keywords: antimicrobial resistance, microplastics, heavy metals, plastisphere, emerging technologies, antibiotics

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****Correspondence:***
Simona Bartkova
simona.bartkova@taltech.ee;
simonabartkova86@hotmail.com
Margit Heinlaan
margit.heinlaan@kbfi.ee

† These authors have contributed equally and share the last authorship

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INTRODUCTION

The increasing resistance of pathogenic bacteria to common antibiotics (AB) found in human and veterinary settings worldwide (WHO, 2018) highlights the urgent need for improved surveillance programs (Dadgostar, 2019) and research to hinder further escalation of antimicrobial resistance (AMR) (Interagency Coordination Group on Antimicrobial Resistance, 2019). Although the number is debatable (de Kraker et al., 2016), according to O'Neill (2016), the global annual death toll due to AMR could rise to 10 million by 2050.

The emerging contaminant—plastic—has potential to further enhance AMR by providing porous micro ecosystems termed "plastisphere" (Keswani et al., 2016). In the environment, plastic does not biodegrade but fragmentizes into smaller fractions such as microplastics (MPs) (1 μm–5 mm) (Frias and Nash, 2019) or further into nanoplastics (NPs) (≤1 μm) (Gigault et al., 2018). MPs have been increasingly detected in all the ecosystems, though due to rapid microbial colonization and subsequent density changes, about 70% of the MPs in the aquatic environment sedimentates and thus the sediments, along with soils that receive MPs contamination from sludge application, have been considered as the sinks of MPs (Nizzetto et al., 2016; Corradini et al., 2019; Schmiedgruber et al., 2019). Plastic is also ingested and inhaled by humans (Cox et al., 2019; de Wit and Bigaud, 2019) as indicated by detection of plastic in stool samples (Schwabl et al., 2019)

and human lung tissue (Pauly et al., 1998), respectively. Compared to MPs, NPs have been scarcely studied due to limitations of current analytical techniques (Nguyen B. et al., 2019), yet Besseling et al. (2019) have speculated future NP concentrations in mass may become 10^{14} times higher than currently measured MP concentrations.

The plastisphere creates a habitat that promotes attachment of and subsequent biofilm production by microbes (Zettler et al., 2013). In this habitat, the microbes are also in close vicinity of MP-associated pollutants, such as (ABs) and heavy metals (HMs) (**Figure 1**). This combination of being surrounded by pollutants while being protected by biofilm can lead to possible change in the microbial species distribution (Munier and Bendell, 2018; Imran et al., 2019). ABs are considered to be the primary drivers of AMR (Kraemer et al., 2019), originating largely from inefficient wastewater treatment processes and pharmaceutical discharge (Wilkinson and Boxall, 2019). HMs are accumulating in the environment *via* waste flows from industrial activities (mining, smelting, fertilizer use, sewage sludge application), but may also be mobilized due to natural processes (e.g., bedrock weathering) (Ali et al., 2019; Zhou et al., 2020). HM pollution drives the selection for metal resistance genes (MRGs) and correlates with increased occurrences and amount of antibiotic resistance genes (ARGs) (**Figure 1**; Baker-Austin et al., 2006; Li et al., 2017; Nguyen C.C. et al., 2019).

It is hypothesized that weathering can intensify both HM (Prunier et al., 2019) and AB association (Zhou et al., 2020) with MPs and potential migration of additives (Commission Regulation, 2011) from the polymer. Indeed, the European Chemical Agency has identified 1,550 additives (European Chemical Agency, 2019), many of which are known to leach into the environment (De-la-Torre et al., 2020; Bolívar-Subirats et al., 2021) as they are generally not chemically bound to polymers and can thus potentially migrate (Hahladakis et al., 2018). Metals and metal-based additives are mostly used as colorants and fillers, and research on release rate of toxic HM additives (e.g., Cd, Pb, Sb, Sn) in plastics during recycling is ongoing (Hahladakis et al., 2018). Long-term impact of MP pollution on the development of AMR is yet unclear, but AB retention, ARG presence, and exchange of ARGs through horizontal gene transfer among bacteria on MPs has been shown (Imran et al., 2019; Yang et al., 2019; Zhu et al., 2019).

In this mini-review, we discuss the role of plastisphere in the development of AMR, and the current technologies used to address various aspects of AB-HM-MP pollution and highlight the data gaps, novel techniques, and approaches.

CHARACTERIZATION OF PLASTISPHERE-ASSOCIATED ANTIBIOTICS AND HEAVY METALS

MP abundance and polymer type are determined by microscopy and spectroscopy methods (**Figure 2**). The first steps(s) in analyzing plastic from environmental samples usually comprises of different separation and/or purification procedures. Separation frequently consists of passing samples through sieves or filter membranes (Fu et al., 2020). The purification process commonly involves treatment with, for example, ethanol (Zettler et al., 2013; Dussud et al., 2018), purified sea water (Dussud et al., 2018), or strong acidic and/or alkaline solutions (Cole et al., 2014; Imhof et al., 2016). Stereomicroscopes are used for the general estimate of MPs in environmental samples but also to characterize their surface, size, and shape (Gimiliani et al., 2020; Zhang Y. et al., 2020). Roughness and hydrophobicity of MPs is evaluated by tensiometry, measuring the contact angle of water drops (Dussud et al., 2018; Hossain et al., 2019). For visualization with a greater resolving power, scanning electron microscopy (SEM) (Arias-Andres et al., 2018; Li et al., 2018), or atomic force microscopy (AFM) (Dussud et al., 2018) are used. The main difference between stereomicroscopy and SEM is their resolution limit of around 200 and 2 nm, respectively. AFM has a third dimension of magnification (the *z*-axis), enabling constructing landscape maps of surfaces. Spectroscopy methods based on molecular vibration such as Raman spectroscopy (Zettler et al., 2013; Amaral-Zettler et al., 2015; Imhof et al., 2016), Fourier Transform Infrared Spectroscopy (FTIR)(Bryant et al., 2016; Laganà et al., 2019; Zhang Y. et al., 2020), and attenuated total reflection-FTIR (Amaral-Zettler et al., 2015; Viršek et al., 2017) allow to decipher MPs chemical makeup for more precise identification. X-ray diffraction can provide the crystalline structure of MPs (Li et al., 2018). Nevertheless, there is a gap in research, because current technologies still have difficulties in accurately detecting and characterizing the chemical properties of extensively degraded plastics (especially MPs $\leq$50 μm and NPs) from complex environmental samples (Lehner et al., 2019). Examples include lacking a standard procedure for separating and/or purifying samples from different matrices and using purification steps that may damage the plastic (Lö et al., 2017), microscopy techniques not providing information on plastic composition (Müller et al., 2020), and spectroscopy techniques like Raman and FTIR not having the resolution power needed for NP characterization (Imhof et al., 2016; Mason et al., 2018; Fu et al., 2020).

Absorption of light and mass-to-charge ratio are used to measure the content of HMs within and on the surface of MPs *via* atomic absorption spectroscopy (Brennecke et al., 2016; Cabral et al., 2016; Munier and Bendell, 2018) and inductively coupled plasma mass spectrometry (ICP-MS) (Rochman et al., 2014; Cabral et al., 2016; Imhof et al., 2016), respectively (**Figure 2**). The latter being the gold standard for detecting and characterizing metals. AB affinity for MP has been studied in batch adsorption experiments in laboratory settings. High-performance liquid chromatography (HPLC) coupled to a diode array detector (Guo et al., 2019; Guo and Wang, 2019) and to a triple quadruple detector (Bolívar-Subirats et al., 2021) and ultra-performance liquid chromatography coupled to a photodiode array detector (Zhang et al., 2018) but also UV-visible spectroscopy have been used to determine MP-sorbed AB (Wan et al., 2019; Yu et al., 2020a,b; **Figure 2**). In addition, FTIR has been used to characterize the interaction mechanisms between MPs and ABs (Wan et al., 2019; Yu et al., 2020a,b; **Figure 2**). Although technology has allowed in-depth analysis of HMs and ABs, there is a knowledge gap on how the affinity for pollutants differs from MPs and NPs

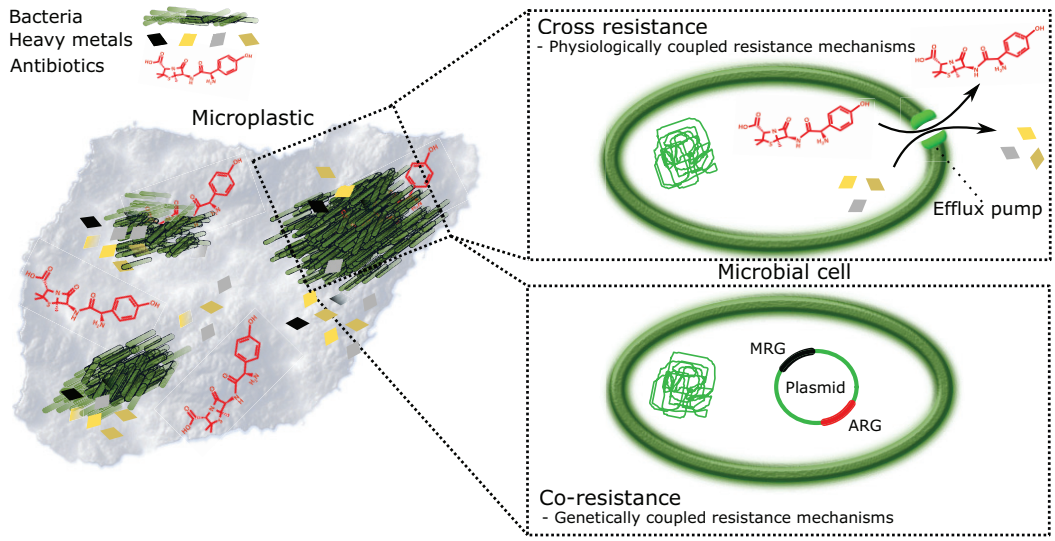

FIGURE 1 | Plastisphere is a potential hotspot for evolution of antimicrobial resistance (AMR). Persistence and surface characteristics of microplastics make it an excellent reservoir of microbes and pollutants, such as heavy metals (HMs) and antibiotics (ABs). Together they form miniature ecosystems, plastispheres, where AMR is promoted by (i) cross-resistance where resistance mechanisms to HMs and ABs are physiologically coupled, for example, efflux pumps (Baker-Austin et al., 2006; Seiler and Berendonk, 2012); and (ii) co-resistance where antibiotic resistance genes (ARGs) and metal resistance genes (MRGs) are present on the same mobile genetic element and thus genetically coupled, whereby selection for metal resistance in, for example, animal gut, and anthropogenically contaminated soils and water bodies lead to automatic selection of ARGs (Baker-Austin et al., 2006; Seiler and Berendonk, 2012; Li et al., 2017).

degraded from larger plastic due to weathering processes to primary MPs and NPs.

CHARACTERIZATION OF PLASTISPHERE-INHABITING BACTERIA

MPs in water bodies form an ideal substratum for bacterial biofilm formation as they adsorb nutrients and organic matter from the essentially nutrient-poor water habitat supporting the growth of bacteria. Generally, the colonization of MPs is a very rapid process (within 24 h) depending on a variety of factors (Oberbeckmann et al., 2015) of which environmental factors and not the plastic type have recently been shown to be the most significant influencer for microbial composition on MPs (Wright et al., 2020).

Bacterial association with MPs is analyzed by SEM and fluorescence microscopy) (Zettler et al., 2013; Bryant et al., 2016; Arias-Andres et al., 2018; Dussud et al., 2018; Hossain et al., 2019; **Figure 2**). Fluorescence can further examine the gene exchange rate within biofilm communities and planktonic bacteria (Arias-Andres et al., 2018), accomplished *via* fluorescent self-transmissible plasmids (Arias-Andres et al., 2018). To study the extracellular polymeric substances of the biofilm matrix, confocal laser scanning microscopy (CLSM) is used (Hossain et al., 2019; **Figure 2**). This is due to CLSM's ability of obtaining high-resolution images in various depths of a sample, usually 50–100 μm in biological samples (Jonkman et al., 2020). Overall, research in this area has greatly expanded due to the above-mentioned technologies; however, there is a gap in analysis and modeling of microbial colonization of both MPs and NPs in different environmental settings. Change in species diversity is mostly investigated by sequencing variable regions from the conserved 16S ribosomal RNA (16S rRNA) (Knapp et al., 2017; Zhao et al., 2018; Zhao Y. et al., 2019; Chen et al., 2019; Learman et al., 2019; Meier et al., 2020; **Figure 2**). Sequencing methods, including whole genome sequencing (WGS), are also ideal for studying effect of HMs on resistance-related genes in bacteria (Pathak et al., 2020; **Figure 2**).

Metagenomics with possible combination of metatranscriptomics permits analysis of the species present in the microbial community, including non-culturable bacteria, while simultaneously studying regulation of ARGs, MRGs, and other genes at the mRNA/functional level within the whole community (Cabral et al., 2016; Meier et al., 2020; **Figure 2**). Functional metagenomics is useful for screening of resistance genes that are expressed in specific environments such as HM polluted sites, while further allowing discovery of possible genes with novel functions (Cheng et al., 2012; Staley et al., 2015). Further methods for routine examination are polymerase chain reaction (PCR) techniques (Medardus et al., 2014; Knapp et al., 2017; Chen et al., 2019), although here, only a limited number of genes are investigated (Zhang Y. et al., 2020). This can be overcome by high-throughput qPCR chip technologies (Zhao et al., 2018; Zhao Y. et al., 2019) and WGS (Learman et al., 2019; **Figure 2**). Alternations in gene expression levels caused by HM exposure can be determined by reverse transcription PCR for specific genes (Leng et al., 2019) or with metatranscriptomics for the whole transcriptome (Cabral et al., 2016). Many different ecosystems have now been investigated for ARGs and MRGs,

General research area	Research objective	Focus point(s)	Technologies	Reference
Characterization of plastisphere	Polymer type	MPs	FTIR + Raman	(1)(2)
		NPs	**AFM-IR/Raman**	(3)
		NPs and MPs	**Py-GCToF**	(4)
	Abundance	General estimate	LM	(5)(1)
		Aqueous samples	FCM	(6)
	Properties	Roughness/hydrophobicity	Tensiometry	(7)(8)
		Chemical make-up	FTIR + Raman	(1)(2)
		Crystalline Structure	XRD	(9)
		Visualization	EM + AFM	(9)(7)
Characterization of plastisphere-associated heavy metals and antibiotics	Heavy metals	Detection/characterization	AAS + ICP-MS	(10)(2)
	Antibiotics	AB affinity for MPs	HPLC + UPLC + UV-VIS	(11)(12)(13)
		MP - AB interaction mechanisms	FTIR	(13)
Characterization of plastisphere-inhabiting bacteria	Presence	Bacterial MP colonization	SEM + FM + **Microfluidics**	(8)(7)(14)
		Attachment	**Microfluidics**	(15)
		Gene exchange rate	FM	(7)
		Extracellular polymeric substances	CLSM	(8)
	Species composition	Basic species analysis	RT-PCR + 16S rRNA	(16)(17)
		Extensive species analysis	WGS + HT-qPCR + Metagenomics	(18)(19)(17)
		Evolution and mutations	WGS + GREACE	(20)(21)
Analysis of antimicrobial resistance in plastispehere	Species composition	Basic species analysis	RT-PCR + 16S rRNA	(16)(17)
		Extensive species analysis	WGS + HT-qPCR + Metagenomics	(18)(19)(17)
		Evolution and mutations	WGS + GREACE	(20)(21)
	Resistance genes	Non-culturable bacteria and gene expression	**DeepARG**	(22)
		Gene expression in single-cell bacteria	**microSPLIT**	(23)
		Hetero-resistance	**Droplet microfluidics**	(24)
	Proteins	Basic protein analysis	Western blot + 2D-PAGE	(16)(25)
		Cost-effective protein identification	**1D-LC-MS/MS**	(26)
		Detect low-abundance proteins	**2D-LC-MS/MS**	(26)
	Metabolites	Detect a wide variety of metabolites	GC-MS	(27)
		Investigate complex samples	**MALDI-MSI/FISH**	(28)

FIGURE 2 | Flow chart with current and novel (written in bold and blue color) approaches for studying the effects of plastisphere-associated pollutants (heavy metals, antibiotics) on antimicrobial resistance. Abbreviations for technologies in alphabetical order: AAS, Atomic absorption spectroscopy; AFM, Atomic Force Microscopy; AFM-IR/Raman, Atomic force microscopy infrared/Raman; CLSM, Confocal Laser Scanning Microscopy; DeepARG, Deep learning model for antibiotic resistance genes; EM, Electron microscopy; FCM, Flow cytometry; PFGE, Pulsed-field gel electrophoresis; FM, Fluorescence microscopy; FTIR, Fourier transform infrared microscopy; GC-MS, Gas chromatography–mass spectrometry; GREACE, Genome Replication Engineering Assisted Continuous Evolution; HPLC, High-performance liquid chromatography; HT-qPCR, High-throughput qPCR; ICP-MS, Inductively coupled plasma mass spectrometry; LM, Light microscopy; MALDI-MSI/FISH, Matrix assisted laser desorption/ionization–Mass spectrometry imaging/Fluorescence *in situ* hybridization; microSPLIT, Microbial Split-Pool Ligation Transcriptomics; Py-GCToF, Pyrolysis–Gas Chromatography Time of Flight Mass Spectrometry; RT-PCR, Reverse transcription polymerase chain reaction; SEM, Scanning Electron Microscopy; UPLC, Ultra-performance liquid chromatography; UV-VIS, Ultraviolet–visible spectrophotometry; XRD, X-ray diffraction; WGS, Whole genome sequencing; 1D/2D-LC-MS/MS, One dimensional/Two dimensional online separation-liquid chromatography-tandem mass spectrometry; 2D-PAGE, Two-dimensional gel electrophoresis. References in numerical order: (1) = (Zhang Y. et al., 2020), (2) = (Imhof et al., 2016), (3) = (Fu et al., 2020), (4) = (Sullivan et al., 2020), (5) = (Gimiliani et al., 2020), (6) = (Kaile et al., 2020), (7) = (Dussud et al., 2018), (8) = (Hossain et al., 2019, (9) = (Li et al., 2018), (10) = (Munier and Bendell, 2018), (11) = (Bolívar-Subirats et al., 2021), (12) = (Zhang et al., 2018), (13) = (Yu et al., 2020b), (14) = (Pousti et al., 2019), (15) = (Secchi et al., 2020), (16) = (Leng et al., 2019), (17) = (Meier et al., 2020), (18) = (Pathak et al., 2020), (19) = (Zhao Y. et al., 2019), (20) = (Li X. et al., 2019), (21) = (Qin et al., 2019), (22) = (Cuadrat et al., 2020), (23) = (Kuchina et al., 2020), (24) = (Scheler et al., 2020), (25) = (Bar et al., 2007), (26) = (Hinzke et al., 2019), (27) = (Li W. et al., 2019), (28) = (Geier et al., 2020).

yet a comprehensive overview of the ARG and MRG prevalence remains to be done. Another gap is single-cell bacterial research, as the effect of HMs and plastic on AMR at the level of single-bacterium is virtually non-existent. This is mainly due to lack of technologies being able to extract and analyze their genetic material (Kuchina et al., 2020).

Sequencing in combination with long-term experiments can detect mutations that occur in bacteria during prolonged growth in HM rich environments (Chi et al., 2017; Li X. et al., 2019; Qin et al., 2019). These experiments include serial long-term culturing of resistant mutants exposed to subtoxic levels of HMs, followed by WGS (Li X. et al., 2019). Genome Replication Engineering Assisted Continuous Evolution is another alternative, in which evolution of resistant mutants is accelerated before sequencing (Qin et al., 2019; **Figure 2**). *In vivo* experiments with mice being exposed to HMs *via* oral administration followed by sequencing of the gut microbiota have shed light on the effect of HMs in mammals *in vivo* (Chi et al., 2017). There are several ways to explore how HMs can have an effect on the protein and metabolite level in bacterial monocultures. A frequently used method for proteomics is two-dimensional gel electrophoresis (Bar et al., 2007; **Figure 2**). Another way to explore protein expression is through liquid chromatography-tandem mass spectrometry (LC-MS/MS) where proteins are first separated by LC and then ionized and characterized by mass-to-charge ratio and relative abundance (Li W. et al., 2019; **Figure 2**). Western blotting is another widely applied technique (Leng et al., 2019). For studying metabolites, gas chromatography-mass spectrometry is usually performed (Leng et al., 2019; **Figure 2**). Although working with monoculture bacteria does not mimic the true situation of microbial interaction in the environment, possible impact of HMs and plastics on the proteomics/metabolomics level in different types of bacteria is currently still a research gap in need of investigation. This gap needs to be addressed in the light of recent studies, such as the one by Li W. et al. (2019), showing that an alternation in bacterial

metabolic pathways may affect their AMR. Exposure to HMs has already shown the ability to alter metabolic pathways of bacteria in the gut (Chi et al., 2017); nonetheless, there is still much to be learned on possible influence of HMs and perhaps plastics on the different pathways and the interplay with AMR.

OVERCOMING THE CHALLENGES TO THE PLASTISPHERE CHARACTERIZATION RESEARCH GAPS

The most optimal solution for future development of a standard method for quantifying and characterizing the composition of smaller fractions of MPs, including NPs (<1 μm), might be to merge completely new analytical methods with the existing technologies (Nguyen B. et al., 2019; Fu et al., 2020). For now, combining AFM with infrared spectroscopy or Raman seems promising, since AFM offers relatively simple sample preparation, and samples can be conserved during analysis (Fu et al., 2020; **Figure 2**). One disadvantage is, however, that obtaining quality imaging of the sample depends on how flat and smooth the sample is (Fu et al., 2020), making it difficult for simultaneous investigation of NPs and larger MPs. To obtain a more complete overview, at least in aqueous samples, we recommend analysis of plastics on PTFE membranes combined with Pyrolysis-Gas Chromatography Time of Flight Mass Spectrometry (Py-GCToF) (Sullivan et al., 2020; **Figure 2**). This analytical method is based on analyzing thermal degradation products, and it has shown to be fast, reliable, and have high resolution (Sullivan et al., 2020). A second option for aqueous samples that might be more easily standardized for future environmental identification and quantification of MPs and NPs (0.2–2 μm), is flow cytometry in combination with staining and cell sorting (Kaile et al., 2020).

Change in MPs and NPs composition and their affinity for pollutants and microbes can be uncovered by merging analytical and sequencing technology with *in situ* and *ex situ* experiments. *Ex situ* batch sorption experiments provide the opportunity to focus on specific parameters (Li et al., 2018; Zhang H. et al., 2020), while *in situ* studies are necessary to observe the real-life complex interaction of MPs and NPs with their surroundings (Oberbeckmann et al., 2017). Depending on the environment investigated, suitable analytical techniques for detection and characterization of MPs and NPs in such experiments could be either Py-GCToF (Sullivan et al., 2020) or micro-FTIR and Raman spectroscopy (Lö et al., 2017; **Figure 2**). HPLC and ICP-MS could further be used for AB and HM detection, respectively (Cabral et al., 2016; Zhang H. et al., 2020; **Figure 2**). Finally, microbial analysis in the experiments would need to include both analytical tools for studying biofilm (e.g., CLSM) and metagenomic sequencing for discovering possible ARGs and MRGs as well as species diversity (Cabral et al., 2016; Hossain et al., 2019).

Accumulation of MPs in the food chain and the effect on spread of AMR should be investigated by long-term *in vivo* studies combined with multidisciplinary tools such as NGS sequencing, ICP-MS, and vibrational spectroscopy methods. Previous *in vivo* studies focusing on influence of MP accumulation are inconsistent in their methods and yield conflicting results (Van Raamsdonk et al., 2020). One issue with standardizing *in vivo* studies is the complexity of the sample material, making it difficult to detect MPs. This could be solved by an enzymatic purification method for MPs/NPs developed by Lö et al. (2017), which can remove organic and inorganic material from different matrices while not affecting the polymers and couple it to micro-FTIR and Raman spectroscopy (**Figure 2**). Lö et al. (2017) provides a step-by-step guide to the enzymatic purification, which includes optional subdivision of samples, usage of specific buffers, and lipase and amylase for samples with high lipid or polysaccharide content.

NOVEL APPROACHES AND METHODS FOR ADDRESSING AMR KNOWLEDGE GAPS IN THE PLASTISPHERE

Obtaining a wider overview of microbial communities, including spread of ARGs and MRGs in different habitats, is feasible with modern NGS approaches. There are two aspects that should be considered in future analyses: (1) presence of non-culturable bacteria and (2) expression level of resistant genes in the bacterial communities. Integrating metagenomics and metatranscriptomics with machine-learning tools such as DeepARG, trained to find the existing and novel ARGs and MRGs, is a suitable option for this challenge (Arango-Argoty et al., 2018; Cuadrat et al., 2020; **Figure 2**). Studying heterogeneous modulation of gene expression by HMs (and MPs/NPs) in a single bacterium is possible, but single-cell RNA sequencing (scRNA-seq) studies are still scarce due to differences from eukaryotic cells such as low mRNA content and lack of polyadenylation. This challenge could be overcome by the scRNA-seq platform Microbial Split-Pool Ligation Transcriptomics (Kuchina et al., 2020; **Figure 2**). The approach was recently adapted for *Bacillus subtilis* and *Escherichia coli* by Kuchina et al. (2020) and has advantages such as: (1) no need for single cell physical isolation, (2) compatibility with a wide range of cell shapes and sizes; and (3) enables use of un-encapsulated and fixed cells (Ma et al., 2019).

Proteomic and metabolomic pathways in bacteria play an important role in AMR (Li W. et al., 2019). 1D and 2D-LC-MS/MS spectrometry and mass spectrometry imaging (MSI) can be used for analyzing the impact that MPs and HMs potentially have on proteomics/metabolomics activity levels in the bacteria. In their extensive testing of LC-MS/MS spectrometry, Hinzke et al. (2019) suggest the most cost-effective method for maximizing the number of identified proteins by MS is online separation by 1D-LC. For a more precise guidance for specific objectives, we refer to the flow chart and overall work of Hinzke et al. (2019) (**Figure 2**). Though, regardless of technology used, it is essential that proteome bioinformatics progresses in parallel with the recent advances in MS methods; otherwise, proteomic analysis will remain limited (Ameen and Raza, 2017; Petriz and Franco, 2017). In congruence, technology needed for analysis of metabolites is restricted as

well (Dunham et al., 2017). One promising method is MSI, because it provides chemical and spatial analysis and methods can easily be adapted to specific environmental samples. MSI works by distinguishing chemical compounds *via* their mass-to-charge ratio, and currently, there are three MSI methods commercially available for analyzing bacteria (Dunham et al., 2017). Though the main limiting factor is that any single MSI experiment only gives a fraction of the metabolites present in samples (Dunham et al., 2017). Nevertheless, we believe that when combined with other technologies, it could pave the way for future metabolomic research as exemplified by the work of Geier et al. (2020), where matrix-assisted laser desorption/ionization MSI was combined with FISH microscopy (**Figure 2**). This enabled linking metabolomes to groups of 50–100 microbial cells in complex environmental samples and be able to resolve single-cell bacteria in the near future (Geier et al., 2020).

Microbial colonization and ability to form biofilm are also heterogenous characteristics of microbes, and they play a key role in AMR. Microfluidic platforms show great potential for enabling complex biofilm studies (Yawata et al., 2016; Pousti et al., 2019), including the scarcely researched effect of flow rate and motility of bacteria on attachment (Secchi et al., 2020; **Figure 2**). Bacteria communities, both hetero and isogenic, can contain cells with diverse range of resistance (El-Halfawy and Valvano, 2015). Droplet microfluidic technology could be the most promising tool for such investigation because it allows high-throughput culturing of bacteria at wide range of isolated conditions (Kaminski et al., 2016; Scheler et al., 2020; **Figure 2**).

CONCLUSION

In this mini-review, we highlighted technologies that have been used for analyzing different aspects of plastisphere-associated AMR. Although we found that many different aspects of AMR have been explored through multiple studies using advanced methods, knowledge gaps remain. To address these gaps, we summarize currently available technologies potentially suitable for future research. This should provide analytical tools for scientists of diverse backgrounds seeking answers for complex urgent problems: HM- and AB-contaminated plastisphere-associated promotion of AMR.

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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REFERENCES

Ali, H., Khan, E., and Ilahi, I. (2019). Environmental chemistry and ecotoxicology of hazardous heavy metals: environmental persistence, toxicity, and bioaccumulation. *J. Chem.* 2019:6730305. doi: 10.1155/2019/6730305

Amaral-Zettler, L. A., Zettler, E. R., Slikas, B., Boyd, G. D., Melvin, D. W., Morrall, C. E., et al. (2015). The biogeography of the plastisphere: implications for policy. *Front. Ecol. Environ.* 13:541–546. doi: 10.1890/150017

Ameen, A., and Raza, S. (2017). Metaproteomics approaches and techniques: a review. *Int. J. Adv. Sci. Res.* 3:49. doi: 10.7439/ijasr.v3i5.4167

Arango-Argoty, G., Garner, E., Pruden, A., Heath, L. S., Vikesland, P., and Zhang, L. (2018). DeepARG: a deep learning approach for predicting antibiotic resistance genes from metagenomic data. *Microbiome* 6:23. doi: 10.1186/s40168-018-0401-z

Arias-Andres, M., Klümper, U., Rojas-Jimenez, K., and Grossart, H. P. (2018). Microplastic pollution increases gene exchange in aquatic ecosystems. *Environ. Pollut.* 237, 253–261. doi: 10.1016/j.envpol.2018.02.058

Baker-Austin, C., Wright, M. S., Stepanauskas, R., and McArthur, J. V. (2006). Co-selection of antibiotic and metal resistance. *Trends Microbiol.* 14, 176–182. doi: 10.1016/J.TIM.2006.02.006

Bar, C., Patil, R., Doshi, J., Kulkarni, M. J., and Gade, W. N. (2007). Characterization of the proteins of bacterial strain isolated from contaminated site involved in heavy metal resistance-A proteomic approach. *J. Biotechnol.* 128, 444–451. doi: 10.1016/j.jbiotec.2006.11.010

Besseling, E., Redondo-Hasselerharm, P., Foekema, E. M., and Koelmans, A. A. (2019). Quantifying ecological risks of aquatic micro- and nanoplastic. *Crit. Rev. Environ. Sci. Technol.* 49, 32–80. doi: 10.1080/10643389.2018.1531688

Bolívar-Subirats, G., Rivetti, C., Cortina-Puig, M., Barata, C., and Lacorte, S. (2021). Occurrence, toxicity and risk assessment of plastic additives in Besos river, Spain. *Chemosphere* 263:128022. doi: 10.1016/j.chemosphere.2020.128022

Brennecke, D., Duarte, B., Paiva, F., Caçador, I., and Canning-Clode, J. (2016). Microplastics as vector for heavy metal contamination from the marine environment. *Estuar. Coast. Shelf Sci.* 178, 189–195. doi: 10.1016/J.ECSS.2015.12.003

Bryant, J. A., Clemente, T. M., Viviani, D. A., Fong, A. A., Thomas, K. A., Kemp, P., et al. (2016). Diversity and activity of communities inhabiting plastic debris in the north pacific gyre. *mSystems* 1:e00024–16. doi: 10.1128/msystems.00024-16

Cabral, L., Júnior, G. V. L., Pereira de Sousa, S. T., Dias, A. C. F., Lira Cadete, L., Andreote, F. D., et al. (2016). Anthropogenic impact on mangrove sediments triggers differential responses in the heavy metals and antibiotic resistomes of microbial communities. *Environ. Pollut.* 216, 460–469. doi: 10.1016/J.ENVPOL.2016.05.078

Chen, J., Li, J., Zhang, H., Shi, W., and Liu, Y. (2019). Bacterial heavy-metal and antibiotic resistance genes in a copper tailing dam area in northern China. *Front. Microbiol.* 10:1916. doi: 10.3389/fmicb.2019.01916

Cheng, G., Hu, Y., Yin, Y., Yang, X., Xiang, C., Wang, B., et al. (2012). Functional screening of antibiotic resistance genes from human gut microbiota reveals a novel gene fusion. *FEMS Microbiol. Lett.* 336, 11–16. doi: 10.1111/j.1574-6968.2012.02647.x

Chi, L., Bian, X., Gao, B., Tu, P., Ru, H., and Lu, K. (2017). The effects of an environmentally relevant level of arsenic on the gut microbiome and its functional metagenome. *Soc. Toxicol.* 162, 193–204. doi: 10.1093/toxsci/kfx174

Cole, M., Webb, H., Lindeque, P. K., Fileman, E. S., Halsband, C., and Galloway, T. S. (2014). Isolation of microplastics in biota-rich seawater samples and marine organisms. *Sci. Rep.* 4:4528. doi: 10.1038/srep04528

Commission Regulation [EU] (2011). *Commission Regulation (EU) No 10/2011 of 14 January 2011 on Plastic Materials and Articles Intended to Come into Contact with Food Text with EEA Relevance*. Available online at: https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX%3A32011R0010 (accessed August 14, 2020).

Corradini, F., Meza, P., Eguiluz, R., Casado, F., Huerta-Lwanga, E., and Geissen, V. (2019). Evidence of microplastic accumulation in agricultural soils from sewage sludge disposal. *Sci. Total Environ.* 671, 411–420. doi: 10.1016/j.scitotenv.2019.03.368

Cox, K. D., Covernton, G. A., Davies, H. L., Dower, J. F., Juanes, F., and Dudas, S. E. (2019). Human consumption of microplastics. *Environ. Sci. Technol.* 53, 7068–7074. doi: 10.1021/acs.est.9b01517

Cuadrat, R. R. C., Sorokina, M., Andrade, B. G., Goris, T., and Avila, A. M. R. D. (2020). Global ocean resistome revealed: exploring antibiotic resistance gene abundance and distribution in TARA oceans samples. *Gigascience* 9:giaa046. doi: 10.1093/gigascience/giaa046

Dadgostar, P. (2019). Antimicrobial resistance: implications and costs. *Infect. Drug Resist.* 12, 3903–3910. doi: 10.2147/IDR.S234610

de Kraker, M. E. A., Stewardson, A. J., and Harbarth, S. (2016). Will 10 million people die a year due to antimicrobial resistance by 2050? *PLoS Med.* 13:e1002184. doi: 10.1371/journal.pmed.1002184

de Wit, W., and Bigaud, N. (2019). *No Plastic in Nature: Assessing Plastic Ingestion from Nature to People*. Gland: World Wide Fund For Nature.

De-la-Torre, G. E., Dioses-Salinas, D. C., Pizarro-Ortega, C. I., and Saldaña-Serrano, M. (2020). Global distribution of two polystyrene-derived contaminants in the marine environment: a review. *Mar. Pollut. Bull.* 161:111729. doi: 10.1016/j.marpolbul.2020.111729

Dunham, S. J. B., Ellis, J. F., Li, B., and Sweedler, J. V. (2017). Mass spectrometry imaging of complex microbial communities. *Acc. Chem. Res.* 50, 96–104. doi: 10.1021/acs.accounts.6b00503

Dussud, C., Hudec, C., George, M., Fabre, P., Higgs, P., Bruzaud, S., et al. (2018). Colonization of non-biodegradable and biodegradable plastics by marine microorganisms. *Front. Microbiol.* 9:1571. doi: 10.3389/fmicb.2018.01571

El-Halfawy, O. M., and Valvano, M. A. (2015). Antimicrobial heteroresistance: an emerging field in need of clarity. *Clin. Microbiol. Rev.* 28, 191–207. doi: 10.1128/CMR.00058-14

European Chemical Agency (2019). *Plastic Additives Initiative Supplementary Information on Scope and Methods*. Available online at: https://echa.europa.eu/echa-irs (accessed August 14, 2020).

Frias, J. P. G. L., and Nash, R. (2019). Microplastics: finding a consensus on the definition. *Mar. Pollut. Bull.* 138, 145–147. doi: 10.1016/j.marpolbul.2018.11.022.

Fu, W., Min, J., Jiang, W., Li, Y., and Zhang, W. (2020). Separation, characterization and identification of microplastics and nanoplastics in the environment. *Sci. Total Environ.* 721:137561. doi: 10.1016/j.scitotenv.2020.137561

Geier, B., Sogin, E. M., and Michellod, D. (2020). Spatial metabolomics of in situ host–microbe interactions at the micrometre scale. *Nat. Microbiol.* 5, 498–510. doi: 10.1038/s41564-019-0664-6

Gigault, J., ter Halle, A., Baudrimont, M., Pascal, P.-Y., Gauffre, F., Phi, T.-L., et al. (2018). Current opinion: what is a nanoplastic? *Environ. Pollut.* 235, 1030–1034. doi: 10.1016/j.envpol.2018.01.024

Gimiliani, G. T., Fornari, M., Redígolo, M. M., Willian Vega Bustillos, J. O., Moledo de Souza Abessa, D., and Faustino Pires, M. A. (2020). Simple and cost-effective method for microplastic quantification in estuarine sediment: a case study of the Santos and São Vicente Estuarine System. *Case Stud. Chem. Environ. Eng.* 2:100020. doi: 10.1016/j.cscee.2020.100020

Guo, X., Chen, C., and Wang, J. (2019). Sorption of sulfamethoxazole onto six types of microplastics. *Chemosphere* 228, 300–308. doi: 10.1016/j.chemosphere.2019.04.155

Guo, X., and Wang, J. (2019). Sorption of antibiotics onto aged microplastics in freshwater and seawater. *Mar. Pollut. Bull.* 149:110511. doi: 10.1016/j.marpolbul.2019.110511

Hahladakis, J. N., Velis, C. A., Weber, R., Iacovidou, E., and Purnell, P. (2018). An overview of chemical additives present in plastics: migration, release, fate and environmental impact during their use, disposal and recycling. *J. Hazard. Mater.* 344, 179–199. doi: 10.1016/j.jhazmat.2017.10.014

Hinzke, T., Kouris, A., Hughes, R.-A., Strous, M., and Kleiner, M. (2019). More is not always better: evaluation of 1D and 2D-LC-MS/MS methods for metaproteomics. *Front. Microbiol.* 10:238. doi: 10.3389/fmicb.2019.00238

Hossain, M. R., Jiang, M., Wei, Q., and Leff, L. G. (2019). Microplastic surface properties affect bacterial colonization in freshwater. *J. Basic Microbiol.* 59, 54–61. doi: 10.1002/jobm.201800174

Imhof, H. K., Laforsch, C., Wiesheu, A. C., Schmid, J., Anger, P. M., Niessner, R., et al. (2016). Pigments and plastic in limnetic ecosystems: a qualitative and quantitative study on microparticles of different size classes. *Water Res.* 98, 64–74. doi: 10.1016/j.watres.2016.03.015

Imran, M., Das, K. R., and Naik, M. M. (2019). Co-selection of multi-antibiotic resistance in bacterial pathogens in metal and microplastic contaminated environments: an emerging health threat. *Chemosphere* 215, 846–857. doi: 10.1016/J.CHEMOSPHERE.2018.10.114

Interagency Coordination Group on Antimicrobial Resistance (2019). *No Time to Wait: Securing the Future from Drug-Resistant Infections Report to the Secretary-General of the United Nations*. Available online at: https://www.who.int/antimicrobial-resistance/interagency-coordination-group/final-report/en/ (accessed April 3, 2020).

Jonkman, J., Brown, C. M., Wright, G. D., Anderson, K. I., and North, A. J. (2020). Tutorial: guidance for quantitative confocal microscopy. *Nat. Protoc.* 15, 1585–1611. doi: 10.1038/s41596-020-0313-9

Kaile, N., Lindivat, M., Elio, J., Thuestad, G., Crowley, Q. G., and Hoell, I. A. (2020). Preliminary results from detection of microplastics in liquid samples using flow cytometry. *Front. Mar. Sci.* 7:856. doi: 10.3389/fmars.2020.552688

Kaminski, T. S., Scheler, O., and Garstecki, P. (2016). Droplet microfluidics for microbiology: techniques, applications and challenges. *Lab Chip* 16, 2168–2187. doi: 10.1039/C6LC00367B

Keswani, A., Oliver, D. M., Gutierrez, T., and Quilliam, R. S. (2016). Microbial hitchhikers on marine plastic debris: human exposure risks at bathing waters and beach environments. *Mar. Environ. Res.* 118, 10–19. doi: 10.1016/j.marenvres.2016.04.006

Knapp, C. W., Callan, A. C., Aitken, B., Shearn, R., Koenders, A., and Hinwood, A. (2017). Relationship between antibiotic resistance genes and metals in residential soil samples from Western Australia. *Environ. Sci. Pollut. Res.* 24, 2484–2494. doi: 10.1007/s11356-016-7997-y

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

Kuchina, A., Brettner, L. M., Paleologu, L., Roco, C. M., Rosenberg, A. B., Carignano, A., et al. (2020). Microbial single-cell RNA sequencing by split-pool barcoding. *Science* 371:eaba5257. doi: 10.1126/SCIENCE.ABA5257

Laganà, P., Caruso, G., Corsi, I., Bergami, E., Venuti, V., Majolino, D., et al. (2019). Do plastics serve as a possible vector for the spread of antibiotic resistance? First insights from bacteria associated to a polystyrene piece from King George Island (Antarctica). *Int. J. Hyg. Environ. Health* 222, 89–100. doi: 10.1016/J.IJHEH.2018.08.009

Learman, D. R., Ahmad, Z., Brookshier, A., Henson, M. W., Hewitt, V., Lis, A., et al. (2019). Comparative genomics of 16 Microbacterium spp. that tolerate multiple heavy metals and antibiotics. *PeerJ* 6:e6258. doi: 10.7717/peerj.6258

Lehner, R., Weder, C., Petri-Fink, A., and Rothen-Rutishauser, B. (2019). Emergence of nanoplastic in the environment and possible impact on human health. *Environ. Sci. Technol.* 53, 1748–1765. doi: 10.1021/acs.est.8b05512

Leng, F., Li, Y., Luo, W., Wei, Q., Jing, Y., Wang, X., et al. (2019). Cloning, expression, and bioinformatics analysis of heavy metal resistance gene afe_1862 from *Acidithiobacillus ferrooxidans* L1 in *Escherichia coli*. *Biol. Trace Elem. Res.* 189, 291–300. doi: 10.1007/s12011-018-1462-3

Li, J., Zhang, K., and Zhang, H. (2018). Adsorption of antibiotics on microplastics. *Environ. Pollut.* 237, 460–467. doi: 10.1016/J.ENVPOL.2018.02.050

Li, L.-G., Xia, Y., and Zhang, T. (2017). Co-occurrence of antibiotic and metal resistance genes revealed in complete genome collection. *ISME J.* 11, 651–662. doi: 10.1038/ismej.2016.155

Li, W., Wang, G., Zhang, S., Fu, Y., Jiang, Y., Yang, X., et al. (2019). An integrated quantitative proteomic and metabolomics approach to reveal the negative regulation mechanism of LamB in antibiotics resistance. *J. Proteomics* 194, 148–159. doi: 10.1016/J.JPROT.2018.11.022

Li, X., Gu, A. Z., Zhang, Y., Xie, B., Li, D., and Chen, J. (2019). Sub-lethal concentrations of heavy metals induce antibiotic resistance via mutagenesis. *J. Hazard. Mater.* 369, 9–16. doi: 10.1016/J.JHAZMAT.2019.02.006

Lö, M. G. J., Imhof, H. K., Ladehoff, M., Lö, L. A., Lorenz, C., Mintenig, S., et al. (2017). Enzymatic purification of microplastics in environmental samples. *Environ. Sci. Technol.* 51, 14283–14292. doi: 10.1021/acs.est.7b03055

Ma, Q., Bücking, H., Hernandez, J. L. G., and Subramanian, S. (2019). Single-cell RNA sequencing of plant-associated bacterial communities. *Front. Microbiol.* 10:2452. doi: 10.3389/fmicb.2019.02452

Mason, S. A., Welch, V. G., and Neratko, J. (2018). Synthetic polymer contamination in bottled water. *Front. Chem.* 6:407. doi: 10.3389/fchem.2018.00407

Medardus, J. J., Molla, B. Z., Nicol, M., Morrow, W. M., Rajala-Schultz, P. J., Kazwala, R., et al. (2014). In-feed use of heavy metal micronutrients in U.S. swine production systems and its role in persistence of multidrug-resistant salmonellae. *Appl. Environ. Microbiol.* 80, 2317–2325. doi: 10.1128/AEM.04283-13

Meier, M. J., Dodge, A. E., Samarajeewa, A. D., and Beaudette, L. A. (2020). Soil exposed to silver nanoparticles reveals significant changes in community structure and altered microbial transcriptional profiles. *Environ. Pollut.* 258:113816. doi: 10.1016/j.envpol.2019.113816

Müller, Y. K., Wernicke, T., Pittroff, M., Witzig, C. S., Storck, F. R., Klinger, J., et al. (2020). Microplastic analysis—are we measuring the same? Results on the first global comparative study for microplastic analysis in a water sample. *Anal. Bioanal. Chem.* 412, 555–560. doi: 10.1007/s00216-019-02311-1

Munier, B., and Bendell, L. I. (2018). Macro and micro plastics sorb and desorb metals and act as a point source of trace metals to coastal ecosystems. *PLoS One* 13:e0191759. doi: 10.1371/journal.pone.0191759

Nguyen, B., Claveau-Mallet, D., Hernandez, L. M., Xu, E. G., Farner, J. M., and Tufenkji, N. (2019). Separation and analysis of microplastics and nanoplastics in complex environmental samples. *Acc. Chem. Res.* 52, 858–866. doi: 10.1021/acs.accounts.8b00602

Nguyen, C. C., Hugie, C. N., Kile, M. L., and Navab-Daneshmand, T. (2019). Association between heavy metals and antibiotic-resistant human pathogens in environmental reservoirs: a review. *Front. Environ. Sci. Eng.* 13:46. doi: 10.1007/s11783-019-1129-0

Nizzetto, L., Futter, M., and Langaas, S. (2016). Are agricultural soils dumps for microplastics of urban origin? *Environ. Sci. Technol.* 50, 10777–10779. doi: 10.1021/acs.est.6b04140

Oberbeckmann, S., Kreikemeyer, B., and Labrenz, M. (2017). Environmental factors support the formation of specific bacterial assemblages on microplastics. *Front. Microbiol.* 8:2709. doi: 10.3389/FMICB.2017.02709

Oberbeckmann, S., Löder, M. G. J., and Labrenz, M. (2015). Marine microplastic-associated biofilms – a review. *Environ. Chem.* 12, 551–562. doi: 10.1071/EN15069

O'Neill, J. (2016). *Tackling Drug-Resistant Infections Globally: Final Report and Recommendations the Review on Antimicrobial Resistance*. Available online at: https://amr-review.org/ (accessed April 3, 2020).

Pathak, A., Jaswal, R., and Chauhan, A. (2020). Genomic characterization of a mercury resistant Arthrobacter sp. H-02-3 reveals the presence heavy metal and antibiotic resistance determinants. *Front. Microbiol.* 10:3039. doi: 10.3389/fmicb.2019.03039

Pauly, J. L., Stegmeier, S. J., Allaart, H. A., Cheney, R. T., Zhang, P. J., Mayer, A. G., et al. (1998). Inhaled cellulosic and plastic fibers found in human lung tissue. *Cancer Epidemiol. Biomarkers Prev.* 7, 419–428.

Petriz, B. A., and Franco, O. L. (2017). Metaproteomics as a complementary approach to gut microbiota in health and disease. *Front. Chem.* 5:4. doi: 10.3389/fchem.2017.00004

Pousti, M., Zarabadi, M. P., Abbaszadeh Amirdehi, M., Paquet-Mercier, F., and Greener, J. (2019). Microfluidic bioanalytical flow cells for biofilm studies: a review. *Analyst* 144, 68–86. doi: 10.1039/c8an01526k

Prunier, J., Maurice, L., Perez, E., Gigault, J., Pierson Wickmann, A. C., Davranche, M., et al. (2019). Trace metals in polyethylene debris from the North Atlantic subtropical gyre. *Environ. Pollut.* 245, 371–379. doi: 10.1016/j.envpol.2018.10.043

Qin, W., Zhao, J., Yu, X., Liu, X., Chu, X., Tian, J., et al. (2019). Improving cadmium resistance in *Escherichia coli* through continuous genome evolution. *Front. Microbiol.* 10:278. doi: 10.3389/fmicb.2019.00278

Rochman, C. M., Hentschel, B. T., and Teh, S. J. (2014). Long-term sorption of metals is similar among plastic types: implications for plastic debris in aquatic environments. *PLoS One* 9:85433. doi: 10.1371/journal.pone.0085433

Scheler, O., Makuch, K., Debski, P. R., Horka, M., Ruszczak, A., Pacocha, N., et al. (2020). Droplet-based digital antibiotic susceptibility screen reveals single-cell clonal heteroresistance in an isogenic bacterial population. *Sci. Rep.* 10:3282. doi: 10.1038/s41598-020-60381-z

Schmiedgruber, M., Hufenus, R., and Mitrano, D. M. (2019). Mechanistic understanding of microplastic fiber fate and sampling strategies: synthesis and utility of metal doped polyester fibers. *Water Res.* 155, 423–430. doi: 10.1016/j.watres.2019.02.044

Schwabl, P., Koppel, S., Konigshofer, P., Bucsics, T., Trauner, M., Reiberger, T., et al. (2019). Detection of various microplastics in human stool: a prospective case series. *Ann. Intern. Med.* 171, 453–457. doi: 10.7326/M19-0618

Secchi, E., Vitale, A., Miño, G. L., Kantsler, V., Eberl, L., Rusconi, R., et al. (2020). The effect of flow on swimming bacteria controls the initial colonization of curved surfaces. *Nat. Commun.* 11:2851. doi: 10.1038/s41467-020-16620-y

Seiler, C., and Berendonk, T. U. (2012). Heavy metal driven co-selection of antibiotic resistance in soil and water bodies impacted by agriculture and aquaculture. *Front. Microbiol.* 3:399. doi: 10.3389/fmicb.2012.00399

Staley, C., Johnson, D., Gould, T. J., Wang, P., Phillips, J., Cotner, J. B., et al. (2015). Frequencies of heavy metal resistance are associated with land cover type in the Upper Mississippi River. *Sci. Total Environ.* 511, 461–468. doi: 10.1016/j.scitotenv.2014.12.069

Sullivan, G. L., Gallardo, J. D., Jones, E. W., Hollliman, P. J., Watson, T. M., and Sarp, S. (2020). Detection of trace sub-micron (nano) plastics in water samples using pyrolysis-gas chromatography time of flight mass spectrometry (PY-GCToF). *Chemosphere* 249:126179. doi: 10.1016/j.chemosphere.2020.126179

Van Raamsdonk, L. W. D., Van Der Zande, M., Koelmans, A. A., Hoogenboom, P. L. A., Peters, R. J. B., Groot, M. J., et al. (2020). Current insights into monitoring, bioaccumulation, and potential health effects of microplastics present in the food chain. *Foods* 9:72. doi: 10.3390/foods9010072

Viršek, M. K., Lovšin, M. N., Koren, Š, Kržan, A., and Peterlin, M. (2017). Microplastics as a vector for the transport of the bacterial fish pathogen species *Aeromonas salmonicida*. *Mar. Pollut. Bull.* 125, 301–309. doi: 10.1016/J.MARPOLBUL.2017.08.024

Wan, T., Lu, S., Cheng, W., Ren, J., Wang, M., Hu, B., et al. (2019). A spectroscopic and theoretical investigation of interaction mechanisms of tetracycline and polystyrene nanospheres under different conditions. *Environ. Pollut.* 249, 398–405. doi: 10.1016/j.envpol.2019.03.049

WHO (2018). *High Levels of Antibiotic Resistance Found Worldwide, New Data Shows*. Available online at: https://www.who.int/news-room/detail/29-01-2018-high-levels-of-antibiotic-resistance-found-worldwide-new-data-shows (accessed February 19, 2019).

Wilkinson, A., and Boxall, J. (2019). "*The first global study of pharmaceutical contamination in riverine environments*," in *Proceedings of the SETAC Europe 29th Annual Meeting*, Helsinki.

Wright, R. J., Langille, M. G. I., and Walker, T. R. (2020). Food or just a free ride? A meta-analysis reveals the global diversity of the Plastisphere. *ISME J.* 15, 789–806. doi: 10.1038/s41396-020-00814-9

Yang, Y., Liu, G., Song, W., Ye, C., Lin, H., Li, Z., et al. (2019). Plastics in the marine environment are reservoirs for antibiotic and metal resistance genes. *Environ. Int.* 123, 79–86. doi: 10.1016/J.ENVINT.2018.11.061

Yawata, Y., Nguyen, J., Stocker, R., and Rusconi, R. (2016). Microfluidic studies of biofilm formation in dynamic environments. *J. Bacteriol.* 198, 2589–2595. doi: 10.1128/JB.00118-16

Yu, F., Li, Y., Huang, G., Yang, C., Chen, C., Zhou, T., et al. (2020a). Adsorption behavior of the antibiotic levofloxacin on microplastics in the presence of different heavy metals in an aqueous solution. *Chemosphere* 260:127650. doi: 10.1016/j.chemosphere.2020.127650

Yu, F., Yang, C., Huang, G., Zhou, T., Zhao, Y., and Ma, J. (2020b). Interfacial interaction between diverse microplastics and tetracycline by adsorption in an aqueous solution. *Sci. Total Environ.* 721:137729. doi: 10.1016/j.scitotenv.2020.137729

Zettler, E. R., Mincer, T. J., and Amaral-Zettler, L. A. (2013). Life in the "plastisphere": microbial communities on plastic marine debris. *Environ. Sci. Technol.* 47, 7137–7146. doi: 10.1021/es401288x

Zhang, H., Liu, F. F., Wang, S. C., Huang, T. Y., Li, M. R., Zhu, Z. L., et al. (2020). Sorption of fluoroquinolones to nanoplastics as affected by surface functionalization and solution chemistry. *Environ. Pollut.* 262:114347. doi: 10.1016/j.envpol.2020.114347

Zhang, H., Wang, J., Zhou, B., Zhou, Y., Dai, Z., Zhou, Q., et al. (2018). Enhanced adsorption of oxytetracycline to weathered microplastic polystyrene: kinetics, isotherms and influencing factors. *Environ. Pollut.* 243, 1550–1557. doi: 10.1016/j.envpol.2018.09.122

Zhang, Y., Lu, J., Wu, J., Wang, J., and Luo, Y. (2020). Potential risks of microplastics combined with superbugs: enrichment of antibiotic resistant

bacteria on the surface of microplastics in mariculture system. *Ecotoxicol. Environ. Saf.* 187:109852. doi: 10.1016/j.ecoenv.2019.109852

Zhao, Y., Cocerva, T., Cox, S., Tardif, S., Su, J.-Q., Zhu, Y.-G., et al. (2019). Evidence for co-selection of antibiotic resistance genes and mobile genetic elements in metal polluted urban soils. *Sci. Total Environ.* 656, 512–520. doi: 10.1016/J.SCITOTENV.2018.11.372

Zhao, Y., Su, J. Q., An, X. L., Huang, F. Y., Rensing, C., Brandt, K. K., et al. (2018). Feed additives shift gut microbiota and enrich antibiotic resistance in swine gut. *Sci. Total Environ.* 621, 1224–1232. doi: 10.1016/j.scitotenv.2017.10.106

Zhou, Q., Yang, N., Li, Y., Ren, B., Ding, X., Bian, H., et al. (2020). Total concentrations and sources of heavy metal pollution in global river and lake water bodies from 1972 to 2017. *Glob. Ecol. Conserv.* 22:e00925. doi: 10.1016/j.gecco.2020.e00925

Zhu, F., Zhu, C., Wang, C., and Gu, C. (2019). Occurrence and ecological impacts of microplastics in soil systems: a review. *Bull. Environ. Contam. Toxicol.* 102, 741–749. doi: 10.1007/s00128-019-02623-z

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Antibiotics and Antibiotic Resistance Genes in Animal Manure – Consequences of Its Application in Agriculture

*Magdalena Zalewska, Aleksandra Błażejewska, Agnieszka Czapko and Magdalena Popowska**

Department of Bacterial Physiology, Institute of Microbiology, Faculty of Biology, University of Warsaw, Warsaw, Poland

Antibiotic resistance genes (ARGs) are a relatively new type of pollutant. The rise in antibiotic resistance observed recently is closely correlated with the uncontrolled and widespread use of antibiotics in agriculture and the treatment of humans and animals. Resistant bacteria have been identified in soil, animal feces, animal housing (e.g., pens, barns, or pastures), the areas around farms, manure storage facilities, and the guts of farm animals. The selection pressure caused by the irrational use of antibiotics in animal production sectors not only promotes the survival of existing antibiotic-resistant bacteria but also the development of new resistant forms. One of the most critical hot-spots related to the development and dissemination of ARGs is livestock and poultry production. Manure is widely used as a fertilizer thanks to its rich nutrient and organic matter content. However, research indicates that its application may pose a severe threat to human and animal health by facilitating the dissemination of ARGs to arable soil and edible crops. This review examines the pathogens, potentially pathogenic microorganisms and ARGs which may be found in animal manure, and evaluates their effect on human health through their exposure to soil and plant resistomes. It takes a broader view than previous studies of this topic, discussing recent data on antibiotic use in farm animals and the effect of these practices on the composition of animal manure; it also examines how fertilization with animal manure may alter soil and crop microbiomes, and proposes the drivers of such changes and their consequences for human health.

Keywords: antibiotic resistance genes, antibiotic-resistant bacteria, antibiotic use, animal agriculture, fecal matter, manure resistome, soil resistome, plant resistome

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****Correspondence:***
Magdalena Popowska
magdapop@biol.uw.edu.pl

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ANTIBIOTIC USE IN ANIMAL FARMS

The spread of antibiotic-resistant bacteria (ARB) is a growing problem worldwide. In 2017, the most important ARB were included in a list published by the World Health Organization (WHO). The ARB families that were believed to pose the greatest threat to human health were categorized as critical, high or medium priority, depending on the need to seek effective treatment options.

It has been estimated that antimicrobial resistance (AMR) is responsible for 25,000 deaths/year in the European Union (EU), and 700,000 worldwide. It has also been predicted that by 2050, AMR will be responsible for more deaths than cancer (European Commission, 2018). With the

discovery of penicillin, in 1928, many life-threatening or even lethal diseases became curable, with clear benefits for veterinarians and animal breeders; however, since the 1960s, antibiotics have been widely applied in sub-lethal doses as growth promoters for food-producing animals (Hassan et al., 2018). Such extensive, uncontrolled use may result in the presence of low, sub-inhibitory concentrations in the tissues and guts of treated animals (Lim et al., 2020) and in the environment (Khan et al., 2013). Although the precise complete mechanisms of action remain unclear, it has been hypothesized that when applied in sub-lethal doses, antibiotics stimulate the intestinal synthesis of vitamins, lower the total amount of bacteria in the intestinal tract by reducing competition between microorganism and host for nutrients, inhibits the growth of harmful bacteria, and modifies the microbial metabolism of the rumen (Economou and Gousia, 2015).

However, the chronic application of such sub-therapeutic doses favors the selection of ARB by promoting their growth or introducing *de novo* mutations. One of the first reviews of the effect of sublethal doses of antibiotics on the bacterial community was published by Lorian (1975). The presence of ARB may enhance the transfer of antibiotic-resistant genes (ARGs) between enteric bacteria in the intestinal tract of the host animal, and affect quorum sensing (e.g., azithromycin in *Pseudomonas aeruginosa*, *Staphylococcus aureus,* or *Streptococcus pneumoniae*). In addition, the presence of antibiotics can stimulate biofilm formation and horizontal gene transfer (HGT) in some bacteria; for example, the transfer of azithromycin, ciprofloxacin or tigecycline resistance has been observed in *Enterococcus faecalis* and *P. aeruginosa*. It can also increase the rate of recombination and selected gene expression in the bacterial community, such as ciprofloxacin, norfloxacin, rifampicin, gentamycin, and tetracycline in *Escherichia coli*, *P. aeruginosa,* or *S. aureus* (Lorian, 1975). The mechanisms of action of sublethal levels of antibiotics on bacteria are described in detail by Andersson and Hughes (2014). In food-producing animals, antibiotic use influences the functions of enteric bacteria and can temporarily increase antibiotic resistance in the fecal microbiome (Chee-Sanford et al., 2009; Broom, 2017).

The majority of ARG transmission occurs *via* HGT, i.e., whereby mobile genetic elements (MGE), such as plasmids or transposons coding for ARGs, are exchanged between bacterial species, even those that are not closely related (Redondo-Salvo et al., 2020). The dynamics of transfer depend not only on the presence of positive selection, i.e., antibiotic concentrations in the environment ranging from values much higher than minimal inhibitory concentrations to those several hundred-fold lower, but also the spatial structure of the community and the presence of predators (Cairns et al., 2018). The WHO, World Organization for Animal Health (OIE), and the Food and Agriculture Organization (FAO) have officially stated that the non-human use of different types of antimicrobials may have harmful consequences for human health (So et al., 2015). Although some countries have officially restricted the use of antimicrobials in livestock to only medical purposes (e.g., the EU in 2006 in accordance to 1831/2003/EC legislation), they are still overused on some regions characterized by highly-intensive

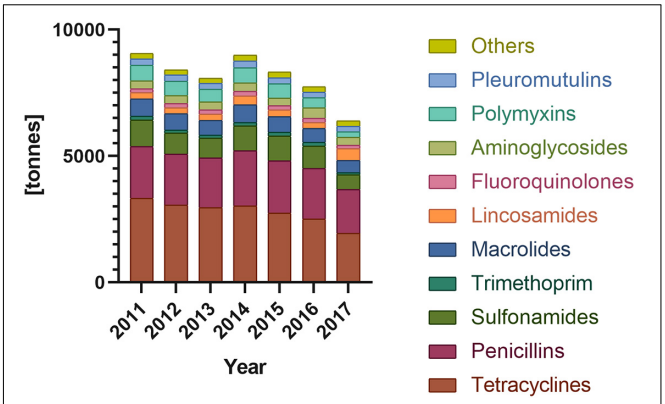

FIGURE 1 | Antibiotic sales for food-producing animals in Europe sort by antibiotic classes (according to ECDC/EFSA/EMA second joint report on the integrated analysis of the consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from humans and food-producing animals, 2017).

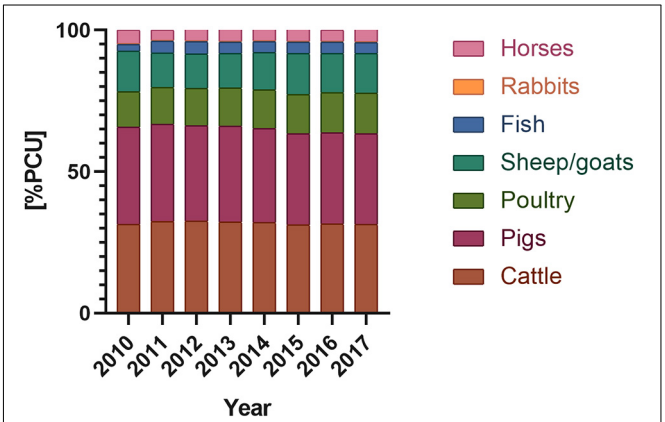

FIGURE 2 | Antimicrobials used in domestic animals (according to according to ECDC/EFSA/EMA second joint report on the integrated analysis of the consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from humans and food-producing animals, 2017) [PCU] – population corrected unit; PCU a technical unit of measurement, used only to estimate sales corrected by the animal population in the individual countries; 1 PCU = 1 kg of different categories of livestock and slaughtered animals.

livestock production, such as the United States, Russia, India, China, and South Africa. In the US, antimicrobial treatment in food-producing animals has been estimated to account for approximately 80% of total annual use; the vast majority of these antimicrobials are believed to include essential human medicines used for the treatment of common infections, or which are necessary for performing surgeries, organ transplantations or chemotherapy in humans (Van Boeckel et al., 2015) (**Figures 1**, **2**).

A study of antimicrobial use in Southeast Asia indicated that, excluding feed, farmers in this region administered 46 mg of different antimicrobial compounds per kg of live pigs and 52–276 mg per kilogram of live chickens per year (Nhung et al., 2016). In addition, a 2014 study in the EU found the average

intake of antimicrobials to be 123.7 mg/kg in humans and 151.5 mg/kg in animals (European Centre for Disease Prevention, and Control [ECDC], European Food Safety Authority [EFSA], and European Medicines Agency [EMA], 2017).

The use of antibiotics for non-medical purposes, or for treating entire extensive animal production facilities when a single animal falls ill, has resulted in a growth in ARB in the animal production chain. It has been estimated that 84% of the antimicrobial compounds administered in chicken farms in the Mekong Delta region were given only for prophylactic purposes; of these, the most common were penicillins, lincosamides, quinolones, and combinations of sulfonamides with trimethoprim (Nhung et al., 2016). Elsewhere, the most common antibiotics found to be present in pig, beef and turkey manure were tetracyclines (oxytetracycline and chlortetracycline), tylosin, sulfamethazine, amprolium, monensin, virginiamycin, penicillin, and nicarbazin (De Liguoro et al., 2003; Kumar et al., 2005).

The Food and Drug Administration (FDA) has approved 18 classes of antimicrobials for use in food-producing animals (Medicine, 2019). However, the WHO reports that 57% of all antimicrobials used in animal production are essential for human medicine, including amoxicillin, colistin, tetracyclines, neomycin, lincomycin, and bacitracin. Of the FDA classes, the most widely used in human medicine worldwide are the penicillins, macrolides, and fluoroquinolones, while the tetracyclines, penicillins, and sulfonamides were the highest selling for food-producing animals.

In the EU/EEA (the European Union/European Economic Area) carbapenems and monobactams are not approved for use in food-producing animals, and pleuromutilins are not authorized for use in humans. In addition, higher total consumption of the penicillins, cephalosporins (all generations) and fluoroquinolones was demonstrated by humans than food-producing animals (European Centre for Disease Prevention, and Control [ECDC], European Food Safety Authority [EFSA], and European Medicines Agency [EMA], 2017). A complete list of antibiotics allowed for use in food production animals, divided according to veterinary importance (Critically Important, Highly Important and Important) was first prepared in 2007 by the OIE in consultation with the FAO and WHO, and is constantly being revised; the most recent update was released in May 2018 by the World Assembly of OIE Delegates (International Office of Epizootics, 2015; Góchez et al., 2019).

Although environmental factors have long been known to affect human health, the relationship between the spread of AMR and antibiotic use in agriculture was first recorded in the 1969 Swann report (Soulsby, 2007). Even though antibiotics and ARGs/ARB are ancient and naturally-occurring substances, they are nevertheless considered emerging pollutants associated closely with human-impacted environments (He et al., 2019; Zhang et al., 2019). The global debate on the scale of the threat posed by antimicrobial use in livestock was intensified by the recent finding of a plasmid-mediated colistin resistance gene (*mcr-1*) in commensal *E. coli* from pigs, pork products and humans in China (Liu et al., 2016).

In 2008, the FAO, WHO, OIE, the United Nations Children's Fund (UNICEF), United Nations System Influenza Coordination, and the World Bank developed a holistic approach entitled "Contributing to One World, One Health-A Strategic Framework for Reducing Risks of Infectious Diseases at the Animal-Human-Ecosystems Interface" including a recommendation for a One Health action plan for global health. In 2017, the European Commission adopted the WHO One Health framework, naming AMR as one of the main concerns. The proposed approach holistically regards the health of humans, animals, and different environmental compartments, such as water or soil, as a single network, where one part inseparably depends on another. This framework has three major aims: (1) making the EU a region with best practice in AMR management, (2) boosting research, development, and innovation in AMR, and (3) shaping the global approach to addressing AMR. Each aim has been divided into smaller tasks that address existing gaps in knowledge concerning AMR in the environment, such as the role of the environment in ARGs/ARB transmission, the routes of ARGs/ARB transmission, the most critical hot spots, human health risk assessment, and the establishment of tracking and detection systems.

The current limitations on antimicrobial use in livestock are explained in the document "Council conclusions on the next steps under a One Health approach to combat AMR" (2016). In addition, a good review of the existing global policies on antibiotics use in livestock is provided by Walia et al. (2019).

THE USE OF ANTIBIOTICS IN THE POULTRY INDUSTRY AND ITS EFFECT ON THE POULTRY MANURE RESISTOME

One of the most significant sectors of meat and egg production worldwide is that of poultry production. In 2018, global poultry population (laying hens and broilers) had reached 23.7 billion, growing from 22.85 billion in 2017 and 14.38 billion in 2000. The largest chicken producer in 2018 was China, with 5274.48 million animals, followed by Indonesia with 2384.15 million, and the US with 1973.38 million. China is also the largest egg producer globally, with 529 billion eggs, followed by the US with 106.7 billion. Moreover, in 2018, in the US alone, 22.6 million tons of broiler meat was produced. It has been predicted that in 2020, the global export of poultry meat will increase by 3.7% to reach 13.8 million tones. Thailand, Brazil, Turkey, Ukraine, the US, and the EU are the leading poultry exporters, from whom almost 80% of exported poultry meat originates (FAOSTAT, 2020).

Since the discovery of antibiotics, strict biosecurity guidelines and prevention techniques have been employed by the poultry and meat production industry to not only increase its production efficiency and eliminate the risk of zoonotic diseases, but enhance the growth and weight of the broilers (Collett et al., 2019). Although the use of antibiotics is strongly regulated in food-producing animals in the EU and the US (European Commission, 2005; World Health Organization and WHO Advisory Group on Integrated Surveillance of Antimicrobial Resistance (AGISAR), 2017), they are often used for disease prevention in many poultry

exporting countries, such as Brazil and China (Roth et al., 2019). A few classes of antibiotics are approved as additions to animal fodder in Canada, such as bambermycin, bacitracin, salinomycin, penicillin, virginiamycin, and chlortetracycline (Diarra and Malouin, 2014).

In addition, in a 2017 study of eight farms in Thailand, where 14,000 3 kg broilers were raised, it was estimate that 303 mg of antibiotics were used per chicken as routine prophylaxis; this mixture included amoxicillin, colistin, doxycycline, oxytetracycline, and tilmicosin. According to the WHO, amoxicillin and colistin are critically important for human medicine, with colistin being considered the primary therapeutic option to treat diseases caused by resistant gram-negative bacteria, particularly enterobacteria (Wongsuvan et al., 2018).

In survey-based studies in Ghana in 2016, farmers from 400 poultry farms reported using antibiotics for prophylactic purposes, including penicillins (26.22%), tetracyclines (24.13%), and aminoglycosides (20.51%) (Andoh et al., 2016).

Besides growth promotion and disease prevention, antibiotics are also prescribed by veterinarians for treating illnesses. In 2015, a total of 8361 tons of antimicrobial agents were sold for veterinary use in the EU (European Medicines Agency, 2017).

Tetracyclines, with their broad spectrum of action, are one of the most frequently-used antimicrobials in the poultry industry, not only for prophylaxis or antibacterial therapy but also as growth promoters (Ljubojević et al., 2017). Interestingly, while tetracyclines constitute two-thirds of the antibiotics applied to animals in the US (Gonzalez Ronquillo and Angeles Hernandez, 2017), they accounted for less than 40% in the EU between 2015 and 2017 (OIE, 2018). However, the tetracyclines are poorly absorbed by the animal, and their active residues can concentrate in meat, urine, and feces. A study of broiler farms in Egypt, found high levels of chlortetracycline and oxytetracycline in chicken litter and droppings, these values being, respectively, 6.05 and 2.47 μg/g for chlortetracycline and 5.9 and 1.33 μg/g for oxytetracycline. Slightly lower amounts of tetracycline and doxycycline were found: 1.9 μg/g in litter and 0.46 μg/g in droppings for tetracycline, and 0.87 μg/g and 0.02 μg/g for doxycycline. More importantly, the researchers report the presence of three tetracycline resistance genes (*tetM*, *tetW*, *tetQ*) in the intestinal contents: *tetW* was observed in all analyzed samples, *tetQ* in 42% of them, and *tetM* in only 17% (Mahmoud and Abdel-Mohsein, 2019).

Despite being banned as growth promoters in the early 1970s (EC directive 70/524), the total consumption of tetracyclines in food-producing animals in Europe was still estimated at 3138 tons in 2012. Carballo et al. (2016) report that oxytetracycline was present in 37% of tested manure samples, with a maximum concentration of 0.88 mg/kg, and doxycycline in one sample, with a concentration of 0.53 mg/kg. The existence of antibiotic residues in the environment creates on-site selection pressure, may result in the presence of ARB, which are often isolated from manure samples. In Nigeria, five potential human pathogens were isolated from chicken manure, such as *Salmonella typhi*, *E. coli*, *Shigella dysenteriae*, *S. aureus*, and *Aeromonas hydrophila*, all of which were resistant to tetracycline (Omojowo and Omojasola, 2013).

Arguably, a greater threat to human and animal health is posed by multidrug-resistant (MDR) bacteria, with an alarming increase being observed in the number of MDR strains isolated from the poultry meat production chain (Afridi et al., 2020). Unusually high levels of MDR bacteria, especially those of the *Enterobacteriaceae*, have been isolated from poultry. Portuguese studies based on livestock manure taken from pig, dairy and poultry farms and slaughterhouses found the highest level of resistance to tetracycline, trimethoprim/sulfamethoxazole, chloramphenicol, and amoxicillin/clavulanic acid in strains isolated from poultry farms. In addition, the highest prevalence of ciprofloxacin-resistant bacteria was observed in poultry, which may be related to the growing use of ciprofloxacin to treat infections. In addition, although MDR bacteria were isolated from all samples, the highest number of strains resistant to seven or more antibiotics were isolated from poultry samples. A number of chloramphenicol, quinolone, tetracycline, and sulfonamide resistance genes were identified (Amador et al., 2019).

A similar trend was also noted in Belgium. A study of AMR in commensal *E. coli* isolated from veal calves, young beef cattle, broiler chickens, and slaughtered pigs found the highest number of isolated MDR bacteria to be present in broiler chickens (Hanon et al., 2015).

A high prevalence of *E. coli* and *Klebsiella pneumoniae* resistant to β-lactams with ESBL (Extended-Spectrum Beta-Lactamase) and AmpC (clinically important β-lactamases; cephalosporinases) phenotypes was also identified in a study of MDR gram-negative bacteria isolated from poultry feces kept on farms in the Lebanon. The real-time PCR assay performed on 112 strains of ESBL producers detected bla_{SHV} in 20% of strains, bla_{TEM} in 89%, and $bla_{\mathrm{CTX\text{-}M}}$ in 53%. In addition, more than half (66%) of the isolated ESBL/AmpC strains were also resistant to gentamicin, but only one AmpC β-lactamase gene (bla_{CMY}) was found among all samples (Dandachi et al., 2018).

Resistance to extended spectrum cephalosporins (ESBL genes) has also been observed among *Salmonella* serovars. This could have particularly serious consequences, as these bacteria are key etiological factors in diseases among poultry, and may also cause severe illness in humans; the acquisition of antibiotic resistance may significantly limit the range of treatment options (Büdel et al., 2020). Rayamajhi et al. (2010) report the presence of ampicillin-resistant *Salmonella* isolates in poultry feces, the environment and egg yolks on poultry farms in South Korea (**Table 1**). Among all antibiotics tested, resistance was most commonly observed for sulfamethoxazole (74.7%) and nalidixic acid (63.7%), with streptomycin (38.5%), tetracycline (28.6%), and amoxicillin resistance (18.7%) being less common. Furthermore, the $bla_{\mathrm{TEM\text{-}1}}$ gene was present in all ampicillin-resistant strains and the $bla_{\mathrm{DHA\text{-}1}}$ gene in stains with lower susceptibility to cefoxitin, as indicated by β-lactamase production ability. A 2019 study from Kenya found that almost 50% of *Salmonella* samples isolated from manure from broilers and layers were resistant to amoxicillin. In addition, 28% of *Salmonella* strains were resistant to co-trimoxazole, 11%, to tetracycline and 6% to streptomycin. A search for ARGs revealed the presence of bla_{TEM} in 46%

TABLE 1 | Antibiotic resistance bacteria and genes in poultry manure.

Bacteria species	Phenotype	Resistance genes	Source	Country	References
Poultry manure					
Salmonella spp.	Extended spectrum cephalosporins	bla_{TEM-1}, bla_{DHA-1}	Feces, eggshell, dead egg yolk, cloaca, liver, water, environmental dust	South Korea	Rayamajhi et al., 2010
Escherichia coli		bla_{TEM}, bla_{SHV}, bla_{CTX-M}	Fecal swabs	Lebanon	Dandachi et al., 2018
Klebsiella spp.					
Enterobacteriaceae	Quinolones (CIP)	*qnrB, qnrC, qnrD, qnrS*	Poultry manure	Portugal	Amador et al., 2019
	Tetracyclines (TET)	*tetA, tetB, tetC, tetE, tetK, tetL, tetM, tetO*			
	Sulfonamides	*sul1, sul2, sul3*			
Enterococcus spp.	Vancomycin (VRE)	*vanA*	Poultry manure	Greece	Tzavaras et al., 2012
Enterococcus spp.	Macrolides (Erythromycin)	*ermB, ermA, mrsC*	Poultry manure	United States	Graham et al., 2009
Staphylococcus spp.	Macrolides (Erythromycin)	*ermA, mrsA/B*			
Staphylococcus spp.	MRSA	*mecA; mecR1; mecI*	Poultry manure	South Korea	Lee, 2006

of all isolated bacteria with the bla_{CTX-M} gene present in 18% (Langata et al., 2019).

Together with the high prevalence of the MDR strains among the *Enterobacteriaceae* family, the spread of Vancomycin-Resistant Enterococci (VRE) is widely regarded as one of the greatest threats to human health care. A key factor in the spread of vancomycin resistance is believed to involve a glycopeptide antibiotic called avoparcin, which has been applied in animal husbandry as a growth promoter. Although its use in livestock was banned in the EU early in 1997 (EC directive 97/6/EC), relatively high numbers of VRE are still observed in poultry meat production in the EU, and this can be considered a potential reservoir of vancomycin resistance. One study in Greece found that approximately 14.4% of the *E. faecalis* strains isolated from broilers and poultry fecal samples showed vancomycin resistance, and isolated strains demonstrated resistance to several other antibiotics, such as tetracycline (100%), erythromycin (54.4%), ampicillin (16.5%), and ciprofloxacin (30.4%). Analyses have detected the *vanA* gene in 14.4% of fecal samples from poultry and the *vanC* gene in 8.2% (**Table 1**) (Tzavaras et al., 2012).

Together with the emergence of VRE strains, the preservation of resistant gram-positive bacteria in stored poultry manure is also of great concern. One study by Graham et al. (2009) examined the fate of antibiotic resistant *Enterococcus* spp. and *Staphylococcus* spp. and the persistence of ARGs in litter after a 120-day storage period (**Table 1**). The findings revealed the presence of quinupristin-dalfopristin resistance in 11% of the isolated *Staphylococcus* strains and erythromycin resistance in 57%; in addition, 31% of *E. faecium* were resistant to both antibiotics. The most commonly identified ARGs were *ermB* in *Enterococcus* spp. and *ermA* in *Staphylococcus* spp. Moreover, the *msrC* gene was found in one *Enterococcus* isolate and this was found to be homologous with *msrA* found in *Staphylococcus* spp. It should be stressed that ARGs were found in all collected samples during a 120-day storage period or even later (Graham et al., 2009).

Staphylococcus spp. are considered typical of the chicken microflora. Although they are harmless in most cases, they are known to act as opportunistic pathogens, and can cause severe infections, particularly the bacteria that harbor ARGs. Methicillin-resistant *S. aureus* (MRSA) isolated from poultry may pose a considerable risk for human health, as a high chance of zoonotic transfer exists during the breeding or fattening period (Zaheer et al., 2017). MRSA bacteria were found to be present in almost 90% of fecal chicken swabs taken from small poultry farms in Nigeria, and more worryingly, in 83.3% of nasal swabs taken from personnel working on the farms (Journals and Oke, 2013). MRSA bacteria harboring *mecA* gene were also successfully isolated from chicken samples in South Korea (Lee, 2006) (**Table 1**).

Subtherapeutic doses of antibiotics used in poultry farms exert selection pressure on the bacteria of the intestinal flora of the animals, thus encouraging the spread of ARB into the environment (Suresh et al., 2018). Being located on MGEs such as plasmids, transposons and integrons, ARGs can easily be transmitted between bacteria by HGT. A good example of such spread is that of plasmid-mediated colistin resistance; this can represent a considerable risk for human health, as colistin is one of the strategic drugs used against carbapenem-resistant *Enterobacteriaceae* (Wang et al., 2018). Unfortunately, because of the long history of colistin usage in animals and the complete lack of surveillance until 2014 (Commission Implementing Decision 2013/652/EU), it is difficult to track colistin usage and detect resistance (Kempf et al., 2016). However, what is certain is that the first plasmid carrying the colistin resistance gene *mcr-1* was isolated from *Enterobacteriaceae* bacteria from a pig fattening facility (Liu et al., 2016). In Brazil, of the 280 *Enterobacteriaceae* strains isolated from chicken fecal samples in a study from 2003 to 2015, 113 were found to be colistin resistant; the *mcr-1* gene was identified in 14 *E. coli* strains (Fernandes et al., 2016). Elsewhere, the *mcr-1* gene was detected in eight of 12 (∼66%) tested chicken manure samples (Gao et al., 2019); interestingly, the presence of detected *mcr-1* genes was effectively reduced by 90% during a 30-day composting process.

Class 1 and 2 integrons are also commonly found among MDR *Enterobacteriaceae* isolates, which may indicate that resistance is mediated by plasmid transfer. A study of the *int1* and *int2*

genes in clinical and livestock samples, including poultry, by Goldstein et al. (2001) found the *int1* gene to be present in 79% of *Salmonella* isolates sampled from chickens and *int2* in 2%; however, the *int2* gene was present in only 14% of *E. coli* isolates. Furthermore, a study of 55 *E. coli* strains in broilers and layers in Kenya detected *int1* genes in 26% of isolates, with most coming from β-lactamase positive isolates (Langata et al., 2019).

A full understanding of the development and transmission of AMR in poultry production is essential for creating effective risk management strategies for preventing the spread of resistant bacteria from animals to humans and providing safe sources of food.

THE USE OF ANTIBIOTICS IN THE CATTLE INDUSTRY AND ITS EFFECT ON THE CATTLE MANURE RESISTOME

Global bovine meat production reached 71.1 million tons in 2018, with the largest producers being Brazil, the US, Argentina, the EU, and Australia (FAOSTAT, 2020; Meat Market Review). In addition, there are more than 264 million dairy cows worldwide, producing nearly 600 million tons of milk every year. India has the highest number of dairy cows globally, with over 40 million cows, followed by the US, China, Pakistan, and Brazil (FAOSTAT).

The use of antibiotics in dairy cattle has the potential to stimulate the development and dissemination of ARGs among different types of enteric bacteria characteristic for ruminants, as well as potentially pathogenic species related to the fecal microbiome. Some of these bacteria may be pathogenic to humans, and hence could pose a threat to human health if they additionally acquire ARGs. Moreover, non-pathogenic bacteria from manure might transfer ARGs to pathogens existing in manure, soil, or food consumed by animals or humans (Karami et al., 2007; Brichta-Harhay et al., 2011). Furthermore, manure from dairy cows, commonly used as a soil fertilizer, may harbor diverse new variants of ARGs from the gut microbiota of cattle (Wichmann et al., 2014).

A range of antibiotics are used in the cattle production industry, including aminoglycosides, β-lactams, chloramphenicol, fluoroquinolones, glycolipids, ionophores, macrolides, quinolones, streptogramins, sulfonamides, and tetracyclines. The majority are used in dairy cow husbandry for growth promotion (when allowed), prophylaxis, and treatment of most complex diseases such as mastitis or metritis, or as dry cow therapy: the prophylactic use of antibiotics to avoid mammary gland inflammation in animals after lactation (International Office of Epizootics, 2015). In North America, the major antibiotics administered sub-therapeutically in the diet to beef and dairy cattle are chlortetracycline (16%), tylosin (10%), and sulfamethoxazole (9%). These antibiotics demonstrate quite high excretion rates, being approximately 90% for sulfamethoxazole, 65% for chlortetracycline, and, depending on the form of medication, between 50 and 100% for tylosin. Moreover, it is known that chlortetracycline strongly binds to soil particles and may accumulate in this environment, and that sulfamethoxazole persists in the environment for long periods and may be potentially washed off from the surface (Aust et al., 2008).

The most widely-used antibiotics in cattle production systems are tetracyclines, being used to treat skin, respiratory and gastrointestinal tract diseases. Furthermore, tetracycline resistance may develop rapidly because tetracycline ARGs are often located on MGE (Leclercq et al., 2016). In Europe, macrolides and lincosamides are also commonly used to treat a range of common infections in cattle such as mastitis, foot lesions, respiratory and genital infections. Many studies have examined the abundance of genes conferring resistance to MLSB (macrolides–lincosamides-streptogramin B) and tetracyclines in fecal microbial communities in beef cattle receiving antimicrobial agents, e.g., tylosin in fodder (Chen et al., 2008) (**Table 2**). Alexander et al. (2011) report the presence of five classes of tetracycline resistance genes in bovine fecal samples, as well as two classes of sulfonamide resistance genes and five classes of erythromycin resistance genes (**Table 2**).

The cattle industry also makes considerable use of β-lactams, first- and second-generation cephalosporins for mastitis treatment in dairy cattle. However, in the US, ceftiofur, a third-generation cephalosporin, is most commonly used for treating mastitis, respiratory disease, pododermatitis and metritis, while cefquinome, a fourth-generation cephalosporin, is applied for respiratory disease (Zalewska and Popowska, 2020).

Chambers et al. (2015) report that cows treated with ceftiofur demonstrate an increased proportion of β-lactam resistance and MDR in bacterial isolates compared to untreated controls, as well as a greater prevalence of gene sequences associated with phages, prophages, transposable elements and plasmids. These findings may suggest that treatment with this antibiotic may enhance ARG transfer. After ceftiofur treatment, additional functional shifts were noted, such as an increase in the proportion of gene sequences associated with stress response, chemotaxis and resistance to toxic compounds, and a decrease in those related to cell division, cell cycle and metabolism of aromatic compounds. In addition, measurable taxonomic shifts were observed, characterized by an increase in *Bacteroidia* and decrease in *Actinobacteria*. An increase in the number of *E. coli* isolates carrying $bla_{\text{CMY-2}}$ was also observed during parenteral ceftiofur therapy; this can lead to a higher frequency of plasmid-mediated ARGs transfer by HGT to other enteric bacteria, including potential zoonotic pathogens. However, this would require not only the donor but also the recipient populations to be present in sufficient numbers (Stecher et al., 2012).

Although some previous studies have suggested that carbapenem-resistant bacteria are rare in livestock in the US (Webb et al., 2016; Mollenkopf et al., 2017), Vikram and Schmidt (2018) report the presence of a functional $\text{bla}_{\text{KPC-2}}$ gene (*K. pneumoniae* carbapenemases) in the feces of beef cattle raised with antibiotics and those not; they also indicate that the $bla_{\text{KPC-2}}$ gene may be mobilized. However, Agga et al. (2015) indicate that carbapenem-resistant genes are more frequently detected in effluent from US municipal wastewater treatment plant than in cattle catchment ponds for feedlot runoff or swine waste lagoons. These findings

TABLE 2 | Antibiotic-resistant bacteria and antibiotic resistance genes in cow manure.

Bacteria species	Target antibiotics (major)	Resistance genes	Country	References
na	Macrolide (Erythromycin)	*ermB*, *ermF*, *ermT*, *ermX*	United States	Chen et al., 2008
	Tetracycline	*tetA/C*, *tetG*, and *tetM, tetO, tetP, tetQ, tetS, tetT, tetW*		
na	Tetracycline	*tetB, tetC, tetM*, *tetW*, *tetL*	Canada	Alexander et al., 2011
	Sulfonamide	*sul*1, *sul2*		
	Macrolide (Erythromycin)	*ermA*, *ermB*, *ermT*, *ermF*, *ermX*		
na	Beta-lactam	*bla2*	United States	Wichmann et al., 2014
	Aminoglycoside (Kanamycin)	*nat*, *aph*, *aacA-aphD*		
	Tetracycline	*tetW*, *tetO*		
	Chloramphenicol	*cat*		
na	Tetracycline	*tetC*, *tetM*, *tetW*	Canada	Holman et al., 2019
	Macrolide (Erythromycin)	*ermX*		
	Sulfonamide	*sul*2		
Salmonella spp.	Beta Lactam	bla_{CMY-2}, *ampC*	United States	Winokur et al., 2001
Escherichia coli	Beta Lactam	bla_{CMY-2}, *ampC*	United States	
	Beta Lactam	bla_{TEM}, bla_{CMY-2}, bla_{CTX-M}, bla_{SHV}	Canada	Awosile et al., 2018
	Polymyxin (Colistin)	*mcr-1*	China	He et al., 2017b
	Beta Lactam	bla_{VIM-2}, bla_{NDM-5}		
Klebsiella spp.	Beta Lactam	bla_{TEM-1}, bla_{SHV-1}, bla_{OXA-1}	United States	Winokur et al., 2001
	Aminoglycosides	*rmtB, aac(6)-Ib-cr*		
	Quinoxalines	*oqxAB*		
	Quinolone	*qnrS1, qnrB2*		
	Beta Lactam	bla_{NDM-5}	China	He et al., 2017a
	Beta Lactam	bla_{KPC}, bla_{SHV}, bla_{TEM}	China	Yang et al., 2019
	Multidrug	*tol*C		
	Quinolone	*qnr*A, *qnr*B		
Acinetobacter spp.	Beta Lactam	bla_{OXA-23}	France	Poirel et al., 2012
	Beta Lactam	$bla_{OXA-497}$	USA	Webb et al., 2016
	Beta Lactam	bla_{OXA-23}, bla_{OXA-58}	Lebanon	Al Bayssari et al., 2015
Pseudomonas spp.	Beta Lactam	bla_{VIM-2}	Lebanon	Al Bayssari et al., 2015
Enterococcus spp.	Beta Lactam	*blaZ*	South Africa	Tanih et al., 2017
	Macrolides–lincosamides-streptogramin B	*ermB*		
	Tetracycline	*tetM*		
	Glicopeptide (Vancomycin)	*vanB*, *vanC1*		
	Glicopeptide (Vancomycin)	*vanC, vanA*	French	Haenni et al.,2009

na, not applicable (data obtained from metagenomic study).

may suggest that carbapenem resistance may be more closely related to a close human environment than animal food production facilities.

Nevertheless, the dissemination of carbapenemase-producing bacteria in livestock has become a matter of global concern. Winokur et al. (2001) demonstrated the occurrence of *E. coli* and *Salmonella* spp. strains resistant to cephamycins and third-generation cephalosporins in feces samples isolated from food-producing animals. They also report the presence of carbapenemase-producing *Enterobacteriaceae* in dairy cow feces, e.g., *K. pneumoniae* harboring bla_{NDM-5}, as well as various resistance genes including bla_{TEM-1}, bla_{SHV-1}, bla_{OXA-1}, *rmtB, oqxAB, qnrS1, qnrB2, aac(6)-Ib-cr* (**Table 2**). In addition, carbapenemase-producing non-*Enterobacteriaceae* such as *Acinetobacter* spp. (related to *A. lwoffii*) harboring the bla_{OXA-23} carbapenemase gene have been isolated from dairy cattle feces, as have *Acinetobacter baumannii* with the $bla_{OXA-497}$ gene (Bonardi and Pitino, 2019).

A study of rectal samples of dairy cattle in France by Poirel et al. (2012) identified the presence of *Acinetobacter* spp. isolates that were resistant to penicillins, combinations of penicillins with β-lactamase inhibitors, and carbapenems, but which were fully susceptible to cefotaxime. The identified bacteria also demonstrated reduced susceptibility to ceftazidime, and were resistant to tetracycline, kanamycin, and fosfomycin. The isolates with the bla_{OXA-23} gene were found to express β-lactamase OXA-23, which is widespread among *A. baumannii* (**Table 2**). Furthermore, the main vehicle for the bla_{OXA-23} gene among *Acinetobacter* spp. was identified as transposon Tn2008.

Klebsiella pneumoniae harboring the bla_{NDM-5} gene was also isolated from samples of feces from dairy cows with mastitis in Jiangsu Province, China (**Table 2**). In all isolates, the bla_{NDM-5} gene was found on an approximately 46 kb self-transmissible IncX3 pNDM-MGR194-like plasmid (He et al., 2017a). Additionally, He et al. (2017b) isolated and identified three NDM-5-producing *E. coli* isolates from dairy cows,

including one co-producing the transferrable colistin resistance gene *mcr-1* and another co-harboring the carbapenemase gene $bla_{\text{VIM-2}}$ (**Table 2**). The $bla_{\text{NDM-5}}$- and *mcr-1*-harboring plasmids reduced the fitness of their bacterial hosts but maintained stability in the recipient strain. The *mcr-1*-carrying plasmid could be conjugated into NDM-5-positive *E. coli* isolates *in vitro*, thereby generating strains that eventually achieve pan resistance.

In addition, Al Bayssari et al. (2015) report the detection of VIM2-producing ($bla_{\text{VIM-2}}$) *P. aeruginosa* and OXA-23-producing ($bla_{\text{OXA-23}}$) *A. baumannii* in samples of cattle feces. They also identified the co-occurrence of $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-58}}$ in the same isolate of *A. baumannii* (**Table 2**). The exact origins of these genes remain undefined, but they are known to have previously transferred from environmental bacteria into species with clinical relevance (Jiang et al., 2017; Waglechner and Wright, 2017). Woodford et al. (2014) note that the $bla_{\text{OXA-48}}$ family of enzymes occurs naturally in *Shewanella* spp., a genus that inhabits lake sediments, and that OXA-23 carbapenemase originates from the environmental species *Acinetobacter radioresistens*, suggesting that these carbapenemase genes have probably been transferred from environmental bacteria into the animal microbiome, through the close contact between the host animal and the environment.

Importantly, commensal bacteria from animal intestines may serve as a reservoir of ARGs. *E. coli* is commonly used as an indicator species for monitoring AMR dynamics, especially for critical antimicrobials in veterinary and medicine, such as extended-spectrum cephalosporins (ESC) and fluoroquinolones (Reynolds et al., 2020). A number of studies have found *E. coli* strain O157 isolated from cattle feces from commercial and communal farms to be resistant to erythromycin, tetracycline, sulfamethoxazole, chloramphenicol, kanamycin, ampicillin and streptomycin (Ateba and Bezuidenhout, 2008; Volkova et al., 2012). Most infections caused by *E. coli* O157: H7 result from the consumption of food and water contaminated with the fecal matter of infected animals (Beauvais et al., 2018). Increased ESC resistance has frequently been reported in *E. coli* and *Salmonella enterica* from dairy cattle, and this was found to be caused mainly by ESBL or AmpC; many of these organisms have been classified as MDR (Sawant et al., 2007; Seiffert et al., 2013).

Kanwar et al. (2013) detected the presence of *tetA*, *tetB*, and $bla_{\text{CMY-2}}$ genes in the feces of feedlot cattle; interestingly, the findings indicate that the use of chlortetracycline treatment following ceftiofur therapy could elevate the level of ceftiofur resistance. Jiang et al. (2006) report high resistance to ceftriaxone among various gram-positive and gram-negative bacteria isolated from calf feces.

Another important concern for human medicine is presented by the development of vancomycin resistance in bacterial strains with clinical significance such as *Enterococcus* spp. and *Staphylococcus* spp. Beukers et al. (2017) identified *E. faecium* and *E. faecalis*, which are potentially harmful to humans, in bovine feces; however, the Tn917 transposon conferring MLSB resistance was identified only in *E. faecium* and *E. hirae*. In addition, a study of cattle feces by Tanih et al. (2017) indicated the presence of four *Enterococcus* species, such as *E. hirae, E. faecium, E. durans,* and *E. faecalis* harboring tetracycline, erythromycin, penicillin, and vancomycin resistance genes (**Table 2**).

Jackson et al. (2011) examined AMR in ten different *Enterococcus* species, including *E. hirae*, *E. faecalis*, and *E. faecium,* in feces samples from US dairy cattle. In all species, the most prevalent resistance against an array of 17 antimicrobials was found to be against lincomycin, while all strains were resistant to at least one of the tested antimicrobials. Ten different types of AMR were observed among the *E. hirae* isolates, with one *E. hirae* isolate demonstrating MDR against seven antimicrobials. Interestingly, some *E. hirae* isolates were also resistant to one of the newest antimicrobials, daptomycin. In addition, *E. hirae*, *E. faecalis*, *E. faecium,* and another undetermined *Enterococcus* species demonstrated persistence to four antimicrobials (Jackson et al., 2011). Elsewhere, intrinsic vanC-mediated and acquired vanA-mediated resistance has also been reported in enterococci isolated from French cattle (Haenni et al., 2009) (**Table 2**).

Although pathogens resistant to only one antibiotic may cause severe infections that are very hard to treat, MDR bacteria, such as those isolated from cattle feces, are particularly dangerous. Yang et al. (2019) identified MDR in ESBL^{+} *K. pneumoniae* isolated from cow feces. The strain showed resistance to 13 antibiotics from almost all commonly-used antimicrobial classes. In addition, the following resistance genes were found: bla_{KPC}, bla_{SHV}, bla_{TEM}, *qnr*A, *qnr*B, and *tol*C (**Table 2**). Most carbapenemases present in this *K. pneumoniae* strain belonged to the KPC group.

Studies conducted in Canada have shed light on the ARGs associated with ESC and fluoroquinolone resistance in *Enterobacteriaceae* in dairy calves. Awosile et al. (2018) identified *E. coli* isolates carrying bla_{TEM}, $bla_{\text{CMY-2}}$, bla_{CTXM}, and bla_{SHV} genes in the feces of dairy cattle (**Table 2**). In Germany, Klotz et al. (2019) isolated MDR strains from various non-fermenting bacilli, such as *A. baumannii*; these strains demonstrated resistance to ampicillin, amoxicillin-clavulanic acid, cefalexin, ceftiofur, nitrofurantoin, and chloramphenicol, with intermediate resistance observed for piperacillin (6%) and rifampicin (25%). Interestingly, the highest frequency of *A. baumannii* was observed in dairy cows, followed by beef cattle and calves.

Many studies focusing on the diversity of known and novel ARGs have been conducted. Wichmann et al. (2014) used a combination of functional metagenomics with PacBio sequencing to characterize the resistome of dairy cow manure. The prepared DNA libraries indicated the presence of genes coding resistance to β-lactams, kanamycin, tetracycline, and chloramphenicol (**Table 2**). In addition, the researchers identified 80 unique and functional ARGs, suggesting that the ARGs found in animal manure may originate from other genera such as *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Actinobacteria*. Hence, the full resistome appears to consist of phylogenetically varied organisms; in addition, many ARGs seem to be flanked by MGE such as transposases and insertion sequences, or may be created *de novo* like the newly-identified chloramphenicol resistance genes.

Research data indicates that even a single antimicrobial application can have a strong effect on the diversity and

abundance of ARGs. Holman et al. (2019) found the fecal microbiota of beef cattle to be significantly altered on the second and fifth day after a single injection of either oxytetracycline or tulathromycin, and that they remained at this level until the 34th day after administration. Although five resistance genes (*ermX*, *sul*2, *tetC*, *tetM*, and *tetW*) were detected in the fecal microbiome, only *tetM* and *tetW* differed significantly between the animal groups divided according to the medicine administered: oxytetracycline application increased the prevalence of *tetM* in the fecal sample, while tulathromycin boosted the ratio of *tetM* to *tetW* resistance genes compared to animals receiving no antibiotic treatment. The presence of a single antibiotic in a bacteria-living niche may exert on-site selective pressure, thus facilitating the maintenance of ARGs, not only against this particular antimicrobial but also against unrelated resistance determinants through co-resistance; for example, co-transfer of *ermB* and *tetM* has been observed in the presence of erythromycin in *Streptococcus pyogenes* isolates (Brenciani et al., 2007). Fecal bacteria isolated from conventionally-raised cows, where antibiotics are added either as a growth promoter or for prophylaxis, tend to be more resistant to antibiotics than those from cows raised organically, i.e., where antibiotics application is forbidden or strongly supervised (Sato et al., 2005; Halbert et al., 2006); however, it seems that the observed increase in ARGs may be only temporary (Tragesser et al., 2006; Singer et al., 2008).

Interestingly, even cattle never exposed to antibiotics can also carry ARB and ARGs, such as a fluoroquinolone-, tetracycline- or β-lactam resistance genes (Durso et al., 2011). In addition, Thames et al. (2012) have detected genes coding resistance to tetracycline, sulfonamide, and macrolide in samples from newborn calves. In contrast, Roesch et al. (2006) found similar levels of antibiotic resistance between organic farms and conventional ones.

Reaching a common understanding that uncontrolled and unlimited usage of antibiotics may contribute to environmental pollution seems crucial to stopping the propagation of resistant bacteria in food animal production systems (Call et al., 2013).

THE USE OF ANTIBIOTICS IN THE PIG INDUSTRY AND ITS EFFECT ON THE SWINE MANURE RESISTOME

Worldwide pig production has risen steadily during the last 50 years, reaching approximately 120 million tons in 2018. The leading producers of pig meat are China, with almost 45% of global production, followed by the US, Germany, Spain, and Vietnam. These five countries account for nearly 65% of world pig meat production (FAOSTAT, 2020).

An analysis of commercial pig feed formulation available in Vietnam has revealed that it may contain up to 55.4% antimicrobials, with the most common being bacitracin (24.8%), chlortetracycline (23.9%), and florfenicol (17.4%) (Van Cuong et al., 2016). It is estimated that 286.6 mg of in-feed antimicrobials are used to raise 1 kg of live pig (Van Cuong et al., 2016). Approximately 55.5% of antimicrobials administered to pigs are classified by the WHO as *important*, *very important*, or *critically important* for humans. A list of the antimicrobials intended for use in animals has been published by the OIE as a "list of antimicrobial agents of veterinary importance" (International Office of Epizootics, 2015).

One group of antibiotics considered as essential for human medicine is the polymyxins. Polymyxins are not routinely applied parenterally in animals because of their toxicity; however, one polymyxin, colistin, has been used extensively in pig production outside of North America as an oral treatment for neonatal colibacillosis (Dowling, 2013). In Southeastern Asia (India, China), colistin is used as a last-resort therapeutic option to treat severe infections caused by MDR gram-negative bacteria, including *P. aeruginosa* and *A. baumannii* (Catry et al., 2015). It is widely used in pig production for prophylactic (single animal), metaphylactic (whole pen or herd), and therapeutic purposes.

In addition, *mcr-1*, a plasmid-mediated colistin resistance gene, firstly identified in China (Liu et al., 2016), has also been found in Vietnamese pigs (Van Cuong et al., 2016). Colistin is also widely used in Europe (Catry et al., 2015; Kempf et al., 2016), and this may play a crucial role in the spread of colistin resistance in bacteria: although *mcr-1* has been identified in porcine fecal samples across Europe, it appears to be more predominant in Germany, Netherlands, Belgium, Denmark, Italy, Great Britain (Brauer et al., 2016; Guenther et al., 2017; Pulss et al., 2017), and Spain (Quesada et al., 2016), which may be due to differences in the regional regulations regarding its application. In addition, other variants of this gene have been found: *mcr-2* and *mcr-3* in China (Yin et al., 2017) and *mcr-2* in Germany (Roschanski et al., 2017).

Just as in cattle breeding, the use of ceftiofur and cefquinome in pig farming may be related to the spread of resistance to third and fourth generation cephalosporins among *Salmonella* spp. Resistance to ceftiofur in pigs was first identified in 2002 (bla_{CMY}) (Hanson et al., 2002). A similar observation was also made in 2015 on Danish pig farms, where it was found that frequent use of third and fourth generation cephalosporins appeared to be related to the occurrence of ESBL producing *E. coli*. This resistance was also maintained over a prolonged period in animal herds (Andersen et al., 2015).

Some of the most extensively-studied ARGs are those conferring tetracycline resistance because this antimicrobial is widely administered to pigs. Research conducted on the hog population in the US in 2000 revealed a high level (71%) of tetracycline resistance in isolated *Enterococcus* spp. (Haack and Andrews, 2000). The gene *tetM* is known to confer the widest host range of tetracycline resistance and is often associated with MGEs, enhancing its transfer rate from one bacterium to another. More importantly, a number of tetracycline-resistant genes have been found not only in fecal swabs but also near swine feedlots. The genes found in pig fattening farms may code for a range of resistance mechanisms, such as efflux pumps, ribosomal protection peptides, or enzymatic modifications (Wu et al., 2010). The same genes have also been reported previously in swine lagoons, swine manure, storage pits, and groundwater located near pig production facilities (**Table 3**) (Chee-Sanford et al., 2001; Mackie et al., 2006; Koike et al., 2007). A study of the resistance

TABLE 3 | Antibiotic resistance genes in pig.

Resistance genes	Strain	Source	County	References
mcr-1	*E. coli*	Intensive pig farm	China	Liu et al., 2016
	na	Pig	Vietnam	Van Cuong et al., 2016
		Porcine fecal samples	Germany Netherlands Belgium Denmark Italy Great Britain	Brauer et al., 2016; Guenther et al., 2017; Pulss et al., 2017
mcr-2	*E. coli*	Porcine fecal sample	China	Yin et al., 2017
		Pooled feces and boot swab samples	Germany	Roschanski et al., 2017
mcr-3	*E. coli*	Porcine fecal sample	China	Yin et al., 2017
tetA, tetC, tetE, tetG, tetK, tetL, tetA/P, tetM, tetO, tetQ, tetS, tetT, tetW, tetB/P, tetX	na	Porcine feces swine lagoons manure storage pits groundwater near pig production facilities		Chee-Sanford et al., 2001; Mackie et al., 2006; Koike et al., 2007; Wu et al., 2010
aadA5, *aph(3′)-Ic-like*, *bla*$_{CTX-M-1}$, *dfrA17*, *strA*, *strB*, *sul2-like*, *tet(B)*, *aadA1*, *bla*$_{TEM-1B}$, *dfrA1*, *mcr-1*, *sul1*, *sul2*, *tetA*, *aadA2*, *cmlA1-like*, *mph(A)*, *sul3*, *tet(A)-like*	*E. coli*	Pig manure	Germany	Guenther et al., 2017
bla$_{TEM-1B}$*-like*, *strA-like*, *strB-like*, *tetA*, *aadA1*, *aadA2*, *aph(3′)-Ia-like*, *bla*$_{TEM-1B}$, *cmlA1-like*, *dfrA1*, *mcr-1*, *sul3*		Animal boot swabs		
aadA1, *bla*$_{TEM-1B}$, *dfrA1*, *dfrA14-like*, *mcr-1*, *strA*, *strB*, *sul1*, *sul2*, *tet(A)*		Stable fly		
bla$_{TEM-1B}$*-like*, *dfrA1-like*, *mcr-1*, *strA-like*, *strB-like*, *tetA*		Barn dog feces		
bla$_{CTX-M}$, *ermB, mcr-1, optrA, qnrS, tet(40), aac(6′)-Im*	na	Pig feces	Belgium, Bulgaria, Germany, Denmark, Spain, France, Italy, Netherlands, Poland	Munk et al., 2018
mexF, *oprD, aadA2*, *aadD*, *aacA/adhD*, *aphA1, fox5, tetPB*, *tetQ*, *tet(32)*, *tetL*, *tetO, ermT*, *ermX*, *lnuB*, *vatE, sat4*	na	Pig feces		Zhao et al., 2018
tet36, *tetM*, *tetQ tetX*, *tetP(A), tet40, ermF*, *mefA, sul1*, *sul2, aac(6)-Ie*, *aph(6)-Id, catB3*, *catA16, oxa9*, *cfxa, mexD*	na	Swine fattening facility surroundings (well water, swine wastewater, soil, fishpond)	China	He et al., 2019
sul1, tet(32), tetL, tetM, tetO, tetBP-03, tetQ, tetW, tetX, floR, mexF, aadA, aadA1, aadA2, aadD, aph(2′)-Id, aphA3, ermA, ermB, vatE, sat4	na	Pig slurry	China	Pu et al., 2018
bla$_{OXA-48}$, *bla*$_{TEM-1B}$, *aph(3′)-Ia*, *aadA1*, *aadA2*, *aac(3)-IId*, *lnu*(F), *qnrS1*, *floR*, *cmlA1*, *sul2*, *sul3*, *tet*(A), *tet*(M), *dfrA12, aph(4)-Ia, aadA5, aac(3)-IVa, armA, dfrA17*	*E. coli* pathovars	Porcine fecal samples	Germany, Netherlands Belgium Denmark Italy Great Britain	Pulss et al., 2017
tetM, *tetO*, *bla*$_{TEM-1}$, *bla*$_{SHV-2}$, sul2, *aph(3′′)-Ib*, *aph(6′)-Ic*, *aph(6′)-Ib, ermD*, *mdfA*, *mdtH*, *mdtL*, *rosA*, *tet(B), bcr, adeA*, *armB*, *mdtF, mdtN, mdtO, mdtP, oprA, tolC, acrA*	na	Porcine feces samples	The US	Looft et al., 2012

na, not applicable – dataset was obtained during metagenomic study.

profile of MDR *E. coli* from a pig farm and its surroundings in Germany (**Table 3**) identified similar ARGs in samples taken from stable flies and from the feces of barn dogs, indicating the potential for AMR to spread across the surrounding environment (Guenther et al., 2017).

A study of the resistomes of non-typhoidal *Salmonella* strains isolated from swine fecal samples found the highest levels of AMR to be against tetracycline (80% of isolates), streptomycin (43.4%), and sulfamethoxazole (36%). The results also indicated that the microbes isolated from farms raising animals with antimicrobials tended to demonstrate greater resistance to most classes of antibiotics than antimicrobial-free farms (Gebreyes et al., 2006). Furthermore, MDR *Campylobacter coli* have been found within pig production systems (Thakur and Gebreyes, 2005).

More than 400 ARGs were identified in a study conducted in nine European countries (Belgium, Bulgaria, Germany, Denmark, Spain, France, Italy, Netherlands, and Poland) on 181 pig herds and 178 poultry farms. These studies indicated that fecal pig resistome [*bla*$_{CTX-M}$*, ermB, mcr-1, optrA, qnrS, tet(40), aac(6′)-Im*] differs significantly between countries. This discovery appears to be closely related to the antimicrobial usage policy of each country: countries with a strict policy tend to have a lower rate of antibiotic resistance. Conversely, pig herds located in the same country or countries with a similar

antimicrobial usage policy tend to demonstrate similar ARGs patterns. The newly-identified *optrA* gene (enterococcal linezolid resistance group), which codes resistance to both oxazolidinone and amphenicols (florfenicol), was detected in countries with the highest usage of amphenicols, such as Bulgaria, Italy, and Spain (Munk et al., 2018).

Zhao et al. (2018) identified a total number of 146 ARGs in animal feces from seven large-scale Chinese pig farms, i.e., two with high levels of medication and five with low levels, including eight transposon-transposase genes and two class 1 integron-integrase genes. These ARGs genes represented all major classes of resistance mechanisms: 44.5% conferring antibiotic deactivation, 34.3% coding for efflux pumps, and 19.2% cellular protection. These mechanisms provide resistance for all major antimicrobial classes: aminoglycoside (*aadA2*, *aadD*, *aacA/adhD*, *aphA1* [aka *kanR*] – 20%), β-lactam (*fox5* – 14%), tetracycline [*tetPB*, *tetQ*, *tet(32)*, *tetL*, *tetO* -14%], MLSB (*ermT*, *ermX*, *lnuB*, *vatE* – 14%), vancomycin (8%), sulfonamide (2%), chloramphenicol (2%), and others (*sat4* – 4%). The copy number of the gene ranges from 2.72×10^9 to 1.34×10^{10}, divided mostly between tetracycline (4.77×10^9), MLSB (3.41×10^9), and aminoglycoside (4.02×10^9) ARGs (Zhao et al., 2018).

A wide variety of ARGs have also been found in wastewater from pig farms. Of these, tetracycline resistance genes predominated, followed by MLSB resistance and sulfonamide resistance, with aminoglycoside resistance, phenicol resistance, and β-lactam resistance being slightly less abundant (**Table 3**) (He et al., 2019). In addition, the initially identified genes were also identified in pig wastewater-irrigated soils; however, their type and relative abundance varied depending on the sampling depth.

Another analysis conducted in China in 2018 identified 21 ARGs and three transposons (**Table 3**) in pig slurry originating from a high production livestock facility. The ARGs were divided into four resistance mechanisms: cellular protection (43%), antibiotic deactivation (38%), efflux pump (14%), and others (5%). They thus conferred resistance to seven major classes of antibiotics: tetracycline (38%), florfenicol-chloramphenicol-amphenicol (29%), MLSB (14%), aminoglycoside (9%), sulfonamide (5%), and others (5%) (Pu et al., 2018).

A study conducted by Pulss et al. (2017) also detected many other ARGs in an *E. coli* strain carrying an OXA-181 carbapenemase (**Table 3**).

An analysis of the feces from animals supplemented with typical performance-enhancing antibiotics (chlortetracycline, sulfamethazine, and penicillin) in the US revealed increased abundance and diversity of ARGs in the gut microbiome compared with animals raised without antibiotics. Six types of resistance mechanisms were found to be more abundant in the treated animals: those coding for tetracycline efflux pumps, class A β-lactamases, sulfonamide resistance genes, aminoglycoside *O*-phosphotransferase, and two types of multidrug efflux: a major facilitator superfamily transporter, and a resistance-nodulation-cell division transporter system (**Table 3**). However, some genes were also detected at high frequencies in non-medicated pigs. It is possible that their presence may reflect antibiotic resistance developed by farm animals due to selection pressure exerted by over 50 years of antimicrobial treatment (Looft et al., 2012).

Additionally, heavy metals such as zinc, copper, lead, cadmium, chrome, and nickel have been commonly found in animal manure. It has been proven that high doses of copper and zinc enhance the growth of animals (Poole, 2017), and it is possible that these were supplied as mineral addition to fodder; however, others, such as lead, cadmium, chrome or nickel, may have originated in corroded metal parts of installations (Zhao et al., 2018). It is known that heavy metal tolerance is linked to AMR: it may select for resistant bacteria and facilitate the persistence of resistance genes by cross-resistance. It has also been proven that the presence of sublethal doses of heavy metals in the environment facilitates the conjugative transfer of ARGs (Zhang et al., 2018).

The use of copper and zinc in pig fodder has been found to be closely associated with the presence of MDR *Salmonella* spp. (Barton, 2014). Compared to copper-sensitive strains, copper-resistant strains are more likely to be resistant to ampicillin, tetracycline, chloramphenicol, or sulfonamides, as well as to penicillin, just as nickel-resistant strains are also resistant to ampicillin. Moreover, lead resistance may be linked to resistance to β-lactams, mercury tolerance to streptomycin resistance, or lead and mercury tolerance to ampicillin resistance (Sabry et al., 1997; Bass et al., 1999; Berg et al., 2010). Such cross-resistance between heavy metals and antibiotic resistance in the pig production chain has been confirmed by Hölzel et al. (2012).

An interesting example of a plasmid carrying both ARGs and metal tolerance genes was described by Fang et al. (2016). One of the most common incompatibility groups of plasmids in the *Enterobacteriaceae* is IncHI2. It has been identified in *E. coli*, *S. enterica*, *K. pneumoniae,* and *Enterobacter cloacae* isolated from humans, chickens, and rarely swine. The plasmid may harbor a number of metal tolerance genes, such as *pcoABCDRSE* (efflux systems to detoxify copper), *silESRCBAP* (efflux systems to detoxify silver), ars*CBRH* (efflux systems to detoxify arsenic), *merEDACPTR* (Tn1696-related mercury operon), and *terZABCDEF* and *terY3Y2XY1W* (tellurite resistance systems). It also contains a number of AGRs coding resistance to amphenicols (*floR),* aminoglycosides (*armA*, *aac-Ib*/*aac-Ib-cr*), β-lactams ($bla_{\text{CTX-M}}$, bla_{CMY}, bla_{SHV}, bla_{IMP}, bla_{VIM}), quinolones (*oqxAB*, *qnrA1*, *qnrS1* and *qnrB2*), and fosfomycin (*fosA3*). A plasmid harboring both ARGs and metal tolerance genes, Sal-1457, has been identified in *Salmonella infantis* isolates obtained from the feces and carcasses of goats, cattle and chickens in Australia; it has been found to contain *aph(3″)-I*, *aph(6″)-ld*, $bla_{\text{TEM-1}}$, and *dfrA5,* together with an arsenic resistance operon (Wilson et al., 2019). A very detailed study found almost 17% of analyzed bacterial genomes in samples obtained from multiple environments to carry both ARGs and metal tolerance genes (Pal et al., 2015).

CHANGES IN THE SOIL RESISTOME OCCURRING DUE TO MANURE APPLICATION

The soil has been identified as a critical source of ARGs, not only because of the presence of a diverse range of bacteria able to produce natural antibiotics (Lang et al., 2010) but mainly

because of the application of natural manure on crop fields, which might contain ARGs or antibiotics: only a small amount of antibiotics are absorbed or metabolized by animals, with about 75% of the administered drug being excreted into the feces or urine (Zhang and Zhang, 2011).

Soil fertilization with animal manure is a widespread agricultural practice, not only in Europe but worldwide, especially in less developed countries (Heuer et al., 2011). This kind of manure is known to be a rich source of nutrients and organic matter for fertilizing fields. The use of organic manure as fertilizer can also be a practical approach to animal waste management by lowering the cost of its disposal: a single dairy cow can produce 54 kg of wet manure per day, a pig – 6.4 kg, a sheep – 2.5 kg, and a chicken – 0.2 kg (Girotto and Cossu, 2017). Furthermore, fertilizing arable soil with manure can play an essential role in the active cycling of chemicals which are crucial for optimal crop growth and development, such as phosphorus or nitrogen. However, besides its doubtless advantages, the misuse or overuse of manure field applications may account for the excessive amount of these elements in soils and the accumulation of heavy metals added to feeders.

Antibiotics can react and accumulate in specific ways following manure application depending on physicochemical properties of soil and climate conditions. A study of columns filled with types of soil by Pan and Chu (2017) found that the leaching of antibiotics is generally higher in sandy soil than in clay and silty soil. Antibiotics such as norfloxacin and tetracycline were also found to persist longer at the soil surface, contrary to sulfamethazine and erythromycin that tend to reach deeper layers of soil and groundwater.

The full biotransformation and degradation of antimicrobials may take up to 150 days in bovine manure (De Liguoro et al., 2003). Although manure is an essential and sufficient source of nitrogen for agricultural soil, it is also a rich source of antibiotics and ARB, which may be transferred to the environment and survive there even for several months (Merchant et al., 2012). Therefore, it is extremely important to develop effective treatment strategies for manure used as natural fertilizer, with the aim of eliminating, or at least reducing, the risk of releasing antibiotics, ARGs, and ARB to the environment (Zalewska and Popowska, 2020).

Some antibiotics with high adsorption capacity, such as tetracyclines and quinolones, can actively adhere to soil particles, making them non-biodegradable. They can thus accumulate in agricultural soil amended with manure, changing the natural microbial community structure and promoting the maintenance of resistance genes (Li et al., 2011). There are several possible explanations for the increasing abundance of ARGs observed in manured soils. Firstly, it is possible that, following manure application, any ARGs present in bacteria that will survive in the environment can be simply transferred to new hosts by HGT. Alternatively, residues of active antibiotic compounds in manure can induce new mutations in soil bacteria, or enrich and sustain pre-existing ones. Lastly, animal waste rich in organic matter can enhance the growth of resistant resident bacteria in soil (Udikovic-Kolic et al., 2014; Xie et al., 2018). In addition, heavy metals present in soil act as major stress factors in the environment, and as antibiotics and heavy metals can share the same regulatory responses, their presence may also promote ARG selection *via* co-selection and cross-resistance mechanisms (Imran et al., 2019).

A study in Shandong Province, China examined the abundance of ARB and ARGs in farmland soil fertilized with chicken manure (Zhao et al., 2017). The results indicate the presence of a number of ARB and ten AGRs, such as *tetW, tetO, tetT, tetM, tetA, tetL, tetQ, sul1, sul2,* and *sul3*, in soil samples collected from four fields. In all samples, researchers observed significant correlations between the concentration of applied sulfonamides and the abundance of sulfonamide resistance genes.

Similar studies from Finland examined changes in ARGs and MGE abundance in the soil after fertilization with swine and cow manure. The results identified the presence of ARGs, previously detected in animal waste, in fertilized soil. However, while the abundance of ARGs associated with manure generally decreased following 2 and 6 weeks after fertilization, the levels of some ARGs, such as those conferring resistance to disinfectants, aminoglycosides, and vancomycin, remained elevated compared to unfertilized soil, even after 6 weeks (Muurinen et al., 2017). In addition, Marti et al. (2014) report greater abundance of selected genes related to AMR and gene transfer, such as *sul1*, *ermB, strB*, *int1,* and *repA*, in soils fertilized with cow and swine manure than non-manured soil.

Studies also indicate that the increase in ARB observed in soil treated with cow manure may be due to the bloom of resistant species already present in the soil, rather than resistant bacteria introduced from the manure itself (Udikovic-Kolic et al., 2014). The manure used in this study was obtained from dairy cows that had not been treated with antibiotics.

The length of time for which ARG levels remain elevated varies considerably depends on various factors, such as weather conditions or soil type. Increased levels of *sul1* and *sul2* genes were observed for over 4 months following swine manure application in field plots in Germany (Jechalke et al., 2014), while *intI1* persisted in soil for 10 months after swine manure application in agricultural fields in the United Kingdom (Byrne-Bailey et al., 2011). Tighter regulations regarding manure application are in force in the US: manure application must be performed no less than 120 days before harvesting crops that have direct contact with manured soil and no less than 90 days for crops with no direct contact with the soil (Ferguson and Ziegler, 2004).

CHANGES OF THE RESISTOME IN PLANTS GROWING ON MANURE-FERTILIZED SOIL

Fruits and vegetables often harbor non-pathogenic epiphytic microflora; however, many studies have reported contamination with pathogens or different types of ARGs; such contamination may occur pre-harvest *via* soil and organic fertilizers such as manure, sewage sludge, and irrigation water. This is a serious consideration as any contaminated vegetables intended to be

consumed raw may act as vehicles for the spread of ARB and ARGs to humans (Hölzel et al., 2018).

Sub-inhibitory concentrations of antibiotics in plant tissues have been found to be potential drivers of antibiotic resistance in endophytic bacteria, and small amounts of antibiotics, such as tetracycline, can trigger HGT between different bacteria (Redondo-Salvo et al., 2020).

Antibiotic resistance genes can also be transferred to plants from common soil bacteria *via* root endophytes, and these have been found to survive in the root (Bulgarelli et al., 2012, 2013; Lundberg et al., 2012). Some endophytes appear to be are closely related to human pathogens, particularly opportunistic ones (Rosenblueth and Martínez-Romero, 2006), and this similarity could pose a potential threat to human health. Solomon et al. (2002) demonstrated the transmission of *E. coli* O157: H7 from manure-contaminated soil and irrigation water to lettuce; it is believed that these bacteria can enter the lettuce through the root system and propagate throughout the edible portion of the plant.

The soil may well represent the main source of ARGs to the plant, as indicted by the large degree of overlap between ARGs in the plant microbiome and those in the soil resistome (Yang et al., 2018). ARB associated with soil and manure may enter the plant microbiome by colonizing the roots, which are in direct contact with soil, or the aboveground parts, potentially through air particulates or the motility of root endophytes (Zhu et al., 2017; Guron et al., 2019). In addition, plants have been found to take up antibiotic residues from manure-amended soil; this may exert long-term pressure in the plant, facilitating the acquisition of drug resistance and its spread across the plant resistome (Chen et al., 2019a).

The phyllosphere, i.e., aerial leaf surfaces, is a specific niche harboring diverse species of bacteria and other microbes (Bulgarelli et al., 2013). Many previous studies have examined the impact of manure application on the levels of ARGs in the phyllosphere of leafy vegetables (Chen et al., 2016, 2017), or changes in ARGs abundance and dissemination (Marti et al., 2013; Tien et al., 2017; Murray et al., 2019). MGE such as *int*I1 and genes encoding transposase have been detected in leaf endophytes, as well as in the phyllosphere of lettuce (Wang et al., 2015; Zhu et al., 2017), maize (Chen et al., 2016), *Brassica chinensis* L (Chen et al., 2019a), and *Coriandrum sativum* L (Chen et al., 2019b).

After excretion by animals, pollutants such as antibiotics, antibiotic residues, ARB and ARGs may be transported through the environment *via* runoff, leaching and manure application (McEachran et al., 2015). These can accumulate in soil and increase the risk of selection pressure and crop contamination with ARB and ARGs (Hu et al., 2010). The ability of plants to take up antibiotics depends on several biotic and abiotic factors, as well as the type of crop: fruit and grain demonstrate a lower ability to absorb contaminants than leafy and root vegetables (Christou et al., 2019).

Manure application has been found to increase the abundance of ARB and ARGs in soil (Udikovic-Kolic et al., 2014; McKinney et al., 2018). In addition, manure-amended soils have been associated with increased detection of ARB and ARGs on lettuce and root vegetables; however, this has not been associated with all crops or ARGs (Marti et al., 2013; Rahube et al., 2014; Wang et al., 2015). It is important to note that while fecal bacteria can survive for weeks to months in the environment, depending on species and temperature, genetic elements can persist for much longer, regardless of cell viability (Chee-Sanford et al., 2009). ARB, which are naturally found in manure, may attach to the crops grown in the amended soil and multiply in this new potentially more favorable environment. As a consequence, ARB can become dominant among the natural resident bacterial population (Baquero, 2011; Wichmann et al., 2014).

Humans can become exposed to bacteria carrying ARGs and various pathogens by consuming contaminated vegetables (Berger et al., 2010; van Hoek et al., 2015). Yang et al. (2014) examined the effect of soil amendment with chicken manure on the distribution of antibiotic resistant endophytic bacteria (AREB) and MDR endophytic bacteria in celery, pak choi and cucumber. High numbers of bacteria resistant to at least one antibiotic were identified in all vegetables; however, MDR bacteria were found to be absent from the surface of the tissue or inside the vegetables. However, Yang et al. (2016) report that the application of chicken manure or organic fertilizer increases the populations of MDR bacteria in soil and MDR endophytic bacteria in pak choi. Yang et al. (2014) found the highest amounts of cultivable endophytic ARB in celery roots. This is not surprising, as in most plants, roots generally have the highest rates of total cultivable AREB, and hence may serve as a reservoir for potentially pathogenic bacteria or ARB (Berg et al., 2005).

A study of ARB in wheat by Yang et al. (2009) found that AREB were most prevalent in the roots, followed by the leaves, with no AREB detected in the seeds. A report by Sengeløv et al. (2003) indicated that the occurrence of tetracycline-resistant bacteria in the soil was elevated after pig manure slurry treatment, but this fell to control values over the following 5 months. Furthermore, Schmitt et al. (2006) found that both pig manure and the amended soil contain a wide variety of tetracycline ARGs following fertilization. A number of other publications indicate that ARGs can infiltrate the edible parts of commercial crops and that their presence and concentrations depend upon farming practices (Manaia et al., 2018; Cerqueira et al., 2019a,b).

Marti et al. (2013) evaluated the occurrence of ARB on the surface of vegetables that are often eaten raw, such as tomatoes, cucumbers, peppers, carrots, radishes and lettuce, and compared their presence in vegetables grown in inorganically-fertilized soil with those in soil fertilized with dairy or swine manure. The selected vegetables included a range of roots, fruits, and leafy vegetables with different degrees of presentation to key environmental factors (e.g., sun, rain, or wind). It was found that fertilization only had a significant effect on the abundance of amoxicillin-clavulanic acid, ampicillin, and cefoxitin resistance genes. Interestingly, in the case of carrots, a reduction in the abundance of ampicillin- or cefoxitin-resistant bacteria was observed compared to the control group. Across the sample as a whole, eight gene targets (*sul*2, *tet*B, *tet*T, *erm*A, *erm*F, *qnr*B, bla_{PSE}, and $bla_{\text{OXA-20}}$) were detected on at least one vegetable sample. Plants grown in dairy manure-amended soil carried *sul2*, *erm*F, *qnr*B, bla_{PSE}, and $bla_{\text{OXA-20}}$, while those grown on swine

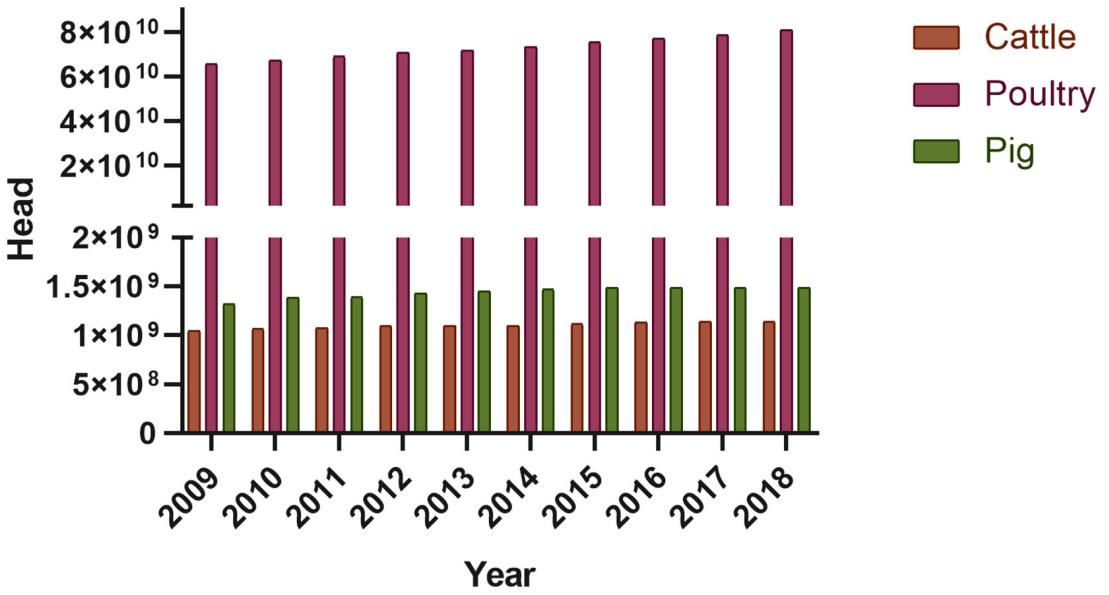

FIGURE 3 | Animal production worldwide (according to FAOSTAT, 2020).

manure-amended soil harbored *sul2*, *tet*B, *tet*T, *erm*A, *erm*F, and $bla_{\text{OXA-20}}$ (Marti et al., 2013).

Cerqueira et al. (2019b) examined the distribution of ARGs in soil, rhizospheric soil, roots, and leaves for tomato, lettuce, and broad beans grown in nine commercial plots. All plots had been fertilized with manure obtained from fairly unusual sources such as horse or pigeon feces. Of the ARGs, the most prevalent was bla_{TEM}, which was detected in all analyzed samples. The data showed that ARGs loads decreased gradually from soil to fruit, resulting in a 100- to 1000-fold dilution for most studied genes; in addition, the ARGs concentration and composition of edible plant parts was strongly dependent on the initial soil ARGs content (Cerqueira et al., 2019b).

Fogler et al. (2019) found the compositions of microbiota and resistomes associated with vegetable surfaces to be strongly influenced by the amendment of soil with raw manure collected from dairy cows during antibiotic administration, especially clindamycin. Their findings indicate that the genes *tetW* and *sul*1 are ubiquitous and present in high abundance in the commensal bacteria inhabiting humans and domestic animals, and that they demonstrate a consequently high representation in waste streams, a low abundance in less-affected environments and a uniform and a highly-conserved DNA sequence; they may therefore serve as valuable markers of soil contamination with ARB, ARGs, and antibiotic residues originating from manure. In addition, lettuce grown in manure-amended soil demonstrated a more diverse bacterial composition than lettuce grown in soil supplied with chemical fertilizers. No significant differences were observed between the relative abundances of detected bacterial phyla/classes based on the type of amendment (Fogler et al., 2019).

Wang et al. (2015) identified ten tetracycline resistance genes (*tetA, tetP, tetC, tetG, tetL, tetB, tetM, tetO, tetW, tetX,*) and two sulfonamide resistance genes (*sul1* and *sul2*), along with one *int1* gene, in root endophytes, leaf endophytes, and phyllosphere microorganisms from vegetable samples grown in manure-amended soil. The prevalence of ARGs was generally lower in leaf endophytes than in the root or phyllosphere microorganisms. Interestingly, it was observed that, for lettuce and endive, the occurrence of ARGs, such as *sul*1, *sul*2, *tetC,* and *tetG*, in root endophytes, leaf endophytes and the phyllosphere members, was also affected by growth period and species of plant (Wang et al., 2015).

A considerable number of studies have reported the presence of significant numbers of ARB on consumer-ready vegetables, such as lettuce and spinach leaves (Abriouel et al., 2008; Bezanson et al., 2008). Zhang et al. (2020) constructed a pot experiment to determine the effect of poultry and cattle manure application on resistome shifts in the plant microbiome of harvested cherry radish. A total of 144 ARGs conferring resistance to eight major classes of antibiotics were detected across all samples. The abundance of MGE was found to be positively correlated with individual ARGs coding for resistance to aminoglycoside, fluoroquinolone, quinolone, florfenicol-chloramphenicol-amphenicol, sulfonamide and tetracycline, and MDR determinants including MLSB. The results suggest that manure application significantly increased the abundance of ARGs in the rhizosphere and phyllosphere but not in the endophytes of the root, i.e., the edible part (Zhang et al., 2020).

The abundance of two MGEs (intI and tnpA-05) also appears to significantly increase after poultry manure application, indicating that not only does manure application directly introduce ARGs to the soil, but also may increase the HGT rate for ARGs; it also suggests that the transmission of ARGs from manure/soil to the surface of vegetables may be the predominant dissemination route. However, although the ARB involved in the

transfer colonize as root endophytes, they demonstrate very little transfer to plant tissues; this suggests that the risk of transmitting external ARGs to the food chain is low. Therefore, root vegetables like cherry radish might be at a lower risk of ARGs contamination than leafy vegetables like lettuce (Zhang et al., 2019, 2020).

The degree to which livestock and agricultural land act as reservoirs of antibiotic-resistant bacteria, and how these two factors interact are relatively unknown (Tyrrell et al., 2019). Many studies have attempted to trace the direction of gene transfer from the environment to manure and determine its implications for future antibiotic resistance management and microbial ecology (Cook et al., 2014; Nesme and Simonet, 2015). In addition, evidence suggests that the primary pathway of gene acquisition from different environments may be HGT, including transfer from soils to the genomes of pathogenic bacteria (Allen et al., 2009; Forsberg et al., 2012; Nesme et al., 2014). Furthermore, DNA element class 1 integrons allow bacteria to adapt and evolve by integrating foreign genes from the environment through the capture of MGEs. This phenomenon has played an essential role in spreading AMR from non-pathogenic bacteria to pathogenic bacteria in the environment (Zhu et al., 2017).

CONCLUDING REMARKS

Although many promising solutions aimed at the reduction of bacterial resistance and overuse of antibiotics in the fields of animal production have emerged (Allen et al., 2013; Cheng et al., 2014; Tabashsum and Biswas, 2019), it seems that no single approach will be able to replace all the antibiotic applications in the animal production sector. Fortunately, public awareness is growing of the harmful effect of antibiotic usage on farm animals, and with it consumer demand for food products with guaranteed quality obtained from animals raised humanely and with minimal environmental impact. However, the demand for animal-derived food products is increasing with rising global population and economic growth (**Figure 3**). This global trend is well illustrated in the FAOSTAT database. Hence, the combination of steadily growing consumption with lack of regulation or intervention strategies, particularly in developing and middle-income countries, will no doubt result in increasing levels of antimicrobial usage, mostly due to the shift from extensive farming to large-scale production systems (Van Boeckel et al., 2015). It is assumed that the global consumption of antimicrobials in livestock and humans will increase by 67% between 2010 (63,151 tons $\pm$ 1560) and 2030 (105,596 $\pm$ 3605), of which approximately two-thirds will be associated with the rising number of food-producing animals (Bloomer and McKee, 2018).

The relationship between antimicrobial use in farm animals and spread of ARB/ARGs is undeniable, as is the risk of their spread to pathogens posing a threat to human health. Antibiotic resistance should be considered a global threat, because neither bacteria nor genes respect geographical or national obstacles.

The most direct way to restrain the spread of AMR is to reduce or optimize their application in animal farming practices. The first step is to improve animal keeping conditions and herd management systems: animals remain healthy when they receive good quality, well-balanced fodder, and are kept in facilities with exceptional hygiene. Moreover, a range of prevention strategies such as vaccination, or the addition of probiotics, prebiotics, or bioactive compounds (e.g., antimicrobial peptides) in fodder can be used to protect vast herds from infection, and subsequently limit antimicrobial usage. In addition, guidelines should be prepared for good practice regarding livestock waste, such as manure management or wastewater treatment strategies (Zhao et al., 2020).

AUTHOR CONTRIBUTIONS

MZ and MP: conceptualization and writing-review and editing. MZ, AB, AC, and MP: writing – original draft preparation. MZ, AB, and AC: visualization. MP: supervision, project administration, and funding acquisition. All authors read and agreed to the published version of the manuscript.

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REFERENCES

Abriouel, H., Omar, N. B., Molinos, A. C., López, R. L., Grande, M. J., Martínez-Viedma, P., et al. (2008). Comparative analysis of genetic diversity and incidence of virulence factors and antibiotic resistance among enterococcal populations from raw fruit and vegetable foods, water and soil, and clinical samples. *Int. J. Food Microbiol.* 123, 38–49. doi: 10.1016/j.ijfoodmicro.2007.11.067

Afridi, O. K., Ali, J., and Chang, J. H. (2020). Next-generation sequencing based gut resistome profiling of broiler chickens infected with multidrug-resistant *Escherichia coli*. *Animals* 10:2350. doi: 10.3390/ani10122350

Agga, G. E., Arthur, T. M., Durso, L. M., Harhay, D. M., and Schmidt, J. W. (2015). Antimicrobial-resistant bacterial populations and antimicrobial resistance genes obtained from environments impacted by livestock and municipal waste. *PLoS One* 10:e0132586. doi: 10.1371/journal.pone.0132586

Al Bayssari, C., Dabboussi, F., Hamze, M., and Rolain, J.-M. (2015). Emergence of carbapenemase-producing *Pseudomonas aeruginosa* and *Acinetobacter baumannii* in livestock animals in Lebanon. *J. Antimicrob. Chemother.* 70, 950–951. doi: 10.1093/jac/dku469

Alexander, T. W., Yanke, J. L., Reuter, T., Topp, E., Read, R. R., Selinger, B. L., et al. (2011). Longitudinal characterization of antimicrobial resistance genes in feces

shed from cattle fed different subtherapeutic antibiotics. *BMC Microbiol.* 11:19. doi: 10.1186/1471-2180-11-19

Allen, H. K., Levine, U. Y., Looft, T., Bandrick, M., and Casey, T. A. (2013). Treatment, promotion, commotion: antibiotic alternatives in food-producing animals. *Trends Microbiol.* 21, 114–119. doi: 10.1016/j.tim.2012.11.001

Allen, H. K., Moe, L. A., Rodbumrer, J., Gaarder, A., and Handelsman, J. (2009). Functional metagenomics reveals diverse beta-lactamases in a remote Alaskan soil. *ISME J.* 3, 243–251. doi: 10.1038/ismej.2008.86

Amador, P., Fernandes, R., Prudêncio, C., and Duarte, I. (2019). Prevalence of antibiotic resistance genes in multidrug-resistant *Enterobacteriaceae* on portuguese livestock manure. *Antibiotics* 8:23. doi: 10.3390/antibiotics8010023

Andersen, V. D., Jensen, V. F., Vigre, H., Andreasen, M., and Agersø, Y. (2015). The use of third and fourth generation cephalosporins affects the occurrence of extended-spectrum cephalosporinase-producing *Escherichia coli* in Danish pig herds. *Vet. J.* 204, 345–350. doi: 10.1016/j.tvjl.2015.03.014

Andersson, D. I., and Hughes, D. (2014). Microbiological effects of sublethal levels of antibiotics. *Nat. Rev. Microbiol.* 12, 465–478. doi: 10.1038/nrmicro3270

Andoh, L. A., Dalsgaard, A., Obiri-Danso, K., Newman, M. J., Barco, L., and Olsen, J. E. (2016). Prevalence and antimicrobial resistance of *Salmonella* serovars isolated from poultry in Ghana. *Epidemiol. Infect.* 144, 3288–3299. doi: 10.1017/S0950268816001126

Ateba, C. N., and Bezuidenhout, C. C. (2008). Characterisation of *Escherichia coli* O157 strains from humans, cattle and pigs in the North-West Province, South Africa. *Int. J. Food Microbiol.* 128, 181–188. doi: 10.1016/j.ijfoodmicro.2008.08.011

Aust, M. O., Godlinski, F., Travis, G. R., Hao, X., McAllister, T. A., Leinweber, P., et al. (2008). Distribution of sulfamethazine, chlortetracycline and tylosin in manure and soil of Canadian feedlots after subtherapeutic use in cattle. *Environ. Pollut.* 156, 1243–1251. doi: 10.1016/j.envpol.2008.03.011

Awosile, B., McClure, J., Sanchez, J., Rodriguez-Lecompte, J. C., Keefe, G., and Heider, L. C. (2018). *Salmonella enterica* and extended-spectrum cephalosporin-resistant *Escherichia coli* recovered from Holstein dairy calves from 8 farms in New Brunswick. *Canada. J. Dairy Sci.* 101, 3271–3284. doi: 10.3168/jds.2017-13277

Baquero, F. (2011). The 2010 Garrod Lecture: the dimensions of evolution in antibiotic resistance: ex unibus plurum et ex pluribus unum. *J. Antimicrob. Chemother.* 66, 1659–1672. doi: 10.1093/jac/dkr214

Barton, M. D. (2014). Impact of antibiotic use in the swine industry. *Curr. Opin. Microbiol.* 19, 9–15. doi: 10.1016/j.mib.2014.05.017

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

Beauvais, W., Gart, E. V., Bean, M., Blanco, A., Wilsey, J., McWhinney, K., et al. (2018). The prevalence of *Escherichia coli* O157: H7 fecal shedding in feedlot pens is affected by the water-to-cattle ratio: a randomized controlled trial. *PLoS One* 13:e0192149. doi: 10.1371/journal.pone.0192149

Berg, G., Eberl, L., and Hartmann, A. (2005). The rhizosphere as a reservoir for opportunistic human pathogenic bacteria. *Environ. Microbiol.* 7, 1673–1685. doi: 10.1111/j.1462-2920.2005.00891.x

Berg, J., Thorsen, M. K., Holm, P. E., Jensen, J., Nybroe, O., and Brandt, K. K. (2010). Cu Exposure under field conditions coselects for antibiotic resistance as determined by a novel cultivation-independent bacterial community tolerance assay. *Environ. Sci. Technol.* 44, 8724–8728. doi: 10.1021/es101798r

Berger, C. N., Sodha, S. V., Shaw, R. K., Griffin, P. M., Pink, D., Hand, P., et al. (2010). Fresh fruit and vegetables as vehicles for the transmission of human pathogens. *Environ. Microbiol.* 12, 2385–2397. doi: 10.1111/j.1462-2920.2010.02297.x

Beukers, A. G., Zaheer, R., Goji, N., Amoako, K. K., Chaves, A. V., Ward, M. P., et al. (2017). Comparative genomics of *Enterococcus* spp. isolated from bovine feces. *BMC Microbiol.* 17:52. doi: 10.1186/s12866-017-0962-1

Bezanson, G. S., MacInnis, R., Potter, G., and Hughes, T. (2008). Presence and potential for horizontal transfer of antibiotic resistance in oxidase-positive bacteria populating raw salad vegetables. *Int. J. Food Microbiol.* 127, 37–42. doi: 10.1016/j.ijfoodmicro.2008.06.008

Bloomer, E., and McKee, M. (2018). Policy options for reducing antibiotics and antibiotic-resistant genes in the environment. *J. Public Health Policy.* 39, 389–406. doi: 10.1057/s41271-018-0144-x

Bonardi, S., and Pitino, R. (2019). Carbapenemase-producing bacteria in food-producing animals, wildlife and environment: a challenge for human health. *Ital. J. Food Saf.* 8:7956. doi: 10.4081/ijfs.2019.7956

Brauer, A., Telling, K., Laht, M., Kalmus, P., Lutsar, I., Remm, M., et al. (2016). Plasmid with colistin resistance gene mcr-1 in extended-spectrum-β-lactamase-producing *Escherichia coli* strains isolated from pig slurry in Estonia. *Antimicrob. Agents Chemother.* 60, 6933–6936. doi: 10.1128/AAC.00443-16

Brenciani, A., Bacciaglia, A., Vecchi, M., Vitali, L. A., Varaldo, P. E., and Giovanetti, E. (2007). Genetic elements carrying erm(B) in *Streptococcus pyogenes* and association with tet(M) tetracycline resistance gene. *Antimicrob. Agents Chemother.* 51, 1209–1216. doi: 10.1128/AAC.01484-06

Brichta-Harhay, D. M., Arthur, T. M., Bosilevac, J. M., Kalchayanand, N., Shackelford, S. D., Wheeler, T. L., et al. (2011). Diversity of multidrug-resistant *salmonella enterica* strains associated with cattle at harvest in the United States. *Appl. Environ. Microbiol.* 77, 1783–1796. doi: 10.1128/AEM.01885-10

Broom, L. J. (2017). The sub-inhibitory theory for antibiotic growth promoters. *Poult. Sci.* 96, 3104–3108. doi: 10.3382/ps/pex114

Büdel, T., Kuenzli, E., Campos-Madueno, E. I., Mohammed, A. H., Hassan, N. K., Zinsstag, J., et al. (2020). On the island of Zanzibar people in the community are frequently colonized with the same MDR Enterobacterales found in poultry and retailed chicken meat. *J. Antimicrob. Chemother.* 75, 2432–2441. doi: 10.1093/jac/dkaa198

Bulgarelli, D., Rott, M., Schlaeppi, K., Ver Loren van Themaat, E., Ahmadinejad, N., Assenza, F., et al. (2012). Revealing structure and assembly cues for *Arabidopsis* root-inhabiting bacterial microbiota. *Nature* 488, 91–95. doi: 10.1038/nature11336

Bulgarelli, D., Schlaeppi, K., Spaepen, S., Ver Loren van Themaat, E., and Schulze-Lefert, P. (2013). Structure and functions of the bacterial microbiota of plants. *Annu. Rev. Plant Biol.* 64, 807–838. doi: 10.1146/annurev-arplant-050312-120106

Byrne-Bailey, K. G., Gaze, W. H., Zhang, L., Kay, P., Boxall, A., Hawkey, P. M., et al. (2011). Integron prevalence and diversity in manured soil. *Appl. Environ. Microbiol.* 77, 684–687. doi: 10.1128/AEM.01425-10

Cairns, J., Ruokolainen, L., Hultman, J., Tamminen, M., Virta, M., and Hiltunen, T. (2018). Ecology determines how low antibiotic concentration impacts community composition and horizontal transfer of resistance genes. *Commun. Biol.* 1:35. doi: 10.1038/s42003-018-0041-7

Call, D. R., Matthews, L., Subbiah, M., and Liu, J. (2013). Do antibiotic residues in soils play a role in amplification and transmission of antibiotic resistant bacteria in cattle populations? *Front. Microbiol.* 4:193. doi: 10.3389/fmicb.2013.00193

Carballo, M., Aguayo, S., González, M., Esperon, F., and de la Torre, A. (2016). Environmental assessment of tetracycline's residues detected in pig slurry and poultry manure. *J. Environ. Prot.* 7, 82–92. doi: 10.4236/jep.2016.71008

Catry, B., Cavaleri, M., Baptiste, K., Grave, K., Grein, K., Holm, A., et al. (2015). Use of colistin-containing products within the European Union and European Economic Area (EU/EEA): development of resistance in animals and possible impact on human and animal health. *Int. J. Antimicrob. Agents* 46, 297–306. doi: 10.1016/j.ijantimicag.2015.06.005

Cerqueira, F., Matamoros, V., Bayona, J., Elsinga, G., Hornstra, L. M., and Piña, B. (2019a). Distribution of antibiotic resistance genes in soils and crops. A field study in legume plants (*Vicia faba* L.) grown under different watering regimes. *Environ. Res.* 170, 16–25. doi: 10.1016/j.envres.2018.12.007

Cerqueira, F., Matamoros, V., Bayona, J. M., Berendonk, T. U., Elsinga, G., Hornstra, L. M., et al. (2019b). Antibiotic resistance gene distribution in agricultural fields and crops. A soil-to-food analysis. *Environ. Res.* 177:108608. doi: 10.1016/j.envres.2019.108608

Chambers, L., Yang, Y., Littier, H., Ray, P., Zhang, T., Pruden, A., et al. (2015). Metagenomic analysis of antibiotic resistance genes in dairy cow feces following therapeutic administration of third generation cephalosporin. *PLoS One* 10:e0133764. doi: 10.1371/journal.pone.0133764

Chee-Sanford, J. C., Aminov, R. I., Krapac, I. J., Garrigues-Jeanjean, N., and Mackie, R. I. (2001). Occurrence and diversity of tetracycline resistance genes in lagoons and groundwater underlying two swine production facilities. *Appl. Environ. Microbiol.* 67, 1494–1502. doi: 10.1128/AEM.67.4.1494-1502.2001

Chee-Sanford, J. C., Mackie, R. I., Koike, S., Krapac, I. G., Lin, Y.-F., Yannarell, A. C., et al. (2009). Fate and transport of antibiotic residues and antibiotic resistance genes following land application of manure waste. *J. Environ. Qual.* 38, 1086–1108. doi: 10.2134/jeq2008.0128

Chen, J., Fluharty, F. L., St-Pierre, N., Morrison, M., and Yu, Z. (2008). Technical note: occurrence in fecal microbiota of genes conferring resistance to both macrolide-lincosamide-streptogramin B and tetracyclines concomitant with feeding of beef cattle with tylosin. *J. Anim. Sci.* 86, 2385–2391. doi: 10.2527/jas.2007-0705

Chen, Q., An, X., Li, H., Su, J., Ma, Y., and Zhu, Y.-G. (2016). Long-term field application of sewage sludge increases the abundance of antibiotic resistance genes in soil. *Environ. Int.* 92–93, 1–10. doi: 10.1016/j.envint.2016.03.026

Chen, Q.-L., An, X.-L., Zhu, Y.-G., Su, J.-Q., Gillings, M. R., Ye, Z.-L., et al. (2017). Application of struvite alters the antibiotic resistome in soil, rhizosphere, and phyllosphere. *Environ. Sci. Technol.* 51, 8149–8157. doi: 10.1021/acs.est.7b01420

Chen, Q.-L., Cui, H.-L., Su, J.-Q., Penuelas, J., and Zhu, Y.-G. (2019a). Antibiotic resistomes in plant microbiomes. *Trends Plant Sci.* 24, 530–541. doi: 10.1016/j.tplants.2019.02.010

Chen, Q.-L., Zhu, D., An, X.-L., Ding, J., Zhu, Y.-G., and Cui, L. (2019b). Does nano silver promote the selection of antibiotic resistance genes in soil and plant? *Environ. Int.* 128, 399–406. doi: 10.1016/j.envint.2019.04.061

Cheng, G., Hao, H., Xie, S., Wang, X., Dai, M., Huang, L., et al. (2014). Antibiotic alternatives: the substitution of antibiotics in animal husbandry? *Front. Microbiol.* 5:217. doi: 10.3389/fmicb.2014.00217

Christou, A., Papadavid, G., Dalias, P., Fotopoulos, V., Michael, C., Bayona, J. M., et al. (2019). Ranking of crop plants according to their potential to uptake and accumulate contaminants of emerging concern. *Environ. Res.* 170, 422–432. doi: 10.1016/j.envres.2018.12.048

Collett, S. R., Smith, J. A., Boulianne, M., Owen, R. L., Gingerich, E., Singer, R. S., et al. (2019). "Principles of disease prevention, diagnosis, and control," in *Diseases of Poultry*, eds D. E. Swayne, et al. (Hoboken, NJ: John Wiley & Sons, Ltd), 1–78. doi: 10.1002/9781119371199.ch1

Cook, K. L., Netthisinghe, A. M. P., and Gilfillen, R. A. (2014). Detection of pathogens, indicators, and antibiotic resistance genes after land application of poultry litter. *J. Environ. Qual.* 43:1546. doi: 10.2134/jeq2013.10.0432

Dandachi, I., Sokhn, E. S., Dahdouh, E. A., Azar, E., El-Bazzal, B., Rolain, J.-M., et al. (2018). Prevalence and characterization of multi-drug-resistant gram-negative bacilli isolated from lebanese poultry: a nationwide study. *Front. Microbiol.* 9:550. doi: 10.3389/fmicb.2018.00550

De Liguoro, M., Cibin, V., Capolongo, F., Halling-Sørensen, B., and Montesissa, C. (2003). Use of oxytetracycline and tylosin in intensive calf farming: evaluation of transfer to manure and soil. *Chemosphere* 52, 203–212. doi: 10.1016/S0045-6535(03)00284-4

Diarra, M. S., and Malouin, F. (2014). Antibiotics in Canadian poultry productions and anticipated alternatives. *Front. Microbiol.* 5:282. doi: 10.3389/fmicb.2014.00282

Dowling, P. M. (2013). "Peptide antibiotics," in *Antimicrobial Therapy in Veterinary Medicine*, eds S. Giguère, J. F. Prescott, and P. M. Dowling (Hoboken, NJ: John Wiley & Sons, Ltd), doi: 10.1002/9781118675014.ch11

Durso, L. M., Harhay, G. P., Bono, J. L., and Smith, T. P. L. (2011). Virulence-associated and antibiotic resistance genes of microbial populations in cattle feces analyzed using a metagenomic approach. *J. Microbiol. Methods* 84, 278–282. doi: 10.1016/j.mimet.2010.12.008

Economou, V., and Gousia, P. (2015). Agriculture and food animals as a source of antimicrobial-resistant bacteria. *Infect. Drug Resist.* 8, 49–61. doi: 10.2147/IDR.S55778

European Centre for Disease Prevention, and Control [ECDC], European Food Safety Authority [EFSA], and European Medicines Agency [EMA] (2017). ECDC/EFSA/EMA second joint report on the integrated analysis of the consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from humans and food-producing animals. *EFSA J.* 15:e04872. doi: 10.2903/j.efsa.2017.4872

European Commission (2005). *Regulation 1831/2003/EC on Additives for Use in Animal Nutrition, Replacing Directive 70/524/EEC on Additives in Feeding-stuffs*.

European Commision (2018). *A European One Health Action Plan Against Antimicrobial Resistance (AMR); 2017/C 212/01*. Available online at: https://ec.europa.eu/health/antimicrobial-resistance/eu-action-on-antimicrobial-resistance_en

European Medicines Agency (2017). *ESVAC Report – Ninth ESVAC Report: Sales of Veterinary Antimicrobial Agents in 31 European Countries in 2017*. Available online at: https://www.ema.europa.eu/en/documents/report/sales-veterinary-antimicrobial-agents-31-european-countries-2017_en.pdf

Fang, L., Li, X., Li, L., Li, S., Liao, X., Sun, J., et al. (2016). Co-spread of metal and antibiotic resistance within ST3-IncHI2 plasmids from *E. coli* isolates of food-producing animals. *Sci. Rep.* 6:25312. doi: 10.1038/srep25312

FAOSTAT (2020). Available online at: http://www.fao.org/faostat/en/#home

Ferguson, J. J., and Ziegler, M. R. (2004). *Guidelines for Purchase and Application of Poultry Manure for Organic Crop Production. EDIS 2004*. Available online at: https://journals.flvc.org/edis/article/view/111168 (accessed March 12, 2020).

Fernandes, M. R., Moura, Q., Sartori, L., Silva, K. C., Cunha, M. P., Esposito, F., et al. (2016). Silent dissemination of colistin-resistant *Escherichia coli* in South America could contribute to the global spread of the mcr-1 gene. *Eurosurveillance* 21:30214. doi: 10.2807/1560-7917.ES.2016.21.17.30214

Fogler, K., Guron, G. K. P., Wind, L. L., Keenum, I. M., Hession, W. C., Krometis, L.-A., et al. (2019). Microbiota and antibiotic resistome of lettuce leaves and radishes grown in soils receiving manure-based amendments derived from antibiotic-treated cows. *Front. Sustain. Food Syst.* 3:22. doi: 10.3389/fsufs.2019.00022

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

Gao, Y., Lu, C., Shen, D., Liu, J., Ma, Z., Yang, B., et al. (2019). Elimination of the risks of colistin resistance gene (mcr-1) in livestock manure during composting. *Environ. Int.* 126, 61–68. doi: 10.1016/j.envint.2019.02.015

Gebreyes, W. A., Thakur, S., and Morgan Morrow, W. E. (2006). Comparison of prevalence, antimicrobial resistance, and occurrence of multidrug-resistant *Salmonella* in antimicrobial-free and conventional pig production. *J. Food Prot.* 69, 743–748. doi: 10.4315/0362-028X-69.4.743

Girotto, F., and Cossu, R. (2017). "Animal waste: opportunities and challenges," in *Sustainable Agriculture Reviews Sustainable Agriculture Reviews*, ed. E. Lichtfouse (Cham: Springer International Publishing), 1–13. doi: 10.1007/978-3-319-48006-0_1

Góchez, D., Raicek, M., Pinto Ferreira, J., Jeannin, M., Moulin, G., and Erlacher-Vindel, E. (2019). OIE annual report on antimicrobial agents intended for use in animals: methods used. *Front. Vet. Sci.* 6:317. doi: 10.3389/fvets.2019.00317

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

Gonzalez Ronquillo, M., and Angeles Hernandez, J. C. (2017). Antibiotic and synthetic growth promoters in animal diets: review of impact and analytical methods. *Food Control* 72, 255–267. doi: 10.1016/j.foodcont.2016.03.001

Graham, J. P., Evans, S. L., Price, L. B., and Silbergeld, E. K. (2009). Fate of antimicrobial-resistant enterococci and staphylococci and resistance determinants in stored poultry litter. *Environ. Res.* 109, 682–689. doi: 10.1016/j.envres.2009.05.005

Guenther, S., Falgenhauer, L., Semmler, T., Imirzalioglu, C., Chakraborty, T., Roesler, U., et al. (2017). Environmental emission of multiresistant *Escherichia coli* carrying the colistin resistance gene mcr-1 from German swine farms. *J. Antimicrob. Chemother.* 72, 1289–1292. doi: 10.1093/jac/dkw585

Guron, G. K. P., Arango-Argoty, G., Zhang, L., Pruden, A., and Ponder, M. A. (2019). Effects of dairy manure-based amendments and soil texture on lettuce- and radish-associated microbiota and resistomes. *mSphere* 4:e239-19. doi: 10.1128/mSphere.00239-19

Haack, B. J., and Andrews, R. E. Jr. (2000). Isolation of Tn916-like conjugal elements from swine lot effluent. *Can. J. Microbiol.* 46, 542–549.

Haenni, M., Saras, E., Châtre, P., Meunier, D., Martin, S., Lepage, G., et al. (2009). vanA in *Enterococcus* faecium, *Enterococcus* faecalis, and *Enterococcus* casseliflavus detected in French cattle. *Foodborne Pathog. Dis.* 6, 1107–1111. doi: 10.1089/fpd.2009.0303

Halbert, L. W., Kaneene, J. B., Ruegg, P. L., Warnick, L. D., Wells, S. J., Mansfield, L. S., et al. (2006). Evaluation of antimicrobial susceptibility patterns in *Campylobacter* spp isolated from dairy cattle and farms managed organically and conventionally in the midwestern and northeastern United States. *J. Am. Vet. Med. Assoc.* 228, 1074–1081. doi: 10.2460/javma.228.7.1074

Hanon, J.-B., Jaspers, S., Butaye, P., Wattiau, P., Méroc, E., Aerts, M., et al. (2015). A trend analysis of antimicrobial resistance in commensal *Escherichia coli* from several livestock species in Belgium (2011–2014). *Prev. Vet. Med.* 122, 443–452. doi: 10.1016/j.prevetmed.2015.09.001

Hanson, R., Kaneene, J. B., Padungtod, P., Hirokawa, K., and Zeno, C. (2002). Prevalence of *Salmonella* and *E. coli*, and their resistance to antimicrobial agents, in farming communities in northern Thailand. *Se. Asian. J. Trop. Med.* 33, 120–126.

Hassan, Y. I., Lahaye, L., Gong, M. M., Peng, J., Gong, J., Liu, S., et al. (2018). Innovative drugs, chemicals, and enzymes within the animal production chain. *Vet. Res.* 49:71. doi: 10.1186/s13567-018-0559-1

He, L.-Y., He, L.-K., Liu, Y.-S., Zhang, M., Zhao, J.-L., Zhang, Q.-Q., et al. (2019). Microbial diversity and antibiotic resistome in swine farm environments. *Sci. Total Environ.* 685, 197–207. doi: 10.1016/j.scitotenv.2019.05.369

He, T., Wang, Y., Sun, L., Pang, M., Zhang, L., and Wang, R. (2017a). Occurrence and characterization of blaNDM-5-positive *Klebsiella pneumoniae* isolates from dairy cows in Jiangsu. *China. J. Antimicrob. Chemother.* 72, 90–94. doi: 10.1093/jac/dkw357

He, T., Wei, R., Zhang, L., Sun, L., Pang, M., Wang, R., et al. (2017b). Characterization of NDM-5-positive extensively resistant *Escherichia coli* isolates from dairy cows. *Vet. Microbiol.* 207, 153–158. doi: 10.1016/j.vetmic.2017.06.010

Heuer, H., Schmitt, H., and Smalla, K. (2011). Antibiotic resistance gene spread due to manure application on agricultural fields. *Curr. Opin. Microbiol.* 14, 236–243. doi: 10.1016/j.mib.2011.04.009

Holman, D. B., Yang, W., and Alexander, T. W. (2019). Antibiotic treatment in feedlot cattle: a longitudinal study of the effect of oxytetracycline and tulathromycin on the fecal and nasopharyngeal microbiota. *Microbiome* 7:86. doi: 10.1186/s40168-019-0696-4

Hölzel, C. S., Müller, C., Harms, K. S., Mikolajewski, S., Schäfer, S., Schwaiger, K., et al. (2012). Heavy metals in liquid pig manure in light of bacterial antimicrobial resistance. *Environ. Res.* 113, 21–27. doi: 10.1016/j.envres.2012.01.002

Hölzel, C. S., Tetens, J. L., and Schwaiger, K. (2018). Unraveling the role of vegetables in spreading antimicrobial-resistant bacteria: a need for quantitative risk assessment. *Foodborne Pathog. Dis.* 15, 671–688. doi: 10.1089/fpd.2018.2501

Hu, X., Zhou, Q., and Luo, Y. (2010). Occurrence and source analysis of typical veterinary antibiotics in manure, soil, vegetables and groundwater from organic vegetable bases, northern China. *Environ. Pollut.* 2992–2998. doi: 10.1016/j.envpol.2010.05.023

Imran, M., Das, K. R., and Naik, M. M. (2019). Co-selection of multi-antibiotic resistance in bacterial pathogens in metal and microplastic contaminated environments: an emerging health threat. *Chemosphere* 215, 846–857. doi: 10.1016/j.chemosphere.2018.10.114

International Office of Epizootics (ed.) (2015). *OIE standards, Gidelines and Resolution on Antimicrobial Resistance and the Use of Antimicrobial Agents*. Paris: OIE.

Jackson, C. R., Lombard, J. E., Dargatz, D. A., and Fedorka-Cray, P. J. (2011). Prevalence, species distribution and antimicrobial resistance of enterococci isolated from US dairy cattle. *Lett. Appl. Microbiol.* 52, 41–48. doi: 10.1111/j.1472-765X.2010.02964.x

Jechalke, S., Heuer, H., Siemens, J., Amelung, W., and Smalla, K. (2014). Fate and effects of veterinary antibiotics in soil. *Trends Microbiol.* 22, 536–545. doi: 10.1016/j.tim.2014.05.005

Jiang, X., Ellabaan, M., Charusanti, P., Munck, C., Blin, K., Tong, Y., et al. (2017). Dissemination of antibiotic resistance genes from antibiotic producers to pathogens. *Nat. Commun.* 8:15784. doi: 10.1038/ncomms15784

Jiang, X., Yang, H., Dettman, B., and Doyle, M. P. (2006). Analysis of fecal microbial flora for antibiotic resistance in ceftiofur-treated calves. *Foodborne Pathog. Dis.* 3, 355–365. doi: 10.1089/fpd.2006.3.355

Journals, I., and Oke, A. A. (2013). Incidence of methicillin-resistant *Staphylococcus aureus* (MRSA) in a small poultry in south West, Nigeria. *IOSR J. Agric. Vet. Sci.* 5, 53–55. doi: 10.9790/2380-0535355

Kanwar, N., Scott, H. M., Norby, B., Loneragan, G. H., Vinasco, J., McGowan, M., et al. (2013). Effects of ceftiofur and chlortetracycline treatment strategies on antimicrobial susceptibility and on tet(A), tet(B), and bla CMY-2 resistance genes among *E. coli* isolated from the feces of feedlot cattle. *PLoS One* 8:e80575. doi: 10.1371/journal.pone.0080575

Karami, N., Martner, A., Enne, V. I., Swerkersson, S., Adlerberth, I., and Wold, A. E. (2007). Transfer of an ampicillin resistance gene between two *Escherichia coli* strains in the bowel microbiota of an infant treated with antibiotics. *J. Antimicrob. Chemother.* 60, 1142–1145. doi: 10.1093/jac/dkm327

Kempf, I., Jouy, E., and Chauvin, C. (2016). Colistin use and colistin resistance in bacteria from animals. *Int. J. Antimicrob. Agents* 48, 598–606. doi: 10.1016/j.ijantimicag.2016.09.016

Khan, G. A., Berglund, B., Khan, K. M., Lindgren, P.-E., and Fick, J. (2013). Occurrence and abundance of antibiotics and resistance genes in rivers, canal and near drug formulation facilities – a study in Pakistan. *PLoS One* 8:e62712. doi: 10.1371/journal.pone.0062712

Klotz, P., Higgins, P. G., Schaubmar, A. R., Failing, K., Leidner, U., Seifert, H., et al. (2019). Seasonal occurrence and carbapenem susceptibility of bovine *Acinetobacter baumannii* in Germany. *Front. Microbiol.* 10:272. doi: 10.3389/fmicb.2019.00272

Koike, S., Krapac, I. G., Oliver, H. D., Yannarell, A. C., Chee-Sanford, J. C., Aminov, R. I., et al. (2007). Monitoring and source tracking of tetracycline resistance genes in lagoons and groundwater adjacent to swine production facilities over a 3-year period. *Appl. Environ. Microbiol.* 73, 4813–4823. doi: 10.1128/AEM.00665-07

Kumar, K., C. Gupta, S., Chander, Y., and Singh, A. K. (2005). Antibiotic use in agriculture and its impact on the terrestrial environment. *Adv. Agron.* 87, 1–54. doi: 10.1016/S0065-2113(05)87001-4

Lang, K. S., Anderson, J. M., Schwarz, S., Williamson, L., Handelsman, J., and Singer, R. S. (2010). Novel florfenicol and chloramphenicol resistance gene discovered in alaskan soil by using functional metagenomics. *Appl. Environ. Microbiol.* 76, 5321–5326. doi: 10.1128/AEM.00323-10

Langata, L. M., Maingi, J. M., Musonye, H. A., Kiiru, J., and Nyamache, A. K. (2019). Antimicrobial resistance genes in *Salmonella* and *Escherichia coli* isolates from chicken droppings in Nairobi, Kenya. *BMC Res. Notes* 12:22. doi: 10.1186/s13104-019-4068-8

Leclercq, S. O., Wang, C., Zhu, Y., Wu, H., Du, X., Liu, Z., et al. (2016). Diversity of the tetracycline mobilome within a Chinese pig manure sample. *Appl. Environ. Microbiol.* 82, 6454–6462. doi: 10.1128/AEM.01754-16

Lee, J. H. (2006). Occurrence of methicillin-resistant *Staphylococcus aureus* strains from cattle and chicken, and analyses of their mecA, mecR1 and mecI genes. *Vet. Microbiol.* 114, 155–159. doi: 10.1016/j.vetmic.2005.10.024

Li, Y.-W., Wu, X.-L., Mo, C.-H., Tai, Y.-P., Huang, X.-P., and Xiang, L. (2011). Investigation of sulfonamide, tetracycline, and quinolone antibiotics in vegetable farmland soil in the Pearl River Delta Area, Southern China. *J. Agric. Food Chem.* 59, 7268–7276. doi: 10.1021/jf1047578

Lim, S.-K., Kim, D., Moon, D.-C., Cho, Y., and Rho, M. (2020). Antibiotic resistomes discovered in the gut microbiomes of Korean swine and cattle. *GigaScience* 9:giaa043. doi: 10.1093/gigascience/giaa043

Liu, Y.-Y., Wang, Y., Walsh, T. R., Yi, L.-X., Zhang, R., Spencer, J., et al. (2016). Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infect. Dis.* 16, 161–168. doi: 10.1016/S1473-3099(15)00424-7

Ljubojević, D., Pelić, M., Puvača, N., and Milanov, D. (2017). Resistance totetracycline in *Escherichia coli* isolates from poultry meat: epidemiology, policy and perspective. *Worlds Poult. Sci. J.* 73, 409–417. doi: 10.1017/S0043933917000216

Looft, T., Johnson, T. A., Allen, H. K., Bayles, D. O., Alt, D. P., Stedtfeld, R. D., et al. (2012). In-feed antibiotic effects on the swine intestinal microbiome. *Proc. Natl. Acad. Sci. U. S. A.* 109, 1691–1696. doi: 10.1073/pnas.1120238109

Lorian, V. (1975). Some effects of subinhibitory concentrations of antibiotics on bacteria. *Bull. N. Y. Acad. Med.* 51, 1046–1055.

Lundberg, D. S., Lebeis, S. L., Paredes, S. H., Yourstone, S., Gehring, J., Malfatti, S., et al. (2012). Defining the core *Arabidopsis* thaliana root microbiome. *Nature* 488, 86–90. doi: 10.1038/nature11237

Mackie, R. I., Koike, S., Krapac, I., Chee-Sanford, J., Maxwell, S., and Aminov, R. I. (2006). Tetracycline residues and tetracycline resistance genes in groundwater impacted by swine production facilities. *Anim. Biotechnol.* 17, 157–176. doi: 10.1080/10495390600956953

Mahmoud, M. A. M., and Abdel-Mohsein, H. S. (2019). Hysterical tetracycline in intensive poultry farms accountable for substantial gene resistance, health

and ecological risk in Egypt- manure and fish. *Environ. Pollut.* 255:113039. doi: 10.1016/j.envpol.2019.113039

Manaia, C. M., Rocha, J., Scaccia, N., Marano, R., Radu, E., Biancullo, F., et al. (2018). Antibiotic resistance in wastewater treatment plants: tackling the black box. *Environ. Int.* 115, 312–324. doi: 10.1016/j.envint.2018.03.044

Marti, R., Scott, A., Tien, Y.-C., Murray, R., Sabourin, L., Zhang, Y., et al. (2013). Impact of manure fertilization on the abundance of antibiotic-resistant bacteria and frequency of detection of antibiotic resistance genes in soil and on vegetables at harvest. *Appl. Environ. Microbiol.* 79, 5701–5709. doi: 10.1128/AEM.01682-13

Marti, R., Tien, Y.-C., Murray, R., Scott, A., Sabourin, L., and Topp, E. (2014). Safely coupling livestock and crop production systems: how rapidly do antibiotic resistance genes dissipate in soil following a commercial application of swine or dairy manure? *Appl. Environ. Microbiol.* 80, 3258–3265. doi: 10.1128/AEM.00231-14

McEachran, A. D., Blackwell, B. R., Hanson, J. D., Wooten, K. J., Mayer, G. D., Cox, S. B., et al. (2015). Antibiotics, bacteria, and antibiotic resistance genes: aerial transport from cattle feed yards via particulate matter. *Environ. Health Perspect.* 123, 337–343. doi: 10.1289/ehp.1408555

McKinney, C. W., Dungan, R. S., Moore, A., and Leytem, A. B. (2018). Occurrence and abundance of antibiotic resistance genes in agricultural soil receiving dairy manure. *FEMS Microbiol. Ecol.* 94:fiy010. doi: 10.1093/femsec/fiy010

Medicine (2019). *Committee on Human Health Risk Assessment of Using Subtherapeutic Antibiotics in Animal Feeds*. Washington, DC: The National Academies Press.

Merchant, L. E., Rempel, H., Forge, T., Kannangara, T., Bittman, S., Delaquis, P., et al. (2012). Characterization of antibiotic-resistant and potentially pathogenic *Escherichia coli* from soil fertilized with litter of broiler chickens fed antimicrobial-supplemented diets. *Can. J. Microbiol.* 58, 1084–1098. doi: 10.1139/w2012-082

Mollenkopf, D. F., Stull, J. W., Mathys, D. A., Bowman, A. S., Feicht, S. M., Grooters, S. V., et al. (2017). Carbapenemase-producing *Enterobacteriaceae* recovered from the environment of a swine farrow-to-finish operation in the United States. *Antimicrob. Agents Chemother.* 61:e01298-16. doi: 10.1128/AAC.01298-16

Munk, P., Knudsen, B. E., Lukjancenko, O., Duarte, A. S. R., Van Gompel, L., Luiken, R. E. C., et al. (2018). Abundance and diversity of the faecal resistome in slaughter pigs and broilers in nine European countries. *Nat. Microbiol.* 3, 898–908. doi: 10.1038/s41564-018-0192-9

Murray, R., Tien, Y.-C., Scott, A., and Topp, E. (2019). The impact of municipal sewage sludge stabilization processes on the abundance, field persistence, and transmission of antibiotic resistant bacteria and antibiotic resistance genes to vegetables at harvest. *Sci. Total Environ.* 651, 1680–1687. doi: 10.1016/j.scitotenv.2018.10.030

Muurinen, J., Stedtfeld, R., Karkman, A., Pärnänen, K., Tiedje, J., and Virta, M. (2017). Influence of manure application on the environmental resistome under finnish agricultural practice with restricted antibiotic use. *Environ. Sci. Technol.* 51, 5989–5999. doi: 10.1021/acs.est.7b00551

Nesme, J., Cécillon, S., Delmont, T. O., Monier, J.-M., Vogel, T. M., and Simonet, P. (2014). Large-scale metagenomic-based study of antibiotic resistance in the environment. *Curr. Biol.* 24, 1096–1100. doi: 10.1016/j.cub.2014.03.036

Nesme, J., and Simonet, P. (2015). The soil resistome: a critical review on antibiotic resistance origins, ecology and dissemination potential in telluric bacteria. *Environ. Microbiol.* 17, 913–930. doi: 10.1111/1462-2920.12631

Nhung, N. T., Cuong, N. V., Thwaites, G., and Carrique-Mas, J. (2016). Antimicrobial usage and antimicrobial resistance in animal production in southeast asia: a review. *Antibiotics* 5:37. doi: 10.3390/antibiotics5040037

OIE (2018). *OIE Annual Report on Antimicrobial Agents Intended for Use in Animals – Better Understanding of the Global Situation. Third Report*. Available online at: https://rr-africa.oie.int/wp-content/uploads/2019/09/annual_report_amr_3.pdf

Omojowo, F., and Omojasola, F. (2013). Antibiotic resistance pattern of bacterial pathogens isolated from poultry manure used to fertilize fish ponds in new Bussa. Nigeria. *Albanian J. Agric. Sci.* 12, 81–85.

Pal, C., Bengtsson-Palme, J., Kristiansson, E., and Larsson, D. G. J. (2015). Co-occurrence of resistance genes to antibiotics, biocides and metals reveals novel insights into their co-selection potential. *BMC Genomics* 16:964. doi: 10.1186/s12864-015-2153-5

Pan, M., and Chu, L. M. (2017). Transfer of antibiotics from wastewater or animal manure to soil and edible crops. *Environ. Pollut.* 231, 829–836. doi: 10.1016/j.envpol.2017.08.051

Poirel, L., Berçot, B., Millemann, Y., Bonnin, R. A., Pannaux, G., and Nordmann, P. (2012). Carbapenemase-producing *Acinetobacter* spp. in Cattle. *France. Emerg. Infect. Dis.* 18, 523–525. doi: 10.3201/eid1803.111330

Poole, K. (2017). At the nexus of antibiotics and metals: the impact of Cu and Zn on antibiotic activity and resistance. *Trends Microbiol.* 25, 820–832. doi: 10.1016/j.tim.2017.04.010

Pu, C., Liu, H., Ding, G., Sun, Y., Yu, X., Chen, J., et al. (2018). Impact of direct application of biogas slurry and residue in fields: in situ analysis of antibiotic resistance genes from pig manure to fields. *J. Hazard. Mater.* 344, 441–449. doi: 10.1016/j.jhazmat.2017.10.031

Pulss, S., Semmler, T., Prenger-Berninghoff, E., Bauerfeind, R., and Ewers, C. (2017). First report of an *Escherichia coli* strain from swine carrying an OXA-181 carbapenemase and the colistin resistance determinant MCR-1. *Int. J. Antimicrob. Agents* 50, 232–236. doi: 10.1016/j.ijantimicag.2017.03.014

Quesada, A., Ugarte-Ruiz, M., Iglesias, M. R., Porrero, M. C., Martínez, R., Florez-Cuadrado, D., et al. (2016). Detection of plasmid mediated colistin resistance (MCR-1) in *Escherichia coli* and *Salmonella enterica* isolated from poultry and swine in Spain. *Res. Vet. Sci.* 105, 134–135. doi: 10.1016/j.rvsc.2016.02.003

Rahube, T. O., Marti, R., Scott, A., Tien, Y.-C., Murray, R., Sabourin, L., et al. (2014). Impact of fertilizing with raw or anaerobically digested sewage sludge on the abundance of antibiotic-resistant coliforms, antibiotic resistance genes, and pathogenic bacteria in soil and on vegetables at harvest. *Appl. Environ. Microbiol.* 80, 6898–6907. doi: 10.1128/AEM.02389-14

Rayamajhi, N., Jung, B. Y., Cha, S. B., Shin, M. K., Kim, A., Kang, M. S., et al. (2010). Antibiotic resistance patterns and detection of blaDHA-1 in *Salmonella* species isolates from chicken farms in South Korea. *Appl. Environ. Microbiol.* 76, 4760–4764. doi: 10.1128/AEM.02536-09

Redondo-Salvo, S., Fernández-López, R., Ruiz, R., Vielva, L., de Toro, M., Rocha, E. P. C., et al. (2020). Pathways for horizontal gene transfer in bacteria revealed by a global map of their plasmids. *Nat. Commun.* 11:3602. doi: 10.1038/s41467-020-17278-2

Reynolds, L. J., Sala-Comorera, L., Martin, N. A., Nolan, T. M., Stephens, J. H., Gitto, A., et al. (2020). Correlation between antimicrobial resistance and faecal contamination in small urban streams and bathing waters. *Sci. Total Environ.* 739:140242. doi: 10.1016/j.scitotenv.2020.140242

Roesch, M., Perreten, V., Doherr, M. G., Schaeren, W., Schällibaum, M., and Blum, J. W. (2006). Comparison of antibiotic resistance of udder pathogens in dairy cows kept on organic and on conventional farms. *J. Dairy Sci.* 89, 989–997. doi: 10.3168/jds.S0022-0302(06)72164-6

Roschanski, N., Falgenhauer, L., Grobbel, M., Guenther, S., Kreienbrock, L., Imirzalioglu, C., et al. (2017). Retrospective survey of mcr-1 and mcr-2 in German pig-fattening farms, 2011–2012. *Int. J. Antimicrob. Agents* 50, 266–271. doi: 10.1016/j.ijantimicag.2017.03.007

Rosenblueth, M., and Martínez-Romero, E. (2006). Bacterial endophytes and their interactions with hosts. *Mol. Plant Microbe Interact.* 19, 827–837. doi: 10.1094/MPMI-19-0827

Roth, N., Käsbohrer, A., Mayrhofer, S., Zitz, U., Hofacre, C., and Domig, K. J. (2019). The application of antibiotics in broiler production and the resulting antibiotic resistance in *Escherichia coli*: a global overview. *Poult. Sci.* 98, 1791–1804. doi: 10.3382/ps/pey539

Sabry, S. A., Ghozlan, H. A., and Abou-Zeid, D.-M. (1997). Metal tolerance and antibiotic resistance patterns of a bacterial population isolated from sea water. *J. Appl. Microbiol.* 82, 245–252. doi: 10.1111/j.1365-2672.1997.tb03580.x

Sato, K., Bartlett, P. C., and Saeed, M. A. (2005). Antimicrobial susceptibility of *Escherichia coli* isolates from dairy farms using organic versus conventional production methods. *J. Am. Vet. Med. Assoc.* 226, 589–594. doi: 10.2460/javma.2005.226.589

Sawant, A. A., Hegde, N. V., Straley, B. A., Donaldson, S. C., Love, B. C., Knabel, S. J., et al. (2007). Antimicrobial-resistant enteric bacteria from dairy cattle. *Appl. Environ. Microbiol.* 73, 156–163. doi: 10.1128/AEM.01551-06

Schmitt, H., Stoob, K., Hamscher, G., Smit, E., and Seinen, W. (2006). Tetracyclines and tetracycline resistance in agricultural soils: microcosm and field studies. *Microb. Ecol.* 51, 267–276. doi: 10.1007/s00248-006-9035-y

Seiffert, S. N., Hilty, M., Perreten, V., and Endimiani, A. (2013). Extended-spectrum cephalosporin-resistant Gram-negative organisms in livestock: an

emerging problem for human health? *Drug. Resist. Updat.* 16, 22–45. doi: 10.1016/j.drup.2012.12.001

Sengeløv, G., Agersø, Y., Halling-Sørensen, B., Baloda, S. B., Andersen, J. S., and Jensen, L. B. (2003). Bacterial antibiotic resistance levels in Danish farmland as a result of treatment with pig manure slurry. *Environ. Int.* 28, 587–595. doi: 10.1016/s0160-4120(02)00084-3

Singer, R. S., Patterson, S. K., and Wallace, R. L. (2008). Effects of therapeutic ceftiofur administration to dairy cattle on *Escherichia coli* dynamics in the intestinal tract. *Appl. Environ. Microbiol.* 74, 6956–6962. doi: 10.1128/AEM.01241-08

So, A. D., Shah, T. A., Roach, S., Chee, Y. L., and Nachman, K. E. (2015). An integrated systems approach is needed to ensure the sustainability of antibiotic effectiveness for both humans and animals. *J. Law. Med. Ethics* 43, 38–45. doi: 10.1111/jlme.12273

Solomon, E. B., Yaron, S., and Matthews, K. R. (2002). Transmission of *Escherichia coli* O157:H7 from contaminated manure and irrigation water to lettuce plant tissue and its subsequent internalization. *Appl. Environ. Microbiol.* 68, 397–400. doi: 10.1128/aem.68.1.397-400.2002

Soulsby, L. (2007). Antimicrobials and animal health: a fascinating nexus. *J. Antimicrob. Chemother.* 60, i77–i78. doi: 10.1093/jac/dkm164

Stecher, B., Denzler, R., Maier, L., Bernet, F., Sanders, M. J., Pickard, D. J., et al. (2012). Gut inflammation can boost horizontal gene transfer between pathogenic and commensal *Enterobacteriaceae*. *Proc. Natl. Acad. Sci. U. S. A.* 109, 1269–1274. doi: 10.1073/pnas.1113246109

Suresh, G., Das, R. K., Brar, S. K., Rouissi, T., Ramirez, A. A., Chorfi, Y., et al. (2018). Alternatives to antibiotics in poultry feed: molecular perspectives. *Crit. Rev. Microbiol.* 44, 318–335. doi: 10.1080/1040841X.2017.1373062

Tabashsum, Z., and Biswas, D. (2019). "Chapter 17 - acceptable alternative growth promoters for organic farm animal production," in *Safety and Practice for Organic Food*, eds D. Biswas and S. A. Micallef (Cambridge, MA: Academic Press), 345–363. doi: 10.1016/B978-0-12-812060-6.00017-9

Tanih, G. N., Okoh, A. I., Ndip, R. N., Nwodo, U. U., and Green, E. (2017). Multidrug resistance in *Enterococcus* species of faecal origin from commercial dairy lactating cattle: public health concern. *Asian Pac. J. Trop. Dis.* 7, 665–670. doi: 10.12980/apjtd.7.2017D7-154

Thakur, S., and Gebreyes, W. A. (2005). Prevalence and antimicrobial resistance of *Campylobacter* in antimicrobial-free and conventional pig production systems. *J. Food Prot.* 68, 2402–2410. doi: 10.4315/0362-028X-68.11.2402

Thames, C. H., Pruden, A., James, R. E., Ray, P. P., and Knowlton, K. F. (2012). Excretion of antibiotic resistance genes by dairy calves fed milk replacers with varying doses of antibiotics. *Front. Microbiol.* 3:139. doi: 10.3389/fmicb.2012.00139

Tien, Y.-C., Li, B., Zhang, T., Scott, A., Murray, R., Sabourin, L., et al. (2017). Impact of dairy manure pre-application treatment on manure composition, soil dynamics of antibiotic resistance genes, and abundance of antibiotic-resistance genes on vegetables at harvest. *Sci. Total Environ.* 581–582, 32–39. doi: 10.1016/j.scitotenv.2016.12.138

Tragesser, L. A., Wittum, T. E., Funk, J. A., Winokur, P. L., and Rajala-Schultz, P. J. (2006). Association between ceftiofur use and isolation of *Escherichia coli* with reduced susceptibility to ceftriaxone from fecal samples of dairy cows. *Am. J. Vet. Res.* 67, 1696–1700. doi: 10.2460/ajvr.67.10.1696

Tyrrell, C., Burgess, C. M., Brennan, F. P., and Walsh, F. (2019). Antibiotic resistance in grass and soil. *Biochem. Soc. Trans.* 47, 477–486. doi: 10.1042/BST20180552

Tzavaras, I., Siarkou, V. I., Zdragas, A., Kotzamanidis, C., Vafeas, G., Bourtzi-Hatzopoulou, E., et al. (2012). Diversity of vanA-type vancomycin-resistant *Enterococcus* faecium isolated from broilers, poultry slaughterers and hospitalized humans in Greece. *J. Antimicrob. Chemother.* 67, 1811–1818. doi: 10.1093/jac/dks166

Udikovic-Kolic, N., Wichmann, F., Broderick, N. A., and Handelsman, J. (2014). Bloom of resident antibiotic-resistant bacteria in soil following manure fertilization. *Proc. Natl. Acad. Sci. U.S.A.* 111, 15202–15207. doi: 10.1073/pnas.1409836111

Van Boeckel, T. P., Brower, C., Gilbert, M., Grenfell, B. T., Levin, S. A., Robinson, T. P., et al. (2015). Global trends in antimicrobial use in food animals. *Proc. Natl. Acad. Sci. U.S.A.* 112, 5649–5654. doi: 10.1073/pnas.1503141112

Van Cuong, N., Nhung, N. T., Nghia, N. H., Mai Hoa, N. T., Trung, N. V., Thwaites, G., et al. (2016). Antimicrobial consumption in medicated feeds in vietnamese pig and poultry production. *EcoHealth* 13, 490–498. doi: 10.1007/s10393-016-1130-z

Vikram, A., and Schmidt, J. W. (2018). Functional blaKPC-2 sequences are present in U.S. beef cattle feces regardless of antibiotic use. *Foodborne Pathog. Dis.* 15, 444–448. doi: 10.1089/fpd.2017.2406

Volkova, V. V., Lanzas, C., Lu, Z., and Gröhn, Y. T. (2012). Mathematical model of plasmid-mediated resistance to ceftiofur in commensal enteric *Escherichia coli* of cattle. *PLoS One* 7:e36738. doi: 10.1371/journal.pone.0036738

Waglechner, N., and Wright, G. D. (2017). Antibiotic resistance: it's bad, but why isn't it worse? *BMC Biol.* 15:84. doi: 10.1186/s12915-017-0423-1

van Hoek, A. H. A. M., Veenman, C., van Overbeek, W. M., Lynch, G., de Roda Husman, A. M., and Blaak, H. (2015). Prevalence and characterization of ESBL- and AmpC-producing Enterobacteriaceae on retail vegetables. *Int. J. Food Microbiol.* 204, 1–8. doi: 10.1016/j.ijfoodmicro.2015.03.014

Walia, K., Sharma, M., Vijay, S., and Shome, B. R. (2019). Understanding policy dilemmas around antibiotic use in food animals & offering potential solutions. *Indian J. Med. Res.* 149, 107–118. doi: 10.4103/ijmr.IJMR_2_18

Wang, F.-H., Qiao, M., Chen, Z., Su, J.-Q., and Zhu, Y.-G. (2015). Antibiotic resistance genes in manure-amended soil and vegetables at harvest. *J. Hazard. Mater.* 299, 215–221. doi: 10.1016/j.jhazmat.2015.05.028

Wang, R., Dorp, L., van, Shaw, L. P., Bradley, P., Wang, Q., et al. (2018). The global distribution and spread of the mobilized colistin resistance gene mcr-1. *Nat. Commun.* 9:1179. doi: 10.1038/s41467-018-03205-z

Webb, H. E., Bugarel, M., den Bakker, H. C., Nightingale, K. K., Granier, S. A., Scott, H. M., et al. (2016). Carbapenem-resistant bacteria recovered from faeces of dairy cattle in the high plains region of the USA. *PLoS One* 11:e0147363. doi: 10.1371/journal.pone.0147363

Wichmann, F., Udikovic-Kolic, N., Andrew, S., and Handelsman, J. (2014). Diverse antibiotic resistance genes in dairy cow manure. *mBio* 5:e01017. doi: 10.1128/mBio.01017-13

Wilson, A., Fox, E. M., Fegan, N., and Kurtböke, D. Í (2019). Comparative genomics and phenotypic investigations into antibiotic, heavy metal, and disinfectant susceptibilities of *Salmonella enterica* strains isolated in Australia. *Front. Microbiol.* 10:1620. doi: 10.3389/fmicb.2019.01620

Winokur, P. L., Vonstein, D. L., Hoffman, L. J., Uhlenhopp, E. K., and Doern, G. V. (2001). Evidence for transfer of CMY-2 AmpC beta-lactamase plasmids between *Escherichia coli* and *Salmonella* isolates from food animals and humans. *Antimicrob. Agents Chemother.* 45, 2716–2722. doi: 10.1128/AAC.45.10.2716-2722.2001

Wongsuvan, G., Wuthiekanun, V., Hinjoy, S., Day, N. P., and Limmathurotsakul, D. (2018). Antibiotic use in poultry: a survey of eight farms in Thailand. *Bull. World Health Organ.* 96, 94–100. doi: 10.2471/BLT.17.195834

Woodford, N., Wareham, D. W., Guerra, B., and Teale, C. (2014). Carbapenemase-producing *Enterobacteriaceae* and non-*Enterobacteriaceae* from animals and the environment: an emerging public health risk of our own making? *J. Antimicrob. Chemother.* 69, 287–291. doi: 10.1093/jac/dkt392

World Health Organization and WHO Advisory Group on Integrated Surveillance of Antimicrobial Resistance (AGISAR) (2017). *Critically Important Antimicrobials for Human Medicine: Ranking of Antimicrobial Agents for Risk Management of Antimicrobial Resistance Due to Non-Human Use, 5th Revision*. Avialable online at: https://apps.who.int/iris/handle/10665/255027

Wu, N., Qiao, M., Zhang, B., Cheng, W.-D., and Zhu, Y.-G. (2010). Abundance and diversity of tetracycline resistance genes in soils adjacent to representative swine feedlots in China. *Environ. Sci. Technol.* 44, 6933–6939. doi: 10.1021/es1007802

Xie, W.-Y., Shen, Q., and Zhao, F. J. (2018). Antibiotics and antibiotic resistance from animal manures to soil: a review. *Eur. J. Soil Sci.* 69, 181–195. doi: 10.1111/ejss.12494

Yang, F., Deng, B., Liao, W., Wang, P., Chen, P., and Wei, J. (2019). High rate of multiresistant *Klebsiella pneumoniae* from human and animal origin. *Infect. Drug Resist.* 12, 2729–2737. doi: 10.2147/IDR.S219155

Yang, L., Liu, W., Zhu, D., Hou, J., Ma, T., Wu, L., et al. (2018). Application of biosolids drives the diversity of antibiotic resistance genes in soil and lettuce at harvest. *Soil Biol. Biochem.* 122, 131–140. doi: 10.1016/j.soilbio.2018.04.017

Yang, Q., Ren, S., Niu, T., Guo, Y., Qi, S., Han, X., et al. (2014). Distribution of antibiotic-resistant bacteria in chicken manure and manure-fertilized

vegetables. *Environ. Sci. Pollut. Res. Int.* 21, 1231–1241. doi: 10.1007/s11356-013-1994-1

Yang, Q., Zhang, H., Guo, Y., and Tian, T. (2016). Influence of chicken manure fertilization on antibiotic-resistant bacteria in soil and the endophytic bacteria of pakchoi. *Int J Environ Res Public Health* 13:662. doi: 10.3390/ijerph13070662

Yang, Q., Zhang, J., Zhu, K., and Zhang, H. (2009). Influence of oxytetracycline on the structure and activity of microbial community in wheat rhizosphere soil. *J. Environ. Sci.* 21, 954–959. doi: 10.1016/S1001-0742(08)62367-0

Yin, W., Li, H., Shen, Y., Liu, Z., Wang, S., Shen, Z., et al. (2017). Novel plasmid-mediated colistin resistance gene mcr-3 in *Escherichia coli*. *mBio* 8:e543-17. doi: 10.1128/mBio.00543-17

Zaheer, Z., Rahman, S. U., Zaheer, I., Abbas, G., and Younas, T. (2017). Methicillin-resistant *Staphylococcus aureus* in poultry- an emerging concern related to future epidemic. *Matrix Sci. Medica* 1, 15–18. doi: 10.26480/msm.01.2017.15.18

Zalewska, M., and Popowska, M. (2020). "Antimicrobial/antibiotic resistance genes due to manure and agricultural waste applications," in *Antibiotics and Antimicrobial Resistance Genes: Environmental Occurrence and Treatment Technologies Emerging Contaminants and Associated Treatment Technologies*, ed. M. Z. Hashmi (Cham: Springer International Publishing), 139–161. doi: 10.1007/978-3-030-40422-2_6

Zhang, X.-X., and Zhang, T. (2011). Occurrence, abundance, and diversity of tetracycline resistance genes in 15 sewage treatment plants across China and other global locations. *Environ. Sci. Technol.* 45, 2598–2604. doi: 10.1021/es103672x

Zhang, Y., Gu, A. Z., Cen, T., Li, X., He, M., Li, D., et al. (2018). Sub-inhibitory concentrations of heavy metals facilitate the horizontal transfer of plasmid-mediated antibiotic resistance genes in water environment. *Environ. Pollut.* 237, 74–82. doi: 10.1016/j.envpol.2018.01.032

Zhang, Y.-J., Hu, H.-W., Chen, Q.-L., Singh, B. K., Yan, H., Chen, D., et al. (2019). Transfer of antibiotic resistance from manure-amended soils to vegetable microbiomes. *Environ. Int.* 130:104912. doi: 10.1016/j.envint.2019.104912

Zhang, Y.-J., Hu, H.-W., Chen, Q.-L., Yan, H., Wang, J.-T., Chen, D., et al. (2020). Manure application did not enrich antibiotic resistance genes in root endophytic bacterial microbiota of cherry radish plants. *Appl. Environ. Microbiol.* 86:e2106-19. doi: 10.1128/AEM.02106-19

Zhao, X., Wang, J., Zhu, L., Ge, W., and Wang, J. (2017). Environmental analysis of typical antibiotic-resistant bacteria and ARGs in farmland soil chronically fertilized with chicken manure. *Sci. Total Environ.* 593–594, 10–17. doi: 10.1016/j.scitotenv.2017.03.062

Zhao, Y., Su, J.-Q., An, X.-L., Huang, F.-Y., Rensing, C., Brandt, K. K., et al. (2018). Feed additives shift gut microbiota and enrich antibiotic resistance in swine gut. *Sci. Total Environ.* 621, 1224–1232. doi: 10.1016/j.scitotenv.2017.10.106

Zhao, Y., Yang, Q. E., Zhou, X., Wang, F.-H., Muurinen, J., Virta, M. P., et al. (2020). Antibiotic resistome in the livestock and aquaculture industries: status and solutions. *Crit. Rev. Environ. Sci. Technol.* 1–38. doi: 10.1080/10643389.2020.1777815

Zhu, B., Chen, Q., Chen, S., and Zhu, Y.-G. (2017). Does organically produced lettuce harbor higher abundance of antibiotic resistance genes than conventionally produced? *Environ. Int.* 98, 152–159. doi: 10.1016/j.envint.2016.11.001

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Do Long-Term Conservation Pasture Management Practices Influence Microbial Diversity and Antimicrobial Resistant Genes in Runoff?

Yichao Yang[1], Amanda J. Ashworth[2], Lisa M. Durso[3], Mary Savin[1], Jennifer M. DeBruyn[4], Kimberly Cook[5], Philip A. Moore Jr.[2] and Phillip R. Owens[6]*

[1]*Department of Crop, Soil, and Environmental Sciences, University of Arkansas, Fayetteville, AR, United States,* [2]*USDA-ARS, Poultry Production and Product Safety Research Unit, Fayetteville, AR, United States,* [3]*USDA-ARS, Agroecosystem Management Research Unit, Lincoln, NE, United States,* [4]*Department of Biosystems Engineering & Soil Science, University of Tennessee, Knoxville, Knoxville, TN, United States,* [5]*USDA-ARS, Nutrition, Food Safety/Quality, Office of National Programs, Beltsville, MD, United States,* [6]*USDA-ARS, Dale Bumpers Small Farms Research Center, Booneville, AR, United States*

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***Correspondence:**
Amanda J. Ashworth
amanda.ashworth@usda.gov

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Runoff from land-applied manure and poultry litter is one mechanism by which manure-borne bacteria are transported over large distances in the environment. There is a global concern that antimicrobial resistant (AMR) genes may be transmitted through the food chain from animal manures to soil to surface water. However, details are lacking on the ecology of AMR genes in water runoff as well as how conservation management practices may affect the runoff microbiome or minimize the movement of AMR genes. The aim of this study was to identify microbial community structure and diversity in water runoff following 14-years of poultry litter and cattle manure deposition and to evaluate the amount of AMR genes under five conventional and conservation pasture management strategies. Since 2004, all watersheds received annual poultry litter at a rate of 5.6 Mg ha^{-1} and were consistently managed. Surface runoff samples were collected from each watershed from 2018 to 2019, characterized using Illumina 16S rRNA gene amplicon sequencing and enumerated for four AMR-associated genes (*ermB*, *sull*, *intlI*, and $bla_{ctx\text{-}m\text{-}32}$) using quantitative PCR. Overall, long-term pasture management influenced water microbial community structure, with effects differing by year ($p < 0.05$). Bacterial richness (Chao1 index) was influenced by pasture management, with the lowest richness occurring in the control (nearby non-agricultural water source) and the greatest under fields that were hayed (no cattle presence). Runoff bacterial richness in watersheds increased following poultry litter applications, indicating poultry litter is a possible source of bacteria and altered runoff community structure. The $bla_{ctx\text{-}m\text{-}32}$ gene was not detected in any surface water sample. The remaining three AMR genes were absent in the non-agricultural control, but present in agricultural samples. However, there was no impact ($p > 0.05$) from pasture management on the abundance of these genes, indicating both conventional and conservation practices have similar ecologies for these targets; however, there was a greater detection of *sull* genes from runoff in continuously grazed systems in 2019, with hay being lowest in 2019. Results illustrate that the edge of field buffer strips may increase bacterial richness in water runoff, but these changes in richness do not greatly impact target AMR genes in the United States largest land-use category.

Keywords: antimicrobial resistance, runoff microbiome, conservation agriculture, animal manure, microbial abundance

INTRODUCTION

Livestock manure and byproducts are valuable fertilizer sources [namely nitrogen (N), phosphorus (P), and potassium (K)] in grassland systems, but their soil and water microbial ecologies may be affected by management. Previous work by Yang et al. (2019) evaluated how pasture management [hayed (H), continuously grazed (CG), rotationally grazed (R), rotationally grazed with a buffer strip (RB), rotationally grazed with a fenced riparian buffer (RBR), and a control (represented by nearby non-agricultural water samples)] affected soil bacterial diversity and found that CG systems had greater community richness, which corresponded with greater soil pH and nutrients. Consequently, continuously grazed systems reportedly increase soil microbial diversity, owing to continuous nutrient-rich manure deposition; however, this management strategy may adversely affect aboveground plant communities and water quality. In an additional study, Yang et al. (2020) quantified four antimicrobial resistant (AMR) genes in these soils after 14-years of continuous management and found that *ermB*, *sulI*, and *intlI* genes were the greatest under long-term CG (relative to the conservation best management practices), suggesting continuous cattle manure deposition may increase AMR gene presence. Therefore, soil is a natural reservoir of AMR bacteria and genes (Forsberg et al., 2012). However, other studies have indicated that AMR genes can be found in un-grazed and non-agricultural soils (Durso et al., 2012, 2016). Similarly, Cadena et al. (2018) identified tetracycline and sulfonamide antibiotic resistance genes in soils from organic farming operations.

In addition to nutrients, runoff from land-applied manures can carry bacteria and genes originating from both manure and soil. Large-scale rainfall events have been linked to decreases in microbial water quality, with 51% of waterborne disease outbreaks occurring following precipitation events (Hrudey et al., 2003; USEPA, 2007). Runoff may also contain AMR bacteria and genes (Barrios et al., 2020; Meyers et al., 2020). Following land application of poultry litter, antibiotics, AMR bacteria, and AMR genes may move from soil through runoff, leaching, and particle adsorbed runoff (Kay et al., 2004; Leal et al., 2013; Sun et al., 2013), thus potentially ending up in surface and groundwater (He et al., 2014). However, the extent of this is largely unknown in the United States largest land-use category.

Manure management practices have been shown to impact runoff. Kreuzig et al. (2005) found that litter incorporation reduced AMR concentrations in runoff relative to surface applications. Kay et al. (2004) identified that land application methods (i.e., chisel plowing compared to no-tillage) affected AMR surface water runoff. Thurston-Enriquez et al. (2005) also confirmed that bacterial and pathogenic fecal levels in runoff were elevated in a rainfall simulation study, which was attributed to manure land application. Numerous factors affect how soil and fecal microorganisms are transported in manure-amended fields, although little is known about how specific management practices (e.g., rotational grazing) affect runoff microbial communities or the composition of AMR genes. Conservation management practices may help to mitigate AMR gene distribution (Heinonen-Tanski and Uusi-Kamppa, 2001; Tate et al., 2006), although the degree of this is unknown.

Here, we test the hypothesis that pasture management will impact the runoff microbial communities of long-term watersheds (14-years) receiving poultry litter amendments and determine if conservation agricultural practices can minimize the dissemination of four AMR genes chosen to represent targets important in human health, agriculture, and environmental AMR surveillance.

Abbreviations: ARG, Antibiotic resistant genes; AMR, Antimicrobial resistance; PERMANOVA, Permutational analysis of variance; ANOVA, Analysis of variance; CG, Continuously grazed; H, Hayed system; RBR, Rotational grazing with a fenced riparian buffer; OTU, Operational taxonomic unit; PCoA, Principle coordinate analysis; qPCR, Quantitative-polymerase chain reaction; *ermB*, Erythromycin resistance gene; *sulI*, Sulfonamide resistance gene integrase gene; *intlI*, Integrase gene; $bla_{ctx\text{-}m\text{-}32}$, β-Lactams resistance gene.

MATERIALS AND METHODS

Treatment Implementation and Sample Collection

A field study was initiated in 2004 at the USDA-ARS Dale Bumpers Small Farms Research Center in Booneville, Arkansas (N 35°06′12″ W 93°56′05″ 150 m altitude) to evaluate the impact of conservation pasture management on water quality (Pilon et al., 2017a,b, 2018; Yang et al., 2019, 2020; Anderson et al., 2020). Fifteen watersheds were constructed on a site with an average slope of 8% and on an Enders (fine, mixed, active, and thermic Typic Fragiudults) and Leadvale silt loam (fine-silty, siliceous, semiactive, and thermic Typic Fragiudults). Each watershed was 25 × 57 m for a total area of 0.14 ha, where common bermudagrass (*Cynodon dactylon* L.) was the dominant forage.

Four agricultural management strategies were evaluated, along with one non-agricultural set of control samples. Grazing management strategies were implemented from 2004 to 2019 with three replications: hayed (H), continuously grazed (CG), rotationally grazed (R), rotationally grazed with a buffer strip (RB), and rotationally grazed with a fenced riparian buffer (RBR; **Figure 1**). The H treatment was hayed three times annually (April, June, and October) to a height of 10 cm with a rotary hay mower (no cattle in these watersheds). The CG watersheds were continuously grazed by one or two calves throughout the year. The R watersheds were rotationally grazed by three steers turned into the paddocks when forage height was 20–25 cm (10–15 cm using a disc meter) and removed when forage height was 10–15 cm (5 cm using disc meter). The RB watersheds were rotationally grazed with a 15.3-m buffer strip composed of the same vegetation (total area of 283 m^2) at the base of these watersheds. The RBR watersheds were rotationally grazed with a fenced riparian buffer area to exclude cattle and planted with four sapling white oak (*Quercus alba* L.), four green ash (*Fraxinus pennsylvanica* Marshall), and four pecan [*Carya illinoinensis* (Wangenh.) K. Koch] trees in 2003 (Pilon et al., 2017a; **Figure 1**). Each watershed

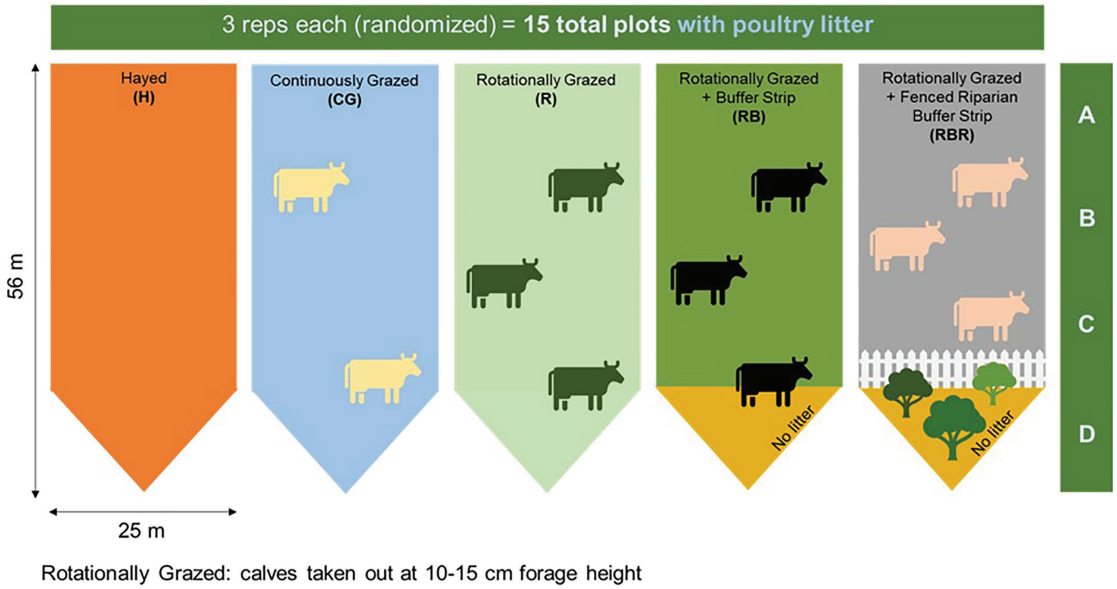

FIGURE 1 | Schematic representation of the experimental set-up. Randomized complete block design with three replications (15 watersheds total) from 2004 to 2019. All areas have received annual poultry litter applications. Figure credit: Amorim et al. (2020). Continuous treatments included: hayed (H), continuously grazed (CG), rotationally grazed (R), rotationally grazed with a buffer strip (RB), and rotationally grazed with a fenced riparian buffer (RBR). Water samples were collected from 2018–2019 at Booneville, AR. For both years, both pre and post samples were used in 16S sequencing (n = 60 total), but only post samples were used in qPCR analyses (n = 30 total). Therefore, only 16S methods evaluated poultry litter timing effects.

was divided, perpendicular to the slope into three zones (corresponding to shoulder, upper backslope, and lower backslope positions), whereas the RBR consist of four zones. Broiler litter was land applied at a rate of 5.6 Mg ha^{-1} in April or May of each year to each watershed (excluding the riparian buffer strip). Since poultry litter was omitted in the buffered area of the RB and RBR treatment, application rates were identical on an aerial basis (RB and RBR watersheds received 658 kg plot^{-1}, whereas H, R, and CG received 794 kg plot^{-1}). Broiler litter was obtained annually from a commercial broiler farm near Booneville, AR.

Watersheds were hydrologically isolated from each other by earthen berms that were constructed with offsite soil. Briefly, the bottom of each watershed narrows to a point containing a covered 30.5-cm H-series fiberglass flume equipped with a pressure transducer for measuring runoff volumes (Pilon et al., 2017a). The transducer was connected to a housed automatic water sampler (American Sigma Corporation), which was programed to automatically collect 100 ml of sample for analysis from every 94.7 L of surface runoff. Surface runoff water samples (one per watershed) were collected after each rain event (typically within 24 h). Runoff samples collected during 2018 and 2019 were utilized for this study. For both years, there were 60 water runoff samples [30 runoff samples collected 3 months prior to poultry litter application ("pre"), and 30 runoff samples collected within 3 months following poultry litter application ("post")]. For both years, both pre and post samples were used in 16S sequencing (n = 60 total), but only post samples were used in qPCR analyses (n = 30 total). Therefore, only 16S methods evaluated poultry litter timing effects.

Since all watersheds used in this study received animal inputs (poultry litter and cattle manure), water samples from a more pristine water source (the Mulberry River) were included to serve as a control. Evaluation of AMR in non-agricultural samples provides background data on AMR occurring in similar soils without agricultural impacts. These background data are used to contextualize study results and provides insight on the impact of agricultural management practices on AR within agroecosystems (Rothrock et al., 2016). The Mulberry River is a 110 km long tributary of the Arkansas River in northwest Arkansas and has been designated as a National Wild and Scenic River since 1992. Samples were collected (Latitude: 35.6693 Longitude: −93.6676; n = 3 per year) and stored as described above.

DNA Extraction, qPCR Amplification, and Sequencing

Each water sample was filtered by placing a sterile member filter (45 mm, 0.45 µm pore size, polycarbonate) on the filter base, grid-side up, and then placing another filter (47 mm, 1.2 µm, cellulose) on top of the 0.45 µm polycarbonate filter. After 250 ml of water passed through, filters were removed from the filter base and sterile forceps were used to aseptically discard the 1.2 µm filter. The 0.45-µm isopore filter was folded and transferred to corresponding labeled Lysing Matrix E tube. DNA was extracted from each water sample using the extraction

kit of MpBio FastDNA Spin Kit for Soil (MpBio Laboratories, SKU 116560200-CF) according to the manufacturer's directions. Extracted DNA was quantified using Quant-It™ PicoGreen® (Invitrogen) dsDNA quantitation assay and stored at 20°C.

Bacterial community composition was determined using Illumina Miseq sequencing of 16S rRNA gene amplicons. Extracted DNA was sent to the University of Tennessee Genomic Services Laboratory, where the V4 region of the 16S rRNA gene was amplified with barcoded primers 515F and 806R (Caporaso et al., 2011). Amplicon libraries were pooled and 291 base-paired end sequences were obtained on the Illumina MiSeq Platform, resulting in a total of 5,997,907 sequence reads. Reads were processed using the open source bioinformatics software Mothur V 1.40.0 following the Miseq SOP protocol (Kozich et al., 2013). Sequences that did not match the primers were eliminated from demultiplexed sequence reads. These ambiguous base sequences with a length less than 100 bp were deleted and chimeric sequences were removed using the UCHIME algorithm implemented in Mothur. After the quality control pipeline, 5,969,039 sequence reads remained using a 97% similarity threshold to define ribotypes in Mothur (21.27% were deleted). Taxonomic assignment was performed using the Greengenes database. Microbial alpha diversity in observed operational taxonomic unit (OTU) level including Chao1, Shannon index, and Simpson index were calculated using Mothur. To detect significant differences based on fixed effects of pasture management (H, CG, R, RB, and RBR), timing of sampling (pre or post poultry litter application), and random effects (year and replication), an analysis of variance (ANOVA) was used by the JMP software (JMP®12; SAS Institute, 2007). Probability values less than 0.05 were considered as significant. Beta-diversity was measured using Bray-Curtis index. Principle coordinate analysis (PCoA) plots were generated based on weighted and unweighted UniFrac distance metrics by using MicrobiomeAnalyst (Dhariwal et al., 2017). Bacterial community structure was quantified in a matrix of Bray-Curtis similarities, which was then analyzed in a permutational analysis of variance (PERMANOVA) to compare bacterial communities at the phylum level in PRIMER-E.

Detection and Analysis of Four AMR Genes Following Long-Term Management Using qPCR

The extracted DNA from runoff following poultry litter applications (n = 30; 15 from October of 2018 and the other 15 from October 2019) were used directly in the quantitative PCR (qPCR) for detection of four genes associated with AMR described in clinical isolates, which includes *ermB* (Florez et al., 2014), *sulI* (Barraud et al., 2010), *intlI* (Pei et al., 2006), and $bla_{\text{ctx-m-32}}$ (Szczepanowski et al., 2009), using previously published primers (Yang et al., 2019). Each PCR amplification was performed in triplicate. The positive control (named as gBlock2 4G with 16S *ermB* Florez 1-18-17) is an 808 bp double stranded synthetic gBlocks® gene fragment synthesized by Integrated DNA technologies, lnc. (Blazejewski et al., 2019). It contains four genes of *ermB*, *sulI*, *intlI*, and $bla_{\text{ctx-m-32}}$. The standard curves consisted of a serial dilution of known copy numbers of the gene fragment, ranging from 1.15×10^5 to 1.15×10^{11} copies per 5 µl. The quantities of gene copy numbers were calculated based on the standard curve using Quant Studio 3 real-time PCR system. (ThermoFisher Scientific). As a negative control, all sets of primers were tested with sterile water as the template, and all of them were below threshold. Each 20 µl qPCR reaction included 5 µl of extracted DNA (approximately 100 ng), 10 µl of SYBR Green PCR Master Mix, and 100 mM of each primer. The following cycling conditions were used: an initial denaturation step of 15 min at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s at annealing temperature specific for each gene (Yang et al., 2019), and 10 s at 72°C, followed by 60–95°C of melting curve. The amplification efficiency was between 92 and 105%, and the R^2 value is above 0.98. Baseline and threshold calculations were performed using QuantStudio® Design & Analysis software. The quantities of gene copy numbers were determined using standard curves. Gene copy abundances were normalized per volume of water. Finally, the gene copy numbers per volume were transformed into log10 values for further statistical analysis, as they were not normally distributed (Ganger et al., 2017). To detect significant differences for the fixed effect (pasture management) and random effect (year), an ANOVA was used by the JMP software (JMP®12; SAS Institute, 2007), with replicate as a random effect. Probability values less than 0.05 were considered significant.

This set of targets was chosen to cover clinically relevant, environmentally relevant, and agriculturally relevant antibiotic resistance determinants. The specific targets were chosen by a panel of scientists working on antibiotic resistance in agriculture, and they aligned with an environmental antibiotic resistance gene surveillance effort in Europe. The blaCTX-M gene codes for third-generation cephalosporin resistance, one type of β-lactamase resistant drug. These drugs are classified as "Critically Important" (the top category) by WHO. Most individual drugs in the class are limited to use on humans, pets (dog/cat), and horses; however, two (cefquinome and ceftiofur) are indicated for use in food animals, though they are not administered to groups of animals *via* food or water. The ermB gene codes for resistance to macrolide drugs such as erythromycin. These drugs are classified as "Critically Important" (the top category) by the WHO. Erythromycin is used in large and small animals, is FDA approved for use in cattle, swine, and poultry, and is administered to food animals *via* food and water. A related macrolide, azithromycin, is also approved for use in sick food animals and is individually listed on the 2013 CDC AR Threat list as a concern for some foodborne pathogens. The sul1 gene codes for sulfonamide-resistance. It is one of the most commonly studied resistance genes in environmental samples. Sulfonamides are classified as "Highly Important" (the second category) by the WHO. Three drugs in this class are used in food animals and administered to groups of animals *via* food and water. Finally, the intl1 gene codes for an integron-integrase gene that helps AR genes to spread from cell to cell. It has been proposed as a gene that will help to identify resistance that is associated with human activities, and as a marker for "pollutants" including AR, heavy metals, and disinfectants.

RESULTS

Effects of Pasture Management, Poultry Litter Application Timing, and Sampling Year on Alpha Diversity of Runoff Bacterial Communities

Comparisons of Runoff Among Pasture Management Systems (Control Excluded)

Control samples were excluded in analyses to evaluate how agricultural practices influenced explanatory variables (within watersheds) but are included in the next section. Conservation pasture management including grassed buffer strips and riparian buffers affected bacterial richness (Chao1 estimate), with greater richness occurring in H, RB, and RBR and the lowest richness in CG and R ($p < 0.05$; **Table 1**; **Figure 2A**). However, there was no impact on bacterial diversity estimated by Shannon and Simpson indexes, when control samples were excluded in the analysis ($p > 0.05$; **Figures 2B,C**; **Table 1**). Bacterial richness increased in runoff following poultry litter application ($p < 0.05$). However, poultry litter application timing had no impact on runoff bacterial diversity ($p > 0.05$; **Table 1**). There was an interaction between year and poultry litter application timing on bacterial richness, with pre-poultry litter applications in 2018 being the lowest and post applications in 2019 being the greatest ($p < 0.05$; **Table 1**). Although there was no year effect on richness ($p > 0.05$), diversity varied by year ($p < 0.05$; **Table 1**). Overall, runoff bacterial diversity was lower in 2018 and greater in 2019 (**Table 1**).

To evaluate differences in alpha diversity between water runoff samples under pasture management and the control, a comparison between these two datasets was conducted. Bacterial richness in control samples were lower than samples collected from watersheds under pasture management ($p < 0.05$; **Figure 2D**). However, there were no diversity differences between control samples and pasture management based on Shannon and Simpson indices (**Table 1; Figures 2E,F**). Year also had no influence on bacterial richness, however, there was an impact of year on bacterial diversity between runoff and control samples ($p < 0.05$). An interaction between pasture management and poultry litter application timing (pre vs. post) on richness was also detected, with pre in the control being lowest and post in the H treatment the greatest ($p < 0.05$; **Table 1**).

Bacterial Community Composition Based on Pasture Management, Poultry Litter Application Timing, and Sampling Years

Runoff water bacterial community composition was not altered based on pasture management (H, CG, R, RB, and RBR;

TABLE 1 | ANOVA of richness (Chao1) and diversity (Shannon) in alpha diversity influenced by pasture management and year following 13 years of pasture management and year.

	Parameter	Factor	*df*	*F*-value	*p*
With Control Samples	Richness	Pasture management[4]	5	3.1464	0.0235*
		Timing	1	0.0884	0.7673
		Year	1	0.262	0.6106
		Pasture management × Year	5	0.7954	0.5347
		Pasture management × Timing	5	2.7036	0.0428*
		Year × Timing	1	3.6696	0.0601
		Pasture × Year × Timing	5	1.1895	0.3291
	Diversity	Pasture management	5	1.1805	0.3329
		Timing	1	1.08	0.2854
		Year	1	4.9233	0.0302*
		Pasture management × Year	5	0.1155	0.9763
		Pasture management × Timing	5	1.2247	0.3145
		Year × Timing	1	0.0304	0.862
		Pasture × Year × Timing		0.8642	0.4931
Without Control Samples	Richness	Pasture management	4	2.9211	0.0332*
		Timing	1	1.2636	0.0005*
		Year	1	0.0988	0.7549
		Pasture management × Year	4	0.7385	0.5715
		Pasture management × Timing	4	2.5099	0.0573
		Year × Timing	1	6.1145	0.0176*
		Pasture × Year × Timing	4	1.1043	0.3683
	Diversity	Pasture management	5	1.0732	0.3829
		Timing	1	1.0907	0.3028
		Year	1	9.7051	0.0034*
		Pasture management × Year	5	0.1050	0.9801
		Pasture management × Timing	5	1.1134	0.3641
		Year × Timing	1	0.0238	0.8782
		Pasture × Year × Timing	5	0.7856	0.5415

[4]*Treatments included: hayed (H), continuously grazed (CG), rotationally grazed (R), rotationally grazed with a buffer strip (RB), rotationally grazed with a fenced riparian buffer (RBR); Timing = poultry litter application timing, pre and post; Year = 2018 and 2019.*

*$p < 0.05$.

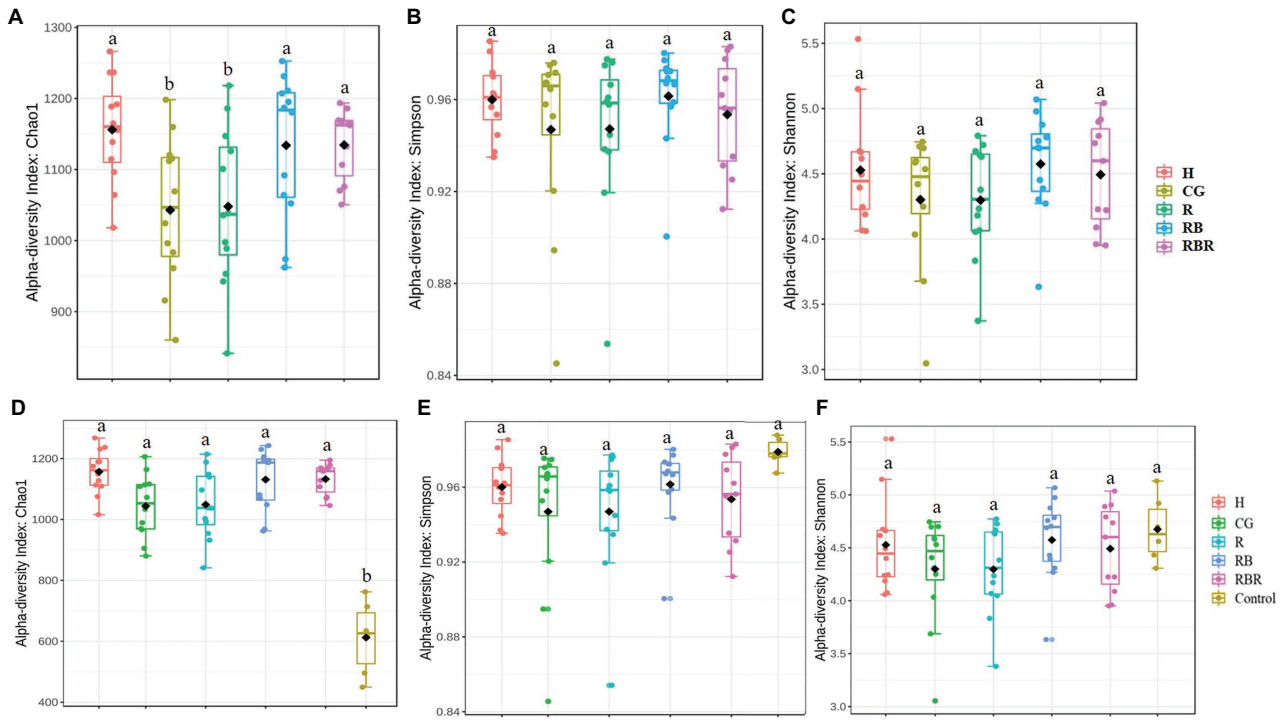

FIGURE 2 | Alpha diversity of bacterial communities in runoff water from different pasture management treatments. Treatments included: hayed (H), continuously grazed (CG), rotationally grazed (R), rotationally grazed with a buffer strip (RB), and rotationally grazed with a fenced riparian buffer (RBR). Illustrates results from only pasture management included three indices of Chao1 **(A)**, Simpson **(B)** and Shannon **(C)**. The factor includes non-agricultural samples (control), Chao1**(D)**, Simpson **(E)**, and Shannon **(F)**.

$p > 0.05$) at the OTU or order level (**Table 2**; **Figure 3**). However, there was a difference in bacterial community composition between water runoff and control samples at both levels (OTU and order; PERMANOVA $p < 0.05$; **Table 2**; **Figure 3B**). Year also had an impact on bacterial communities (with or without control samples; **Table 2**; **Figure 3**), as it differed in water bacterial communities at the phyla level between sampling years (2018 and 2019; $p < 0.05$; **Figure 3**).

The bacterial community composition was analyzed at two different levels (phylum and order). The following top 10 phyla dominated agricultural runoff bacterial communities: Proteobacteria (mean relative abundance of all libraries was 50.7%), Bacteriodetes (30.2%), Verrucomicrobia (6.4%), Firmicutes (3.1%), Actinobacteria (2.7%), Fibrobacteres (2.0%), Acidobacteria (1.2%), Saccharibacteria (TM7; 0.9%), Chloroflexi (0.6%), and Nitrospirae (0.3%; **Figure 3A**). The following top 10 orders were Burkholderiales (22.9%), Sphingobacteriales (18.1%), Flavobacteriales (11.8%), Pseudomonadales (11.5%), Saprospirales (4.4%), Caulobacterales (4.1%), Rhizobiales (4.0%), Verrucomicrobiales (3.9%), Actinomycetales (3.5%), and Bacillales (3.2%; **Figure 3B**).

PCoA of Bray-Curtis distance of the bacterial community visualized differences between pasture management systems and non-agricultural samples (control). However, the bacterial community composition was not influenced by pasture management (when pristine samples were excluded from metadata), suggesting there was no difference among H, CG, R, RB, and RBR (**Figure 4A**) in terms of runoff water bacterial communities. However, the bacterial community composition was different between pasture management and control samples (**Figure 4B**).

Beta diversity was influenced by poultry litter application timing, with greater beta diversity in runoff samples collected after poultry litter application and lower diversity in runoff samples collected before poultry litter application (**Figure 4C**). Year also had an influence on the beta diversity of runoff, resulting in greater diversity in 2018 and lower diversity in 2019 (**Figure 4D**).

Distribution of Four AMR Associated Genes in Runoff Based on Pasture Management and Sampling Time

Quantification of ARG Targets

Pasture management (H, CG, R, RB, and RBR) and year (2018 and 2019; $P > 0.05$) had no impact on the abundance of *ermB* gene (**Table 3; Figure 5**), nor was there an interaction for pasture management × year on the abundance of *ermB* genes ($p > 0.05$). The $bla_{\text{ctx-m-32}}$ gene was not detected in any water runoff samples, and $bla_{\text{ctx-m-32}}$ was therefore not included in Tables or Figures. Similarly, there was no amplification of the four AMR-associated genes from the control water samples.

TABLE 2 | PERMANOVA in bacterial community structure by pasture management and year with or without non-agricultural samples (control).

	Factor	Pseudo-F	*p*
Including Control Samples (OTU level)	Pasture management[4]	0.8921	0.0095*
	Year	7.1399	0.0001*
	Pasture management × Year	−0.6100	0.7671
Including Control Samples (Order level)	Pasture management	11.871	0.0001*
	Year	12.048	0.0001*
	Pasture management × Year	0.4976	0.5596
No Control Samples (OTU level)	Pasture management	0.8184	0.5156
	Year	7.3945	0.0001*
	Pasture management × Year	−1.0893	0.5358
No Control Samples (Order level)	Pasture management	0.6904	0.701
	Year	12.532	0.0001*
	Pasture management × Year	−1.1535	0.6112

[4]*Treatments included: hayed (H), continuously grazed (CG), rotationally grazed (R), rotationally grazed with a buffer strip (RB), rotationally grazed with a fenced riparian buffer (RBR); Year = 2018 and 2019.*
$p < 0.05$.

Pasture management (H, CG, R, RB, and RBR) had no impact on the abundance of *intlI* gene ($p > 0.05$), however, differences in abundance of *intlI* gene was identified between 2018 and 2019, with a greater abundance of *intlI* occurring in 2018 than 2019 ($p < 0.05$; **Figure 5**). There was no interaction for pasture management × year on the abundance of *intlI* genes ($p > 0.05$). Similarly, there was no difference in *sulI* gene detection based on pasture management (H, CG, R, RB, and RBR), and year (2018 and 2019; $p > 0.05$). However, the interaction between pasture management and year had an influence on the abundance of *sulI* genes detected from runoff samples ($p < 0.05$; **Table 3**). Overall, CG was the greatest in 2019 and H being the lowest in 2019.

DISCUSSION

Effect of Pasture Management, Poultry Litter Application, and Year on Alpha Diversity of Water Runoff Bacterial Communities

The least bacterial richness occurred in water runoff collected from CG, and the greatest from H (when control samples were excluded from the analysis). This result indicated that continuous grazing decreased bacterial richness in runoff, with no grazing increasing the bacterial richness in runoff samples (without control samples). This was not expected, as consistent grazing leads to less vegetative cover than other pasture management systems, thus causing greater bacterial movement and subsequent increased bacterial richness (Yang et al., 2020). Compared with non-agricultural samples, the bacterial richness was greater in pasture systems. This suggests bacterial richness in the oligotrophic river is lower than agricultural runoff samples. One explanation is that runoff samples from agricultural pastures included greater soil, soil bound nutrients, and manure sources, which are sources of greater microbial diversity (Ashworth et al., 2017; Yang et al., 2019).

Greater bacterial richness occurred in the runoff samples following poultry litter application to watersheds. Therefore, it is likely that poultry litter included sources of bacteria and introduced its own suite of bacteria to the watersheds, which had attenuating effects over time (Ashworth et al., 2017). Previous studies found similar results in that poultry litter applications increased bacterial richness in soil (Yang et al., 2019). Year effects also caused differences in runoff bacterial diversity, with greater diversity in 2019 than in 2018. However, no differences were found for bacterial richness between 2018 and 2019. Overall, the best management practices of grassed buffer strips and riparian buffers increased bacterial richness relative to overgrazed or continuously grazed systems, thus richness is improved by edge of field filter strips. In a previous study at this site, Pilon et al. (2018) found that these best management strategies increased carbon and nitrogen in long-term runoff, therefore, increased richness could be owing to greater microbial substrate (C and N) following these conservation practices. Such increases in runoff C, N, and richness could be due to greater plant mass in runoff from vegetative filter strips.

Bacterial Community Composition Based on Pasture Management, Poultry Litter Application, and Sampling Year (With Control Samples)

Results illustrated the importance of evaluating water bacterial community across pasture management and sampling year, as well as the impact from poultry litter application timing. Beta diversity analysis indicated that bacterial community composition was different between runoff samples collected from our experimental watersheds and the control samples (Mulberry River, the National Wild and Scenic River). Water runoff samples included the dominant phylum Proteobacteria, however, the most abundance bacteria in control samples was Acidobacteria. Proteobacteria is a major phylum of Gram-negative bacteria including a variety of pathogenic genera, such as *Escherichia*, *Salmonella*, *Helicobacteria*, and many others (Madigan and Martinko, 2005). Proteobacteria has also been estimated as the most abundant phylum in most soils, where their functional roles are connected to such processes as nitrogen fixation and

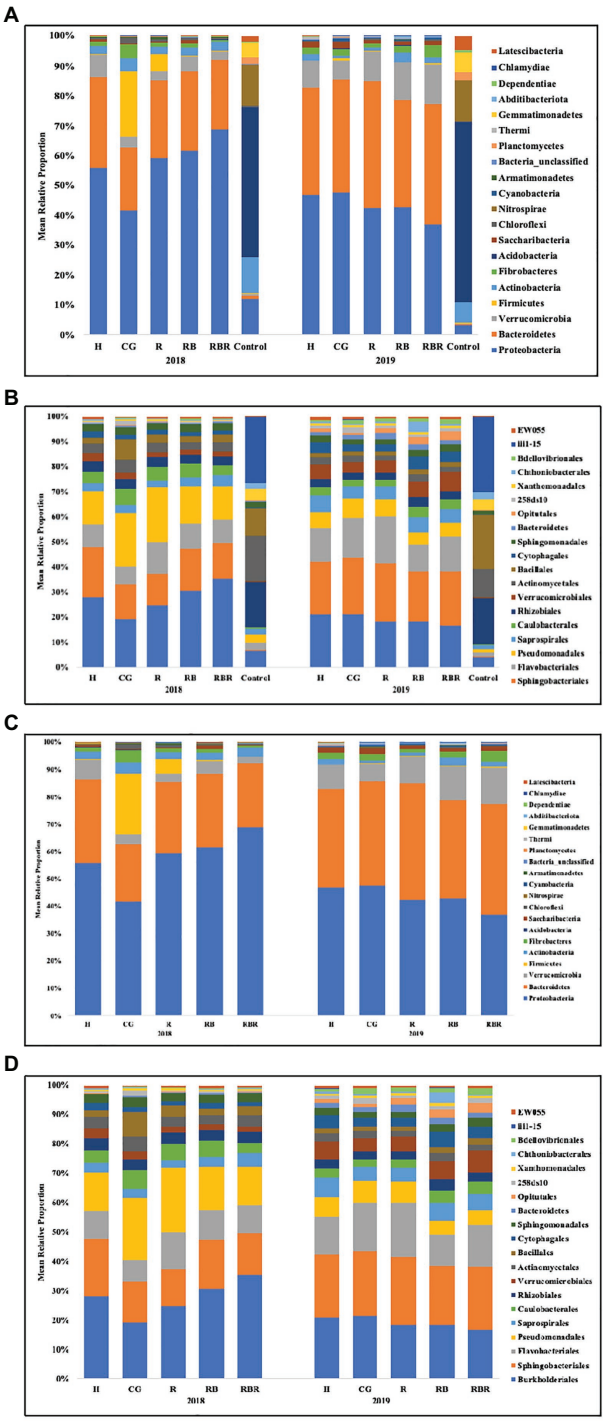

FIGURE 3 | Mean relative proportion of bacterial taxa at the phylum **(A)** and order **(B)** level found in runoff (with or without control samples included in the analysis) following long-term conservation pasture management. Treatments included: hayed (H), continuously grazed (CG), rotationally grazed (R), rotationally grazed with a buffer strip (RB), and rotationally grazed with a fenced riparian buffer (RBR). **(A)** Bacterial community composition at the phylum level found in runoff with control samples. **(B)** Bacterial community composition at the order level found in runoff with control samples. **(C)** Bacterial community structure in phylum level found in runoff without control samples. **(D)** Bacterial community structure in order level found in runoff without control samples.

oxidation of iron, sulfur, and methane (Spain et al., 2009; Itavaara et al., 2016). Many studies also identified Proteobacteria as the dominant phylum in drinking water and sediment (Spring et al., 2000; Zhang et al., 2017). Considering the phylogenetic breadth of the Acidobacteria, it is similar to the metabolically diverse Proteobacteria, albeit they both fill different niches. Acidobacteria plays an important role in using nitrite as a N source, respond to soil macro-, micro-nutrients, and soil acidity, as well as expressing multiple active transporters, degrading gellan gum, and producing exopolysaccharide (Kielak et al., 2016).

The bacterial community composition was different pre- and post-poultry litter application, with diversity increasing directly following poultry litter land applications, which indicated that poultry litter was a possible source of bacteria and altered runoff community structure. The bacterial community structure also varied between 2018 and 2019, which indicated that bacterial communities in these watersheds may vary annually and interannually.

Distribution of Four AMR Associated Genes in Runoff Samples Based on Pasture Management and Sampling Year

Overall, the main effect of pasture management (H, CG, R, RB, and RBR) had no effect on the four AMR-associated genes (*ermB*, *sulI*, *intlI*, and $bla_{\text{ctx-m-32}}$) in water runoff samples ($p > 0.05$). Although there was a greater detection of *sulI* genes from runoff samples in the CG system in 2019, with H being the lowest in 2019. The $bla_{\text{ctx-m-32}}$ gene codes for beta-lactamase resistance and is one of the top AMR global health priorities. The $bla_{\text{ctx-m-32}}$ variant has been previously associated with cattle, cattle feces, and poultry (Cottell et al., 2013; Bevan et al., 2017; Palmeira and Ferreira, 2020), but was not detected in any of our post-poultry litter application runoff samples. A related study evaluating AMR associated genes in soils, indicated *ermB*, *sulI*, and *intlI* genes in soil were the highest under continuous grazing (relative to the conservation best management practices), suggesting overgrazing and continuous cattle manure deposition may increase AMR gene presence (Yang et al., 2020). In this same study, metagenomic shotgun sequencing revealed a greater total number of AMR genes in soils under long-term CG, while fewer AMR genes were found in H (no cattle manure; Yang et al., 2020). In the current study, we did not observe an increase in the macrolide resistance gene *ermB*, sulfonamide resistance gene *sul1*, or the integrase genes *intI1* in runoff based on pasture management. Of note, there was no amplification of these four genes from control water samples. Therefore, although bacterial community composition has been shown to impact bacterial resistomes in many habitats (Fosberg et al., 2014), the runoff bacterial community changes observed with different pasture management strategies was not linked with significant changes in the four AMR-associated genes quantified in this study. A study by Hall et al. (2020) found that a filter strip to surface water setback distance between 34 and 67 m is required to allow manure-borne antibiotics and ARGs in runoff to reach background levels;

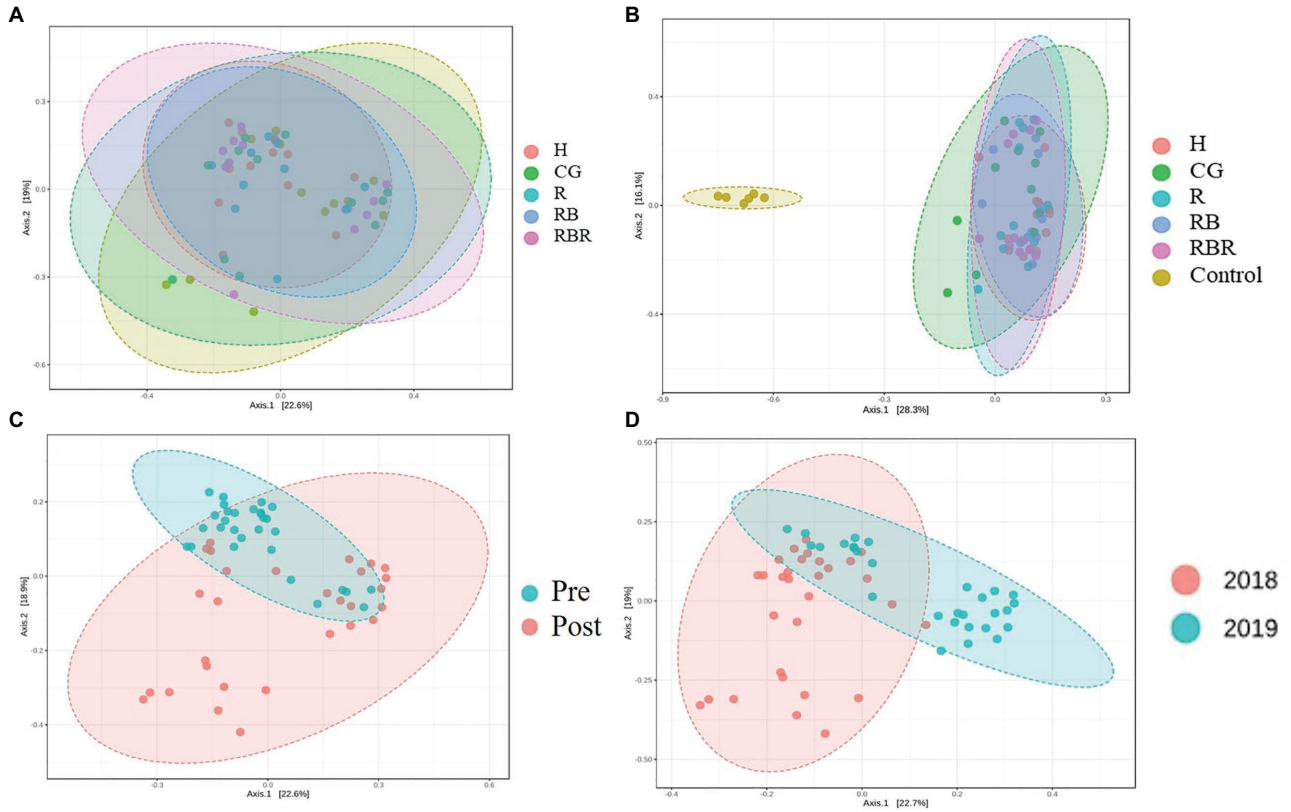

FIGURE 4 | The beta diversity of bacterial community impact by pasture management [without **(A)** or with control samples **(B)**], sampling collection timing (pre and post poultry litter surface application; **C**) and year (2018 and 2019; **D**) in water samples. Treatments included: hayed (H), continuously grazed (CG), rotationally grazed (R), rotationally grazed with a buffer strip (RB), and rotationally grazed with a fenced riparian buffer (RBR).

TABLE 3 | ANOVA results testing for differences in quantities of three AMR genes following poultry litter soil applications (runoff samples collected within 3 months) without control samples by pasture management, as well as the interaction between these two factors at Booneville, AR from 2018 to 2019.

Parameter	Factor	df	F-value	*p*
ermB	Pasture management[4]	4	1.74	0.17
	Year	1	0.08	0.78
	Pasture management × Year	4	0.52	0.72
sull	Pasture management	4	1.30	0.30
	Year	1	0.54	0.47
	Pasture management × Year	4	2.98	0.04*
intII	Pasture management	4	0.83	0.52
	Year	1	6.76	0.01*
	Pasture management × Year	4	1.45	0.25

[4]*Treatments included: hayed (H), continuously grazed (CG), rotationally grazed (R), rotationally grazed with a buffer strip (RB), and rotationally grazed with a fenced riparian buffer (RBR); Year = 2018 and 2019.*

*$p < 0.05$.

therefore, given that this experimental set up was less (<10 m) filter strip benefits on AMR gene reduction was not fully realized.

CONCLUSION

Pasture management and animal manure additions had an influence on bacterial richness in water runoff, with the lowest bacterial richness occurring in CG and the greatest in H (when non-agricultural samples were excluded). These results could be attributed to a greater contribution of the poultry litter bacterial community in runoff in the absence of grazing, as more poultry litter remains on the soil surface and less incorporation occurs without cattle hooves acting as tillage in these systems. Further, bacterial richness was lower in control samples (Mulberry River, a National Wild and Scenic River)

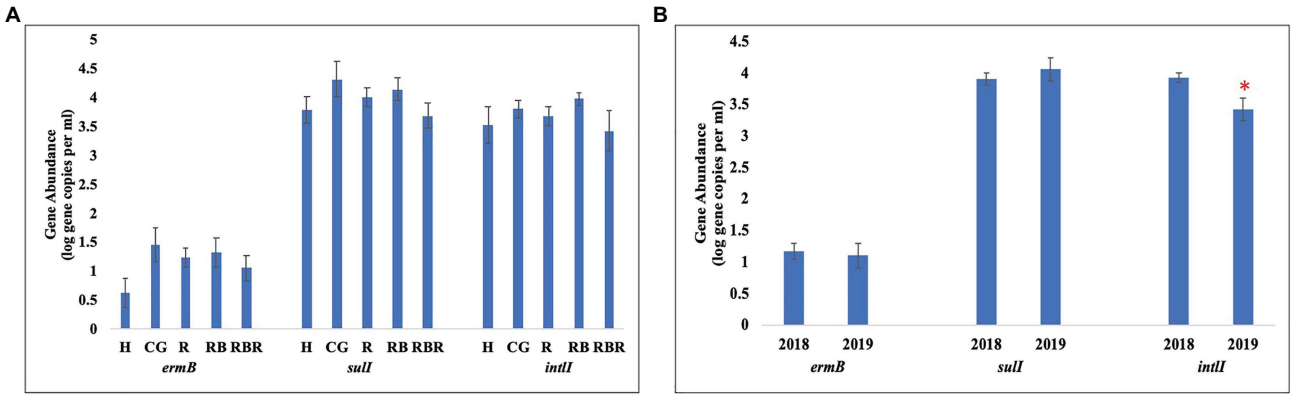

FIGURE 5 | Mean abundances of three AMR associated genes, *ermB*, *sull*, and *intll* amplified from water genomic DNA samples based on **(A)** pasture management (H, CG, R, RB, and RBR), **(B)** sampling year (2018 and 2019). Treatments included: hayed (H), continuously grazed (CG), rotationally grazed (R), rotationally grazed with a buffer strip (RB), and rotationally grazed with a fenced riparian buffer (RBR). Error bars represent standard errors. The star indicates a significant difference at an alpha level of 0.05.

than experimental pasture watersheds. This suggests bacterial richness is greater in agricultural runoff samples. One possible explanation is that runoff samples from pastures included greater soil, soil bound nutrients, and manure sources, which are sources of microbial diversity. Finally, beta diversity was influenced by poultry litter application timing, with greater diversity occurring directly after poultry litter land applications, therefore it is likely that poultry litter introduced its own suite of bacteria to watersheds. Overall, conservation pasture management including grassed buffer strips and riparian buffers increased bacterial richness (Chao1) relative to "business as usual" or continuously grazed systems.

Long-term (14 years) pasture management resulted in detection of three AMR-associated genes (*ermB*, *sulI*, and *intlI*), but not in the non-agricultural (control) water body, which received runoff from soil, but not animal manure inputs (poultry litter). This suggests that runoff from recently applied poultry litter and cattle manure may act as sources of AMR-associated genes in runoff. Overall, these results highlight the importance of monitoring pasture management and poultry litter application timing on bacterial community analysis and AMR-associated genes in water runoff for the development of the best management or conservation agricultural strategies.

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/, PRJNA717042.

AUTHOR CONTRIBUTIONS

AA received the funding, organized the experiment, and drafted the manuscript. YY conducted the laboratory and data analyses and developed the figures. LD, JD, and KC provided the guidance on analyses and data presentation. LD drafted the manuscript and assisted with data analysis. PM and PO contributed to the overall experimental management of long-term watersheds. MS provided the manuscript editing. All authors contributed to the article and approved the submitted version.

REFERENCES

Amorim, H. C. S., Ashworth, A. J., Moore, P. A. Jr., Wienhold, B. J., Savin, M. S., Owens, P. R., et al. (2020). Soil quality indices following long-term conservation pasture management practices. *Agric. Ecosyst. Environ.* 301:107060. doi: 10.1016/j.agee.2020.107060

Anderson, K., Moore, P. A. Jr., Pilon, C., Martin, J., Pote, D. H., Owens, P. R., et al. (2020). Long-term effects of grazing management and buffer strips on phosphorus runoff from pastures fertilized with poultry litter. *J. Environ. Qual.* 49, 85–96. doi: 10.1002/jeq2.20010

Ashworth, A. J., DeBruyn, J., Allen, F. L., Radiosevich, M. A., and Owens, P. R. (2017). Microbial community structure is affected by cropping sequences and poultry litter under long-term no-tillage. *Soil Biol. Biochem.* 114, 210–219. doi: 10.1016/j.soilbio.2017.07.019

Barraud, O., Baclet, M. C., Denis, F., and Ploy, M. C. (2010). Quantitative multiplex real-time PCR for detecting class 1, 2, and 3 integrons. *J. Antimicrob. Chemother.* 65, 1642–1645. doi: 10.1093/jac/dkq167

Barrios, R. E., Khuntia, H. K., Bartelt-Hunt, S. L., Gilley, J. E., Schmidt, A. M., Snow, D. D., et al. (2020). Fate and transport of antibiotics and antibiotic resistance genes in runoff and soil as affected by the timing of swine manure slurry application. *Sci. Total Environ.* 712:136505. doi: 10.1016/j.scitotenv.2020.136505

Bevan, E. R., Jones, A. M., and Hawkey, P. M. (2017). Global epidemiology of CTX-M B-lactamases: temporal and geographical shifts in genotype. *J. Antimicrob. Chemother.* 72, 2145–2155. doi: 10.1093/jac/dkx146

Blazejewski, T., Ho, H. I., and Wang, H. H. (2019). Synthetic sequence entanglement augments stability and containment of genetic information in cells. *Science* 365, 595–598. doi: 10.1126/science.aav5477

Cadena, M., Durso, L. M., Miller, D. N., Waldrip, H. M., Castleberry, B. L., Drijber, R. A., et al. (2018). Tetracycline and sulfonamide antibiotic resistance genes in soils from Nebraska organic farming operations. *Front. Microbiol.* 9:1283. doi: 10.3389/fmicb.2018.01283

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., et al. (2011). Global patterns of 16S rRNA diversity at a

depth of millions of sequences per sample. *Proc. Natl. Acad. Sci. U. S. A.* 108(Suppl. 1), 4516–4522. doi: 10.1073/pnas.1000080107

Cottell, J. L., Kanwar, N., Castillo-Courtade, L., Chalmers, G., Scott, H. M., Norby, B., et al. (2013). *bla*$_{CTX-M-32}$ on an IncN plasmid in *Escherichia coli* from beef cattle in the United States. *Antimicrob. Agents Chemother.* 57, 1096–1097. doi: 10.1128/AAC.01750-12

Dhariwal, A., Chong, J., Habib, S., King, I. L., Agellon, L. B., and Xia, J. (2017). MicrobiomeAnalyst: a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. *Nucleic Acids Res.* 45, W180–W188. doi: 10.1093/nar/gkx295

Durso, L. M., Miller, D. N., and Wienhold, B. J. (2012). Distribution and quantification of antibiotic resistant genes and bacteria across agricultural and non-agricultural metagenomes. *PLoS One* 7:e48325. doi: 10.1371/journal.pone.0048325

Durso, L. M., Wedin, D. A., Gilley, J. E., Miller, D. N., and Marx, D. B. (2016). Assessment of selected antibiotic resistances in ungrazed native Nebraska prairie soils. *J. Environ. Qual.* 45, 454–462. doi: 10.2134/jeq2015.06.0280

Florez, A. B., Alegria, A., Rossi, F., Delgado, S., Felis, G. E., Torriani, S., et al. (2014). Molecular identification and quantification of tetracycline and erythrocycline resistance genes in Spanish and Italian retail cheeses. *BioMed. Res. Intern.* 2014:746859. doi: 10.1155/2014/746859

Fosberg, K. J., Patel, S., Gibson, M. K., Lauber, C. L., Knight, R., Fierer, N., et al. (2014). Bacterial phylogeny structures soil resistomes across habitats. *Nature* 509, 612–616. doi: 10.1038/nature13377

Forsberg, K. J., Reyes, A., Wang, B., Selleck, E. M., Sommer, M. O., and Dantas, G. (2012). The shared antibiotic resistome of soil bacteria and human pathogens. *Science* 6098, 1107–1111. doi: 10.1126/science.1220761

Ganger, M. T., Dietz, G. D., and Ewing, S. J. (2017). A common base method for analysis of qPCR data and the application of simple blocking in qPCR experiments. *BMC Bioinformatics* 18:534. doi: 10.1186/s12859-017-1949-5

Hall, M. C., Noelle, A., Mware, J. E., Gilley, S. L., Bartelt-Hunt, D. D., Snow, A. M., et al. (2020). Influence of setback distance on antibiotics and antibiotic resistance genes in runoff and soil following the land application of swine manure slurry. *Environ. Sci. Technol.* 54, 4800–4809. doi: 10.1021/acs.est.9b04834

He, L. Y., Liu, Y. S., Su, H. C., Zhao, J. L., Liu, S. S., Chen, J., et al. (2014). Dissemination of antibiotic resistance genes in representative broiler feedlots environments: identification of indicator ARGs and correlations with environmental variables. *Environ. Sci. Technol.* 48, 13120–13129. doi: 10.1021/es5041267

Heinonen-Tanski, H., and Uusi-Kamppa, J. (2001). Runoff of faecal microorganisms and nutrients from perennial grass ley after application of slurry and mineral fertilizer. *Water Sci. Technol.* 43, 143–146. doi: 10.2166/wst.2001.0726

Hrudey, S. E., Payment, P., Huck, P. M., Gillham, R. W., and Hrudey, E. J. (2003). A fatal waterborne disease epidemic in Walkerton, Ontario: comparison with other waterborne outbreaks in the developed world. *Water Sci. Technol.* 47, 7–14. doi: 10.2166/wst.2003.0146

Itavaara, M., Salavirta, H., Marjamaa, K., and Ruskeeniemi, T. (2016). Geomicrobiology and metagenomics of terrestrial deep subsurface microbiomes. *Adv. Appl. Microbiol.* 94, 1–77. doi: 10.1016/bs.aambs.2015.12.001

Kay, P., Blackwell, P. A., and Boxall, A. B. A. (2004). Fate of veterinary antibiotics in a macroporous tile drained clay soil. *Environ. Toxicol. Chem.* 23, 1136–1144. doi: 10.1897/03-374

Kielak, A. M., Barreto, C. C., Kowalchuk, G. A., van Veen, J. A., and Kuramae, E. E. (2016). The ecology of Acidobacteria: moving beyond genes and genomes. *Front. Microbiol.* 7:744. doi: 10.3389/fmicb.2016.00744

Kozich, J. J., Westcott, S. L., Baxter, N. T., Highlander, S. K., and Schloss, P. D. (2013). Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Appl. Environ. Microbiol.* 79, 5112–5120. doi: 10.1128/AEM.01043-13

Kreuzig, R., Holtge, S., Brunotte, J., Berenzen, N., Wogram, J., and Schulz, R. (2005). Test-plot studies on runoff of sulfonamides from manured soils after sprinkler irrigation. *Environ. Toxicol. Chem.* 24, 777–781. doi: 10.1897/04-019r.1

Leal, R. M. P., Alleoni, L. R. F., Tornisielo, V. L., and Regitano, J. B. (2013). Sorption of fluoroquinolones and sulfonamides in 13 Brazilian soils. *Chemosphere* 92, 979–985. doi: 10.1016/j.chemosphere.2013.03.018

Meyers, M. A., Durso, L. M., Gilley, J. E., Miller, D. N., Li, X., and Schmidt, A. M. (2020). Setback distance impacts on transport and antibiotic resistance phenotypes of fecal indicators. *Agrosyst. Geosci. Environ.* 3:e220081. doi: 10.1002/agg2.20081

Madigan, M., and Martinko, J. (eds.) (2005). *Brock biology of microorganisms. 11th Edn*. Upper Saddle River, NJ: Prentice Hall.

Palmeira, J. D., and Ferreira, H. M. N. (2020). Extended-spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae in cattle production – a threat around the world. *Heliyon* 6:e03206. doi: 10.1016/j.heliyon.2020.e03206

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

Pilon, C., Moore, P. A., Pote, D. H., Pennington, J. H., Martin, J. W., Brauer, D. K., et al. (2017a). Long-term effects of grazing management and buffer strips on soil erosion from pastures. *J. Environ. Qual.* 46, 364–372. doi: 10.2134/jeq2016.09.0378

Pilon, C., Moore, P. A., Pote, D. H., Martin, J. W., and DeLaune, P. B. (2017b). Effects of grazing management and buffer strips on metal runoff from pastures fertilized with poultry litter. *J. Environ. Qual.* 46, 402–410. doi: 10.2134/jeq2016.09.0379

Pilon, C., Moore, P. A., Pote, D. H., Martin, J. W., Owens, P. R., Ashworth, A. J., et al. (2018). Grazing management and buffer strip impact on nitrogen runoff from pastures fertilized with poultry litter. *J. Environ. Qual.* 48, 297–304. doi: 10.2134/jeq2018.04.0159

Rothrock, M. J., Keen, P. L., Cook, K. L., Durso, L. M., Franklin, A. M., and Dungan, R. S. (2016). How should we be determining background and baseline antibiotic resistance levels in agroecosystem research? *J. Environ. Qual.* 45, 420–431. doi: 10.2134/jeq2015.06.0327

SAS Institute (2007). *SAS/STAT 9.4 user's guide*. Cary: SAS Institute.

Spain, A., Krumholz, L., and Elshahed, M. (2009). Abundance, composition, diversity and novelty of soil Proteobacteria. *ISME J.* 3, 992–1000. doi: 10.1038/ismej.2009.43

Spring, S., Schulze, R., Overmann, J., and Schleifer, K. H. (2000). Identification and characterization of ecologically significant prokaryotes in the sediment of freshwater lakes: molecular and cultivation studies. *FEMS Microbiol. Rev.* 24, 573–590. doi: 10.1111/j.1574-6976.2000.tb00559.x

Sun, P., Barmaz, D., Cabrera, M. L., Pavlostathis, S. G., and Huang, C. H. (2013). Detection and quantification of ionophore antibiotics in runoff, soil and poultry litter. *J. Chromatogr. A* 1312, 10–17. doi: 10.1016/j.chroma.2013.08.044

Szczepanowski, R., Linke, B., Krahn, I., Gartemann, K., Gutzkow, T., Eicher, W., et al. (2009). Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing reduced susceptibility to selected antibiotics. *Microbiology* 155, 2306–2319. doi: 10.1099/mic.0.028233-0

Tate, K. W., Atwill, E. R., Bartolome, J. W., and Nader, G. (2006). Significant Escherchia coli attenuation by vegetative buffers on annual grasslands. *J. Environ. Qual.* 35, 795–805. doi: 10.2134/jeq2005.0141

Thurston-Enriquez, J. A., Gilley, J. E., and Eghball, B. (2005). Microbial quality of runoff following land application of cattle manure and swine slurry. *J. Water Health* 3, 157–171. doi: 10.2166/wh.2005.0015

USEPA (2007). National water quality inventory: Report to congress 2002 reporting cycle. EPA 841-R-07-001. Available at http://epa.gov/305bn (Accessed september 4, 2018). USEPA: Washington, DC.

Yang, Y., Ashworth, A. J., DeBruyn, J. M., Willett, C., Durso, L. M., Cook, K. L., et al. (2019). Soil biodiversity is driven by long-term pasture management, poultry litter, and cattle manure inputs. *PeerJ* 7:e7839. doi: 10.7717/peerj.7839

Yang, Y., Ashworth, A. J., DeBruyn, J. M., Durso, L. M., Savin, M., Cook, K. L., et al. (2020). Antimicrobial resistant gene prevalence in soils due to animal manure deposition and long-term pasture management. *PeerJ* 8:e10258. doi: 10.7717/peerj.10258

Zhang, J., Zhu, C., Guan, R., Xiong, Z., Zhang, W., Shi, J., et al. (2017). Microbial profiles of a drinking water resource based on different 16S rRNA V regions during a heavy cyanobacterial bloom in Lake Taihu, China. *Environ. Sci. Pollut. Res.* 24, 12796–12808. doi: 10.1007/s11356-017-8693-2

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Prevalence and Genetic Analysis of Chromosomal *mcr-3/7* in *Aeromonas* From U.S. Animal-Derived Samples

*Yan Wang[1,2], Naxin Hou[1], Reuven Rasooly[1], Yongqiang Gu[1] and Xiaohua He[1]**

[1] Western Regional Research Center, Agricultural Research Service, United States Department of Agriculture, Albany, CA, United States, [2] State Key Laboratory of Infectious Disease Prevention and Control, National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing, China

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John P. Brooks,
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Independent Researcher, Wunstorf, Germany
Cátia Marques,
University of Lisbon, Portugal

***Correspondence:**
Xiaohua He
Xiaohua.he@usda.gov

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The prevalence of *mcr*-positive bacteria in 5,169 domestic animal-derived samples collected by USDA Food Safety and Inspection Service between October 2018 and May 2019 was investigated. A procedure including enriched broth culture and real-time PCR targeting *mcr-1* to *mcr-8* were used for the screening. Fifteen positive isolates were identified, including one plasmid-borne *mcr-1*-positive *Escherichia coli* strain, EC2492 (reported elsewhere) and 14 *mcr-3/7*-positive strains from poultry (1), catfish (2), and chicken rinse (11) samples, resulting in an overall prevalence of *mcr*-positive bacteria 0.29% in all meat samples tested. Analysis of 16S rRNA and whole genome sequences revealed that all 14 strains belonged to *Aeromonas*. Data from phylogenetic analysis of seven housekeeping genes, including *gyrB, rpoD, gyrA, recA, dnaJ, dnaX,* and *atpD*, indicated that nine strains belonged to *Aeromonas hydrophila* and five strains belonged to *Aeromonas jandaei*. Antimicrobial tests showed that almost all *mcr*-positive strains exhibited high resistance to colistin with MICs $\geq$ 128mg/L, except for one *A. jandaei* strain, which showed a borderline resistance with a MIC of 2 mg/L. A segment containing two adjacent *mcr-3* and *mcr-3*-like genes was found in two *A. hydrophila* and one *A. jandaei* strains and a variety of IS-like elements were found in the flanking regions of this segment. A *mcr-3*-related lipid A phosphoethanolamine transferase gene was present in all 14 *Aeromonas* strains, while an additional *mcr-7*-related lipid A phosphoethanolamine transferase gene was found in 5 *A. jandaei* strains only. In addition to *mcr* genes, other antimicrobial resistance genes, including $bla_{OXA-12/OXA-724}$, *aqu-2, tru-1*, *cepS*, *cphA*, *imiH*, *ceph-A3*, *ant(3″)-IIa*, *aac(3)-Via,* and *sul1* were observed in chromosomes of some *Aeromonas* strains. The relative high prevalence of chromosome-borne *mcr-3/7* genes and the close proximity of various IS elements to these genes highlights the need for continued vigilance to reduce the mobility of these colistin-resistance genes among food animals.

Keywords: *Aeromonas*, antimicrobial resistance, colistin resistance, food-producing animals, prevalence, whole genome sequence

INTRODUCTION

The polymyxins, including polymyxin B and polymyxin E (colistin), are a group of cationic lipopeptide antibiotics against most Gram-negative bacteria by interacting with lipid A to disrupt the outer membrane and cause cell death (Falagas and Rafailidis, 2008). It was discontinued for routine use in humans due to its kidney toxicity. The increased emergence of multidrug resistant (MDR) bacteria has caused the reintroduction of polymyxins as the last-resort antibiotics in clinic. However, the recent discovery of mobile colistin resistance genes (*mcr-1* to *mcr-10*) in a broad range of sources has jeopardized the clinical efficacy of colistin (Liu et al., 2016; Xavier et al., 2016; Borowiak et al., 2017; Carattoli et al., 2017; Yin et al., 2017; AbuOun et al., 2018; Wang et al., 2018; Yang et al., 2018; Carroll et al., 2019; Wang C. et al., 2020).

To minimize further spread of colistin resistance, investigations on the prevalence of *mcr*-positive bacteria from humans, retail meat, and environmental samples have been performed globally (Luo et al., 2020). However, reports on systemic screening of *mcr*-positive bacteria in animal-derived food products in the United States were rare.

In this study, we screened 5,169 food-producing animal samples including chicken rinse, ground beef, beef trim, pork, poultry, and catfish collected by the Food Safety and Inspection Service (FSIS, USDA) to investigate the prevalence of *mcr*-positive Gram-negative bacteria; characterize *mcr*-positive bacterial strains based on the analysis of whole genome sequences; identify *mcr* variants and analyze their genetic environment in bacterial genomes.

MATERIALS AND METHODS

Sample Collection, Processing, and Analysis

A total of 5,169 samples, including 1,787 chicken rinses, 1,369 ground beef, 1,057 beef trim, 416 pork, 363 poultry, and 177 catfish, randomly collected at various locations across the U.S. between October 10, 2018 and May 10, 2019, were sent to the FSIS Western Laboratory and processed as described, previously (Wang Y. et al., 2020). Briefly, samples were subjected to a non-selective enrichment at 35 or 42°C overnight with one part meat in three parts media. A neutralizing Buffered Peptone Water (nBPW) was used for chicken rinse samples and modified Tryptone-Soy Broth (mTSB) was used for all other samples. Overnight cultures (200 μL) were then transferred to a 96 deep-well block pre-filled with 800 μl/well of TSB selection medium containing $CaCl_2$ (5 mM), colistin (4 μg/mL) and vancomycin (50 μg/mL, to reduce Gram-positive bacteria) and further incubated overnight at 37°C. These samples constitute approximately 14–29% of each sample type analyzed by FSIS during this time period according to FSIS Annual Sampling Program Plan at file:///C:/Users/Xiaohua.He/OneDrive%20-%20USDA/Desktop/fsis-annual-sampling-plan-fy2021.pdf (accessed on 3/25/2021). A listing of FSIS Inspected Establishments can be found at https://www.fsis.usda.gov/inspection/establishments/meat-poultry-and-egg-product-inspection-directory (accessed on 3/25/2021).

Identification and Isolation of *mcr*-positive Bacteria

Following enrichments of samples in non-selective and then selective media, wells with visible bacterial growth were analyzed by real-time PCR targeting on *mcr-1* to *mcr-8* using the Rotor-Gene Q (Qiagen) system. A total of five pairs of primers were designed for amplification of *mcr-1* to *mcr-8* fragments (**Table 1**). The primers for *mcr-1/2/6* were designed based on the alignment of *mcr-1.1* (NG_050417), *mcr-2.1* (NG_051171), and *mcr-6.1* (MF176240). The primers for *mcr-3/7* were designed based on the alignment of *mcr-3.1* (NG_056184) and *mcr-7.1* (NG_056413). The primers for *mcr-4*, *mcr-5*, and *mcr-8* were designed based on the *mcr-4.1* (MG459156), *mcr-5.1* (NG_055658) and *mcr-8.1* (NG_061399) genes, respectively. All primers listed in **Table 1** were designed using Primer-BLAST, and the specificity of each pair of primers was evaluated using the Basic Local Alignment Search Tool (BLAST) on NCBI. For real-time PCR analysis, cells from 100 μL of overnight cultures were collected by centrifugation and resuspended in 100 μL of water and then lysed by boiling for 10 min. After removing cell debris by centrifugation, the clear lysate was used as DNA template. A 20 μL of PCR reaction included 2 μL of cell lysate (∼100 ng DNA template), 0.5 μM of each primer, 1x SYBR Green Master Mix. The thermal cycling conditions for all PCRs were: 1 cycle of 95°C for 2 min (polymerase activation); 40 cycles at 95°C for 5 s (melting), followed by 60°C for 20 s (annealing and signal acquisition). For melting curve analysis, the default setting was used: rise 1°C each step from 72 to 95°C, wait for 90 s on the first step of the pre-melting condition and then wait for 5 s for each step afterward. When PCR cycle threshold (Ct) value is less than 30 at melting temperature of the amplification products $T_m = 84.06 \pm 0.21$°C, the sample was considered as positive.

PCR-positive cultures were plated on TSA plates containing 2 μg/mL of colistin. Candidate colistin-resistant colonies (5–10) were picked from plates with an appropriate serial dilution (∼ 50 colonies/plate) and dipped into a microcentrifuge tube with 50 μL of water. After boiling for 10 min, 2 μL of the lysate

TABLE 1 | Primers used in this study.

Primer name	Sequence	Product size (bp)
MCR-1/2/6-F	GTCGTCGGTGAGACGGC	198
MCR-1/2/6-R	GTATTTGGCGGTATCGACATCA	
MCR-3/7-F	AACACATGCTATGACGAGGTTGT	228
MCR-3/7-R	GGTGTAGCGGATGGTGTTGTC	
MCR-4-F	TGCGAAGAATGCCAGTCGTA	169
MCR-4-R	GCCGCATGAGCTAGTATCGT	
MCR-5-F	TGCGCAACTACGGGGTTTAT	328
MCR-5-R	CGAATGCCCGAGATGACGTA	
MCR-8-F	CCTGCATGTTCTCGCGAATG	486
MCR-8-R	GCATCCCGGAATAACGTTGC	

was further analyzed by PCR to confirm the presence of the *mcr* gene. *mcr*-positive strains were preserved in TSB containing 30% glycerol and stored at −80°C.

Whole Genome Sequencing

Genomic DNA from *mcr*-positive strains were extracted for single-molecule real-time (SMRT) sequencing using the Blood and Cell Culture DNA Midi kit (Qiagen cat. no. 13343, CA) and Genomic DNA Buffer Set (Qiagen cat. no. 19060, CA), according to manufacturer's protocols for preparation of Gram-negative bacteria sample and isolation of genomic DNA from bacteria. The 20-kb DNA libraries were prepared using the BluePippin size selection system following manufacturer's instructions. High-throughput sequencing was performed on a PacBio RSII platform using the 360-min data collection protocol. The PacBio reads were assembled using the Hierarchical Genome Assembly Process 3 (HGAP3, SMRT Analysis v2.3.0). The completed genome sequences were submitted to NCBI Prokaryotic Genome Annotation Pipeline (PGAP) for annotation.

Species Identification and Genetic Analysis of *mcr*-positive Strains

The multilocus phylogenetic analysis (MLPA) was used to identify the species of each *Aeromonas* strain based on seven housekeeping genes, including *gyrB, rpoD, gyrA, recA, dnaJ, dnaX*, and *atpD* (Martinez-Murcia et al., 2011). Strains isolated in this study, along with 25 other *Aeromonas* strains were analyzed by MLPA. Phylogenetic tree was constructed based on the concatenated sequences in the same order using MEGA 5.0 and maximum likelihood method with 1000 bootstrap replicates. The phylogenetic trees of *mcr-3* variants, *mcr-3*-like genes, *mcr-3*-related phosphoethanolamine-lipid A transferase coding genes and *mcr-7*-related phosphoethanolamine-lipid A transferase coding genes were also constructed using MEGA 5.0 and maximum likelihood method with 1000 bootstrap replicates. Alignment of gene clusters was performed using Mauve (version snapshot_2015-02-25) and Easyfig (version 2.2.2) (Darling et al., 2004; Sullivan et al., 2011). Searching of antimicrobial resistance genes in the whole genome sequence of each strain was performed by ABRicate[1] against the comprehensive antibiotic resistance database (CARD).

Antimicrobial Susceptibility Testing

The minimum inhibitory concentration (MIC) of colistin for each strain was determined using the broth microdilution (BMD) method recommended by the Clinical and Laboratory Standards Institute (CLSI). Briefly, BMD is performed using cation-adjusted Mueller-Hinton broth, a range of 2-fold dilutions of colistin (ranging from 0.25 to 128 mg/L), and a bacterial inoculum density of 500,000 cfu/mL per well. *E. coli* strains, AR-Bank #0346 (MIC of colistin = 4 mg/L) and AR-Bank #0349 (MIC of colistin = 2–4 mg/L), were used as positive controls, and *E. coli* reference strain, ATCC25922, was used as a negative control. Resistant breakpoint for colistin was adopted from CLSI with MIC $\leq$ 2 mg/L as the susceptibility breakpoint and MIC $>$ 2 mg/L as the resistance breakpoint.

Nucleotide Sequence Accession Number

The whole-genome nucleotide sequences of 14 *Aeromonas* strains have been submitted to DDBJ/EMBL/GenBank. The accession numbers were listed in **Table 2**.

RESULTS

Prevalence of *mcr*-positive Bacteria in U.S. Beef, Pork, Poultry, and Catfish

The prevalence of *mcr* in U.S. food-producing animal samples was investigated by enrichment culture and real-time PCR using a total of 5,169 samples, including 1,787 chicken rinse, 1,369 ground beef, 1,057 beef trim, 416 pork, 363 poultry, and 177 catfish. One pork sample was identified to be PCR-positive for *mcr-1/2/6*, and 14 samples were found to be PCR-positive for

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

TABLE 2 | Some characteristics of *mcr-3/7*-positive *Aeromonas* strains isolated in this study.

Isolate	Isolate source	Species	*mcr* variant and *mcr-3*-like gene	Colistin MIC (mg/L)	Accession No.
1805	Catfish	*A. hydrophila*	*mcr-3-related*	128	CP038515
2359	Chicken rinse	*A. hydrophila*	*mcr-3-related*	128	CP043324
2692	Chicken rinse	*A. hydrophila*	*mcr-3.27, mcr-3-like1, mcr-3-related*	128	CP038513-CP038514
2961	Chicken rinse	*A. hydrophila*	*mcr-3-related*	128	VHIX00000000
3019	Catfish	*A. hydrophila*	*mcr-3-related*	128	CP053885
3036	Chicken rinse	*A. jandaei*	*mcr-3-related, mcr-7-related*	2	CP053882
3206	Chicken rinse	*A. hydrophila*	*mcr-3-related*	128	CP043323
3299	Chicken rinse	*A. jandaei*	*mcr-3-related, mcr-7-related*	128	CP043322
3384	Chicken rinse	*A. jandaei*	*mcr-3-related, mcr-7-related*	128	CP043321
3924	Chicken rinse	*A. hydrophila*	*mcr-3-related*	128	CP053884
4484	Chicken rinse	*A. hydrophila*	*mcr-3.27, mcr-3-like2, mcr-3-related*	128	VHIW00000000
4608	Poultry	*A. jandaei*	*mcr-3, mcr-3-like3, mcr-3-related, mcr-7-related*	128	CP053881
4956	Chicken rinse	*A. jandaei*	*mcr-3-related, mcr-7-related*	128	CP053879-CP053880
4960	Chicken rinse	*A. hydrophila*	*mcr-3-related*	128	CP053883

mcr-3/7. A *mcr-1*-positive *Escherichia coli* strain, EC2492, was isolated from the pork sample that was PCR-positive for *mcr-1/2/6* and characterized phenotypically and genomically in details (Wang Y. et al., 2020). Fourteen *mcr-3/7*-positive isolates were identified from the corresponding samples that were positive by PCR. **Table 2** shows some basic information about these isolates. The prevalence of *mcr-3/7* were 0.28% (1/363) in poultry, 1.13% (2/177) in catfish, and 0.62% (11/1787) in chicken rinse, respectively. The overall prevalence of *mcr-3/7* was 0.27% in all meat samples tested. No other *mcr* genes, including *mcr-2*, *mcr-4*, *mcr-5*, *mcr-6*, and *mcr-8* were found in this survey.

Identification and Genomic Characterization of *mcr*-3/7-positive Isolates

All 14 *mcr-3/7*-positive isolates were identified as *Aeromonas* species based on results from BLAST searching the database of 16S ribosomal RNA sequences. Nine strains, including AH1805, AH2359, AH2692, AH2961, AH3019, AH3206, AH3924, AH4484, and AH4960, were identified as *Aeromonas hydrophila,* and five strains, including AJ3036, AJ3299, AJ3384, AJ4608, and AJ4956, were identified as *Aeromonas jandaei* based on the MLPA (**Figure 1**). The genomes of six *A. hydrophila* strains were assembled into one scaffold with sizes varied from 4.8 Mb to 5.2 Mb and the GC contents varied from 60.96 to 61.58%. The genome of strain AH2692 was assembled into two scaffolds, including one 5.0 Mb chromosome and one 10 kb plasmid. The genome of strain AH2961 was assembled into three scaffolds, including one 4.8 Mb-chromosome and two plasmids with sizes of 35 and 12 kb in length, respectively. The genome of strain AH4484 was assembled into 12 scaffolds with a total length of 4.8 Mb and average GC content of 61.63%. The GC contents of the five *A. jandaei* strains varied from 58.87 to 59.01% and the genomes of four were assembled into one scaffold with sizes varied from 4.5 to 4.6 Mb, and one (AJ4956) was assembled into two scaffolds, including a 4.5 Mb-chromosome and an 147 kb-plasmid with a GC content of 55.4%.

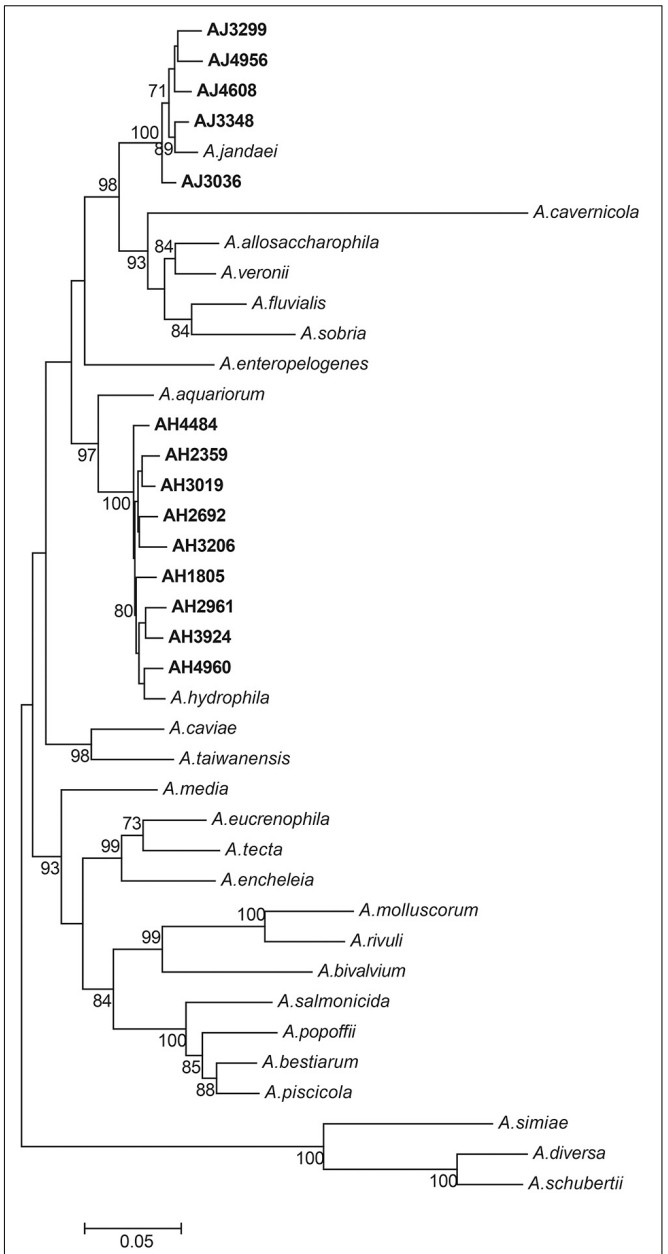

FIGURE 1 | Phylogenetic tree constructed using maximum likelihood method based on the concatenate sequences of seven housekeeping genes from 14 *Aeromonas* strains isolated in this study and 25 publicly available *Aeromonas* strains. The strains isolated in this study were written in bold font.

mcr-3-*mcr*-3-like Segment in *Aeromonas* Strains

Two *A. hydrophila* strains, AH2692 and AH4484, and one *A. jandaei* strain, AJ4608, harbored a *mcr-3-mcr-3*-like segment, which was flanked by an *eamA* gene (EamA family transporter) and a *dgkA* gene (diacylglycerol kinase). The *dgkA* genes in strains AH2692 and AH4484 were interrupted by the IS*Kpn10* family transposase genes at different positions. An IS element, IS*As17*, was found upstream of the *eamA* gene in strain AH2692, just like what was found in an *A. dhakensis* strain isolated from human peritoneal fluid (accession no. AOBN01000008.1). In addition, two IS4 family transposase genes, IS*Apu1* and IS*Apu2* were found downstream of the *mcr-3-mcr-3*-like segment (**Figure 2**). Unlike in *A. hydrophilla* strains, no IS elements were present adjacent to the *mcr-3-mcr-3*-like segment in strain AJ4608. The *mcr*-3 variant gene in strain AH2692 was 1623 bp long and coded for a protein of 540 amino acids that was identical to the MCR-3.27 (WP_017778762.1). The *mcr-3* variant gene in strain AH4484 was 1557 bp long and coded for a protein of 518 amino acids that was almost identical to the MCR-3.27 except for missing the first 22 amino acids and having 2 amino acid substitutions. The *mcr-3* gene in strain AJ4608 was 1623 bp long and coded for a protein of 540 amino acids that was 100% identical to MCR-3, a phosphoethanolamine-lipid A transferase (WP_118854326.1) from an *Aeromonas veronnni* strain and highly similar to MCR-3.8 (WP_099156048.1) with 2 amino acid substitutions. The three *mcr-3*-like genes adjacent to the

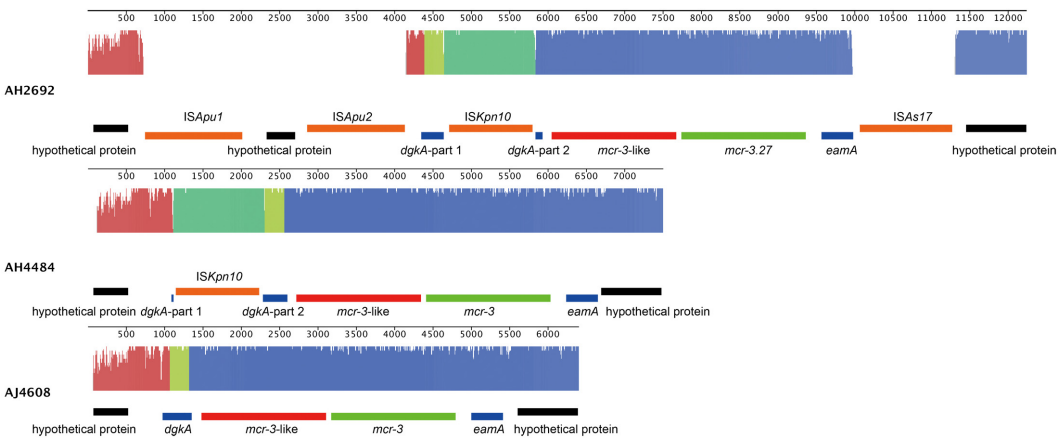

FIGURE 2 | Alignment and genomic organization of *mcr-3-mcr-3*-like clusters in *A. hydrophila* strains, AH2692, AH4484, and *A. jandaei* strain AJ4608. The annotation information is shown above or under each gene block.

mcr-3 genes were more divergent to all reported *mcr-3* gene variants (**Figure 3**). The insertion regions harboring the *mcr-3-mcr-3*-like cluster, were located between genes *rimO* (ribosomal protein S12 methylthiotransferase) and HD domain-containing protein coding gene with sizes of 52 and 30 kb in strain AH2692 and AH4484, respectively. Additionally, the insertion region of strain AH2692 harbored a Tn3 family transposon associated with mercuric resistance. In strain AJ4608, the insertion region harboring the *mcr-3-mcr-3-like* cluster was 36 kb long and inserted in a *ligA* (DNA ligase) gene.

mcr-3-related Genes in *Aeromonas* Strains

A *mcr-3*-related gene encoding for an MCR-3-related phosphoethanolamine-lipid A transferase was identified in all *Aeromonas* strains isolated in this study. The genetic environment of *mcr-3*-related genes in *A. hydrophila* strains consisted of upstream genes for a MFS (Major Facilitator Superfamily) transporter and a hypothetical protein and downstream genes for another hypothetical protein, a MprF (Multiple Peptide Resistance Factor) and a virulence factor (**Figure 4A**). Additionally, there was a sequence coding for a type III secretion system inserted between a tRNA and a MFS transporter gene in strains AH1805, AH2359, AH3019, AH3924, and AH4960. In strain AH1805, there was another gene cluster associated with tellurium resistance next to the sequence for a type III secretion system (**Figure 4A**). The genetic environment of *mcr-3*-related genes in *A. jandaei* strains consisted of an upstream gene for a hypothetical protein, and downstream genes for a DGKA, a virulence factor and a MprF protein (**Figure 4A**). Notably, all the *mcr-3* related genes present in *A. hydrophila* clustered into one genetic group, while all the *mcr-3* related genes present in *A. jandaei* clustered into another distinct group. Compared with the deduced amino acid sequence of *mcr-3.1*, the *mcr-3*-related genes shared 70.65–70.96% identity in *A. hydrophila,* and 72.74–73.42% identity in *A. jandaei* (**Figure 4B**).

mcr-7-related Genes in *A. jandaei* Strains

A *mcr-7*-related gene encoding for a MCR-7-related phosphoethanolamine-lipid A transferase was found in all *A. jandaei* strains. The *mcr-7*-related genes in *A. jandaei* shared 81.79–82.34% identity to *mcr-7.1* at nucleic acid level. **Figure 5A** shows genes in flanking regions of the *mcr-7*-related gene, which were conserved in all *A. jandaei* strains. **Figure 5B** shows the phylogenetic tree of *mcr-7*-related genes in *A. jandaei* strains and the *mcr-7.1* gene, with the *mcr-3.1* gene as an outgroup (**Figure 5B**).

Colistin Susceptibility Test

Based on results from BMD tests, all 14 *mcr-3/7*-positive *Aeromonas* strains were resistant to colistin. Except for strain AJ3036 that showed a borderline resistance to colistin (MIC = 2 mg/L), all other 13 strains exhibited very high resistance to colistin with MIC $\geq$ 128 mg/L.

Other Antimicrobial Resistance Genes

In addition to colistin resistance genes, a variety of other antimicrobial resistance genes (ARGs) involved in resistance to cephalosporin, carbapenem, aminoglycoside, and sulfonamide were identified in 14 colistin-resistant *Aeromonas* isolates (**Table 3**). The $bla_{\mathrm{OXA-12}}$ and $bla_{\mathrm{OXA-724}}$ genes, encoding Oxacillin-hydrolyzing (OXA)-type β-lactamases for resistance to cephalosporin, were observed in all *A. jandaei* and *A. hydrophila* strains. Another three ARGs, *tru-1, cepS,* and *aqu-2* responsible for resistance to cephalosporin were found in one, five, and four *Aeromonas* strains, respectively. Four *cphA* variant genes, including *cphA2*, *cphA4*, *cphA7*, and *cphA8*, were identified in 5 *A. hydrophila* and 3 *A. jandaei* strains. These *cphA* genes code for carbapenem-hydrolyzing metallo-β-lactamases responsible for resistance to carbapenem. The *imiH* and *ceph-A3* genes accounting for resistance to carbapenem were found in three *A. hydrophila* and two *A. jandaei* strains, respectively. A strain (AH2359) harboring six ARGs responsible for resistance to cephalosporins, carbapenems, aminoglycosides,

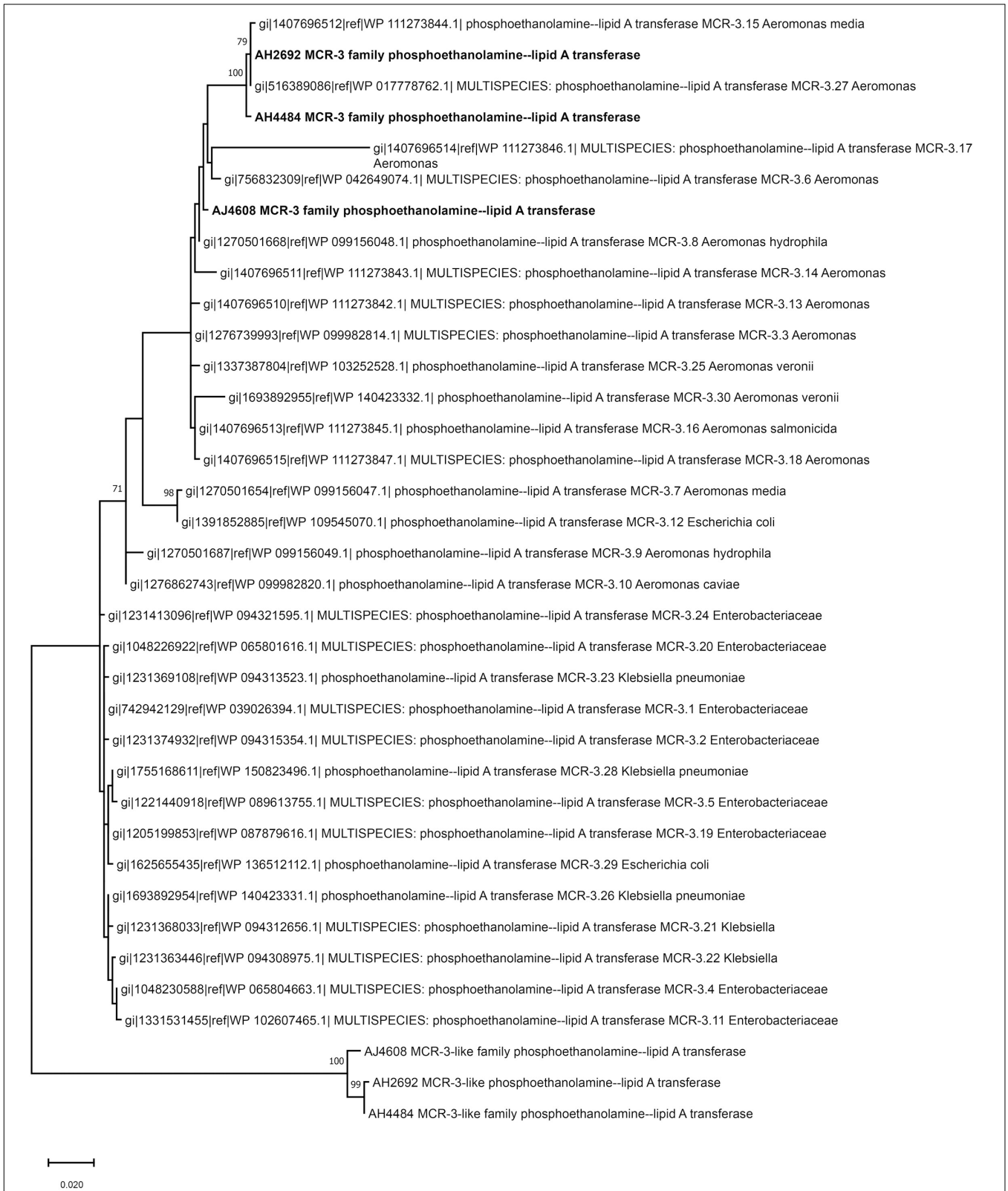

FIGURE 3 | Phylogenetic tree constructed based on amino acid sequences of *mcr-3* variants, *mcr-3*-like genes identified in this study and *mcr-3* variants available in public database.

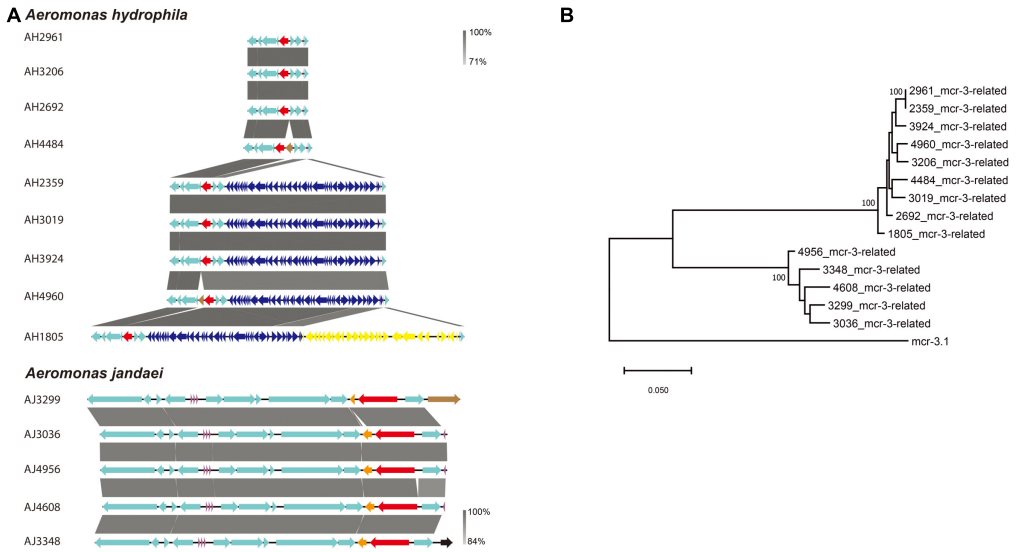

FIGURE 4 | (A). Alignment of *mcr-3*-related phosphoethanolamine-lipid A transferase coding gene in *A. hydrophila* and *A. jandaei* strains isolated in this study. The levels of nucleotide identity were shown in gray shaded regions by color intensity. The *mcr-3*-related genes were marked in red. The transposase genes were marked in brown. tRNAs were marked in purple. The type III secretion system were showed in dark blue and the gene cluster associated with tellurium resistance were showed in yellow. **(B)**. Phylogenetic tree of *mcr-3*-related gene, along with the reference sequence of *mcr-3.1*.

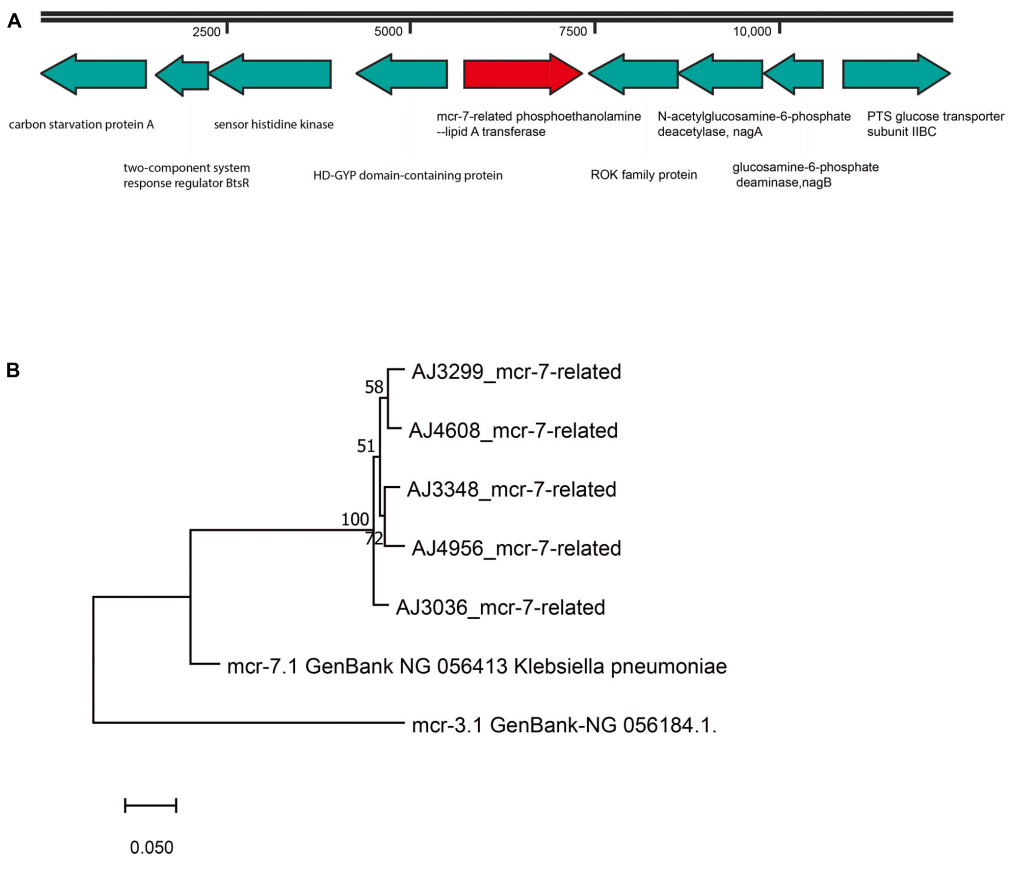

FIGURE 5 | (A). Genetic environment of *mcr-7*-related phosphoethanolamine-lipid A transferase coding gene in *A. jandaei* strains. **(B)**. Phylogenetic tree of *mcr-7*-related gene, along with the reference sequences of *mcr-7.1* and *mcr-3.1*.

TABLE 3 | Other antimicrobial resistance genes identified using the comprehensive antibiotic resistance database (CARD).

ARG	Resistance	AH1805	AH2359	AH2692	AH2961	AH3019	AJ3036	AH3206	AJ3299	AJ3384	AH3924	AH4484	AJ4608	AJ4956	AH4960
bla_{OXA-12}/ $bla_{OXA-724}$	cephalosporin	+	+	+	+	+	+	+	+	+	+	+	+	+	+
tru-1	cephalosporin	-	-	-	-	-	-	-	-	-	-	-	+	-	-
cepS	cephalosporin	+	+	+	-	-	-	+	-	-	+	-	-	-	-
aqu-2	cephalosporin	-	-	-	+	-	-	-	-	+	-	+	-	-	+
imiH	carbapenem	-	+	-	+	-	-	-	-	-	-	+	-	-	-
cphA	carbapenem	-	-	+	-	+	+	+	+	+	+	-	-	-	+
ceph-A3	carbapenem	-	-	-	-	-	-	-	-	-	-	-	+	+	-
ant(3″)-IIa	aminoglycoside	-	+	-	-	-	-	-	-	-	-	-	-	-	-
aac(3)-VIa	aminoglycoside	-	+	-	-	-	-	-	-	-	-	-	-	-	-
sul1	sulfonamide	-	+	-	-	-	-	-	-	-	-	-	-	-	-

and sulfonamides was identified. All ARGs mentioned above were located on the chromosome.

DISCUSSION

This is the second report on the prevalence of *mcr* genes in U.S. domestic animal-origin samples following our first report (Wang Y. et al., 2020). Among 5,169 samples, one *E. coli* strain carrying a plasmid-borne *mcr-1* gene was isolated from a raw pork sample. The characteristics of the strain was described, previously (Wang Y. et al., 2020). Here, we report 14 chromosome-borne *mcr*-positive strains isolated from 1 poultry, 2 catfish and 11 chicken rinse samples. No *mcr*-positive samples were found in ground beef or beef trim samples. Based on data obtained from this survey, the prevalence of chromosome-borne *mcr* (0.27%) was much higher than that of plasmid-borne *mcr* (0.02%) in the U.S. animal-derived samples.

It was found that all 14 *mcr*-positive strains belonged to *Aeromonas.* Among them, nine *A. hydrophila* strains harbored a *mcr-3*-related gene, while five *A. jandaei* strains harbored a *mcr-3*-related gene plus a *mcr-7*-related gene. In addition, a *mcr-3-mcr-3*-like segment was present in three *Aeromonas strains*, including AH2692, AH4484, and AJ4608. This *mcr-3-mcr-3*-like segment was originally reported in *A. veronni* 172 isolated from chicken meat (accession no. KY924928.1) (Ling et al., 2017). Subsequently, the *mcr-3.6-mcr-3*-like, *mcr-3.8-mcr-3*-like, and *mcr-3.9-mcr-3*-like segments were reported in *Aeromonas allosaccharophila*, *A. jandaei*, and *A. hydrophila*, respectively (Eichhorn et al., 2018). The widespread of *mcr-3* genes in *Aeromonas* species suggests that *Aeromonas* may be the origin of *mcr-3* genes (Shen et al., 2018). In this study, a variety of complete or truncated IS elements, including IS*AS17*, IS*Kpn10*, IS*Apu1*, and IS*Apu2* were identified in proximity to the *mcr-3.27-mcr-3*-like segments in strains AH2692 and AH4484, suggesting these IS elements may play a role in the mobility of these colistin resistance genes. However, no IS elements, were identified near the *mcr-3-mcr-3*-like segment in strain AJ4608. It has been reported that a *dgkA* gene, coding for a diacylglycerol kinase involving in the phosphatidic acid pathway, was frequently present immediately downstream of *mcr* genes in both chromosome and plasmid (Ling et al., 2017; Yin et al., 2017). In-depth analysis of the flanking regions, a *dgkA* gene was found downstream of the *mcr-3-mcr-3*-like segments in AH2692, AH4484, and AJ4608 strains and downstream of the *mcr-3* related genes in 5 *A. jandaei* strains. In addition, an IS element with 91% nucleotide identity to IS*Aeme13* (IS4 family) was located next to a hypothetical protein immediately upstream of the *mcr-3*-related gene in *A. jandaei* strain AJ3299. It is possible that this IS element involves in transferring *mcr-3* genes between chromosome and plasmid. As *Aeromonas* species are routine microflora in poultry and fish, the presence of potentially transferrable *mcr* genes in *Aeromonas* could facilitate the spread of these genes to other bacteria species living in the same habitat.

It was reported that *A. hydrophila* had low level of resistance to colistin but showed increased MIC values following preculture with low dose of colistin, while *A. jandaei* exhibited intrinsic resistance to colistin (Fosse et al., 2003). A research from 479 unrelated *Aeromonas* isolates showed that only 0.84% of *Aeromonas* strains carried a *mcr* gene, suggesting that *mcr* is not the intrinsic genes in *Aeromonas* (Eichhorn et al., 2018). In this study, almost all *Aeromonas* strains (13 out of 14) isolated showed much higher resistance to colistin (MICs $\geq$ 128 mg/L) than the *Escherichia coli* strain WJ1 (MIC = 8 mg/L) and *Klebsiella pneumoniae* strain SC20141012 (MIC = 4 mg/L), the original strains reported to harbor the plasmid-borne *mcr*-3.1 and *mcr*-7.1, respectively (Yin et al., 2017; Yang et al., 2018). Whether the high level of colistin resistance is due to a synergistic effect from *mcr* and other ARG genes remains unknown. Surprisingly, strain AJ3036, an *Aeromonas* species with innate resistance to colistin showed borderline resistance to colistin, although it carried genes responsible for colistin-resistance, including *mcr-3*- and *mcr-7*-related genes. The correlation between *mcr-3-/mcr-7*-related genes and colistin resistance, or the machinery on neutralizing the resistance to colistin in this strain is awaiting to be elucidated.

Based on the whole genome sequences, ten ARGs involving resistance to four kinds of antimicrobials were identified in 14 *Aeromonas* strains. The OXA-type β-lactamases that belong to class D β-lactams and are responsible for much of the β-lactams like penicillin and cephalosporins resistance are widely identified in *Aeromonas* species. Consistently, the bla_{OXA-12} and $bla_{OXA-724}$ (also named *ampH*) genes, originated from *A. jandaie*

AER 14 and *A. hydrophila* T429125 strains, respectively, were found in all 14 *Aeromonas* isolates (Rasmussen et al., 1994; Avison et al., 2000). Carbapenems are usually regarded as the most effective antibiotics for serious infections, however, their usage was compromised by the appearance of carbapenemases.

In conclusion, a total of 14 *mcr-3/7*-positive *Aeromonas* strains with high level of colistin resistance were isolated in a study of 5,169 food-producing animal samples collected by FSIS. Results based on whole genome sequences of these isolates indicated that *A. hydrophila* strains only carried *mcr-3* genes on their chromosome, while *A. jandaie* strains carried both chromosome-borne *mcr-3* and *mcr-7* genes. A set of *mcr-3*-*mcr-3*-like segment was identified in two *A. hydrophila* and one *A. jandaie* isolates. A variety of IS elements were observed in proximity to some *mcr-3/7* genes. In accordance with other studies (Ling et al., 2017; Shen et al., 2018), our findings further confirmed that *mcr-3/7* are more common in *Aeromonas* than in other bacterial species although they are not intrinsic genes in *Aeromonas*. In addition to *mcr* genes, 10 other ARGs involving in resistance to cephalosporin, carbapenem, aminoglycoside and sulfonamide were found in these strains. Of note, one *A. hydrophila* strain harbored six ARGs responsible for four types of antimicrobial resistance, simultaneously. Food contamination with colistin-resistance genes that have the potential to transfer among different bacterial species both vertically and horizontally could pose huge risk to animal and human health, global surveillance and collaborations are needed to combat or prevent such threat.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

XH and YW contributed to the conception and design of the work, ensuring any part of the work are appropriately investigated and resolved, critically revised the final version to be published, and were responsible for the integrity of the work. NH, YG, YW, and RR contributed by performing sequencing, analyzing the data, and revising the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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REFERENCES

AbuOun, M., Stubberfield, E. J., Duggett, N. A., Kirchner, M., Dormer, L., Nunez-Garcia, J., et al. (2018). mcr-1 and mcr-2 (mcr-6.1) variant genes identified in *Moraxella* species isolated from pigs in Great Britain from 2014 to 2015. *J. Antimicrob. Chemother.* 73:2904. doi: 10.1093/jac/dky272

Avison, M. B., Niumsup, P., Walsh, T. R., and Bennett, P. M. (2000). Aeromonas hydrophila AmpH and CepH beta-lactamases: derepressed expression in mutants of *Escherichia coli* lacking creB. *J. Antimicrob. Chemother.* 46, 695–702. doi: 10.1093/jac/46.5.695

Borowiak, M., Fischer, J., Hammerl, J. A., Hendriksen, R. S., Szabo, I., and Malorny, B. (2017). Identification of a novel transposon-associated phosphoethanolamine transferase gene, mcr-5, conferring colistin resistance in d-tartrate fermenting *Salmonella enterica* subsp. enterica serovar Paratyphi B. *J. Antimicrob. Chemother.* 72, 3317–3324. doi: 10.1093/jac/dkx327

Carattoli, A., Villa, L., Feudi, C., Curcio, L., Orsini, S., Luppi, A., et al. (2017). Novel plasmid-mediated colistin resistance mcr-4 gene in *Salmonella* and *Escherichia coli*, Italy 2013, Spain and Belgium, 2015 to 2016. *Euro Surveill.* 22:30589. doi: 10.2807/1560-7917.ES.2017.22.31.30589

Carroll, L. M., Gaballa, A., Guldimann, C., Sullivan, G., Henderson, L. O., and Wiedmann, M. (2019). Identification of novel mobilized colistin resistance gene mcr-9 in a multidrug-resistant, colistin-susceptible *Salmonella enterica* serotype typhimurium isolate. *mBio* 10:e00853-19. doi: 10.1128/mBio.00853-19

Darling, A. C., Mau, B., Blattner, F. R., and Perna, N. T. (2004). Mauve: multiple alignment of conserved genomic sequence with rearrangements. *Genome Res.* 14, 1394–1403. doi: 10.1101/gr.2289704

Eichhorn, I., Feudi, C., Wang, Y., Kaspar, H., Fessler, A. T., Lubke-Becker, A., et al. (2018). Identification of novel variants of the colistin resistance gene mcr-3 in *Aeromonas* spp. from the national resistance monitoring programme GERM-Vet and from diagnostic submissions. *J. Antimicrob. Chemother.* 73, 1217–1221. doi: 10.1093/jac/dkx538

Falagas, M. E., and Rafailidis, P. I. (2008). Re-emergence of colistin in today's world of multidrug-resistant organisms: personal perspectives. *Expert Opin. Investig. Drugs* 17, 973–981. doi: 10.1517/13543784.17.7.973

Fosse, T., Giraud-Morin, C., and Madinier, I. (2003). Induced colistin resistance as an identifying marker for *Aeromonas* phenospecies groups. *Lett. Appl. Microbiol.* 36, 25–29. doi: 10.1046/j.1472-765x.2003.01257.x

Ling, Z., Yin, W., Li, H., Zhang, Q., Wang, X., Wang, Z., et al. (2017). Chromosome-Mediated mcr-3 variants in *Aeromonas veronii* from Chicken Meat. *Antimicrob. Agents Chemother.* 61:e01272-17. doi: 10.1128/AAC.01272-17

Liu, Y.-Y., Wang, Y., Walsh, T. R., Yi, L.-X., Zhang, R., Spencer, J., et al. (2016). Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infect. Dis.* 16, 161–168. doi: 10.1016/s1473-3099(15)00424-7

Luo, Q., Wang, Y., and Xiao, Y. (2020). Prevalence and transmission of mobilized colistin resistance (mcr) gene in bacteria common to animals and humans. *Biosaf. Health* 2, 71–78. doi: 10.1016/j.bsheal.2020.05.001

Martinez-Murcia, A. J., Monera, A., Saavedra, M. J., Oncina, R., Lopez-Alvarez, M., Lara, E., et al. (2011). Multilocus phylogenetic analysis of the genus *Aeromonas*. *Syst. Appl. Microbiol.* 34, 189–199. doi: 10.1016/j.syapm.2010.11.014

Rasmussen, B. A., Keeney, D., Yang, Y., and Bush, K. (1994). Cloning and expression of a cloxacillin-hydrolyzing enzyme and a cephalosporinase from Aeromonas sobria AER 14M in *Escherichia coli*: requirement for an *E. coli* chromosomal mutation for efficient expression of the class D enzyme. *Antimicrob. Agents Chemother.* 38, 2078–2085. doi: 10.1128/aac.38.9.2078

Shen, Y., Xu, C., Sun, Q., Schwarz, S., Ou, Y., Yang, L., et al. (2018). Prevalence and genetic analysis of mcr-3-positive *Aeromonas* species from humans, retail meat, and environmental water samples. *Antimicrob. Agents Chemother.* 62, e404–e418. doi: 10.1128/AAC.00404-18

Sullivan, M. J., Petty, N. K., and Beatson, S. A. (2011). Easyfig: a genome comparison visualizer. *Bioinformatics* 27, 1009–1010. doi: 10.1093/bioinformatics/btr039

Wang, C., Feng, Y., Liu, L., Wei, L., Kang, M., and Zong, Z. (2020). Identification of novel mobile colistin resistance gene mcr-10. *Emerg. Microbes Infect.* 9, 508–516. doi: 10.1080/22221751.2020.1732231

Wang, X., Wang, Y., Zhou, Y., Li, J., Yin, W., Wang, S., et al. (2018). Emergence of a novel mobile colistin resistance gene, mcr-8, in NDM-producing *Klebsiella pneumoniae*. *Emerg. Microbes Infect.* 7:122. doi: 10.1038/s41426-018-0124-z

Wang, Y., Hou, N., Johnston, J., Sarreal, C., Jarosh, J., Hughes, A. C., et al. (2020). Low prevalence of mobile colistin-resistance in U.S. meat, catfish, poultry and genomic characterization of a mcr-1 positive *Escherichia coli* strain. *Food Control* 118:107434. doi: 10.1016/j.foodcont.2020.107434

Xavier, B. B., Lammens, C., Ruhal, R., Kumar-Singh, S., Butaye, P., Goossens, H., et al. (2016). Identification of a novel plasmid-mediated colistin-resistance gene, mcr-2, in *Escherichia coli*, Belgium, June 2016. *Euro Surveill.* 21, doi: 10.2807/1560-7917.ES.2016.21.27.30280

Yang, Y. Q., Li, Y. X., Lei, C. W., Zhang, A. Y., and Wang, H. N. (2018). Novel plasmid-mediated colistin resistance gene mcr-7.1 in *Klebsiella pneumoniae*. *J. Antimicrob. Chemother.* 73, 1791–1795. doi: 10.1093/jac/dky111

Yin, W., Li, H., Shen, Y., Liu, Z., Wang, S., Shen, Z., et al. (2017). Novel Plasmid-mediated colistin resistance gene mcr-3 in *Escherichia coli*. *mBio* 8:e00543-17.

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Antimicrobial Resistance in *Escherichia coli* and Enterococcal Isolates From Irrigation Return Flows in a High-Desert Watershed

Robert S. Dungan and David L. Bjorneberg*

Northwest Irrigation and Soils Research Laboratory, Agricultural Research Service, United States Department of Agriculture, Kimberly, ID, United States

Irrigation return flows (IRFs) collect surface runoff and subsurface drainage, causing them to have elevated contaminant and bacterial levels, and making them a potential source of pollutants. The purpose of this study was to determine antimicrobial susceptibility among *Escherichia coli* and enterococcal isolates that were collected from IRFs in a south-central Idaho watershed. Environmental isolates can be a potentially important source of antimicrobial resistance (AMR) and IRFs may be one way resistance genes are transported out of agroecosystems. Water samples were collected from nine IRFs and one background site (canal water from Snake River) on a biweekly basis during 2018. *Escherichia coli* and enterococci were enumerated *via* a most probable number (MPN) technique, then subsamples were plated on selective media to obtain isolates. Isolates of *E. coli* (187) or enterococci (185) were tested for antimicrobial susceptibility using Sensititre broth microdilution plates. For *E. coli*, 13% (25/187) of isolates were resistant to tetracycline, with fewer numbers being resistant to 13 other antimicrobials, with none resistant to gentamicin. While 75% (141/187) of the *E. coli* isolates were pan-susceptible, 12 multidrug resistance (MDR) patterns with 17 isolates exhibiting resistance to up to seven drug classes (10 antimicrobials). For the enterococcal species, only 9% (16/185) of isolates were pan-susceptible and the single highest resistance was to lincomycin (138/185; 75%) followed by nitrofurantoin (56/185; 30%) and quinupristin/dalfopristin (34/185; 18%). In addition, 13 enterococcal isolates belonging to *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus casseliflavus*, and *Enterococcus thailandicus*, were determined to be MDR to up to six different antimicrobial drug classes. None of the enterococcal isolates were resistant to gentamycin, linezolid, tigecycline, and vancomycin.

Keywords: antimicrobial susceptibility, *Escherichia coli*, enterococci, irrigation return flow, fecal indicator bacteria, watershed

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Abd El-Latif Hesham, Assiut University, Egypt
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Rui Pacheco, Escola Secundária de Fonseca Benevides, Portugal

****Correspondence:***
Robert S. Dungan
robert.dungan@usda.gov

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INTRODUCTION

Antimicrobials are used as the first line of defense in treating bacterial infections, greatly reducing morbidity and mortality associated with diseases in humans and animals. However, antimicrobial resistance (AMR) in bacteria is on the rise and a growing threat to the future welfare of countries around the world if not mitigated (O'Neill, 2016). In agroecosystems, the prevalence of AMR is

often attributed to questionable livestock production practices that began in the 1950s, such as use of subtherapeutic amounts of antimicrobials in feed or water for the purpose of enhancing the production performance of livestock (Franklin et al., 2016). For example, the majority of *Escherichia coli* in United Kingdom swine herds had become tetracycline-resistant after 18 years of feeding low doses of this antimicrobial (Smith, 1970). Chickens fed chlortetracycline, either intermittently at a high concentration or at a low concentration throughout life, were found to carry tetracycline-resistant *Enterococcus faecalis* (Elliott and Barnes, 1959). While livestock production practices like this are certainly a driving force in the evolution of antimicrobial-resistant bacteria (ARB), AMR is an ancient bacterial trait that did not originate as a product of agricultural antimicrobial use (Allen and Stanton, 2014). Nonetheless, a temporal analysis of archived agricultural soils from 1940 to 2008 in Netherlands suggests that the antimicrobial era is responsible for increasing environmental AMR, as the number of antimicrobial resistance genes (ARGs) from major antimicrobial classes significantly increased during that period (Knapp et al., 2010). In agricultural soils, the application of manure, wastewater, or biosolids have been demonstrated to increase ARG levels, but not under all conditions (Marti et al., 2013; Rothrock et al., 2016; Dungan et al., 2018; McKinney et al., 2018). It is also important to consider that ARGs are likely horizontally transferred from manure-borne bacteria to indigenous soil bacteria (Smalla et al., 2000), thus increasing the persistence of resistance genes in soil (Binh et al., 2008; Heuer et al., 2011).

Both *E. coli* and enterococci are used as indicator organisms of fecal pollution in surface waters because they are highly abundant, easy to culture, and widely associated with the mammalian intestinal tract. Although fecal indicator bacteria (FIB) may not directly cause human illness, high levels in recreational waters were found to be associated with an increased risk for gastrointestinal illness (United States EPA, 1986). In a rural mixed-use watershed, FIB enter surface waters from human and non-human sources through a variety of pathways, which could be on a continuous basis, such as discharges from a wastewater treatment plant or failing septic system, episodically during storm or irrigation events, or by direct defecation by animals into waterways. In an agricultural watershed in southern Alberta, significant positive correlations were observed between FIB levels in river samples and farm size, total land area used for irrigation, and total land area used for manure application (Jokinen et al., 2012). Scott et al. (2017) found that *E. coli* levels in the Illinois River watershed in northwest Arkansas increased significantly once the percentage of pasture in the drainage area exceeded 55%. In a California agricultural watershed in the San Joaquin Valley, three irrigation return flows (IRFs) were found to exceed *E. coli* and enterococci standards in approximately 50 and 100% of water samples collected, respectively (Diaz et al., 2010). Runoff from fields does contribute a significant load of fecal bacteria to waterways, increasing the threat of pathogens in water supplies used by humans for recreational, irrigation, and consumption purposes (Ferguson et al., 2003; Jokinen et al., 2012). However, FIB can survive for extended periods in stream sediments and become resuspended in the water column when the sediments are disturbed (Jamieson et al., 2003; Rehmann and Soupir, 2009; Cho et al., 2010; Brinkmeyer et al., 2015). As a result, when water samples test positive for FIB, it could either be an indication of fecal contamination or that naturalized extraintestinal populations have been released from soils or streambed sediments (Ishii et al., 2006; Field and Samadpour, 2007).

There is growing evidence that the environment is an important reservoir and source of ARGs for bacteria infecting humans and animals, and that manure-impacted water may be one way that antimicrobials, resistant bacteria, and resistance genes are transported out of agroecosystems (Pruden et al., 2012; Rothrock et al., 2016; Dungan et al., 2017b). Given that *E. coli* and enterococci are of fecal origin, can adapt to live for extended periods in the environment, and can be opportunistic or true pathogens causing a variety of illnesses in humans, their role with respect to AMR in aquatic environments is worthy of investigation. Potential human exposures can occur when fecally contaminated surface waters are used for irrigation of crops or recreational purposes. Various studies to date have investigated antimicrobial resistant *E. coli* and *Enterococcus* spp. in food, animals and humans (Bell et al., 1998; Klein, 2003; Jackson et al., 2011; Frye and Jackson, 2013; Adenipekun et al., 2015; Agga et al., 2016), with some consideration of isolates from surface waters (Chen et al., 2016; Gomi et al., 2016; Cho et al., 2020b) and other related environmental matrices (Sayah et al., 2005; Ibekwe et al., 2011; Maal-Bared et al., 2013). In the present study, our objective was to increase knowledge of the abundance and AMR of *E. coli* and enterococci in IRFs the Upper Snake River watershed in south-central Idaho. The IRFs, which return excess irrigation water back to the Snake River, as well as subsurface drainage and surface runoff, represent a potential conduit for the transfer of chemical and biological contaminants (Bjorneberg et al., 2015; Dungan et al., 2017b). Results from antimicrobial susceptibility tests were also used to determine the prevalence of multidrug resistance (MDR; resistance to three or more antimicrobial drug classes) among the FIB isolates.

MATERIALS AND METHODS

Watershed in South-Central Idaho

The Twin Falls tract (820 km^2) of the Upper Snake Rock (USR) watershed, is located along the south side of the Snake River in south-central Idaho, United States, and has been part of the USDA Conservation Effects Assessment Project (CEAP) since 2005 (Bjorneberg et al., 2008). In this watershed, water from the Snake River is used to irrigate crops, which would otherwise not grow in this semiarid region due to lack of precipitation during the crop growing season. Snake River water is diverted into canals from mid-April to late October, increasing IRF. Some IRF streams continue to flow after the irrigation season due to water flowing from subsurface drainage. Water samples were collected from eight IRF sampling sites: Cedar Draw (CD), Deep Creek (DC), Hansen Coulee (HC), I Coulee (IC), Mud Creek (MC), N Coulee (NC), Rock Creek Poleline (RCP), and Twin Falls Coulee (TFC). A sample was also collected from the Main

Line Canal (MLC), which contains diverted Snake River water and supplies more than 75% of the water to the Twin Falls tract (Bjorneberg et al., 2020), and for the purposes of the present study was designated as a background site. The sampling sites were selected to match those from previous USR watershed studies (Dungan et al., 2017b; Dungan and Bjorneberg, 2020).

Water Sample Collection

Water samples were collected on a biweekly basis from 9 January to 18 December in 2018 with some exceptions because not all sites have water outside the growing season. Specifically, samples could only be collected from NC, TFC, HC, and MCL when the irrigation water was available starting in mid-April, with the last samples collected from these sites on 23 October. Samples could be collected from DC, MC, IC, CD, and RCP, before and after the irrigation season, since these streams flow all year due to subsurface drainage. On collection day, surface water samples were collected in sterile 500 mL polypropylene bottles, then stored in a cooler until delivered to the laboratory. At the laboratory the water samples were immediately placed under refrigeration at 5°C and subsequently processed within 24 h.

Enumeration of *Escherichia coli* and Enterococci

Water samples (100 mL) were either processed directly or diluted when necessary into IDEXX (Westport, ME, United States) Colilert or Enterolert substrate for enumeration of total coliforms/*E. coli* and enterococci, respectively, as recommended by the manufacturer. In brief, the mixtures were then transferred to an IDEXX Quanti-Tray/2000, sealed using the IDEXX Quanti-Tray Sealer PLUS, and then, respectively, incubated at 35 and 41°C for 24 h as recommended by the manufacturer. Positive control organisms, consisting of *Klebsiella pneumoniae* subsp. *pneumoniae* (ATCC [American Type Culture Collection] 13883) and *Escherichia coli* (ATCC 25922) for Colilert and *E. faecalis* (ATCC 29212) for Enterolert, were utilized to confirm the effectiveness of each new substrate lot. After the 24-h incubation period, the Colilert and Enterolert trays were viewed under a 365-nm UV light, with blue fluorescing wells marked positive for *E. coli* and enterococci. In Colilert trays, yellow wells under normal lighting were marked positive for total coliforms. The number of positive wells were converted to most probable number (MPN) using the IDEXX result interpretation table and the dilution factor.

Isolation of *Escherichia coli* and Enterococci

The back of each Quanti-Tray/2000 was wiped with 70 % ethanol, then a flamed surgical scalpel was used to create a small opening in a positive well. A 10 μL loopful of solution from the well was then transferred to a 150 mm plate containing CHROMagar *E. coli* (CHROMagar, Paris) for the Colilert wells and m Enterococcus Agar (Becton, Dickinson and Co., Franklin Lakes, NJ, United States) for the Enterolert wells. Up to five plates were inoculated from separate wells on each tray. The CHROMagar plates were incubated at 35°C for 24 h, while m Enterococcus plates were incubated at the same temperature for up to 48 h. Afterward, a well-isolated colony (blue from CHROMagar E. coli, red from m Enterococcus) was removed from each plate and streaked for isolation. *Escherichia coli* were streaked onto 2× Yeast Extract Tryptone (2× YT) medium plus agar plates (Becton, Dickinson and Co.), while enterococci were streaked onto Brain Heart Infusion (BHI) agar plates (Becton, Dickinson and Co.), followed by incubation at 35°C for 24 h. Select colonies were then placed in 2-mL cryovials with a solution of either 2× YT or BHI broth and 10% glycerol and stored at −80°C.

Preparation of DNA From Isolates

Prior to cryopreservation, a colony from all *E. coli* and enterococcal isolates was transferred to a well of a 96-well PCR plate containing 100 μL of either molecular biology grade water (Hyclone, Logan, UT, United States) for *E. coli* or Tris-EDTA buffer, pH 8.0 (Sigma-Aldrich, St. Louis, MO, United States) for enterococci. The plate was sealed with foil sealing film (Microseal F Foil, Bio-Rad) and then placed into a thermocycler and heated at 100°C for 10 min. The cellular debris was pelleted by centrifugation at 1,000 × *g* for 2 min, then the plates were stored at −20°C until PCR was performed. *Escherichia coli* (ATCC 25922) and *E. faecalis* (ATCC 29212) were used as positive control organisms.

Genetic Analysis of *Escherichia coli*

The phylotyping method as modified by Doumith et al. (2012) was used to assign *E. coli* isolates into one of seven phylotypes (A0, A1, B1, B2.2, B2.3, D1, and D2) as described by Escobar-Paramo et al. (2004b). Briefly, 3 μL of template DNA was used in a multiplex PCR assay that utilized 12.5 μL of AmpliTaq Gold 360 Master Mix (Applied Biosystems, Carlsbad, CA, United States), forward and reverse primers (600 nM *chuA*, 400 nM *gadA*, 200 nM *yjaA*, and 200 nM TSPE4.CA), and molecular biology grade water to a final volume of 25 μL. DNA from *Escherichia coli* ATCC 25922 was used as template in positive controls and molecular biology grade water was used for no-template controls. The primer sequences (5′–3′) were: *chuA*-F, ATGATCATCGCGGCGTGCTG; *chuA*-R, AAACGCGCTCGCGCCTAAT; *gadA*-F, GATGAAATGGCGTT GGCGCAAG; *gadA*-R, GGCGGAAGTCCCAGACGATATCC; *yjaA*-F, TGTTCGCGATCTTGAAAGCAAACGT; *yjaA*-R, ACC TGTGA CAAACCGCCCTCA; TSPE4.CA-F, GCGGGTGAGA CAGAAACGCG; TSPE4.CA-R, TTGTCGTGAGTTGCGAAC CCG (Doumith et al., 2012). The respective amplicon lengths for *chuA*, *gadA*, *yjaA*, and TSPE4.A were 281, 373, 216, and 152 bp. The thermocycler (T100, Bio-Rad, Hercules, CA, United States) conditions consisted of (i) one initial denaturation cycle at 95°C for 10 min; (ii) 30 amplification cycles at 95°C for 30 s, 65°C for 30 s, and 72°C for 30 s; and (iii) a final extension at 72°C for 7 min. PCR products were electrophoresed on 2% agarose gels in 1 × Tris-Borate-EDTA (TBE). Afterward, the gels were stained for 30 min in a solution containing SYBR Green 1 (Invitrogen, Eugene, OR, United States), then visualized under UV (302 nm) in a Gel Doc XR+ System (Bio-Rad) and photographed using a SYBR photographic filter. A 50 bp DNA ladder (Invitrogen,

Waltham, MA, United States) was used to confirm the size of amplification products.

To determine if any of the *E. coli* isolates were enterohemorrhagic, quantitative real-time PCR was utilized for the detection of Shiga toxin (i.e., *stx*1, *stx*2) and intimin (i.e., *eae*) genes as described by Dungan et al. (2012). The primer sequences (5′–3′) were: *stx1*-F, GACTGCAAAGACGTATGTAGATTCG; *stx*1-R, ATCTATCCCTCTGACATCAACTGC; *stx*2-F, ATTAA CCACACCCCACCG; *stx*2-R, GTCATGGAAACCGTT GTCAC; *eae*-F, GTAAGTTACACTATAAAAGCACCGTCG; *eae*-R, TCT GTGTGGATGGTAATAAATTTTTG.

Identification of Enterococcal Isolates

Polymerase chain reaction was performed on the enterococci lysis products for subsequent sequencing and identification of the isolates. Individual PCR reactions consisted of 12.5 μL of Invitrogen Platinum Green Hot Start PCR 2× Mastermix (ThermoFisher Scientific, Waltham, MA, United States), 300 nM of forward (Ent-ES-211-233-F-bio; 5′-GHACAGAAGTRAAATAYGAAGG-3′) and reverse (Ent-EL-74-95-R; 5′-GGNCCTAABGTHACTTTNACTG-3′) primers (Zaheer et al., 2012), 2 mL of DNA template (lysis product), and molecular biology grade water to a final volume of 25 μL. The thermocycler conditions consisted of one cycle at 94°C for 2 min, 35 cycles of 94°C for 30 s, 51°C for 30 s and 72°C for 30 s and one cycle at 72°C for 5 min. *E. faecalis* (ATCC 29212) lysis product was run as a positive control and molecular biology grade water was run as a negative control. The PCR products were loaded directly onto 2% agarose gels and electrophoresed in 1× TBE. The gels were stained with SYBR and visualized as described above. A 50 bp DNA ladder was used to confirm the size of the amplification products, which were about 200 bp. The remaining PCR product was shipped to TACGen (Richmond, CA, United States) and subsequently pyrosequenced using the degenerate sequencing primer Ent-sp-seq (5′-GCAAATTTVAWHTCTTTTGCCAT-3′) (Zaheer et al., 2012). Raw sequences were handled and trimmed using Chromas 2.6.6 (Technelysium Pty Ltd., South Brisbane) and then checked for putative chimeric sequences using DECIPHER (Wright et al., 2012). Sequences were compared to those in GenBank using BLAST (Basic Local Alignment Search Tool) of NCBI (National Center for Biotechnology Information, Bethesda, MD, United States).

Antimicrobial Susceptibility Testing

Escherichia coli and enterococcal isolates were tested for their susceptibility to a panel of 14 and 16 antimicrobials by broth microdilution using the National Antimicrobial Resistance Monitoring System (NARMS) Gram Negative CMV3AGNF and Gram positive CMV3AGPF plates (Sensititre, ThermoFisher Scientific), respectively. Prior to the Sensititre analyses, a small amount of frozen material was aseptically removed from selected isolate cryovials, then it was streaked on 2× YT and BHI agar plates for *E. coli* and enterococcus, respectively. The agar plates were incubated for 16–24 h at 35°C. Colonies were transferred to 5 mL of sterile demineralized water (Cat no. T3339, ThermoFisher Scientific), followed by vortexing until the cells were completely dispersed, then measured on a DEN-1B densitometer (Grant Instruments Ltd., Cambridgeshire) and adjusted accordingly by adding more colonies until it reached 0.5 McFarland. This suspension (10 μL) was then added into a vial containing 11 mL of Mueller-Hinton broth (Cat no. T3462, ThermoFisher Scientific) and mixed by vortexing. The vial contents were transferred to a sterile 10 mL reagent reservoir and 50 μL was transferred into each well of a Sensititre plate using an 8-channel pipette. As recommended by the manufacturer, *Escherichia coli* (ATCC 25922) and *E. faecalis* (ATCC 29212) were used as quality control organisms for CMV3AGNF and CMV3AGPF plates, respectively. After inoculation, the plates were sealed with plate film and incubated at 35°C for 18 h. The plates were read manually using a Sensititre Manual Viewbox (ThermoFisher Scientific).

Sensititre plate results were interpreted according to NARMS-established breakpoints available on the United States Food and Drug Administration website[1]. For *E. coli*, the panel of 14 antimicrobials and breakpoints for classification as resistant were as follows: amoxicillin/clavulanic acid, ≥ 32/16 μg mL^{-1}; ampicillin, ≥ 32 μg mL^{-1}; azithromycin, ≥ 16 μg mL^{-1}; cefoxitin, ≥ 32 μg mL^{-1}; ceftiofur, ≥ 8 μg mL^{-1}; ceftriaxone, ≥ 4 μg mL^{-1}; chloramphenicol, ≥ 32 μg mL^{-1}; ciprofloxacin, ≥1 μg mL^{-1}; gentamicin, ≥16 μg mL^{-1}; nalidixic acid, ≥32 μg mL^{-1}; streptomycin, ≥32 μg mL^{-1}; sulfisoxazole, ≥256 μg mL^{-1}; tetracycline, ≥16 μg mL^{-1}; and trimethoprim/sulfamethoxazole, ≥4/76 μg mL^{-1}. Since the CMV3AGNF plate has maximum concentrations of 16 and 256 μg mL^{-1} for azithromycin and sulfisoxazole, we could not evaluate the NARMS resistance breakpoints of ≥32 and ≥512 μg mL^{-1}, respectively. For enterococci, the panel of 16 antimicrobials and breakpoints for classification as resistant were as follows: chloramphenicol, ≥32 μg mL^{-1}; ciprofloxacin, ≥4 μg mL^{-1}; daptomycin, ≥8 μg mL^{-1}; erythromycin ≥ 8 μg mL^{-1}; gentamicin, ≥512 μg mL^{-1}; kanamycin, ≥1,024 μg mL^{-1}; lincomycin, ≥8 μg mL^{-1}; linezolid, ≥8 μg mL^{-1}; nitrofurantoin, ≥64 μg mL^{-1}; penicillin, ≥16 μg mL^{-1}; quinupristin/dalfopristin, ≥4 μg mL^{-1}; streptomycin, ≥1,024 μg mL^{-1}; tetracycline, ≥16 μg mL^{-1}; tigecycline, ≥0.5 μg mL^{-1}; tylosin, ≥32 μg mL^{-1}; and vancomycin, ≥32 μg mL^{-1}. The CMV3AGPF plate has a maximum concentration of 64 μg mL^{-1} for nitrofurantoin, thus we could not evaluate the NARMS resistance breakpoint of ≥128 μg mL^{-1}.

RESULTS

Enumeration of Fecal Indicator Bacteria

In 2018, 161 and 13 water samples were collected from the IRFs and background MLC site between 9 January and 18 Dec, respectively. In **Figure 1**, the MPN results for total coliforms, *E. coli*, and enterococci are presented as box plots. In the IRF samples, the respective mean levels were $\log_{10}$ 3.8, 2.3, and 2.5 MPN 100 mL^{-1}, while in MLC samples the mean levels were

[1]https://www.fda.gov/media/108180/download

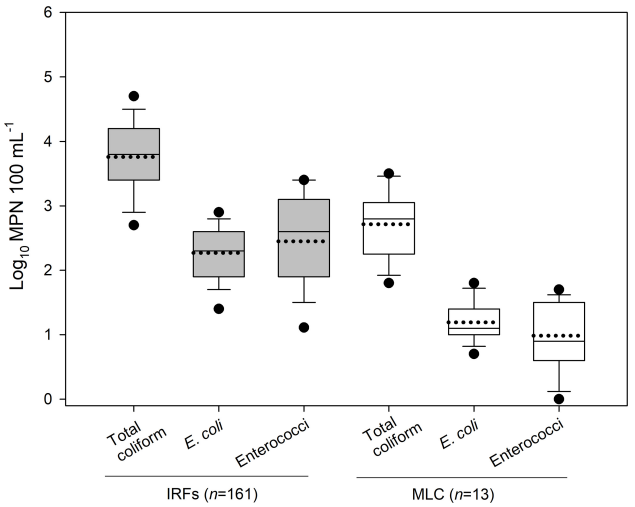

FIGURE 1 | The level (log_{10} MPN 100 mL^{-1}) of total coliform, *E. coli*, and enterococci in the irrigation return flows (IRFs) and background canal site (MLC) in the Upper Snake Rock watershed in south-central Idaho. The black horizontal lines in the box plots, from bottom to top including the whisker caps, represent the 10th, 25th, 50th, 75th, and 90th percentiles, while the black dotted line is the mean and black circles are the 5th and 95th percentiles.

TABLE 1 | Summary of total coliform, *E. coli,* and enterococci levels in irrigation return waters in the Upper Snake Rock watershed in south-central Idaho.

		Log_{10}MPN 100 mL^{-1}		
Site		**Total coliforms**	***E. coli***	**Enterococci**
NC	Min	2.8	1.7	1.9
	Max	4.9	2.8	3.7
	Mean	4.4	2.3	3.1
DC	Min	1.9	1.8	0.0
	Max	4.2	3.4	3.4
	Mean	3.7	2.9	2.6
MC	Min	3.4	1.4	1.5
	Max	4.5	2.8	3.4
	Mean	4.1	2.3	2.8
IC	Min	2.5	1.6	1.4
	Max	4.8	2.9	3.5
	Mean	4.1	2.4	2.9
CD	Min	2.6	1.3	0.8
	Max	4.5	3.2	3.6
	Mean	4.0	2.4	2.8
RCP	Min	2.7	1.0	1.4
	Max	4.4	2.7	3.5
	Mean	3.9	2.2	2.8
TFC	Min	3.2	1.1	1.8
	Max	4.7	2.8	3.7
	Mean	4.3	2.5	3.1
HC	Min	2.9	1.9	1.9
	Max	4.7	2.9	3.4
	Mean	4.1	2.6	3.1
MLC	Min	1.8	0.7	0.0
	Max	3.5	1.8	1.7
	Mean	3.0	1.3	1.2

lower at log_{10} 2.7, 1.2, and 1.0 MPN 100 mL^{-1}. **Table 1** shows the minimum, maximum, and mean levels for the FIB at each of the sampling sites, with 1 CFU 100 mL^{-1} being the lowest level detected during the entire study. The levels for total coliforms, *E. coli*, and enterococci in the IRFs ranged from log_{10} 1.9 to 4.9, 1.0 to 3.4, and 0 to 3.7 MPN 100 mL^{-1}, respectively. At the MLC site, the respective ranges were log_{10} 1.8 to 3.5, 0.7 to 1.8, and 0 to 1.7 MPN mL^{-1}. The sampling sites with the greatest mean levels of total coliforms and *E. coli* were NC and DC at log_{10} 4.4 and 2.9 MPN 100 mL^{-1}, respectively, while NC, TFC, and HC had the greatest mean levels of enterococci at log_{10} 3.1 MPN 100 mL^{-1}. For all FIB, mean levels at each of the IRF sites were determined to be statistically greater ($P < 0.05$) than mean levels at MLC.

To illustrate how the MPN levels changed throughout the year across all IRFs and at MLC, the mean MPN 100 mL^{-1} for the FIB on each collection day (total of 26 d) is presented in **Figure 2**. Total coliform levels were lower at approximately log_{10} 3 MPN 100 mL^{-1} from January through May, then on the first sampling date in June the levels began to increase above log_{10} 4 MPN 100 mL^{-1} until the greatest mean level of log_{10} 4.6 MPN 100 mL^{-1} was noted on 6 August, followed by a gradual decline thereafter (**Figure 2A**). In the MLC samples, total coliform levels from 17 April to 9 October were, on average, 1.5-fold lower than IFR levels. *Escherichia coli* mean levels in the IRFs tended to fluctuate between log_{10} 2 and 3 MPN 100 mL^{-1} throughout much of the year, with the highest levels generally occurring during summer and early fall (**Figure 2B**). The *E. coli* levels in MLC samples were 2.3-fold lower on average than in IFR samples. Enterococci mean levels were approximately log_{10} 2 MPN 100 mL^{-1} from 23 January to 2 April, then increased on 17 April and reached a maximum level of log_{10} 3.4 MPN 100 mL^{-1} on 6 August and then decreased to background levels on 13 November (**Figure 2C**). In MLC samples, enterococci levels were 3.5-fold lower on average than in IRF samples.

Characterization of *Escherichia coli* and Enterococcal Isolates

A total of 806 *E. coli* and 605 enterococcal isolates were obtained from the water samples, but only 187 and 185 isolates were selected for antimicrobial susceptibility testing. More specifically, 171 and 16 *E. coli* and 173 and 12 enterococci were chosen from the IRFs and MLC samples, respectively. Phylotyping and sequencing results for *E. coli* and enterococci, respectively, were used to select a representative subset of isolates from each sampling site and water collection event. Phylotyping of *E. coli* revealed that all seven phylogroups were represented at almost all of the sampling sites, except for three sites lacking the following phylogroups: A1 at DC, B2.2 at RCP, and D2 at IC (**Table 2**). Across all sampling sites, phylotype B1 (47/187; 25%) was the most abundant, while D2 (14/187; 8%) was the least abundant. At MLC, phylotype B1 was also the most abundant (4/16; 25%).

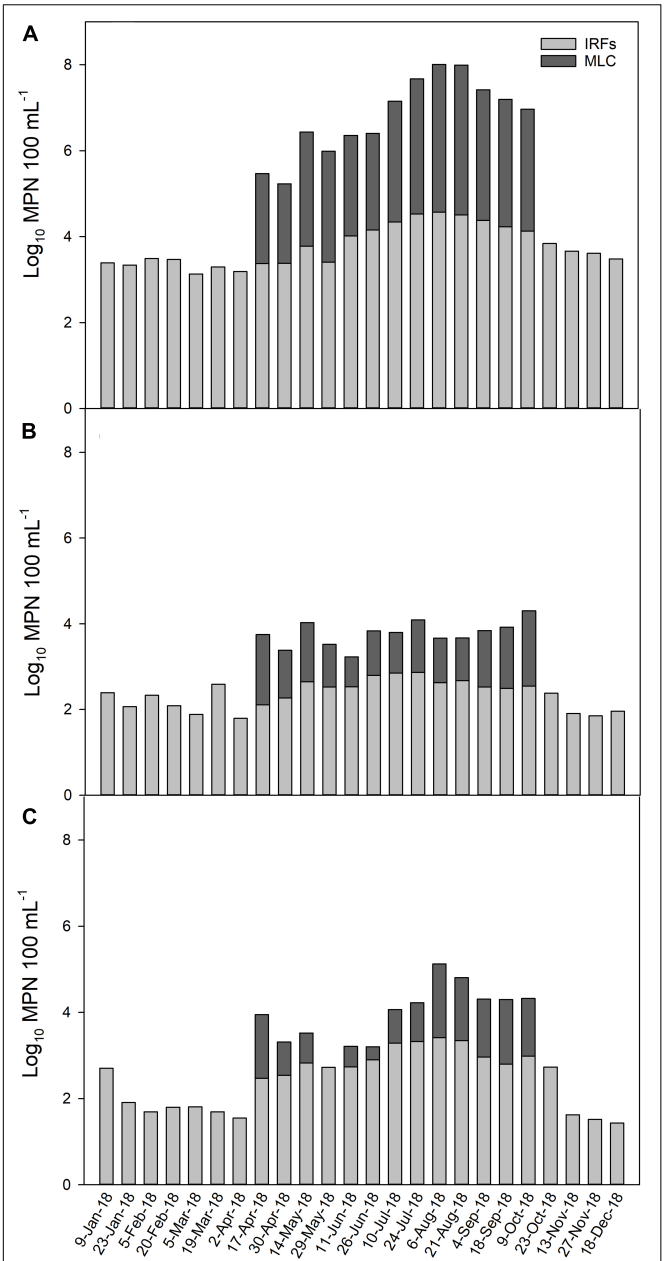

FIGURE 2 | The average level (log_{10} MPN 100 mL^{-1}) of **(A)** total coliform, **(B)** *E. coli*, and **(C)** enterococci in the irrigation return flows (IRFs) and background canal site (MLC) on each sampling day from January to December 2018. Snake River water was diverted into MLC from 10 April to 21 October, 2018.

TABLE 2 | Phylogroups of *E. coli* isolates (n = 187) from the irrigation return flows and background canal site (MLC).

Site	Phylogentic group						
	AO	**AI**	**BI**	**B2.2**	**B2.3**	**DI**	**D2**
NC	2	2	4	2	2	1	2
DC	2	0	6	2	2	6	1
MC	2	5	6	1	4	6	1
IC	2	4	6	1	3	4	0
CD	4	2	7	1	6	6	3
RCP	6	4	6	0	6	4	2
TFC	3	1	4	5	1	1	2
HC	3	1	4	4	1	3	2
MLC	1	3	4	2	2	3	1
Σ=	25	22	47	18	27	34	14

Based on 16S rRNA gene sequencing, the enterococcal isolates were determined to be most closely aligned with the following *Enterococcus* species: *E. casseliflavus*, *E. faecalis*, *E. faecium*, *E. hirae*, *E. malodoratus*, *E. mundtii*, *E. phoeniculicola*, and *E. thailandicus* (**Table 3**). The species that were most prevalent were *E. casseliflavus* (38/185; 21%), *E. faecalis* (37/185; 20%), *E. faecium* (55/185; 30%) and *E. mundtii* (40/185; 22%), which were detected at all sampling sites with occurrences ranging from 1 to 10 per site. *Enterococcus hirae*, *E. malodoratus*, *E. phoeniculicola*, and *E. thailandicus* only represented 8% (15/185) of all enterococcal isolates and they were detected at some of the IFR sampling sites, but not at MLC.

Antimicrobial Resistance of *Escherichia coli*

Escherichia coli isolates from the IRFs and MLC were resistant to 12 of 14 antimicrobials (**Table 4**). For IRF isolates, resistance to tetracycline (21/171; 12%) was the most commonly encountered, followed by ampicillin (13/171; 8%), sulfisoxazole (12/171; 7%), amoxicillin/clavulanic acid (10/171; 6%), and cefoxitin (8/171; 5%). Overall resistance to azithromycin, ceftiofur, chloramphenicol, ciprofloxacin, nalidixic acid, streptomycin, and trimethoprim/sulfamethoxazole was 9.4% of the IRF *E. coli* isolates, with none being resistant to gentamicin. The *E. coli* isolates from MLC were resistant to all but azithromycin and gentamicin, with resistance to nalidixic acid and sulfisoxazole (both 5/16; 31%) being the most common, while resistance to amoxicillin/clavulanic acid, cefoxitin, ceftiofur, and ceftriaxone (all 2/16; 13%) being the least common.

Twenty-five resistance patterns were detected in the *E. coli* isolates (**Table 5**). None of the *E. coli* were pan-resistant, but 7% (12/171) from the IRFs and 25% (4/16) from MLC were MDR. A total of nine MDR patterns with resistance to three and five antimicrobials were observed in *E. coli* from the IRFs. The other resistant IRF *E. coli*, however, were only resistant to 1 (13/171; 8%) and 2 (13/171; 8%) different classes of antimicrobials. The most common resistance patterns among the IRF *E. coli* were Tet and Aug2AmpFox. In *E. coli* from MLC, three MDR patterns were observed with four isolates being resistant to 5, 7, or 10 antimicrobials. In the latter case, one isolate was resistant to AmpXnlAxoChlCipNalStrFisTetSxt, which consists of seven antimicrobial drug classes. Seventy-eight percent (133/171) and 50% (8/16) of the IRF and MLC isolates were pan-susceptible, respectively.

The distribution of the 38 IRF and eight MLC resistant *E. coli* isolates by phylogroup and resistance pattern is presented in

TABLE 3 | The number of *Enterococcus* spp. isolated from the irrigation return flows and background canal site (MLC) that v/ere characterized for antibiotic resistance in the present study.

Site	*n*	No. of isolates							
		E. casseliflavus	*E. faecalis*	*E. faecium*	*E. hirae*	*E. malodoratus*	*E. mundtii*	*E. phoeniculicola*	*E. thailandicus*
NC	13	3	3	4	0	0	2	0	1
DC	23	5	5	4	3	0	4	1	1
MC	26	6	8	7	1	0	4	0	0
IC	26	5	8	7	1	0	5	0	0
CD	28	7	4	8	0	0	7	1	1
RCP	26	4	2	10	1	1	6	0	2
TFC	15	3	3	4	0	0	5	0	0
HC	16	2	3	4	1	0	6	0	0
MLC	12	3	1	7	0	0	1	0	0
Σ=	185	38	37	55	7	1	40	2	5

Table 6. All seven phylogroups were represented in isolates from the IRFs, while five out of seven were represented in the MLC isolates. In IRF isolates, B1 (eight isolates) and D1/D2 (three isolates each) were the most and least abundant groups, respectively. In MLC isolates, the phylogroups represented were A1 (three isolates), B1 (one isolate), B2.3 (two isolates), D1 (one isolate), and D2 (one isolate). Among the IRF isolates, the greatest number of resistances was to five antimicrobials, and was noted in isolates belonging to groups A1 and D1, while an MLC isolate belonging to group A1 was resistant to 10 antimicrobials. Two other MLC isolates belonging to groups A1 and B2.3 were also found to be resistant to the same seven antimicrobials (i.e., ChlCipNalStrFisTetSxt).

Antimicrobial Resistance of Enterococci

Enterococcus spp. from the IRFs were resistant to 10 of 16 antimicrobials (**Table 7**), with none being resistant to gentamicin, linezolid, tigecycline, and vancomycin. Resistance to lincomycin (130/173; 75%) was the most prevalent, followed by nitrofurantoin (50/173; 29%) and tetracycline (20/173; 12%). Resistance to chloramphenicol, ciprofloxacin, daptomycin, erythromycin, kanamycin, penicillin, streptomycin, and tylosin tartrate was relatively uncommon at only 8% of the IRF enterococcal isolates. Enterococcal isolates from MLC were only resistant to four of 16 antimicrobials, specifically lincomycin (8/12; 67%), nitrofurantoin (6/12; 50%), quinupristin/dalfopristin (1/12; 8%), and tetracycline (2/12; 17%).

TABLE 4 | Antimicrobial resistance of *E. coli* isolates from the irrigation return flows (IRFs) and background canal site (MLC).

Antimicrobial	Breakpoint (μg mL^{-1})	No. of resistant (%)	
		IRFs (*n* = 171)	MLC (*n* = 16)
Amoxicillin/clavulanic acid	≥32/16	10 (5.8)	2 (12.5)
Ampicillin	≥32	13 (7.6)	3 (18.8)
Azithromycin	≥16	1 (0.6)	0 (0)
Cefoxitin	≥32	8 (4.7)	2 (12.5)
Ceftiofur	≥8	1 (0.6)	2 (12.5)
Ceftriaxone	≥4	2 (1.2)	2 (12.5)
Chloramphenicol	≥32	4 (2.3)	3 (18.8)
Ciprofloxacin	≥1	4 (2.3)	5 (31.3)
Gentamicin	≥16	0 (0)	0 (0)
Nalidixic Acid	≥32	4 (2.3)	5 (31.3)
Streptomycin	≥32	9 (5.3)	3 (18.8)
Sulfisoxazole	≥256	12 (7)	5 (31.3)
Tetracycline	≥16	21 (12.3)	4 (25)
Trimethoprim/sulfamethoxazole	≥4/76	2 (1.2)	4 (25)

Seventeen resistance patterns were detected in the enterococcal isolates across all species (**Table 8**). In IRF enterococcal isolates, the most common resistance patterns were Lin (71/173; 41%), LinSyn (30/173; 17%), Nit (18/173; 10%), and LinNit (15/173; 9%). A total of six MDR patterns were observed with 12 isolates being resistant to 3, 4, or 6 antimicrobial drug classes, with LinNitTet (5/173; 3%) as the most abundant MDR pattern. In MLC isolates, Lin (4/12; 33%) and Nit (3/12; 25%) were the most common drug resistances, while LinNetTet (2/12; 17%) was the only MDR pattern. Only 9% (15/173) and 8% (1/12) of the IRF and MLC isolates were pan-susceptible, respectively.

To better understand AMR patterns among the enterococcal isolates, the results were broken down according to species (**Table 9**). Only one isolate from an IRF was identified as *E. malodoratus* and it was pan-susceptible. Very few resistant isolates were identified as *E. hirae* (five Lin, one LinSyn), *E. phoeniculicola* (two Lin), and *E. thailandicus* (three LinNit, one LinTet, and one LinNitTet). The majority of the *Enterococcus* spp. were resistant to Lin (138/185; 75%). The species with the greatest to smallest percentage of lincomycin-resistant isolates were *E. thailandicus* (5/5; 100%), *E. phoeniculicola* (2/2; 100%), *E. mundtii* (35/40; 88%), *E. faecalis* (33/37; 89%), *E. hirae* (6/7; 86%), *E. casseliflavus* (30/38; 79%), and *E. faecium* (27/55; 49%). The second most abundant resistance pattern was Nit (56/185; 30%) and 75% (42/56) of the isolates were identified as *E. faecium*. Overall, 13 enterococcal isolates, predominantly *E. faecalis*, *E. faecium*, *E. casseliflavus*, and *E. thailandicus*, were determined to be MDR to up to six different drug classes. MDR was more common among *E. faecium* than the other enterococcal

TABLE 5 | Single and multidrug resistance patterns in *E. coli* from the irrigation return flows (IRFs) and background canal site (MLC).

Resistance pattern	No. of antimicrobials	No. of drug classes	No. of isolates	
			IRFs (*n* = 171)	MLC (*n* = 16)
Pan-susceptible	0	0	133	8
Azi	1	1	1	0
Fis	1	1	1	1
Fox	1	1	1	0
Str	1	1	1	0
Tet	1	1	9	0
AmpTet	2	2	1	0
Aug2Fis	2	2	1	0
ChlTet	2	2	1	0
CipNal	2	2	1	1
StrTet	2	2	1	0
XnlNal	2	2	1	0
AmpStrTet	3	3*	1	0
AxoCipFis	3	3*	1	0
Aug2AmpFox	3	2	7	1
Aug2FisTet	3	3*	2	0
CipTetStr	3	3*	1	0
FisTetStr	3	3*	1	0
AmpNalFisSxt	4	3*	1	0
AxoCipNalFis	4	3*	1	0
FisStrSxtTet	4	3*	1	0
AmpChlStrFisTet	5	5*	3	0
Aug2AmpAxoFo Xnl	5	2	0	1
CipNalFisTetSxt	5	3*	0	1
ChlCipNalStrFis TetSxt	7	5*	0	2
AmpXnlAxoChlCip NalStrFisTetSxt	10	7*	0	1

Aug2, amoxicillin/clavulanic acid; Amp, ampicillin; Azi, azithromycin; Fox, cefoxitin; XnL. ceftiofur; Axo, ceftriaxone; Chl, chloramphenicol; Cip, ciprofloxacin; Gen, gentamicin; Nal, nalidixic acid; Str, streptomycin; Fis, sulfisoxazole; Tet, tetracyline; Sxt, trimethoprim/ sulfamethoxazole; Pan-susceptible, susceptible to all antimicrobials tested.
**Denotes multidrug resistance based on resistance to antimicrobials in three or more drug classes.*

TABLE 6 | The distribution of resistant *E. coli* isolates from the irrigation return flows (IRFs) and background canal site (MLC) by phylogroup and resistance pattern.

Source	No. of isolates	Phylogenetic group	Resistance pattern
IRFs	1	A0	Fis
	1	A0	Tet
	1	A0	AxoCipFis
	1	A0	AmpStrTet
	1	A0	Aug2AmpFox
	1	A0	AxoCipNalFis
	3	A1	Tet
	1	A1	Aug2Fis
	1	A1	StrTet
	2	A1	AmpChlStrFisTet
	1	B1	Azi
	1	B1	Tet
	1	B1	AmpTet
	1	B1	ChlTet
	1	B1	XnlNal
	1	B1	Aug2AmpFox
	1	B1	Aug2AmpFox
	1	B1	FisTetStr
	1	B2.2	Fox
	1	B2.2	Tet
	2	B2.2	Aug2AmpFox
	1	B2.2	CipTetStr
	1	B2.3	Str
	1	B2.3	Tet
	1	B2.3	CipNal
	1	B2.3	Aug2AmpFox
	2	B2.3	Aug2FisTet
	1	D1	Tet
	1	D1	AmpNalFisSxt
	1	D1	AmpChlStrFisTet
	1	D2	Tet
	1	D2	Aug2AmpFox
	1	D2	FisStrSxtTet
MLC	1	A1	Aug2AmpFox
	1	A1	ChlCipNalStrFisTetSxt
	1	A1	AmpXnlAxoChlCipNalStrFisTetSxt
	1	B1	CipNal
	1	B2.3	ChlCipNalStrFisTetSxt
	1	B2.3	Fis
	1	D1	Aug2AmpAxo FoxXnl
	1	D2	CipNalFisTetSxt

species; eight isolates had MDR patterns of LitNetTet (five isolates), CipLinNitTet (two isolates), KanNitTet (one isolate), and LinNitPenStrTet (one isolate).

DISCUSSION

The purpose of this study was to characterize resistance to one or more antimicrobials in *E. coli* and enterococcal isolates obtained from IFRs and a background canal site in the USR watershed. This mixed-use watershed is located in a high-desert region that supports intensive agricultural production, with a large portion utilized for cash crops, forages and dairy production, but it is also populated with small towns and cities, as well as an extensive rural population where households use on-site underground septic systems for wastewater treatment. Land application of manure, compost, and wastewater from dairy operations is a common practice, which can improve soil quality and provide nutrients for crops (Dungan et al., 2011, 2017a). The USR watershed presents a unique opportunity to study waterborne contaminants because rainfall is limited, and thus the vast majority of water that is cycled within the watershed is a direct result of crop irrigation during the growing season

TABLE 7 | Antimicrobial resistance of enterococcal isolates from the irrigation return flows (IRFs) and background canal site (MLC).

Antimicrobial	Breakpoint (μ g mL^{-1})	No. of resistant (%)	
		IRFs (*n* = 173)	MLC (*n* = 12)
Chloramphenicol	≥32	2 (1.2)	0 (0)
Ciprofloxacin	≥4	2 (1.2)	0 (0)
Daptomycin	≥8	1 (0.6)	0 (0)
Erythromycin	≥8	1 (0.6)	0 (0)
Gentamicin	≥512	0 (0)	0 (0)
Kanamycin	≥1024	2 (1.2)	0 (0)
Lincomycin	≥8	130 (75.1)	8 (66.7)
Linezolid	≥8	0 (0)	0 (0)
Nitrofurantoin	≥64	50 (28.9)	6 (50)
Penicillin	≥16	1 (0.6)	0 (0)
Quinupristin/dalfopristin	≥4	33 (19.1)	1 (8.3)
Streptomycin	≥1024	2 (1.2)	0 (0)
Tetracycline	≥16	20 (11.6)	2 (16.7)
Tigecycline	≥0.5	0 (0)	0 (0)
Tylosin tartrate	≥32	2 (1.2)	0 (0)
Vancomycin	≥32	0 (0)	0 (0)

TABLE 8 | Single and multidrug resistance patterns in enterococcal isolates from the irrigation return flows (IRFs) and background canal site (MLC).

Resistance pattern	No. of antimicrobials	No. of drug classes	No. of isolates	
			IRFs (*n* = 173)	MLC (*n* = 12)
Pan-susceptible	0	0	15	1
Lin	1	1	71	4
Nit	1	1	18	3
Tet	1	1	1	0
ChlLin	2	2	1	0
DapLin	2	2	1	0
LinNit	2	2	15	1
LinSyn	2	2	30	1
LinTet	2	2	1	0
LinTylt	2	2	1	0
NitSyn	2	2	1	0
NitTet	2	2	7	0
KanNitTet	3	3*	1	0
LinNitTet	3	3*	5	2
ChlLinSynTet	4	4*	1	0
CipLinNitTet	4	4*	2	0
LinNitPenStrTet	5	4*	1	0
EryKanLinStrSynTetTylt	7	6*	1	0

Chl, chloramphenicol; Cip, ciprofloxacin; Dap, daptomycin; Ery, erythromycin; Kan, kanamycin; Lin, lincomycin; Lzd, linezolid; Nit, nitrofurantoin, Pen, penicillin; Str, streptomycin; Syn, quinupristin/dalfopristin; Tet, tetracyline; Tylt, tylosin tartrate; Pan-susceptible, susceptible to all antimicrobials tested.

**Denotes multidrug resistance based on resistance to antimicrobials in three or more drug classes.*

(Bjorneberg et al., 2020). Previous research in the watershed has shown that antimicrobial residue and ARG levels in IFRs were slightly elevated compared with the incoming surface water from the Snake River that is diverted into canals, laterals, and ditches (Dungan et al., 2017b; Dungan and Bjorneberg, 2020).

Escherichia coli and enterococci are important indicators for understanding the impact of fecal pollution on water resources, but reports on AMR among these organisms in IRFs are lacking. On average, the FIB levels in the IRFs were found to be greater than in water samples from the background site, which can be expected since the irrigation returns receive water from surface and subsurface drainage (Bjorneberg et al., 2008). The influence of irrigation on the FIB levels in the IRFs was evident as the levels increased when the irrigation season commenced in mid-April and stayed elevated until irrigation ceased in mid-October. An earlier Investigation of a rangeland watershed in southwest Idaho suggested that IRFs were responsible for flushing total and fecal coliforms from some fields into a stream during the irrigation season (Stephenson and Street, 1978). While FIB are likely carried into IRFs in surface and subsurface drainage from a variety of sources (e.g., fields, septic systems, urban drainage), it is also possible that the increased flow/turbulence in the irrigation returns during the irrigation season resuspends FIB that reside in sediments. As a result, the irrigation return FIB could be derived from fecal contamination or naturalized extraintestinal populations that populate soils and sediments (Bradshaw et al., 2016). Using AMR analysis of fecal coliforms in water samples from a mixed-use watershed in Georgia, human sources were determined to contribute a majority of the baseflow isolates in urbanized areas, while livestock sources were responsible for the majority of the baseflow isolates in rural areas (Burnes, 2003).

Phylotyping was performed on *E. coli* to assess the diversity among the isolates, with a large proportion (92/187; 49%) belonging to group B and a nearly equal split between groups A (47/187; 25%) and D (48/187; 26%). Of the seven phylogroups that were encountered, B1 (47/187; 25%) was the most abundant, while the least abundant was D2 (14/187; 7%). The existence of distinct phylogroups within *E. coli* has long been recognized and phylogroup determination is a useful characterization tool since a strain's ecological niche and pathogenicity vary with its phylogenetic origins (Gordon et al., 2008; Clermont et al., 2015). Many studies have shown that isolates responsible for extraintestinal disease in humans belong mainly to group B2 and to a lesser extent group D (Picard et al., 1999; Tenaillon et al., 2010). In contrast, Escobar-Paramo et al. (2004a) found that *E. coli* responsible for acute and severe diarrhea were not found in groups B2 and D, while those causing chronic and mild diarrhea were distributed among all phylogroups. In surface water samples and animal feces collected in the Upper Midwest of the United States, 57 and 51% of *E. coli* isolates were found to belong to group B1, respectively, with substantially fewer isolates belonging to all other phylogroups (Johnson et al., 2017). However, they reported that about 95% of the strains among water and fecal isolates were non-extraintestinal, with those determined to be extraintestinal to be predominantly from group B2. In humans, group A strains were predominant (41%) [followed by group B2 (26%), then B1 and D (17% each)], while in animals, group B1 strains were predominant (41%) [followed

TABLE 9 | Resistance patterns of *Enterococcus* spp. from the irrigation return flows and background canal site.

Resistance pattern	No. of isolates							
	***E. casseliflavus* (*n* = 38)**	***E. faecalis* (*n* = 37)**	***E. faecium* (*n* = 55)**	***E. hirae* (*n* = 7)**	***E. malodoratus* (*n* = 1)**	***E. mundtii* (*n* = 40)**	***E. phoeniculicola* (*n* = 2)**	***E. thailandicus* (*n* = 5)**
Lin	24	3	9	5	0	32	2	0
Nit	0	2	17	0	0	2	0	0
Tet	1	0	0	0	0	0	0	0
ChlLin	1	0	0	0	0	0	0	0
DapLin	0	0	0	0	0	1	0	0
LinNit	2	0	9	0	0	2	0	3
LinSyn	1	28	1	1	0	0	0	0
LinTet	0	0	0	0	0	0	0	1
LinTylt	1	0	0	0	0	0	0	0
NitSyn	0	1	0	0	0	0	0	0
NitTet	0	0	7	0	0	0	0	0
KanNitTet	0	0	1	0	0	0	0	0
LinNitTet	1	0	5	0	0	0	0	1
ChlLinSynTet	0	1	0	0	0	0	0	0
CipLinNitTet	0	0	2	0	0	0	0	0
LinNitPenStrTet	0	0	1	0	0	0	0	0
EryKanLinStrSynTetTylt	0	1	0	0	0	0	0	0

Chl, chloramphenicol; Cip, ciprofloxacin; Dap, daptomycin; Ery, erythromycin; Kan, kanamycin; Lin, lincomycin; Lzd, linezolid; Nit, nitrofurantoin; Pen, penicillin; Str, streptomycin; Syn, quinupristin/dalfopristin; Tet, tetracyline; Tylt, tylosin tartrate.

by group A (22%), B2 (21%), and D (16%)] (Tenaillon et al., 2010). In a study of 300 *E. coli* isolates from a variety of animal production systems across the United States, all seven of the phylogroups were represented with phylogroups B1 (107 isolates or 36%) and B2.2 (7 isolates or 2.3%) being the most and least abundant, respectively (Ducey et al., 2020). Given the distribution of the *E. coli* strains among the phylogroups in the present study with B1 being the most abundant group (i.e., 47/187; 25%), the evidence would suggest that many have originated from animals.

Although commensal bacteria are generally considered harmless, they can act as a reservoir of many ARGs, which may be organized within genetic elements such as integrons (Bailey et al., 2010; Fard et al., 2011; Lebreton et al., 2013). A growing body of evidence indicates that ARGs can readily be transferred among microbial species (including between commensals and pathogens) mainly *via* transformation and conjugation events (Marshall et al., 2009; Stokes and Gillings, 2011). It is not known if the *E. coli* and enterococcal isolates from the present study are pathogenic to humans and other animals; however, none of the *E. coli* were found to contain genes encoding for intimin and Shiga toxins, thus confirming that they were not enterohemorrhagic *E. coli* (data not shown) (Fagan et al., 1999; Sharma and Dean-Nystrom, 2003). The present data can be of value to determine the possible spread of these bacteria and their ARGs to the Snake River, which is a recreational waterbody and the 9th longest river in the United States. Because water can move substantial distances in rivers, waterways could be a dominant route by which ARGs are disseminated throughout the environment (Pruden et al., 2012; Keen et al., 2018).

From the susceptibility testing results for *E. coli*, 75% of the 187 isolates were pan-susceptible, while 16% were resistant to antimicrobials from one or two drug classes and 9% were MDR. When just considering the number of resistant isolates per individual antimicrobial, tetracycline resistance was the most prevalent (13%), followed by sulfisoxazole (9%) and ampicillin (9%) resistance. The high rate of tetracycline resistance is not surprising given that tetracycline resistance genes [e.g., *tet*(B), *tet*(M), and *tet*(X)] have been detected in IRF and MLC samples (Dungan and Bjorneberg, 2020). While we have not detected tetracycline and ampicillin residues, trace quantities of certain sulfonamides have been detected (Dungan et al., 2017b). Tetracycline resistance genes and other ARGs are generally more abundant in riverine environments impacted by urban and agricultural activities (Pei et al., 2006; Storteboom et al., 2010). Keen et al. (2018) found that tetracycline resistance genes were more abundant in the Sumas River agricultural watershed of British Columbia than in a forested headwater control site. It was speculated that higher intensity rainfall events, agricultural activities, and land use practices contributed to elevated gene levels in the watershed mainly as a result of soil erosion. Many ARGs are present in native soils, but the abundance of the genes is dramatically greater in cropland soils, especially those that have a history of being treated with animal manures and biosolids (Knapp et al., 2010; Dungan et al., 2019). Fecal coliforms from cattle, humans, and other domesticated animals and wildlife are known to be resistant to a variety of antimicrobials and their resistance patterns have been used for source tracking in watersheds (Whitlock et al., 2002; Burnes, 2003; Ducey et al., 2020).

Multidrug resistance was observed among the *E. coli* from both the IRFs and MLC. Twelve MDR patterns with resistance to up to seven drug classes (10 antimicrobials) was noted in 17 isolates that were found among all of the phylogroups. Similarly, Cho et al. (2018) found that a small percentage of *E. coli* isolates

(15/496; 3%) were MDR in surface waters from the Upper Oconee watershed, which is a mixed-used watershed in northern Georgia. Eleven totally different MDR patterns were detected, including one isolate that was resistant to seven antimicrobials (i.e., AmpCipNalStrFisTetSxt) and belonged to phylogroup B1. In a survey of *E. coli* isolates from surface water in the Grand River watershed in Waterloo, Canada, 17 out of 93 isolates (18%) were found to be resistant to 2–5 antimicrobial classes, but the MDR patterns were not provided (Kadykalo et al., 2020). In the Cho et al. (2018) and Kadykalo et al. (2020) studies, it should be noted that the researchers used the CMV3AGNF Sensititre plate as used in the present study. In a study of *E. coli* from aquatic environments in Rio de Janeiro, Brazil, 66 out of 178 isolates (37%) were resistant to at least one of 11 antimicrobials tested and 17 isolates were MDR (de Luca Rebello and Regua-Mangia, 2014). The lowest percentage of MDR *E. coli* were recovered from agricultural wastewaters (4%) with higher percentages in recreational waters (13%) and residential (8%), industrial (11%), and hospital (17%) wastewaters (de Luca Rebello and Regua-Mangia, 2014). Similarly, MDR prevalence among *E. coli* from the Seine river watershed in France was lowest in agricultural (8%) and forest (1%) non-point sources and higher in municipal wastewaters (34%), rivers (35%), and hospital wastewaters (65%) (Servais and Passerat, 2009).

Surface waters contain a wide variety of enterococcal species, which are influenced by anthropogenic activities in the surrounding environment and wild/domesticated animals (Messi et al., 2006; Meinersmann et al., 2008; Furtula et al., 2013). Commonly isolated enterococcal species from surface waters are *E. faecalis* and *E. faecium*, as well as *E. casseliflavus*, *E. gallinarum*, *E. durans*, *E. hirae,* and *E. mundtii* (Švec and Sedláèek, 1999; Łuczkiewicz et al., 2010). In contrast to the *E. coli* isolates, the majority of the enterococcal isolates in the present study were found to be AMR and the single highest resistance was to lincomycin. In surface water samples collected in an area with intensive poultry production, the highest level of resistance among 36 enterococcal isolates was to lincomycin (88%), followed by tetracycline (24%) (Furtula et al., 2013). Although lincomycin is commonly used in poultry production, we have not previously detected lincomycin residues in the USR watershed (Dungan et al., 2017b), which is not surprising given that it is not approved for use in dairy cattle in the United States. Jackson et al. (2011) showed that the most common AMR phenotype among enterococcal isolates from dairy cattle was to lincomycin (587/636; 92%). In addition, they detected at least 10 enterococcal species and lincomycin-resistant isolates were abundant among each of the different species Jackson et al. (2011). Similarly, 10 different enterococcal species were detected in surface water samples from the Upper Oconee watershed and the majority (564/637; 89%) were resistant to lincomycin (Cho et al., 2020a). In the present study, seven out of eight enterococcal species (except *E. malodoratus*) were resistant to lincomycin (range of 49–100% isolates per species). The high rate of lincomycin resistance among enterococci is likely not a result of exposure to the antimicrobial, but due to the fact that most enterococci, with the exception of *E. durans*, are intrinsically resistant to lincomycin (Gilmore et al., 2002).

While the majority of the enterococcal isolates in the present study were AMR, 7% (13/185) were MDR to as many as six drug classes with six different patterns, and these MDR isolates were only found among four of eight species: *E. casseliflavus*, *E. faecalis*, *E. faecium*, and *E. thailandicus*. Similarly, Cho et al. (2020a) found that 8% (51/637) of enterococcal isolates from an agricultural watershed were MDR to as many as five drug classes with 18 different patterns; six of nine species were found to be MDR, including *E. casseliflavus*, *E. faecalis*, *E. faecium*, *E. hirae*, *E. gallinarum*, and *E. mundtii*, and no MDR was detected among *E. avium*, *E. durans*, and *E. pallens*. The differences between the results of Cho et al. (2020a) and the present study are likely related to a number of factors such as watershed characteristics, regional climates, and *Enterococcus* spp. source. *Enterococcus faecalis* and *faecium* are commensals from the gastrointestinal tract of warm-blood animals, but can cause life-threating infections in humans and account for about 80–90% and 5–15% of all clinical isolates, respectively (Cetinkaya et al., 2000). *Enterococcus hirae* and *durans* are infrequently isolated from human clinical samples and are known pathogenic agents in young animals (Gilmore et al., 2002). *Enterococcus casseliflavus* and *gallinarum* are intrinsically resistant to low levels of vancomycin (Dutka-Malen et al., 1994) and are also rarely isolated in clinical samples (Reid et al., 2001), but they are detected in horse, cattle, and bird feces (Thal et al., 1995; Haenni et al., 2009) and cause urinary tract infections in canines (Simjee et al., 2002). Given that the AMR enterococcal isolates in the present study could potentially cause disease in exposed humans, an additional concern is that therapeutic treatments could fail. All of the AMR enterococci were resistant to antimicrobials that are deemed "important," "highly important," or "critically important" for human medicine by the World Health Organization (WHO, 2017).

In conclusion, this is the first report to our knowledge to address resistance phenotypes of FIB from IRFs, which are an important conduit of surface waters in agroecosystems. The results indicate that the IRFs are polluted with material of fecal origin, which would not be surprising given that this is a mixed-use watershed and livestock manures are commonly applied to cropland soils. However, some of the *E. coli* and enterococcal isolates could be from naturalized extraintestinal populations that were released from sediments and soils. Regardless of FIB source, a wide variety of resistance patterns were found among many of the isolates, suggesting the potential for horizontal transfer of ARGs in the aquatic environment. Although *E. coli* and enterococci are intrinsically resistant to some of the antimicrobials tested, it may be possible that AMR among the isolates has also emerged in response to selection from the presence of antimicrobial residues or other chemical agents. While the FIB levels did increase during the irrigation season, resistant isolates were obtained during each sampling event throughout the year, indicating a permanent presence of ARB in the watershed. Since the IRFs do discharge into the Snake River, there is a potential opportunity for human contact with resistant *E. coli* and enterococci when the river is used for recreational purposes. Additional monitoring, as well as source tracking, of *E. coli* and enterococci in the IRFs will be necessary to understand

the long-term trends of AMR and sources of FIB in this mixed-use watershed.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

RD conceived and designed the study, analyzed the data, and wrote the manuscript. DB provided technical support and reviewed the manuscript. Both authors contributed to the article and approved the submitted version.

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REFERENCES

Adenipekun, E. O., Jackson, C. R., Oluwadun, A., Iwalokun, B. A., Frye, J. G., Barrett, J. B., et al. (2015). Prevalence and antimicrobial resistance in *Escherichia coli* from food animals in Lagos, Nigeria. *Microb. Drug Resist.* 21, 358–365.

Agga, G. E., Schmidt, J. W., and Arthur, T. M. (2016). Effects of in-feed chlortetracycline prophylaxis in beef cattle on animal health and antimicrobial-resistant *Escherichia coli*. *Appl. Environ. Microbiol.* 82, 7197–7204. doi: 10.1128/aem.01928-16

Allen, H. K., and Stanton, T. B. (2014). Altered egos: antibiotic effects on food animal microbiomes. *Annu. Rev. Microbiol.* 68, 297–315. doi: 10.1146/annurev-micro-091213-113052

Bailey, J. K., Pinyon, J. L., Anantham, S., and Hall, R. M. (2010). Commensal *Escherichia coli* of healthy humans: a reservoir for antibiotic-resistance determinants. *J. Med. Microbiol.* 59, 1331–1339. doi: 10.1099/jmm.0.022475-0

Bell, J. M., Paton, J. C., and Turnidge, J. (1998). Emergence of vancomycin-resistant enterococci in Australia: phenotypic and genotypic characteristics of isolates. *J. Clin. Microbiol.* 36, 2187–2190. doi: 10.1128/jcm.36.8.2187-2190.1998

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

Bjorneberg, D. L., King, B. A., and Koehn, A. C. (2020). Watershed water balance changes as furrow irrigation is converted to sprinkler irrigation in an arid region. *J. Soil Water Conserv.* 75, 254–262. doi: 10.2489/jswc.75.3.254

Bjorneberg, D. L., Leytem, A. B., Ippolito, J. A., and Koehn, A. C. (2015). Phosphorus losses from an irrigated watershed in the northwestern United States: case study of the upper snake rock watershed. *J. Environ. Qual.* 44, 552–559. doi: 10.2134/jeq2014.04.0166

Bjorneberg, D. L., Westermann, D. T., Nelson, N. O., and Kendrick, J. H. (2008). Conservation practice effectiveness in the irrigated Upper Snake River/Rock Creek watershed. *J. Soil Water Conserv.* 63, 487–495. doi: 10.2489/jswc.63.6.487

Bradshaw, J. K., Snyder, B. J., Oladeinde, A., Spidle, D., Berrang, M. E., Meinersmann, R. J., et al. (2016). Characterizing relationships among fecal indicator bacteria, microbial source tracking markers, and associated waterborne pathogen occurrence in stream water and sediments in a mixed land use watershed. *Water Res.* 101, 498–509. doi: 10.1016/j.watres.2016.05.014

Brinkmeyer, R., Amon, R. M., Schwarz, J. R., Saxton, T., Roberts, D., Harrison, S., et al. (2015). Distribution and persistence of *Escherichia coli* and Enterococci in stream bed and bank sediments from two urban streams in Houston, TX. *Sci. Total Environ.* 502, 650–658. doi: 10.1016/j.scitotenv.2014.09.071

Burnes, B. S. (2003). Antibiotic resistance analysis of fecal coliforms to determine fecal pollution sources in a mixed-use watershed. *Environ. Monit. Assess* 85, 87–98.

Cetinkaya, Y., Falk, P., and Mayhall, C. G. (2000). Vancomycin-resistant enterococci. *Clin. Microbiol. Rev.* 13, 686–707.

Chen, P. A., Hung, C. H., Huang, P. C., Chen, J. R., Huang, I. F., Chen, W. L., et al. (2016). Characteristics of CTX-M extended-spectrum beta-Lactamase-producing *Escherichia coli* strains isolated from multiple rivers in Southern Taiwan. *Appl. Environ. Microbiol.* 82, 1889–1897. doi: 10.1128/aem.03222-15

Cho, K. H., Pachepsky, Y. A., Kim, J. H., Guber, A. K., Shelton, D. R., and Rowland, R. (2010). Release of *Escherichia coli* from the bottom sediment in a first-order creek: experiment and reach-specific modeling. *J. Hydrol.* 391, 322–332. doi: 10.1016/j.jhydrol.2010.07.033

Cho, S., Hiott, L. M., Barrett, J. B., Mcmillan, E. A., House, S. L., Humayoun, S. B., et al. (2018). Prevalence and characterization of *Escherichia coli* isolated from the Upper Oconee Watershed in Northeast Georgia. *PLoS One* 13:e0197005. doi: 10.1371/journal.pone.0197005

Cho, S., Hiott, L. M., Mcdonald, J. M., Barrett, J. B., Mcmillan, E. A., House, S. L., et al. (2020a). Diversity and antimicrobial resistance of Enterococcus from the Upper Oconee Watershed, Georgia. *J. Appl. Microbiol.* 128, 1221–1233. doi: 10.1111/jam.14550

Cho, S., Jackson, C. R., and Frye, J. G. (2020b). The prevalence and antimicrobial resistance phenotypes of *Salmonella*, *Escherichia coli* and *Enterococcus* sp. in surface water. *Lett. Appl. Microbiol.* 71, 3–25. doi: 10.1111/lam.13301

Clermont, O., Gordon, D., and Denamur, E. (2015). Guide to the various phylogenetic classification schemes for *Escherichia coli* and the correspondence among schemes. *Microbiol.* 161, 980–988. doi: 10.1099/mic.0.000063

de Luca Rebello, R. C., and Regua-Mangia, A. H. (2014). Potential enterovirulence and antimicrobial resistance in *Escherichia coli* isolates from aquatic environments in Rio de Janeiro, Brazil. *Sci. Total Environ.* 490, 19–27. doi: 10.1016/j.scitotenv.2014.04.040

Diaz, F. J., O'geen, A. T., and Dahlgren, R. A. (2010). Efficacy of constructed wetlands for removal of bacterial contamination from agricultural return flows. *Agric. Water Manag.* 97, 1813–1821. doi: 10.1016/j.agwat.2010.06.015

Doumith, M., Day, M. J., Hope, R., Wain, J., and Woodford, N. (2012). Improved multiplex PCR strategy for rapid assignment of the four major *Escherichia coli* phylogenetic groups. *J. Clin. Microbiol.* 50, 3108–3110. doi: 10.1128/jcm.01468-12

Ducey, T. F., Durso, L. M., Ibekwe, A. M., Dungan, R. S., Jackson, C. R., Frye, J. G., et al. (2020). A newly developed *Escherichia coli* isolate panel from a cross section of U.S. animal production systems reveals geographic and commodity-based differences in antibiotic resistance gene carriage. *J. Hazard. Mater.* 382:120991. doi: 10.1016/j.jhazmat.2019.120991

Dungan, R. S., and Bjorneberg, D. L. (2020). Antibiotic resistance genes, class 1 integrons, and IncP-1/IncQ-1 plasmids in irrigation return flows. *Environ. Pollut.* 257:113568. doi: 10.1016/j.envpol.2019.113568

Dungan, R. S., Bjorneberg, D. L., and Leytem, A. B. (2011). Effect of sprinkler pressure and spray plate on culturable microorganism concentrations during simulated irrigation of dairy wastewater. *Trans. ASABE* 54, 1669–1673. doi: 10.13031/2013.39853

Dungan, R. S., Klein, M., and Leytem, A. B. (2012). Quantification of bacterial indicators and zoonotic pathogens in dairy wastewater ponds. *Appl. Environ. Microbiol.* 78, 8089–8095.

Dungan, R. S., Leytem, A. B., Tarkalson, D. D., Ippolito, J. A., and Bjorneberg, D. L. (2017a). Greenhouse gas emissions from an irrigated dairy forage rotation as influenced by fertilizer and manure applications. *Soil Sci. Soc. Am. J.* 81, 537–545. doi: 10.2136/sssaj2016.08.0254

Dungan, R. S., Mckinney, C. W., and Leytem, A. B. (2018). Tracking antibiotic resistance genes in soil irrigated with dairy wastewater. *Sci. Total Environ.* 635, 1477–1483. doi: 10.1016/j.scitotenv.2018.04.020

Dungan, R. S., Snow, D. D., and Bjorneberg, D. L. (2017b). Occurrence of antibiotics in an agricultural watershed in South-Central Idaho. *J. Environ. Qual.* 46, 1455–1461. doi: 10.2134/jeq2017.06.0229

Dungan, R. S., Strausbaugh, C. A., and Leytem, A. B. (2019). Survey of selected antibiotic resistance genes in agricultural and non-agricultural soils in south-central Idaho. *FEMS Microbiol. Ecol.* 95:fiz071.

Dutka-Malen, S., Blaimont, B., Wauters, G., and Courvalin, P. (1994). Emergence of high-level resistance to glycopeptides in Enterococcus gallinarum and *Enterococcus casseliflavus*. *Antimicrob. Agents Chemother.* 38, 1675–1677. doi: 10.1128/aac.38.7.1675

Elliott, S. D., and Barnes, E. M. (1959). Changes in serological type and antibiotic resistance of Lancefield group D streptococci in chickens receiving dietary chlortetracycline. *J. Gen. Microbiol.* 20, 426–433. doi: 10.1099/00221287-20-2-426

Escobar-Paramo, P., Clermont, O., Blanc-Potard, A. B., Bui, H., Le Bouguenec, C., and Denamur, E. (2004a). A specific genetic background is required for acquisition and expression of virulence factors in *Escherichia coli*. *Mol. Biol. Evol.* 21, 1085–1094. doi: 10.1093/molbev/msh118

Escobar-Paramo, P., Grenet, K., Le Menac'h, A., Rode, L., Salgado, E., Amorin, C., et al. (2004b). Large-scale population structure of human commensal *Escherichia coli* isolates. *Appl. Environ. Microbiol.* 70, 5698–5700. doi: 10.1128/aem.70.9.5698-5700.2004

Fagan, P. K., Hornitzky, M. A., Bettelheim, K. A., and Djordjevic, S. P. (1999). Detection of Shiga-like toxin (stx1 andstx2), intimin (eaeA), and enterohemorrhagic *Escherichia coli* (EHEC) hemolysin (EHEC hlyA) genes in animal feces by multiplex PCR. *Appl. Environ. Microbiol.* 65, 868–872. doi: 10.1128/aem.65.2.868-872.1999

Fard, R. M., Heuzenroeder, M. W., and Barton, M. D. (2011). Antimicrobial and heavy metal resistance in commensal enterococci isolated from pigs. *Vet. Microbiol.* 148, 276–282. doi: 10.1016/j.vetmic.2010.09.002

Ferguson, C., Husman, A. M. D., Altavilla, N., Deere, D., and Ashbolt, N. (2003). Fate and transport of surface water pathogens in watersheds. *Crit. Rev. Environ. Sci. Technol.* 33, 299–361. doi: 10.1080/10643380390814497

Field, K. G., and Samadpour, M. (2007). Fecal source tracking, the indicator paradigm, and managing water quality. *Water Res.* 41, 3517–3538. doi: 10.1016/j.watres.2007.06.056

Franklin, A. M., Aga, D. S., Cytryn, E., Durso, L. M., Mclain, J. E., Pruden, A., et al. (2016). Antibiotics in agroecosystems: introduction to the special section. *J. Environ. Qual.* 45, 377–393. doi: 10.2134/jeq2016.01.0023

Frye, J. G., and Jackson, C. R. (2013). Genetic mechanisms of antimicrobial resistance identified in *Salmonella enterica*, *Escherichia coli*, and *Enteroccocus* spp. isolated from U.S. food animals. *Front. Microbiol.* 4:135. doi: 10.3389/fmicb.2013.00135

Furtula, V., Jackson, C. R., Farrell, E. G., Barrett, J. B., Hiott, L. M., and Chambers, P. A. (2013). Antimicrobial resistance in *Enterococcus* spp. isolated from environmental samples in an area of intensive poultry production. *Int. J. Environ. Res. Public Health* 10, 1020–1036. doi: 10.3390/ijerph10031020

Gilmore, M. S., Clewell, D. B., Courvalin, P., Dunny, G., Murray, B., and Rice, L. (2002). *The Enterococci: Pathogenesis, Molecular Biology, and Antibiotic Resistance*. Washington, DC: ASM Press.

Gomi, R., Matsuda, T., Matsumura, Y., Yamamoto, M., Tanaka, M., Ichiyama, S., et al. (2016). Whole-genome analysis of antimicrobial-resistant and extraintestinal pathogenic *Escherichia coli* in river water. *Appl. Environ. Microbiol.* 83:e02703-16.

Gordon, D. M., Clermont, O., Tolley, H., and Denamur, E. (2008). Assigning *Escherichia coli* strains to phylogenetic groups: multi-locus sequence typing versus the PCR triplex method. *Environ. Microbiol.* 10, 2484–2496. doi: 10.1111/j.1462-2920.2008.01669.x

Haenni, M., Saras, E., Chatre, P., Meunier, D., Martin, S., Lepage, G., et al. (2009). vanA in *Enterococcus faecium*, *Enterococcus faecalis*, and *Enterococcus casseliflavus* detected in French cattle. *Foodborne Pathog. Dis.* 6, 1107–1111.

Heuer, H., Schmitt, H., and Smalla, K. (2011). Antibiotic resistance gene spread due to manure application on agricultural fields. *Curr. Opin. Microbiol.* 14, 236–243. doi: 10.1016/j.mib.2011.04.009

Ibekwe, A. M., Murinda, S. E., and Graves, A. K. (2011). Genetic diversity and antimicrobial resistance of *Escherichia coli* from human and animal sources uncovers multiple resistances from human sources. *PLoS One* 6:e20819. doi: 10.1371/journal.pone.0020819

Ishii, S., Ksoll, W. B., Hicks, R. E., and Sadowsky, M. J. (2006). Presence and growth of naturalized *Escherichia coli* in temperate soils from Lake Superior watersheds. *Appl. Environ. Microbiol.* 72, 612–621. doi: 10.1128/aem.72.1.612-621.2006

Jackson, C. R., Lombard, J. E., Dargatz, D. A., and Fedorka-Cray, P. J. (2011). Prevalence, species distribution and antimicrobial resistance of enterococci isolated from US dairy cattle. *Lett. Appl. Microbiol.* 52, 41–48. doi: 10.1111/j.1472-765x.2010.02964.x

Jamieson, R. C., Gordon, R. J., Tattrie, S. C., and Stratton, G. W. (2003). Sources and persistence of fecal coliform bacteria in a rural watershed. *Water Qual. Res. J. Can.* 38, 33–47. doi: 10.2166/wqrj.2003.004

Johnson, J. R., Johnston, B. D., Delavari, P., Thuras, P., Clabots, C., and Sadowsky, M. J. (2017). Phylogenetic backgrounds and virulence-associated traits of *Escherichia coli* isolates from surface waters and diverse animals in Minnesota and Wisconsin. *Appl. Environ. Microbiol.* 83:e01329-17.

Jokinen, C. C., Edge, T. A., Koning, W., Laing, C. R., Lapen, D. R., Miller, J., et al. (2012). Spatial and temporal drivers of zoonotic pathogen contamination of an agricultural watershed. *J. Environ. Qual.* 41, 242–252. doi: 10.2134/jeq2011.0203

Kadykalo, S., Thomas, J., Parmley, E. J., Pintar, K., and Fleury, M. (2020). Antimicrobial resistance of *Salmonella* and generic *Escherichia coli* isolated from surface water samples used for recreation and a source of drinking water in southwestern Ontario, Canada. *Zoonoses Public Health* 67, 566–575. doi: 10.1111/zph.12720

Keen, P. L., Knapp, C. W., Hall, K. J., and Graham, D. W. (2018). Seasonal dynamics of tetracycline resistance gene transport in the Sumas River agricultural watershed of British Columbia, Canada. *Sci. Total Environ.* 628-629, 490–498. doi: 10.1016/j.scitotenv.2018.01.278

Klein, G. (2003). Taxonomy, ecology and antibiotic resistance of enterococci from food and the gastro-intestinal tract. *Int. J. Food Microbiol.* 88, 123–131. doi: 10.1016/s0168-1605(03)00175-2

Knapp, C. W., Dolfing, J., Ehlert, P. A., and Graham, D. W. (2010). Evidence of increasing antibiotic resistance gene abundances in archived soils since 1940. *Environ. Sci. Technol.* 44, 580–587. doi: 10.1021/es901221x

Lebreton, F., Van Schaik, W., Mcguire, A. M., Godfrey, P., Griggs, A., Mazumdar, V., et al. (2013). Emergence of epidemic multidrug-resistant *Enterococcus faecium* from animal and commensal strains. *mBio* 4:e00534-13.

Łuczkiewicz, A., Jankowska, K., Kurlenda, J., and Olańczuk-Neyman, K. (2010). Identification and antimicrobial resistance of *Enterococcus* spp. isolated from surface water. *Water Sci. Technol.* 62, 466–473. doi: 10.2166/wst.2010.909

Maal-Bared, R., Bartlett, K. H., Bowie, W. R., and Hall, E. R. (2013). Phenotypic antibiotic resistance of *Escherichia coli* and *E. coli* O157 isolated from water, sediment and biofilms in an agricultural watershed in British Columbia. *Sci. Total Environ.* 443, 315–323. doi: 10.1016/j.scitotenv.2012.10.106

Marshall, B. M., Ochieng, D. J., and Levy, S. B. (2009). Commensals: underappreciated reservoir of antibiotic resistance. *Microbe* 4, 231–238. doi: 10.1128/microbe.4.231.1

Marti, R., Scott, A., Tien, Y. C., Murray, R., Sabourin, L., Zhang, Y., et al. (2013). Impact of manure fertilization on the abundance of antibiotic-resistant bacteria and frequency of detection of antibiotic resistance genes in soil and on vegetables at harvest. *Appl. Environ. Microbiol.* 79, 5701–5709. doi: 10.1128/aem.01682-13

McKinney, C. W., Dungan, R. S., Moore, A., and Leytem, A. B. (2018). Occurrence and abundance of antibiotic resistance genes in agricultural soil receiving dairy manure. *FEMS Microbiol. Ecol.* 94:fiy010.

Meinersmann, R. J., Berrang, M. E., Jackson, C. R., Fedorka-Cray, P., Ladely, S., Little, E., et al. (2008). *Salmonella*, *Campylobacter* and *Enterococcus* spp.: their

antimicrobial resistance profiles and their spatial relationships in a synoptic study of the Upper Oconee River basin. *Microb. Ecol.* 55, 444–452. doi: 10.1007/s00248-007-9290-6

Messi, P., Guerrieri, E., De Niederhausern, S., Sabia, C., and Bondi, M. (2006). Vancomycin-resistant enterococci (VRE) in meat and environmental samples. *Int. J. Food Microbiol.* 107, 218–222. doi: 10.1016/j.ijfoodmicro.2005.08.026

O'Neill, J. (2016). *The Review on Antimicrobial Resistance. Tackling Drug-Resistant Infections Globally: Final Report and Recommendations*. London: HM Government.

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

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

Pruden, A., Arabi, M., and Storteboom, H. N. (2012). Correlation between upstream human activities and riverine antibiotic resistance genes. *Environ. Sci. Technol.* 46, 11541–11549. doi: 10.1021/es302657r

Rehmann, C. R., and Soupir, M. L. (2009). Importance of interactions between the water column and the sediment for microbial concentrations in streams. *Water Res.* 43, 4579–4589. doi: 10.1016/j.watres.2009.06.049

Reid, K. C., Cockerill, I. F., and Patel, R. (2001). Clinical and epidemiological features of *Enterococcus casseliflavus*/flavescens and *Enterococcus gallinarum* bacteremia: a report of 20 cases. *Clin. Infect. Dis.* 32, 1540–1546. doi: 10.1086/320542

Rothrock, M. J., Keen, P. L., Cook, K. L., Durso, L. M., Franklin, A. M., and Dungan, R. S. (2016). How should we be determining background and baseline antibiotic resistance levels in agroecosystem research? *J. Environ. Qual.* 45, 420–431. doi: 10.2134/jeq2015.06.0327

Sayah, R. S., Kaneene, J. B., Johnson, Y., and Miller, R. (2005). Patterns of antimicrobial resistance observed in *Escherichia coli* isolates obtained from domestic- and wild-animal fecal samples, human septage, and surface water. *Appl. Environ. Microbiol.* 71, 1394–1404. doi: 10.1128/aem.71.3.1394-1404.2005

Scott, E. E., Leh, M. D. K., and Haggard, B. E. (2017). Spatiotemporal variation of bacterial water quality and the relationship with pasture land cover. *J. Water Health* 15, 839–848. doi: 10.2166/wh.2017.101

Servais, P., and Passerat, J. (2009). Antimicrobial resistance of fecal bacteria in waters of the Seine river watershed (France). *Sci. Total Environ.* 408, 365–372. doi: 10.1016/j.scitotenv.2009.09.042

Sharma, V. K., and Dean-Nystrom, E. A. (2003). Detection of enterohemorrhagic *Escherichia coli* O157 : H7 by using a multiplex real-time PCR assay for genes encoding intimin and Shiga toxins. *Vet. Microbiol.* 93, 247–260. doi: 10.1016/s0378-1135(03)00039-7

Simjee, S., White, D. G., Mcdermott, P. F., Wagner, D. D., Zervos, M. J., Donabedian, S. M., et al. (2002). Characterization of Tn1546 in vancomycin-resistant *Enterococcus faecium* isolated from canine urinary tract infections: evidence of gene exchange between human and animal enterococci. *J. Clin. Microbiol.* 40, 4659–4665. doi: 10.1128/jcm.40.12.4659-4665.2002

Smalla, K., Heuer, H., Gotz, A., Niemeyer, D., Krogerrecklenfort, E., and Tietze, E. (2000). Exogenous isolation of antibiotic resistance plasmids from piggery manure slurries reveals a high prevalence and diversity of IncQ-like plasmids. *Appl. Environ. Microbiol.* 66, 4854–4862. doi: 10.1128/aem.66.11.4854-4862.2000

Smith, H. W. (1970). Effect of antibiotics on bacterial ecology in animals. *Am. J. Clin. Nutr.* 23, 1472–1479. doi: 10.1093/ajcn/23.11.1472

Stephenson, G. R., and Street, L. V. (1978). Bacterial variations in streams from a Southwest Idaho Rangeland Watershed. *J. Environ. Qual.* 7, 150–157. doi: 10.2134/jeq1978.00472425000700010032x

Stokes, H. W., and Gillings, M. R. (2011). Gene flow, mobile genetic elements and the recruitment of antibiotic resistance genes into Gram-negative pathogens. *FEMS Microbiol. Rev.* 35, 790–819. doi: 10.1111/j.1574-6976.2011.00273.x

Storteboom, H., Arabi, M., Davis, J. G., Crimi, B., and Pruden, A. (2010). Tracking antibiotic resistance genes in the South Platte River basin using molecular signatures of urban, agricultural, and pristine sources. *Environ. Sci. Technol.* 44, 7397–7404. doi: 10.1021/es101657s

Švec, P., and Sedláèek, I. (1999). Occurrence of *Enterococcus* spp. in waters. *Folia Microbiol.* 44, 3–10. doi: 10.1007/bf02816212

Tenaillon, O., Skurnik, D., Picard, B., and Denamur, E. (2010). The population genetics of commensal *Escherichia coli*. *Nat. Rev. Microbiol.* 8, 207–217. doi: 10.1038/nrmicro2298

Thal, L. A., Chow, J. W., Mahayni, R., Bonilla, H., Perri, M. B., Donabedian, S. A., et al. (1995). Characterization of antimicrobial resistance in enterococci of animal origin. *Antimicrob. Agents Chemother.* 39, 2112–2115. doi: 10.1128/aac.39.9.2112

United States EPA (1986). *Ambient Water Quality Criteria for Bacteria*, 1986th Edn. Washington, DC: U.S. EPA Office of Water.

Whitlock, J. E., Jones, D. T., and Harwood, V. J. (2002). Identification of the sources of fecal coliforms in an urban watershed using antibiotic resistance analysis. *Water Res.* 36, 4273–4282. doi: 10.1016/s0043-1354(02)00139-2

WHO (2017). *Critically Important Antimicrobials for Human Medicine: Ranking of Antimicrobial Agents for Risk Management of Antimicrobial Resistance Due to Non-Human Use*. Geneva: World Health Organization.

Wright, E. S., Yilmaz, L. S., and Noguera, D. R. (2012). DECIPHER, a search-based approach to chimera identification for 16S rRNA sequences. *Appl. Environ. Microbiol.* 78, 717–725. doi: 10.1128/aem.06516-11

Zaheer, R., Yanke, L. J., Church, D., Topp, E., Read, R. R., and Mcallister, T. A. (2012). High-throughput species identification of enterococci using pyrosequencing. *J. Microbiol. Methods* 89, 174–178. doi: 10.1016/j.mimet.2012.03.012

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Evaluation of Metagenomic-Enabled Antibiotic Resistance Surveillance at a Conventional Wastewater Treatment Plant

Haniyyah J. Majeed[1], Maria V. Riquelme[1†], Benjamin C. Davis[1], Suraj Gupta[2], Luisa Angeles[3], Diana S. Aga[3], Emily Garner[1†], Amy Pruden[1] and Peter J. Vikesland[1]**

[1] Department of Civil & Environmental Engineering, Virginia Polytechnic Institute and State University, Blacksburg, VA, United States, [2] Interdisciplinary Ph.D Program in Genetics, Bioinformatics, and Computational Biology, Virginia Polytechnic Institute and State University, Blacksburg, VA, United States, [3] Department of Chemistry, University at Buffalo, Buffalo, NY, United States

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Yu Xia, Southern University of Science and Technology, China
Johan Bengtsson-Palme, University of Gothenburg, Sweden

***Correspondence:**
Amy Pruden
apruden@vt.edu
Peter J. Vikesland
pvikes@vt.edu

† Present address:
Maria V. Riquelme, Diversigen, Inc., Houston, TX, United States
Emily Garner, Department of Civil and Environmental Engineering, West Virginia University, Morgantown, WV, United States

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Wastewater treatment plants (WWTPs) receive a confluence of sewage containing antimicrobials, antibiotic resistant bacteria, antibiotic resistance genes (ARGs), and pathogens and thus are a key point of interest for antibiotic resistance surveillance. WWTP monitoring has the potential to inform with respect to the antibiotic resistance status of the community served as well as the potential for ARGs to escape treatment. However, there is lack of agreement regarding suitable sampling frequencies and monitoring targets to facilitate comparison within and among individual WWTPs. The objective of this study was to comprehensively evaluate patterns in metagenomic-derived indicators of antibiotic resistance through various stages of treatment at a conventional WWTP for the purpose of informing local monitoring approaches that are also informative for global comparison. Relative abundance of total ARGs decreased by ∼50% from the influent to the effluent, with each sampling location defined by a unique resistome (i.e., total ARG) composition. However, 90% of the ARGs found in the effluent were also detected in the influent, while the effluent ARG-pathogen taxonomic linkage patterns identified in assembled metagenomes were more similar to patterns in regional clinical surveillance data than the patterns identified in the influent. Analysis of core and discriminatory resistomes and general ARG trends across the eight sampling events (i.e., tendency to be removed, increase, decrease, or be found in the effluent only), along with quantification of ARGs of clinical concern, aided in identifying candidate ARGs for surveillance. Relative resistome risk characterization further provided a comprehensive metric for predicting the relative mobility of ARGs and likelihood of being carried in pathogens and can help to prioritize where to focus future monitoring and mitigation. Most antibiotics that were subject to regional resistance testing were also found in the WWTP, with the total antibiotic load decreasing by ∼40–50%, but no strong correlations were found between antibiotics and corresponding ARGs. Overall, this study provides insight into how metagenomic data can be collected and analyzed for surveillance

of antibiotic resistance at WWTPs, suggesting that effluent is a beneficial monitoring point with relevance both to the local clinical condition and for assessing efficacy of wastewater treatment in reducing risk of disseminating antibiotic resistance.

Keywords: shotgun sequencing, resistome, microbiome, antibiotics, antibiograms, resistome (ARGs and MGEs)

INTRODUCTION

Antibiotic resistance is a complex health threat that requires both global and local action. Wastewater treatment plants (WWTPs) are a promising point of surveillance and mitigation, as they receive a confluence of sewage containing antibiotics, other antimicrobials, antibiotic resistant bacteria (ARB), antibiotic resistance genes (ARGs), and pathogens (Bürgmann et al., 2018). However, clear guidance is lacking with respect to which stage(s) of wastewater treatment and which monitoring targets are most informative with respect to assessing the ARG content of a given sewage. Of particular interest are the potential for ARGs to mobilize and spread and the efficacy of WWTPs in reducing ARG loads and associated human health risks prior to discharge or reuse (Aarestrup and Woolhouse, 2020).

Recent research demonstrates that ARGs that enter a given WWTP are reflective of various attributes of the local population, including antibiotic use patterns and socioeconomic factors (Hendriksen et al., 2019). These ARGs may exist on mobile genetic elements (MGEs), such as plasmids and transposons (Kim et al., 2014), which can facilitate their spread between different bacteria, including human pathogens. Further, ARGs may vary in clinical relevance of the antibiotic to which they encode resistance (e.g., front-line versus last-resort antibiotics) and may exist intracellularly (i.e., within ARB) or extracellularly as naked DNA that could potentially be later assimilated via transformation (Woegerbauer et al., 2020). Ideally, an effective surveillance scheme will serve to capture the breadth and depth of the full ARG profile as it changes through each stage of treatment, while also providing the ability to link the observed ARG patterns to clinical antibiotic resistance concerns, both on a local and global scale (Huijbers et al., 2019).

Conventional WWTPs rely on the dense, highly microbially active biological treatment step of activated sludge to efficiently remove organic matter and attenuate pathogens present in sewage. This reliance on activated sludge has brought about concern that this stage of treatment may serve as a "hotspot" for the mobilization of ARGs (Baquero et al., 2008; Zhang et al., 2009; Rizzo et al., 2013). This concern largely stems from the potential for the conditions within activated sludge to facilitate horizontal transfer of ARGs to pathogenic host bacteria (Zhang et al., 2011). Numerous studies have reported tracking ARB and ARGs through WWTPs (Bréchet et al., 2014; Yang et al., 2014; Mao et al., 2015; Guo et al., 2017; Joseph et al., 2019; Ju et al., 2019). Shotgun metagenomic sequencing is a promising means to gain such insight, as it enables direct profiling of total ARGs representative of a given sample (i.e., the "resistome," Wright, 2007), without biases associated with culture or primer-directed gene amplification methods (e.g., quantitative polymerase chain reaction (qPCR) or qPCR array).

A challenge of metagenomic surveillance approaches for antibiotic resistance is that they support generation of data for up to thousands of ARGs, making it difficult to systematically and meaningfully assess and compare resistomes, both locally for a given WWTP with time, and globally with other WWTPs (Aarestrup and Woolhouse, 2020). The quantitative capacity of metagenomics is also not well defined, which is important for informing human health risk assessment (Manaia et al., 2018). Furthermore, consensus is lacking with respect to ideal locations within the WWTP to sample, frequency of sampling, and which ARG targets are most informative with respect to potential for ARGs to mobilize, efficacy of treatment for reducing ARGs, and relevance to human health risk assessment. A few recent studies have attempted to classify sewage (Hendriksen et al., 2019; Pärnänen et al., 2019) and final effluent (Pärnänen et al., 2019) by geographical region to predict antibiotic resistance burden. Comprehensive metagenomic analysis within individual WWTPs over time and comparison with trends relative to other WWTPs can help clarify which ARGs and groups of ARGs are most informative for linking to clinical resistance in a given community, for assessing WWTP ARG removal performance, and identifying potential anomalies that warrant further attention. Given the monetary cost of metagenomic sequencing, comprehensive longitudinal studies of WWTPs can help to narrow down key sampling locations, sampling frequencies, and replication needed for global comparative studies.

The overarching objective of this study was to comprehensively assess the composition of the resistome through the various stages of treatment and with time at a conventional WWTP to identify candidate metagenomic-derived targets for antibiotic resistance surveillance. This was achieved through eight sampling events over an 18-month period at a local WWTP followed by metagenomic sequencing. These samples were then compared and bench-marked to various dimensions of the resistome as they related to independent quantitative measures of target ARGs, factors associated with antibiotic use in the community and ARG selection (e.g., antibiotics), mobility (e.g., associations with MGEs), and local clinical resistance information. Specifically, we examined the core resistome (i.e., the full complement of ARGs detectable across all treatment stages), discriminatory resistomes (i.e., ARGs that separate the influent from effluent), specific ARGs of clinical concern, and resistome risk scores (i.e., the extent to which ARGs are predicted to occur on MGEs and in pathogens; Martínez et al., 2015; Oh et al., 2018) and compared these to independent qPCR measurements of target ARGs, antibiotic measurements, and local clinical resistance data. The findings provide insight into informative sampling locations, frequencies, and targets suitable for monitoring of antibiotic resistance flowing into and emanating from WWTPs.

MATERIALS AND METHODS

Site Description, Sample Collection, and Sample Preservation

A three million gallons per day (MGD), on average, conventional (anoxic/oxic process with enhanced nitrogen removal) WWTP serving a population of approximately 21,500 in southwestern Virginia, United States was subjected to approximately bi-monthly sampling over the course of 18 months. The design capacity of the WWTP is six MGD, treating approximately 95% municipal wastewater (as % of COD) and 5% pre-treated industrial wastewater. Two industries contribute to the inflow at the WWTP: (1) a machine and fabrication plant discharging an average of 15,000 gallons per day (GPD) and (2) an environmental waste industry discharging an average of 1,600 GPD out of an allowed 14,000 GPD (varies greatly depending on weather conditions). Prior to discharge to a local river, the final effluent is subject to ultraviolet disinfection, with secondary effluent samples collected prior to UV and final effluent samples collected following this treatment step. To explore seasonality in subsequent analyses, samples were separated from the influent (n = 8) and secondary effluent (n = 8) and containing two sampling events in each season. Influent and secondary effluent samples were also separated into grouped seasons as such: winter/spring and summer/fall to capture a gradual transition from one extreme season (i.e., summer or winter) to the next. Details about the eight sampling events are provided in **Supplementary Table 1**.

Grab samples were collected using sterile materials at each stage of treatment and stored on ice until further processing. Temperature, dissolved oxygen (DO), and pH were measured on site for each aqueous sample. Upon receipt at the lab, aqueous samples were divided in triplicate by mixing vigorously and subsequently measuring the same mass of water for each replicate prior to concentrating on a 0.22-μm mixed-cellulose ester membrane filter (Millipore, Billerica, MA, United States). The volume of water (determined by mass) for the first replicate to clog the filter was recorded. Filters were folded and stored in a 50% ethanol solution in 2-mL O-ring tubes and preserved at −20°C. At a later date, the filters were torn into ∼0.1 cm^2 pieces using sterile forceps, transferred to lysing tubes and DNA extraction was performed using a FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH, United States).

Shotgun Metagenomic Analysis

Twenty-two samples were selected for shotgun metagenomic sequencing, with pooling of triplicate DNA extracts in equal mass proportions. These included influent and secondary effluent (i.e., prior to disinfection) samples from each sampling event and a cross section of each WWTP process (i.e., influent, primary effluent, activated sludge, secondary effluent, and final effluent) for representative summer (August 2018) and winter (February 2018) events. Sequencing was performed by Diversigen, Inc. (Houston, TX, United States) on an Illumina NovaSeq 6000 utilizing the NexteraXT DNA Flex library preparation kit (Illumina, San Diego, CA, United States). The target depth was 7 gigabases per sample, corresponding to approximately 47 million reads (2 × 150 paired-end). Metagenomic read statistics are detailed in **Supplementary Table 2**. The samples were uploaded to the MetaStorm (Arango-Argoty et al., 2016) pipeline whereby they were quality filtered prior to annotation with the following databases: Comprehensive Antibiotic Resistance Database (CARD) version 2.0.1 (Jia et al., 2017) and Metagenomic Phylogenetic Analysis 2 (MetaPhlAn2) (Truong et al., 2015). The manual curation of CARD used in this study, as described in the Supplementary Material, can be found in the **Supplementary Data 1**. CARD output from MetaStorm is available in **Supplementary Data 6**, reported as relative abundance (i.e., ARG copies per copies of 16S rRNA genes identified from metagenomic data; Li et al., 2015). To determine calculated absolute abundance (i.e., ARG copies/mL; **Supplementary Data 7**), relative abundance values were multiplied by 16S rRNA gene copies as determined via qPCR (Garner et al., 2018). To help inform monitoring targets, four categories of ARGs were specified within the core resistome according to their absolute differences in the influent and effluent: **(Category 1)** detected only in the influent across all sampling events, **(Category 2)** increased in the secondary effluent with respect to the influent across all sampling events, **(Category 3)** decreased across all sampling events in the secondary effluent with respect to the influent, or **(Category 4)** detected only in secondary effluent across all sampling events.

Reads were assembled in MetaStorm using the IDBA-UD *de novo* assembler (Peng et al., 2012) according to default parameters to generate contigs for gene contextualization and clinically relevant pathogen-ARG screening. Contigs were filtered for sequences ≥1000 bps then protein-coding open reading frames (ORFs) were predicted using Prodigal version 2.6.3 with the "-p meta" option (Hyatt et al., 2010). Predicted ORFs were annotated with CARD and an in-house constructed MGE dataset (Arango-Argoty et al., 2019) using blastp in Diamond version 0.9.24 (Buchfink et al., 2015). Diamond alignments were filtered for stringent ARG and MGE annotation (80% identity, aa length ≥ 100, e-value ≤ 1e-10, bitscore ≥ 50). Each contig was assigned taxonomy using Kraken2 version 2.0.7 (Wood and Salzberg, 2014) with the Kraken2 standard database of complete bacterial, archaeal, and viral genomes in RefSeq.

The core resistome of the influent and secondary effluent was determined as any ARG with a non-zero value relative abundance detected across all sampling events. ExtrARG (Gupta et al., 2019), established based on the extremely randomized tree algorithm, was utilized to identify discriminatory ARGs (i.e., ARGs that collectively distinguish different wastewater samples) taking relative abundance into account.

Assessment of Relative Resistome Risk

Contigs were submitted to the MetaCompare pipeline (Oh et al., 2018). MetaCompare assigns relative resistome risk (i.e., the cumulative potential for ARGs to occur on MGEs and in human pathogens, as inferred from assembled metagenomic data; Martínez et al., 2015) scores based on the distance each sample point is from an established theoretical, maximum risk score in a 3-dimensional "hazard space." A sample's location

in this 3-dimensional space is determined as: (1) the number of occurrences of ARGs on assembled contigs, (2) the number of co-occurrences of ARGs and MGEs, and (3) the number of co-occurrences of ARGs, MGEs, and human pathogen-like sequences, all of which were normalized to the total number of contigs.

Local, Clinical Resistance Isolates

According to the Virginia Healthcare Emergency Management Program, the WWTP in this study is located in the "Near Southwest" region. Fourteen out of fifteen hospitals from this region participated in the 2017 Virginia state and regional cumulative antibiogram (Virginia Department of Health, 2017). The 2017 regional data was used to identify the number of resistant isolates by antibiotic and resistance class corresponding to each species tested.

Quantitative Polymerase Chain Reaction

All qPCR assays were performed on a CFX96 Real Time System (BioRad, Hercules, CA, United States) on triplicate DNA extracts. Gene copies of total bacterial 16S rRNA genes (Suzuki et al., 2000) and the following indicators ARGs of anthropogenic and clinical relevance were quantified in triplicate reactions based on previously published protocols: *bla*TEM (Bibbal et al., 2007), *erm*B (Chen et al., 2007), *sul*1 (Pei et al., 2006), *van*A (Dutka-Malen et al., 1995), and *int*I1 (Hardwick et al., 2008). A 100-fold dilution was determined as optimal to minimize inhibition and applied to the corresponding influent, secondary, and final effluent DNA extracts. On each qPCR plate, a triplicate negative control and standard curve ranging from 10^7 to 10^1 gene copies/μL were included for each target gene. Melt curves were assessed to confirm specificity of the amplicons and standards. Primers, R^2 values, and efficiencies of the standard curves of each assay are reported in **Supplementary Table 9**.

Antibiotic Analysis

Aqueous samples were concentrated using solid phase extraction, and the cartridges shipped to the University at Buffalo for analysis using high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS), as described previously (Singh et al., 2019). The analysis included the following antibiotics: acetylsulfamethoxazole, anhydro erythromycin, anhydrochlorotetracycline, azithromycin, chlorotetracycline, ciprofloxacin, clarithromycin, erythromycin, enrofloxacin, norfloxacin, oxolinic acid, oxytetracycline, roxithromycin, sarafloxacin, spiramycin I, spiramycin II, spiramycin III, sulfachlorpyridazine, sulfadiazine, sulfamethoxine, sulfamerazine, sulfamethazine, sulfamethizole, sulfamethoxazole, sulfamethoxydiazine, sulfathiazole, tetracycline, tilmicosin, trimethoprim, and tylosin. Water samples were collected in pre-combusted (at a temperature of 500°C) amber glass bottles. Because sample collection and solid phase extraction protocols required the use of amber glass bottles, the ability to detect tetracyclines was lost due to their tendency to sorb to glass.

Statistical Analysis and Data Visualization

To determine differences between stages of treatment, a paired Wilcoxon rank-sum test was utilized using the wilcox.test in R version 3.5.1 (R Core Team, 2018) with the built-in stats package. To compare differences among groups based on sampling event, stage of treatment, and seasonality one-way analysis of similarities (ANOSIM) based on Bray–Curtis dissimilarity was conducted in R using the anosim function of the *vegan* (Oksanen et al., 2019) R package. Procrustes analysis was conducted using the procrustes function in the *vegan* R package. Correlation analyses were performed using the Spearman option in rcorr contained within the *Hmisc* R package. A significance level of $\alpha = 0.05$ was used for all statistical analyses. Coefficients of variation, utilized to assess relative variability on resistance class abundance in the influent or secondary effluent, was calculated by dividing the standard deviation by the mean. Nonmetric multi-dimensional scaling analysis was applied to visualize and compare relative abundances of ARGs and taxonomic ranks across samples. Graphics were generated using the *ggplot2* (Wickham, 2016), *circlize* (Gu et al., 2014), and *RColorBrewer* (Neuwirth, 2014) packages in R.

RESULTS

Wastewater Physiochemical Parameters

Correlations were examined between several wastewater physiochemical parameters measured at the time of sampling or monitored by the WWTP (e.g., daily TSS measurements, **Supplementary Table 1**). Influent water temperature, ambient temperature, DO, pH, and TSS were not strongly correlated with any individual ARGs (**Supplementary Data 5**).

Assessment of Metagenomic Sequencing Data and Comparison to qPCR

Metagenomic sequencing yielded an average of approximately 37 million (range: 3.7–54.5 million) paired-end reads per sample (**Supplementary Table 2**). A total of 953 ARGs were identified across all 22 samples collected over the eight sampling events. A Spearman rank order correlation analysis was performed to explore the relationship between ARG abundance as determined by qPCR (**Supplementary Figure 1**) and the calculated absolute abundance (i.e., ARG copies/mL) of ARGs derived from metagenomic data (**Supplementary Data 7**). Significant correlations were observed for *erm*B ($R = 0.87$, Bonferroni-corrected $p < 0.001$), *sul*1 ($R = 0.83$, Bonferroni-corrected $p < 0.001$), and aggregate *bla*TEM genes (*bla*TEM-17, *bla*TEM-57, *bla*TEM-75, *bla*TEM-91, *bla*TEM-166, *bla*TEM-176, *bla*TEM-194, *bla*TEM-195, *bla*TEM-207, *bla*TEM-215) ($R = 0.84$, Bonferroni-corrected $p < 0.001$) versus corresponding *erm*B, *sul*1, and *bla*TEM measurements by qPCR. *van*A, a vancomycin ARG, was only detected by metagenomics in one effluent sampling event (December 2017) and therefore could not be correlated to qPCR data. These results supported

further quantitative evaluation of metagenomically derived measurements of the resistome.

Trends in ARG Abundance Detected in Influent and Effluent

There were 859 ARGs detected across all influent samples and an average total ARG (i.e., all ARGs detected) relative abundance (i.e., normalized per 16S rRNA gene copies) of 2.35 (minimum = 2.15, maximum = 2.76). ARGs corresponding to the following classes represented the majority of ARGs detected: multidrug (range: 26.7–30.7% of total ARGs), macrolide-lincosamide-streptogramin (MLS, 25.3–34.1%), and beta-lactam (9.9–12.4%) (**Figure 1**). Across all secondary effluent samples, there were 637 ARGs detected and an average total ARG relative abundance of 1.20 (minimum = 0.48, maximum = 1.75). ARGs corresponding to the following resistance classes were most abundant: multidrug (range: 24.7–39.6% of total ARGs),

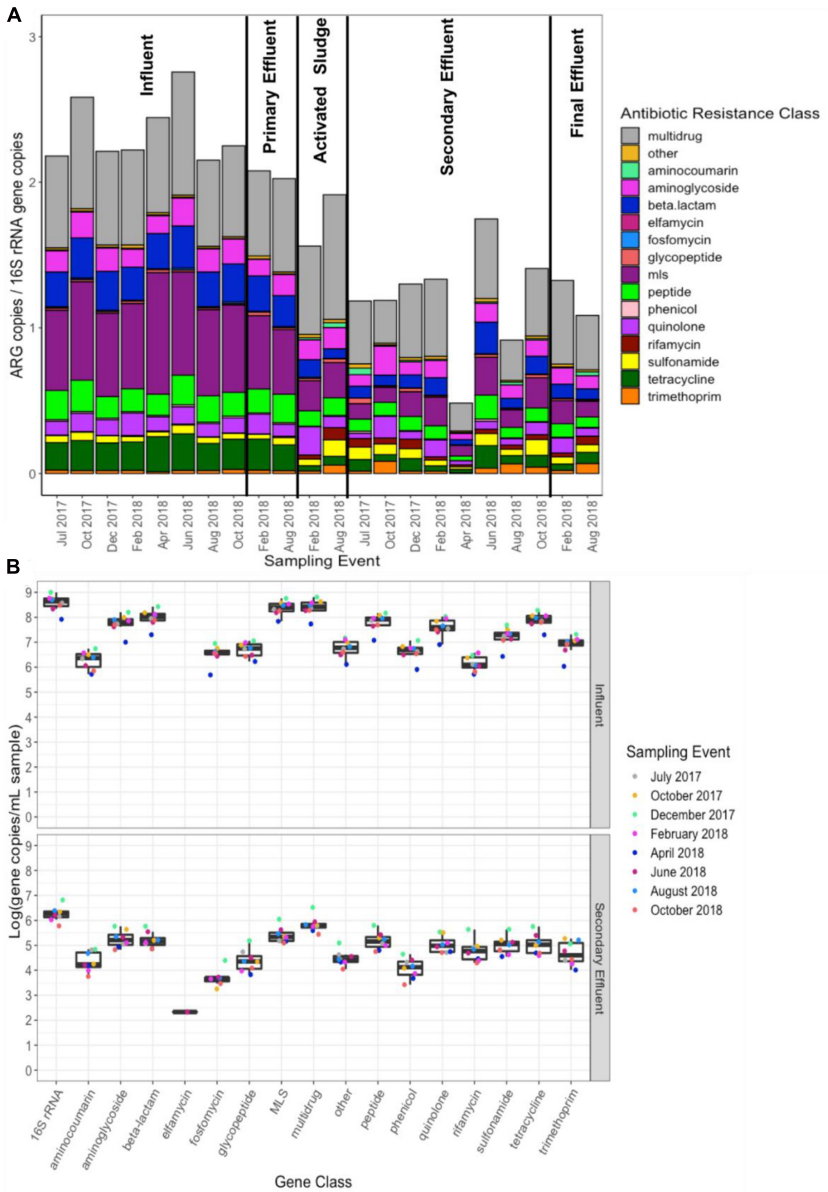

FIGURE 1 | (A) Total ARG (i.e., all ARGs detected) relative abundance [copies per copies of 16S rRNA normalized as previously described (Li et al., 2015)], grouped by antibiotic resistance class, across all 22 samples subject to shotgun metagenomic sequencing and **(B)** calculated absolute abundance (units: $\log_{10}$[gene copies/mL sample]; relative abundance multiplied by 16S rRNA gene copies quantified by qPCR) of each resistance class. ARGs were identified via annotation against CARD version 2.0.1 (Jia et al., 2017). "Multi-drug" represents ARGs conferring resistance to antibiotics corresponding to at least two drug classes, whereas the "other" category comprises genes conferring resistance to non-antibiotics (e.g., antimicrobials, antifungals). MLS indicates resistance to macrolides, lincosamides, and streptogramins.

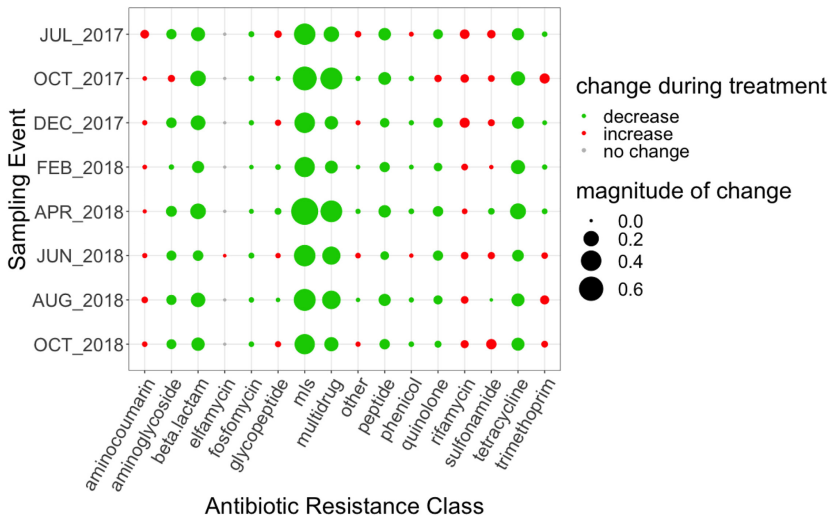

FIGURE 2 | Magnitude of change (based on absolute difference) between influent and secondary effluent for each ARG grouped by antibiotic resistance class. Change was calculated as the difference between the relative abundance of the secondary effluent and the relative abundance of the influent. MLS indicates resistance to macrolides, lincosamides, and streptogramins.

MLS (8.6–14.9%), aminoglycoside (6.6–16.7%), beta-lactam (6.1–12.4%) and quinolone (3.0–12.4%) (**Figure 1**).

ANOSIM confirmed that there was a distinct shift in the resistome composition from influent to effluent, while there was no significant separation between the secondary versus final effluent (**Supplementary Tables 4, 5**). Focus on secondary effluent provides a more consistent DNA yield and presents the advantage of comparability across WWTPs, as not all WWTPs employ effluent disinfection. For each sampling event, there was a decrease in total ARG relative abundance from influent to secondary effluent (Wilcoxon, paired; $p = 0.007813$), corresponding to an average removal of 1.15 ARG copies/16S rRNA gene copies or approximately 50% reduction (**Figure 1**).

The change in relative abundance between the influent to the secondary effluent by antibiotic resistance class ranged between 8.15×10^{-5} to 0.76 ARG copies/16S rRNA gene copies (**Figure 2**). The total number of mapped reads by resistance class in the influent and secondary effluent can be found in **Supplementary Table 10**. On average, the magnitude of observed changes was ∼2,200 reads. MLS ARGs decreased to the greatest extent, with an average removal of 0.49 ARG copies/16S rRNA gene copies. However, a net increase in the relative abundance of ARGs conferring resistance to several classes of antibiotics was indicated through the secondary clarification process for some sampling events (# events): aminocoumarin (8), glycopeptide (4), phenicol (2), rifamycin (8), sulfonamide (6), trimethoprim (4), and other (4) (**Figure 2**).

There was a similar magnitude of decrease in absolute abundance (i.e., ARG copies/mL) of various ARG classes across the WWTP (**Figure 1**). Absolute abundances were calculated from relative abundances of ARGs (ARGs/16S rRNA genes) based on independent measurements of 16S rRNA gene copies per mL via qPCR, which decreased more than 2-$\log_{10}$ from influent to secondary effluent (**Figure 1**).

Core Resistome Composition and Behavior

The "core WWTP" resistome, defined as ARGs detected across each treatment process and all sampling events, consisted of 111 ARGs. However, when considering only the influent and secondary effluent, there were 143 ARGs identified across all sampling events. The 25 ARGs with the highest mean relative abundance in the influent and secondary effluent are presented in **Tables 1**, **2**, respectively. There were 34 ARGs that overlapped between the top 25 ARGs in the influent and the top 25 ARGs in the effluent (**Supplementary Figure 2**). There was an increase in relative abundance of 26 of these 34 ARGs from influent to effluent in all or certain sampling events.

TABLE 1 | Top 25 most abundant core influent ARGs (detected in all sampling events), based on mean relative abundance, that were also detected in secondary effluent core resistome determined by metagenomics.

Antibiotic resistance class	Antibiotic resistance genes
Aminocoumarin	*par*Y in *Streptomyces rishiriensis*
Aminoglycoside	*aac*(6′)-Ib7
Beta-lactam	*bla*OXA-210
MLS	*mac*B, *mph*D, *mph*G, *msr*B, *msr*E, *erm*B, *erm*F
Peptide	*pmr*E, *ros*B
Quinolone	*qac*H, *qnr*S2
Sulfonamide	*sul*1, *sul*2
Tetracycline	*tet*39, *tet*Q
Multidrug	*ade*J, *ade*K, *cpx*R in *Pseudomonas aeruginosa*, *crp*, *mdt*B, *mex*K, *msb*A, *mux*B

TABLE 2 | Top 25 most abundant core secondary effluent ARGs (detected in all sampling events), based on mean relative abundance, that were also detected in the influent core resistome determined by metagenomics.

Antibiotic resistance class	Antibiotic resistance genes
Aminocoumarin	*parY* in *Streptomyces rishiriensis*
Aminoglycoside	*aac*(6′)-Ib7, *aac*(6′)-Ib8, *kdp*E
MLS	*mac*B, *mph*D, *msr*B, *msr*E
Peptide	*pmr*E, *ros*B
Quinolone	*qac*H, *qnr*S2
Sulfonamide	*sul*1, *sul*2
Multidrug	*ade*F, *cpx*R in *Pseudomonas aeruginosa*, *crp*, *mdt*B, *mdt*C, mexK, *msb*A, *mux*B, *mtr*A, *sme*R, *oqx*B
Other	*ile*S in bifidobacteria

For example, *sul*1 increased in six sampling events, excluding April 2018 and August 2018, when the relative abundance decreased. Likewise, the relative abundance of *sul*2 increased in six sampling events, excluding February 2018 and April 2018, when the relative abundance decreased. Interestingly, the calculated absolute abundances (i.e., ARG copies/mL) of *sul*1 and *sul*2 were perfectly correlated ($R = 1.00$, $p < 0.001$). February 2018 represented the sampling event with the greatest number of ARGs exhibiting an increase in relative abundance from influent to secondary effluent (**Figure 3**).

Categorizing Core ARGs Based on Tendency to Increase or Decrease During Treatment

An average of approximately half (41.1–68.0%) of the total ARGs that entered the WWTP on any given sampling date persisted through the secondary effluent (**Table 3**). There were only a relatively small number of ARG types that appeared in the WWTP secondary effluent that were not detected in the influent. Notably (in 7/8 events), ∼90% of the ARG types detected in the secondary effluent were also found in the influent (i.e., were not removed by the treatment train). There were only two ARGs in Category 1: *aph*(3′)-VI (aminoglycoside) and *opr*Z (multidrug). Eleven ARGs fell into Category 2 belonging to the following classes: one beta-lactam (*bla*OXA-46), three rifamycin (*arr*-1, *rph*A, *rph*B), two tetracycline [*tap*, *tet*A(48)], four multidrug (*efp*A, *mex*N, *mtr*A, *mux*C), and one mupirocin, classified as "other" (*ile*S in bifidobacteria). Sixty-one ARGs were included in Category 3 (**Supplementary Table 3**) and no ARGs were included in Category 4 when collectively considering all sampling events. However, between 22 and 93 ARGs uniquely appeared in the secondary effluent when considering individual sampling dates. The greatest number of unique ARGs (i.e., highest diversity) that were detected in the secondary effluent, but not the influent, corresponded to the October 2018 sampling event. Thus, we analyzed the shared ARGs between the October 2018 sampling event and all other sampling events (**Table 4**). A substantial number of these shared ARGs encoded resistance to beta-lactams, although the total relative abundance of this resistance class decreased with each sampling event.

Discriminatory Resistome Composition and Behavior

Thirty-two ARGs were found to most effectively distinguish the influent versus the secondary effluent based on their magnitude of change in relative abundance: one aminocoumarin (*novA*), three aminoglycoside (*acr*D, *ant*(3″)-IIc, *aph*(6)-Id), ten beta-lactam (*bla*ADC-15, *car*O, *bla*FOX-10, *bla*GES-22, *opr*D in *Acinetobacter baumannii,* *bla*OXA-211, *bla*OXA-212, *bla*OXA-309, *bla*OXA-333, *bla*OXA-334), two MLS (*mph*D, *msr*E), seven tetracycline (*ade*A, *tet*32, *tet*39, *tet*40, *tet*O, *tet*Q, *tet*W), eight multidrug (*abe*M, *ade*I, *ade*J, *ade*K, *ade*N, *emr*A, *emr*B), and *far*B, conferring resistance to antibacterial free fatty acids (other) (**Figure 4**). All of the aforementioned ARGs decreased in relative abundance from influent to effluent in each sampling event,

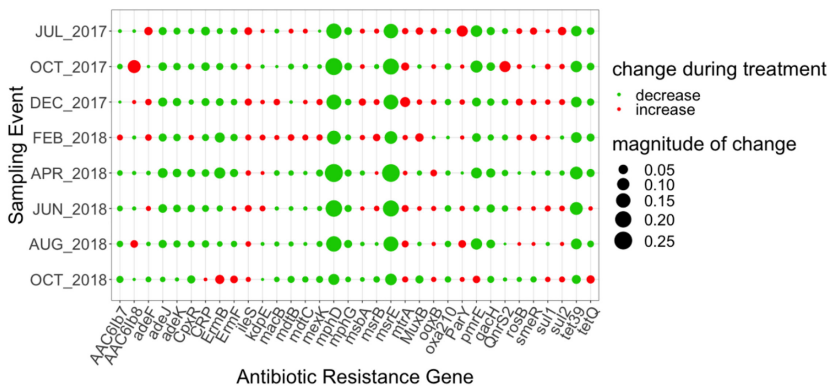

FIGURE 3 | Magnitude of change (based on absolute difference) in relative abundance between influent and secondary effluent of top 34 ARGs pertaining to the core resistome (ARGs detected across each treatment process and all sampling events). Change was calculated as the difference between the relative abundance of the secondary effluent and the relative abundance of the influent.

TABLE 3 | ARGs detected in the influent only, effluent only, or shared between influent and effluent determined by metagenomics.

Sampling event (total ARGs in influent/effluent)	# of unique ARGs detected in the influent (% total ARGs in influent)	# of unique ARGs detected in the effluent (% total ARGs in effluent)	# of ARGs detected in influent *and* effluent (% total ARGs in influent/effluent)
July 2017 (576/271)	334 (58.0)	29 (10.7)	242 (42.0/89.3)
October 2017 (670/331)	318 (47.5)	39 (11.8)	292 (52.5/88.2)
December 2017 (596/330)	309 (51.8)	43 (13.0)	287 (49.2/87.0)
February 2018 (585/309)	298 (50.9)	22 (7.1)	287 (41.1/92.9)
April 2018 (546/326)	253 (46.3)	33 (10.1)	293 (53.7/89.9)
June 2018 (643/441)	250 (38.9)	48 (10.9)	393 (61.1/89.1)
August 2018 (551/381)	219 (39.7)	49 (12.9)	332 (60.3/87.1)
October 2018 (341/325)	109 (32.0)	93 (28.6)	232 (68.0/71.4)

except *nov*A and *tet*Q. *mph*D and *msr*E, which underwent the greatest decrease in relative abundance between influent and effluent, followed by *tet*39.

Clinically Relevant ARGs

A database of 931 ARGs known in clinical isolates was compiled to help prioritize selection of core and discriminatory ARGs for further monitoring (**Supplementary Data 2**). Eleven clinically relevant ARGs (*bla*OXA-3, *bla*OXA-5, *bla*OXA-16, *bla*OXA-46, *bla*OXA-74, *bla*OXA-118, *bla*OXA-129, *bla*OXA-145, *bla*OXA-205, *bla*OXA-210, *qnr*S2) (**Supplementary Table 7**) were detected in the core resistome across all samples. Within the discriminatory resistome of the influent and secondary effluent, there were four clinically relevant ARGs (*bla*GES-22, *bla*OXA-212, *bla*OXA-309, *bla*OXA-333) (**Supplementary Table 7**) detected. Notably, 189 of the 954 total ARGs detected across all samples were also found in the database of clinically relevant ARGs.

Shift in Microbiome Through the WWTP

In most influent samples, *Arcobacter*, *Acinetobacter*, and *Enhydrobacter* comprised greater than 50% of the genera detected (**Figure 5**). This was not the case during the months of December 2017 and February 2018, when *Enhydrobacter* was much lower in relative abundance compared to other sampling events. While the influent and primary effluent were quite similar, there was a sharp shift in the composition of the microbial community in the activated sludge stage. Thereafter, the composition of the secondary effluent and final effluent mirrored that of the activated sludge (for the two events where activated sludge was analyzed. Some of the more dominant genera found from activated sludge onward included: *Thiomonas, Thauera, Nitrospira, Polaromonas,* and *Limnohabitans*. Notably, there appeared to be more variability in the abundance of the top 20 genera in activated sludge, secondary effluent, and final effluent as compared to influent and primary effluent (**Figure 5**).

TABLE 4 | ARGs detected only in the effluent that were also detected in the October 2018 sampling, which yielded the highest number of unique ARG detections determined by metagenomics.

Sampling event (# ARGs shared with October 2018 sampling)	ARGs shared
July 2017 (8)	*bla*F, *bla*CARB-12, *bla*VIM-23, *bla*LRA-19, *bla*OXA-224, *bla*OXA-29, *dfr*B2, *vat*A
October 2017 (15)	*AAC*(6′)-IIb, *bla*F, *bla*IMP-19, *bla*IMP-44, *bla*LRA-10, *bla*VIM-23, *dfr*A2d, *dfr*B2, *dfr*B6, *ole*C, *srm*B, *sul*3, *van*M, *van*XO, *vat*A
December 2017 (16)	*AAC*(3)-IIIc, *abe*S, *arr*-3, *bla*F, *bla*IMP-19, *bla*LRA-10, *bla*LRA-19, *bla*VIM-23, *dfr*A15, *dfr*B2, *dfr*B6, *mfp*A, *ole*C, *tlr*C, *vat*A, *mur*A in *Chlamydia trachomatis*
February 2018 (8)	*bla*F, *bla*IMP-19, *bla*LRA-19, *bla*VIM-2, *bla*VIM-23, *fos*A7, *tet*(Y), *van*M
April 2018 (17)	*AAC*(6′)-IIb, *bla*F, *bla*FEZ-1, *bla*LRA-10, *bla*LRA-19, *bla*OXA-29, *bla*VIM-2, *bla*VIM-23, *dfr*A16, *dfr*B2, *dfr*B6, *iri, ole*B, *ole*C, *qnr*B72, *vat*I, *mur*A in *Chlamydia trachomatis*
June 2018 (11)	*AAC*(3)-IIIc, *bla*FEZ-1, *bla*IMP-19, *bla*LRA-10, *bla*OXA-29, *bla*VIM-2, *bla*VIM-23, *dfr*A2d, *dfr*B6, *ole*B, *sul*3
August 2018 (10)	*arr*-2, *arr*-3, *arr*-5, *bla*LRA-10, *dfr*B6, *ole*C, *qep*A, *qep*A3, *sul*3, *tet*B(48)

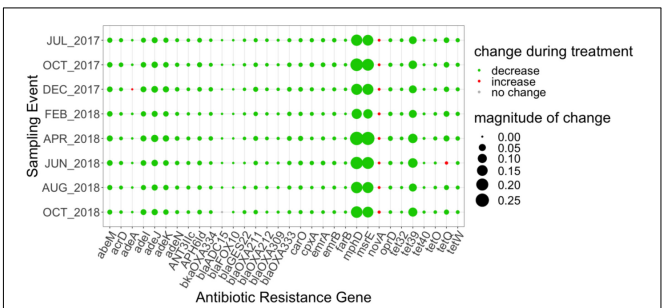

FIGURE 4 | Magnitude of change (based on absolute difference) in relative abundance between influent and secondary effluent of ARGs (n = 32) detected in the discriminatory resistome (ARGs which most effectively distinguish the influent versus the secondary effluent) as determined by ExtrARG (Gupta et al., 2019). Change was calculated as the difference between the relative abundance of the secondary effluent and the relative abundance of the influent.

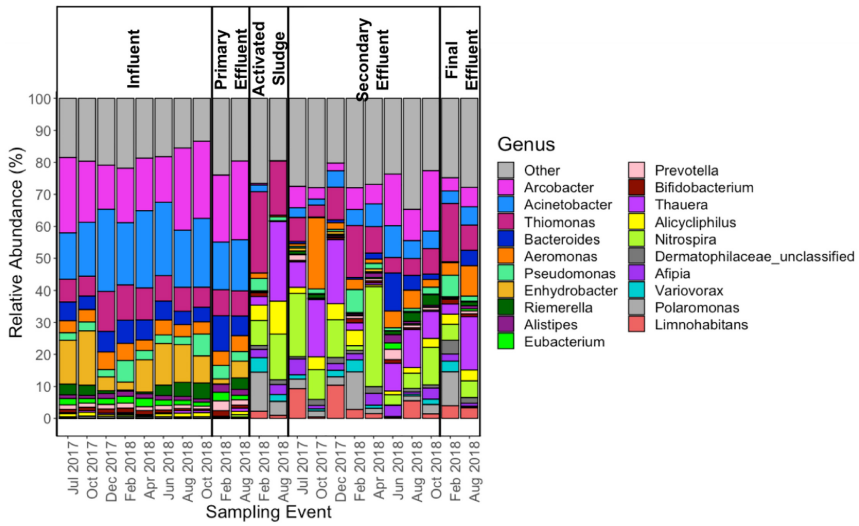

FIGURE 5 | Relative abundance (as percentage) of the top 20 and "other" genera, as annotated using MetaPhlAn2 (Truong et al., 2015) across all 22 samples subject to metagenomic sequencing.

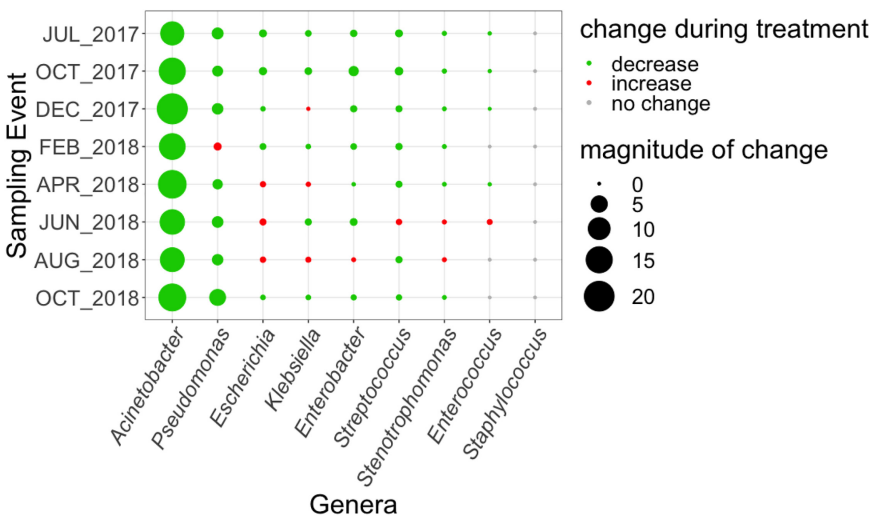

FIGURE 6 | Magnitude of change (based on absolute difference) in relative abundance, as a percentage of corresponding genetic markers quantified from metagenomics data using MetaPhlAn2 (Truong et al., 2015) in influent versus secondary effluent, of pathogen-containing genera tested in the 2017 Virginia state and regional cumulative antibiogram (Virginia Department of Health, 2017).

Trends in Taxonomic Annotations Corresponding to Locally Monitored Clinical Pathogenic Bacteria

As a means of linking the WWTP and local clinical data, we compared the abundance of genetic markers corresponding to the nine genera containing pathogens monitored by the regional antibiogram using MetaPhlAn2 (Truong et al., 2015) in the influent and secondary effluent across the eight sampling events (**Figure 6**). Genetic material corresponding to several of these organisms could still be found in the effluent, including: *Acinetobacter*, *Escherichia, Klebsiella, Enterobacter, Pseudomonas, Enterococcus, Streptococcus,* and *Stenotrophomonas*. Of these, all but *Acinetobacter* increased in relative abundance from influent to effluent in at least one sampling event.

Variation of Resistome and Microbiome Through the WWTP and With Time

According to the coefficients of variation, as percent of the total ARG relative abundance of influent (9.1%) versus secondary effluent (30%) samples, the influent exhibited less variability in resistome composition with time than the secondary effluent (**Table 5**). Influent samples were consistently less variable than

TABLE 5 | Coefficient of variation, as percent (%), in influent and secondary effluent metagenomic samples.

Antibiotic resistance class	Influent (n^a = 8)	Secondary effluent (n^a = 8)
Total ARG relative abundance	9.1	30
Aminocoumarin	24	91
Aminoglycoside	16	43
Beta-lactam	8.7	54
Elfamycin	ND[b]	NA[c]
Fosfomycin	22	66
Glycopeptide	25	67
MLS	15	42
Peptide	15	40
Phenicol	12	63
Quinolone	19	60
Rifamycin	41	48
Sulfonamide	21	45
Tetracycline	12	58
Trimethoprim	20	77
Multidrug	11	30
Other	20	34

[a] *n = number of samples.*
[b] *ARGs conferring resistance to this antibiotic class not detected in any samples.*
[c] *ARGs conferring resistance to this antibiotic class detected in only one sample.*

secondary effluent samples across all antibiotic resistance classes. Variability ranged from: 8.7–41% in the influent and from 30 to 91% in secondary effluent (**Table 5**).

NMDS analysis of total ARG relative abundance indicated distinct resistomes associated with each stage of treatment (ANOSIM; #ARGs = 953, $R = 0.708$, $p = 0.001$; **Figure 7**). Influent samples were separated from activated sludge (ANOSIM; #ARGs = 953, $R = 1$, $p = 0.022$), secondary effluent (ANOSIM; #ARGs = 953, $R = 0.933$, $p = 0.001$), and final effluent (ANOSIM; #ARGs = 953, $R = 1$, $p = 0.022$), but not from primary effluent (ANOSIM; #ARGs = 953, $R = 0.228$, $p = 0.178$). Activated sludge samples were not separated from secondary effluent (ANOSIM; #ARGs = 953, $R = 0.151$, $p = 0.267$) or final effluent (ANOSIM; #ARGs = 953, $R = 0.25$, $p = 0.333$). The ANOSIM R statistic and significance level of each pairwise test between each stage of treatment based on relative abundance is available in **Supplementary Table 4**. ARGs that were not detected in at least one sample in a subset were excluded from ANOSIM analysis. When only influent and secondary effluent samples were grouped by stage of treatment, ARG profiles were separated based on relative abundance (ANOSIM; #ARGS = 916, $n = 8$, $R = 0.933$, $p = 0.001$).

Taxonomic profiles at the genus level followed a similar trend as ARGs and were also uniquely separated by stage of treatment (ANOSIM; $R = 0.6547$, $p = 0.001$). Influent samples were separated from activated sludge (ANOSIM; $R = 1$, $p = 0.019$), secondary effluent (ANOSIM; $R = 0.801$, $p = 0.002$), and final effluent (ANOSIM; $R = 1$, $p = 0.024$), but not from primary effluent (ANOSIM; $R = 0.31$, $p = 0.136$). Activated sludge samples were not separated from secondary effluent (ANOSIM; $R = 0.289$, $p = 0.15$) or final effluent (ANOSIM; $R = 0.25$, $p = 0.667$). The ANOSIM R statistic and significance level of each pairwise test between each stage of treatment based on genus level relative abundance is available in **Supplementary Table 5**.

A Procrustes analysis was performed on the ARG and taxonomic NMDSs of all 22 samples, assuming symmetry, resulting in a Procrustes Sum of Squares value equal to 0.261 ($R = 0.86$, $p = 0.001$). This low value suggests that ARG and taxonomic profiles behave similarly across the WWTP with time. Influent samples did not exhibit distinct separation based on relative abundance of ARGs when grouped by season (ANOSIM; #ARGS = 859, $R = 0.1458$, $p = 0.241$) or grouped seasons (ANOSIM; #ARGS = 859, $R = 0.2396$, $p = 0.064$). Abundance of genera also did not exhibit distinct separation based on grouped seasons (ANOSIM; $R = 0.1354$, $p = 0.213$). However, more distinct separation of the taxonomic profile as a whole was observed by season (ANOSIM; $R = 0.5208$, $p = 0.04$). Similar trends of no distinct separation of resistome or microbiome were observed based on relative abundances in secondary effluent samples: ARG profiles grouped by season (ANOSIM; #ARGS = 637, $R = -0.1667$, $p = 0.785$), ARG profiles based on grouped seasons (ANOSIM; #ARGS = 637, $R = -0.04167$, $p = 0.63$), taxonomic profiles grouped by season (ANOSIM; $R = -0.1458$, $p = 0.775$), and taxonomic profiles based on grouped seasons (ANOSIM; $R = -0.1146$, $p = 0.789$).

Relative Resistome Risk

As would be expected based on the WWTP achieving its intended purpose of reducing pathogens, MetaCompare indicated that there was a higher resistome risk associated with influent and primary effluent samples relative to activated sludge, secondary effluent, and final effluent (**Figure 8**). Influent relative resistome risk scores were significantly higher than those of the secondary effluent (Wilcoxon, paired; $p = 0.007813$). Overall, there was a consistent and high rate of metagenomic assembly incorporated into the resistome risk score determination (**Supplementary Table 6**). The percent of sequences successfully assembled across samples ranged from 24 to 69% (only four samples with <40%). A higher percent assembly was generally achieved for secondary effluent samples (Kruskal–Wallis, $p = 0.0229$).

Comparison to Locally Available Clinical Resistance Data

Overall, there were 45 co-occurrences of ARGs, MGEs, and human pathogen-like sequences on assembled contigs recovered from the influent and 38 from the effluent that corresponded to antibiotic resistant pathogens tested in the regional antibiogram (**Supplementary Data 3**). Among the assembled contigs, there were none found in both the influent and effluent. Nonetheless, the same resistance classes associated with human pathogen-like sequences were found in the effluent, including tetracycline resistance in *E. faecium* and *S. aureus*, beta-lactam resistance in *K. pneumoniae*, and aminoglycoside resistance in *P. aeruginosa*. Multidrug resistance genes were excluded from subsequent analysis because antibiogram data were only available based on single organism-antibiotic combinations.

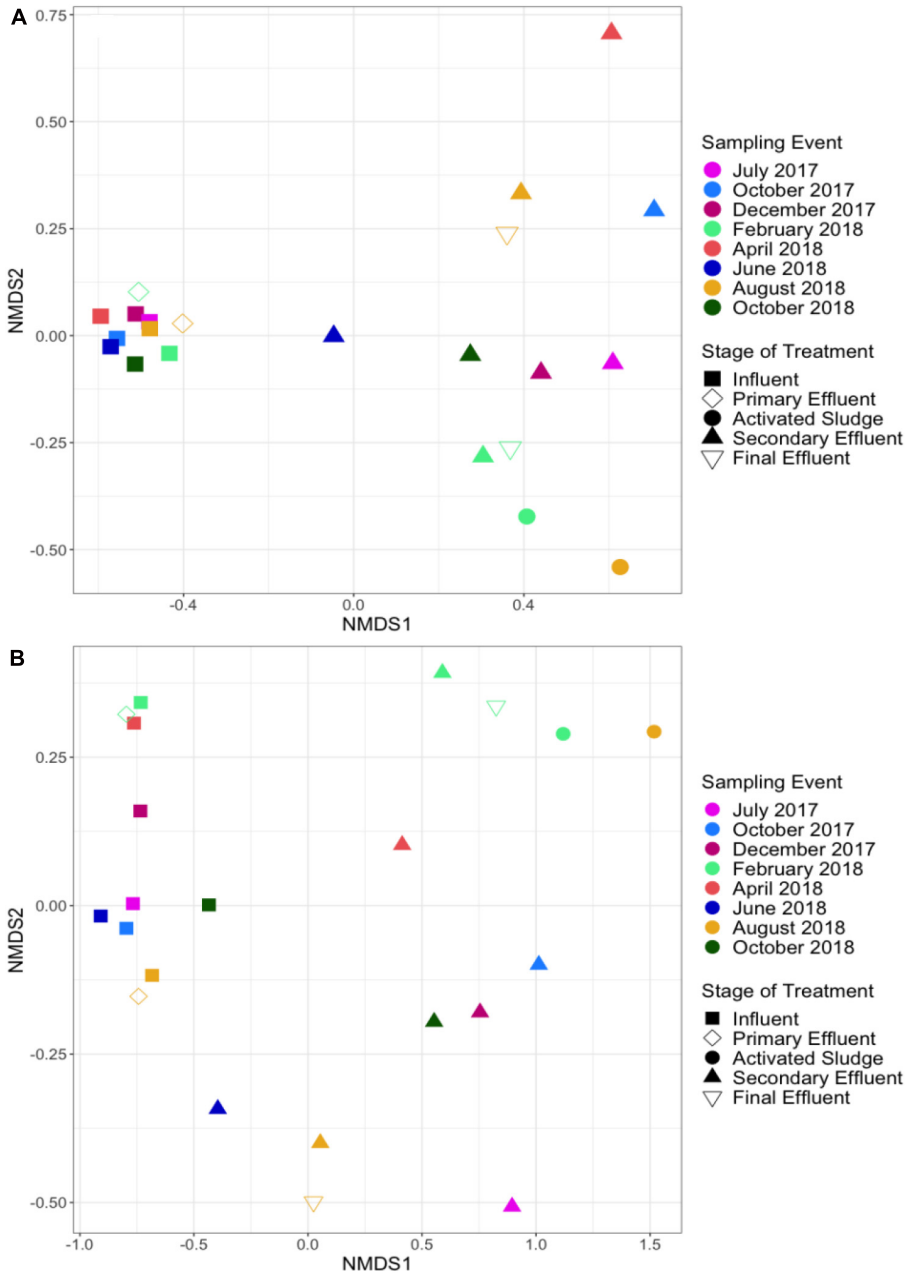

FIGURE 7 | NMDS analysis of **(A)** ARG profiles (ANOSIM; $R = 0.708$, $p = 0.001$) and **(B)** taxonomic profiles (ANOSIM; $R = 0.6547$, $p = 0.001$) across WWTP sampling locations and sampling dates according to shotgun metagenomic sequencing. Influent samples were separated from activated sludge (ANOSIM; $R = 1$, $p = 0.022$), secondary effluent (ANOSIM; $R = 0.933$, $p = 0.001$), and final effluent (ANOSIM; $R = 1$, $p = 0.022$), but not from primary effluent (ANOSIM; $R = 0.228$, $p = 0.178$). Secondary and final effluent samples were not separated (ANOSIM; $R = -0.306$, $p = 0.911$). ARGs were annotated via CARD (Jia et al., 2017) and the microbiome was annotated via MetaPhlAn2 (Truong et al., 2015).

The greatest number of co-occurrences of ARGs with taxonomic markers of antibiogram pathogens in the influent were approximately equal among the MLS (11 co-occurrences), beta-lactam (10 co-occurrences), and aminoglycoside (9 co-occurrences) resistance classes (**Figure 9**). *E. faecium* contained the greatest number of co-occurrences, associated with glycopeptide and MLS resistance classes, followed by *K. pneumoniae*, associated with aminoglycoside and beta-lactam resistance classes. In contrast, the effluent was largely dominated by beta-lactam (14 co-occurrences) and quinolone (8 co-occurrences) resistance classes, with fewer co-occurrences associated with aminoglycoside and MLS resistance classes. *E. coli* comprised the greatest number of co-occurrences in the effluent and, unlike in the influent, was associated

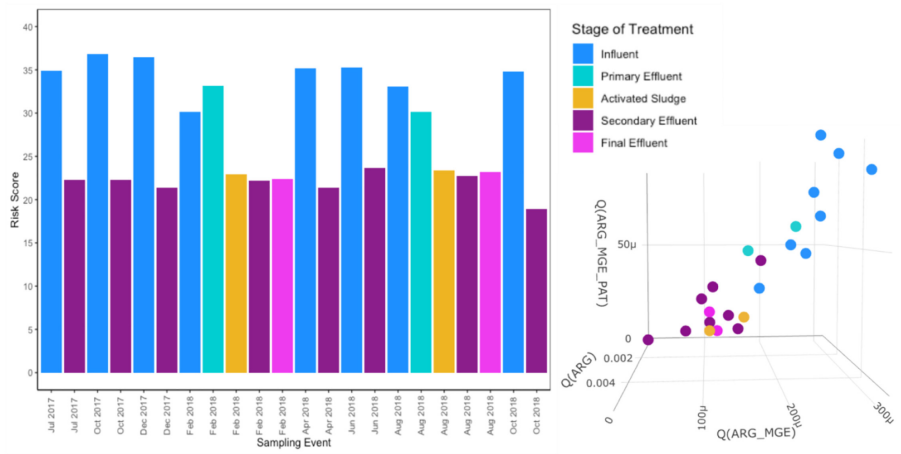

FIGURE 8 | Resistome risk scores and projection in 3D hazard space for each sample as determined by MetaCompare (Oh et al., 2018) using assembled metagenomic data. Risk scores are calculated using: (1) the number of occurrences of ARGs on assembled contigs normalized to the total number of contigs in a given sample, (2) the number of co-occurrences of ARGs and MGEs normalized to the total number of contigs, and (3) the number of co-occurrences of ARGs, MGEs, and human pathogen-like sequences normalized to the total number of contigs.

with quinolone, beta-lactam, and aminoglycoside resistance classes (in descending order). Remarkably, the trends apparent in the effluent were more similar to those of the regional antibiogram data than those apparent in the influent, especially the aforementioned associations with *E. coli* and MLS resistance in *S. aureus* (**Figure 9**). The number of resistant isolates determined from the regional antibiogram can be found in **Supplementary Data 4**.

Antibiotic Detection and Correlation With Corresponding ARG Classes

All antibiotic classes tested in the regional antibiogram were detectable in the WWTP samples, except beta-lactams, due to analytical challenges. Analysis of targeted antibiotics indicated a tendency to reduce from the influent to the final effluent (**Figure 10** and **Supplementary Table 8**). However, among the antibiotics tested, the MLS and trimethoprim antibiotics appeared to be the most persistent and difficult to remove. Cumulative total measured antibiotic loading in the influent ranged between 2,614 and 12,780 ng/L, while final effluent ranged between 1,045 and 7,665 ng/L. The highest antibiotic loading in the influent occurred in the month of February 2018 (12,780 ng/L), closely followed by December 2017 (12,584 ng/L). The highest antibiotic loading in the final effluent occurred in December 2017 (7,665 ng/L), corresponding to a removal efficiency of approximately 39%. Total antibiotic concentration between the two stages of treatment were significantly different (Wilcoxon, paired; $p = 0.0078$), exhibiting an approximate 40–50% removal efficiency.

Sulfamethoxazole and acetylsulfamethoxazole were the only sulfonamides detected among the influent and effluent samples, however, the concentration of sulfamethoxazole was never greater than the proposed no effect concentration (PNEC) (Bengtsson-Palme and Larsson, 2016), below which no selection of antibiotic resistance bacteria is anticipated (**Supplementary Table 8**). The concentration of acetylsulfamethoxazole was greater than sulfamethoxazole in all sampling events, except August 2018, and the two were not strongly correlated ($R = 0.1557$, $p > 0.05$). Aggregate sulfonamide antibiotics and sulfonamide ARGs were not correlated ($R = 0.14$, Bonferroni-corrected $p > 0.05$). Sulfonamides and trimethoprim antibiotics showed a strong correlation in the influent ($R = 0.95$, Bonferroni-corrected $p = 0.0021$). Trimethoprim exceeded the PNEC in seven out of eight sampling events (**Supplementary Table 8**), although of the ARGs detected within the trimethoprim resistant dihydrofolate reductase (dfr) gene family, only two ARGs correlated in the influent at $R > 0.5$, although the associated Bonferroni-corrected p-values were > 0.05. All Spearman rank correlation coefficients and p-values are available in **Supplementary Data 5**.

Of the MLS antibiotics, azithromycin was the most persistent from influent to final effluent, furthermore, azithromycin and clarithromycin appeared to be somewhat negatively correlated ($R = -0.69$, Bonferroni-corrected $p > 0.05$). In five out of eight sampling events, azithromycin or clarithromycin were not detected simultaneously. Anhydro erythromycin and respective MLS ARGs did not correlate, while erythromycin was not detected. Aggregate values of MLS ARGs and MLS antibiotics were not strongly correlated ($R = 0.52$, Bonferroni-corrected $p > 0.05$).

There were no strong correlations of ciprofloxacin, the only quinolone antibiotic detected of those screened, with individual or aggregate quinolone ARGs in the influent (Bonferroni-corrected $p > 0.05$). This was the case even though the concentration of ciprofloxacin exceeded the PNEC in all sampling events in which it was detected (**Supplementary Table 8**).

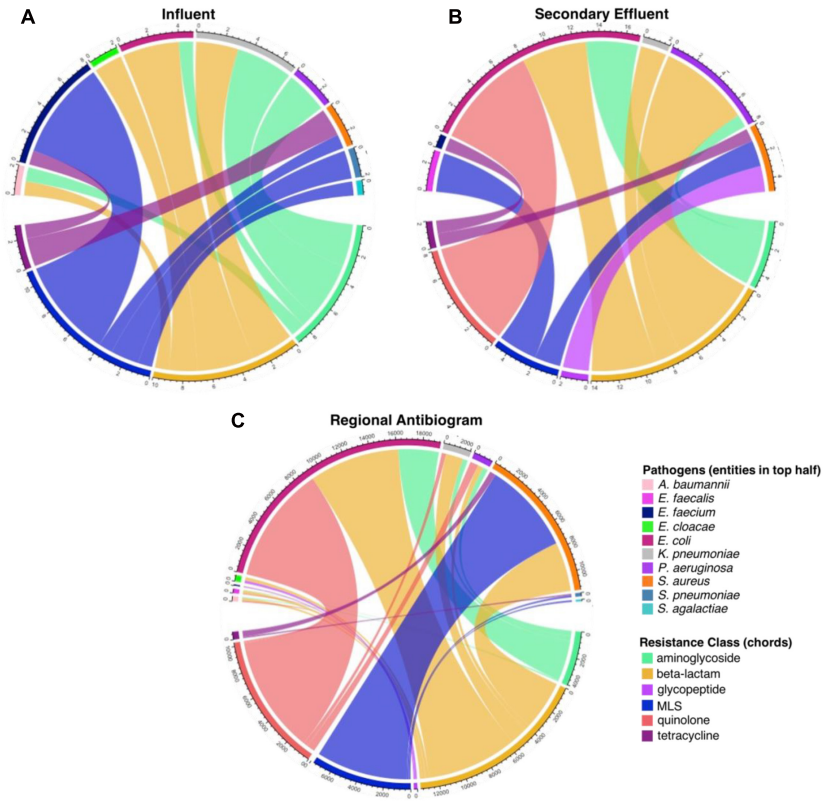

FIGURE 9 | Comparison of ARG-pathogen combinations identified in the assembled metagenomic data and in the 2017 corresponding state and regional cumulative antibiogram (Virginia Department of Health, 2017). The values on the outer rings indicate the frequency of co-occurring antibiotic resistance genes (ARGs) grouped by resistance class, mobile genetic elements (MGEs), and human pathogen-like sequences in all **(A)** influent and **(B)** secondary effluent samples (**Supplementary Data 3**). **(C)** The values on the outer ring represent each antibiotic-pathogen combination which was tested in the regional antibiogram, aggregated by antibiotic resistance class (i.e., a single connection is indicative of one isolate conferring resistance to one antibiotic within a given class; **Supplementary Data 4**).

Percent Resistance to Antibiotics Among Regional Clinical Isolates

Given that sulfamethoxazole/trimethoprim, ciprofloxacin, and macrolides (anhydro-erythromycin, azithromycin, and clarithromycin) were readily detectable in the wastewater samples, resistances to these classes of antibiotics were further examined in the available regional clinical resistance data (Virginia Department of Health, 2017). As a percentage of isolates across multiple medical facilities in the near-southwest region of Virginia, *Acinetobacter baumannii* (36% of 239 isolates), *Enterobacter cloacae* (15% of 454), *Escherichia coli* (23% of 23,761 isolates), *Klebsiella pneumoniae* (11% of 3,759 isolates) and *Staphylococcus aureus* (4% of 7,455 isolates) conferred resistance to sulfamethoxazole/trimethoprim. Similar percentages were observed for resistance to ciprofloxacin: *Acinetobacter baumannii* (33% of 151 isolates), *Enterobacter cloacae* (11% of 442 isolates), *Escherichia coli* (23% of 23,387 isolates), *Klebsiella pneumoniae* (5% of 3,274 isolates), and *Pseudomonas aeruginosa* (18% of 2,245 isolates). Erythromycin was the least effective against tested isolates: *Staphylococcus aureus* (64% of 7,692 isolates), *Streptococcus pneumoniae* (46% of 232 isolates, and *Streptococcus agalactiae* (65% of 122 isolates). Although erythromycin was not directly detected, its secondary metabolite was readily detected and at levels exceeding the PNEC (Oct 2018). As expected, antibiotics being prescribed in the local clinical environment were detectable in wastewater along with gene markers of organisms resistant to those antibiotics. While selection pressure in the WWTP is a possibility, this likely indicates these antibiotics and ARGs originate from the same sources.

DISCUSSION

This study provides comprehensive insight into the composition and variance of the antibiotic resistome across eight sampling events conducted at a small conventional WWTP over an 18-month period. Metagenomic sequencing targeting various stages of treatment, along with core and discriminatory ARG analysis, enabled assessment of which ARGs are most effectively removed (i.e., Categories 1 and 3) versus which increase in or are unique to the effluent (Categories 2 and 4, respectively). Further, observed patterns in composition and removal of ARGs, as well as genera containing key pathogens of antibiotic resistance

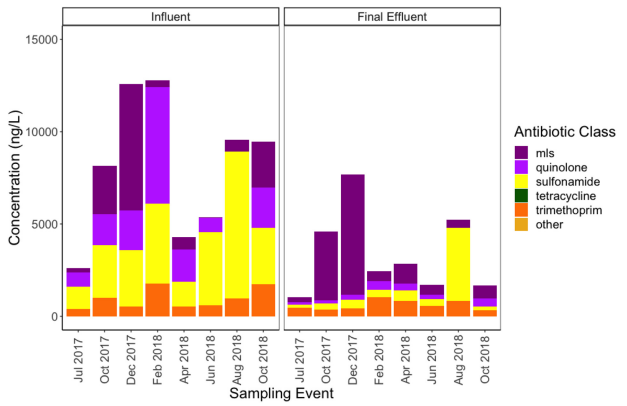

FIGURE 10 | Concentrations of antibiotics (ng/L) detected via high performance liquid chromatography-tandem mass spectrometry in influent and final effluent samples grouped by resistance class. The methods applied were as described previously (Singh et al., 2019). MLS indicates antibiotics belonging to the macrolide, lincosamide, and streptogramin classes. Tetracycline and other (i.e., tylosin) antibiotics were tested but not detected in wastewater samples.

concern, were compared to antibiotic measurements and regional clinical resistance patterns. The approach provided insight into the efficacy of each stage of treatment for ARG removal, while also identifying indicator ARGs and other metagenomic-derived metrics (e.g., resistome risk) relevant to treatment and clinical resistance that should be considered as future candidates for metagenomic surveillance of WWTPs. Notably, strong correlations between all targeted ARGs (excluding *van*A) measured by qPCR are an encouraging indication that shotgun metagenomic sequencing yields quantitative information that can be compared within and across WWTPs with time. However, the discrepancy between *van*A measured via qPCR and metagenomics suggests that caution should be taken in recognizing that there could be biases in metagenomic sequencing. We also recognize that there are inherent limitations in the detection limit of metagenomics and note that the sequencing applied in this study was deeper than most prior WWTP resistome studies. Finally, given that the number of features detected by metagenomics will always be greater than the sample size, there are inherent challenges in making statistical inferences (Jonsson et al., 2016; Bengtsson-Palme et al., 2017).

About 50% reduction in total ARG relative abundance was observed from influent to final effluent (i.e., 2-fold difference) across all sampling events, along with a distinct shift in the resistome composition. Approximately the same magnitude of reduction in total ARG relative abundance was reported in a previous study of three Swedish WWTPs serving 150,000–800,000 people, each sampled once (Bengtsson-Palme et al., 2016). Changes in relative abundance are a useful indicator of the relative degree of selection pressure for carriage of ARGs across a microbial community, although it is important to acknowledge that unrelated factors shaping taxonomy will also affect the resistome composition. Overall, this study is consistent with others that suggest that, while some ARGs persist or increase during wastewater treatment, the general tendency is loss or reduction in ARGs through activated sludge treatment and secondary settling (Gao et al., 2012; Bengtsson-Palme et al., 2016; Lira et al., 2020). Further, it is important to consider that there is a sharp decrease in total bacterial loads from influent to effluent, which will further decrease loads of ARGs in the effluent and likely any associated risks. In the present study, bacterial abundances (16S rRNA gene numbers) decreased by 2-orders of magnitude. Bengtsson-Palme et al. (2016) noted that accounting for the removal of total bacteria resulted in a ∼50-fold decrease in absolute abundance (i.e., ARG copies/mL) of total ARGs from influent to effluent, while 2–3 $\log_{10}$ removal in absolute abundance was observed in the present study. Here we find that both relative abundance and absolute abundance estimations are useful, with the latter considered to be more informative for risk assessment.

The number of ARGs (i.e., ARG diversity) detected from influent to secondary effluent was observed to decrease across all sampling events, from 859 to 637 (a loss in detection of 25% of ARGs). Lira et al. (2020) examined the metagenome at a 9.5 MGD conventional WWTP in Portugal over three sampling events and observed a much sharper loss in detection of ARG types from influent to final effluent (post-UV disinfection) of about 75% of ARGs, although only 259 ARGs were detected across the study. This lower detection rate is likely due to the much shallower sequencing depth obtained via MiSeq sequencing (maximum 8.2 million reads per sample reported in the Lira study, versus 37 million average reads per sample in present study) and higher stringency applied in ARG annotation (95% identity, versus 80% identity). While general trends in ARG removal determined via metagenomics were consistent across the present study and others, this highlights that differences in sequencing approach (e.g., platform, library prep, sequencing depth, sequence length) and analysis approach (e.g., databases, search criteria) will influence precisely which ARGs are detected and could lead to false positives or false negatives, especially for less abundant ARGs.

Based on this study, multidrug ARGs were found to be the most persistent, accounting for up to 40% of total ARG relative abundance in the secondary effluent. The most substantial reduction occurred in the MLS resistance class. Aminocoumarin, glycopeptide, phenicol, rifamycin, sulfonamide, trimethoprim, and other resistance classes experienced an overall increase in ARG relative abundance in at least four sampling events. February 2018 exhibited the greatest number of individual core ARGs that increased from influent to effluent (Category 2 ARGs), while June 2018 accounted for the least reduction in total ARG relative abundance.

The discriminatory resistome was comprised of ARGs that most effectively distinguished the secondary effluent from the influent. Important to note is that the discriminatory ARGs identified by the ExtrARG randomized tree algorithm (Gupta et al., 2019) are distinct from those identified based on the above comparisons of ARG detections in the secondary effluent versus influent. For example, there were several abundant core ARGs that appeared in the discriminatory resistome due to the magnitude of change in relative abundance from influent to

secondary effluent. These shared ARGs included: *mph*D, *msr*E, *tet*39, *tet*Q, *ade*J, *ade*K, of which all but *tet*Q decreased in relative abundance during each sampling event (the relative abundance of *tet*Q increased in June 2018). Such genes could be monitored in future to verify consistent removal of these ARGs. Further, clinically relevant ARGs of concern that are also discriminatory could also be prioritized, including multiple OXA beta-lactamases, as well as the MLS ARGs *mph*D and *msr*E.

The relatively tight clustering of influent samples, visualized by NMDS analysis, indicated that the incoming resistome was relatively stable across the 18-month study period. Greater variability was observed in the resistome of the secondary effluent, which was further indicated by the higher COVs for each antibiotic resistance class. These results suggest that the sewage resistome of a given community served by a WWTP is relatively stable compared to that of the effluent, which is influenced by day-to-day treatment variability. Furthermore, this finding suggests locational reproducibility, and thus relatively infrequent influent sampling may be sufficient for the purpose of comparison of global trends across WWTPs. For the two sampling events when activated sludge was sequenced in this study (February 2018 and August 2018), it was noted that there was a sharp decrease in subsequent total ARG relative abundance, emphasizing the efficacy of this biological treatment stage for ARG attenuation. Still, although it was difficult to link precise changes in the influent to precise shifts in the effluent, it is important to note that ∼90% of secondary effluent ARGs were also found in the influent. The effluent also largely reflected the activated sludge, suggesting that it is heavily influenced by microbes escaping settling. The finding demonstrates that the activated sludge barrier does not completely erase the influence of the influent on the effluent resistome. This suggests that policies aimed at restricting what is discharged to WWTPs could in fact influence input of ARGs to receiving environments. Future studies aimed at assessing how influent resistomes shape effluent resistomes would be of interest.

Considering the results in terms of clinical resistance information helps to further refine potential metagenomic monitoring approaches. For example, ARGs of known clinical concern that are also found in the core and discriminatory resistomes, primarily OXA beta-lactamases, could be prioritized for monitoring and compared against the typical trends denoted by Categories 1–4 as an indicator of potential local resistance concerns and overall WWTP performance. Further, it was quite remarkable that the ARG-pathogen linkages noted in the assembled effluent metagenomes were more similar to regional patterns of clinical resistance than those found in the influent. This was especially the case in June 2018 and August 2018, both summer sampling events, with the most occurrences of increased abundance of pathogen-containing genera of local clinical importance. The tendency of achieving greater percent assembly of secondary effluent metagenomes could have attributed to higher frequency of identification of co-occurring ARG, MGE, and pathogen-like annotations than in the influent. We also acknowledge that MGE annotation is challenging due to out-of-date, incomplete, and disparate public databases, thus improving the accuracy of databases used for annotation should be prioritized in future research. Regardless, these results highlight that WWTP effluent monitoring also has the potential to shed light on local clinical antibiotic resistance concerns, as has been recently proposed with respect to monitoring sewage influents (Aarestrup and Woolhouse, 2020). Effluent monitoring also presents the advantage that it can aid in assessing WWTP performance and provides a measure of loading to the environment and potential associated exposures. Given that the variability of the effluent was higher than the influent, this suggests that it may also be possible to better detect and act upon anomalies of concern than by influent monitoring alone.

In terms of a comprehensive indicator of the potential for ARGs to spread and be present in human pathogens, MetaCompare showed promise in this study. In particular, MetaCompare analysis provided a comprehensive metric that takes into account ARGs, their mobility, and potential presence in pathogens. In this way, relative comparisons of resistome risk can be made in time or space for a given system to prioritize further investigation or action. Consistent with reduction of pathogens from influent, resistome risk scores also consistently decreased through the WWTP in this study. Still, taxonomic markers corresponding to the genus-level of all nine pathogens monitored in the local clinical antibiotic surveillance could be detected in the effluent and sometimes even increased in relative abundance. *Escherichia* stood out in this study both as having the most ARG connections on assembled contigs in common with local antibiogram data and tending to increase in relative abundance in the effluent. Extended-spectrum beta-lactamase producing *E. coli* are regularly isolated from treated wastewater effluent globally and are being considered by the World Health Organization (2021) and others as a standardized monitoring target for antibiotic resistance in wastewater (Diallo et al., 2013; Bréchet et al., 2014; Li et al., 2019; Marano et al., 2020).

There did not appear to be distinct grouping of influent resistomes by season (winter, spring, summer, fall) or grouped seasons (winter/spring, summer/fall) based on ARG profiles. This finding is consistent with no observed seasonality in the monthly sampling of activated sludge at a Hong Kong WWTP over 9 years, although the authors hypothesized that this was due to minimal local shifts in temperature (Yin et al., 2019). The distinct separation of influent from activated sludge, secondary effluent, and final effluent in the NMDS plots is consistent with the trends in mobile ARGs and plasmid replicons observed among three sampling events over 6 months at the Portuguese WWTP noted above (Lira et al., 2020), consistent with the above observation of relatively stable resistome in the influent that subsequently shifts and increases in variability in the effluent. Interestingly, it was noted that the activated sludge resistome shifted in composition every 2–3 years at the Hong Kong WWTP (Yin et al., 2019), a turnover period that would not have been captured over the duration of the present study. Still, the fact that the effluent resistome was generally more variable over the period of this study than the influent resistome suggests that there may have been some variability in the activated sludge resistome of the prior treatment stage as well (although it was only sequenced on two sampling dates).

While the microbiome was also distinct in composition for each stage of treatment, its stability mirrored that of the resistome throughout the 18-month sampling period. The dominance of *Arcobacter* and *Acinetobacter* in the influent was not unlike a previous metagenomic study comparing the resistome and microbiome of coastal beach versus sewage waters in Uruguay (Fresia et al., 2019). In contrast, *Pseudomonas* was the most abundant genera in the sewage waters in this study, whereas *Pseudomonas* was one of the lesser dominant genera in the top 20 most abundant. *Enhydrobacter* was not a discussed in the Uruguayan study. Although the relative abundances of *Arcobacter* and *Acinetobacter* decreased from the influent through activated sludge, their average abundances remained similar to the core OTUs found in activated sludge compartments globally (Wu et al., 2019): *Arcobacter* (0.28%, versus 0.43% in global study) and *Acinetobacter* (1.07%, versus 0.22% in global study). Of the more dominant genera found from activated sludge onward, only *Nitrospira* was present as a core global OTU (Wu et al., 2019). Fresia et al. (2019) also utilized MetaPhlAn2 for taxonomic annotation, while Wu et al. (2019) relied on 16S rRNA amplicon sequencing.

Total measured antibiotic load also decreased by ∼40–50%, by the same order of magnitude of total ARG relative abundance. Otherwise, the fate of antibiotics was not predictive of the fate of respective ARGs. For example, MLS antibiotics, especially azithromycin, were the most persistent among the measured antibiotic classes and sometimes even increased in concentration from influent to final effluent. MLS ARGs, on the other hand, consistently decreased from influent to secondary effluent. Even for antibiotic concentrations greater than the PNEC, in most cases there were not significant correlations with ARGs. For example, the concentration of ciprofloxacin was greater than the PNEC in seven sampling events, yet no significant correlations with corresponding quinolone ARGs were observed. We acknowledge that difficulty detecting clinically relevant quinolone ARGs, due to exclusion of resistance due to point mutation (which is often the case for clinically relevant quinolone ARGs) in the metagenomic analysis, could have influenced this analysis. One limitation of the current study is difficulty in detecting beta-lactams, which represented a large portion of the discriminatory resistome and Category 4 ARGs. However, beta-lactams degrade rapidly under environmental conditions and few studies have reported their detection in WWTPs (Singh et al., 2019). Generally, other studies have similarly noted lack of correlation between antibiotic residues and ARG abundance (Bengtsson-Palme et al., 2016; Hendriksen et al., 2019; Pärnänen et al., 2019; Riquelme et al., in preparation). Thus, growing consensus among several studies supports the overall conclusion that selection pressures for carriage of ARGs across the microbial community are generally diminished through the WWTP. This is consistent with the reduced total ARG relative abundance from influent to effluent consistently observed across this study. Still, monitoring antibiotics can be informative to gain insight into which antibiotics are being used in a given community, which is difficult to ascertain from publicly available data (Morgan et al., 2011; Auta et al., 2019), is also important surveillance information.

CONCLUSION

Based on this intense 18-month study of a conventional WWTP and comparison to locally available clinical resistance information, metagenomic analysis was found to yield rich information about resistomes and associated microbiomes that can be mined to inform effective strategies for antibiotic resistance surveillance. In terms of metrics that are worthy of consideration for future monitoring efforts, both core and discriminatory resistome analysis revealed several ARGs of clinical concern, while MetaCompare analysis provided a comprehensive metric for relative comparison of the degree to which ARGs are predicted to be mobile and carried in pathogens (i.e., resistome risk).

Overall, it was observed that most antibiotic resistance indicators of concern decreased during wastewater treatment, including: measured antibiotics (although some macrolides increased), relative abundance of total ARGs, absolute abundance of total ARGs, and resistome risk. Together with Category 1 and Category 3 ARGs, which are either completely removed or decrease during treatment, such metrics could routinely be monitored to verify that a given WWTP is performing according to baseline. On the other hand, monitoring Category 2 ARGs, which tend to increase during treatment, could be informative for optimizing WWTP for maximal attenuation of ARGs. Further, this study provided insight into the behavior of specific ARGs of clinical relevance. Clinically relevant ARGs that are also discriminatory (e.g., *bla*OXA) could be prioritized for surveillance, either through metagenomics or qPCR-based methods.

Remarkably, it was observed that linked ARG-MGE-taxonomic information found on assembled contigs in the WWTP effluent were more similar to antibiotic resistant pathogens identified to be of local concern, relative to those found on contigs from the influent sewage. These results suggest that monitoring WWTP effluent can also provide valuable surveillance information, as has recently been proposed for WWTP influents. The fact that effluent resistomes were also more variable than influent resistomes further suggests that more frequent monitoring of effluents may be warranted than for influents and could serve to identify important upsets or anomaly events. Effluent is also valuable to monitor given that it also represents the worst case of what can be discharged to the environment and result in exposure, either through recreation in receiving waters or water reuse. Based on the findings of this study, we emphasize the following specific considerations for metagenomic-based surveillance of antibiotic resistance in WWTPs:

- Metagenomic-based surveillance of antibiotic resistance can be economized by less frequent sampling of sewage influents if the purpose is global comparison.
- Metagenomic surveillance of effluents is a promising approach to assessing both efficacy of WWTP for mitigation, but also for assessing the local clinical antibiotic resistance condition.

- Core and discriminatory resistomes can be compared within and among WWTPs with time to identify similarities and differences. About 50% reduction in relative abundance of total ARGs was achieved across the WWTP and several specific ARG types were consistently observed to be removed or to increase. Diversity of ARGs consistently decreased. Such measures could be used to benchmark WWTP performance and assess potential operational deficiencies or upsets.
- Core and discriminatory ARG analysis comparing influent and secondary effluent identified a collection of "indicator" ARGs that are also clinically relevant; such as *bla*OXA, *bla*GES, *mph*D, *msr*E, and *qnr*S, that should be further considered as targets for antibiotic resistance monitoring in WWTPs.
- Metagenomic analysis of co-occurrences of ARGs, MGEs, and pathogen gene markers was notably consistent with patterns in resistance of locally available *E. coli* monitoring data, suggesting value in coordinating with recent initiatives to target *E. coli* for antibiotic resistance surveillance of water environments. *S. aureus* harboring MLS ARGs also emerged as a target worth exploring in future research.
- While antibiotics generally tended to decrease across the WWTP, they were not generally predictive of patterns observed in corresponding ARG increase or removal.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the NCBI Short Read Archive (SRA) under the BioProject ID PRJNA683044.

AUTHOR CONTRIBUTIONS

HM led the writing of this manuscript, along with AP, MR, BD, and PV. HM, MR, PV, and AP contributed to the experimental design. HM, AP, BD, SG, and EG contributed to the rationale of the metagenomic analysis approach. HM, BD, and SG conducted sampling at the WWTP. DA and LA carried out the antibiotic analysis. All authors read and commented on the manuscript.

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SUPPLEMENTARY MATERIAL

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REFERENCES

Aarestrup, F. M., and Woolhouse, M. E. J. (2020). Using sewage for surveillance of antimicrobial resistance. *Science* 367, 630–632. doi: 10.1126/science.aba3432

Arango-Argoty, G., Singh, G., Heath, L. S., Pruden, A., Xiao, W., and Zhang, L. (2016). MetaStorm: a public resource for customizable metagenomics annotation. *PLoS One* 11:e0162442. doi: 10.1371/journal.pone.0162442

Arango-Argoty, G. A., Dai, D., Pruden, A., Vikesland, P., Heath, L. S., and Zhang, L. (2019). NanoARG: a web service for detecting and contextualizing antimicrobial resistance genes from nanopore-derived metagenomes. *Microbiome* 7:88. doi: 10.1186/s40168-019-0703-9

Auta, A., Hadi, M. A., Oga, E., Adewuyi, E. O., Abdu-Aguye, S. N., Adeloye, D., et al. (2019). Global access to antibiotics without prescription in community pharmacies: a systematic review and meta-analysis. *J. Infect.* 78, 8–18. doi: 10.1016/j.jinf.2018.07.001

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

Bengtsson-Palme, J., Hammarén, R., Pal, C., Östman, M., Björlenius, B., Flach, C.-F., et al. (2016). Elucidating selection processes for antibiotic resistance in sewage treatment plants using metagenomics. *Sci. Total Environ.* 572, 697–712. doi: 10.1016/j.scitotenv.2016.06.228

Bengtsson-Palme, J., and Larsson, D. G. J. (2016). Concentrations of antibiotics predicted to select for resistant bacteria: proposed limits for environmental regulation. *Environ. Int.* 86, 140–149. doi: 10.1016/j.envint.2015.10.015

Bengtsson-Palme, J., Larsson, D. G. J., and Kristiansson, E. (2017). Using metagenomics to investigate human and environmental resistomes. *J. Antimicrob. Chemother.* 72, 2690–2703. doi: 10.1093/jac/dkx199

Bibbal, D., Dupouy, V., Ferre, J. P., Toutain, P. L., Fayet, O., Prere, M. F., et al. (2007). Impact of three ampicillin dosage regimens on selection of ampicillin resistance in *Enterobacteriaceae* and excretion of bla(TEM) genes in swine feces. *Appl. Environ. Microbiol.* 73, 4785–4790. doi: 10.1128/AEM.00252-07

Bréchet, C., Plantin, J., Sauget, M., Thouverez, M., Talon, D., Cholley, P., et al. (2014). Wastewater treatment plants release large amounts of extended-spectrum β-lactamase–producing *Escherichia coli* into the environment. *Clin. Infect. Dis.* 58, 1658–1665. doi: 10.1093/cid/ciu190

Buchfink, B., Xie, C., and Huson, D. H. (2015). Fast and sensitive protein alignment using DIAMOND. *Nat. Methods* 12, 59–60. doi: 10.1038/nmeth.3176

Bürgmann, H., Frigon, D., Gaze, W., Manaia, C., Pruden, A., Singer, A. C., et al. (2018). Water & sanitation: an essential battlefront in the war on antimicrobial resistance. *FEMS Microb. Ecol.* 95:fiy101. doi: 10.1093/femsec/fiy101

Chen, J., Zhongtang, Y., Michel, F. C. Jr., Wittum, T., and Morrison, M. (2007). Development and application of real-time PCR assays for quantification of erm genes conferring resistance to macrolides-lincosamides-streptogramin b in livestock manure and manure management systems. *Appl. Environ. Microbiol.* 73, 4405–4416. doi: 10.1128/AEM.02799-06

Diallo, A. A., Brugère, H., Kérourédan, M., Dupouy, V., Toutain, P.-L., Bousquet-Mélou, A., et al. (2013). Persistence and prevalence of pathogenic and extended-spectrum beta-lactamase-producing *Escherichia coli* in municipal wastewater

treatment plant receiving slaughterhouse wastewater. *Water Res.* 47, 4719–4729. doi: 10.1016/j.watres.2013.04.047

Dutka-Malen, S., Evers, S., and Courvalin, P. (1995). Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. *J. Clin. Microbiol.* 33, 24–27. doi: 10.1128/JCM.33.1.24-27.1995

Fresia, P., Antelo, V., Salazar, C., Gimeìnez, M., D'Alessandro, B., Afshinnekoo, E., et al. (2019). Urban metagenomics uncover antibiotic resistance reservoirs in coastal beach and sewage waters. *Microbiome* 7:35. doi: 10.1186/s40168-019-0648-z

Gao, P., Munir, M., and Xagoraraki, I. (2012). Correlation of tetracycline and sulfonamide antibiotics with corresponding resistance genes and resistant bacteria in a conventional municipal wastewater treatment plant. *Sci. Total Environ.* 421-422, 171–173. doi: 10.1016/j.scitotenv.2012.01.061

Garner, E., Chen, C., Xia, K., Bowers, J., Engelthaler, D. M., McLain, J., et al. (2018). Metagenomic characterization of antibiotic resistance genes in full-scale reclaimed water distribution systems and corresponding potable systems. *Environ. Sci. Technol.* 52, 6113–6125. doi: 10.1021/acs.est.7b05419

Gu, Z., Gu, L., Eils, R., Schlesner, M., and Brors, B. (2014). Circlize implements and enhances circular visualization in R. *Bioinformatics* 30, 2811–2812. doi: 10.1093/bioinformatics/btu393

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

Gupta, S., Arango-Argoty, G., Zhang, L., Pruden, A., and Vikesland, P. J. (2019). Identification of discriminatory antibiotic resistance genes among environmental resistomes using extremely randomized tree algorithm. *Microbiome* 7:123. doi: 10.1186/s40168-019-0735-1

Hardwick, S. A., Stokes, H. W., Findlay, S., Taylor, M., and Gillings, M. R. (2008). Quantification of class 1 integron abundance in natural environments using real-time quantitative PCR. *FEMS Microbiol. Lett.* 278, 207–212.

Hendriksen, R. S., Munk, P., Njage, P., van Bunnik, B., McNally, L., Lukjancenko, O., et al. (2019). Global monitoring of antimicrobial resistance based on metagenomics analyses of urban sewage. *Nat. Commun.* 10:1124. doi: 10.1038/s41467-019-08853-3

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

Hyatt, D., Chen, G. -L., LoCascio, P. F., Land, M. L., Larimer, F. W., and Hauser, L. J. (2010).Prodigal: prokaryotic gene recognition and translation initiation site identification. *BMC Bioinform.* 11:119. doi: 10.1186/1471-2105-11-119

Jia, B., Raphenya, A. R., Alcock, B., Waglechner, N., Guo, P., Tsang, K. K., et al. (2017). CARD 2017: expansion and model-centric curation of the comprehensive antibiotic resistance database. *Nucleic Acids Res.* 45, D566–D573. doi: 10.1093/nar/gkw1004

Jonsson, V., Österlund, T., Nerman, O., and Kristiansson, E. (2016). Statistical evaluation of methods for identification of differentially abundant genes in comparative metagenomics. *BMC Genomics* 17:78. doi: 10.1186/s12864-016-2386-y

Joseph, S. M., Battaglia, T., Maritz, J. M., Carlton, J. M., and Blaser, M. J. (2019). Longitudinal comparison of bacterial diversity and antibiotic resistance genes in New York City sewage. *mSystems* 4:e00327-19. doi: 10.1128/mSystems.00327-19

Ju, F., Beck, K., Yin, X., Maccagnan, A., McArdell, C. S., Singer, H. P., et al. (2019). Wastewater treatment plant resistomes are shaped by bacterial composition, genetic exchange, and upregulated expression in the effluent microbiomes. *ISME J.* 13, 346–360. doi: 10.1038/s41396-018-0277-8

Kim, S., Yun, Z., Ha, U.-H., Lee, S., Park, H., Kwon, E. E., et al. (2014). Transfer of antibiotic resistance plasmids in pure and activated sludge cultures in the presence of environmentally representative micro-contaminant concentrations. *Sci. Total Environ.* 468–469, 813–820. doi: 10.1016/j.scitotenv.2013.08.100

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

Li, Q., Chang, W., Zhang, H., Hu, D., and Wang, X. (2019). The role of plasmids in the multiple antibiotic resistance transfer in ESBLs-producing *Escherichia coli* isolated from wastewater treatment plants. *Front. Microbiol.* 10:633. doi: 10.3389/fmicb.2019.00633

Lira, F., Vaz-Moreira, I., Tamames, J., Manaia, C. M., and Martines, J. L. (2020). Metagenomic analysis of an urban resistome before and after wastewater treatment. *Sci. Rep.* 10:8174. doi: 10.1038/s41598-020-65031-y

Manaia, C. M., Rocha, J., Scaccia, N., Marano, R., Radu, E., Biancullo, F., et al. (2018). Antibiotic resistance in wastewater treatment plants: tackling the black box. *Environ. Int.* 115, 312–324. doi: 10.1016/j.envint.2018.03.044

Mao, D., Yu, S., Rysz, M., Luo, Y., Yang, F., Li, F., et al. (2015). Prevalence and proliferation of antibiotic resistance genes in two municipal wastewater treatment plants. *Water Res.* 85, 458–466. doi: 10.1016/j.watres.2015.09.010

Marano, R., Fernandes, T., Manaia, C. M., Nunes, O., Morrison, D., Berendonk, T. U., et al. (2020). A global multinational survey of cefotaxime-resistant coliforms in urban wastewater treatment plants. *Environ. Int.* 144:106035. doi: 10.1016/j.envint.2020.106035

Martínez, J., Coque, T., and Baquero, F. (2015). What is a resistance gene? Ranking risk in resistomes. *Nat. Rev. Microbiol.* 13, 116–123. doi: 10.1038/nrmicro3399

Morgan, D. J., Okeke, I. N., Laxminarayan, R., Perencevich, E. N., and Weisenberg, S. (2011). Non-prescription antimicrobial use worldwide: a systematic review. *Lancet Infect. Dis.* 11, 692–701. doi: 10.1016/S1473-3099(11)70054-8

Neuwirth, E. (2014). *RColorBrewer: ColorBrewer Palettes. R Package Version 1.1-2.*

Oh, M., Pruden, A., Chen, C., Heath, L. S., Xia, K., and Zhang, L. (2018). MetaCompare: a computational pipeline for prioritizing environmental resistome risk. *FEMS Microbiol. Ecol.* 94:fiy079. doi: 10.1093/femsec/fiy079

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., et al. (2019). *vegan: Community Ecology Package. R package version 2.5-6.*

Pärnänen, K. M. M., Narciso-da-Rocha, C., Kneis, D., and Berendonk, T. U. (2019). Antibiotic resistance in European wastewater treatment plants mirrors the pattern of clinical antibiotic resistance prevalence. *Sci. Adv.* 5:eaau9124. doi: 10.1126/sciadv.aau9124

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

Peng, Y., Leung, H. C., Yiu, S. M., and Chin, F. Y. (2012). IDBA-UD: a de novo assembler for single-cell and metagenomic sequencing data with highly uneven depth. *Bioinformatics* 28, 1420–1428. doi: 10.1093/bioinformatics/bts174

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

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

Singh, R. R., Angeles, L. F., Butryn, D. M., Metch, J. W., Garner, E., Vikesland, P. J., et al. (2019). Towards a harmonized method for the global reconnaissance of multi-class antimicrobials and other pharmaceuticals in wastewater and receiving surface waters. *Environ. Int.* 124, 361–369. doi: 10.1016/j.envint.2019.01.025

Suzuki, M. T., Taylor, L. T., and DeLong, E. F. (2000). Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 5′- nuclease assays. *Appl. Environ. Microbiol.* 66, 4605–4614. doi: 10.1128/AEM.66.11.4605-4614.2000

Truong, D. T., Franzosa, E. A., Tickle, T. L., Scholz, M., Weingart, G., Pasolli, E., et al. (2015). MetaPhlAn2 for enhanced metagenomic taxonomic profiling. *Nat. Methods* 12, 902–903. doi: 10.1038/nmeth.3589

Virginia Department of Health (2017). *Data from: 2017 Virginia State and Regional Cumulative Antibiogram*. Richmond, VI: Virginia Department of Health.

Wickham, H. (2016). *ggplot2: Elegant Graphics for Data Analysis*. New York, NY: Springer-Verlag New York.

Woegerbauer, M., Bellanger, X., and Merlin, C. (2020). Cell-free DNA: an underestimated source of antibiotic resistance gene dissemination at the interface between human activities and downstream environments in the context of wastewater reuse. *Front. Microbiol.* 11:671.

Wood, D. E., and Salzberg, S. L. (2014). Kraken: ultrafast metagenomic sequence classification using exact alignments. *Genome Biol.* 15:R46. doi: 10.1186/gb-2014-15-3-r46

World Health Organization (2021). *WHO Integrated Global Surveillance on ESBL-Producing E. coli Using a "One Health" Approach: Implementation and Opportunities. Licence: CC BY-NC-SA 3.0 IGO*. Geneva: World Health Organization.

Wright, G. D. (2007). The antibiotic resistome: the nexus of chemical and genetic diversity. *Nat. Rev. Microbiol.* 5, 175–186. doi: 10.1038/nrmicro1614

Wu, L., Ning, D., Zhang, B., Li, Y., Zhang, P., Shan, X., et al. (2019). Global diversity and biogeography of bacterial communities in wastewater treatment plants. *Nat. Microbiol.* 4, 1183–1195. doi: 10.1038/s41564-019-0426-5

Yang, Y., Li, B., Zou, S., Fang, H. H. P., and Zhang, T. (2014). Fate of antibiotic resistance genes in sewage treatment plant revealed by metagenomic approach. *Water Res.* 62, 97–106. doi: 10.1016/j.watres.2014.05.019

Yin, X., Deng, Y., Ma, L., Wang, Y., Chan, L. Y. L., and Zhang, T. (2019). Exploration of the antibiotic resistome in a wastewater treatment plant by a nine-year longitudinal metagenomic study. *Environ. Int.* 133:105270. doi: 10.1016/j.envint.2019.105270

Zhang, T., Zhang, X. X., and Ye, L. (2011). Plasmid metagenome reveals high levels of antibiotic resistance genes and mobile genetic elements in activated sludge. *PLoS One* 6:e26041. doi: 10.1371/journal.pone.0026041

Zhang, Y. L., Marrs, C. F., Simon, C., and Xi, C. W. (2009). Wastewater treatment contributes to selective increase of antibiotic resistance among *Acinetobacter* spp. *Sci. Total Environ.* 407, 3702–3706. doi: 10.1016/j.scitotenv.2009.02.013

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Prevalence of *Escherichia coli* and Antibiotic-Resistant Bacteria During Fresh Produce Production (Romaine Lettuce) Using Municipal Wastewater Effluents

*Harvey N. Summerlin III[1], Cícero C. Pola[2], Eric S. McLamore[3], Terry Gentry[4], Raghupathy Karthikeyan[3] and Carmen L. Gomes[1,2]**

[1]Department of Biological and Agricultural Engineering, Texas A&M University, College Station, TX, United States, [2]Department of Mechanical Engineering, Iowa State University, Ames, IA, United States, [3]Department of Agricultural Sciences, Clemson University, Clemson, SC, United States, [4]Department of Soil and Crop Sciences, Texas A&M University, College Station, TX, United States

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***Correspondence:**
Carmen L. Gomes
carmen@iastate.edu

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High demand for food and water encourages the exploration of new water reuse programs, including treated municipal wastewater usage. However, these sources could contain high contaminant levels posing risks to public health. The objective of this study was to grow and irrigate a leafy green (romaine lettuce) with treated wastewater from a municipal wastewater treatment plant to track *Escherichia coli* and antibiotic-resistant microorganisms through cultivation and post-harvest storage to assess their fate and prevalence. Contamination levels found in the foliage, leachate, and soil were directly ($p < 0.05$) related to *E. coli* concentrations in the irrigation water. Wastewater concentrations from 177 to 423 CFU ml^{-1} resulted in 15–25% retention in the foliage. Leachate and soil presented means of 231 and 116% retention, respectively. *E. coli* accumulation on the foliage was observed ($p < 0.05$) and increased by over 400% during 14-day storage (4°C). From randomly selected *E. coli* colonies, in all four biomass types, 81 and 34% showed resistance to ampicillin and cephalothin, respectively. Reclaimed wastewater usage for leafy greens cultivation could pose potential health risks, especially considering the bacteria found have a high probability of being antibiotic resistance. Successful reuse of wastewater in agriculture will depend on appropriate mitigation and management strategies to guarantee an inexpensive, efficient, and safe water supply.

Keywords: wastewater, produce irrigation, water reuse, fecal coliforms prevalence, antibiotic-resistant bacteria, food safety

INTRODUCTION

As world population and demand for food increase, safe water for agricultural use has become increasingly scarce. The water footprint of humanity is estimated at 9,087 km^3 year^{-1}, of which agriculture accounts for 92% (Hoekstra and Mekonnen, 2012). In some areas, surface water is not readily available, and other options, such as drilling a well, are not cost-effective.

Non-traditional water supplies, such as treated municipal wastewater for irrigation, have the potential to meet increasing water demands and conserve current potable supplies; however, wastewaters often have microbial and chemical contaminants that may affect public health and/or environmental quality. Wastewater treatment (WWT) strategies and advanced irrigation systems may limit exposure of crops, animals, humans, and groundwater to contaminants. Several irrigation practices, such as drip, flood, and subsurface irrigation techniques used with treated wastewater, have been reported to mitigate the risk of contamination (Solomon et al., 2002; Pavione et al., 2013). However, due to the morphology of certain plants, such as lettuce or spinach, commercial-scale production requires canopy (or spray) irrigation. This irrigation process involves water coming into direct contact with the edible foliage, which poses a higher risk of contamination (Robinson, 2002).

A review by De Keuckelaere et al. (2015) reports that there are few site-specific data points available for risk assessment related to use of water and food safety of fresh produce. Specific parameters lacking hard data include rate of pathogen transfer from irrigation water to crops, and pathogen fate, transport, and survival in or on food crops. Furthermore, precise information regarding fecal coliforms, pathogens, and antibiotic-resistant bacteria (ARB) accumulation during and after harvest and their potential effect on future crops along with risks posed to human health are scarce. Therefore, in order to create adequate risk management practices and guidelines for wastewater irrigation in agriculture, site-specific studies of bacteria motility and accumulation are critical, as it is becoming an increasingly popular alternative.

Nowadays, consumption of fresh produce is on the rise, due to its associated health and nutritional benefits. At the same time, fresh produce is one of the leading causes of foodborne illnesses (Rai and Tripathi, 2007) with 377 outbreaks reported by the United States Centers for Disease Control and Prevention (CDC) from 2004 to 2012 (Callejon et al., 2015). Foodborne illnesses can emerge from poor water quality used during fresh produce production. For instance, a multistate outbreak of *E. coli* O157:H7 infections linked to romaine lettuce that infected 210 people from 36 states and caused five deaths indicated that the source of *E. coli* O157:H7 was likely from the canal water (Yuma growing region) used to irrigate the romaine lettuce (CDC, 2018). Moreover, the overuse of antibiotics can be directly related to the occurrence and propagation of ARB, which have been increasing rapidly over the past decades (Edberg et al., 2000; WHO, 2006; Bitton, 2010). The ARB issue has been identified by many global public health entities including the World Health Organization and CDC as a critical concern (Bitsch et al., 2014).

Several studies have examined the effects and risks of using wastewater effluents to irrigate fresh produce such as lettuce, spinach, rocket, and tomato (Assadian et al., 2005; Ribera and McCorkle, 2012; De Keuckelaere et al., 2015). Throughout these studies, multiple factors were tested to observe their effects on the prevalence of fecal indicator bacteria (FIB), which were used to estimate the levels of harmful pathogens for risk assessment (Mena and Pillai, 2008; Alam et al., 2014). Additionally, these previous studies reported on the levels of crop contamination; however, these studies did not show how the entire system of foliage, soil, and leachate is affected over time when using wastewater irrigation.

Reclaimed wastewater in agriculture has the potential to provide alternative irrigation and nutrient sources in water-scarce regions and consequently promote water conservation. However, to develop mitigation plans to protect public health, site-specific evaluation of bacteria movement and persistence is needed. In this study, lettuce was irrigated with secondary treated wastewater to track the fate and prevalence of *E. coli* and ARB throughout the entire system (foliage, soil, and leachate) during cultivation and post-harvest storage.

MATERIALS AND METHODS

Escherichia coli Monitoring in Fresh Produce Materials

Wastewater

Wastewater was obtained weekly from the Texas A&M WWT Plant, College Station, TX, United States. The wastewater was collected after solids removal and secondary clarification processes. Three liters were collected using a beaker affixed to a pole and placed into sterile plastic jugs for transport to the laboratory. A sample (10 ml) of the wastewater was reserved for further analysis (described below).

Leafy Greens

Twelve young 15-cm romaine lettuce plants (*Lactuca sativa* var. longifolia, Bonnie Plants, Union Springs, AL, United States) were purchased from a local nursery. The plants were placed into 20-cm diameter plastic pots and filled with EcoScraps moisture retaining potting soil (EcoScraps Co., South Jordan, UT), leaving a 2-cm lip to the top. The potting soil was sterilized in an autoclave for 90 min at 121°C and analyzed by the Texas A&M Department of Soil and Crop Sciences Laboratory (College Station, TX) generating the following results: pH: 7.2, nitrate: 0 ppm, phosphorus: 95 ppm, potassium: 441 ppm, moisture content: 4.44%, soil composition with sand: 91.2%, clay: 2.6%, silt: 6.1%, and total solids: 55.63%. A suggested supplement of nitrogen was applied in the amount of 0.68 g cm^{-2}.

Lettuce plants were transplanted and grown using sterile reverse osmosis (RO) water for 14 days prior to the irrigation experiment. Each row of six plants was grown under two 2-Light T12 fluorescent shop lights (Lithonia Lighting, Conyers, GA) containing four 1.22 m 40-watt fluorescent tube light bulbs (General Electric, Fairfield, CT). The bulbs provided 2,900 lumens each and consisted of two 6,500 K and two 3,000 K color temperature bulbs to resemble natural daylight. The lighting fixtures were plugged into a wall outlet timer that allowed 14 h of continuous light located 15 cm above the plants. Temperature (23 ± 2 C) and relative humidity (55 ± 4%) were kept constant throughout the experiment.

Inoculation

Once a week, for 3 weeks, approximately 15 ml of wastewater was applied directly to each plant's foliage using a 150-ml sterile spray bottle (Apothecary Products, Inc., Minneapolis, MN) with the nozzle on mist position, thoroughly covering each side of all leaves from a 15-cm distance. Then, 150 ml of the wastewater was poured into each plant's pot, completely soaking the soil following good agricultural practice recommendations. These irrigation methods were applied to mimic sprinkler and furrow irrigation, which are typically used for leafy greens cultivation (Masabni et al., 2009). Plants were also supplemented with 50 ml of RO water each day for the rest of the week to prevent drying out and wilting. The experimental design is summarized in **Figure 1**. All procedures were performed inside a biosafety cabinet.

Sample Collection and Analysis

A 10-ml sample of the wastewater was taken and placed into a sterile conical centrifuge tube (VWR International, Radnor, PA). After wastewater irrigation, 10 ml of leachate from each plant was immediately collected from the pot saucer. Foliage samples were collected 1 h after irrigation, by cutting the outermost leaves from their stems. Leaf blades were removed from the vein and cut into 2.5-cm strips. From each plant, 5 g of foliage was stored into sterile Whirl-Pak® bags (eNasco, Fort Atkinson, WI). Then, 10 ml of buffered peptone water (BPW, Becton, Dickinson and Company, Franklin Lakes, NJ, United States) was added to each foliage sample to create a 1:2 ratio of foliage to buffer suspension, and the bags were homogenized for 2 min. Post-harvest foliage samples were collected at the same time as the pre-harvest day 14 samples and stored at 4°C in sterile plastic bags (low-density polyethylene, Whirl-Pak® bags) for 7–14 days when BPW was then added and samples were processed accordingly. Dry weight for foliage samples was obtained by moisture content measurement following AOAC method 930.04 (AOAC, 1990).

Soil samples were collected 6 h after irrigation to allow adequate drainage. From each pot, a sterile 2-cm-diameter core tube was inserted 5 cm deep to collect 2 g of soil. Then, 8 ml of BPW was added to create a 1:4 ratio of soil to buffer suspension. The collected samples were then vortexed for 30 s to homogenize the contents. Subsequently, these samples were allowed to settle for 10 min to separate the soil from buffer.

Aliquots (0.1 ml) of the samples were then plated on MacConkey Agar (Hardy Diagnostics, Santa Maria, CA) using the spread plating method. Several serial dilutions of all samples were plated to ensure samples were within the limits of detection. Plates were incubated overnight at 35°C. Two plates per dilution of each sample were plated, counted, and reported in either CFU g^{-1} or CFU ml^{-1} of sample (USFDA, 1998).

Antibiotic-Resistant Bacteria

Ten *E. coli* colonies from each of the four materials collected (i.e., wastewater, soil, leachate, and foliage) for a total of 40 *E. coli* isolates were randomly selected per pre-harvest sampling time, and similarly, 10 *E. coli* colonies from foliage per post-harvest sampling time. Individual colonies were collected, suspended in BPW, streaked on Tryptic soy agar (TSA, Becton, Dickinson and Company, Franklin Lakes, NJ, United States), and incubated overnight at 35°C in accordance with the Kirby-Bauer method (USFDA, 1998). Next, *E. coli* cell suspensions were then prepared by placing two isolates into tubes containing 5 ml of Tryptic soy broth (TSB, Becton, Dickinson and Company, Franklin Lakes, NJ, United States)

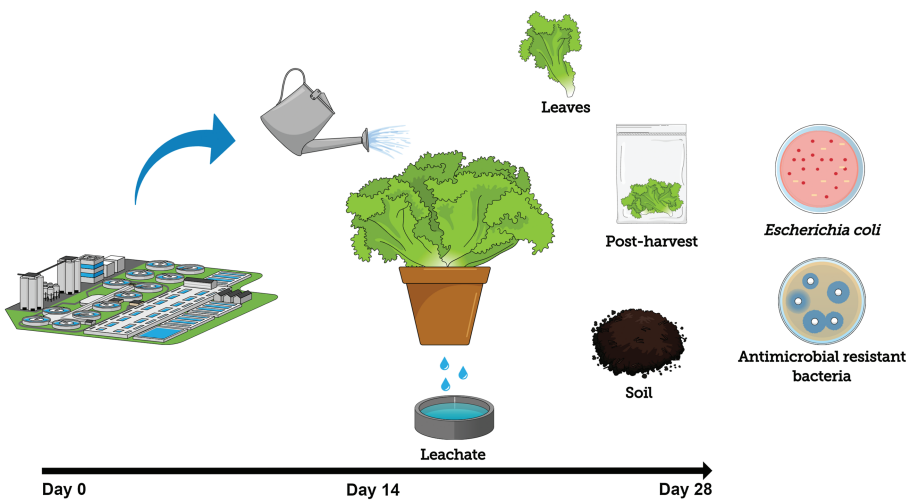

FIGURE 1 | Experimental design schematic showing lettuce cultivation using secondary-treated wastewater as irrigation source to track the fate and prevalence of *Escherichia coli* and antibiotic-resistant bacteria (ARB) throughout the entire system (foliage, soil, and leachate) during cultivation (14-day period) and post-harvest storage at 4°C (14-day period).

and incubating at 35°C for 3 h while shaking at 150 rpm in a water bath (VWR International). Tubes were checked for appropriate turbidity with 0.5 McFarland standard, which corresponds to a 10^8 CFU ml^{-1} bacterial cell count (USFDA, 1998).

Escherichia coli suspensions were then re-streaked onto Muller Hinton Agar (MHA, Becton, Dickinson and Company, Franklin Lakes, NJ, United States) plates. Then, antibiotic resistance of the colonies was determined by the Kirby-Bauer method for antibiotic susceptibility. Eight antibiotic susceptibility disks (Becton, Dickinson and Company, Franklin Lakes, NJ) of ampicillin (10 μg), cefoperazone (75 μg), cephalothin (30 μg), ciprofloxacin (5 μg), gentamicin (120 μg), imipenem (10 μg), sulfamethoxazole/trimethoprim (23.75/1.25 μg), and tetracycline (30 μg) were stamped onto each MHA plate using a BBL® Sensi-Disc® 8-place Dispenser (Becton, Dickinson and Company, Franklin Lakes, NJ, United States). The stamped MHA plates were incubated for 24 h at 35°C. Then, the zones of inhibition (ZOI) were measured to determine resistance, intermediate resistance, or susceptibility to each antibiotic, according to the Clinical and Laboratory Standards Institute standards (Bauer et al., 1966).

Data Analysis

All experiments were performed in triplicate as independent experiments, and the results are expressed as mean ± standard deviation. Differences among variables were tested using one-way ANOVA with a significance level of 5%, and significantly, different means were separated by the Tukey HSD test. All data were analyzed using JMP®Pro statistical software (SAS, Cary, NC 27513). Due to the rain event prior to day 14, large input (wastewater) variability of *E. coli* contamination levels in foliage, soil, and leachate was normalized by transforming CFU ml^{-1} to percentage of *E. coli* retention. Response material (i.e., soil, leachate, and foliage) *E. coli* concentration (CFU ml^{-1}) was divided by wastewater *E. coli* concentration (CFU ml^{-1}) to yield retention as a percentage. Beginning with pre-harvest data, statistical analysis was performed using a Tukey HSD *post-hoc* test to compare *E. coli* prevalence over time among all materials (wastewater, leachate, foliage, and soil). Then, differences in means were analyzed among materials for each sampling time. Finally, post-harvest foliage samples were analyzed to detect mean differences over storage time.

Antibiotic-resistant bacteria samples were analyzed, with focus on the three most common resistance patterns observed from ARB analysis: ampicillin (10 μg), cephalothin (30 μg), and ciprofloxacin (5 μg). In this study, intermediate resistant and resistant bacteria were combined and expressed as "resistant" to simplify the results. Resistance among samples was expressed as a percentage of each sampling population. ARB results were compared over time among all materials. Then, mean differences were analyzed among materials for each sampling time. All analyses were performed by using Tukey HSD *post-hoc* test to separate differences in means and Levene's test to test for homoscedasticity.

RESULTS AND DISCUSSION

Fresh Produce Materials and *E. coli* Monitoring

The concentrations of *E. coli* present in the irrigation water varied and were recorded in log CFU ml^{-1} as 2.3 ± 0.0, 2.6 ± 0.2, and 5.1 ± 0.1 log CFU ml^{-1} for days 0, 7, and 14, respectively. There was a large rain event of 32.8 mm (Weather Underground, 2016) in the Bryan/College Station (Texas) area on November 6, 2016, 1 day prior to the collection of the day 14 sample. It was also observed that the WWT plant was not operating at full effectiveness because of a failure in the aeration system, which provides oxygen to microorganisms in the solids removal tank. The WWT plant reported an *E. coli* concentration of 2.5 ± 0.6 log CFU 100 ml^{-1} in UV-treated effluent on day 14 and an average of 2.6 ± 2.0 log CFU 100 ml^{-1} in the 4 days following the rain event and system failure. These are significantly higher concentrations than the average for the rest of the month which was 1.0 ± 0.7 log CFU 100 ml^{-1}, which consequently affected the results by introducing very large concentrations of *E. coli* that were significantly different ($p < 0.05$) than the previous two irrigations. The EPA standard for final effluent discharge is a geometric mean of 2.1 log CFU 100 ml^{-1} (USEPA, 2017).

For irrigation water, including alternative water sources, the *E. coli* concentration must not exceed 126 CFU 100 ml^{-1} (geometric mean or 2.10 log CFU 100 ml^{-1}) without triggering a responsive action (FDA, 2017; EC, 2020; EPA, 2021). This study was carried out with an initial *E. coli* concentration that exceeded regulatory requirements in order to track the fate and prevalence of *E. coli* and ARB throughout the entire system (foliage, soil, and leachate) during cultivation and post-harvest storage, and also to demonstrate the worst-case scenario in the event of high *E. coli* levels in irrigation water. Recent reviews have addressed the technological challenges of implementing these federal guidelines for the specific case of alternative water sources (e.g., treated wastewater and brackish water; Markland et al., 2017; Rock et al., 2019) that would benefit with data-informed decision support tools being actively used to monitor microbial contamination (McLamore et al., 2019; Giacobassi et al., 2021).

A recent study conducted by Solaiman et al. (2020) assessed the prevalence of bacteria indicating water quality, fecal contamination and crop contamination risk (*E. coli*, total coliforms, *Enterococcus*, and *Aeromonas*) over a 26-month longitudinal study in the mid-Atlantic region of the United States. For all water types, higher *E. coli* counts ($p < 0.05$) were observed in the vegetable crop growing (May-October) than non-growing (November-April) season. Additionally, this study found that bacterial counts in reclaimed water generally met microbial standards by federal guidelines or needed minimal mitigation (Solaiman et al., 2020). Another recent work studied the prevalence of Shigatoxigenic *E. coli* (STEC) and atypical enteropathogenic *E. coli* (aEPEC) in untreated surface water and reclaimed water in the mid-Atlantic United States (Haymaker et al., 2019). These pathogenic strains were selected

since they have been responsible for several outbreaks of infections associated with leafy greens consumption recently. The study found that 2.35% (12/510) of water samples contained STEC isolates, while 9.0% (46/510) contained aEPEC isolate. The authors pose that STEC isolates at reclaimed water sites may have been introduced after WWTs (Haymaker et al., 2019), which reinforces the need to monitor irrigation water quality to minimize the risk of foodborne illnesses associated with leafy greens.

Initially, soil and leachate displayed higher concentrations of *E. coli* than foliage until day 14 when foliage surpassed the soil concentrations (**Figures 2A–C**). Overall, each material showed an increase in *E. coli* concentration from the previous sampling time except for soil on day 7 (**Figure 3B**). Foliage consistently increased the concentration throughout cultivation and post-harvest storage (**Figure 3A**). Meanwhile, leachate samples had the largest concentrations at each sampling time (**Figure 3C**). Foliage displayed a positive trend in retention with 16, 31, and 43% on days 0, 7, and 14, respectively (**Figure 3D**). This shows that there was accumulation of *E. coli* on the foliage throughout the cultivation process. The bacteria were able to survive and persist on foliage for more than 1 week. Similar results were observed by Alam et al. (2014), which studied cessation of irrigation prior to harvest, and how the elapsed time affected *E. coli* concentration. In a recent study by Allard et al. (2019), fecal indicators, pathogenic bacteria, and total bacterial communities were tracked from a creek water irrigation source used to irrigate fresh produce *via* drip irrigation to assess the impact of irrigation events on soil and produce microbiota. The study reported that total coliforms in soil were significantly increased immediately and 3 days post-irrigation compared to pre-irrigation, and *E. coli* level in soil increased after irrigation; however, the difference was not significant, and bacterial die-off was not observed neither in soil nor on produce (Allard et al., 2019).

Soil and leachate retention rates were often higher than 100% (**Figures 3E,F**) which shows that the soil was not completely sterile prior to the first irrigation of wastewater on day 0. Possibly, the soil in the lettuce transplants was contaminated with *E. coli*, which propagated during the first 2 weeks of sterile irrigation prior to day 0. Prior to any wastewater application, there was no detectable *E. coli* on the foliage, and the autoclaved soil had 13 ± 10 CFU g^{-1} of *E. coli*, presenting a 25-fold increase after first irrigation. These findings are similar to Orlofsky et al. (2016), which studied the correlation of FIB and pathogens found on fresh crops irrigated with different types of water, including potable, secondary-treated wastewater (TWW), and tertiary TWW, and found *E. coli* in soil, which had only been irrigated with potable water. Conversely, in the present study, soil concentrations were relatively consistent but displayed a negative trend in retention with 188, 53, and 2% on days 0, 7, and 14, respectively (**Figures 3B,E**). This result suggests a maximum contamination load in the soil and that the excess of *E. coli* will stay in the irrigation water to become leachate. Such results are particularly important for low-growing crops, since they have a closer contact with the ground,

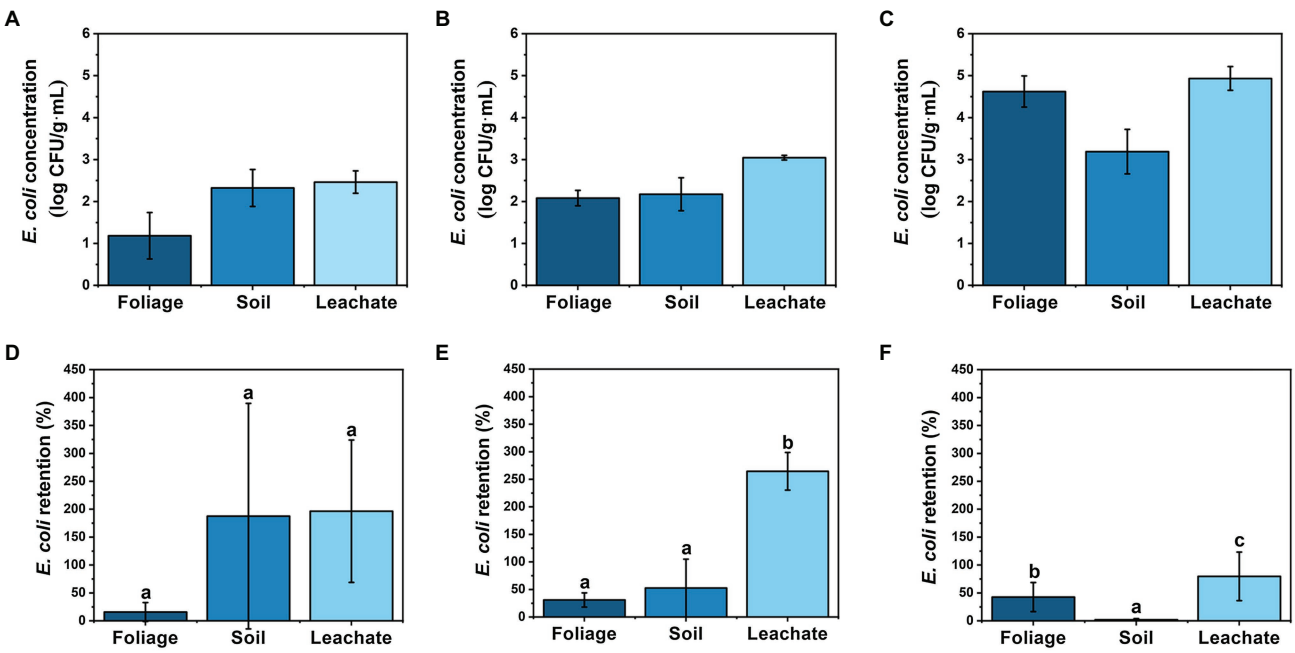

FIGURE 2 | Comparison of *E. coli* concentration at **(A)** day 0, **(B)** day 7, and **(C)** day 14 in log CFU g^{-1} or log CFU ml^{-1} (wet weight basis) and retention on **(D)** day 0, **(E)** day 7, and **(F)** day 14 in % for foliage, soil, and leachate samples, respectively. Retention was calculated by dividing *E. coli* sample concentration (CFU ml^{-1} or CFU g^{-1}) by weekly *E. coli* irrigation water concentration (CFU ml^{-1}). Moisture content of foliage = 91.3% and soil = 4.44%. Sample sizes: wastewater = 10 ml, leachate = 10 ml, foliage = 5 g, and soil = 2 g. Error bars denote standard deviation for arithmetic mean (n = 6 for day 0 foliage and n = 12 for all others). Different letters indicate statistical difference using Tukey-Kramer HSD, α = 0.05.

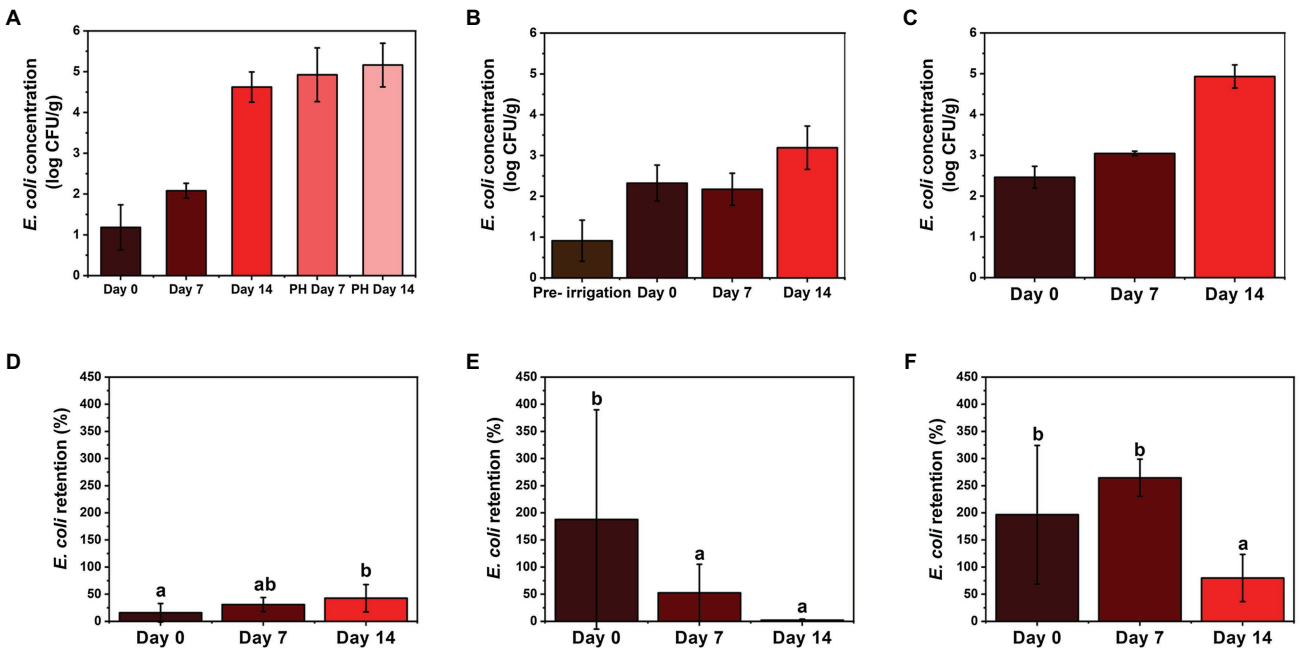

FIGURE 3 | Comparison of *E. coli* concentration on **(A)** foliage, **(B)** soil, and **(C)** leachate in log CFU g^{-1} or log CFU ml^{-1} (wet weight basis) and retention on **(D)** foliage, **(E)** soil, and **(F)** leachate in % over time (days 0, 7, and 14 and post-harvest (PH) days 7 and 14), respectively. Retention was calculated by dividing *E. coli* sample concentration (CFU ml^{-1} or CFU g^{-1}) by weekly *E. coli* irrigation water concentration (CFU ml^{-1}). Moisture content of foliage = 91.3% and soil = 4.44%. Sample sizes: wastewater = 10 ml, leachate = 10 ml, foliage = 5 g, and soil = 2 g. Error bars denote standard deviation for arithmetic mean (n = 6 for day 0 foliage and n = 12 for all others). Different letters indicate statistical difference using Tukey-Kramer HSD, α = 0.05.

increasing the infection risk coming from the contaminated soil (Pavione et al., 2013). Allard et al. (2019) showed that when using drip irrigation to cultivate kale and radish from creek water as irrigation source, even though target pathogens (*Salmonella enterica* and *Listeria monocytogenes*) were detected in irrigation water, they were not likely transferred to the field *via* drip irrigation (i.e., only one post-irrigation kale sample was positive for *S. enterica*). However, this study reported that elevated total coliforms and *E. coli* levels in surface water irrigation influenced bacterial communities in soil and on produce (Allard et al., 2019).

Leachate exhibited the largest retention rates among response materials during the cultivation process (**Figures 2D–F**). The leachate collected the existing *E. coli* in the soil in addition to the *E. coli* introduced by the irrigation water, yielding a retention rate greater than 100% for days 0 and 7 (**Figure 3F**). According to Dwivedi et al. (2016), the saturated water content of the soil is an important parameter in subsurface *E. coli* transport, and in this study, sterile supplemental water was provided during cultivation to avoid drying out and wilting of the lettuce. Similar to soil, day 14 leachate retention was affected by large input concentration and was significantly less than the previous two sampling times, dropping to 80% (**Figure 3F**), even though accumulation increased over time (**Figure 3C**). Our results show that contaminated water can penetrate through 15 cm of soil, but further investigation is needed to determine *E. coli's* fate as water percolates down to groundwater reservoirs. According to Stall et al. (2014), depth of soil has a positive effect on reducing *E. coli* concentrations in leachate.

Escherichia coli concentration in foliage increased during post-harvest storage at 4°C (**Figure 3A**). Similar results were reported by Lopez-Velasco et al. (2010), which studied the effect of post-harvest storage temperatures (4 and 10°C) and times (5, 10, and 15 days) on *E. coli*-contaminated spinach. Even though this is not the ideal temperature for *E. coli* growth (35 ± 2°C), stress response mechanisms can trigger the expression of genes, such as RpoS which is believed to be directly related to the synthesis of internal trehalose in the bacterial cell resulting in the increase of cold resistance (Battesti et al., 2011). *E. coli* counts increased from 4.6 ± 0.4 log CFU g^{-1} on the harvest day to 4.9 ± 0.7 log CFU g^{-1} after 7 days of refrigerated storage. After 14 days of storage, 5.2 ± 0.5 log CFU g^{-1} was observed, a 200% increase from day 7 of post-harvest storage. Days 7 and 14 were significantly different ($p < 0.05$) than day 0, but not significantly different from each other ($p > 0.05$, **Figure 3A**). These results support the importance of fresh produce being free of any pathogenic microbial contamination during cultivation and processing, as *E. coli* left on the surface can quickly propagate at recommended storage temperature (4°C) and pose health risks to consumers if no disinfection treatments are applied prior consumption (Lopez-Velasco et al., 2010). For post-harvest, there is a requirement of no detectable generic *E. coli* in 100 ml of water used in direct contact with produce or on food contact surfaces (FDA, 2017). Additionally, the

United States Food and Drug Administration Food Safety Modernization Act established standards in a Produce Safety Rule specific to pre-harvest agricultural water that will come in direct contact with edible portions of fresh produce crops during cultivation including mitigation measure of allowing up to 4 days elapse between irrigation and harvest to allow for bacterial die-off (Havelaar et al., 2017; FDA, 2020, 2021a). These requirements in combination with the "hold and test" policy adopted in 2012 by the United States Department of Agriculture (USDA) significantly reduce the risk of consumer exposure to unsafe products *via* food recalls (USDA-FSIS, 2013). Notably, to date, there have been no reported foodborne illnesses resulting from the use of reclaimed water (tertiary treated) in irrigation practices in the United States (EPA, 2021; FDA, 2021b).

Antibiotic-Resistant Bacteria

A total of 140 *E. coli* isolates across all sampling times and materials were tested for antibiotic resistance against eight antibiotics. Ampicillin had the highest recorded resistance among isolates at 81% (n = 114), followed by cephalothin at 34% (n = 47; **Figure 4**). Silva et al. (2006) reported that WWT plants have generally been ineffective at removing certain strains of resistant bacteria, specifically *Enterococcus* isolates resistant to the antibiotics ciprofloxacin, erythromycin, and tetracycline, and that the prevalence of ciprofloxacin resistance increased throughout the treatment process. Recently, Chopyk et al. (2020) characterized the taxonomic and functional variations in microbial communities of untreated surface and reclaimed water used in irrigation applications in the mid-Atlantic region of the United States. Among their findings, antimicrobial resistance genes to commonly used antibiotics (aminoglycosides, sulfonamides, rifamycins, macrolides, cephalosporins, fluoroquinolones, and tetracyclines) were found with the highest diversity and abundance in samples from a reclamation facility and a wastewater-impacted freshwater creek (Chopyk et al., 2020). Additionally, the authors reported that bacterial community characteristics varied depending on the date sampled and the specific site (Chopyk et al., 2020), which corroborates with this study findings. Gentamicin and imipenem displayed the lowest rate of resistance, with 1% (n = 1) and 0%, respectively. Several antibiotics including ampicillin (n = 13), cefoperazone (n = 11), cephalothin (n = 14), and ciprofloxacin (n = 13) displayed larger intermediate rates of resistance, ranging from 8 to 10% of all isolates. These findings are important because there is a high probability that these organisms will adapt to their environment and become more resistant, as suggested by Lagacé-Wiens et al. (2013). For this reason, isolates displaying intermediate resistance were categorized as resistant for the remainder of analysis, similar to Laird (2016).

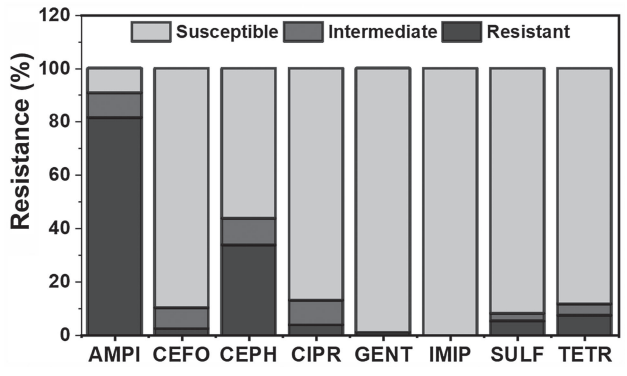

FIGURE 4 | Distribution of antibiotic resistance (resistant, intermediate, and susceptible) among all materials (wastewater, foliage, leachate, and soil) for eight different antibiotics tested (AMPI, ampicillin; CEFO, cefoperazone; CEPH, cephalothin; CIPR, ciprofloxacin; GENT, gentamicin; IMIP, imipenem; SULF, sulfamethoxazole; and TETR, tetracycline) throughout romaine lettuce production (days 0, 7, and 14, post-harvest day 7, and post-harvest day 14). Total *E. coli* isolates were n = 140.

Three antibiotics with the highest combined prevalence of resistance and intermediate resistance were selected to further investigate their fate and transport throughout fresh produce production (**Figure 5**). These antibiotics were ampicillin, cephalothin, and ciprofloxacin with 90% (n = 127), 44% (n = 61), and 13% (n = 18) rate of resistance in all isolates, respectively. For an *E. coli* isolate to be categorized as resistant or intermediate resistant, the bacterial lawn on the Kirby-Bauer plate had to show little to no ZOI around a given antibiotic disc (Bauer et al., 1966). As shown in **Figure 5A**, ampicillin had the highest overall resistance prevalence in foliage. There was no distinct trend or significant differences in antibiotic resistance over the duration of the experiment. Of the 20 post-harvest foliage samples, 17 samples (85%) were resistant to ampicillin. Conversely, only 5% (n = 1) of post-harvest foliage isolates were resistant to cephalothin and 0% to ciprofloxacin. In the United States, ampicillin and ciprofloxacin are two of the top five antibiotics prescribed to adults (Shapiro et al., 2014). These antibiotics have been found in WWTPs in varying concentrations and treatment plant designs (Batt et al., 2007) due to their frequent use in the past and today's society, which suggests that treatment plants may be contributing to the prevalence of ARB found downstream.

Day 0 sampling time displayed the largest resistance in isolates from ampicillin (n = 34), followed by cephalothin (n = 27) and ciprofloxacin (n = 8), for all four materials tested (**Figure 6**). Furthermore, ampicillin was the most prevalent isolate resistance among each material throughout sampling times (n = 127). Ciprofloxacin showed the least resistance (13%) of the three selected antibiotics, for all materials for each sampling day, except for day 7 for soil samples, where both cephalothin and ciprofloxacin showed 0% isolate resistance. For overall ampicillin resistance, soil and wastewater were significantly different from each other (p = 0.049); however, no significant differences were observed from foliage nor leachate. There were no other significant differences among sample materials for cephalothin and ciprofloxacin.

Tracking ARB in leachate and soil in addition to foliage is equally important since these bacteria can make their way back into water sources like rivers and creeks, *via* runoff and leaching. Furthermore, moist soil provides an optimal environment for resistant bacteria to propagate and pass along resistant genes (Orlofsky et al., 2016). Consequently, water

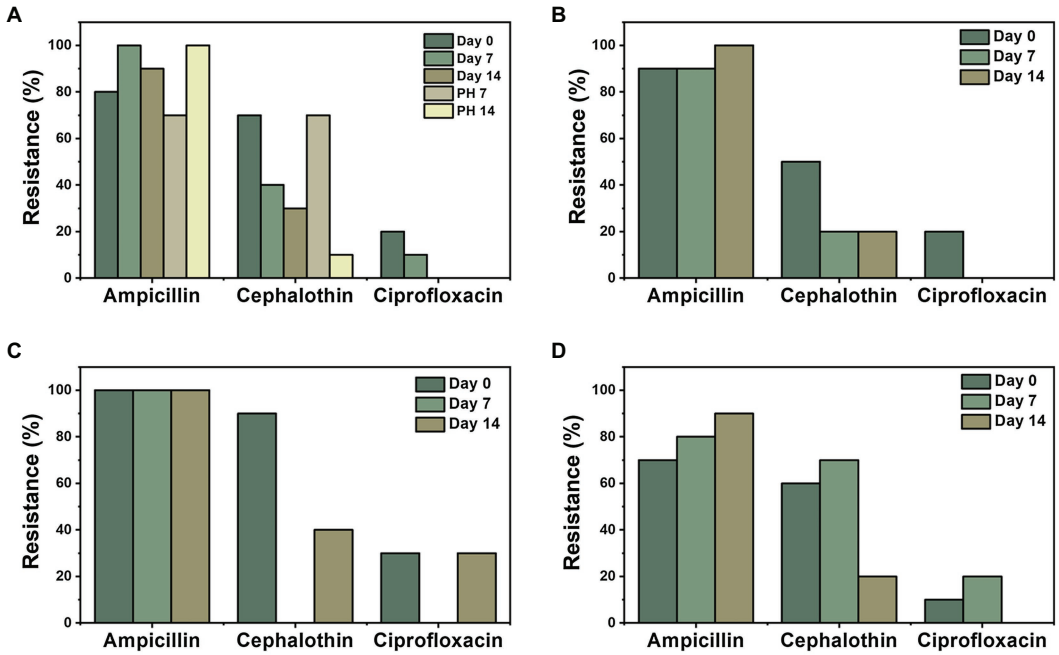

FIGURE 5 | Distribution of ARB over time (days 0, 7, and 14 and post-harvest (PH) days 7 and 14) for three antibiotics that displayed the highest prevalence of resistance (ampicillin, cephalothin, and ciprofloxacin) among response materials: **(A)** foliage, **(B)** leachate, **(C)** soil, and **(D)** wastewater source.

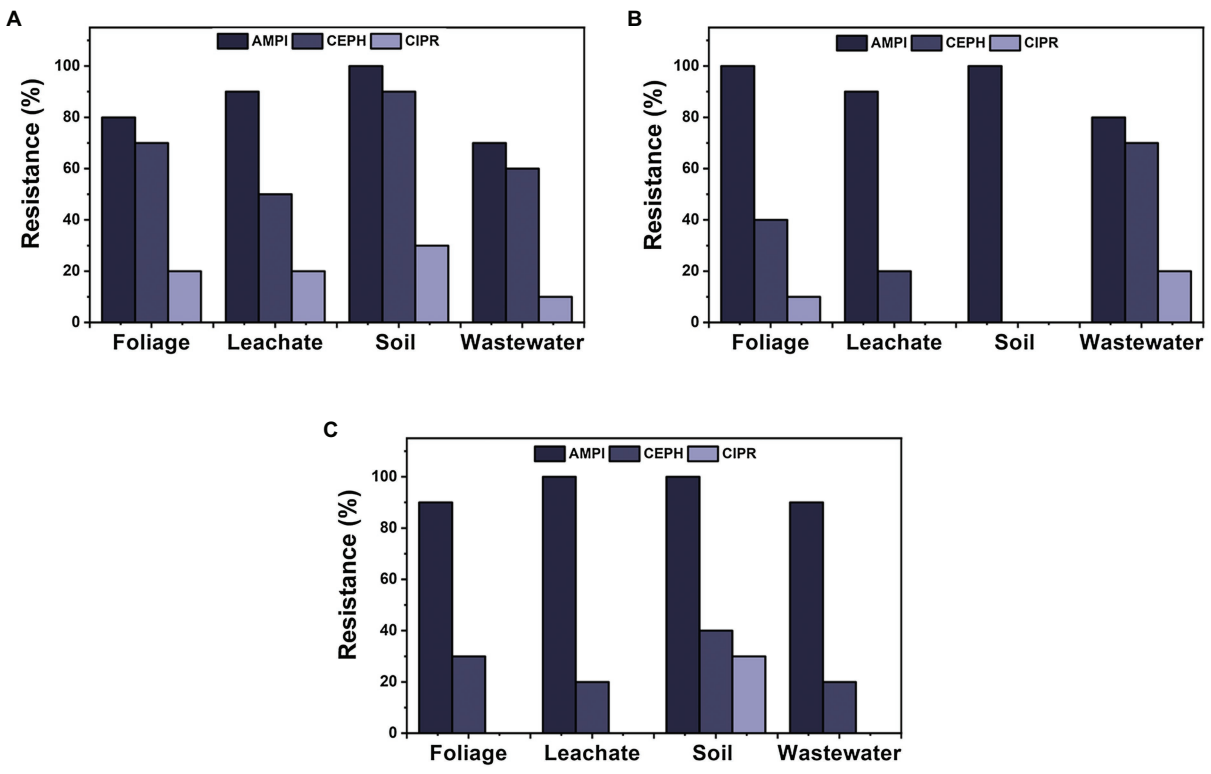

FIGURE 6 | Comparison of ARB for three antibiotics that displayed the highest prevalence of resistance (AMPI, ampicillin; CEPH, cephalothin; and CIPR, ciprofloxacin) among materials over sampling times during cultivation: **(A)** day 0, **(B)** day 7, and **(C)** day 14.

systems are a key vehicle for these bacteria containing antibiotic resistant traits to propagate, multiply, and transfer their resistant genes (Pei et al., 2006). Extensive research on the fate and transport of ARB in water sources resulting from livestock production has been carried out (Addison, 1984; Humphrey et al., 2005). A recent study by Malayil et al. (2020) described the metabolically active bacteria diversity and abundance from reclaimed water and agricultural ponds used as alternative irrigation water sources from the mid-Atlantic United States region. The study observed that antimicrobial resistance and virulence gene profiles appeared to be more diverse and abundant in relic (inactive) DNA than in viable cells (metabolically active) in the tested water types with *Actinobacteria*, *Flavobacterium* spp., *Pseudomonas* spp., and *Aeromonas* spp. being the most abundant and metabolic-active microorganisms (Malayil et al., 2020). Our study presented some baseline information on the prevalence of viable ARB during fresh produce production irrigated with treated municipal wastewater. Further studies are needed to identify potential mitigation and intervention points in the farm-to-fork continuum when treated wastewater effluents for irrigation of fresh produce.

This study has shown the existence of a direct relationship between the bacterial contamination of irrigation water and the contamination levels of subsequent biomass such as foliage, soil, and leachate. Contaminated soil and leachate can generate health risks for future generations of crops, especially those with low growing foliage that have direct contact with the ground. There are potential public health risks from using non-disinfected wastewater effluent to irrigate crops. The results show that leafy greens irrigated with treated wastewater effluents could pose health risks to humans, especially considering the bacteria found have a high probability of being resistant to one or more antibiotic. Overall, the reuse of wastewater as irrigation source for crops attracts enormous interest, mainly in water scarce regions, and its successful application will depend on management strategies to guarantee an inexpensive, efficient, and safe water supply.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

Conceptualization and funding acquisition: TG, RK, EM, and CG. Methodology: HS, CP, TG, RK, and CG. Formal analysis: HS, CP, TG, EM, RK, and CG. Resources: TG, RK, and CG. Data curation, original draft preparation, and visualization: HS, CP, and CG. Review and editing: CP, RK, and CG. Supervision: CG. All authors contributed to the article and approved the submitted version.

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REFERENCES

Addison, J. B. (1984). Antibiotics in sediments and run-off waters from feedlots. *Residue Rev.* 92, 1–28. doi: 10.1007/978-1-4612-5266-5_1

Alam, M., Ahlström, C., Burleigh, S., Olsson, C., Ahrné, S., El-Mogy, M., et al. (2014). Prevalence of *Escherichia coli* O157:H7 on spinach and rocket as affected by inoculum and time to harvest. *Sci. Hort.* 165, 235–241. doi: 10.1016/j.scienta.2013.10.043

Allard, S. M., Callahan, M. T., Bui, A., Ferelli, A. M. C., Chopyk, J., Chattopadhyay, S., et al. (2019). Creek to table: tracking fecal indicator bacteria, bacterial pathogens, and total bacterial communities from irrigation water to kale and radish crops. *Sci. Total Environ.* 666, 461–471. doi: 10.1016/j.scitotenv.2019.02.179

AOAC (1990). Moisture in Plants. Association of Official Analytical Collaboration Official Method 930:4.

Assadian, N. W., Di Giovanni, G. D., Enciso, J., Iglesias, J., and Lindemann, W. (2005). The transport of waterborne solutes and bacteriophage in soil subirrigated with a wastewater blend. *Agric. Ecosyst. Environ.* 111, 279–291. doi: 10.1016/j.agee.2005.05.010

Batt, A. L., Kim, S., and Aga, D. S. (2007). Comparison of the occurrence of antibiotics in four full-scale wastewater treatment plants with varying designs and operations. *Chemosphere* 68, 428–435. doi: 10.1016/j.chemosphere.2007.01.008

Battesti, A., Majdalani, N., and Gottesman, S. (2011). The RpoS-mediated general stress response in *Escherichia coli*. *Annu. Rev. Microbiol.* 65, 189–213. doi: 10.1146/annurev-micro-090110-102946

Bauer, A. W., Kirby, W. M., Sherris, J. C., and Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. *Am. J. Clin. Pathol.* 45, 493–496. doi: 10.1093/ajcp/45.4_ts.493

Bitsch, V., Koković, N., and Rombach, M. (2014). Risk communication and market effects during foodborne illnesses: a comparative case study of bacterial outbreaks in the US and in Germany. *Int. Food Agribus. Man.* 17, 97–114. doi: 10.22004/ag.econ.183451

Bitton, G. (2010). *Wastewater Microbiology: Bitton/Wastewater Microbiology 4E*. Hoboken: Wiley-Blackwell.

Callejon, R. M., Rodriguez-Naranjo, M. I., Ubeda, C., Hornedo-Ortega, R., Garcia-Parrilla, M. C., and Troncoso, A. M. (2015). Reported foodborne outbreaks due to fresh produce in the United States and European Union: trends and causes. *Foodborne Pathog. Dis.* 12, 32–38. doi: 10.1089/fpd.2014.1821

CDC (2018). Multistate outbreak of *E.coli* O157:H7 infections linked to Romaine lettuce (final update). Available at: https://www.cdc.gov/ecoli/2018/o157h7-04-18/index.html (Accessed October 25, 2018).

Chopyk, J., Nasko, D. J., Allard, S., Bui, A., Treangen, T., Pop, M., et al. (2020). Comparative metagenomic analysis of microbial taxonomic and functional variations in untreated surface and reclaimed waters used in irrigation applications. *Water Res.* 169:115250. doi: 10.1016/j.watres.2019.115250

De Keuckelaere, A., Jacxsens, L., Amoah, P., Medema, G., McClure, P., Jaykus, L.-A., et al. (2015). Zero risk does not exist: lessons learned from microbial risk assessment related to use of water and safety of fresh produce. *Compr. Rev. Food Sci. Food Saf.* 14, 387–410. doi: 10.1111/1541-4337.12140

Dwivedi, D., Mohanty, B. P., and Lesikar, B. J. (2016). Impact of the linked surface water-soil water-groundwater system on transport of *E. coli* in the subsurface. *Water Air Soil Pollut.* 227, 1–16. doi: 10.1007/s11270-016-3053-2

EC (2020). European Commision. Water reuse. Regulation on minimim requirements for water reuse enters into force. Available at: https://ec.europa.eu/environment/water/reuse.htm (Accessed March 20, 2021).

Edberg, S. C., Rice, E. W., Karlin, R. J., and Allen, M. J. (2000). *Escherichia coli*: the best biological drinking water indicator for public health protection. *J. Appl. Microbiol.* 88, 106S–116S. doi: 10.1111/j.1365-2672.2000.tb05338.x

EPA (2021). Water Reuse and Recycling. United States Environmental Protection Agency. Available at: https://www.epa.gov/waterreuse (Accessed April 02, 2021).

FDA (2017). Food ad Drug Administration. Standards for the growing, harvesting, packing, and holding of produce for human consuption: what you need to know about FDA Regulation: Guidance for industry small entity compliance guide. Available at: https://www.fda.gov/media/107298/download (Accessed March 20, 2021).

FDA (2020). Food and Drug Administration. Standard for the growing, harvesting, packing, and holding of produce for human consuption. Department of Health and Human Services. Available at: https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfCFR/CFRSearch.cfm?CFRPart=112 (Accessed March 22, 2021).

FDA (2021a). Food and Drug Administration. FSMA Final Rule on Produce Safety. Standards for the growing, harvesting, paking, and holding of produce for human consumption. Available at: https://www.fda.gov/food/food-safety-modernization-act-fsma/fsma-final-rule-produce-safety (Accessed March 25, 2021).

FDA (2021b). Outbreak of foodborne illness. U.S. Food and Drug Administration. Available at: https://www.fda.gov/food/recalls-outbreaks-emergencies/outbreaks-foodborne-illness (Accessed April 02, 2021).

Giacobassi, C. A., Oliveira, D. A., Pola, C. C., Xiang, D., Tang, Y., Datta, S. P. A., et al. (2021). Sense–Analyze–Respond–Actuate (SARA) paradigm: proof of concept system spanning nanoscale and macroscale actuation for detection of *Escherichia coli* in aqueous media. *Actuators* 10:2. doi: 10.3390/act10010002

Havelaar, A. H., Vazquez, K. M., Topalcengiz, Z., Muñoz-Carpena, R., and Danyluk, M. D. (2017). Evaluating the U.S. food safety modernization act produce safety rule standard for microbial quality of agricultural water for growing produce. *J. Food Prot.* 80, 1832–1841. doi: 10.4315/0362-028X.JFP-17-122

Haymaker, J., Sharma, M., Parveen, S., Hashem, F., May, E. B., Handy, E. T., et al. (2019). Prevalence of Shiga-toxigenic and atypical enteropathogenic *Escherichia coli* in untreated surface water and reclaimed water in the Mid-Atlantic U.S. *Environ. Res.* 172, 630–636. doi: 10.1016/j.envres.2019.02.019

Hoekstra, A. Y., and Mekonnen, M. M. (2012). The water footprint of humanity. *Proc. Natl. Acad. Sci. U. S. A.* 109, 3232–3237. doi: 10.1073/pnas.1109936109

Humphrey, T. J., Jorgensen, F., Frost, J. A., Wadda, H., Dominque, G., Elviss, N. C., et al. (2005). Prevalence and subtypes of ciprofloxacin-resistant *Campylobacter* spp. in commercial poultry flocks before, during, and after treatment with fluoroquinolones. *Antimicrob. Agents Chemother.* 49, 690–698. doi: 10.1128/AAC.49.2.690-698.2005

Lagacé-Wiens, P. R. S., Adam, H. J., Low, D. E., Blondeau, J. M., Baxter, M. R., Denisuik, A. J., et al. (2013). Trends in antibiotic resistance over time among pathogens from Canadian hospitals: results of the CANWARD study 2007–11. *J. Antimicrob. Chemother.* 68, i23–i29. doi: 10.1093/jac/dkt023

Laird, D. (2016). Characterization of antibiotic resistance profiles of surface water bacteria in an urbanizing watershed. master's thesis. Texas A&M University.

Lopez-Velasco, G., Davis, M., Boyer, R. R., Williams, R. C., and Ponder, M. A. (2010). Alterations of the phylloepiphytic bacterial community associated with interactions of *Escherichia coli* O157:H7 during storage of packaged spinach at refrigeration temperatures. *Food Microbiol.* 27, 476–486. doi: 10.1016/j.fm.2009.12.010

Malayil, L., Ramachandran, P., Chattopadhyay, S., Cagle, R., Hittle, L., Ottesen, A., et al. (2020). Metabolically-active bacteria in reclaimed water and ponds revealed using bromodeoxyuridine DNA labeling coupled with 16S rRNA and shotgun sequencing. *Water Res.* 184:116185. doi: 10.1016/j.watres.2020.116185

Markland, S. M., Ingram, D., Kniel, K. E., and Sharma, M. (2017). Water for agriculture: the convergence of sustainability and safety. *Microbiol. Spectrum* 5, 143–157. doi: 10.1128/microbiolspec.PFS-0014-2016

Masabni, J., Dainello, F., and Cotner, S. (2009). *The Texas vegetable growers handbook*. Texas, USA: Texas A&M University, college station.

McLamore, E. S., Palit Austin Datta, S., Morgan, V., Cavallaro, N., Kiker, G., Jenkins, D. M., et al. (2019). SNAPS: Sensor Analytics Point Solutions for detection and decision support systems. *Sensors* 19:4935. doi: 10.3390/s19224935

Mena, K. D., and Pillai, S. D. (2008). An approach for developing quantitative risk-based microbial standards for fresh produce. *J. Water Health* 6, 359–364. doi: 10.2166/wh.2008.047

Orlofsky, E., Nirit, B., Mollie, S., Ahuva, V., Maya, B., Arti, K., et al. (2016). Comparable levels of microbial contamination in soil and on tomato crops after drip irrigation with treated wastewater or potable water. *Agric. Ecosyst. Environ.* 215, 140–150. doi: 10.1016/j.agee.2015.08.008

Pavione, D. M., Bastos, R. K., and Bevilacqua, P. D. (2013). Quantitative microbial risk assessment applied to irrigation of salad crops with waste stabilization pond effluent. *Water Sci. Technol.* 67, 1208–1215. doi: 10.2166/wst.2013.674

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

Rai, P. K., and Tripathi, B. D. (2007). Microbial contamination in vegetables due to irrigation with partially treated municipal wastewater in a tropical city. *Int. J. Environ. Health Res.* 17, 389–395. doi: 10.1080/09603120701628743

Ribera, L. A., and McCorkle, D. (2012). Economic impact estimate of irrigation water shortages on the Lower Rio Grande Valley agriculture. Available at: http://agecoext.tamu.edu/files/2013/08/EconImpactIrrigWaterShortLRGV.pdf (Accessed July 15, 2015).

Robinson, J. (2002). Alternative approaches to estimate the impact of irrigation water shortages on Rio Grande Valley agriculture. Texas Water Resources Institute. Available at: http://twri.tamu.edu/reports/2002/2002-015/sr2002-015.pdf (Accessed August 20, 2016).

Rock, C. M., Brassill, N., Dery, J. L., Carr, D., McLain, J. E., Bright, K. R., et al. (2019). Review of water quality criteria for water reuse and risk-based implications for irrigated produce under the FDA Food Safety Modernization Act, produce safety rule. *Environ. Res.* 172, 616–629. doi: 10.1016/j.envres.2018.12.050

Shapiro, D. J., Hicks, L. A., Pavia, A. T., and Hersh, A. L. (2014). Antibiotic prescribing for adults in ambulatory care in the USA, 2007–09. *J. Antimicrob. Chemother.* 69, 234–240. doi: 10.1093/jac/dkt301

Silva, J., Castillo, G., Callejas, L., Lopez, H., and Olmos, J. (2006). Frequency of transferable multiple antibiotic resistance amongst coliform bacteria isolated from a treated sewage effluent in Antofagasta, Chile. *Electron. J. Biotechnol.* 9, 533–540. doi: 10.2225/vol9-issue5-fulltext-7

Solaiman, S., Allard, S. M., Callahan, M. T., Jiang, C., Handy, E., East, C., et al. (2020). Longitudinal assessment of the dynamics of *Escherichia coli*, total coliforms, *Enterococcus* spp., and *Aeromonas* spp. in alternative irrigation water sources: a CONSERVE study. *Appl. Environ. Microbiol.* 86:e00342-20. doi: 10.1128/AEM.00342-20

Solomon, E. B., Potenski, C. J., and Matthews, K. R. (2002). Effect of irrigation method on transmission to and persistence of *Escherichia coli* O157:H7 on lettuce. *J. Food Prot.* 65, 673–676. doi: 10.4315/0362-028X-65.4.673

Stall, C., Amoozegar, A., Lindbo, D., Graves, A., and Rashash, D. (2014). Transport of *E. coli* in a sandy soil as impacted by depth to water table. *J. Environ. Health* 76, 92–100.

USDA-FSIS (2013). United Stats Department of Agriculture Food Safety and Inspection Services. FSIS compliance guideline for controlling meat and poultry products pending FSIS test results. Available at: https://www.fsis.usda.gov/guidelines/2013-0003 (Accessed March 25, 2021).

USEPA (2017). U.S. Environmental Protection Agency. Title 40. Protection of Environment. Part 131.Water quality standards. Accessed on 07/19/2017. U.S. Government Publishing Office. Available at: http://www.ecfr.gov/cgi-bin/retrieveECFR?gp=&SID=e47d88969d2c1ee17f098f238adf00a9&mc=true&r=PART&n=pt40.22.131 (Accessed July 19, 2017).

USFDA (1998). *U.S. Food and Drug Administration. Bacteriological Analytical Manual. 8th Edn.* USFDA (USA: College Station, TX).

Weather Underground (2016). "Easterwood, College Station, TX". Available at: https://www.wunderground.com/history/airport/KCLL/2016/11/6/DailyHistory.html?req_city=College+Station&req_state=TX&req_statename=&reqdb.zip=77840&reqdb.magic=1&reqdb.wmo=99999 (Accessed July 11, 2016).

WHO (2006). *WHO Guidelines for the Safe Use of Wastewater, Excreta, and Greywater: Policy and Regulatory Aspects.* Geneva, France: World Health Organization.

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Increased Antimicrobial and Multidrug Resistance Downstream of Wastewater Treatment Plants in an Urban Watershed

Maitreyee Mukherjee[1,2], Edward Laird[2], Terry J. Gentry[2], John P. Brooks[3] and Raghupathy Karthikeyan[4]*

[1] School of Biological, Environmental, and Earth Sciences, The University of Southern Mississippi, Long Beach, MS, United States, [2] Department of Soil and Crop Sciences, Texas A&M University, College Station TX, United States, [3] USDA-ARS, Mississippi State, Starkville, MS, United States, [4] Department of Agricultural Sciences, Clemson University, Clemson, SC, United States

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***Correspondence:**
Maitreyee Mukherjee
Maitreyee.Mukherjee@usm.edu; maitreyee25@gmail.com

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Development and spread of antimicrobial resistance (AMR) and multidrug resistance (MDR) through propagation of antibiotic resistance genes (ARG) in various environments is a global emerging public health concern. The role of wastewater treatment plants (WWTPs) as hot spots for the dissemination of AMR and MDR has been widely pointed out by the scientific community. In this study, we collected surface water samples from sites upstream and downstream of two WWTP discharge points in an urban watershed in the Bryan-College Station (BCS), Texas area, over a period of nine months. *E. coli* isolates were tested for resistance to ampicillin, tetracycline, sulfamethoxazole, ciprofloxacin, cephalothin, cefoperazone, gentamycin, and imipenem using the Kirby-Bauer disc diffusion method. Antimicrobial resistant heterotrophic bacteria were cultured on R2A media amended with ampicillin, ciprofloxacin, tetracycline, and sulfamethoxazole for analyzing heterotrophic bacteria capable of growth on antibiotic-containing media. In addition, quantitative real-time polymerase chain reaction (qPCR) method was used to measure eight ARG – *tetA, tetW, aacA, ampC, mecA, ermA, blaTEM,* and *intI1* in the surface water collected at each time point. Significant associations ($p < 0.05$) were observed between the locations of sampling sites relative to WWTP discharge points and the rate of *E. coli* isolate resistance to tetracycline, ampicillin, cefoperazone, ciprofloxacin, and sulfamethoxazole together with an increased rate of isolate MDR. The abundance of antibiotic-resistant heterotrophs was significantly greater ($p < 0.05$) downstream of WWTPs compared to upstream locations for all tested antibiotics. Consistent with the results from the culture-based methods, the concentrations of all ARG were substantially higher in the downstream sites compared to the upstream sites, particularly in the site immediately downstream of the WWTP effluent discharges (except *mecA*). In addition, the Class I integron (*intI1*) genes were detected in high amounts at all sites and all sampling points, and were about ∼20 times higher in the downstream sites (2.5×10^7 copies/100 mL surface water) compared to the upstream sites (1.2×10^6 copies/100 mL surface water). Results suggest that the treated WWTP

effluent discharges into surface waters can potentially contribute to the occurrence and prevalence of AMR in urban watersheds. In addition to detecting increased ARG in the downstream sites by qPCR, findings from this study also report an increase in viable AMR (HPC) and MDR (*E. coli*) in these sites. This data will benefit establishment of improved environmental regulations and practices to help manage AMR/ MDR and ARG discharges into the environment, and to develop mitigation strategies and effective treatment of wastewater.

Keywords: antimicrobial resistance, antibiotic resistance genes, wastewater treatment plant, antibiotic resistant bacteria, multidrug resistance

INTRODUCTION

Incidences of antimicrobial resistance (AMR) in previously susceptible pathogenic bacteria are on the rise (Jones et al., 2008). Another related issue of serious consequences to public health is the proliferation of multidrug resistance (MDR) within both pathogenic and non-pathogenic bacterial populations (Levy and Marshall, 2004). This has been identified as a critical issue of profound concern by several global organizations such as the World Health Organization, U.S. Center for Disease Control, the National Academy of Science's Institute of Medicine, the Federal Interagency Task Force on Antimicrobial Resistance, the Infectious Diseases Society of America and numerous other worldwide public health authorities (Spellberg et al., 2008; Allen et al., 2010; Bush et al., 2011; Pruden, 2014). AMR and MDR development in pathogenic bacteria results in several issues concerning public health including limited treatment options, increased morbidity and mortality rates, increased hospital stays, high treatment costs, and the increased necessity for novel antibacterial agents (Goossens et al., 2005; Lim and Webb, 2005; Chopra et al., 2008; Kemper, 2008; Blot et al., 2010; Lye et al., 2012; Nikaido and Pagès, 2012; Naqvi et al., 2013; Worthington and Melander, 2013).

Major contributors to the spread of antibiotic resistance include excessive use in humans and animals, overcrowding and increased rates of transmission between people in communities and hospitals, and the failure of implementing and executing proper hygiene and disinfection practices (Gopal Rao, 2012). AMR and MDR can rapidly spread within bacterial populations of related and unrelated species (Davison, 1999; Pepper and Gentry, 2015) by horizontal gene transfer (Dzidic and Bedeković, 2003) of antibiotic resistance genes (ARG) present in plasmids, transposons, and integrons, or through development of spontaneous mutations (Courvalin, 1994). In recent years, understanding the sources of AMR, MDR and ARG distribution has been deemed critical to eventually control and regulate the spread of ARG (Allen et al., 2010). Yet, enormous gaps still remain in our current knowledge about the occurrence, spread and distribution of AMR, MDR and ARG in the reservoirs found in natural and artificial environments (Allen et al., 2010; Wright, 2010; Rizzo et al., 2013).

While the mechanisms by which antibiotic resistant bacteria (ARB) and ARG are transported and spread through the environment are still not fully understood, previous studies have predicted connections between human activity and the conveyance of resistance traits through agricultural operations, aquatic environments, and sediments (Pei et al., 2006; Baquero et al., 2008; Zhang X. et al., 2009; Huijbers et al., 2015). Pharmaceutical compounds and resistant bacteria may also be introduced to wastewater treatment systems through hospital, industrial, and residential wastewater discharge, and then introduced to the environment (Zuccato et al., 2010; Amador et al., 2015; Verlicchi et al., 2015).

The evolution and development of resistance in clinically important bacteria could be a result of increased opportunities of genetic exchanges with the environmental ARG pool (Bouki et al., 2013; Rizzo et al., 2013; Huijbers et al., 2015, 2019; Chu et al., 2018). Urban WWTPs are increasingly being suspected to be one of the major reservoirs of AMR, MDR and ARG and their mobilization into the environment through effluents (Iwane et al., 2001; Jindal et al., 2006; Szczepanowski et al., 2009; Zhang Y. et al., 2009; Bouki et al., 2013; Korzeniewska et al., 2013; Rizzo et al., 2013; Huijbers et al., 2015; Chu et al., 2018). Contemporary municipal WWTPs are typically incapable of specifically addressing the influx of antibiotics (Adams et al., 2002; Rizzo et al., 2013). Wastewater treatment has also been found to be generally ineffective against certain strains of resistant enterococci, specifically with resistance to ciprofloxacin, erythromycin, and tetracycline (da Silva et al., 2006), with the prevalence of ciprofloxacin resistance actually increasing through the treatment process. The presence of sulfonamide resistance genes in a river environment was found to increase significantly downstream of a swine feedlot WWTP (Hsu et al., 2014). Iwane et al. (2001) found that *Escherichia coli* isolates obtained along the Tama River in Tokyo, Japan expressed increasing resistance to antibiotic agents as sampling moved downstream, and was attributed to treatment plant discharge. Studies tend to vary with respect to the efficiency in which resistant organisms are removed during the treatment process, the microbial species expressing resistance in the effluent, and the antimicrobial agents to which the organisms express resistance. It also should be noted that different WWTP unit operations will affect the overall removal efficiency and ultimately antibiotic resistant bacteria discharge to the environment (Sayah et al., 2005; Janezic et al., 2013; Hamilton et al., 2020). Czekalski et al. (2012) found that while WWTPs reduced total bacterial loads in the effluent, there was an observed increase in

multidrug resistant bacteria and ARG which were then found to accumulate in the sediment of the plant outlet. *Aeromonas* and *Pseudomonas aeruginosa* isolates obtained from some water reservoirs were found to express 50 and 100% multi-drug resistance, respectively (Blasco et al., 2008). A recent review also suggests the importance of accounting for stormwater as a key source of ARG propagation considering the cumulative impact stormwater runoff may have as it comes in contact with overflows from untreated wastewater, sewer and sanitary discharges, among others (Hamilton et al., 2020). Understanding of the impacts of urbanization and wastewater effluent on the presence of antibiotic resistance in the environment will aid in future efforts to address antibiotic resistance through treatment plant process design - establishing more informed guidelines and proper regulations surrounding WWTP practices. We investigated the relationship between urban development and the occurrence and persistence of antimicrobial resistance in the surrounding aquatic environment using antimicrobial resistance data produced by culture-based and quantitative PCR methods. In this study, we collected surface water samples from six sites in an urban watershed – upstream and downstream of two wastewater treatment plants in the Bryan - College Station (BCS), Texas, over a period of nine months. Heterotrophic bacteria capable of growing on antibiotic amended media (HPC-Ab) and *E. coli* were isolated from the six sampling sites and evaluated for resistance to selected antibiotics. Surface water samples were also analyzed and quantified for the presence of eight different ARG targets. Rates of antimicrobial resistance for *E. coli* isolates and antimicrobial resistant heterotrophic communities were compared by sampling site and their relative position with respect to WWTPs to determine if WWTP discharge may affect the antimicrobial resistance profiles of surface water bacteria in the surrounding environment.

MATERIALS AND METHODS

Study Area

Six sampling sites were established within the boundaries of the Carters Creek watershed in BCS area on the main stems of Carters Creek and Burton Creek (**Figure 1**). Sites were selected to represent areas up and downstream of two WWTPs. Sites 1, 3, 5, and 6 were located on the main stem of Carters Creek, and sites 2 and 4 located on the main stem of Burton Creek. Most sites (all but site 3) were located at the intersection of the respective creek and an overpassing bridge. Sites 2, 4, 5, and 6 were sampled upstream of the bridge crossing, and site 1 was sampled directly underneath the overpass. Site 3 was sampled on the stream stem of Carters Creek running adjacent to the highway, upstream of its confluence with Burton Creek. Site 2 was located at the outlet of a channelized stretch of Burton Creek, characterized by shallow flow with substantial algal growth on the concrete surface. All of the sampling sites selected in this study are regular water quality monitoring sites for the Texas Commission on Environmental Quality (TCEQ, 2006) since the commencement of an ongoing Carters Creek watershed Total Maximum Daily Load (TMDL) project in August, 2007.

The Burton Creek WWTP is located upstream and Carters Creek WWTP downstream. Burton Creek WWTP permitted discharge is 8 MGD. Carters Creek WWTP was built in the late 50's to treat about 0.6 MGD to meet the population of 6,000 people. Now the treatment capacity is 9.5 MGD,

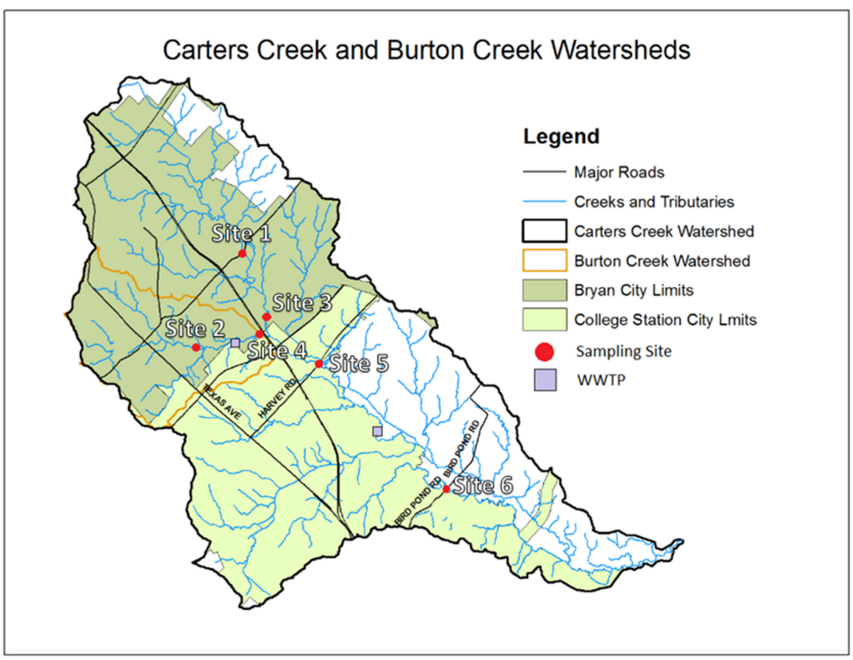

FIGURE 1 | Map of the Carters Creek Watershed and locations of the six sampling sites and the two WWTPs.

even though it reaches only about 7 MGD maximum. Both the treatment plants use activated sludge process to remove carbonaceous and nitrogenous BOD. They both do not use any tertiary treatments. Burtons Creek WWTP uses chlorination to remove pathogens while Carters Creek WWTP applies UV as a disinfection method. Dewatered sludge is digested anaerobically at Burton Creek and aerobic digestion is used in Carters Creek. To meet the population growth another 2 MGD plant was constructed (Lick Creek WWTP) and in operation since early 90's. Additional information on this watershed and the water quality standards and compliance is available at https://www.tceq.texas.gov/waterquality/tmdl/85-carterscreek.html. Urbanization-related maps are provided in **Supplementary Figure 4**. Specific information concerning the sampling sites is included in **Supplementary Table 1** (see **Supplementary Material**).

Sample Collection

A total of six separate sampling events were conducted over a nine-month period between July 2015 and April 2016. Surface water samples were collected using ∼500 mL Whirl-Pak® sterile bags (eNasco, Fort Atkinson, WI, United States) attached to a sampling pole. Water samples were collected from the mid-point of the stream flow approximately 3 cm below the surface. Samples were immediately transferred on ice back to the laboratory and processed within 6 h of collection.

Escherichia coli Isolation and Antibiotic Susceptibility Testing

Inhibition zone diameters were measured and recorded in millimeters and compared to CLSI (Clinical and Laboratory Standards Institute) standards to determine if each isolate was susceptible or resistant to each antibiotic. Isolates were subsequently confirmed as *E. coli* through PCR amplification of *uidA* with *E. coli*-specific primers (data included in **Supplementary Material**).

Initially, four concentrations of each water sample were prepared (1.0, 0.1, 0.01, and 0.001) by ten-fold serial dilutions in phosphate-buffered saline solution (PBS). Ten mL of each dilution was then filtered through a 0.45 μm filter membrane (Millipore, Billerica, MA, United States) by vacuum filtration. Filter membranes were placed on 47 mm Difco® Modified mTEC agar plates (Becton, Dickinson and Company, Sparks, MD, United States) and incubated at 35°C for 2 h and then 44.5°C for 24 h in accordance with EPA Method 1603 (USEPA, 2005). Following incubation, ten presumed *E. coli* (magenta) colonies for each of the six sites were randomly selected, transferred to Difco® Tryptic Soy agar (Becton, Dickinson and Company, Sparks, MD, United States) using a sterile loop, and incubated at 35°C for 24 h. *E. coli* cell suspensions were prepared by transferring two colonies of each isolate into tubes with 5 mL of BBL® Tryptic Soy Broth (Becton, Dickinson and Company, Sparks, MD, United States) and incubating at 35°C for 3 h while shaking at 150 rpm. Tubes were checked for turbidity against a pre-prepared 0.5 McFarland standard corresponding to a 10^7–10^8 CFU/mL bacterial cell count in the broth.

After incubation, sterile swabs were used to inoculate 100 mm Mueller Hinton Agar (MHA) plates (Neogen Corporation, Lansing, MI, United States). Antibiotic resistance of the *E. coli* isolates was determined by the Kirby-Bauer method for antibiotic susceptibility (Bauer et al., 1966). Eight antibiotic susceptibility discs (Becton, Dickinson and Company, Franklin Lakes, NJ, United States) of tetracycline (30 μg), ampicillin (10 μg), ciprofloxacin (5 μg), imipenem (10 μg), sulfamethoxazole/trimethoprim (23.75/1.25 μg), gentamicin (120 μg), cefoperazone (75 μg), and cephalothin (30 μg) were stamped onto each MHA plate using a BBL® Sensi-Disc® 8-place Dispenser (Becton, Dickinson and Company, Franklin Lakes, NJ, United States). The MHA plates were then incubated at 35°C for 16–24 h and the diameters of the inhibition zones measured to determine resistance or susceptibility of each isolate to the antibiotics according the Clinical and Laboratory Standards Institute (CLSI) standards. Control organisms, *E. coli* 25922, *Staphylococcus aureus* 25923, and *Pseudomonas aeruginosa* 27852, were used to ensure consistency during the antibiotic disc diffusion process.

PCR Isolate Confirmation

PCR amplification of the *E. coli* specific *uidA* sequence was used to confirm all isolates collected as *E. coli* (Bower et al., 2005). Cell suspensions of each presumed *E. coli* isolate were prepared by suspending bacterial growth from the MHA agar in 100 μL of sterile, distilled water. PCR mixtures (50 μL) were prepared consisting of 25 μL of GoTaq® G2 Green Master Mix (Promega, Madison, WI, United States), 1.75 μL (350 nM) each of the forward (uidA1318F) and reverse (uidA1698R) primers (Integrated DNA Technologies, Coralville, IA, United States), 5 μL of cell suspension as the template DNA, and 16.5 μL of sterile nuclease-free water. *E. coli* 25922 isolates were used for the positive control. Primer sequences, target, and reference are shown in **Table 1A**.

PCR conditions included one initial heating cycle at 94°C for 4 min; followed by 35 cycles at 94°C for 30 s, 60°C for 30s, and 72°C for 30 s; a final cycle at 72°C for 6 min, and then held at 4°C. DNA electrophoresis was performed in a 2% agarose gel (Amresco, Solon, OH, United States) stained with ethidium bromide (Sigma-Aldrich, St. Louis, MO, United States) and a 1X Tris-Borate-EDTA (TBE) (Fisher BioReagents, Fair Lawn, NJ, United States) buffer solution. A 100 bp ExACTGene™ DNA ladder (Fisher BioReagents, Fair Lawn, NJ, United States) was used as the marker (Data in **Supplementary Material**, Section "PCR Isolate Confirmation Results" and **Supplementary Figure 1**).

Heterotrophic Plate Counts

Heterotrophic bacteria capable of growth on antibiotic-containing media was analyzed using the following protocol. This method potentially captures both intrinsic and acquired resistant bacteria, but does not differentiate between the populations. Four ten-fold serial dilutions of each of the six water samples were prepared (1.0, 0.1, 0.01, and 0.001) by diluting in PBS. Thirty microliters of each dilution were spread-plated onto five sets of 47 mm plate Bacto® Reasoner's 2A

TABLE 1 | A. PCR and B. qPCR primers and conditions used in this study.

Target	Primer sequence (Forward-F, Reverse-R)	Amplicon size and Annealing temperature	References
A. **A. PCR conditions**			
E. coli β –glucuronidase	F- 5′CCGATCACCTGTGT CAATGT 3′ 5′GTTACCGCCAACGCGC AATA 3′	400 bp 60°C	Bower et al., 2005
B. **B. qPCR conditions**			
Class I integron (*intI1*)	F- CTGGATTTCGATCACGG CACG R- ACATGCGTGTAAATCAT CGTCG	473 bp 60°C	Hardwick et al., 2008
Tetracycline (*tetA*)	F- GCTACATCCTGCTTGC CTTC R- CATAGATCGCCGTGA AGAGG	210 bp 62°C	Fan et al., 2007
Tetracycline (*tetW*)	F- GAGAGCCTGCTATATG CCAGC R- GGGCGTATCCACAAT GTTAAC	168 bp 64°C	Aminov et al., 2001
Ampicillin (*ampC*)	F- TTCTATCAAMACTG GCARCC R- CCYTTTTATGTACCC AYGA	550 bp 55°C	Schwartz et al., 2003
Erythromycin (*ermA*)	F- GAAATYGGRTCAGGAA AAGG R- AAYAGYAAACCYAAA GCTC	332 bp 55°C	Chen et al., 2010
Methicillin (*mecA*)	F- AAAACTAGGTGTTGGTGA AGATATACC R- GAAAGGATCTGTACTGG GTTAATCAG	146 bp 55°C	Sabet et al., 2007
Aminoglycoside (*aacA*)	F- TCCTTACTTAATGACCG ATGTACTCT R- TCTTCGCTTTCGC CACTTTGA	146 bp 55°C	Sabet et al., 2007
β-lactamase (*blaTEM*-Univ)	F- CACTATTCTCAGAATGA CTTGGT R- TGCATAATTCTCTTACTG TCATG	84 bp 60°C	Lachmayr et al., 2009

(R2A) agar (Difco Laboratories, Detroit, MI, United States) amended with the following antibiotics: 32 μg/mL ampicillin (HPC-Am) (Ward's Science, Rochester, NY, United States), 16 μg/mL tetracycline (HPC-Te) (Alfa Aesar, Ward Hill, MA, United States), 4 μg/mL ciprofloxacin (HPC-Cpr) (TCI America, Portland, OR, United States), 50.4 μg/mL sulfamethoxazole (HPC-Su) (Chem-Impex International Inc., Wood Dale, IL, United States), and un-amended R2A (HPC) with no antibiotic. Antibiotic concentrations in the agar were determined based on prior studies and are generally around half the strength of either the IV or oral dosage concentrations (Pei et al., 2006; Gao et al., 2012; Garcia-Armisen et al., 2013). Plates also contained 200 μg/mL of cycloheximide (Amresco, Solon, OH, United States) as a fungicide to suppress any fungal growth. All plates were incubated at 28°C for 5 days before obtaining bacterial CFU plate counts. The limit of detection was one CFU in 30 μL of undiluted sample, or 1.52 $\log_{10}$ CFU/mL. There were five instances in which no bacteria were culturable within the sample volume and concentration limit; four of the ciprofloxacin-amended plates, and one of the tetracycline-amended plates. These results were reported as below the limit of detection, and were represented as ½ the limit of detection (16.67 CFU/mL) for statistical analysis.

DNA Extraction and Quantification

From each sample, 100 mL of water was filtered through sterile 0.22 μm Millipore membrane filters of 47 mm diameter and stored in sealed sterile petri plates with the biomass facing up at −80°C for further analyses. The environmental DNA was extracted from these membrane filters using a MoBio PowerWater® DNA Isolation Kit (currently Qiagen, Germantown, MD, United States), following the manufacturer's instructions. Biomass from the filters were prepared and lysed using the PowerWater® bead tubes on a MoBio vortex adapter. For DNA extraction, the centrifugation method was used to bind DNA onto the provided spin filter, washed and finally eluted using the kit's elution buffer. The final elution volume for each DNA sample was 100 μl. The DNA thus obtained was analyzed for concentration using a NanoDrop spectrophotometer (Thermo Fisher Scientific) and used for further quantitative PCR analysis.

Quantitative PCR Analysis

The DNA from each sample was analyzed for the presence of eight ARG: *intI1, tetA, tetW, ampC, blaTEM, mecA, aacA,* and *ermA*. The primer pairs and conditions used for each qPCR analysis is listed in **Table 1**. Each primer pair was tested with each qPCR standard for accuracy of product size and annealing temperature by confirming with end point PCR and agarose gel electrophoresis before using for final qPCR analyses. For qPCR analysis, each sample was run in duplicate. Each standard (standard detail listed in **Supplementary Table 2**, see **Supplementary Material**) was prepared by (i) extracting DNA from the control cultures using the MoBio microbial DNA isolation kit (currently Qiagen, Germantown, MD), (ii) PCR amplification using respective primers, (iii) confirming purity and amplicon size using agarose gel, and (iv) purifying using the Qiagen PCR purification kit (Germantown, MD, United States). The standard DNA was then quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific) and serially diluted to generate qPCR standard curves for each primer pair. Each qPCR reaction had a final volume of 25 μL and consisted of the following reagents: 12.5 μL of 2X SYBR Green (Applied Biosystems), 1.25 μL of 10 μM of each primer, and 10 μL of environmental DNA from each site. The reactions were run in duplicates on 96-well Eppendorf green-skirted plates (Fisher Scientific), and sealed with a plate sealer using qPCR sealing films (BioRad) before analysis using an Eppendorf Realplex2 Mastercycler system. The results were analyzed using the Eppendorf Realplex^2 software and converted into genomic units per 100 mL (GU 100 mL^{-1}) of water sample (Brooks et al., 2014).

Statistical Analyses

Escherichia coli isolate responses to antibiotic susceptibility disc diffusion were categorized as either susceptible or resistant (including intermediate resistance) and assigned a binary value

for each response: 1 for resistant and 0 for susceptible. Then, isolates and isolate responses were grouped into a number of various categories and tested for significant associations by chi-square analysis. Groupings were generally done by pairing binary data from two individual sampling sites or two groups of sampling sites, generating two-by-two grids with one degree of freedom. Significant differences were determined by Chi square sums of 3.84 or greater, or $p < 0.05$ for one degree of freedom. Post hoc multi-comparison tests were carried out for sample site, where appropriate, by conducting pairwise Chi square tests with Bonferonni adjusted p-values. Statistical analysis of the HPC-Ab and box plot generation was done using SAS® University Edition (Cary, NC, United States). Significant differences in the abundance and normalized resistance rates of heterotrophic ARB were evaluated using one-way ANOVA by least-significant-difference (LSD) comparison. Significant differences were checked for homogeneity of variance by Levene's test. In cases where significant differences in homogeneity were found in the data set, it was (then) determined by Welch's ANOVA. Relationships were considered to be significant at $p < 0.05$.

Antibiotic resistance gene levels per 100 mL (GU 100^{-1} mL) were $\log_{10}$ transformed prior to statistical analysis. Differences in response variables (stream position and creek) was assessed for all measured ARG using the mixed procedure in SAS Enterprise Guide 7.1 (SAS Institute). Creek was used as a random variable in the mixed model. Residuals were normally distributed and means were post-hoc adjusted and compared using least square means. All differences were significant at $p < 0.05$, unless otherwise noted.

RESULTS

E. coli Resistance Patterns to Individual Antibiotics

The number of isolates expressing resistance to individual antimicrobial agents by sampling site are displayed in **Figure 2** and **Supplementary Table 3**. Twelve percent of all isolates were susceptible to all 8 antibiotics. A large proportion (84%) of all isolates expressed resistance to cephalothin, with rates of resistance at each individual sampling site falling consistently between 77 and 90% of the isolates collected. The next highest rates of resistance after cephalothin occurred with ampicillin and tetracycline at 15 and 14% of all isolates, respectively. Resistance to ampicillin was expressed in 41 isolates, resistance to tetracycline was expressed in 38 isolates, and resistance to cefoperazone, gentamycin, ciprofloxacin, and sulfamethoxazole/trimethoprim was found in a fewer number of isolates, at rates of 3, 3, 4, and 5%, respectively. All 280 isolates were susceptible to imipenem.

A column chart of isolate resistance responses by sampling site and antibiotic shows an increase in the total number of resistant responses in the downstream sampling sites (**Figure 2**). Isolates collected from the downstream sampling sites expressed resistance more frequently and to diverse antimicrobial agents than the upstream sites. Sampling site 1 displays the most diversity in resistance to different agents in the upstream group, due to one isolate sampled during event two expressing resistance to six agents. The number of total resistant responses also appears to increase as the sampling sites are farther away downstream. For ampicillin, resistance rates fell between 2 and 13% for isolates obtained upstream of WWTP discharges and 17–28% for isolates obtained downstream of WWTP discharges. For tetracycline, resistance rates fell between 0 and 9% for isolates obtained upstream of WWTP discharges and 17–28% for isolates obtained downstream of WWTP discharges. Resistance to cefoperazone, gentamycin, ciprofloxacin and sulfamethoxazole were found more frequently in the isolates obtained from downstream sampling sites. Gentamycin resistance was the only instance in which isolate resistance was found to occur more frequently in one of the upstream sites than in one of the downstream sites (site 1 vs. site 4). Cephalothin resistance is presented separately in **Figure 2B** as to not visually overwhelm the less frequently occurring antibiotic resistances. Cephalothin resistance occurred at a greater frequency and more consistently across all sampling sites than the observed resistance to other antibiotics, irrespective of proximity to WWTP.

Chi-square tests for isolate resistance by individual sampling site (**Supplementary Figure 2**) showed significant differences ($p < 0.003$) between at least one pair of sites for ampicillin, sulfamethoxazole, tetracycline, and ciprofloxacin. The majority of these occurred between site pairings in which one site was upstream of a WWTP and the other site was downstream of a WWTP. Only one test reported a significant difference between two sites with the same relative location to a WWTP. This result was reported for the rate of isolate resistance to tetracycline between sites 2 and 3, corresponding to the Burton Creek site upstream of the WWTP and the Carters Creek site upstream of its confluence with Burton Creek, respectively. When sampling sites were categorized into either an upstream (sites 1–3) or downstream (sites 4–6) group, a significant difference ($p < 0.05$) was found to exist in isolate rates of resistance to ampicillin, tetracycline, cefoperazone, ciprofloxacin, and sulfamethoxazole. While cefoperazone resistance did not increase significantly between any individual sampling sites, there was a significant increase when rates of isolate resistance were categorized and compared between the upstream and downstream.

E. coli Multi-Drug Resistance Patterns

Binomial resistance values determined by the number of resistant responses of each isolate were tallied, organized by sampling site, and sorted into five groups – isolates resistant to 1, 2, 3, or $\geq$4 agents (**Table 2**). Out of the 280 isolates, the majority (88%) showed resistance to at least 1 antibiotic agent. A total of 28 isolates (10% of total) showed resistance to 2 agents, 9 (3% of total) showed resistance to 3 agents, and 17 (6% of total) showed resistance to 4 or more agents. Resistance responses were also sorted by type of antibiotic and number of agents that each isolate was resistant to (**Table 3**). Cephalothin resistance was again the most frequently occurring (95%) antibiotic resistance in the sample set of multi-drug resistant isolates (resistant to two or more agents). Out of all isolates that were resistant to at least one antibiotic, 74% were only resistant to cephalothin,

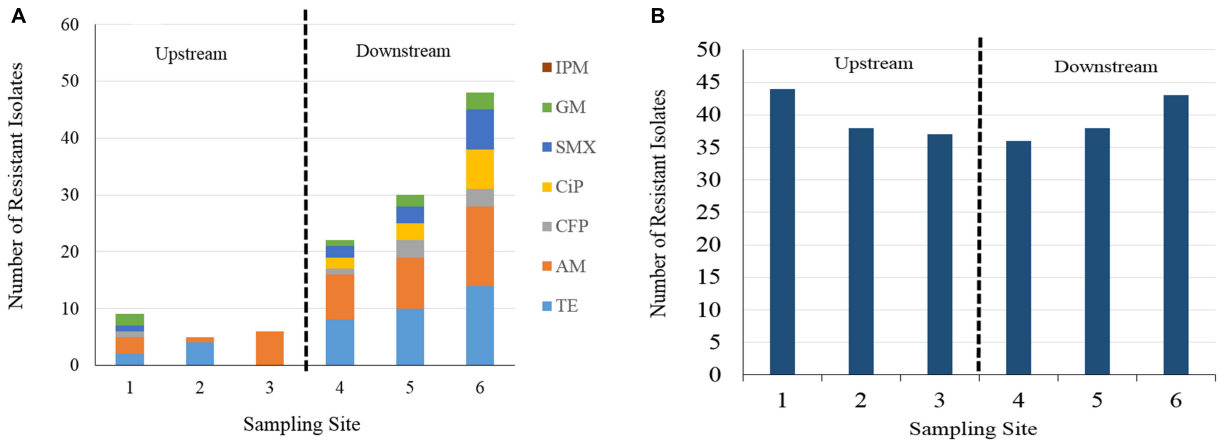

FIGURE 2 | (A) Number of resistant isolate responses to seven of eight antibiotics at different sampling site by Kirby Bauer disc diffusion method. Cephalothin is excluded for visibility of less frequently occurring AB resistances. IPM, imipenem, GM, gentamycin, SMX, sulfamethoxazole, CiP, ciprofloxacin, CFP, cefoperazone, AM, ampicillin, TE, tetracycline. **(B)** Column chart of cephalothin resistant *E. coli* isolate responses. Resistance occurred at a greater frequency than other antibiotic resistances, more consistently across upstream and downstream sampling sites.

and cephalothin resistance accounted for 95% of all single-drug resistant isolates. Isolates only resistant to tetracycline, ampicillin, or cefoperazone were found sparingly, each representing less than 2% of the single-drug resistant isolates. No isolates were only resistant to ciprofloxacin, sulfamethoxazole, gentamycin, or imipenem. Isolates resistant to two or more agents were generally resistant to cephalothin and either tetracycline (41%), or ampicillin (48%). Resistance to three agents occurred less frequently than resistance to four or more agents, at only 4% of resistant isolates. All isolates showing resistance to 4 or more antibiotics were resistant to cephalothin, over 80% of these isolates were also resistant to tetracycline, and 90% to ampicillin. Sulfamethoxazole resistance was only found in isolates resistant to three or more agents. Resistance to cefoperazone, ciprofloxacin, sulfamethoxazole, and gentamycin was generally accompanied by several other resistances (**Table 3**).

Of the 54 multi-drug resistant isolates collected (resistant to at least 2 agents), 41 (76%) were obtained from downstream sites (sites 4–6). All isolates resistant to 3 agents and all but one of the isolates that were resistant to four agents were collected from one of the downstream sites.

TABLE 2 | Percentage (%) of multi-drug resistant *E. coli* isolates by sampling site.

Site number	Number (% by site) of Isolates with Resistance to *n* agents:					Total
	n = 0	*n* = 1	*n* = 2	*n* = 3	*n* ≥ 4	
1	5 (10)	39 (80)	4 (8)	0 (0)	1 (2)	49
2	5 (11)	35 (80)	4 (9)	0 (0)	0 (0)	44
3	7 (15)	35 (76)	4 (9)	0 (0)	0 (0)	46
4	8 (17)	29 (62)	5 (11)	2 (4)	3 (6)	47
5	6 (14)	24 (55)	6 (14)	3 (7)	5 (11)	44
6	2 (4)	31 (62)	5 (10)	4 (8)	8 (16)	50
All sites	33 (12)	193 (69)	28 (10)	9 (3)	17 (6)	280

Chi-square analysis revealed significant associations between several sampling site pairings for when isolates were classified according to multi-drug resistance (**Supplementary Figure 3**). The most significant factor contributing to differences in isolate multi-drug resistance was associated when isolates were grouped according to relative upstream and downstream position. A significant association ($p < 0.001$) was found to exist between the number of isolates expressing resistance to 1, 2, 3, and ≥4 antibiotic agents and whether the isolate was collected upstream of any WWTP (sites 1, 2, and 3) vs. downstream of at least 1 WWTP (sites 4, 5, and 6).

Heterotrophic Plate Counts and HPC-Ab Populations

Heterotrophic bacterial plate counts were obtained during six sampling events to examine the antibiotic resistance profiles of

TABLE 3 | Number of *E. coli* isolates expressing resistance to each antibiotic, grouped by the number of agents the isolate was resistant to.

Antibiotic	Number (%) of Resistant Isolates when Isolate is Resistant to:				Total
	1 agent	2 agents	3 agents	≥4 agents	
	n = 193	*n* = 29	*n* = 9	*n* = 17	*n* = 248
Tetracycline	4 (2)	12 (41)	8 (89)	14 (82)	38 (15)
Ampicillin	3 (1.5)	14 (48)	8 (89)	16 (94)	41 (17)
Cefoperazone	1 (0.5)	0 (0)	0 (0)	7 (41)	8 (3)
Ciprofloxacin	0 (0)	2 (7)	0 (0)	10 (59)	12 (5)
Sulfamethoxazole/ Trimethoprim	0 (0)	0 (0)	3 (33)	13 (76)	13 (5)
Gentamycin	0 (0)	1 (3)	1 (11)	6 (35)	8 (3)
Cephalothin	184 (95)	28 (97)	7 (78)	17 (100)	236 (95)
Imipenem	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

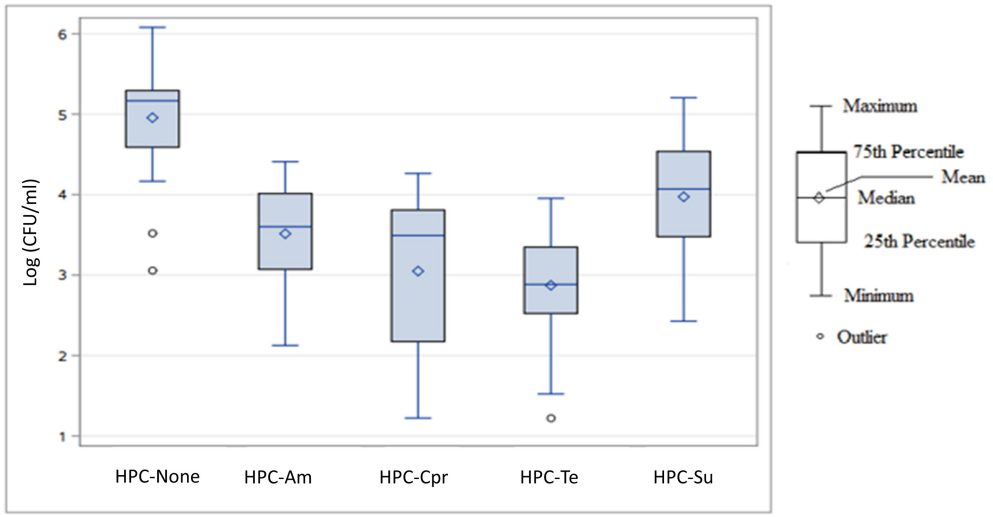

FIGURE 3 | Box plot of log-transformed concentration distributions (log_{10} CFU/mL) of heterotrophic bacteria capable of growing on antibiotic amended agar by antibiotic agent across all sampling events and sampling sites. HPC-none, no antibiotic added; HPC-Am, HPC on ampicillin amended R2A plate; HPC-Cpr, HPC on ciprofloxacin amended R2A plate; HPC-Te, HPC on tetracycline amended R2A plate; HPC-Su, HPC on sulfamethoxazole amended R2A plate.

the culturable, HPC-Ab community in the watershed. The log-transformed bacterial concentrations of each treatment category for all sampling events and sampling sites are displayed in **Supplementary Table 4** and **Figure 3**.

For the total concentrations of each subset of heterotrophic bacterial populations, the R2A agar with no antibiotic produced the highest overall concentration with a median value of 1.47×10^5 CFU/mL and a mean value of 1.68×10^5 CFU/mL (**Figure 3**). Sulfamethoxazole HPC-Su were the next highest with a median concentration of 1.18×10^4 CFU/mL, followed by HPC-Am and HPC-Cpr with median concentrations of 4.00×10^3 CFU/mL and 3.10×10^3 CFU/mL, respectively. HPC-Te had the lowest overall concentration in the study area with a median concentration of 7.67×10^2 CFU/mL. Variance in the total population for each treatment was considerably large, with standard deviations larger than the mean values.

Abundance of HPC-Am varied significantly between sampling sites ($p < 0.0001$), primarily due to the variance occurring between sites 1, 2, 3, and 6 compared to sites 4 and 5 (**Figure 4A**). HPC did not vary significantly by sampling event ($p > 0.65$). HPC-Am were found in significantly greater ($p < 0.0001$) concentrations in the downstream group relative to WWTP discharge, with mean concentrations of 1.3×10^4 and 1.2×10^4 CFU/mL from sites 4 and 5, respectively.

HPC-Te produced a few outliers due to an atypically compact distribution of concentrations at site 2 (**Figure 4B**), in contrast to an otherwise expansive distribution and large standard deviations as seen in the other treatments. Standard deviations of HPC-Te concentrations at sites 2 and 3 were lower by one order of magnitude or more than what was typically seen in other resistant bacterial population. The abundance of HPC-Te varied significantly by sampling site ($p < 0.007$) and sampling event ($p < 0.02$), mainly due to considerably higher concentrations sampled during event 6.

HPC-Su were the most prominent across all sampling sites in this study with the highest mean concentration of resistant bacteria at any sampling site of 6.67×10^4 CFU/mL (**Figure 4C**). Sampling site had a significant influence ($p < 0.0001$) on the concentration of HPC-Ab, mainly due to consistently higher concentrations found at sites downstream from WWTPs.

The mean concentrations HPC-Ab obtained upstream of a WWTP in the tetracycline and sulfamethoxazole amended media were an order of magnitude below the mean concentrations in their respective downstream sites. Significant differences in the abundance HPC-Ab were found to exist between upstream and downstream sites for both the tetracycline ($p < 0.0001$) and sulfamethoxazole amended media ($p < 0.0001$).

Quantitative Monitoring of Antibiotic Resistance Gene Prevalence and Distribution

Overall, analysis of the frequency, distribution, and quantity of the tested ARG reveal a significantly higher measure of ARG in the sites downstream of the WWTP discharge than that found in the upstream sites (**Figure 5**). Overall, *blaTEM*, *ermA*, *intI1*, *tetA*, and *tetW* were significantly greater in downstream compared with upstream sites ($p < 0.05$). Except for *mecA, aacA, emrA*, and *blaTEM* genes at specific sites and sampling events, all other ARG were detected in all sites at all location at all sampling events (**Figure 5**).

Overall, the highest level of ARG was associated with the combined tetracycline resistant genes (*tetA* and *tetW*) – averaged at 7.3×10^4 and 9.9×10^5 copies100 mL^{-1} surface water in the upstream and downstream sites, respectively (**Figure 5** and **Supplementary Table 5**).

A significantly high concentration of the integrase (*intI1*) gene was noted amongst all sites – 1.25×10^6 and 2.5×10^7 gene

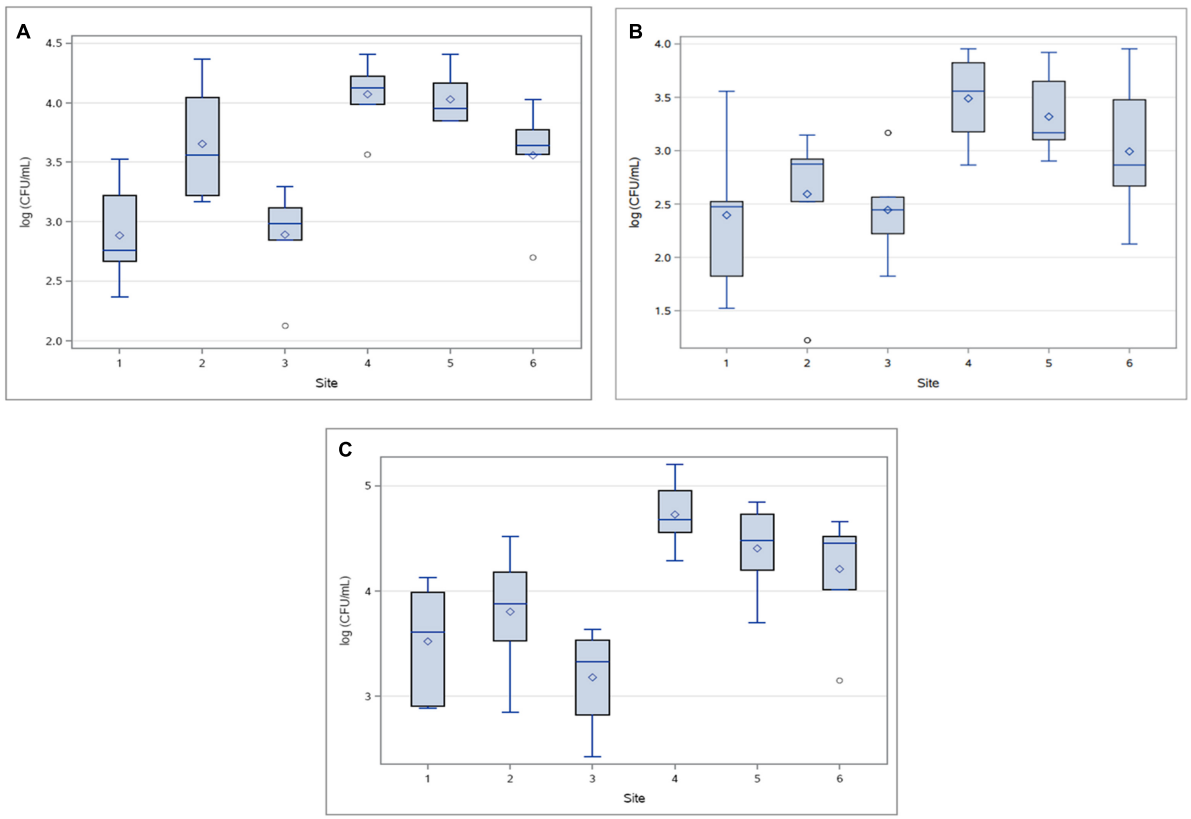

FIGURE 4 | Concentrations (log_{10} CFU/mL) of **(A)** HPC-Am, **(B)** HPC-Tet and **(C)** HPC-Su across sampling sites for all sampling events.

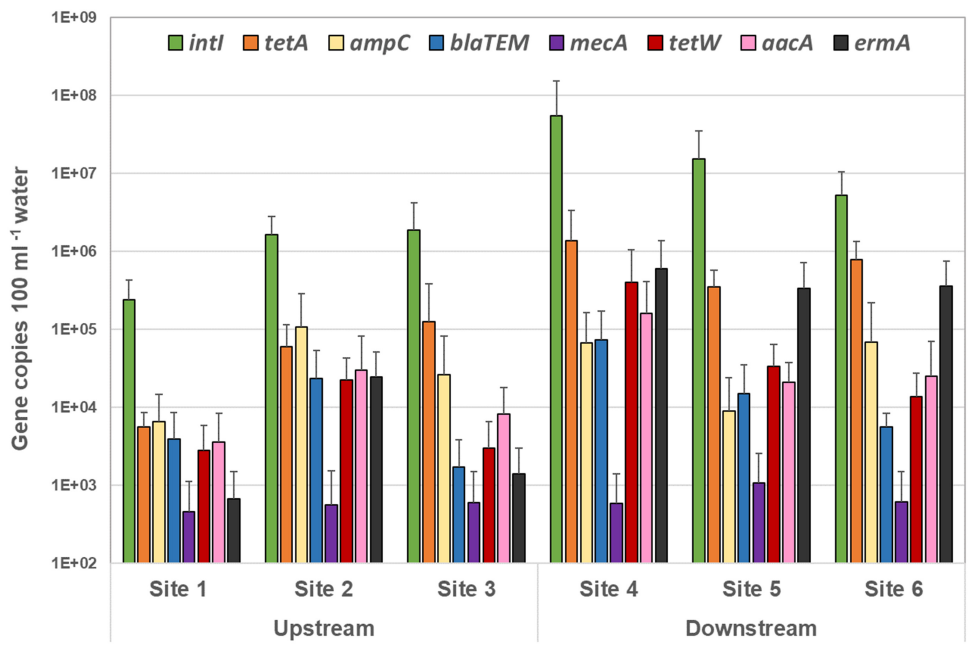

FIGURE 5 | Quantity of each antibiotic resistance gene over all sampling events detected by quantitative PCR in the upstream sites and downstream sites. The target gene values (gene copies 100 ml^{-1} sample) are displayed in mean of all sampling events, and the standard deviations represent the variation among the six sampling events.

copies 100 mL^{-1} surface water in the upstream and downstream sites respectively ($p < 0.0001$) (**Supplementary Table 5** and **Figure 5**). In addition, copies of all ARG tested in this study (except for *mecA*) were found to be considerably greater in site 4 – the site immediately downstream of the BCWWTP (Burton Creek Wastewater Treatment Plant) (**Supplementary Table 5**). This is a notable result and may suggest a role of the WWTP in dissemination of the ARG into the immediate surrounding environment. Additionally, the high prevalence in ARG copies within the immediate downstream site taken together with 40 times higher prevalence of *intI1* genes in the same site (**Supplementary Table 5**) also suggest that the WWTP may provide a favorable setting for genetic exchanges that lead to development of AMR and MDR in resident bacterial populations.

Among the other tested ARG, erythromycin resistant genes (*ermA*) were found to be substantially high in all downstream sites (4.3×10^5 copies 100 mL^{-1} water) ($p < 0.0001$), particularly high in site 4 (6×10^5 copies 100 mL^{-1} water) – 48 times higher when compared to the upstream sites (9×10^3 copies 100 mL^{-1} water) (**Supplementary Table 5**). A similar trend, though not significant, was noticed with the broad-spectrum aminoglycoside resistance gene target (*aacA*) – 1.4×10^4 copies 100 mL^{-1} water compared to 1.6×10^5 in site 4 (**Supplementary Table 5**). Similarly, copies of *blaTEM* genes targeting the TEM type β-lactamases (Bradford, 2001) were significantly greater downstream compared with upstream sites ($p = 0.0115$), for instance, in site 4 (7.33×10^4 copies 100 mL^{-1} water) compared to the combined average of all upstream sites (9.6×10^3 copies 100 mL^{-1} water) (**Supplementary Table 5**).

DISCUSSION

Antimicrobial Resistance to Specific Antibiotics

Ampicillin, sulfamethoxazole, and ciprofloxacin, or closely related drugs (amoxicillin), are among the top 5 antibiotics prescribed for use for adults in the United States (Shapiro et al., 2013; Van Boeckel et al., 2014; Hicks et al., 2015), and all have been found to occur in WWTPs in varying concentrations and design conditions (Batt et al., 2007). In the current study, a significant association ($p < 0.05$) was found to exist between the location of sampling sites relative to WWTPs (upstream group vs. downstream group) and isolates expressing resistance to ampicillin, ciprofloxacin, cefoperazone, sulfamethoxazole, and tetracycline. This supports the hypothesis that WWTP effluent may be contributing to the conveyance of antibiotic resistant bacteria downstream from discharge points. The absence of significant associations between rates of isolate resistance among upstream sites indicates that these differences are not solely dependent on variations between all sampling sites, but also their relative location to WWTP discharge points.

The occurrence of antibiotic resistance may not always imply an outside effect and can be an intrinsic property of the natural environment. In this study, cephalothin represented the highest rate (84%) of resistance found in all isolates, irrespective of their collection location. The high rate of resistance to cephalothin and the ubiquity of its presence suggests that this resistance trait may be partially due to natural occurrence in the watershed. This is consistent with results from other studies performed with *E. coli* isolates obtained from surface waters in Michigan and Illinois, rates of isolate resistance to cephalothin at 80.6 and 80%, respectively (Sayah et al., 2005; Janezic et al., 2013). Cephalothin resistance also represented 95% of the 193 isolates resistant to only 1 antibiotic, dramatically inflating the abundance of isolates classified as resistant to at least 1 antibiotic. If cephalothin was excluded from the AMR data for this study, an additional 184 isolates (66% of total) would be classified as susceptible to all agents.

The rate of isolate resistance to tetracycline (14% of all isolates) was found to be lower than expected when compared to similar research (Jindal et al., 2006; Rajić et al., 2006; Brooks and McLaughlin, 2009; Sullivan and Karthikeyan, 2012). Previous studies have found the occurrence of tetracycline resistance to be prevalent in watersheds associated with agricultural and animal feed lot operations (Jindal et al., 2006; Rajić et al., 2006), with resistance rates of over 90% found in *E. coli* isolates obtained from swine lagoon effluent (Brooks and McLaughlin, 2009). Sullivan and Karthikeyan (2012) reported the prevalence of tetracycline resistant bacteria and tetracycline resistant genes in sediment and surface water samples collected from Carters Creek watershed; however, the majority were found in greater abundance bound in stream sediment samples than in surface water samples. In another study on the Carters Creek watershed, Sullivan and Karthikeyan (2012) found that while the occurrence of tetracycline resistance genes increased downstream of WWTPs, concentrations of tetracycline resistant bacteria were not significantly affected.

Imipenem is a group 2 carbapenem generally reserved as the last line of defense against particularly resilient Gram-negative pathogens and not widely prescribed (Nicolau et al., 2012). Out of all the isolates collected from all sampling sites, all 280 *E. coli* isolates were susceptible to imipenem. Imipenem resistance was not detected in the surface water bacteria of the Carters Creek watershed.

Multidrug Resistance

A substantial fraction (19%) of all 280 *E. coli* isolates expressed resistance to two or more antibiotics. Multi-drug resistance was found to increase significantly ($p < 0.05$) in the sites downstream of a WWTP for isolates resistant to $\geq$2, $\geq$3, and $\geq$4 agents. Other studies have observed high rates in the development of multidrug resistance in *E. coli* isolates in WWTP processes (Korzeniewska et al., 2013; Amador et al., 2015), found to be primarily driven by the transfer of conjugative plasmids (da Silva et al., 2006). A number of WWTP disinfection practices had negligible effects on reducing rates of multidrug resistant bacteria, and in a number of cases increasing it (Bouki et al., 2013). Even if the WWTP effluents in this study had considerably low levels of multidrug resistant bacteria, in other studies (Rizzo et al., 2013) these low concentrations have been shown to persist and propagate in the environment once discharged. ARG not necessarily bound to culturable organisms

are also likely escaping treatment processes and contributing to the development of multidrug resistance (Kümmerer, 2009). Resistance to cefoperazone, sulfamethoxazole, ciprofloxacin, and gentamycin was more frequently found in multidrug resistant isolates, and rarely as the only type of resistance. This suggests that resistance to these agents is either driven by similar modes of defense coded by resistance genes to other agents, or that the acquisition of resistance to these agents usually occurs in tandem with other antibiotic resistances.

The high rates of resistance to cephalothin across all six sampling sites inflated multi-drug resistance rates, present in 95% of the 248 isolates resistant to at least one antibiotic. This increases the importance of the multidrug resistance classifications of isolates resistant to three or more and four or more agents, due to cephalothin resistance effectively acting as a resistance baseline for this data set. While the strictest definition for multidrug resistance is resistant to two or more agents, "resistance to three or more classes" has become increasingly standard for defining multidrug resistance in Gram-positive and Gram-negative bacteria (Magiorakos et al., 2012). Still, a substantial number, 9% of all 280 isolates, expressed resistance to at least 3 antibiotics. This rate is more in line with other reports of the prevalence of multidrug resistant *E. coli* in surface waters (Blaak et al., 2015), though these rates likely differ considerably as a function of antibiotics tested and sampling site. A large majority (86%) of these isolates were collected downstream of a WWTP, and all significant increases in rates of isolate resistance to three or more agents occurred when comparing an upstream site to a downstream site. While some degree of multidrug resistance appears to exist naturally, the results suggest that WWTPs in the watershed might be contributing significantly to multidrug resistant bacteria in the surface water.

HPC-Ab

A significant increase in the concentrations of HPC-Ab downstream of WWTP discharge was found for all four agents tested against the total HPC community. Heterotrophic bacteria populations are diverse and possess a considerable amount of intrinsic variability in the way they occur and interact in the environment (Garcia-Armisen et al., 2013). By normalizing the abundance of HPC-Ab in the study area to the total heterotrophic bacteria population, a better understanding can be made concerning the extent of HPC-Ab relative to total numbers. Unfortunately, this diversity also makes it difficult to establish a reliable standard for which to compare resistance rates against. Additionally, the levels of HPC-Am, -Cpr, and -Te bacteria were generally confined to a range of 1–10% of the total heterotrophic community when compared to the control, though in some instances spiking to between 20 and 40% of the total population. However, these large spikes in the ratios of HPC-Ab to the control CFU were generally due to significantly lower counts in the control during a sampling event or at a sampling site and not because the CFU of HPC-Ab increased. In contrast, HPC-Su were frequently found to represent from 20 to 80% of the total heterotrophic population, ratios significantly ($p < 0.001$) higher than all other HPC-Ab. This same trend in HPC-Su was found to exist throughout numerous processes sampled in a municipal wastewater treatment plant (Gao et al., 2012), also finding that while the total abundance of resistant bacteria were reduced in the effluent, that reduction was consistent with the reduction in total HPC-Ab populations. The similarities in HPC-Su observed in the downstream sites in this study may indicate contribution of resistance traits originating from WWTP effluent.

The occurrence of a significant increase in the concentrations of HPC-Te bacteria downstream of WWTPs in the total heterotrophic populations appears to contradict the findings of Sullivan and Karthikeyan (2012), research also conducted in the Carters Creek watershed. Sullivan and Karthikeyan (2012) found no effect of WWTP location on the prevalence of HPC-Te in surface water, but did see an increase in the abundance of tetracycline ARG. While molar concentrations of tetracycline used in both studies were similar, the discrepancy might be explained by differences in the cultivation media: Sullivan and Karthikeyan (2012) used nutrient-rich agar and this study used nutrient-limited R2A agar. Differences in cultivation media can significantly affect the counts of culturable HPC even from identical samples (Garcia-Armisen et al., 2013). Sullivan and Karthikeyan (2012) also found no seasonal variability in the occurrence of tetracycline resistant genes or bacteria.

While there was a significant increase in the abundance of HPC-Ab in the downstream sites, there was no significant increase when the concentrations were normalized to total heterotrophic bacteria with no antibiotic in the cultivation media. This indicates that while the total amount of resistant bacteria is increasing downstream through the watershed, it is increasing proportionately with the total population. This can be due to several plausible reasons. Viable bacteria from treated effluents may be a reason to increase total abundance without increasing normalized rates of resistance in the watershed. Suspended solids, dissolved organic carbon, and nutrients in WW effluents may facilitate the growth of pre-existing HPC-Ab downstream. Favorable growth conditions and increased total heterotrophic population proportionately increased the abundance of resistant bacteria.

Total heterotrophic population CFUs on control plates during antimicrobial studies can vary dramatically (3 orders of magnitude) (Pei et al., 2006), making it difficult to normalize results of antibiotic bacteria within the population. Additionally, the methodology used to capture HPC-Ab is known to capture both intrinsic and acquired resistance traits (Brooks et al., 2007); thus, these values may represent an over representation of the antibiotic resistant population.

Antibiotic Resistance Gene Prevalence

The scope of this project was not limited to only cultivable bacteria, the study also evaluated the prevalence of ARG by qPCR methods. Both the cultivation-based approach and the qPCR approach reveal a major difference in the AMR bacterial numbers in the downstream vs. the upstream sites. The detected dominance of tetracycline resistant genes is not surprising as several other studies that focused on the distribution of ARG in urban waters, wastewaters, and WWTPs have previously found elevated levels of various tetracycline resistance genes (Szczepanowski et al., 2009; Gao et al., 2012; Brooks et al., 2014; Laht et al., 2014; Mao et al., 2015; Makowska et al., 2016; Ng et al., 2016). In addition, these results are in agreement

with the culture-based results presented in the previous sections and an earlier study in the Carters Creek watershed where Sullivan et al. (2013) found an abundance of a variety tetracycline resistant genes and tetracycline resistant bacterial groups in several sampling sites within the watershed.

Class I integrons are genetic elements that are tangentially associated with the distribution of AMR and development of MDR among Gram-negative bacteria in a variety of environments (Cambray et al., 2010; Domingues et al., 2012; Gillings, 2014; Strugeon et al., 2016). The integron integrase gene (*intI1*) is the key fragment of the functional structure of Class I integrons as it is responsible for antibiotic resistance element-containing gene cassettes to be acquired, expressed and disseminated across bacterial species (Stokes and Hall, 1989; Collis and Hall, 1992; Domingues et al., 2012; Strugeon et al., 2016). Several previous studies have linked the presence of *intI1* with prevalence of MDR in different environments (Leverstein-van Hall et al., 2002; Stalder et al., 2012; Brooks et al., 2014; Hultman et al., 2018). Furthermore, an elevated quantity of Class I integrase genes has been found to be present in studies that examined the occurrence of *intI1* specifically in WWTPs (Makowska et al., 2016; McConell, 2017) and further associated with anthropogenic environmental influence (Gillings et al., 2015). Therefore, the considerably high abundance of the *intI1* genes observed in all sites within this study is consistent with the observation of increased instances of AMR and MDR bacterial population in this site. It is important to note that we did not measure 16S rRNA while we measured ARG. Thus, we were not able to normalize our data to 16S rRNA which would provide for proportional context to the data set.

CONCLUSION

Results from this study find a considerably greater AMR and MDR in the downstream sites of the WWTPs carrying the wastewater effluents compared to the upstream sites. Downstream sites showed an increased resistance to the antibiotics ampicillin, ciprofloxacin, cefoperazone, sulfamethoxazole, and tetracycline, and were more often resistant to a higher number of different antibiotics. These effects were mirrored in the total HPC-Ab community, with a significant increase in the abundance of HPC-Am, -Cpr, -Su, and -Te bacteria in the surface water downstream of WWTP discharge points Quantitative PCR analysis of eight ARG in the samples also revealed similar results. In addition, we also noted in this study that the class 1 integron integrase gene, previously widely reasoned in similar studies to be associated with mobile genetic elements responsible for the movement of AMR and MDR between bacterial species in several environments, was detected at significantly high concentration in all sites at all times. We also detected a markedly higher abundance of most of the tested genes, including the Class I integrons, in the site immediately downstream of the WWTP.

Antibiotic resistance and increased rates of resistance can be attributed by several factors, including WWTP discharges as reported here. A more specific future investigation and more constrained system focusing on the inflows, outflows, and process components at the WWTPs would be beneficial in determining the extent of its contribution to resistance in the environment. Occurrence and persistence of antibiotic resistance and maintenance of the resistome are complex to describe in natural settings such as watersheds with varying hydrology, land use changes, and anthropogenic activities. Several watershed processes including overland runoff, stormwater outflow, and runoff from impermeable surfaces will affect the dissemination of antibiotic resistant bacteria. In watersheds, particularly in urban settings, WWTPs play a critical mitigation point for antibiotic resistance. Effective treatments should decrease further spread of resistance. It is important to note that only a particular fragment of potential ARG were tested in our analysis; additional insights could be obtained into these interpretations with a more extensive antibiotic "resistome" study. Further understanding of the interrelationships among ARB concentrations, ARG concentrations, antibiotic agents, microbial species, and environmental media will help modeling of antibiotic resistance transfer in terrestrial and aquatic environment.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

MM, TG, EL, JB, and RK: conceptualization. MM, TG, EL, and JB: methodology. MM and EL: formal analysis and data curation. MM: writing—original draft preparation. MM, TG, JB, and RK: writing—review and editing. TG: supervision, project administration, and funding acquisition. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.657353/full#supplementary-material

REFERENCES

Adams, C., Wang, Y., Loftin, K., and Meyer, M. (2002). Removal of antibiotics from surface and distilled water in conventional water treatment processes. *J. Environ. Eng.* 128, 253–260. doi: 10.1061/(asce)0733-9372(2002)128:3(253)

Allen, H. K., Donato, J., Wang, H. H., Cloud-Hansen, K. A., Davies, J., and Handelsman, J. (2010). Call of the wild: antibiotic resistance genes in natural environments. *Nat. Rev. Microbiol.* 8, 251–259. doi: 10.1038/nrmicro2312

Amador, P. P., Fernandes, R. M., Prudêncio, M. C., Barreto, M. P., and Duarte, I. M. (2015). Antibiotic resistance in wastewater: occurrence and fate of *Enterobacteriaceae* producers of Class A and Class C β-lactamases. *J. Environ. Sci. Health A* 50, 26–39. doi: 10.1080/10934529.2015.964602

Aminov, R. I., Garrigues-Jeanjean, N., and Mackie, R. I. (2001). Molecular ecology of tetracycline resistance: development and validation of primers for detection of tetracycline resistance genes encoding ribosomal protection proteins. *Appl. Environ. Microbiol.* 67, 22–32. doi: 10.1128/AEM.67.1.22-32.2001

Baquero, F., Martınez, J. M., and Canto, R. (2008). Antibiotics and antimicrobial resistance in water environments. *Curr. Opin. Biotech.* 19, 260–265.

Batt, A. L., Kim, S., and Aga, D. S. (2007). Comparison of the occurrence of antibiotics in four full-scale wastewater treatment plants with varying designs and operations. *Chemosphere* 68, 428–435. doi: 10.1016/j.chemosphere.2007.01.008

Bauer, A., Kirby, W., Sherris, J. C., and Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. American Journal of Clinical Pathology 45, 493–496.

Blaak, H. G., Lynch, R., Italiaander, R. A., Hamidjaja, F. M., and de Roda Husman, A. M. (2015). Multidrug-resistant and extended spectrum beta-lactamase-producing *Escherichia coli* in Dutch surface water and wastewater. *PLoS One* 10:e0127752. doi: 10.1371/journal.pone.0127752

Blasco, M. D., Esteve, C., and Alcaide, E. (2008). Multiresistant waterborne pathogens isolated from water reservoirs and cooling systems. *J. App. Microbiol.* 105, 469–475. doi: 10.1111/j.1365-2672.2008.03765.x

Blot, S., Vandijck, D., Lizy, C., Annemans, L., and Vogelaers, D. (2010). Estimating the length of hospitalization attributable to multidrug antibiotic resistance. *Antimicro. Agric. Chemother.* 54, 4046–4047. doi: 10.1128/aac.00689-10

Bouki, C., Venieri, D., and Diamadopoulos, E. (2013). Detection and fate of antibiotic resistant bacteria in wastewater treatment plants: a review. *Ecotoxicol. Environ. Saf.* 91, 1–9. doi: 10.1016/j.ecoenv.2013.01.016

Bower, P. A., Scopel, C. O., Jensen, E. T., Depas, M. M., and McLellan, S. L. (2005). Detection of genetic markers of fecal indicator bacteria in Lake Michigan and determination of their relationship to *Escherichia coli* densities using standard microbiological methods. *App. Environ. Microbiol.* 71, 8305–8313. doi: 10.1128/aem.71.12.8305-8313.2005

Bradford, P. A. (2001). Extended-spectrum ß-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. *Clin. Microbiol. Rev.* 14, 933–951. doi: 10.1128/cmr.14.4.933-951.2001

Brooks, J. P., Adeli, A., and McLaughlin, M. R. (2014). Microbial ecology, bacterial pathogens, and antibiotic resistant genes in swine manure wastewater as influenced by three swine management systems. *Wat. Res.* 57, 96–103. doi: 10.1016/j.watres.2014.03.017

Brooks, J. P., Maxwell, S. L., Rensing, C., Gerba, C. P., and Pepper, I. L. (2007). Occurrence of antibiotic-resistant bacteria and endotoxin associated with the land application of biosolids. *Can. J Microbiol.* 53, 616–622. doi: 10.1139/w07-021

Brooks, J. P., and McLaughlin, M. R. (2009). Antibiotic resistant bacterial profiles of anaerobic swine lagoon effluent. *J. Environ. Qual.* 38, 2431–2437. doi: 10.2134/jeq2008.0471

Bush, K., Courvalin, P., and Dantas, G. (2011). Tackling antibiotic resistance. *Nat. Rev. Microbiol.* 9, 894–896.

Cambray, G., Guerout, A. M., and Mazel, D. (2010). Integrons. *Ann. Rev. Genet.* 44, 141–166.

Chen, J., Michel, F. C., Sreevatsan, S., Morrison, M., and Yu, Z. (2010). Occurrence and persistence of erythromycin resistance genes (erm) and tetracycline resistance genes (tet) in waste treatment systems on swine farms. *Microb. Ecol.* 60, 479–486. doi: 10.1007/s00248-010-9634-5

Chopra, I., Schofield, C., and Everett, M. (2008). Treatment of health-care-associated infections caused by Gram-negative bacteria: a consensus statement. *Lancet Infect. Dis.* 8, 133–139. doi: 10.1016/s1473-3099(08)70018-5

Chu, B. T. T., Petrovich, M. L., Chaudhary, A., Wright, D., Murphy, B., Wells, G., et al. (2018). Metagenomics reveals the impact of wastewater treatment plants on the dispersal of microorganisms and genes in aquatic sediments. *App. Environ. Microbiol.* 84:e002168-17.

Collis, C. M., and Hall, R. M. (1992). Site-specific deletion and rearrangement of integron insert genes catalyzed by the integron DNA integrase. *J. Bacteriol.* 174, 1574–1585. doi: 10.1128/jb.174.5.1574-1585.1992

Courvalin, P. (1994). Transfer of antibiotic resistance genes between gram-positive and gram-negative bacteria. *Antimicro. Agric. Chemother.* 38, 1447–1451. doi: 10.1128/aac.38.7.1447

Czekalski, N., Berthold, T., Caucci, S., Egli, A., and Bürgmann, H. (2012). Increased levels of multiresistant bacteria and resistance genes after wastewater treatment and their dissemination into Lake Geneva, Switzerland. *Front. Microbiol.* 3:106. doi: 10.3389/fmicb.2012.00106

da Silva, M. F., Tiago, I., Veríssimo, A., Boaventura, R. A. R., Nunes, O. C., and Manaia, C. M. (2006). Antibiotic resistance of enterococci and related bacteria in an urban wastewater treatment plant. *FEMS Microbiol. Ecol.* 55, 322–329. doi: 10.1111/j.1574-6941.2005.00032.x

Davison, J. (1999). Genetic exchange between bacteria in the environment. *Plasmid* 42, 73–91. doi: 10.1006/plas.1999.1421

Domingues, S., da Silva, G. J., and Nielsen, K. M. (2012). Integrons: vehicles and pathways for horizontal dissemination in bacteria. *Mob. Genet. Element* 2, 211–223. doi: 10.4161/mge.22967

Dzidic, S., and Bedeković, V. (2003). Horizontal gene transfer-emerging multidrug resistance in hospital bacteria. *Acta. Pharmacol. Sin.* 24, 519–526.

Fan, W., Hamilton, T., Webster-Sesay, S., Nikolich, M. P., and Lindler, L. E. (2007). Multiplex real-time SYBR Green I PCR assay for detection of tetracycline efflux genes of Gram-negative bacteria. *Mol. cellular probes* 21, 245–256. doi: 10.1016/j.mcp.2006.12.005

Gao, P., Munir, M., and Xagoraraki, I. (2012). Correlation of tetracycline and sulfonamide antibiotics with corresponding resistance genes and resistant bacteria in a conventional municipal wastewater treatment plant. *Sci. Total. Environ.* 421, 173–183. doi: 10.1016/j.scitotenv.2012.01.061

Garcia-Armisen, T., Anzil, A., Cornelis, P., Chevreuil, M., and Servais, P. (2013). Identification of antimicrobial resistant bacteria in rivers: Insights into the cultivation bias. *Wat. Res.* 47, 4938–4947. doi: 10.1016/j.watres.2013.05.036

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

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

Goossens, H., Ferech, M., Vander Stichele, R., Elseviers, M., and ESAC Project Group (2005). Outpatient antibiotic use in Europe and association with resistance: a cross-national database study. *Lancet* 365, 579–587. doi: 10.1016/s0140-6736(05)17907-0

Gopal Rao, G. (2012). Risk factors for the spread of antibiotic-resistant bacteria. *Drugs* 55, 323–330. doi: 10.2165/00003495-199855030-00001

Hamilton, K. A., Garner, E., Joshi, S., Ahmed, W., Ashbolt, N., Medema, G., et al. (2020). Antimicrobial-resistant microorganisms and their genetic determinants in stormwater: a systematic review. *Curr. Opin. Environ. Sci. Health* 16, 101–112. doi: 10.1016/j.coesh.2020.02.012

Hardwick, S. A., Stokes, H. W., Findlay, S., Taylor, M., and Gillings, M. R. (2008). Quantification of class 1 integron abundance in natural environments using real-time quantitative PCR. *FEMS Microbiol. Lett.* 278, 207–212. doi: 10.1111/j.1574-6968.2007.00992.x

Hicks, L. A., Bartoces, M. G., Roberts, R. M., Suda, K. J., Hunkler, R. J., Taylor, T. H., et al. (2015). US outpatient antibiotic prescribing variation according to geography, patient population, and provider specialty in 2011. *Clin. Infect Dis.* 60, 1308–1316.

Hsu, J. T., Chen, C. Y., Young, C. W., Chao, W. L., Li, M. H., Liu, Y. H., et al. (2014). Prevalence of sulfonamide-resistant bacteria, resistance genes and integron-associated horizontal gene transfer in natural water bodies and soils adjacent to a swine feedlot in northern Taiwan. *J. Haz. Mat.* 277, 34–40. doi: 10.1016/j.jhazmat.2014.02.016

Huijbers, P. M., Blaak, H., de Jong, M. C., Graat, E. A., Vandenbroucke-Grauls, C. M., and de Roda Husman, A. M. (2015). Role of the environment in the transmission of antimicrobial resistance to humans: a review. *Environ. Sci. Technol.* 49, 11993–12004. doi: 10.1021/acs.est.5b02566

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

Hultman, J., Tamminen, M., Pärnänen, K., Cairns, J., Karkman, A., and Virta, M. (2018). Host range of antibiotic resistance genes in wastewater treatment plant influent and effluent. *FEMS Microbiol. Ecol.* 94:fiy038.

Iwane, T., Urase, T., and Yamamoto, K. (2001). Possible impact of treated wastewater discharge on incidence of antibiotic resistant bacteria in river water. *Wat. Sci. Technol.* 43, 91–99. doi: 10.2166/wst.2001.0077

Janezic, K. J., Ferry, B., and Hendricks, E. W. (2013). Phenotypic and genotypic characterization of *Escherichia coli* isolated from untreated surface waters. *Open Microbiol. J.* 7, 9–19. doi: 10.2174/1874285801307010009

Jindal, A., Kocherginskaya, S., Mehboob, A., Robert, M., Mackie, R. I., Raskin, L., et al. (2006). Antimicrobial use and resistance in swine waste treatment systems. *Appl. Environ. Microbiol.* 72, 7813–7820. doi: 10.1128/aem.01087-06

Jones, K. E., Patel, N. G., Levy, M. A., Storeygard, A., Balk, D., Gittleman, J. L., et al. (2008). Global trends in emerging infectious diseases. *Nature* 451, 990–993.

Kemper, N. (2008). Veterinary antibiotics in the aquatic and terrestrial environment. *Ecol. Indicat.* 8, 1–13. doi: 10.1016/j.ecolind.2007.06.002

Korzeniewska, E., Korzeniewska, A., and Harnisz, M. (2013). Antibiotic resistant *Escherichia coli* in hospital and municipal sewage and their emission to the environment. *Ecotoxicol. Environ. Saf.* 91, 96–102. doi: 10.1016/j.ecoenv.2013.01.014

Kümmerer, K. (2009). Antibiotics in the aquatic environment – A review – Part II. *Chemosphere* 75, 435–441. doi: 10.1016/j.chemosphere.2008.12.006

Lachmayr, K. L., Kerkhof, L. J., DiRienzo, A. G., Cavanaugh, C. M., and Ford, T. E. (2009). Quantifying nonspecific TEM β-lactamase (blaTEM) genes in a wastewater stream. *Appl. Environ. Microbiol.* 75, 203–211. doi: 10.1128/AEM.01254-08

Laht, M., Karkman, A., Voolaid, V., Ritz, C., Tenson, T., Virta, M., et al. (2014). Abundances of tetracycline, sulphonamide and beta-lactam antibiotic resistance genes in conventional wastewater treatment plants (WWTPs) with different waste load. *PLoS One* 9:e103705. doi: 10.1371/journal.pone.0103705

Leverstein-van Hall, M. A., Paauw, A., Box, A. T. A., Blok, H. E. M., Verhoef, J., and Fluit, A. C. (2002). Presence of integron-associated resistance in the community is widespread and contributes to multidrug resistance in the hospital. *J. Clin. Micobiol.* 40, 3038–e3040.

Levy, S. B., and Marshall, B. (2004). Antibacterial resistance worldwide: causes, challenges, and responses. *Nat. Med.* 10, 122–129.

Lim, S. M., and Webb, S. A. R. (2005). Nosocomial bacterial infections in intensive care units. I: organisms and mechanisms of antibiotic resistance. *Anaesthesia* 60, 887–902. doi: 10.1111/j.1365-2044.2005.04220.x

Lye, D. C., Earnest, A., Ling, M. L., Lee, T. E., Yong, H. C., Fisher, D. A., et al. (2012). The impact of multidrug resistance in healthcare-associated and nosocomial Gram-negative bacteraemia on mortality and length of stay: cohort study. *Clin. Microbiol. Infect* 18, 502–508. doi: 10.1111/j.1469-0691.2011.03606.x

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

Makowska, N., Koczura, R., and Mokracka, J. (2016). Class 1 integrase, sulfonamide and tetracycline resistance genes in wastewater treatment plant and surface water. *Chemosphere* 144, 1665–1673. doi: 10.1016/j.chemosphere.2015.10.044

Mao, D., Yu, S., Rysz, M., Luo, Y., Yang, F., Li, F., et al. (2015). Prevalence and proliferation of antibiotic resistance genes in two municipal wastewater treatment plants. *Wat. Res.* 85, 458–466. doi: 10.1016/j.watres.2015.09.010

McConell, M. (2017). *Abundance of Antibiotic Resistance Genes in Two Municipal Wastewater Treatment Plants and Receiving Water in Atlantic Canada*. Ph. D.thesis. Halifax: Dalhousie University.

Naqvi, S., Kiran, U., Ali, M. I., Jamal, A., Hameed, A., Ahmed, S., et al. (2013). Combined efficacy of biologically synthesized silver nanoparticles and different antibiotics against multidrug-resistant bacteria. *Int. J. Nanomed.* 8, 3187–3195. doi: 10.2147/ijn.s49284

Ng, C., Tan, B., Jiang, X., Gu, X., Chen, H., Schmitz, B. W., et al. (2016). Metagenomic and resistome analysis of a full-scale municipal wastewater treatment plant in Singapore containing membrane bioreactors. *Nat. Microbiol.* 161, 1–7.

Nicolau, D. P., Carmeli, Y., Crank, C. W., Goff, D. A., Graber, C. J., Lima, A. L. L., et al. (2012). Carbapenem stewardship: does ertapenem affect *Pseudomonas* susceptibility to other carbapenems? A review of the evidence. *Int. J. Antimicrob. Agric.* 39, 11–15. doi: 10.1016/j.ijantimicag.2011.08.018

Nikaido, H., and Pagès, J. M. (2012). Broad-specificity efflux pumps and their role in multidrug resistance of Gram-negative bacteria. *FEMS. Microbiol. Rev.* 36, 340–363. doi: 10.1111/j.1574-6976.2011.00290.x

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

Pepper, I. L., and Gentry, T. J. (2015). "Chapter 2 - Microorganisms found in the environment," in *Environmental Microbiology*, 3rd Edn, eds I. Pepper, C. Gerba, and T. Gentry (San Diego: Academic Press), 9–36. doi: 10.1016/b978-0-12-394626-3.00002-8

Pruden, A. (2014). Balancing water sustainability and public health goals in the face of growing concerns about antibiotic resistance. *Environ. Sci. Technol.* 48, 5–14. doi: 10.1021/es403883p

Rajić, A., Reid-Smith, R., Deckert, A. E., Dewey, C. E., and McEwen, S. A. (2006). Reported antibiotic use in 90 swine farms in Alberta. *Can. Vet. J.* 47, 446–452.

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

Sabet, N. S., Subramaniam, G., Navaratnam, P., and Sekaran, S. D. (2007). Detection of methicillin-and aminoglycoside-resistant genes and simultaneous identification of *S. aureus* using triplex real-time PCR Taqman assay. *J. Microbiol. Methods* 68, 157–162. doi: 10.1016/j.mimet.2006.07.008

Sayah, R. S., Kaneene, J. B., Johnson, Y., and Miller, R. S. (2005). Patterns of antimicrobial resistance observed in *Escherichia coli* isolates obtained from domestic- and wild-animal fecal samples, human septage, and surface water. *Appl. Envron. Microbiol.* 71, 1394–1404. doi: 10.1128/aem.71.3.1394-1404.2005

Schwartz, T., Kohnen, W., Jansen, B., and Obst, U. (2003). Detection of antibiotic-resistant bacteria and their resistance genes in wastewater, surface water, and drinking water biofilms. *FEMS Microbiol. Ecol.* 43, 325–335. doi: 10.1111/j.1574-6941.2003.tb01073.x

Shapiro, D. J., Hicks, L. A., Pavia, A. T., and Hersh, A. L. (2013). Antibiotic prescribing for adults in ambulatory care in the USA, 2007–09. *J. Antimicrob. Chemother.* 69, 234–240. doi: 10.1093/jac/dkt301

Silva, J., Castillo, G., Callejas, L., López, H., and Olmos, J. (2006). Frequency of transferable multiple antibiotic resistance amongst coliform bacteria isolated from a treated sewage effluent in Antofagasta, Chile. *Electron. J. Biotechnol.* 9:8.

Spellberg, B., Guidos, R., Gilbert, D., Bradley, J., Boucher, H. W., Scheld, W. M., et al. (2008). The epidemic of antibiotic-resistant infections: a call to action for the medical community from the Infectious Diseases Society of America. *Clin. Infect. Dis.* 46, 155–164. doi: 10.1086/524891

Stalder, T., Barraud, O., Casellas, M., Dagot, C., and Ploy, M. C. (2012). Integron involvement in environmental spread of antibiotic resistance. *Front. Microbiol.* 3:119. doi: 10.3389/fmicb.2012.00119

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

Strugeon, E., Tilloy, V., Ploy, M. C., and Da Re, S. (2016). The stringent response promotes antibiotic resistance dissemination by regulating integron integrase expression in biofilms. *mBio* 7:e0868-16.

Sullivan, B. A., Gentry, T. J., and Karthikeyan, R. (2013). Characterization of tetracycline-resistant bacteria in an urbanizing subtropical watershed. *J. Appl. Microbiol.* 115, 774–785. doi: 10.1111/jam.12283

Sullivan, B. A., and Karthikeyan, R. (2012). Occurrence and prevalence of tetracycline resistant bacteria in a rapidly urbanizing subtropical watershed. *J. Nat. Environ. Sci.* 2, 25–31.

Szczepanowski, R., Linke, B., Krahn, I., Gartemann, K. H., Gützkow, T., Eichler, W., et al. (2009). Detection of 140 clinically relevant antibioticresistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing reduced susceptibility to selected antibiotics. *Microbiology* 155, 2306–2319. doi: 10.1099/mic.0.028233-0

TCEQ (2006). *Water Use Permit No. 5912*. Bryan, TX: TCEQ.

USEPA (2005). *Method 1603: Escherichia coli (E. coli) in Water by Membrane Filtration Using Modified Membrane-Thermotolerant Escherichia coli Agar (Modified mTEC)*. Washington, DC: Environmental Protection Agency.

Van Boeckel, T. P., Gandra, S., Ashok, A., Caudron, Q., Grenfell, B. T., Levin, S. A., et al. (2014). Global antibiotic consumption 2000 to 2010: an analysis of national pharmaceutical sales data. *Lanc. Infect. Dis.* 14, 742–750. doi: 10.1016/s1473-3099(14)70780-7

Verlicchi, P., Al Aukidy, M., and Zambello, E. (2015). What have we learned from worldwide experiences on the management and treatment of hospital effluent? — An overview and a discussion on perspectives. *Sci. Total Environ.* 514, 467–491. doi: 10.1016/j.scitotenv.2015.02.020

Worthington, R. J., and Melander, C. (2013). Combination approaches to combat multidrug-resistant bacteria. *Trends Biotechnol.* 31, 177–184. doi: 10.1016/j.tibtech.2012.12.006

Wright, G. D. (2010). Antibiotic resistance in the environment: a link to the clinic? *Curr. Opin. Microbiol.* 13, 589–594. doi: 10.1016/j.mib.2010.08.005

Zhang, X., Zhang, T., and Fang, H. H. P. (2009). Antibiotic resistance genes in water environment. *Appl. Microbiol. Biotechnol.* 82, 397–414.

Zhang, Y., Marrs, C. F., Simon, C., and Xi, C. (2009). Wastewater treatment contributes to selective increase of antibiotic resistance among *Acinetobacter spp*. *Sci. Total Environ.* 407, 3702–3706. doi: 10.1016/j.scitotenv.2009.02.013

Zuccato, E., Castiglioni, S., Bagnati, R., Melis, M., and Fanelli, R. (2010). Source, occurrence and fate of antibiotics in the Italian aquatic environment. *J. Haz. Mat.* 179, 1042–1048. doi: 10.1016/j.jhazmat.2010.03.110

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Integrated Metagenomic Assessment of Multiple Pre-harvest Control Points on Lettuce Resistomes at Field-Scale

Lauren Wind[1], Ishi Keenum[2], Suraj Gupta[3], Partha Ray[4,5], Katharine Knowlton[4], Monica Ponder[6], W. Cully Hession[1], Amy Pruden[2] and Leigh-Anne Krometis[1]*

[1] Department of Biological Systems Engineering, Virginia Tech, Blacksburg, VA, United States, [2] Department of Civil and Environmental Engineering, Virginia Tech, Blacksburg, VA, United States, [3] The Interdisciplinary PhD Program in Genetics, Bioinformatics, and Computational Biology, Virginia Tech, Blacksburg, VA, United States, [4] Department of Dairy Science, Virginia Tech, Blacksburg, VA, United States, [5] Department of Animal Sciences, University of Reading, Reading, United Kingdom, [6] Department of Food Science and Technology, Virginia Tech, Blacksburg, VA, United States

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***Correspondence:**
Lauren Wind
wlauren@vt.edu

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An integrated understanding of factors influencing the occurrence, distribution, and fate of antibiotic resistance genes (ARGs) in vegetable production systems is needed to inform the design and development of strategies for mitigating the potential for antibiotic resistance propagation in the food chain. The goal of the present study was to holistically track antibiotic resistance and associated microbiomes at three distinct pre-harvest control points in an agroecosystem in order to identify the potential impacts of key agricultural management strategies. Samples were collected over the course of a single growing season (67 days) from field-scale plots amended with various organic and inorganic amendments at agronomic rates. Dairy-derived manure and compost amendment samples (n = 14), soil samples (n = 27), and lettuce samples (n = 12) were analyzed *via* shotgun metagenomics to assess multiple pre-harvest factors as hypothetical control points that shape lettuce resistomes. Pre-harvest factors of interest included manure collection during/post antibiotic use, manure composting, and soil amended with organic (stockpiled manure/compost) versus chemical fertilizer. Microbial community resistome and taxonomic compositions were unique from amendment to soil to lettuce surface according to dissimilarity analysis. The highest resistome alpha diversity (i.e., unique ARGs, n = 642) was detected in amendment samples prior to soil application, while the composted manure had the lowest total ARG relative abundance (i.e., 16S rRNA gene-normalized). Regardless of amendment type, soils acted as an apparent ecological buffer, i.e., soil resistome and taxonomic profiles returned to background conditions 67 d-post amendment application. Effects of amendment conditions surprisingly re-emerged in lettuce phyllosphere resistomes, with the highest total ARG relative abundances recovered on the surface of lettuce plants grown in organically-fertilized soils (i.e., compost- and manure-amended soils). Co-occurrence analysis identified 55 unique ARGs found both in the soil amendments and on lettuce surfaces. Among these, *arnA* and *pmrF* were the most abundant ARGs co-occurring with mobile genetic elements (MGE). Other prominent ARG-MGE

co-occurrences throughout this pre-harvest lettuce production chain included: *Tet*M to transposon (*Clostridiodies difficile*) in the manure amendment and *Tri*C to plasmid (*Ralstonia solanacearum*) on the lettuce surfaces. This suggests that, even with imposing manure management and post-amendment wait periods in agricultural systems, ARGs originating from manure can still be found on crop surfaces. This study demonstrates a comprehensive approach to identifying key control points for the propagation of ARGs in vegetable production systems, identifying potential ARG-MGE combinations that could inform future surveillance. The findings suggest that additional pre-harvest and potentially post-harvest interventions may be warranted to minimize risk of propagating antibiotic resistance in the food chain.

Keywords: antibiotic resistance genes, antibiotic resistome, antimicrobial resistance, agriculture, manure, lettuce, metagenomics, next-generation sequence

INTRODUCTION

Antibiotic resistance is recognized as a critical threat to human health, food security, and global development (O'Neill, 2016). In 2015, the WHO strongly encouraged the development of holistic action plans to preserve drug efficacy for usage in humans and animals (WHO, 2017). Antibiotic use in agriculture has especially been scrutinized, given that the majority of antibiotics used world-wide is devoted to livestock production. The use of antibiotics as growth promoters on EU farms was banned in 2006 (European Commission, 2005) and more recently (2017) on United States farms as part of the Veterinary Feed Directive (final rule #213), which requires veterinary authorization and oversight, rather than simple over-the-counter distribution, for the use of antimicrobials in livestock (FDA, 2013, 2017, 2018). Despite this progress in antibiotic stewardship, it is worth noting that antibiotic resistance genes (ARGs) and antibiotic resistant bacteria (ARB) are still regularly recovered from EU and United States livestock herds and the United States and some EU countries remain the top users of antibiotics for livestock (Aarestrup et al., 2001; Van Boeckel et al., 2015; Elliott et al., 2017). In addition, antibiotics are widely used to promote livestock growth in nations beyond the United States and EU, with much less regulatory oversight. As the need for food and agricultural products (e.g., crops, livestock, and fiber) increases to meet the demands of a growing global population (FAO, 2020), there is concern that the emergence and dissemination of environmentally-linked antibiotic resistant infections could intensify, especially in nations that have not yet adopted environmental antibiotic resistance mitigation policies (Van Boeckel et al., 2015). Knowledge is particularly lacking with respect to how use of livestock manure-derived soil amendments in vegetable production systems could influence carriage of antibiotic resistance in the food-chain.

A variety of agricultural best management practices are employed to prevent contamination of consumer products with pathogens (e.g., manure management, post-harvest washing), but these practices were generally developed to be effective against standard sentinels of disease risk (e.g., coliforms, *Salmonella* spp.), rather than for the control of antibiotic resistance. For example, the United States Food and Drug Administration (FDA) Food Safety and Modernization Act (FSMA) recommends composting manure prior to amending soil as a best management practice to decrease the transfer of pathogens to crops (FDA, 2015). Previous research emphasizes that composting can effectively reduce fecal indicator organisms, bacterial pathogens, and up to 100% of detected antibiotics (Storteboom et al., 2007; Wu et al., 2010; Ray et al., 2017; Zhang M. et al., 2019). However, observed reductions of pathogens and/or indicator bacteria do not necessarily ensure a subsequent decrease in ARBs and/or ARGs (Storteboom et al., 2007; Edrington et al., 2009; Xu et al., 2019). The potential for ARGs linked to mobile genetic elements (MGEs) to persist and propagate in soil amendments is especially of concern, as MGEs can facilitation ARG sharing and amplification among pathogenic and non-pathogenic ARBs (Partridge et al., 2018). In particular, class 1 integrons have been identified as key markers of anthropogenic sources of ARGs and their potential to spread across species (Gillings et al., 2015). Other MGEs, such as transposases, have been linked to HGT of ARGs through co-occurrence analysis of agricultural soil using high throughput quantitative polymerase chain reaction (HT-qPCR) (Xie et al., 2018).

Tracking movement of ARGs through environmental systems is complicated due to inherent microbial diversity (Finley et al., 2013; Graham et al., 2019) and the lack of comprehensive, or consistent, analytical strategies. Multiple quantification methods are used to evaluate antibiotic resistance in agroecosystems (culture-, molecular-, etc.) and, as a consequence, analytical endpoints are not directly comparable (Davies and Davies, 2010; Rocha et al., 2019, 2020; Pelley, 2020; Suttner et al., 2020; Wind et al., 2020). Given the biological complexity inherent in the acquisition, amplification, and mobilization of ARGs, the concept of the "resistome," i.e., all ARGs carried across a microbial community, is often useful in assessing resistance potential (Wright, 2007). Shotgun metagenomic sequencing is an attractive approach for avoiding biases of culture- and qPCR-based methods and capturing the full range of genes, including ARGs, MGEs, metal resistance genes, and taxonomic markers, across a bacterial community.

A handful of studies to date have begun to apply metagenomic sequencing toward characterizing the influence of pre-harvest

factors on amendment (Gou et al., 2018; Zhang et al., 2020), soil (Fang et al., 2015; Chen et al., 2019; Macedo et al., 2021), and vegetable (Guron et al., 2019) resistomes, but most studies only examine such factors in isolation. Gou et al. (2018) observed that composting livestock-manure decreased resistome diversity as compared to raw (source) manure prior to land application. However, examinations of soils amended with either inorganic or organic-derived fertilizers indicates that, although there may be an initial increase in some measures of resistance, agricultural soil resistomes generally return to background levels after a single growing season (Gou et al., 2018; Han et al., 2018; Chen et al., 2019; Zhang Y. J et al., 2019). In contrast, after 25-years of repeated pig manure fertilization to agricultural soils in Hunan Province, China, Xie et al. (2018) did observe that organic fertilization altered abundance and diversity of soil resistomes compared to control and inorganically fertilized soils. Interestingly, there was no observed difference in taxonomic α-diversity (i.e., total unique operational taxonomic units) between the different soil treatments (Xie et al., 2018), suggesting ARG mobilization might have contributed to a disconnect between the resistome and microbial community composition. Examinations of interactions between agricultural soils and the surfaces of crops *via* metagenomics have been limited to a single growing season, suggesting that soil amendment type can affect crop surface resistome compositions, e.g., specific drug classes and specific ARGs (Tien et al., 2017; Guron et al., 2019). For example, in a greenhouse study, Guron et al. (2019) observed that lettuce grown in soils amended with raw manure carried 2.3 and 11.1 times more aminoglycoside- and triclosan-associated ARGs on their surfaces as compared to those grown in soils amended with composted manure. Field-scale examination of the fate and transport of ARGs through agroecosystems, from manure collection and management through soil amendment and crop harvest, is essential in order identify critical control points that represent the greatest risk of antibiotic resistance spread and further refine management practices as necessary (Bengtsson-Palme, 2017; Vikesland et al., 2017; Larsson et al., 2018).

Demand for livestock-origin organic fertilizer (e.g., manure, slurry, and compost) is anticipated to continue to increase in order to support increasing global agricultural demands (Byrnes and Bumb, 2017). The aim of this study was to perform an integrated assessment of the influence of multiple preharvest factors as hypothetical critical control points for the control of lettuce resistomes at field-scale. Potential control points included: antibiotic use in cows generating manure-derived (i.e., organic) fertilizers, composting versus stockpiling manure, and amendment of soil with organic versus inorganic fertilizer. Shotgun metagenomic sequencing analysis served to characterize: (1) composition of resistomes (ARG abundance and diversity); (2) ARG-MGE co-occurrences; (3) ARG linkages with key taxonomic indicators, and (4) relative potential for ARGs to mobilize to pathogens (i.e., "resistome risk") across amendments, amended soils, and lettuce phyllosphere samples. The integrated analysis carried out in this study provides a means to simultaneously compare and optimize the roles of various on-farm management practices for mitigating the potential for antibiotic resistance to spread through the food chain.

MATERIALS AND METHODS

The aims of this study were achieved through integrated analysis of previously published archival soil and lettuce phyllosphere metagenomic sequencing datasets described in Wind et al. (2020) and Fogler et al. (2019), as well as metagenomic sequencing of amendments carried out specifically for the purpose of this study. The amendment, soil and crop surface shotgun metagenomic sequences used in this study are publicly available (NCBI BioProject: PRJNA506850). The broader experimental design is described in the following sections.

Generation of Manure, Composting, and Stockpiling

The methods for cattle selection and manure collection were described previously (Ray et al., 2017; Wind et al., 2018). Briefly, healthy dairy cows (n = 10) selected for their similar body weights, milk yield, and with no history of antibiotic treatment were treated with pirlimycin or cephapirin according to label recommendations. Beginning on 3 days following antibiotic administration, all manure (mixed feces and urine) produced was collected for six consecutive days to generate the "antibiotic manure." To generate "control manures," manure was collected in the same fashion from similar cows receiving no antibiotics. Antibiotic manure was subject to stockpiling [stacked outdoors in large metal bins (6 m × 2.4 m × 1.2 m)] and both antibiotic and control manures were composted in piles (5.8 m × 0.9 m × 0.8 m) in similar bins and aerated *via* a perforated polyvinyl chloride (PVC) pipe system and air pump at the bottom of the bin. Stockpiled manure and compost samples were collected at two time points: initial compost/stockpile mixture (0 day) and at the time of compost completion (63 days, **Supplementary Figure 1**). Thermophilic conditions (US FDA FSMA-recommended temperature of > 55°C for at least 3 days) was reached in both control and antibiotic composts on day 2 (FDA, 2015). Samples were collected from multiple locations are various depths of the manure and compost piles (3–4 m^3) using a soil probe.

Field Study Conditions and Lettuce Cultivation

Lettuce (*lactuca sativa*) was cultivated during the 2016 growing season (March–July) at the Virginia Tech Urban Horticulture Center (UHC) in Blacksburg, VA, United States on soil categorized as a Remus fine sandy loam and documented to have remained fallow for at least a decade. Lettuce was studied as it a popular leafy crop that is often eaten raw in the United States and has been the focus of recent foodborne illness outbreaks (CDC, 2021). Individually-bordered soil plots (3 m × 3 m) were constructed as described in Wind et al. (2018) to compare lettuce cultivation conditions in triplicate with two organic soil amendments: stockpiled antibiotic manure and antibiotic

compost. All organic amendments were applied at appropriate agronomic rates (6.72 Mg ha^{-1}; Day 0), and supplemental inorganic fertilizer was added to reach the required nutritional content for optimal lettuce growth. Three additional plots received inorganic fertilizer (N-P-K) only, at rates recommended for the optimal growth of lettuce (140-112-112 kg ha^{-1}; Virginia Cooperative Extension, 2015) and three more plots served as a no amendment control (**Supplementary Figure 1**). Lettuce was grown in a mix of peat and perlite for 8 weeks, with inorganic fertilizer to avoid exogenous ARB or ARG contamination, and transplanted to the plots 30 days after the amendments were applied. The no amendment control plots were not subject to lettuce cultivation due to insufficient nutrients for crop growth. Further study details, including descriptions of plot construction, field preparation, lettuce cultivation, and patterns of culturable fecal ARB, heavy metals, and metal resistance genes in soils (Wind et al., 2018, 2020), release of targeted ARGs quantified *via* qPCR in surface runoff during natural storm events (Jacobs et al., 2019), and recovery of ARGs by qPCR from harvested vegetables (Fogler et al., 2019) are available in prior reports.

Soil samples were collected at three time points relative to amendment application: background soil conditions (−1 day), amendment application (0 days), and lettuce harvest (67 days, **Supplementary Figure 1**). In-depth description of soil collection and processing are available in Wind et al. (2020). In brief, at each sampling time, four 2-cm soil cores from a 5-cm depth were collected from each soil plot at a randomly generated location and homogenized into one sample on site. Lettuce plants were collected from each plot at the time of harvest (67 days, **Supplementary Figure 1**) and immediately processed to recover surficial microbes and their DNA from the phyllosphere (Fogler et al., 2019).

DNA Extraction

Amendment and soil samples were stored at −80°C until DNA extraction occurred, whereas lettuce sample DNA was extracted immediately upon harvest from the field (<2 h). DNA was extracted from 0.5 g compost or manure samples of the homogenized amendment samples using the FASTDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH, United States) which followed the same extraction methods used previously described for this soil (Wind et al., 2020). Inhibition was minimized using the ZYMO PCR OneStep PCR Inhibitor Removal Kit (ZYMO Research, Irvine, CA, United States). To obtain microbial DNA from lettuce, leaves were washed in a sterile 0.1% peptone and 0.1% Tween solution and hand massaged to remove surficial phyllosphere bacteria, while avoiding release of plant DNA (Fogler et al., 2019). The subsequent lettuce wash diluent was filtered through 0.22-μm filters (EMD Millipore, Merck Group, Darmstadt, Germany), which were subject to the same DNA extraction and clean-up procedures described above for manure, compost, and soil samples. All DNA extracts were stored at −80°C for up 24 months prior to subsequent metagenomic analyses.

Shotgun Metagenomic Sequencing Analysis

Amendment (n = 14), soil (n = 27), and lettuce (n = 12) DNA extracts were subject to shotgun metagenomic sequencing (**Supplementary Figure 1** and **Supplementary Table 1**). For most of the treatment and time factors, samples were sequenced in triplicate. The amendments prior to manure management (0 day) and the soils prior to amendment (−1 day) were considered homogenous across the treatments and were grouped together as the control replicates. Samples and corresponding replicates were selected for sequencing depending on treatment and time effects of interest. Details on samples subject to metagenomic sequencing are reported in **Supplementary Table 1**.

Amendment metagenomes were prepared using the Nextera XT library prep (Illumina, San Diego, CA, United States) and sequenced on an Illumina NextSeq 500 (2 bp × 75 bp, paired-end) at the Scripps Center for Computational Biology and Bioinformatics (La Jolla, CA, United States), which is the same preparation and sequencing methods of the amended soils. The lettuce samples were prepared using the Accel-NGS 2S DNA kit (SwiftBio, Ann Arbor, MI, United States) and sequenced on an Illumina HiSeq 2500 (2 bp × 100 bp, paired-end) at the Biocomplexity Institute of Virginia Tech (Blacksburg, VA, United States). Complete sequencing preparation details are available in Wind et al. (2020) and Fogler et al. (2019). Raw reads and associated metadata were uploaded to the NCBI BioProject: PRJNA506850 for each pre-harvest critical control point of interest.

ARG Annotation and Normalization

Paired-end raw sequences were processed using MetaStorm using the read-matching pipeline (Arango-Argoty et al., 2016). On average, each amendment, soil, and lettuce sample contained 24,650,830, 23,610,275, and 12,292,992 raw reads (see **Supplementary Table 2** for all metagenomic sample sequencing metrics), respectively, after TRIMMOMATIC quality filtering (< 0.16% reads dropped *via* filtering, Bolger et al., 2014). ARGs were annotated against the Comprehensive Antibiotic Resistance Database (CARD v2.0.1; Jia et al., 2017) with at least 80% identity using the read match sequence pipeline of MetaStorm. Known housekeeping genes were removed prior to gene abundance analysis (Wind et al., 2020). To account for variable read and gene lengths, gene counts analyzed from MetaStorm were normalized to 16S rRNA gene copy number to report relative abundance using Greengenes (DeSantis et al., 2006; Li et al., 2015). A list of clinically-relevant ARGs, defined as genes that confer resistance to key clinically-important antibiotics (e.g., peptides, glycopeptides, beta-lactams, and macrolides), were further analyzed (Keenum et al., 2021).

ARG-MGE Co-occurrence and Resistome Risk Analysis

De novo assembled scaffolds were generated and annotated using the MetaStorm assembly pipeline for downstream ARG-MGE co-occurrence analyses (Arango-Argoty et al., 2016). In brief, MetaStorm uses IDBA-UD (Peng et al., 2012) to assemble

scaffolds and employs PRODIGAL (Hyatt et al., 2010) to predict genes within each scaffold. Downstream taxonomic and functional gene annotation details are described in-depth in Arango-Argoty et al. (2016). Assembled scaffolds averaged 1,102, 504, and 1,066 bp for amendments, soils, and lettuce surfaces, respectively. Coverage of assembly varied among each sample type, from 0.2–74.5% of the total number of contigs. The average coverage of assembly of amendment and lettuce samples was 36.8 and 41%, respectively. This was much higher than the average assembly of soil samples, which was only 0.64% (Wind et al., 2020). Estimated average coverage of the amendment, soil, and lettuce samples were 40, 20.62 and 63%, as determined *via* Non-pareil (Rodriguez-R and Konstantinidis, 2014). Due to low assembly of the soil samples, which is to be expected giving its high complexity (Howe et al., 2014; Myrold et al., 2014; Chen et al., 2019); the ARG-MGE co-occurrences along each point of the pre-harvest vegetable production are most comparable within each sample type (i.e., amendment, soil, vegetable surface). Using MetaCompare, an emerging metagenomic risk computational tool (Oh et al., 2018), ARG-MGE co-occurrences were analyzed from assembled contigs and hypothetical resistome risk scores were calculated for each metagenome. The risk score, Q, was calculated by evaluating the occurrence of a scaffold that contained one or more co-occurring ARG, MGE, or pathogen gene marker (Oh et al., 2018). To reduce influence of potentially incorrectly assembled contigs, a threshold was set in which only ARG-MGE contig that were identified in two or more sequence replicates per sample type (i.e., amendment, soil, and vegetable surface) were included. This threshold was not achieved for any soil samples and they were therefore excluded from further assembly-based analysis.

Taxonomic Analysis

Taxonomy was annotated *via* Kraken2 (Wood and Salzberg, 2014). Once taxonomy was assigned, genera abundance was determined *via* Bracken [Bayesian Re-estimation of Abundance with KrakEN, (Lu and Salzberg, 2020)]. Genera relative abundance files produced *via* Bracken were analyzed for microbial communities using R "vegan" package (Oksanen et al., 2015).

Statistical Analysis

All statistical analyses were conducted using R (v. 4.0.3, R Core Team, 2020) with the alpha threshold of 0.05 ($p < 0.05$) applied as the level of significance. All metagenomic datasets were confirmed as non-parametric *via* the Shapiro-Wilkes normality test ($p < 0.05$). ARG relative abundance varied based on gene amplified and a Kruskal-Wallis rank sum test followed by a *post hoc* Dunn's test assessed differences between time points, treatments, and among the amendment, soil, and lettuce surface metagenomes. On the assumption that prior to amendment application the field plots represented the same condition, the background (−1 day) soil metagenomic samples from the inorganic chemical fertilizer control, compost with antibiotics, and stockpiled manure with antibiotics amended soils were grouped with the no amendment control amended soil samples at the time of background, which resulted in six replicate samples during the statistical analysis (**Supplementary Table 1**). Bray-Curtis dissimilarity and a one-way analysis of similarities (ANOSIM) statistical analysis was used to compare resistome profiles (ARG type and ARG abundance).

The microbial community alpha diversity was measured using Shannon diversity index, which takes species proportional abundances into account for species richness. A one-way analysis of variance (ANOVA) followed by a *post hoc* Tukey HSD test assess differences among the amendment, soil, and lettuce surface microbial communities and subsequent treatment and time effects if needed at each point. Indicator species analysis of the significantly differentially abundant species was measured using point biserial correlation coefficient from the R package "indicspecies" (Cáceres, 2013). Figures were produced using R packages "ggplot2," "VennDiagram," and "networkD3" (Wickham, 2009; Gandrud et al., 2017; Chen, 2018).

RESULTS

ARG Profiles

Comparison of Resistomes Across Sample Types

The number of unique ARGs and their relative abundances differed significantly among the three sample types (i.e., amendment, soil, and lettuce; ANOSIM, $R = 0.85$, $p < 0.0001$; **Figure 1**) across the pre-harvest vegetable production chain. Of the 854 unique ARGs annotated across all metagenomes, 302 were detected in all three sample types (**Figure 2A**) and a total of 121, 55, and 114 ARGs were unique to the amendment, soil, and lettuce samples, respectively (Shannon diversity, $p < 0.0001$). Most importantly, 123 of the total ARGs were found to be clinically relevant to pathogens of concern for humans (**Figure 2B**). The greatest number of clinically-relevant ARG types were annotated in samples collected from the lettuce surfaces, with 16 clinically-relevant ARGs common among all amendment, soil, and lettuce samples. The lettuce grown in compost with antibiotics carried the highest clinically-relevant ARG relative abundance, while the lettuce grown in inorganic fertilizer had the least. The clinically-relevant ARGs with the greatest relative abundance overall were *bla*CARB-14, *bla*OXA-322, and *van*A. ARG Shannon diversity was significantly different when comparing amendment and lettuce samples ($p < 0.001$) and the soil and lettuce samples ($p < 0.0001$); but there was no significant difference between the amendment and soil samples ($p = 0.07$).

Comparison of Amendment Resistomes Over Time

Resistome profiles of samples collected from stockpiled manure were distinct from those of the two composts (ANOSIM, $R = 0.595$, $p < 0.001$; **Supplementary Figure 2**). Initial resistome profiles (0 day) of the compost pile and stockpiled manure also were different compared to those at the end (63 days; ANOSIM, $R = 0.462$; $p = 0.011$; **Supplementary Figure 2**). The total ARG relative abundances were greatest in the compost with antibiotic, relative to the control compost or stockpiled manure (Kruskal-Wallis, $p < 0.001$; *post hoc* Dunn Test, $p < 0.001$) at both 0 day and 63 days (Kruskal-Wallis, $p < 0.001$). The control compost

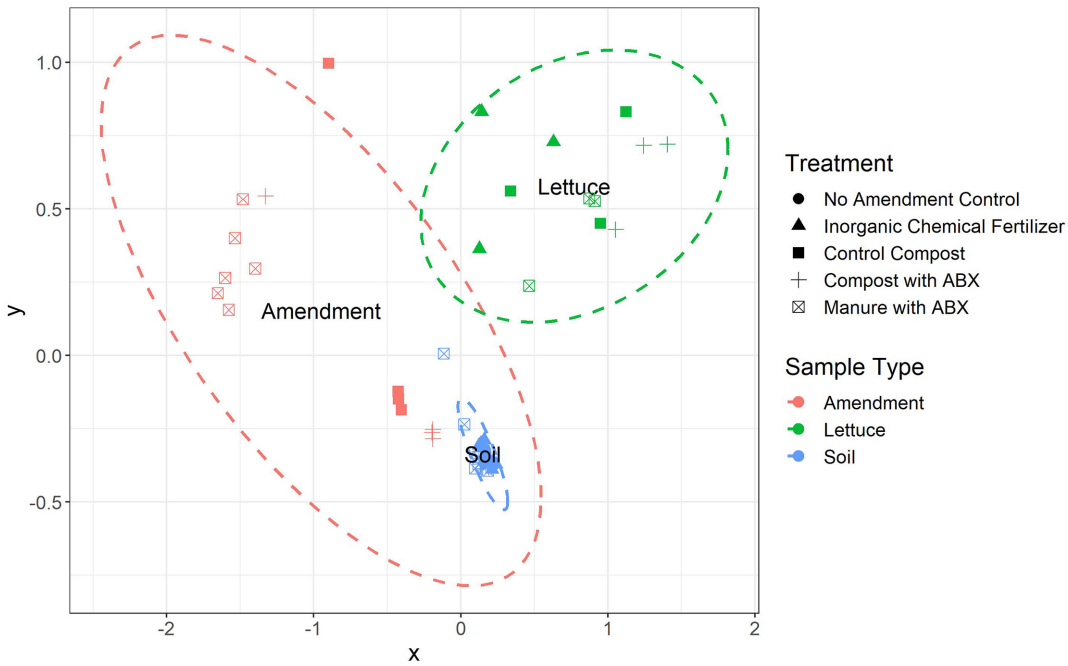

FIGURE 1 | Non-metric multidimensional (NMDS) scaling of the Bray Curtis dissimilarity distances highlight the distinct ARG profiles among amendment, soil, and vegetable samples representing the pre-harvest vegetable production chain (ANOSIM, $R = 0.85$, $p < 0.001$). ARGs were annotated against the CARD v2.0.1 database.

and stockpiled manure with antibiotics were indistinguishable in terms of total ARG relative abundances (*post hoc* Dunn test, $p = 0.63$; **Supplementary Figure 3**).

The ARG classes with the greatest relative abundance among the amendments were multidrug, tetracycline, macrolide-lincosamide-streptogramin (MLS), and glycopeptide (**Supplementary Figure 3**). The three most abundant ARGs were identical between the compost with and without antibiotics at 63 days (*par*Y, *mtr*A, and *van*RO) (**Supplementary Figure 4**), although the most abundant ARGs in the stockpiled raw manure were *tet*W, *Inu*A, and *efr*B (**Supplementary Figure 4**). Overall, 642 ARGs were annotated across the amendment samples and almost half, 316 ARGs, were common among all samples (**Supplementary Figure 5**). The control compost contained the most unique ARGs ($n = 555$), followed by the compost with antibiotics ($n = 475$) and the stockpiled manure ($n = 405$). The control compost also had the most ARGs distinct to a given sample type ($n = 88$), followed by the compost and stockpiled manure with antibiotics, with 43 and 34 distinct ARGs, respectively.

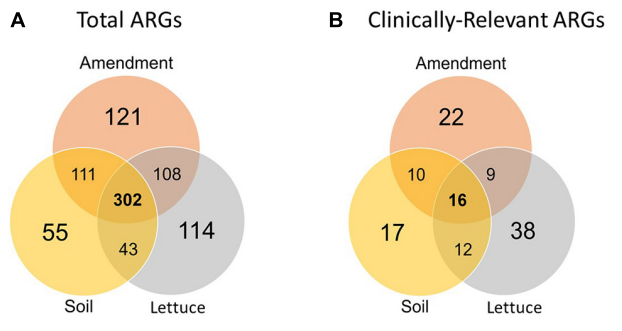

FIGURE 2 | Number of unique **(A)** total and **(B)** clinically-relevant ARGs annotated *via* the CARD v2.0.1 database in the amendment, soil, and lettuce surfaces samples analyzed across the pre-harvest vegetable production chain.

Comparison of Soil Resistomes With Amendment Type and Time

There were distinct differences in resistomes between the no amendment control soils and stockpiled manure-amended soils immediately following amendment (0 days); however, neither inorganic chemical fertilizer or composts amended soils measurably altered the respective soil resistome profiles relative to the control soils. By the time of lettuce harvest (67 days), all soil resistomes were similar (Wind et al., 2020), regardless of initial amendment condition. The total ARG relative abundance in all the soils across all amendment types were much greater than what was measured in the corresponding amendments, indicating that there were substantial naturally-occurring ARGs in the soil before the amendments were applied.

Comparison of Resistomes in Lettuce Grown With Different Amendments

The total ARG relative abundance in samples collected from the surface of lettuce grown in soils amended with compost or raw manure was twice that of the total ARG relative abundances

recovered from the surfaces of the lettuce grown in soils amended with inorganic chemical fertilizer (Fogler et al., 2019). In all cases it is worth noting that the total ARG relative abundance levels recovered from the lettuce surfaces were up to 50% greater than in the amended soils in which they were grown. For example, lettuce grown in soil amended with stockpiled manure carried an average of 2 ARGs/16S rRNA gene, while the corresponding soil contained an average of 1.3 ARGs/16S rRNA gene, immediately after mixing with the amendments.

Co-occurrence of ARGs

Following assembly of metagenomic data, there were 734, 22, and 4,761 unique ARG-MGE co-occurrences among the amendment, soil, and lettuce samples, respectively. After applying the threshold of ARG-MGEs contigs occurring at least twice in a given data set (i.e., across metagenomes from the same sample type), there were 460, 2, and 1,221 unique ARG-MGE co-occurrences among the assembled contigs from amendment, soil, and lettuce samples, respectively (**Figures 3A–C**).

Of the 460 unique ARG-MGE co-occurrences assembled within the amendment metagenomes, the most frequent one was between the rifamycin drug class and plasmids. The ARG *rob*A was the most prevalent on contigs that were also annotated with plasmid markers (**Figure 3A**). Due to low assembly for the soil samples, only 2 unique ARG-MGE co-occurrences were detected, both in the stockpiled manure amended soils (**Figure 3B**). The most frequent ARG-MGE co-occurrences in soil was between the aminoglycoside drug class and plasmids. The ARG *APH*(6)-id, which encodes resistance through antibiotic inactivation, was the most prevalent on plasmids annotated in the soils. Of the 1,221 unique ARG-MGE co-occurrences assembled on the lettuce surfaces, the most frequent co-occurrence was between the multidrug class and plasmids. The ARGs *am*A, *bac*A, and *erm*B were the most frequently co-occurring on the plasmids annotated on the lettuce surfaces.

Relative Resistome Risk Comparison

Among the analyzed samples, all amendment and lettuce samples contained scaffolds that were annotated with co-occurring ARGs, MGEs, and human pathogen markers, which increased their resistome risk scores. Overall, the resistome risk scores were significantly different among the three sample types (Kruskal-Wallis, $p < 0.001$; *post hoc* Dunn, $p < 0.001$ for all iterations; **Supplementary Figure 6**). The average resistome risk scores were 26.9, 22.6, and 60.1 for the amendments, soils, and lettuce, respectively (**Supplementary Table 2**). On average, there were 375, 13, and 1,420 contigs annotated with pathogen gene markers among the three sample types, respectively. Within each sample type, the resistome risk scores were comparable, regardless of associated experimental conditions or time points (Kruskal-Wallis, $p > 0.05$ for all iterations; **Supplementary Figure 7**).

Taxonomic Composition Comparison

Microbial communities recovered from the amendment, soil, and lettuce surface samples were distinct in terms of taxonomic composition (ANOVA, $p < 0.001$; ANOSIM, $R = 0.156$, $p = 0.0104$; **Figure 4**, **Supplementary Figure 8**) and Shannon diversity (*post hoc* Tukey HSD, $p < 0.001$ for all iterations) across the pre-harvest vegetable production chain. Both composts and raw manure amendments were also distinct from each

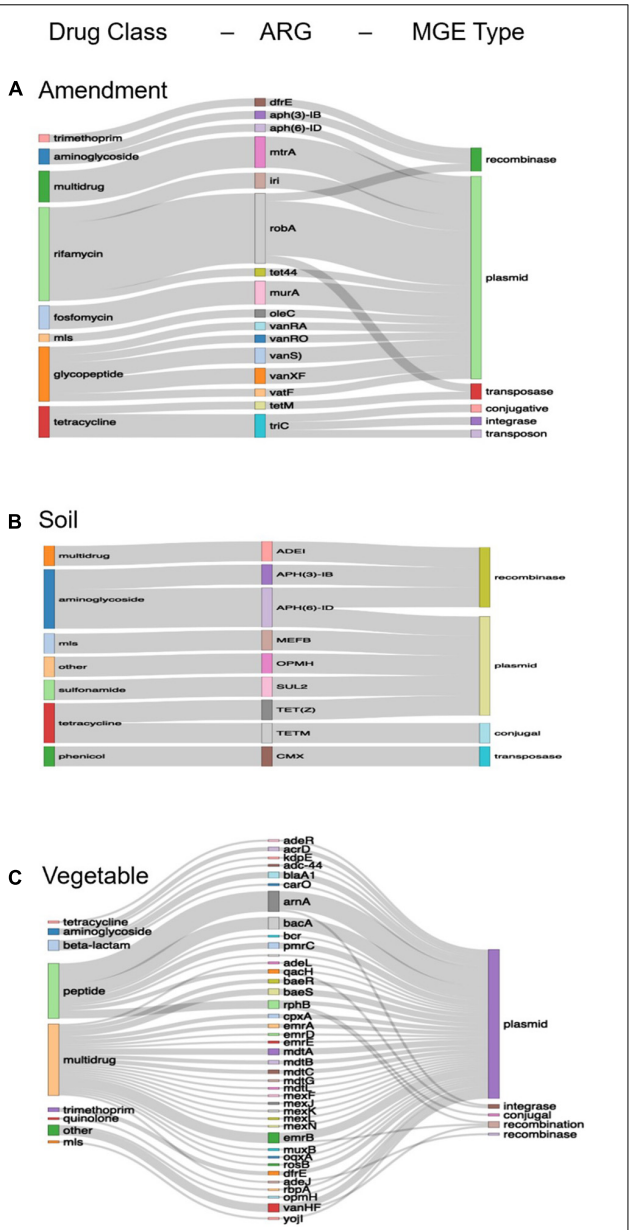

FIGURE 3 | Unique ARG-MGE co-occurrences annotated among assembled contigs of the **(A)** amendment ($n = 460$), **(B)** soil ($n = 22$), and **(C)** lettuce ($n = 1,221$) categorized by drug resistance class, ARG type, and MGE type. The relative height of the bars for each category is proportional to the number of times the category was annotated in the samples and the thickness of the connecting ribbons corresponds to the frequency of the combination. For visualization purposes, the amendments and lettuce represent 460 and 1,221 unique ARG-MGE co-occurrences, respectively, meeting the threshold of having been annotated on at least two contigs within the sample type. However, the soil **(B)** represents all 22 unique ARG-MGE co-occurrences (without applying the threshold of 2 + common ARG-MGE contigs as there were none).

other in terms of Shannon diversity (ANOVA, $p = 0.0114$), but there was not a significant alpha diversity difference in taxonomic composition among the soil samples or among the lettuce samples in terms of the amendment conditions (ANOVA, $p = 0.0687$ and 0.287, respectively) or at different time points (ANOVA, $p = 0.795$).

Bray-Curtis dissimilarity matrixes also indicated differences in beta diversity of taxonomic profiles among the amendments, soils, and lettuce surface samples. Further, differences could be discerned among the amendment samples and soil samples according to corresponding experimental conditions (ANOSIM, $p < 0.001$ for both). Contrary to the resistome analysis, there was no Bray-Curtis dissimilarity difference in taxonomic profiles between the microbial communities before (0 day) versus after (63 days) amendment treatment (ANOSIM, $p = 0.613$) or in the soils over the growing season (−1, 0, and 67 days) (ANOSIM, $p = 0.147$).

Among all the agricultural metagenomes, 567 unique genera were identified, with 25% uniquely found in each sample type. Indicator species analysis *via* the point biserial correlation coefficient highlighted the significant differentially abundant genera at and between the amendments, soils, and lettuce surfaces samples ($p < 0.01$ for all iterations, **Supplementary Table 4**). Amendments had the most diverse set of differentially abundant genera, including 197 unique genera not found in other sample types. Soil and lettuce samples contained 129 and 31 genera that were differentially abundant, respectively. The top indicator genera (i.e., bacteria whose abundances were significantly different between the sample types) for the amendments were *Parageobacillus, Aneurinibacillus,* and *Belliella* ($p < 0.0001$ for all). The top indicator genera of the amended soils were *Bradyrhizobium, Mycobacterium,* and *Rhodoplanes* ($p < 0.0001$ for all). The top indicator genera of the lettuce surfaces were *Pseudomonas, Pantoea,* and *Massilia* ($p < 0.0001$ for all). *Mycobacterium* identified in the soil and the *Pseudomonas* and *Pantoea* identified on the lettuce are considered to be opportunistic pathogens with respect to human health. On average, 15 and 12% of the classified bacteria may be considered pathogenic among the amendment and soil samples, respectively. The most abundant potential pathogenic genera included *Pseudomonas* in the composts and *Clostridium* and *Enterococcus* in the manure amendments, and *Pseudomonas* and *Mycobacterium* in the soils. On average, 36.7% of the classified bacteria may be considered potentially pathogenic among the lettuce surface samples. The most abundant potential pathogenic genera, up to 90% on the majority of lettuce samples, included *Pseudomonas*.

DISCUSSION

This integrated study provided a comprehensive assessment of various pre-harvest factors as critical control points shaping resistomes in vegetable production systems that is not possible from examining points in the system individually. The results highlight the importance of holistically considering multiple control points at which antibiotic resistance might spread, or be controlled, through a vegetable production system. Such points should be considered further as candidates for targeted mitigation strategies; including the use of antibiotics, composting manure prior to land application, and amendment of soils with organic fertilizers, which were all observed to influence the lettuce resistomes in some manner. We found that where effects of these factors were readily measurable in the resistomes of the organic amendments and the harvested lettuce, they were only transiently measurable within the soil following amendment. Remarkably, total ARG relative abundance increased from amendment to soil to lettuce surface across all conditions, except in the inorganic chemical fertilizer condition. The results highlight that, even if attenuation of resistance markers appears to be occurring during manure management and after soil application, that there still could be lingering concerns regarding the potential for ARBs and ARGs to be selectively transferred and enriched on the crop surface.

Effects of Manure Collection During Antibiotic Administration

Freshly collected manure (0 days) from the cows administered pirlimycin or cephapirin contained a higher total ARG relative abundance than manure collected from controls cows. Further, antibiotic administration had a lingering effect on the resistome after composting or stockpiling the manure for 63 days (**Supplementary Figures 3, 4**). Stockpiled manure from cows treated with antibiotics contained substantially more ARG-MGE co-occurrences compared to the compost derived from control cows, suggesting that lack of antibiotic use and composting together reduced ARG mobility. Within the stockpiled manure, the *TET*M to transposon (*Clostridium difficile*) co-occurred 12 times and was the most abundant ARG-MGE annotated across the pre-harvest vegetable production chain. However, the overall resistome risk score of the control compost was higher than both the stockpiled manure and antibiotic compost. The reasons for this are unclear, but could relate to the higher taxonomic beta diversity observed in the control compost (**Figure 3A**) supporting a greater variety of ARG-MGE and potential ARG-MGE-pathogen contigs which aligns with the taxonomic profiles indicating higher beta diversity of the control compost. This finding is counter to the expectation that antibiotic use elevates the risk of spreading resistance.

Effects of Composting

The average total ARG relative abundances were elevated in composted manure as compared to stockpiled manure after 63 days of manure management (i.e., composting). This agrees with previous studies that have observed an increase in ARG abundance following composting (Storteboom et al., 2007; Xu et al., 2019; Keenum et al., 2021). Although composting is recommended to mitigate pathogens (FDA, 2015), the benefits might not extend to ARG attenuation. Interestingly, the control compost was the most diverse in terms of number of ARGs annotated (**Supplementary Figure 5**) among the three amendments, suggesting that the antibiotics had a lingering effect of suppressing taxonomic diversity (**Figure 4** and

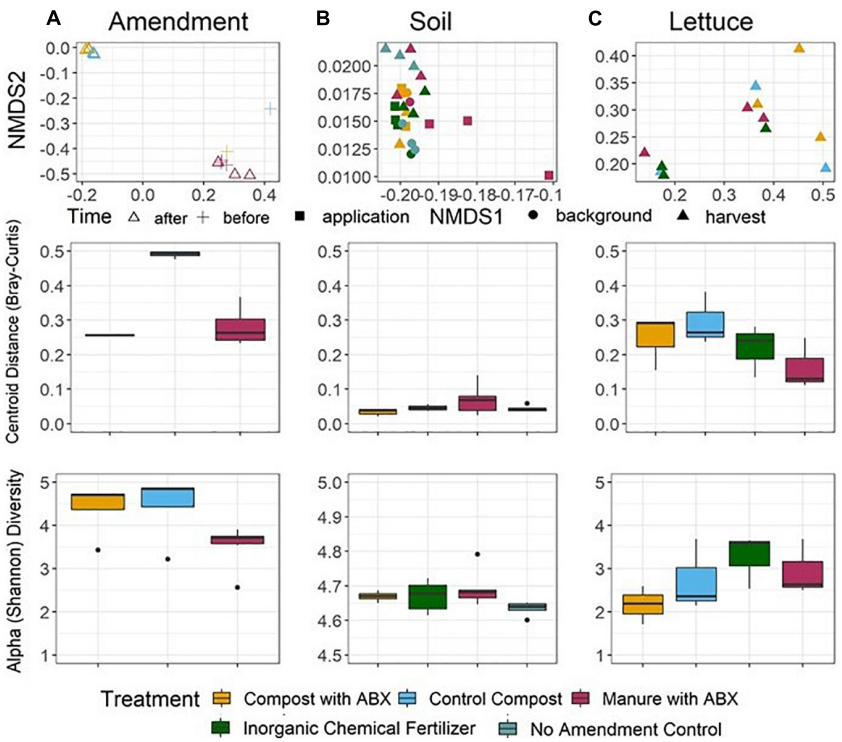

FIGURE 4 | Taxonomic alpha (bottom row) and beta (top and middle rows) diversity across the microbial community's pre-harvest vegetable production chain for the **(A)** amendment, **(B)** soil, and **(C)** lettuce. Alpha diversity was calculated as Shannon diversity. Beta Diversity was determined from Bray-Curtis dissimilarity matrices and visualized as NMDS (top panel) and distance to euclidean centroid (middle panel). Taxonomy was annotated using NBCI taxonomy id using Kraken2 as the metagenomic classification algorithm and Bracken2 to compute species abundance.

Supplementary Figure 8), which carried over into the observed diversity of the resistome. However, the higher diversity of ARGs in the control compost did not translate into higher diversity of ARGs detected in the amended soil or on the cultivated lettuce.

The most prominent classes of antibiotics that the ARGs corresponded to also differed based on manure management practice. By 63 days, the compost had higher relative abundances of glycopeptide ARGs as compared to the stockpiled manure. On the other hand, the stockpiled manure contained higher levels of MLS and tetracycline ARGs than were annotated in the composts (**Supplementary Figure 3**). The differences in ARG profiles categorized by antibiotic class at the end of manure treatment indicate that composting was able to mitigate bacteria conferring resistance to antibiotics that are clinically-important for human health (WHO, 2017). However, there was no significant difference in taxonomic profiles between the stockpiled manure and compost at 63 days, which suggests that the resistome is not merely a function of the taxonomic composition of the microbial community and that there are distinct ecological drivers (e.g., horizontal gene transfer) shaping ARG profiles during composting (Qian et al., 2016; Zhou and Yao, 2020).

Effects of Amending Soil

There were significant resistome (diversity and abundance) differences between the soil amended with stockpiled manure containing antibiotics and the no amendment control soil resistomes; but there was no difference among the total ARG relative abundances quantified between all the amended soils with respect to time (Wind et al., 2020). From this finding, we hypothesize that one growing season was not enough time to predict the long-term impact of potential amendment addition of ARGs in agricultural soils. Recent organically fertilized agricultural soil studies have identified similar patterns of ARG levels and resistomes retuning to background after a single growing season (Zhou et al., 2017; Macedo et al., 2021). Multiple years of application may be required to appreciably alter soil resistomes (Liu et al., 2021).

From a clinical perspective, the soil contained the least ARG-MGE co-occurrences compared to the other points of the pre-harvest vegetable production chain (22 soil ARG-MGE compared to the 734 and 4,761 annotated within the amendment and lettuce surface samples, respectively) and the soil was characterized by significantly lower resistome risk scores (**Supplementary Figures 6, 7**). However, it is important to consider that the lower assembly rate achieved due to the soil complexity likely influenced the resistome risk score. Still, it should be pointed out that the only ARG-MGE co-occurrences that were found in soil were in the stockpiled manure amended condition. This suggests that the stockpiled manure amended soils may ultimately contain more ARG-MGE co-occurrence than the other soils if deeper sequencing is utilized in the future to explore the amended soils.

Lettuce Surface Resistomes

The total ARG abundance recovered from lettuce grown in manure- or compost-amended soil was 2−3 × greater than that collected from lettuce grown in soils amended with inorganic fertilizer. This demonstrated a clear effect of organic soil amendments on lettuce resistomes. Notably, even though measurable differences in soil resistomes associated with organic amendments were lost in soil, they re-emerged on the lettuce. Further, although composting had resulted initially in a larger diversity of ARGs compared to the manure (**Supplementary Figures 3, 5**), there did appear to be benefits in terms of the lettuce grown in control compost-amended soils carrying the lowest ARG relative abundance among the organically-amended soil conditions (Fogler et al., 2019). It is important to highlight that although manure management had an effect on the control compost in terms of ARG abundances compared to the stockpiled manure, no effect was seen when comparing the lettuce grown in the soils amended with control compost to lettuce grown in the soils amended with compost derived from antibiotic-administered cow manure. Lettuce grown in soils amended with compost generated from the manure of treated cows had higher total ARG relative abundance and risk scores compared to the stockpiled manure. In a recent study, Zhang Y. J et al. (2019) similarly found that manure (poultry) application to the soil increased ARG abundance on lettuce surfaces at the greenhouse scale.

Remarkably, when comparing the three sample types (organic amendment, soil, lettuce), there were several concerning features of lettuce resistomes that were identified. For example, the highest abundance of clinically-relevant ARGs (n = 75) and the most ARG-MGE co-occurrences were found in lettuce samples. The *Tri*C to plasmid (*Ralstonia solanacearum*) co-occurred 10 times on the lettuce surfaces grown in the compost and was the second most abundant ARG-MGE. The higher number of ARG-MGE co-occurrences and, at times, higher pathogen detection on the lettuce surface contigs (**Supplementary Table 3**) is concerning from a human health perspective and is an area needing further research due to the potential for horizontal gene transfer (Zhang Y. J et al., 2019). Recent studies have focused specifically on vegetable surface resistomes and have found that post-harvest sanitization practices, including irradiation and washing in water containing sanitizers, reduces surface ARGs (Dharmarha et al., 2019).

Pre-harvest Conditions on Taxonomic Composition

Taxonomic compositions were distinct among each of the three sample types (**Figure 4**). The most common genera detected in the organic amendments belonged to the *Firmicutes* and *Bacteroides* phyla, which is to be expected, given that these are associated with cattle gut microbiomes and fecal bacteria. The top soil genera belonged to the *Proteobacteria* and *Actinobacteria* phyla, which are known to contain pathogenic bacteria relevant to agricultural and terrestrial systems. The top vegetable genera also corresponded to the *Proteobacteria* phyla. Although the vegetables surfaces carried the least indicator genera (n = 31), it should be noted that most of the ones highlighted here are classified as opportunistic pathogens with environmental niches [e.g., *Pseudomonas* (skin, ear, and lung infections), *Panteo* (septic arthritis), and *Massilia,* which poses risk of enteric infections]. The amendment and soil microbial communities were the most similar, containing 124 indicator genera that were significantly associated between the two points ($p < 0.001$). The top three genera shared between amendments and soils included *Spirochete, Acetobacter,* and *Leptospirillum.* Soil and vegetable microbial communities were the least similar to each other, containing only five indicator genera that were significantly associated between the two points ($p < 0.001$). The top three genera between the soil and vegetable surfaces included *Nocardioides, Methylorubrum,* and *Pseudarthrobacter*. The common genera between the pre-harvest vegetable production chain points may be indicative of a transfer of specific microbial taxa, including pathogens, within agroecosystems. Surprisingly, lettuce surface samples had on average almost 3 × and 2.5 × greater percent of the classified bacterial abundance that could be considered pathogenic than the soil and amendments, respectively (**Supplementary Text 1** for list of pathogens). Further microbial community analyses are needed to connect microbial community diversity and ARG attenuation or proliferation, and deeper taxonomic analysis than the genera level may provide insight to potential pathogenicity which will allow for bacterial host identification across the pre-harvest vegetable production chain. Other studies have indicated need to examine this more closely specifically in agricultural soils (Cycoń et al., 2019; Lopatto et al., 2019) and on vegetable surfaces (Zhang Y. J et al., 2019; Shen et al., 2021).

Limitations

To our knowledge, this study is the first of its kind to bridge the pre-harvest vegetable production chain resistomes through a controlled field-scale study applying metagenomics. However, it is critical to explicitly examine analytical and experimental limitations. With any next-generation sequencing approach, there are inherent database bias, sampling processing bias, and varying annotation thresholds to acknowledge. First, due to the archival nature of this study and changing sequencing platforms and methodologies with time, shotgun metagenomic sequencing was carried out on different Illumina instruments for the amendment and soil versus lettuce samples. While we were able to account for such differences in our analysis and interpretation, in the ideal situation, one would conduct such studies in the future with a uniform protocol. However, that will not always be possible, especially as more and more studies seek to harness data analytics toward harvesting the growing volumes of valuable sequencing information available in public databases.

Given the biological richness and physiochemical complexity of the soil ecosystem, it is perhaps not surprising that the soil samples had the lowest percent assemblies of the shotgun metagenomic data, and subsequently contained the least annotated ARGs (n = 511) compared to the amendments (n = 642) and the lettuce surfaces (n = 567). Low sequencing coverage from shotgun metagenomic data in soils is a widely

recognized issue (Myrold et al., 2014; Zaheer et al., 2018). The low sequence coverage and percent assembled contigs from soil metagenomes may have resulted in missing the key ARG-MGE co-occurrences at this specific pre-harvest vegetable production chain point. The low percentage of assembled contigs recovered from soil not only produced two to threefold less ARG-MGE co-occurrences, but also could have been a contributing factor to the low resistome risk score calculated using assembled contigs *via* MetaCompare (Oh et al., 2018). In theory, normalization to total recovered contigs in the determination of the resistome risk score should account for such differences, but this may not be the case for situations of extremely low assembly rates, which may not be representative and thus skew the calculation.

There was some variation in sequencing depth across the sample types, with vegetable samples (18 million reads) averaging ∼80% of reads obtained for amendment and soil (∼23 million reads, **Supplementary Table 2**). Still, there was almost twofold and threefold higher relative abundance of ARGs detected on the lettuce surfaces and amendments, respectively, than in soil. If sequencing coverage would have been a factor, then the opposite trend would have been anticipated. These trends are consistent for soil as soil microbial communities are inherently more diverse than any other environmental microbial community, and therefore the low coverage obtained is anticipated (Myrold et al., 2014; Zaheer et al., 2018). Additionally, the increase in assembled contigs subsequently produced more ARG-MGE co-occurrences and subsequently increased resistome risk estimates for lettuce, compared to the amendment and soil samples. A possible explanation of the increase in assembly is due to the higher sequencing coverage (∼40%) found for the lettuce samples. It is important to mention that the lettuce surface sequences had chloroplast sequences removed prior to annotation (Fogler et al., 2019). Although it is not surprising that the resistome characterized from each environmental sample type (amendment, soil, lettuce surface) was different, the magnitude of these differences was surprising, and suggests a need for future efforts to examine these inconsistencies at a more robust biological level. Overall the differences in sequencing platforms, coverage and assembly across the pre-harvest vegetable production chain points should be acknowledged and accounted for in future when comparing critical control points and environmental studies (Liang et al., 2021).

CONCLUSION AND RECOMMENDATIONS

Agroecosystems are a key reservoir of antibiotic resistance and appropriate mitigation strategies are needed to limit potential for ARGs to spread and negatively influence human and animal health. This study employed metagenomic analysis to provide an integrated assessment of amendment, soil, and lettuce resistomes and highlighted the importance of characterizing multiple hypothetical control points that shape resistome at field-scale, including antibiotic use, composting manure prior to soil amendment, soil composition, and cultivation of lettuce. The findings from the current study emphasize the importance of multiple agricultural best management practices to provide a multi-barrier approach for preventing the spread of ARGs. Antibiotic administration to animals and composting of manure were found to have measurable effects on the harvested lettuce resistomes. The soil was found to be highly stable in the composition of the resistome and corresponding resistomes and has the potential to act as a natural ecological buffer to ARG proliferation, at least following one harvest cycle. Notably, amendment application at standard agronomic rates did not alter soil resistomes or microbial community taxonomic composition after one growing season compared to background levels. Further studies are recommended to determine if there is variance among soil types in their capacity to buffer the potential for ARGs to spread.

Still, total ARG relative abundances were higher in the lettuce phyllosphere than in the soil or amendments and were highest on lettuce grown in soils receiving organic compared to inorganic amendments. This indicates that the transient impacts observed in soil only immediately after amendment had lasting impacts on the phyllosphere resistomes. Lettuce was characterized by the greatest number of ARG-MGE co-occurrences as well as resistome risk scores. Further research examining effects of post-harvest practices, such as washing, packaging, and storage on vegetable resistomes, are needed. Moving forward, it would be beneficial address the clinical ARGs and other forms of antibiotic resistance from a One Health lens (i.e., which clinical ARGs are we most concerned about for humans, animals, and the environment) and then shift detection methods to identifying the most clinically important co-occurring ARG-MGE that are associated with known pathogens to improve future agricultural antibiotic resistance mitigation strategies.

DATA AVAILABILITY STATEMENT

The manure obtained from the dairy cows was from an animal study reviewed and approved by IACUC protocol DASC 13-145.

ETHICS STATEMENT

The animal study was reviewed and approved by IACUC protocol DASC 13–145.

AUTHOR CONTRIBUTIONS

LW: formal analysis, writing—original draft preparation. L-AK, AP, WH, LW, PR, KK, MP, and SG: conceptualization. LW, IK, SG, L-AK, AP, and WH: methodology, writing—review and editing. L-AK and AP: resources, funding acquisition, project administration, and supervision. L-AK, AP, WH, and IK: writing—revising and editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.683410/full#supplementary-material

REFERENCES

Aarestrup, F. M., Seyfarth, A. M., Emborg, H. D., Pedersen, K., Hendriksen, R. S., and Bager, F. (2001). Effect of abolishment of the use of antimicrobial agents for growth promotion on occurrence of antimicrobial resistance in fecal enterococci from food animals in Denmark. *Antimicrob. Agents Chemother.* 45, 2054–2059. doi: 10.1128/AAC.45.7.2054-2059.2001

Arango-Argoty, G., Singh, G., Heath, L. S., Pruden, A., Xiao, W., and Zhang, L. (2016). MetaStorm: A public resource for customizable metagenomics annotation. *PLoS One* 11:1–13. doi: 10.1371/journal.pone.0162442

Bengtsson-Palme, J. (2017). Antibiotic resistance in the food supply chain: where can sequencing and metagenomics aid risk assessment? *Curr. Opin. Food Sci.* 14, 66–71. doi: 10.1016/j.cofs.2017.01.010

Bolger, A. M., Lohse, M., and Usadel, B. (2014). Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics* 30, 2114–2120. doi: 10.1093/bioinformatics/btu170

Byrnes, B. H., and Bumb, B. L. (2017). Population growth, food production and nutrient requirements. *Nutr. Use Crop Prod.* 2017, 1–28. doi: 10.1201/9780203745281

Cáceres, M. (2013). *How to use the indicspecies package (ver. 1.7.1)*. Vienna: R Core Team.

CDC (2021). *Lettuce, other leafy greens, and food safety*. Atlanta: Centers for Disease Control and Prevention.

Chen, C., Pankow, C. A., Oh, M., Heath, L. S., Zhang, L., Du, P., et al. (2019). Effect of antibiotic use and composting on antibiotic resistance gene abundance and resistome risks of soils receiving manure-derived amendments. *Environ. Int.* 128, 233–243. doi: 10.1016/j.envint.2019.04.043

Chen, H. (2018). *VennDiagram: Generate High-Resolution Venn and Euler Plots*. New York, NY: Springer-Verlag.

Cycoń, M., Mrozik, A., and Piotrowska-Seget, Z. (2019). Antibiotics in the Soil Environment—Degradation and Their Impact on Microbial Activity and Diversity. *Front. Microbiol.* 10:00338. doi: 10.3389/fmicb.2019.00338

Davies, J., and Davies, D. (2010). Origins and evolution of antibiotic resistance. *Microbiol. Mol. Biol. Rev.* 74, 417–433. doi: 10.1128/MMBR.00016-10

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., et al. (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl. Environ. Microbiol.* 72, 5069–5072. doi: 10.1128/AEM.03006-05

Dharmarha, V., Guron, G., Boyer, R. R., Niemira, B. A., Pruden, A., Strawn, L. K., et al. (2019). Gamma irradiation influences the survival and regrowth of antibiotic-resistant bacteria and antibiotic-resistance genes on romaine lettuce. *Front. Microbiol.* 10, 1–16. doi: 10.3389/fmicb.2019.00710

Edrington, T. S., Fox, W. E., Callaway, T. R., Anderson, R. C., Hoffman, D. W., and Nisbet, D. J. (2009). Pathogen prevalence and influence of composted dairy manure application on antimicrobial resistance profiles of commensal soil bacteria. *Foodborne Pathog. Dis.* 6, 217–224. doi: 10.1089/fpd.2008.0184

Elliott, K. A., Kenny, C., and Madan, J. (2017). A Global Treaty to Reduce Antimicrobial Use in Livestock. *Cent. Glob. Dev.* 102:27.

European Commission (2005). *Ban on antibiotics as growth promoters in animal feed enters into effect*. Brussels: European Comission.

Fang, H., Wang, H., Cai, L., and Yu, Y. (2015). Prevalence of antibiotic resistance genes and bacterial pathogens in long-term manured greenhouse soils as revealed by metagenomic survey. *Environ. Sci. Technol.* 49, 1095–1104. doi: 10.1021/es504157v

FAO (2020). *Antimicrobial Resistance*. Available online at: http://www.fao.org/antimicrobial-resistance/background/what-is-it/en/ (accessed June 20, 2021).

FDA (2013). *Guidance for Industry #213 New Animal Drugs and New Animal Drug Combination Products Administered in or on Medicated Feed or Drinking Water of Food- Producing Animals: Recommendations for Drug Sponsors for Voluntarily Aligning Product Use Conditions with*. Rome: FDA.

FDA (2015). FSMA Final Rule on Produce Safety: Standards for the Growing, Harvesting, Packing, and Holding of Produce for Human Consumption; Final Rule. *Fed. Regist.* 80, 74353–74568.

FDA (2017). *Summary Report on Antimicrobials sold or distributed for use in food-producing animals*. Rome: FDA.

FDA (2018). *Supporting antimicrobial stewardship in veterinary settings*. Rome: FDA.

Finley, R. L., Collignon, P., Larsson, D. G. J., Mcewen, S. A., Li, X. Z., Gaze, W. H., et al. (2013). The scourge of antibiotic resistance: The important role of the environment. *Clin. Infect. Dis.* 57, 704–710. doi: 10.1093/cid/cit355

Fogler, K., Guron, G. K. P., Wind, L. L., Keenum, I. M., Hession, W. C., Krometis, L. A., et al. (2019). Microbiota and Antibiotic Resistome of Lettuce Leaves and Radishes Grown in Soils Receiving Manure-Based Amendments Derived From Antibiotic-Treated Cows. *Front. Sustain. Food Syst.* 3:00022. doi: 10.3389/fsufs.2019.00022

Gandrud, C., Allaire, J. J., Russell, K., and Yetman, C. J. (2017). *networkD3: Creates 'D3' 'JavaScript' network, tree, dendrogram, and Sankey graphs from 'R'*. New York, NY: Springer-Verlag.

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

Gou, M., Hu, H. W., Zhang, Y. J., Wang, J. T., Hayden, H., Tang, Y. Q., et al. (2018). Aerobic composting reduces antibiotic resistance genes in cattle manure and the resistome dissemination in agricultural soils. *Sci. Total Environ.* 612, 1300–1310. doi: 10.1016/j.scitotenv.2017.09.028

Graham, D. W., Bergeron, G., Bourassa, M. W., Dickson, J., Gomes, F., Howe, A., et al. (2019). Complexities in understanding antimicrobial resistance across domesticated animal, human, and environmental systems. *Ann. N Y. Acad. Sci.* 1441, 17–30. doi: 10.1111/nyas.14036

Guron, G., Arango-Argoty, G., Zhang, L., Pruden, A., and Ponder, M. (2019). Effects of Dairy Manure-Based Amendments and Soil Texture on Lettuce- and Radish-Associated Microbiota and Resistomes Giselle. *mSphere* 4, 1–14.

Han, X. M., Hu, H. W., Chen, Q. L., Yang, L. Y., Li, H. L., Zhu, Y. G., et al. (2018). Antibiotic resistance genes and associated bacterial communities in agricultural soils amended with different sources of animal manures. *Soil Biol. Biochem.* 126, 91–102. doi: 10.1016/j.soilbio.2018.08.018

Howe, A. C., Jansson, J. K., Malfatti, S. A., Tringe, S. G., Tiedje, J. M., and Brown, C. T. (2014). Tackling soil diversity with the assembly of large, complex metagenomes. *Proc. Natl. Acad. Sci. U S A.* 111, 6115. doi: 10.1073/pnas.1405263111

Hyatt, D., Chen, G. L., LoCascio, P. F., Land, M. L., Larimer, F. W., and Hauser, L. J. (2010). Prodigal: Prokaryotic gene recognition and translation initiation site identification. *BMC Bioinformatics* 11:119. doi: 10.1186/1471-2105-11-119

Jacobs, K., Wind, L., Krometis, L.-A., Hession, W. C., and Pruden, A. (2019). Fecal Indicator Bacteria and Antibiotic Resistance Genes in Storm Runoff from Dairy Manure and Compost-Amended Vegetable Plots. *J. Environ. Qual.* 48:1038. doi: 10.2134/jeq2018.12.0441

Jia, B., Raphenya, A. R., Alcock, B., Waglechner, N., Guo, P., Tsang, K. K., et al. (2017). CARD 2017: Expansion and model-centric curation of the comprehensive antibiotic resistance database. *Nucleic Acids Res.* 45, D566–D573. doi: 10.1093/nar/gkw1004

Keenum, I., Williams, R., Ray, P., Garner, E., Knowlton, K., and Pruden, A. (2021). Combined effects of composting and antibiotic administration on cattle manure-borne antibiotic resistance genes. *Microbiome* 9:81.

Larsson, D. G. J., Andremont, A., Bengtsson-Palme, J., Brandt, K. K., de Roda Husman, A. M., Fagerstedt, P., et al. (2018). Critical knowledge gaps and research needs related to the environmental dimensions of antibiotic resistance. *Environ. Int.* 117, 132–138. doi: 10.1016/j.envint.2018.04.041

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

Liang, X., Akers, K., Keenum, I., Wind, L., Gupta, S., Chen, C., et al. (2021). AgroSeek: a system for computational analysis of environmental metagenomic data and associated metadata. *BMC Bioinformatics* 22:1–14. doi: 10.1186/s12859-021-04035-5

Liu, W., Ling, N., Guo, J., Ruan, Y., Wang, M., Shen, Q., et al. (2021). Dynamics of the antibiotic resistome in agricultural soils amended with different sources of animal manures over three consecutive years. *J. Hazard. Mater.* 401:123399. doi: 10.1016/j.jhazmat.2020.123399

Lopatto, E., Choi, J., Colina, A., Ma, L., Howe, A., and Hinsa-Leasure, S. (2019). Characterizing the soil microbiome and quantifying antibiotic resistance gene dynamics in agricultural soil following swine CAFO manure application. *PLoS One* 14:0220770. doi: 10.1371/journal.pone.0220770

Lu, J., and Salzberg, S. L. (2020). Ultrafast and accurate 16S rRNA microbial community analysis using Kraken 2. *Microbiome* 8, 1–11. doi: 10.1186/s40168-020-00900-2

Macedo, G., van Veelen, H. P. J., Hernandez-Leal, L., van der Maas, P., Heederik, D., Mevius, D., et al. (2021). Targeted metagenomics reveals inferior resilience of farm soil resistome compared to soil microbiome after manure application. *Sci. Total Environ.* 770:145399. doi: 10.1016/j.scitotenv.2021.145399

Myrold, D. D., Zeglin, L. H., and Jansson, J. K. (2014). The Potential of Metagenomic Approaches for Understanding Soil Microbial Processes. *Soil Sci. Soc. Am. J.* 78, 3–10. doi: 10.2136/sssaj2013.07.0287dgs

O'Neill, J. (2016). "Tackling Drug-Resistant Infections Globally," in *Final Report and Recommendations*, ed. A. Ro (London: Review on Antimicrobial Resistance).

Oh, M., Pruden, A., Chen, C., Heath, L. S., Xia, K., and Zhang, L. (2018). Meta Compare: A computational pipeline for prioritizing environmental resistome risk. *FEMS Microbiol. Ecol.* 94, 1–9. doi: 10.1093/femsec/fiy079

Oksanen, A. J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., Hara, R. B. O., et al. (2015). *Package 'vegan'—Community Ecology Package, version 2.0-4*. Vienna: R Core Team.

Partridge, S. R., Kwong, S. M., Firth, N., and Jensen, S. O. (2018). Mobile genetic elements associated with antimicrobial resistance. *Clin. Microbiol. Rev.* 31, 1–61. doi: 10.1128/CMR.00088-17

Pelley, J. L. (2020). Backyard bacterial exchange: A one health approach to studying antibiotic resistance transmission. *Environ. Health Perspect.* 128, 1–2. doi: 10.1289/EHP6585

Peng, Y., Leung, H. C. M., Yiu, S. M., and Chin, F. Y. L. (2012). IDBA-UD: A de novo assembler for single-cell and metagenomic sequencing data with highly uneven depth. *Bioinformatics* 28, 1420–1428. doi: 10.1093/bioinformatics/bts174

Qian, X., Sun, W., Gu, J., Wang, X. J., Sun, J. J., Yin, Y. N., et al. (2016). Variable effects of oxytetracycline on antibiotic resistance gene abundance and the bacterial community during aerobic composting of cow manure. *J. Hazard. Mater.* 315, 61–69. doi: 10.1016/j.jhazmat.2016.05.002

R Core Team (2020). *R: A language and environment for statistical computing*. Vienna: R Foundation for Statistical Computing.

Ray, P., Chen, C., Knowlton, K. F., Pruden, A., and Xia, K. (2017). Fate and Effect of Antibiotics in Beef and Dairy Manure during Static and Turned Composting. *J. Environ. Qual.* 46:45. doi: 10.2134/jeq2016.07.0269

Rocha, J., Cacace, D., Kampouris, I., Guilloteau, H., Jäger, T., Marano, R. B. M., et al. (2020). Inter-laboratory calibration of quantitative analyses of antibiotic resistance genes. *J. Environ. Chem. Eng.* 8, 0–1. doi: 10.1016/j.jece.2018.02.022

Rocha, J., Fernandes, T., Riquelme, M. V., Zhu, N., Pruden, A., and Manaia, C. M. (2019). Comparison of culture-and quantitative PCR-based indicators of antibiotic resistance in wastewater, recycled water, and tap water. *Int. J. Environ. Res. Public Health* 16:ijerh16214217. doi: 10.3390/ijerph16214217

Rodriguez-R, L. M., and Konstantinidis, K. T. (2014). Nonpareil: A redundancy-based approach to assess the level of coverage in metagenomic datasets. *Bioinformatics* 30, 629–635. doi: 10.1093/bioinformatics/btt584

Shen, Y., Ryser, E. T., Li, H., and Zhang, W. (2021). Bacterial community assembly and antibiotic resistance genes in the lettuce-soil system upon antibiotic exposure. *Sci. Total Environ.* 778:146255. doi: 10.1016/j.scitotenv.2021.146255

Storteboom, H. N., Kim, S. C., Doesken, K. C., Carlson, K. H., Davis, J. G., and Pruden, A. (2007). Response of antibiotics and resistance genes to high-intensity and low-intensity manure management. *J. Env. Qual.* 36, 1695–1703. doi: 10.2134/jeq2007.0006

Suttner, B., Johnston, E. R., Orellana, L. H., Rodriguez-R, L. M., Hatt, J. K., Carychao, D., et al. (2020). Metagenomics as a public health risk assessment tool in a study of natural creek sediments influenced by agricultural and livestock runoff: Potential and limitations. *Appl. Environ. Microbiol.* 86, 1–51. doi: 10.1128/AEM.02525-19

Tien, Y. C., Li, B., Zhang, T., Scott, A., Murray, R., Sabourin, L., et al. (2017). Impact of dairy manure pre-application treatment on manure composition, soil dynamics of antibiotic resistance genes, and abundance of antibiotic-resistance genes on vegetables at harvest. *Sci. Total Environ.* 581–582, 32–39. doi: 10.1016/j.scitotenv.2016.12.138

U.S. Department of Health & Human Services (2015). FSMA Final Rule on Produce Safety: Standards for the Growing, Harvesting, Packing, and Holding of Produce for Human Consumption; Final Rule. *Fed. Regist.* 80, 74353–74568. doi: 10.1007/s13398-014-0173-7.2

US Food and Drug Administration (2013). *Guidance for Industry #213 New Animal Drugs and New Animal Drug Combination Products Administered in or on Medicated Feed or Drinking Water of Food- Producing Animals: Recommendations for Drug Sponsors for Voluntarily Aligning Product Use Conditions with*. Silver Spring: US Food and Drug Administration.

Van Boeckel, T. P., Brower, C., Gilbert, M., Grenfell, B. T., Levin, S. A., Robinson, T. P., et al. (2015). Global trends in antimicrobial use in food animals. *Proc. Natl. Acad. Sci. U S A.* 2015:1503141112. doi: 10.1073/pnas.1503141112

Vikesland, P. J., Pruden, A., Alvarez, P. J. J., Aga, D., Bürgmann, H., Li, X. D., et al. (2017). Toward a Comprehensive Strategy to Mitigate Dissemination of Environmental Sources of Antibiotic Resistance. *Environ. Sci. Technol.* 51, 13061–13069. doi: 10.1021/acs.est.7b03623

Virginia Cooperative Extension (2015). *Commercial vegetable production recommendations*. Blacksburg: Virginia Cooperative Extension.

WHO (2017). *WHO list of critically important antimicrobials for human medicine (WHO CIA list)*. Geneva: WHO.

Wickham, H. (2009). *ggplot2: Elegant Graphics for Data Analysis*. New York, NY: Springer-Verlag.

Wind, L., Krometis, L. A., Hession, W. C., and Pruden, A. (2020). Cross-comparison of methods for quantifying antibiotic resistance in agricultural soils amended with dairy manure and compost. *Sci. Total Environ.* 766:144321. doi: 10.1016/j.scitotenv.2020.144321

Wind, L., Krometis, L.-A., Hession, W. C., Chen, C., Du, P., Jacobs, K., et al. (2018). Fate of pirlimycin and antibiotic-resistant fecal coliforms in field plots amended with dairy manure or compost during vegetable cultivation. *J. Environ. Qual.* 47:0491. doi: 10.2134/jeq2017.12.0491

Wood, D. E., and Salzberg, S. L. (2014). Kraken: Ultrafast metagenomic sequence classification using exact alignments. *Genome Biol.* 15:r46. doi: 10.1186/gb-2014-15-3-r46

Wright, G. D. (2007). The antibiotic resistome: the nexus of chemical and genetic diversity. *Nat. Rev. Microbiol.* 5, 175–186. doi: 10.1038/nrmicro1614

Wu, N., Qiao, M., Zhang, B., Cheng, W., Da, and Zhu, Y. G. (2010). Abundance and diversity of tetracycline resistance genes in soils adjacent to representative swine feedlots in China. *Environ. Sci. Technol.* 44, 6933–6939. doi: 10.1021/es1007802

Xie, W., Yuan, S., Xu, M., Yang, X., and Shen, Q. (2018). Long-term e ff ects of manure and chemical fertilizers on soil antibiotic resistome. *Soil Biol. Biochem.* 122, 111–119. doi: 10.1016/j.soilbio.2018.04.009

Xu, M., Stedtfeld, R. D., Wang, F., Hashsham, S. A., Song, Y., Chuang, Y., et al. (2019). Science of the Total Environment Composting increased persistence of manure-borne antibiotic resistance genes in soils with different fertilization history. *Sci. Total Environ.* 689, 1172–1180. doi: 10.1016/j.scitotenv.2019.06.376

Zaheer, R., Noyes, N., Ortega Polo, R., Cook, S. R., Marinier, E., Van Domselaar, G., et al. (2018). Impact of sequencing depth on the characterization of the microbiome and resistome. *Sci. Rep.* 8, 1–11. doi: 10.1038/s41598-018-24280-8

Zhang, L., Li, L., Sha, G., Liu, C., Wang, Z., and Wang, L. (2020). Aerobic composting as an effective cow manure management strategy for reducing the dissemination of antibiotic resistance genes: An integrated meta-omics study. *J. Hazard. Mater.* 386:121895. doi: 10.1016/j.jhazmat.2019.121895

Zhang, M., He, L. Y., Liu, Y. S., Zhao, J. L., Liu, W. R., Zhang, J. N., et al. (2019). Fate of veterinary antibiotics during animal manure composting. *Sci. Total Environ.* 650, 1363–1370. doi: 10.1016/j.scitotenv.2018.09.147

Zhang, Y. J., Hu, H. W., Chen, Q. L., Singh, B. K., Yan, H., Chen, D., et al. (2019). Transfer of antibiotic resistance from manure-amended soils to vegetable microbiomes. *Environ. Int.* 130:104912. doi: 10.1016/j.envint.2019.104912

Zhou, X., Qiao, M., Wang, F. H., and Zhu, Y. G. (2017). Use of commercial organic fertilizer increases the abundance of antibiotic resistance genes and antibiotics in soil. *Environ. Sci. Pollut. Res.* 24:7854–z. doi: 10.1007/s11356-016-7854-z

Zhou, Z., and Yao, H. (2020). Effects of composting different types of organic fertilizer on the microbial community structure and antibiotic resistance genes. *Microorganisms* 8, 1–20. doi: 10.3390/microorganisms8020268

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