Investigating the role of heavy metals in the environment as a selective pressure for the dissemination of antimicrobial resistance

Title	Investigating the role of heavy metals in the environment as a selective pressure for the dissemination of antimicrobial resistance
Author(s)	Anedda, Elena
Publication Date	2025-03-25
Publisher	University of Galway

Investigating the role of heavy metals in the environment as a selective pressure for the dissemination of antimicrobial resistance

A thesis submitted to the University of Galway for the degree of Doctor of Philosophy

By

Elena Anedda

Discipline of Bacteriology,

School of Medicine,

University of Galway, Ireland.

Supervisors: Professor Dearbháile Morris, Dr Catherine M. Burgess

Head of Discipline: Professor Dearbháile Morris

September 2024

Table of Contents

List of figures

List of tables

List of abbreviations

AAS	Atomic absorption spectrometry
AFS	Atomic fluorescence spectrometry
AMR	Antimicrobial resistance
ANI	Average nucleotide identity
ARB	Antimicrobial resistant bacteria
ARGs	Antimicrobial resistance genes
AST	Antimicrobial susceptibility test
BPW	Buffered Peptone Water
CFU	Colony forming unit
CIP-RE	Ciprofloxacin resistant Enterobacterales
CLSI	Clinical & Laboratory Standards Institute
CRE	Carbapenem resistant Enterobacterales
DNA	Deoxyribonucleic Acid
EARS-Net	European Antimicrobial Resistance Surveillance Network
ECDC	European Centre for Disease Prevention and Control
EDXRF	Energy-dispersive X-ray fluorescence spectrometry
EPA	Environmental Protection Agency in Ireland
ESBL	Extended-spectrum beta-lactamase
ESBL-PE	Extended-spectrum beta-lactamase producing Enterobacterales
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GLASS	Global Antimicrobial Resistance and Use Surveillance System
GSI	Geological Survey Ireland
HGT	Horizontal gene transfer
HM	Heavy metal
HMR	Heavy metal resistance
HT-qPCR	High throughput quantitative polymerase chain reaction

ICP	Inductively coupled plasma
ICP-MS	Inductively coupled plasma mass spectrometry
ICP-OES	Inductively coupled plasma atomic emission spectrometry
LMIC	Low middle income countries
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization Time of Flight
MDR	Multidrug resistance
MGEs	Mobile genetic elements
MIC	Minimum inhibitory concentration
MRGs	Heavy metal resistance genes
mRNA	Messenger ribonucleic acid
MRSA	Methicillin-resistant *Staphylococcus aureus*
NCBI	National Center for Biotechnology Information
PCR	Polymerase chain reaction
PMQR	Plasmid mediated quinolone resistance
ROS	Reactive oxygen species
STEC	Shiga toxin-producing *Escherichia coli*
VRBGA	Violet Red Bile Glucose Agar
WGS	Whole genome sequencing
WHO	World Health Organisation
WRB	World Reference Base for Soil Resources
ZN	Zinc

Declaration of authorship

I, Elena Anedda, declare that this thesis is entirely my own work, apart from due acknowledgements. No part of this thesis has been previously submitted at this or any other university.

Signed:  Date: 25/09/2024

Printed: Elena Anedda

Acknowledgements

I would like to extend my heartfelt thanks to everyone who made this PhD thesis possible and who, through their support, made this incredible journey unforgettable.

First of all, I want to express my deepest gratitude to my inspiring supervisors, Dr. Kaye Burgess and Professor Dearbháile Morris. Their dedication, guidance, and constant encouragement to strive for improvement contributed significantly to the completion of this thesis. Thank you for your unwavering support, for guiding me through challenging moments, and for always being there whenever I needed you. I know how fortunate I am to have had you both by my side throughout this journey, and for that, I am immensely grateful.

I would like to thank my GRC committee, Dr. Kate Reddington, Dr. Liam Burke, and Professor Martin Cormican, for always listening to me and providing valuable advice from the first to the last meeting. I would also like to thank the One Health European Joint Programme and the Teagasc Walsh Scholarship for funding this research and giving me the opportunity to share my work and meet new colleagues through conferences.

I am grateful to have become part of the Microbiology Department Team at Teagasc, Ashtown, a group of extraordinary people led by the wonderful lab managers Dr. Des Walsh, Mrs. Joan Carroll, and Eoghan McDermott, who have always been ready to help me from my very first day. I am especially thankful to my irreplaceable colleagues who have supported me throughout this journey. A special thanks to Dr. Elena Alexa, Dr. Pilar Marcos and Gaia Streparola, who have been not only excellent mentors but also incredible friends. My gratitude also goes to the future Drs. Leon Maughan, Angelos Papadochristopoulos, Shannon Heapes, Michael Arthur, Artemis Zavitsanou, Grace Kennedy, Jane Kelleghan, Sailusha Kuntamukkula and Natalia Alija, who have been a fundamental part of this thesis. Furthermore, I would like to thank my other Teagasc colleagues, with whom I shared fantastic lunch breaks, train journeys, and sunny and rainy weekends, especially Dr. Laura

Gómez Barrio, Dr. Sara Alvarez Hernandez, Romane Guernalec, and Ailbhe McGurrin.

I also wish to express my thanks to Dr. Niamh Cahill, Dr. Georgios Miliotis, Dr. Brigid Hooban, Dr. Louise O'Connor, and the entire ARME team in Galway for their invaluable contributions. In particular, I want to express my gratitude to Dr. Maeve Louise Farrell, who not only guided me professionally but was a true supporter of every small step throughout this intense journey. You have been an incredible source of motivation.

Finally, I would like to thank my friends and family, who, even from miles away, never made me feel alone. Their unconditional love, support, and encouragement gave me the strength to navigate this intense journey. I am especially grateful to my Irish uncle Mick, who made it possible for me to move to Dublin and always encouraged me to look to the bright side. Thank to my grandmother Giacomina, whose determination and resilience have always inspired me. Heartfelt thanks to my parents, Giorgio and Francesca, who have always supported and motivated me to keep improving. They have been by my side through it all, and with a fig, some grapes, or a slice of cake, brought me the taste and scent of home, giving me strength during the most challenging times. Finally, my deepest gratitude is reserved for my sister Cecilia, who has weathered both the highs and lows of this PhD alongside me. One day, you wiped away my tears, and the next, you celebrated my happiness. You have been my constant anchor throughout this experience, the one who always calmed and encouraged me. You have taught me to live in the present moment, and even if I'm still learning, I promise I'll keep trying.

Abstract

Antimicrobial resistance (AMR) is a growing concern globally. The ability of microorganisms to survive in the presence of antimicrobials and the spread of antimicrobial resistance genes (ARGs) pose a significant risk to human, animal and environmental health. The environment's impact on the emergence and spread of AMR is increasingly acknowledged as critical, with reservoirs like soil and water playing a key role in the persistence and dissemination of antimicrobial-resistant bacteria (ARB) and ARGs. In this regard, the primary food production environment is of considerable importance due to the significant potential of ARB and ARGs to be transmitted to humans and animals through the food chain. Extended-spectrum beta-lactamase-producing Enterobacterales (ESBL-PE) and carbapenem resistant Enterobacterales (CRE) have been recognised by the World Health Organization as critical priority pathogens due to the severe infections they can cause, which are difficult to treat and often require last-resort treatments. Therefore, the surveillance of these bacteria in the environment is fundamental to tackle AMR. Beyond antimicrobials, also other factors, such as heavy metals and biocides, influence AMR. Several mechanisms underlie the association between heavy metals and AMR, including co-resistance, cross-resistance, co-regulation and biofilm formation. Therefore, the aim of this research was to investigate the role of heavy metals, in particular zinc, as a selective pressure for antimicrobial resistant Enterobacterales' occurrence and dissemination in the primary food production environment. Firstly, a scoping review was conducted to identify the knowledge gaps in this area (Chapter Two). A total of 73 studies undertaken over a period from 2008 and 2021 were included, the majority of which were conducted in China. The main environmental samples analysed in these studies were soil, water and manure, collected from different settings, including areas with natural occurrence of heavy metals, areas intentionally amended with heavy metals, and areas close to mines or industries that might release heavy metals. The results underlined the link between heavy metals and AMR, with particular focus on the role of mobile

genetic elements (MGEs) in the dissemination of ARGs and heavy metal resistance genes (MRGs). Following this, a research study was performed to assess the presence and characteristics of antimicrobial resistant Enterobacterales in soil and spinach samples with and without zinc soil amendment (Chapter Three). A total of 160 samples (soil, n = 92, spinach, n = 68) were collected from two different locations, where some of the soil plots were amended with zinc sulphate and other plots were used as control. A total of 20 antimicrobial resistant Enterobacterales were isolated, with *Serratia fonticola* species being the most common (n=16). None of the isolates were resistant to three or more antimicrobials. Genes encoding antimicrobial resistance and genes encoding zinc resistance were identified. However, a direct correlation between zinc amendment of soils and AMR was not identified. A follow on research study was conducted to evaluate the occurrence of antimicrobial resistant Enterobacterales in dairy production (Chapter Four). Fifty soil samples and 29 bovine milk filters were collected from ten different farms (five farms from two different geographical regions in Ireland with low and high zinc concentrations). A total of 40 antimicrobial resistant Enterobacterales were identified, with *Escherichia coli* the most predominant species. Phenotypic analysis identified 17 multidrug resistant (MDR) isolates, including ten *E. coli* isolates collected from the high zinc containing region, and seven MDR out of nine isolates from milk filters. Genotypic analysis identified ARGs and MRGs among the isolates collected from the high zinc containing region; however, no direct correlation between zinc resistance genes and AMR was observed. The advantage of using milk filters for AMR monitoring in dairy production, which allows early detection of ARB before entering the food chain, was recognised. Finally, a metagenomic study on ten composite soil samples and 18 milk filters, collected as part of the research detailed in Chapter Four, was performed to acquire a better understanding of the AMR presence in dairy production in high and low zinc containing regions (Chapter Five). This study identified the dominant microbial communities in both sample types, as well as the diverse profile of ARGs and MRGs present in the different sample

types. Overall, this body of work demonstrates that the primary food production environment can harbour clinically relevant Enterobacterales, serving as a significant reservoir and transmission source of AMR. This highlights the critical need of monitoring strategies to address the spread of AMR within the food chain, to which humans and animals may be exposed, and protect public health. Milk filters provide an ideal opportunity for the surveillance of AMR in dairy production. Although a direct correlation between zinc and AMR occurrence was not observed in the studies undertaken, the potential role of other heavy metals, such as copper, was suggested. Further investigation is needed to understand the impact of other heavy metals on AMR dynamics in the primary food production environment.

Chapter One: Introduction

1.1 Antimicrobial agents and their functions

The term “antimicrobial agents” refers to substances, including antibiotics, antifungals, antivirals and antiparasitics, which are able to kill, inhibit or reduce the growth of their targets (Di Martino, 2022). In addition to these therapeutic substances, the term “antimicrobial agents” also include biocides, which are applied on abiotic surfaces (disinfectants) or on living tissues (antiseptics) (Jones and Joshi, 2021, Maillard and Pascoe, 2024). While antimicrobials cover a broad range of substances, this thesis specifically focuses on antimicrobial resistance (AMR) among bacteria. Antibiotics, which are used to treat or prevent bacterial infections, can be classified in different groups based on their mechanisms of action and/or chemical structure. The mechanisms through which this group of antimicrobials can act include: inhibition of cell wall synthesis (e.g. beta-lactams), inhibition of protein synthesis (e.g. aminoglycosides, tetracyclines, chloramphenicol and macrolides), inhibition of nucleic acid synthesis (e.g. quinolones), lysis of cell membrane (e.g. polymyxins), and inhibition of metabolic pathways (e.g. sulfonamides) (Reygaert, 2018).

The inhibition of the cell wall synthesis is the mechanism employed by beta-lactams. These antimicrobials are characterized by the beta-lactam ring, a four-membered lactam ring which target and inhibit penicillin binding proteins (PBPs), enzymes involved in the biosynthesis of peptidoglycan. Beta-lactams include penicillins, cephalosporins, carbapenems and monobactams. Penicillins were accidentally discovered in 1928 by Alexander Fleming, and with their introduction into therapeutic use in the 1940s the new antimicrobials era began. Penicillins are derived from the fungus *Penicillium chrysogenum*, and they consist of a five-member thiazolidine ring fused to the four-membered lactam ring (Dumancas et al., 2014). Penicillins can be narrow-spectrum, which means that are effective against a specific bacterial group, for example penicillin G and penicillin V, which act mainly against Gram positive cocci and bacilli. They can also be extended-spectrum, which means that are effective against a broader range of bacteria, for

instance ampicillin and amoxicillin, which have greater activity against Gram negative microorganisms (Kumar, 2017). Cephalosporins were discovered in 1945 by Giuseppe Brotzu, an Italian pharmacologist who identified them from the fungus *Cephalosporium acremonium* (Bo, 2000). Cephalosporins have a six-membered dihydrothiazine ring fused to the four-membered lactam ring, and they are classified in five groups, referred as generations, based on their spectrum of activity. As antimicrobial generations advance from first to fifth, the effectiveness against Gram negative bacteria increases, while activity against Gram positive bacteria tends to decrease (Patel and Malangoni, 2009). Cephalexin is a first-generation cephalosporin, whereas cefoxitin is classified as a second-generation cephalosporin. Cefotaxime, ceftazidime, and cefpodoxime are third-generation cephalosporins. The fourth-generation include antimicrobials, such as cefepime, which are effective against bacteria resistant to third-generation cephalosporins (Lin and Kück, 2022). Finally, fifth-generation cephalosporins are effective against MDR bacteria, in particular antimicrobials of this group, such as ceftaroline, are utilised for treating methicillin-resistant *Staphylococcus aureus* (MRSA) and penicillin-resistant *Streptococcus pneumoniae* (Cottagnoud et al., 2013). Carbapenems were discovered in 1976 and their structure is characterised by a carbapenem ring fused to the four-membered lactam ring, which confers stability against beta-lactamase enzymes (Papp-Wallace et al., 2011, Aurilio et al., 2022). Carbapenems possess a wide spectrum of activity and they are defined as last-resort antimicrobials due to their use for treating multidrug resistance (MDR) bacterial infections that can not be treated with other antimicrobials (Sharma et al., 2024). The antimicrobials ertapenem and meropenem are two examples of carbapenems used to treat a variety of serious infections, including urinary infections caused by extended spectrum beta-lactamase-producing Enterobacterales (ESBL-PE) (VanDorf et al., 2024). Finally, monobactams contain only the beta-lactam ring and they have a narrow spectrum of activity, being effective against Gram negative aerobic bacteria

and not against Gram positive and anaerobes (Sauberan et al., 2023). Aztreonam belongs to this category.

The inhibition of protein synthesis is the mechanism employed by several antimicrobial classes, including aminoglycosides, tetracyclines, phenicols and macrolides. The ribosome, which is implicated in protein synthesis, is the target of these antimicrobial classes, and it consists of two subunits, 30S and 50S. Specifically, aminoglycosides bind to the 30S subunit and interfere with the mRNA-tRNA pairing, generating incorrect amino acid sequences and consequently nonfunctional proteins (Sauberan et al., 2023). Aminoglycosides are effective against both Gram positive and negative bacteria, but not against anaerobic bacteria. Kanamycin, streptomycin and gentamycin are part of this group. Tetracyclines bind to the 30S subunit as well as aminoglycosides, but target aminoacyl-tRNAs' movement, interfering with the peptide bonding (Grossman, 2016). Tetracyclines have a broad spectrum of activity and are effective against both Gram positive and negative bacteria. Unlike aminoglycosides and tetracyclines, macrolides bind to the 50S ribosomal subunit, obstructing the site where newly formed proteins are released from the ribosome (Sauberan et al., 2023). Macrolides are mainly used to treat infections caused by Gram positive bacteria, such as *S. aureus* (López-Jacome et al., 2022). Chloramphenicol also binds to the 50S subunit, supressing the peptidyltransferase, thereby inhibiting protein synthesis, and it is active against a wide range of both Gram positive and Gram negative bacteria (Aronson, 2016).

The antimicrobials that inhibit nucleic acid synthesis act by targeting the RNA polymerase, such as rifamycins, or the DNA polymerase, such as quinolones and fluoroquinolones. The rifamycins attach to the channel where the RNA strand is elongated and block its further growth. These antimicrobials act against both Gram positive and Gram negative bacteria, in particular they are used as antituberculosis agents (Ho et al., 2009). Quinolones interfere with DNA synthesis by targeting two enzymes: DNA gyrase or topoisomerase IV. DNA gyrase introduces negative supercoils in the DNA, which is essential

for transcription or replication processes (Dorman, 2019). Topoisomerase IV removes interlocking DNA catenanes generated during replication to facilitate chromosome segregation (Helgesen et al., 2021). Binding the DNA gyrase triggers an immediate effect on the bacterial cell because the DNA gyrase acts before the replication fork, leading to double-stranded DNA breaks, whilst the topoisomerase IV acts after the replication fork; therefore the effect on cell death is not immediate (Sauberan et al., 2023). Quinolones generally target DNA gyrase in Gram negative bacteria, and topoisomerase IV in Gram positive bacteria (Spencer and Panda, 2023). Among the quinolones, fluoroquinolones have a broad spectrum of activity and they are effective for treating several infections, including urinary tract infections, skin infections, and lower respiratory tract infections (Fàbrega et al., 2009).

Sulphonamides and trimethoprim belong to the antimicrobial class that act through the inhibition of metabolic pathways. Sulphonamides and trimethoprim act synergistically by targeting different steps of the folic acid biosynthesis, a fundamental requirement for DNA synthesis. Specifically, sulphonamides target the dihydropteroate synthetase and trimethoprim the dihydrofolate reductase. Therefore, sulphonamides block the dihydropteroate synthetase and consequently the formation of dihydrofolate, while trimethoprim block the reduction of dihydrofolate to tetrahydrofolic acid, the active form of folic acid (Ovung and Bhattacharyya, 2021). Trimethoprim alone is bacteriostatic, therefore it is often used in combination with sulphonamides, which increase the spectrum of activity (Kester et al., 2012).

Finally, some antimicrobials can target the cell membrane, such as polymyxin B and colistin (polymyxin E). Polymyxins bind to the lipid A of the membrane lipopolysaccharide (LPS), altering the permeability, and consequently causing membrane collapse (Bush, 2012). They are utilised as last resort antimicrobials in case of MDR infections in Gram negative pathogens, particularly *Pseudomonas aeruginosa*, *Acinetobacter spp*. and carbapenemase-producing *Klebsiella pneumoniae.* However, due to their

mode of action, polymyxins can be toxic in humans, and their use is limited (Mohapatra et al., 2021).

1.2 Antimicrobial resistance (AMR), mechanisms and transmission

The concept of "antimicrobial resistance" refers to the inefficacy of an antimicrobial to kill or inhibit its target, such as bacteria, viruses or fungi, leading to more difficult to treat infections and increasing the risk of serious illness and death (WHO, 2020). Although AMR is an ancient phenomenon, it has increased progressively in the last decades, becoming one of the greatest public health concerns at a global level (Dhingra et al., 2020). The overuse and misuse of antimicrobials in both humans and animals has accelerated the development and dissemination of AMR (WHO, 2022a). While antimicrobials are primarily used to treat human and animal infections, their applications has extended beyond treatment. For instance, antimicrobials have been used to prevent certain diseases in animals, to protect plants from infections, and even as additives in animal feed to promote animal growth (Pokharel et al., 2020, Verhaegen et al., 2023, Ma et al., 2021). Therefore, this excessive use of antimicrobials has led microorganisms to develop increasingly greater resistance.

The resistance can be natural or acquired (Committee to Study the Human Health Effects of Subtherapeutic Antibiotic Use in Animal Feeds, 1980). In the case of natural resistance, it can be intrinsic, when the microorganism always express the resistance, or induced, when the genes that confer resistance are expressed only in the presence of triggers. Acquired resistance occurs via mutations of the microorganisms' genetic material, or through acquisition of external genetic material from other bacteria (Reygaert, 2018). Specifically, there are five major mechanisms through which bacteria develop resistance.

1. **Alteration of cell membrane**. This mechanism consists of limiting antimicrobial uptake. There is a distinction between Gram negative and Gram positive bacteria; Gram negative bacteria possess an outer membrane that poses a challenge for some antimicrobials that can

not penetrate but can be effective against Gram positive bacteria. However, mutations can impact the membrane permeability and decrease the antimicrobials' absorption in both Gram negative and Gram positive bacteria. Mutations may lead to changes in porins, which are protein channels involved in the influx of external compounds, or on the lipidic components of the cell membrane (Darby et al., 2023). For example, mutations within the *oprD* porin gene in *Pseudomonas aeruginosa* can alter the expression or structure of the OprD porin protein, altering the membrane permeability and inhibiting the permeation of antimicrobials such as beta-lactams (Strateva and Yordanov, 2009).

2. **Alteration of efflux pumps**. Efflux pumps are transmembrane proteins that can expel toxic compounds, such as antimicrobials and heavy metals, from the inside of the bacterial cell to outside. Efflux pumps are classified into families, including the resistance–nodulation–division (RND) family, which plays an important role in the development of MDR in Gram negative bacteria. Mutations can be responsible for overexpression of efflux pumps and efflux pump regulators, resulting in MDR (Darby et al., 2023).
3. **Modification of antimicrobial target**. Mutations can alter the antimicrobial target; this prevents the antimicrobial binding and inhibits the antimicrobial effect. For example, quinolones and rifamycins, which target DNA and RNA polymerase respectively, are ineffective when mutations occur in genes involved in encoding these enzymes which consequently change conformation. Specifically, mutations of *gyrA* and/or *parC* genes confer resistance to quinolones (Fàbrega et al., 2009), and mutations of *rpoB* gene confer resistance to rifamycins (Li et al., 2021).
4. **Protection of antimicrobial target**. A target protection protein can bind to the antimicrobial target and obstruct the antimicrobial. For example, *qnr* genes, commonly found on plasmids of Gram negative bacteria, encode for Qnr proteins which can protect the enzyme

target of quinolones (Yassine et al., 2019). However, high-level resistance typically occurs when both *qnr* genes and mutations of *gyrA* gene are present (Ruiz, 2019).

5. **Inactivation of antimicrobials**. This mechanism consists of the degradation of the antimicrobial itself, or the alteration/transfer of a chemical group to the antimicrobial. The degradation of the antimicrobial is caused by enzymes which damage the antimicrobials. For example, beta-lactamases are enzymes responsible for the hydrolysis of the beta-lactam ring, which cause the inactivation of the beta-lactams (Majiduddin et al., 2002). The enzyme chloramphenicol acetyltransferase adds an acetyl group to the antimicrobial, blocking the bond to the ribosome target (Benjamin et al., 2023).

Bacteria can become resistant to antimicrobials through mutations in their own chromosomes, or through the acquisition of genetic material that harbour antimicrobial resistance genes (ARGs). The transfer of ARGs can be vertical or horizontal. In vertical transfer, ARGs pass from one bacterial generation to the next, while in horizontal transfer, ARGs are transmitted among different bacteria, even those belonging to different species. The mechanisms of horizontal gene transfer (HGT) include transformation, transduction and conjugation. Transformation occurs when bacteria acquire exogenous bacterial DNA fragments from outside. This acquisition occurs when the bacteria express competence to uptake free extracellular DNA and incorporate it in its genome (Hasegawa et al., 2018). Transduction is mediated by viruses, called bacteriophages, which transfer non-viral DNA fragments from one bacterium to another. Finally, conjugation is the transfer of genetic material between two bacteria, a donor and a recipient, that are physically attached through structures known as pili (Di Cesare et al., 2016).

ARGs can be carried on mobile genetic elements (MGEs), small genomic sequences which can move around the genome and can be transmitted

vertically or horizontally among microorganisms, playing an important role in the dissemination of AMR (Squadrone, 2020, Pal et al., 2017). MGEs include insertion sequences, transposons, integrons, integrative conjugative elements (ICEs) and plasmids.

Insertion sequences (IS) are the simplest MGEs. They contain genes that encode for transposases, enzymes that allow them to move from one part of the genome to another. Close to transposase genes, passenger genes can also be transmitted through these genetic elements, including for example ARGs (Siguier et al., 2014). Specifically, ARGs can be carried on composite transposons that are flanked by two different ISs at both ends (Carr et al., 2023). Non-composite transposons are ARGs carriers which are flanked by sequences of inverted repeats (IRs), and not ISs as for the composite transposons (Babakhani and Oloomi, 2018). Overall, the ability of transposons to move around the genome make them important actors in the dissemination of AMR. The genetic element integrons are not able to move themselves, but they are mobile when associated with other MGEs. They have a site-specific recombination system that allow them to incorporate, express and transfer mobile DNA fragments called gene cassettes, which can harbour ARGs (Bhat et al., 2023). Integrative conjugative elements (ICEs) are not able to replicate independently, but they are genetic elements integrated into a bacterial host chromosome to replicate. An excision reaction allow these genetic elements to be released from the host chromosome, and through a conjugation mechanism they can be transferred to another bacteria, spreading genes such as ARGs (Delavat et al., 2017). Finally, plasmids are complex genetic elements able to replicate and transfer independently. Unlike ICEs, plasmids contain an origin for their replication, which is distinct from the bacterial chromosome replication. They usually harbour genes that are not essential for cell functions, including ARGs and/or virulence genes, and are transmitted though conjugation between different bacteria (Hülter et al., 2017). Plasmids are classified in several groups based on their “incompatibility”. The term “incompatibility”

is derived from the inability of plasmids that share the same replication mechanism to occur in the same host cell (Frost et al., 2005). Inc plasmids are commonly found in Enterobacterales, and IncF, A/C, and IncX are the most prevalent in carbapenem-resistant Enterobacterales (CRE) (Chen et al., 2024). Overall, all the MGEs interact with each other and facilitate the spread of AMR among bacteria.

1.3 Antimicrobial resistant Enterobacterales

Enterobacterales are a Gram negative bacterial order that include a vast number of bacterial genera, including *Escherichia*, *Salmonella*, *Klebsiella*, *Enterobacter* and *Shigella*. Enterobacterales are facultative anaerobic bacteria and occur ubiquitously in natural environments. Specifically, their primary habitat is the gastrointestinal tract of humans and animals, and they can be found in several environments, such as soil, vegetation and water. Most of the Enterobacterales are opportunistic pathogens, that means that are not harmful in individuals with a healthy immune system, but they can be responsible for infections and illness in immunocompromised individuals, often associated with nosocomial infections (Anju et al., 2020). Although they are an essential part of the human gut microbiota, some Enterobacterales are associated with foodborne diseases and outbreaks (Wang et al., 2021a).

Antimicrobial resistance in Enterobacterales is a major public health threat, as evidenced by the resistance rates reported by the 2022 European Antimicrobial Resistance Surveillance Network (EARS-Net) report (ECDC, 2023). In the European Centre for Disease Prevention and Control (ECDC) analysis of the health impact of AMR from 2016 to 2020, infections with third-generation cephalosporin-resistant *E. coli* were found to cause the greatest burden of disease (ECDC, 2022). In the EARS-Net 2022 report, the estimated incidence of bloodstream infections caused by third-generation cephalosporin-resistant *E. coli* was reported at 8.67%, with a decreasing trend of 16.8% from 2018; while a significant increase of almost 50% of carbapenem-resistant *Klebsiella pneumoniae* infections was observed

(ECDC, 2023). Gram negative bacteria resistant to critical antibiotics, including third-generation cephalosporins and carbapenems, are recognised as critical priority pathogens by the World Health Organization (WHO) (WHO, 2024). With critical priority pathogens, WHO refers to bacteria that pose a critical public health risk due to varied factors, including limited treatment options, high morbidity and mortality levels, rapid increased resistance, and lack of new treatments. ESBL-PE and CRE, which fall into this category of pathogens, are associated with several infections, such as bloodstream infections, respiratory tract infections and urinary tract infections (Pana and Zaoutis, 2018), and limited treatment options result in increased morbidity and mortality, posing a significant challenge worldwide (Murray et al., 2022).

Enterobacterales can exhibit resistance to beta-lactam antimicrobials, such as cephalosporins and carbapenems, through different mechanisms including alteration of cell membrane, alteration of efflux pumps, modification of antimicrobial target and inactivation of antimicrobials. The alteration of cell membrane in Enterobacterales consists of the modification or reduced production of porin channels, such as OmpF and OmpC in *E. coli*, which consequently reduce the permeability and limit the entry of antimicrobials (Blair et al., 2015). The alteration of efflux pumps occurs in Enterobacterales following the expression of transcription factors, such as MarA, SoxS, RamA or Rob, which induces the overexpression of efflux pumps and confer MDR (Holden and Webber, 2020). The modification of the beta-lactams' target (penicillin-binding proteins) is less prevalent in Enterobacterales compared to Gram positive bacteria. However, the modification of PBP3 in *E. coli* has been observed and considered a resistance mechanism that needs to be monitored (Sethuvel et al., 2023). The inactivation of beta-lactams is the major resistance mechanism in Enterobacterales, and, in combination with the other mechanisms conferring resistance to other antimicrobial classes, it can contribute to the development of MDR. This resistance arises from the production of beta-

lactamases (Kaderabkova et al., 2022). Beta-lactamases are classified based on their functional and molecular characteristics in accordance to Ambler and Bush classification schemes (Ambler, 1980, Bush and Jacoby, 2010). The Ambler classification scheme is based on amino acid sequence similarities, and groups beta-lactamases into classes A, B C and D. The Bush classification scheme uses a functional based system, such as microbiological or enzymatic properties, to categorise beta-lactamases into distinct numerical groups (1, 2, 2d, 3) (Table 1.1).

Table 1.1 Classification of the most clinically relevant beta-lactamases. Adapted from (Bush, 2023).

Active site	Molecular classes (Ambler)	Functional groups (Bush)	Major functional subgroups	Substrates	Inhibition	Major enzymes
Serine	C	1	1	Penicillin* Cephalosporins	AV	AmpC
	A	2	2a 2b 2c	Penicillin Cephalosporins*	AV CA	TEM-1 TEM-2 SHV-1
			2be	Penicillin Cephalosporins Extended-spectrum cephalosporins Monobactam	AV CA	CTX-M ESBLs
			2f	Penicillin Cephalosporins Carbapenem Monobactam	AV CA#	KPC SME
	D	2d	2d 2de	Penicillin Extended-spectrum cephalosporins	AV CA#	OXA-1 OXA-10
			2df	Penicillin Carbapenem Monobactam	AV# CA#	OXA-48 OXA-181
Metallo (Zn^{2+})	B	3	3a	Penicillin Cephalosporins Extended-spectrum cephalosporins Carbapenem	EDTA	NDM VIM

AV = avibactam acid. CA = clavulanic acid. The symbol * indicates substrates weakly hydrolysed by that subgroup, while # indicates that the inhibition depends on specific enzyme.

According to the Ambler classification scheme, classes A, C and D beta-lactamases utilise a serine residue in their active site to form a bond and hydrolyse beta-lactam antimicrobials (Sawa et al., 2020). In contrast, class B beta-lactamases utilise a zinc ion in their active site, which activates a water molecule and breaks the beta-lactam ring (Boyd et al., 2020). The serine beta-lactamases (SBLs) can be inhibited by different inhibitors, such as avibactam and clavulanic acid, with the exception of the enzymes class C which are resistant. In contrast, metallo beta-lactamases (MBLs) are inhibited by different compunds, including EDTA (Bush, 2023). The beta-lactamases belonging to Ambler class C and Bush group 1, such as AmpC, which confer resistance to penicillins, cefoxitin and the inhibitor clavulanic acid, are chromosomally encoded by several Enterobacterales species, including *Citrobacter freundii*, *Enterobacter cloacae*, *Morganella morganii* and *Serratia mercescens*. In these species, the expression of *ampC* is low but inducible following exposure to beta-lactams, or mutations in genes that regulate its expression (Jacoby, 2009). The regulation system that allows this mechanism is not present in other species, such as *E. coli* and *Acinetobacter baumannii*, which poorly express the *ampC* gene; while, species such as *Klebsiella pneumoniae* and *Proteus mirabilis* completely lack the *ampC* gene (Jacoby, 2009, Fisher and Mobashery, 2014). However, plasmid-mediated *ampC* genes can be present in these species, for example, different varieties of *CMY*, *FOX*, *ACT* and *DHA* genes can spread via MGEs among Enterobacterales (Jacoby, 2009). The Ambler class A and Bush subgroup 2a includes beta-lactamases produced by Gram positive cocci, such as staphylococci and enterococci. The Bush subgroup 2b includes TEM-1, TEM-2 and SHV-1, which act against penicillins and early cephalosporins (Bush, 2018); however, point mutations can lead to alterations in their sequences, enabling them to encode resistance to third-generation cephalosporins (Liakopoulos et al., 2016). Bush subgroup 2be includes extended-spectrum beta-lactamases (ESBLs), enzymes that act against penicillins and cephalosporins, but also cefotaxime, ceftazidime and aztreonam. This subgroup also includes less common ESBLs enzymes, such as SFO-1, and the

Cefotaximase-Munich (CTX-M) enzymes, most of which are more resistant to cefotaxime than ceftazidime (Akhtar et al., 2022). Ambler class D and Bush subgroup2d includes the OXA enzymes, the second biggest family of beta-lactamases after ESBLs. The name OXA derived from their characteristic to hydrolyse oxacillin and cloxacillin antimicrobials (Bush, 2023). These enzymes with an extended spectrum are part of the Bush subgroup 2de (Akhtar et al., 2022). The hydrolysis of carbapenems is mediated by enzymes belonging to Ambler class A and Bush subgroup 2f, such as *Klebsiella pneumoniae* carbapenemase (KPC), Ambler class D and Bush subgroup 2df, such as OXA-48, and Ambler class B and Bush subgroup 3a, which include Verona integrin-encoded MBL (VIM) and New Delhi metallo-beta-lactamases (NDM) (Cui et al., 2019). The ability of these enzymes to spread via MGEs and the lack of new treatments against carbapenem-resistant infections pose a significant threat to human health (Tooke et al., 2019). Therefore, ESBL-PE and CRE have become crucial concerns regarding the occurrence and dissemination of AMR.

1.4 Antimicrobial resistant Enterobacterales in the primary food production environment

Many studies have focused on the occurrence of ESBL-PE and CRE in clinical and healthcare settings, but these resistant bacteria have also been found in the environment, such as water, soil, manure and vegetation (Acolatse et al., 2022, Ramatla et al., 2023, Hooban et al., 2021). The presence of these resistant bacteria in the primary food production environment, which encompasses all activities related to producing raw food materials, may be due to several factors. Above all, the use of antimicrobials in livestock represents a substantial driver of AMR dissemination in the environment (Ardakani et al., 2023). Traces of antimicrobials, as well as antimicrobial resistant bacteria, may be present in animal and human waste. This waste may be released into the environment, impacting the soil and water bacterial populations (Ramatla et al., 2023). Agricultural practices may also impact AMR dissemination, for example the application of manure on soil

for fertilisation or the use of crop irrigation water contaminated with antimicrobials, ARB or ARGs (Koutsoumanis et al., 2021, Marutescu et al., 2022). Additionally, the intensive use of antimicrobials in aquaculture systems is a significant source of AMR in the aquatic environment (Kampouris et al., 2022). The spread of AMR in the primary food production environment can lead to the dissemination of ARB and ARGs through the food chain and constitutes a potential threat to public health (Koutsoumanis et al., 2021). Animal-based foods, such as meat, eggs and dairy-products, are recognized as a significant source of ARB in the food chain (Andreoletti et al., 2008). Antimicrobial resistant Enterobacterales have been reported in poultry meat, pork, beef, as well as eggs, milk and ovine meat (Duse et al., 2016, Xu et al., 2022, Abdallah et al., 2022). Similarly, antimicrobial resistant Enterobacterales have been reported in seafood (Dewi et al., 2022, Singh et al., 2020). However, foods of non-animal origin are also potential vectors of ARB/ARGs, including not only resistant Enterobacterales such as *E. coli* and *Salmonella* spp., but also *Listeria* spp., *Bacillus cereus*, *Pseudomonas* spp., and Gram positive bacteria (Hölzel et al., 2018). Raw vegetables, including lettuce, leek, spinach, cucumber and leafy vegetables, have been reported in several studies to be carriers of antimicrobial resistant Enterobacterales (Anokyewaa Appau and Ofori, 2024, Hölzel et al., 2018). Moreover, privately sourced drinking water, as well as recreational water, can also be sources of ARB/ARGs (Alawi et al., 2024, Farrell et al., 2023). The intricate connections between the environment, animals and humans play a critical role in AMR dissemination. The transfer of ARB and ARGs across these three domains intensify the AMR global emergency. To effectively tackle this threat, a “One Heath” approach, where human medicine, veterinary medicine and environmental science collaborate to achieve optimal health in all these sectors, is fundamental (Das et al., 2024). Employing appropriate antimicrobial use policies and guidelines, as well as increasing awareness of AMR, and supporting research in addressing this global issue are essential in order to tackle AMR using a “One Health” approach (Samtiya et al., 2022, Cella et al., 2023). The occurrence and dissemination of AMR in the primary

food production environment may be influenced by several factors. The European Food Safety Authority (EFSA) has highlighted selective pressures that can contribute to the presence and spread of ARB and ARGs in the environment (Koutsoumanis et al., 2021). These selective pressures include not only antimicrobials, but also heavy metals and biocides. Moreover, bacteria's ability to transfer ARGs, form biofilms, and adapt to stress, combined with insufficient biosecurity measures and inadequate food hygiene practices, may further aggravate the AMR issue (Koutsoumanis et al., 2021).

1.5 Heavy metals and heavy metal resistance (HMR) in the environment

The term “heavy metals” refer to a group of metallic chemical elements characterised by a high molecular weight and atomic density (Tchounwou et al., 2012). They can be classified in two main groups: “essential” and “non-essential” metals. The “essential” group includes all metals that are required for biochemical and physiological cell functions, such as calcium (Ca), cobalt (Co), copper (Cu), chromium (Cr), iron (Fe), magnesium (Mg), manganese (Mn), nickel (Ni), potassium (K), selenium (Se), sodium (Na) and zinc (Zn) (Tchounwou et al., 2012). These metals serve as essential components of key enzymes, playing crucial roles in various oxidation-reduction reactions, acting as stabilizers and regulators of cellular osmotic pressure. However, at high concentrations they can be toxic for the cell, affecting cellular organelles, damaging the DNA or cell membrane integrity (Vats et al., 2022). The “non-essential” metals have no biological functions, and this group includes, for example, aluminium (Al), arsenic (As), barium (Ba), cadmium (Cd), lead (Pb), mercury (Hg) and vanadium (V). The “non-essential” metals are highly toxic for living-organisms even at low concentrations (Afzal and Mahreen, 2024).

Heavy metals are natural components of the Earth’s crust. However, several factors impact heavy metal occurrence in soil. Indeed, the geogenic soil composition influences the heavy metal concentrations, where some areas

may naturally have higher concentrations than others, but also atmospheric factors can cause the erosion by winds and water contribute to the presence of heavy metals (Rashid et al., 2023). However, the primary reason for heavy metals' occurrence are anthropogenic activities. Specifically, industrial activities and agricultural practices can cause notable increases of heavy metal concentration in the environment (Vats et al., 2022). In the industrial sector, heavy metals can be released following activities such as mining, metal smelting, fertilizer-producing plants, metal processing in refineries, textiles, plastics and paper manufacturing (Bibi et al., 2023). In agriculture, the use of heavy metals in fertilizers and pesticides, supplementation of animal feeds with heavy metals, land manure application, and use of irrigation water contaminated with heavy metals, promote the dissemination of heavy metals in the agricultural setting (Rashid et al., 2023). Additionally, the use of heavy metals in aquaculture as fish feed and anti-fouling agents to protect fish nets, and use of processed sewage sludge, which derives from municipal or industrial waste, as fertilizer for crops, can also impact the presence of heavy metals in the environment (Yu et al., 2017, Habib et al., 2023, Hudcová et al., 2019). However, when in excess, heavy metals can harm crop health and productivity (Rashid et al., 2023). For example, an excess of copper in soil can induce cytotoxicity, which appears as leaf chlorosis and plant growth limitation, oxidative stress and the production of reactive oxygen species (ROS) (Bibi et al., 2023). Similarly, zinc toxicity arises in plant as leaf chlorosis, curling and rolling leaves, and root growth limitation (Rout and Das, 2009). The toxicity depends on the metal bioavailability and valency, which are influenced by several factors, such as pH and concentration of organic matter (Seiler and Berendonk, 2012). Heavy metal concentration in relation to various industries, including cosmetics, toys, food and agriculture, is widely regulated across Europe (Reg. (EC) 1223/2009, 2009, Directive 2009/48/EC, 2009, Reg. (EU) 2019/1009, 2019, Reg. (EU) 2023/915, 2023). The *Commission Regulation (EU) 2023/915 of 25 April 2023 on maximum levels for certain contaminants in food and repealing Regulation (EC) No 1881/2006* establish maximum levels of arsenic,

cadmium, lead and mercury in food products (Reg. (EU) 2023/915, 2023). Similarly, the *Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed* sets the limits of undesirable substances, such as arsenic, cadmium, lead and mercury, in animal feed (Directive 2002/32/EC, 2002). In the agriculture sector, the *Council Directive 86/278/EEC of 12 June 1986 on the protection of the environment, and in particular of the soil, when sewage sludge is used in agriculture* fixes the limit values for cadmium, chromium, copper, lead, mercury, nickel and zinc in sewage sludge used as a fertiliser (Directive 86/278/EEC, 1986). While, the *Regulation (EU) 2019/1009 of the European Parliament and of the Council of 5 June 2019 laying down rules on the making available on the market of EU fertilising products and amending Regulations (EC) No 1069/2009 and (EC) No 1107/2009 and repealing Regulation (EC) No 2003/2003* sets the limits for heavy metals in fertilisers (Reg. (EU) 2019/1009, 2019). Moreover, the European Commission implemented the EMA recommendation (EMA, 2017) to revoke the market authorisation for the use of therapeutic levels of zinc oxide in animal feed on environmental grounds. This was implemented in 2022. Together, these regulations and directives aim to protect public health, minimizing heavy metal contamination of the environment through setting limits of these compounds in food, feed, and agriculture.

Due to heavy metal ubiquity in the environment, bacteria are constantly exposed to these elements. As a result of heavy metal exposure, bacteria have developed resistance against these compounds, a phenomenon known as heavy metal resistance (HMR) (Halema et al., 2024). There are three main mechanisms of HMR:

- Sequestration of heavy metals, which involves the production of metal binding proteins or chelating metabolites that are able to bind and neutralise the metals. Consequently, the concentration of free metals inside the cell is minimised (Seiler and Berendonk, 2012).

- Alteration of the redox state of metal ions through oxidation-reduction reactions which convert the ions to less harmful forms (Turner et al., 2020).
- Export of heavy metals outside the bacterial cell through efflux pump systems (Huang et al., 2022).

Genes responsible for HMR are known as metal resistance genes (MRGs) and can be harboured on bacterial chromosome or MGEs, such as plasmids (Silver and Phung, 1996). Bacteria carrying MRGs are more commonly found to carry ARGs compared to bacteria that lack MRGs (Samreen et al., 2021). Therefore, exposure to heavy metals may facilitate AMR selection even in the absence of antimicrobial compounds (co-selection) (Murray et al., 2024).

1.6 Heavy metals and AMR in the environment

The co-existence of ARGs and MRGs in bacteria has considerably increased the spread of ARB in the environment, contributing to a rise in MDR (Singh et al., 2024). Different mechanisms contribute to the co-selection of AMR and HMR:

- Co-resistance: ARGs and MRGs are physically linked, most often on the same MGE, therefore when one gene is selected, it indirectly also selects the others (Baker-Austin et al., 2006). For example, the ARGs *oqxAB* and *bla*$_{\text{CTX-M}}$, which encode for quinolones and beta-lactams resistance, were found co-located with *pco* and *sil*, which confer resistance to copper and silver, on IncHI2 plasmids in *E. coli* isolates (Fang et al., 2016). Similarly, Hasman and Aarestrup (2002) demonstrated that copper and macrolide resistance genes co-occurred on a plasmid that was transferred from *Enterococcus facieum* to a susceptible recipient.
- Cross-resistance: a gene provides resistance to different agents, such as antimicrobials and heavy metals. For example, a single mechanism, such as efflux pump system, can target both antimicrobials and heavy metals (Chapman, 2003). Mata et al. (2000)

demonstrated the role of the efflux pump MdrL in *Listeria monocytogenes*, involved in the removal of of heavy metals, such as zinc, cobalt and cadmium, and erythromycin and clindamycin antimicrobials.

- Co-regulation: the same regulatory system interferes with the expression of ARGs and MRGs, providing resistance to both antimicrobials and heavy metals (Yu et al., 2017). For example, it has been shown that in *Pseudomonas aeruginosa* the regulator genes *czcR-czcS* are involved on the regulation of zinc resistance and imipenem resistance (Perron et al., 2004).
- Biofilm formation: development of a structured bacterial mass that adhere to surfaces with a protective matrix, preventing antimicrobial and heavy metal penetration (Flemming and Wingender, 2010).

These mechanisms can lead to the emergence of antimicrobial resistant phenotypes in the presence of heavy metals beyond direct exposure to antimicrobials. Moreover, the spread of MGEs carrying ARGs and MRGs facilitates the occurrence of ARB in different environments, including the primary food production environment (Anedda et al., 2023).

1.7 Monitoring of AMR in Ireland

AMR poses a significant challenge worldwide, including in Ireland, due to its potential impact on public health. Effective monitoring systems to detect emerging pathogens and resistance mechanisms are crucial for tracking changes and understanding the spread of AMR. In Ireland, AMR is monitored through several national programs and strategies, including the Irish National Action Plan on Antimicrobial Resistance (iNAP). The first plan, iNAP (2017-2020), and the second plan, iNAP2 (2021-2025), aim to protect and optimise the health of humans, animals and the environment (Government of Ireland, 2021). iNAP2 places increased emphasis on infection prevention and control, as well as tackling AMR in companion animals. The iNAP2 aligns with the five objectives of the WHO's Global Action Plan on AMR: improving awareness and knowledge, enhancing surveillance, reducing infection

spread, optimizing antimicrobial use, and promoting research and sustainable investment.

Furthermore, iNAP2 underscores collaboration among stakeholders in agriculture, public health, and environmental sectors, reinforcing the importance of a One Health approach to tackle AMR. For example, collaborations between the Department of Agriculture, Food and the Marine (DAFM) and agri-food stakeholders have supported sustainable farming practices while protecting public health and the environment.

Ireland also actively participates to the European Antimicrobial Resistance Surveillance Network (EARS-Net), the largest publicly funded AMR surveillance system in Europe (Health Protection Surveillance Centre (HPSC), 2023). Through EARS-Net, Ireland collects and submits data on resistant pathogens such as *E. coli*, *K. pneumoniae*, and *S. aureus*. This surveillance enables the identification of AMR trends, facilitates comparisons across European countries, and provides critical insights for public health strategies.

These strategies demonstrate Ireland's commitment to addressing AMR through robust monitoring systems, ensuring progress toward mitigating this global health threat.

1.8 Aim and objectives

The overall aim of this project was to investigate and analyse the impact of heavy metals on AMR occurrence in the primary food production environment. The hypothesis of this thesis was that high heavy metal concentrations play a role as a selective pressure in the environment, influencing the composition of ARB and promoting their spread. To accomplish this, a scoping review and laboratory-based research studies were performed.

- The objective of the scoping review (Chapter Two) was to identify the knowledge gaps in the relationship between heavy metals and AMR. Specifically, the study aimed to address the research question: "*Do*

heavy metals have an impact on AMR in the primary food production environment?"

- The objective of the first research study (Chapter Three) was to investigate the presence of antimicrobial resistant Enterobacterales in soil and spinach samples collected from zinc amended and control plots. Phenotypic and genotypic resistant profiles of Enterobacterales from amended and non-amended plots were compared.
- The objective of the second research study (Chapter Four) was to assess antimicrobial resistant Enterobacterales in soil and bovine milk filters samples collected from two different regions, with high and low heavy metal concentrations, across Ireland. This study sought to explore how natural variations in environmental heavy metal concentrations influence the prevalence and distribution of AMR within dairy production.
- The objective of the third research study (Chapter Five) was to characterize the resistomes in low and high naturally occurring heavy metal containing regions across Ireland. This study consisted of a shotgun metagenomic analysis of soil and bovine milk filter samples collected as part of the research detailed in Chapter Four.

Chapter Two

Evaluating the impact of heavy metals on antimicrobial resistance in the primary food production environment: A scoping review.

Published in Environmental Pollution

Anedda E., Farrell M.L., Morris D., Burgess C.M. 2023. Evaluating the impact of heavy metals on antimicrobial resistance in the primary food production environment: A scoping review. *Environmental Pollut*ion, *320,* p.121035. DOI:

https://doi.org/10.1016/j.envpol.2023.121035

Abstract:

Heavy metals are naturally occurring environmental compounds, which can influence antimicrobial resistance (AMR) dissemination. However, there is limited information on how heavy metals may act as a selective pressure on AMR in the primary food production environment. This review aims to examine the literature on this topic in order to identify knowledge gaps. A total of 73 studies, which met pre-established criteria, were included. These investigations were undertaken between 2008 and 2021, with a significant increase in the last three years. The majority of studies included were undertaken in China. Soil, water and manure were the most common samples analysed, and the sampling locations varied from areas with a natural presence of heavy metals, areas intentionally amended with heavy metals or manure, to areas close to industrial activity or mines. Fifty-four per cent of the investigations focused on the analysis of four or more heavy metals, and copper and zinc were the metals most frequently analysed (n= 59, n=49, respectively). The findings of this review highlight a link between heavy metals and AMR in the primary food production environment. Heavy metals impacted the abundance and dissemination of mobile genetic elements (MGEs) and antimicrobial resistance genes (ARGs), with MGEs also observed as playing a key role in the spread of ARGs and metal resistance genes (MRGs). Harmonisation of methodologies used in future studies would increase the opportunity for comparison between studies. Further research is also required to broaden the availability of data at a global level.

2.1 Introduction

Antimicrobial resistance (AMR) is recognised as one of the greatest global threats to human and animal health. It has been estimated that the number of deaths attributed to AMR will reach 10 million per annum by 2050 if no action is taken (O'Neill, 2016). AMR is an ancient natural phenomenon, predating the use of antibiotics (D'Costa *et al.*, 2011). In recent decades, the decreasing pace in the development of new antibiotics, and the overuse and misuse of antibiotics, have caused a rapid increase in the emergence of AMR

(Squadrone, 2020, Acharya and Wilson, 2019, Roca et al., 2015). The inappropriate use of antibiotics, in both human and veterinary medicine, and in food production systems places selective pressure on microorganisms (Acharya and Wilson, 2019, Roca et al., 2015) which has resulted in microorganisms encoding several mechanisms to survive in the presence of antibiotics (Koutsoumanis *et al.*, 2021). These mechanisms may be innate or acquired (Brooks and Brooks, 2014). Bacteria can acquire resistance through the acquisition of mobile genetic elements (MGEs) that carry antimicrobial resistance genes (ARGs) that can be easily transferred among microorganisms via horizontal gene transfer (HGT) (Squadrone, 2020, Pal et al., 2017, Di Cesare et al., 2016).

AMR dissemination is not only influenced by the presence of antibiotics, but it has also been shown that agents in the environment, such as biocides and heavy metals, can facilitate the spread of ARGs and antimicrobial resistant bacteria (ARB) (Seiler and Berendonk, 2012) (Koutsoumanis et al., 2021) (Yazdankhah et al., 2018) . Most heavy metals are naturally occurring elements, and are commonly classified into essential and non-essential metals (Yazdankhah *et al.*, 2018). The first group (essential) includes metals that are necessary for organisms' cellular functions and components (Seiler and Berendonk, 2012), but can be toxic if they are present in excess (Yazdankhah *et al.*, 2018). These metals include copper (Cu), chromium (Cr), cobalt (Co), manganese (Mn), iron (Fe), and zinc (Zn). The second group (non-essential) comprises metals such as barium (Ba), aluminium (Al), and lithium (Li). Depending on the degree of toxicity, heavy metals can be also classified into less toxic, such as tin (Sn), and highly toxic metals, such as mercury (Hg), arsenic (As), cadmium (Cd), lead (Pb), and vanadium (V), which have no biological functions (Squadrone, 2020, Yazdankhah et al., 2018).

There are different ways in which heavy metals can be toxic to bacteria; they can inactivate proteins and enzymes through inappropriate binding of metal-binding sites in enzymes, they can generate reactive oxygen species (ROS), and/or they can interfere with nutrient absorption and cell structure (Yazdankhah et al., 2018, Yu et al., 2017). As a result, bacteria have evolved

resistance mechanisms against these metals, such as metal complex formation or sequestration of toxic metals, detoxification through reduction of intracellular ions, and extrusion of toxic ions by efflux systems (Yazdankhah *et al.*, 2018). Genes that confer resistance to heavy metals, metal resistance genes (MRGs), have been found in both core bacterial genomes and on MGEs of bacteria (Squadrone, 2020). Resistance to heavy metals can co-occur with AMR due to two principal phenomena: co-resistance and cross-resistance (Figure 2.1). Co-resistance occurs when genes responsible for different resistances are located close to each other on the same MGE (Chapman, 2003). This means that MRGs and ARGs are encoded on the same genetic element, such as plasmids, transposons and integrons, thus increasing the potential for co-transfer to other bacteria (Baker-Austin et al., 2006, Yu et al., 2017). Cross-resistance occurs when a single mechanism confers resistance to more than one substance, such as antibiotics and heavy metals (Chapman, 2003). For example, multi-drug efflux pumps can eject both metals and antibiotics from the bacterial cell (Baker-Austin *et al.*, 2006). Additionally, two other phenomena can contribute to the co-selection of AMR and heavy metal resistance (HMR): biofilm formation and co-regulation processes. Biofilm formation allows bacteria to survive in the presence of stress factors, such as heavy metals, because biofilm components can bind or react with metal ions, preventing their diffusion within the biofilm structure. Consequently, mutations in bacterial genomes may be induced and AMR co-selected (Yu et al., 2017). The co-regulation phenomenon occurs when the expression of ARGs changes as a result of a bacterium's exposure to a heavy metal (Yu et al., 2017). Therefore, the presence of heavy metals in the environment can play a crucial role in the dissemination and persistence of AMR.

Heavy metals naturally occur in the environment. They are elements of the earth's crust that derive from pedogenetic processes of erosion of source materials (Wuana and Okieimen, 2011), and cannot be easily degraded because of their stable characteristics (Srivastava et al., 2017, Baker-Austin et al., 2006). Heavy metals can also be released into the environment as a

consequence of anthropogenic activities. Agriculture and aquaculture practices, as well as practices in livestock and horticulture ecosystems, facilitate heavy metal dissemination. Although their application is increasingly restricted due to environmental concerns this varies between countries and regions. Copper compounds are used to protect plants due to their fungicidal functions (Haynes, 2020). In order to prevent accumulation in soil, European legislation has established a ceiling of 28 kg copper per hectare over a period of 7 years (EU Regulation 2018/1981). Additionally, the use of copper compounds in organic agriculture is restricted in the EU, which sets a ceiling of 8 Kg copper per hectare because of its possible long-term consequences if accumulated in the soil (EU regulation 473/2002).

Moreover, to prevent negative effects on human and animal health, as well as in the environment, the use of copper compounds in animal feed is regulated in Europe. EU Regulation 2018/1039, restricts the levels of copper in animal feed. In some parts of the world, copper is used in footbaths on dairy farms to prevent and treat dermatitis, although this practice has been banned in the EU since 2006 (Thomsen *et al.*, 2008). Copper-based antifouling paints are applied in aquaculture to protect cages and nets from the growth of marine microorganisms (Yu et al., 2017). Moreover, heavy metals are added in animal and fish-feed to promote animal health and growth (Seiler and Berendonk, 2012); for instance, zinc oxide, as well as copper sulphate, can be added in pig feed to improve post-weaning performance, although the administration of high levels of zinc oxide was prohibited in the EU from June 2022 (EMA, 2017) (Regulation (EU) 2019/6). Metals added in feed are not fully absorbed, resulting in their presence in animal faeces, which can then be disseminated to the environment through land spreading of agricultural waste. Additionally, the environment can be polluted by heavy metals because of the presence of industrial facilities and factories, or the use of synthetic fertilizers that have heavy metal impurities (Yu et al., 2017). Thus, these factors and practices, including land application of animal manures and biosolids from sewage sludge as fertilizers, direct excretion of faeces on lands, heavy metals use as fungicide, fertilizer, and

additives in animal feed, all contribute to heavy metal pollution worldwide (Yazdankhah *et al.*, 2018). The presence of heavy metals may influence the ARB and ARG dissemination via co- and cross- resistance mechanisms in the primary food production environment, and consequently among plants, animals and humans through transmission routes such as food, water and soil (Koutsoumanis *et al.*, 2021).

Considering that the food production environment is interconnected with human and animal environments, a "One Health" approach, which is a strategy encompassing human, animal and environment concerns (WHO, 2014a), is required to tackle AMR. It is therefore important to understand how agronomic practices and/or the natural presence of heavy metals may influence AMR entering the food chain. This scoping review aims to summarise the evidence regarding the impact of heavy metals as a selective pressure for the dissemination of AMR in the primary food production environment. Whilst a link between HMR and AMR has been demonstrated (Pal et al., 2017, Baker-Austin et al., 2006), there is currently limited information about how this link impacts on AMR in the primary food production environment.

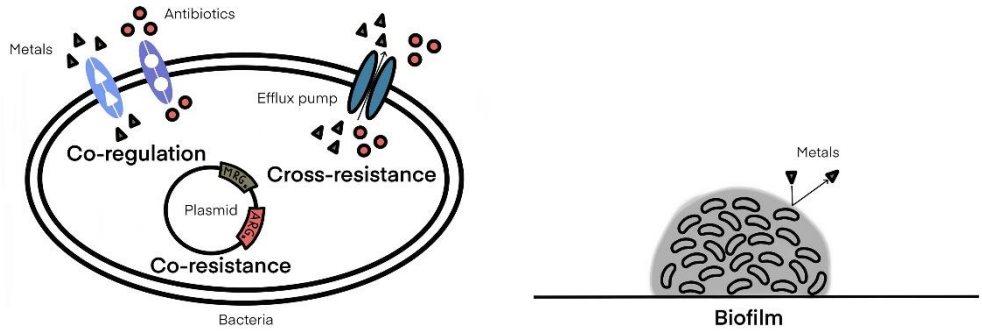

Figure 2.1 Mechanisms associated with co-resistance to heavy metals and antimicrobials.

2.2 Methods

2.2.1 Literature identification: research question and data sources

The review protocol employed for the identification of scientific articles was prepared according to pre-established guidelines (Sargeant and O'Connor, 2020) and adapted from previous studies (Hooban et al., 2020, Farrell et al.,

2021, Chique et al., 2020). Through discussion with a review panel, which included three members, the following research question was formulated:

'Do heavy metals have an impact on AMR in the primary food production environment?'

Based on this question, combined search terms related to 'heavy metals', 'AMR', 'dissemination' and 'primary food production environment' were selected to define search strings. Initially, Scopus was used to assess the terms' efficacy and relevance to the research question. As a result of discussion with the review panel, further terms were added to the search strings, while others were considered not suitable. A list of included and excluded terms is available in Supplementary Material (Appendix A - Table 1). Once a list of the most appropriate terms was compiled, search strings (Supplementary Material, Appendix A - Table 1), with relevant subject headings and Boolean operators such as 'AND' and 'OR', were developed for Scopus, Embase, Medline, PubMed and Biosis (Web of Science) databases. In each database, the field 'TITLE-ABS-KEY' was applied to the search string aiming to explore the terms in these three sections; except for the Biosis database where the terms were searched in the topic field using the field tag 'TS'. Additionally, in the Embase and Medline databases, in order to capture narrower terms, the command 'explode' was used to extend a term which holds more specific terms; whereas in PubMed, 'MeSH' indexing facilitated finding terms that describe the content of an article. Following this, all articles were exported to the reference management software (Endnote 20), and duplicates removed.

Supplementary searches were conducted via grey literature sources using the search term 'antimicrobial resistance and heavy metals' on the following websites: EFSA (www.efsa.europa.eu), FSAI (www.fsai.ie), INAP (www.gov.ie), ECDC (www.ecdc.europa.eu), WHO (https://www.who.int/), CDC (www.cdc.gov), FDA (www.fda.gov), FAO (https://www.fao.org/), OIE(https://www.woah.org/), EPA (www.epa.ie), FSA (www.food.gov.uk) and EMA (www.ema.europa.eu).

2.2.2 Screening and inclusion/exclusion criteria

The article screening consisted of two phases: the first was the evaluation of article titles and abstracts, considering pre-defined inclusion criteria; the second was the assessment of full-texts, after implementing the defined specific exclusion criteria. As shown in Table 2.1, the three main inclusion criteria applied were: (i) focus on antimicrobial resistance; (ii) measurement of heavy metal concentration; (iii) studies undertaken in agricultural/food production environment. Moreover, two limitations were added as inclusion criteria: (iv) articles published since 2006 and (v) texts written in English. Articles published since 2006 was applied as a limitation to ensure the most recent and advanced methodologies, while the English language was chosen for practicality. The articles excluded during the second phase included: studies that analysed samples not related to the primary food production environment; studies that did not investigate AMR or heavy metal concentrations or their association; studies that were focused on a specific process that may have an impact on AMR dissemination and heavy metals but that were not within the scope of this research (e.g. anaerobic thermophilic digestion process); studies that used inappropriate methods; studies that were not primary research (Table 2.1).

Table 2.1 List of inclusion and exclusion criteria applied for article screening.

Inclusion criteria	**Exclusion criteria**
Investigation of antimicrobial resistance	Not relevant to the primary food environment
Determination of heavy metal concentration or detection of heavy metal resistance genes	Heavy metal not investigated
Focus on agricultural/food production environment	Antimicrobial resistance not investigated
Published since 2006 to 2021	Antimicrobial resistance and heavy metal link not investigated
English language	Investigation of AMR and heavy metal for a limited and restricted process not related with the scope of this research (e.g. anaerobic thermophilic digestion process)
	Not primary research (review)
	Inappropriate method used

2.2.3 Data extraction

All relevant data included in the screened research articles were extracted and organised into categories in MS Excel spreadsheets. The main fields included (i) year of publication, (ii) country where analysis was conducted, (iii) type of sample analysed (e.g. soil, water, manure, and other primary food production environmental samples), (iv) sampling location details (e.g. industrial area, mining area, agricultural fields, and other sites), (v) characterization of AMR, (vi) detection of HMR, (vii) measurement of heavy metals, (viii) heavy metals examined and (ix) mobile genetic element

detection (e.g. integrons, plasmids, transposons, and other MGEs) (Supplementary Material, Appendix A, Table 2).

2.3 Results

2.3.1 Literature screening

We identified 2,868 articles (Figure 2.2) by applying the search strings (Supplementary Material, Appendix A - Table 1) in the following databases: Scopus, Embase, Medline, Pubmed and Biosis (Web of Science). Following removal of duplicates that occurred across multiple databases and screening of title and abstract, the articles were subjected to full text review according to pre-established inclusion/exclusion criteria (Table 2.1). The reasons why articles were excluded after full text screening are listed in Figure 2.2.

Several investigations, for example, were excluded because they examined the impact of heavy metal on AMR as part of a specific process outside the scope of this research (n=19). Lu and Lu (2019) for instance, analysed the effect of different parameters, including heavy metal concentration, on AMR during anaerobic composting of swine manure, while Zhang et al. (2018a) investigated the impact of copper on the fate of ARGs, MRGs and MGEs during the aerobic co-composting of tylosin fermentation dregs. Another two articles were excluded due to the use of mathematical modelling to provide a general mechanistic framework (Arya et al., 2021, Gothwal and Thatikonda, 2021), which were therefore incomparable with the other studies. Three articles were considered as a result of the grey literature search, but did not include original data and were therefore not considered in subsequent analysis. The final number of articles considered relevant for inclusion was 73.

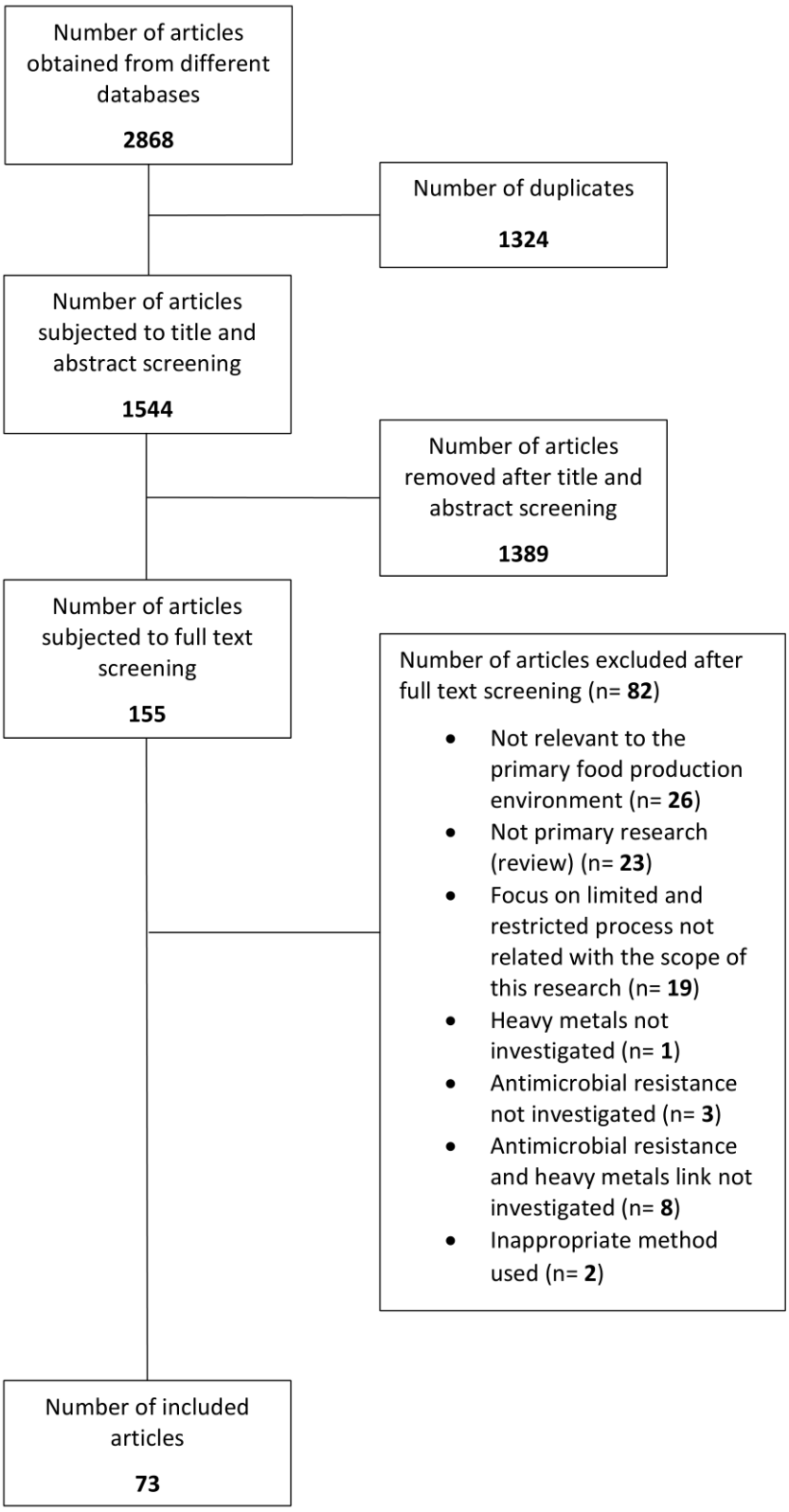

Figure 2.2 Flowchart delineating the review protocol applied for article screening, including exclusion criteria, leading to the final studies included.

2.3.2 Data analysis

The articles included were published over a period of 13 years (from 2008 to 2021) and showed a rapid increase since 2017 (Figure 2.3).

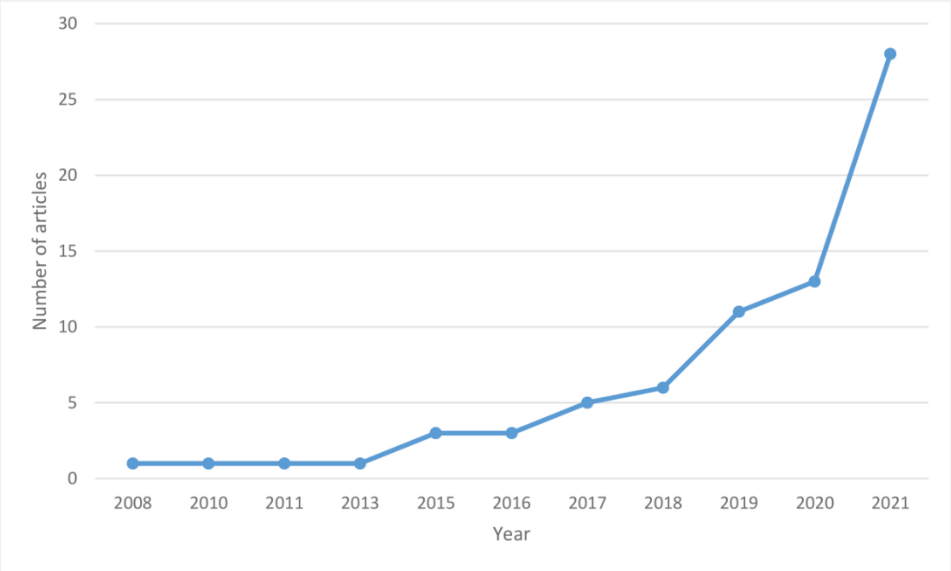

Figure 2.3 Line chart showing the number of included articles per year of publication.

Geographically, most of the investigations were conducted in Asia, with China being the country where the most studies were performed (n= 44), followed by Brazil, India and Iran (n=4 in each country). Three studies were undertaken in Poland and two each in Australia, Portugal and UK, respectively, while one relevant study was conducted in each of the following countries: USA, Korea, Russia, Italy, Spain, Finland, Denmark and Congo (Figure 2.4).

Figure 2.4 Worldwide distribution of included studies.

In terms of the objective of the studies, they were mainly focused either on investigating the impact of heavy metals on AMR (n=50; 68.5%) or on the detection of MRGs and ARGs (n=23; 31.5%). In the first case, different approaches were adopted. For example, Hu et al. (2017) employed a temporal approach to investigate the impact of nickel exposure on the diversity and abundance of ARGs over a period of 4-5 years. Younessi et al. (2020) used a spatial approach to compare the prevalence of ARB in poultry manure between antibiotic-using and antibiotic-free farms; while a quantitative approach was applied in many of the studies, such as the one undertaken by Li et al. (2019) who investigated the effect of sub-lethal levels of heavy metals on antimicrobial resistance.

Overall, there were diverse sample types analysed in the included studies. Soil was the sample analysed most, followed by water (including sea, river and lake water) and manure. Sampling locations also differed between the included articles. In some cases (n=22) samples were collected from areas subjected to treatments, for example soil intentionally amended with heavy metals (Zhao *et al.*, 2020), or with manure for nutrient addition purposes or study purposes. Other studies (n=18) focused on the analysis of samples

collected from areas close to industries or mines. For example, Zhang et al. (2018b) collected soil samples from seven agricultural areas with different distances from a lead-zinc smelting plant which electrolyzes lead, silver, gold, and other metals, while Safari Sinegani and Younessi (2017) analysed soil samples from three different mining sites, two iron ore mines and one lead-zinc mine. Twenty-six investigations were performed on samples collected from areas with no association with either industries or mines, nor were they subjected to treatments with heavy metals or manure, and therefore focused on the natural presence of heavy metals. An example of this is represented by the study conducted by Gallo et al. (2019) on soil samples obtained from sites for which there were no reports of contamination by heavy metals. In seven cases no data regarding sample locations were provided (Figure 2.5).

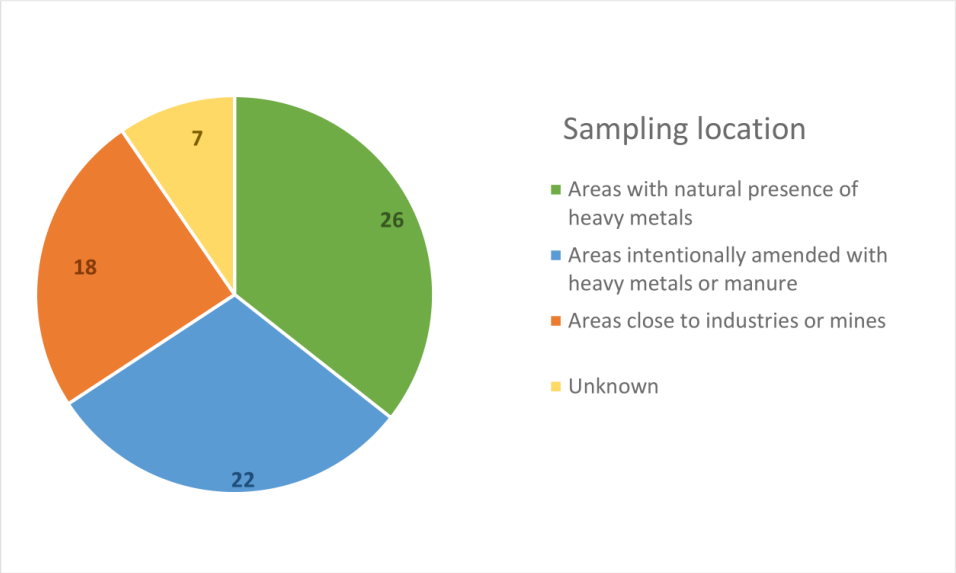

Figure 2.5 Breakdown of sampling location type.

Although AMR was evaluated in all the articles included, HMR was only investigated in a subset of the studies. Some studies examined the heavy metal concentration only. However, in 22 articles both HMR and HM concentration were analysed.

Considering the techniques employed, different approaches for the detection of AMR and HMR were applied; some studies used phenotypic

methods only (n=14), others used a combination of phenotypic and genotypic methods (n=13), while the majority used genotypic methods only (n=46), seven of which used a metagenomics approach. Culture-based analysis, such as minimum inhibitory concentration (MIC) and antibiotic sensitivity testing through disk diffusion, were the phenotypic methods used to detect AMR and HMR. The genotypic methods used to detect ARGs and MRGs were mainly polymerase chain reaction (PCR) and whole genome sequencing (WGS). In a few cases, AMR and HMR were detected using other methods, for example a novel cultivation-independent assay (BrdU-PICT) or a GeoChip hybridization assay (Berg et al., 2010, Zhang et al., 2021).

In one case ARGs were not studied, instead the presence of antibiotics and their correlation with MRGs and MGEs was investigated using high performance liquid chromatography/tandem mass spectrometry system and statistics analysis instead (Lu and Liu, 2021). In two studies the methods used to detect AMR were not specified; both studies conducted ARG conjugative transfer experiments in heavy metal contaminated environments in which the donors harboured plasmids that carried specific ARGs (Pu et al., 2021a, Zhang et al., 2018c). Specifically, Pu et al. (2021a) evaluated the conjugative transfer using the donor *Pseudomonas putida* KT2442, which harboured the lncP plasmid RP4 that carried kanamycin, ampicillin, and tetracycline resistance genes; while, Zhang et al. (2018c) employed the donor *E. coli* S17-1 which harboured the plasmid pCM184-Cm that carried ARGs to ampicillin, tetracycline, and chloramphenicol.

Regarding the methods applied to measure heavy metal concentrations, this analysis was mainly evaluated using a single technique; however, seven studies used two or even three different methods, depending on the type of heavy metal being assessed. Inductively Coupled Plasma (ICP), including ICP-optical emission spectrometry and ICP-optical mass spectrometry, was the method mostly used to assess heavy metals (n=32). Another method applied was Atomic Absorption Spectrometry (AAS), including flame-AAS, Hydra-C-AAS, Hydride generation-AAS and cold vapour-AAS (n=20). While atomic fluorescence spectrometry (liquid chromatography-AFS and cold vapour-

AFS) was used in five studies, X-Ray fluorescence was used in only two studies. HM concentration was not measured in 21 investigations, which instead were focused on HMR analysis. In ten out of these 21 studies, the HM concentration was established in advance. Moreover, data regarding the methods used to detect HM were not provided in two cases. The choice of method applied depends on the type of sample and metal to be analysed. For example, ICP is a method that allows detection of metals at very low concentrations; particularly, it can analyse multiple metals at one time, while AAS is not able (Jin et al., 2020, VELEZ, 2009). AFS, for example, can only be applied for certain metals, such as arsenic and mercury (Jin et al., 2020).

The type and number of heavy metals investigated differed between studies, 54% (n=40) examined four or more heavy metals, 20% (n=15) analysed two or three metals, while the remaining 24% (n=18) focused on a specific single metal, namely copper, zinc, arsenic, cadmium, nickel or mercury. Among all the studies, copper was the most common heavy metal analysed (n=59), followed by zinc, for which concentration and/or resistance were assessed in 49 studies (Figure 2.6B). Both copper and zinc concentrations were determined primarily using ICP and AAS methods, while regarding copper and zinc resistance characterization, MIC was the method mostly used for the detection of resistance phenotypes, followed by PCR and WGS.

The classes of antibiotics tested varied among the studies (Figure 2.6A). The antibiotics mostly tested belonged to the tetracycline class (n=51) and beta-lactam class (n=43). Sulphonamides and quinolones were also commonly tested (n=36), as well as aminoglycosides (n=30) and macrolides (n=28). Most studies (n=54, 73%) focused on resistance to several classes of antibiotics, only in seven cases was the focus on one individual class of antibiotic. For example, Buta et al. (2021) evaluated the presence of ARGs encoding for resistance to eight different antibiotic classes, while Gothwal and Thatikonda (2017) tested seven different antibiotics, but all belonging to the quinolones class.

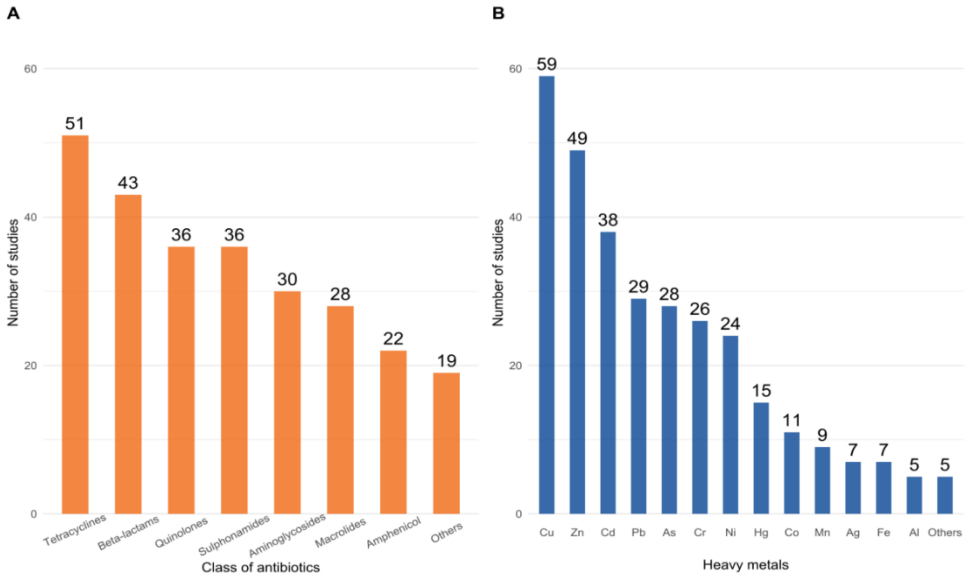

Figure 2.6 (A) Class of antibiotics examined in the included studies. (B) Heavy metals investigated in the studies included in this review.

In most studies, AMR plus HMR were analysed using a genomic approach that did not involve bacterial culture analysis. This meant that the majority of the studies were not focused on the analysis of specific bacterial species or groups. Only a few investigations targeted the analysis of specific bacteria. For instance, Mustafa et al. (2021) studied HMR and its association with AMR in *Salmonella* Typhimurium isolates. In contrast, Laffite et al. (2020) investigated the impact of anthropogenic activities on the occurrence of beta-lactamase and carbapenemase genes in river sediments, although the host bacterial species were not specified.

The majority of studies (n=51, 70%) assessed the presence of MGE, particularly integrons (n=38), plasmids (n=21), transposons (n=18) and insertion sequences (n=8). Among the integrons, class 1, which is frequently linked with ARGs and MRGs (Gillings *et al.*, 2015), was the most commonly detected (n=30), followed by class 2 (n=14), 3 and 4 (n=2 and n=1, respectively). Four studies assessed the presence of plasmid mediated quinolone resistance (PMQR) (Gallo et al., 2019, Siddiqui et al., 2020, Tuo et al., 2018, Xu et al., 2017) and seven examined plasmid mediated beta lactam resistance (Figueiredo et al., 2019, Otinov et al., 2020, Pu et al., 2021a, Pu et

al., 2021b, Wang et al., 2020, Wilson et al., 2019, Zhang et al., 2018c). In some cases (n=9), an experiment was conducted to evaluate the mobility of resistance genes (conjugation transfer of ARGs) (Supplementary Material, Appendix A - Table 2).

All of the included studies identified a link between AMR and heavy metals. Depending on the aim and the type of study conducted, the findings obtained varied among the studies. The majority (n=34) established that heavy metals promote the spread of MGEs and/or ARGs, and a correlation between a specific heavy metal and ARGs or antibiotics has been established. For example, Hao et al. (2021) stated that heavy metals were the most influential environmental factors that affect the distribution of ARGs and MGEs, followed by polycyclic aromatic hydrocarbons and soil features, after statistical analysis. In particular, the authors identified significant positive correlations between the abundance of multidrug ARGs and metals, such as chromium, cobalt and arsenic. Hu et al. (2017) provided evidence that ARG abundance increased as a result of nickel exposure and correlated with its concentration. Overall, copper and/or zinc, which were the main metals analysed, have been associated with tetracycline (Duan et al., 2019, Figueiredo et al., 2019, He et al., 2017, Mazhar et al., 2021, Safari Sinegani and Younessi, 2017, Wu et al., 2020, Zhao et al., 2019a, Zou et al., 2021, Mustafa et al., 2021), sulphonamide (Duan et al., 2019, Figueiredo et al., 2019, He et al., 2017, Wu et al., 2020, Zhao et al., 2019a, Zou et al., 2021, Hubeny et al., 2021), betalactam (Figueiredo et al., 2019, Mazhar et al., 2021, Wu et al., 2020, Zhao et al., 2019b, Zou et al., 2021, Mustafa et al., 2021), aminoglycoside (Figueiredo et al., 2019, Zhao et al., 2019b, Zou et al., 2021, Wang et al., 2021b) and macrolide resistance genes (Zou et al., 2021, Wang et al., 2021b). The same antibiotics have been associated with nickel and arsenic, but nickel has also been associated with multidrug and vancomycin (Hu et al., 2017, Safari Sinegani and Younessi, 2017, Zhao et al., 2019b, Zou et al., 2021), while arsenic was also linked with streptomycin, chloramphenicol and multidrug resistance (Figueiredo et al., 2019, Mazhar et al., 2021, Zhao et al., 2019b, Wang et al., 2021b, Hao et al., 2021).

The role of MGEs on the dissemination of ARGs and MRGs was demonstrated in nine studies. Anjum et al. (2011) demonstrated that bacteria with an IncP plasmid had the capacity to transfer MRGs and ARGs through a conjugative mechanism, therefore they proposed that these plasmids are key for the dissemination of multi-drug resistant bacteria in contaminated alluvial soil. Sixteen investigations showed a positive correlation between HMR and AMR, while 14 studies stated that heavy metals, such as copper and arsenic, apply selective pressure on AMR. For instance, one study revealed a high association between copper tolerance and resistance to antibiotics like ampicillin, erythromycin and vancomycin through statistical correspondence analysis (Glibota *et al.*, 2019), showing a positive correlation between the occurrence of heavy metals and antimicrobial resistance. The role that heavy metals may play as a selective pressure on AMR was demonstrated, for example, in a study which indicated that arsenic exposure exerted a selective pressure on the bacterial resistome in soil (Cao *et al.*, 2020).

2.4 Discussion

This scoping review aimed to interrogate existing literature with regard to the impact of heavy metals on AMR in the primary food production environment. Traditionally much more attention has been given to AMR development and transmission between humans and animals than in natural and agricultural settings; however, this has changed in the last decade (Koutsoumanis *et al.*, 2021). This increased interest in the role of the environment is reflected in the rapid increase in the number of relevant studies since 2016 included in this scoping review. The importance of AMR in environmental settings was highlighted in the 2015 World Health Organization AMR Action Plan (WHO, 2015), that recognized AMR as a worldwide threat to human health. The studies included in this scoping review considered different types of environmental samples: soil, water and manure principally; but also examined the characteristics of the environment where they were collected from, for example the vicinity to

mines or industries, or exposure to a specific treatment. Through the analysis of the included studies, an association between AMR and the presence of heavy metals was generally observed. In some cases, a direct correlation was established, such as promotion of ARGs dissemination induced by heavy metals; while in other cases, the role of MGEs on the spread of ARGs and MRGs was demonstrated.

2.4.1 Limitations in geographical distribution of studies included.

Given the inclusion criteria in the review, there was a limitation in the geographical representativeness. The majority of studies identified were undertaken in China. There may be a number of reasons for this. China is the highest producer and consumer of antibiotics in the world (Duan *et al.*, 2019). In 2013 the annual consumption of antibiotics in China was reported to be 160,000 ton, of which 84,000 ton was used in livestock *(*Yue *et al.*, 2021), with a significant increase in the use of antibiotics in veterinary settings from 46% in 2007 to 52% in 2013 observed (Tiimub *et al.*, 2021). Furthermore, despite the implementation of the Animal Medicine Management Regulations in 2004, it has been reported antibiotics may be overused as treatments and animal growth promoters in China (Zhou *et al.*, 2016). For thousands of years China, like many countries, has applied varied agricultural practices, including raw manure soil amendment and irrigation with wastewater (Zhou *et al.*, 2017). Since most antibiotics are poorly absorbed in the animal and human intestine, they can be released in the environment through faeces and urine (He *et al.*, 2017). Hence, long-term manure application may enhance the levels of ARGs (Tuo *et al.*, 2018). Moreover, heavy metal pollution of soil, especially in the south of China, is a growing concern, notably for cadmium and arsenic which are the most common heavy metals in this area due to the presence of mines and industries (Zhao *et al.*, 2020). This pollution may be caused by industrialization, that lead to the development of mining factories and smelting plants; and anthropogenic activities, such as use of heavy-metal-contained pesticides, application of manure on farmland, and abandonment

of mine tailings (Cao et al., 2020, Qiao et al., 2021). It has been reported that accumulation of heavy metals in agricultural soil in China can be caused by the use of sewage irrigation, which represents an important source of water due to the deficiency of water sources in this country (Wu *et al*., 2022). Additionally, the use of fertilizers and insecticides have increased heavy metal concentration in soil, especially cadmium, nickel, copper and mercury, which are used in crops, such as rice (Shifaw, 2018, Su et al., 2022).

A number of studies were undertaken in mining-affected areas or close to mineral mines and smelting plants where metal areas are more likely to be elevated (Xu et al., 2017, Zou et al., 2021). Heavy metal pollution of water is also of concern; it has been found that heavy metals are responsible for 20% of water pollution incidents in China (Wu *et al.*, 2017). China has acknowledged the growing concern about AMR and heavy metals in the environment, and through its National Action Plan to Contain Antimicrobial Resistance (2016-2020), it has increased research in order to address knowledge gaps (WHO, 2014b).

In accordance with EU legislation (Directive 2003/99/EC, Commission implementing Decision (EU) 2013/652 and 2020/1729), AMR in *Salmonella*, *Campylobacter jejuni* and indicator commensal *E.coli* from major food-production animals is monitored annually by EU member states (Koutsoumanis *et al.*, 2021). Moreover, the use of antibiotics in animal feed as growth promoters has been banned since 2006 in the European Union, as well as in the United States since 2014 (Yue *et al.*, 2021) with more recent restriction on the use of heavy metals in livestock production (EMA, 2017) (EU Regulation 2019/6) (EU Regulation 2018/1039). Despite the presence of these regulations and surveillance systems, there have been limited investigations on AMR and heavy metal linkages in Europe. Anthropogenic activities have caused an increase in heavy metal pollution in Europe; for example, almost 85% of metals discharge into water resources stemmed from human activities (Silva *et al.*, 2021). Additionally, heavy metals are used as animal growth and health promoters, as disinfectants, antiseptics and preservatives in agriculture and aquaculture sectors (Figueiredo *et al.*,

2019), even though their use is increasingly restricted and varies worldwide. Similarly, in low and low middle income countries (LMIC) there is limited monitoring of heavy metal concentrations and AMR; for example, in India the waste generated from industrial, urban and agricultural activities is often discharged into water systems without any treatment (Siddiqui et al., 2020, Laffite et al., 2020), but there is limited understanding of how this may impact on the dissemination of AMR or ARGs.

2.4.2 Diversity of methods applied to detect antimicrobial and heavy metal resistance reflects studies' heterogeneity.

The use of phenotypic and/or genotypic methods to detect AMR or HMR has various advantages and disadvantages. Although the use of culture-based analysis is time-consuming, requires trained personnel, and may be subject to human error (Shanmugakani *et al.*, 2020), it is generally lower cost than genotypic methods, and provides data to guide treatment decisions (Shanmugakani et al., 2020, Boolchandani et al., 2019). However, culture-based analysis can only be applied for cultivable bacteria and not for studying microbial communities that are characterized by uncultivable microorganisms (Boolchandani *et al.*, 2019). In contrast, genotypic methods are more rapid, and provide accurate results on the detection of AMR and HMR genetic mechanisms (Anjum *et al*., 2018). Molecular techniques are supported by the building of resistance gene databases; however, there is still limitation on the harmonization of data feeding into databases, and they may not detect all resistance mechanisms because of the considerable genetic diversity and the continuous discovery of new genes (Boolchandani *et al.*, 2019) .

The methods applied in the studies included in this review included phenotypic, genotypic and a combination of both. This results in a heterogeneity in the studies, which consequently means it is difficult to compare them with each other.

In investigations that demonstrated heavy metals as a selective pressure on AMR dissemination (n=14), several methods were applied. Most of these

articles detected AMR and HMR using only genotypic methods, of which PCR was the most predominant. For example, Hu et al. (2016), observed that AMR in agricultural soil changed considerably after 4-5 years of copper contamination, through the use of high throughput quantitative PCR (HT-qPCR). It would be of interest to determine if this observation also resulted in phenotypic alterations. Three articles employed phenotypic methods to investigate AMR and HMR. For example, MIC analysis was applied to investigate the effects of metal such as arsenate, cooper and zinc on AMR in bacterium LSJC7 (Chen *et al.*, 2015). LSJC7, a sequenced strain of Enterobacterales, was already known to harbour arsenate and tetracycline resistance genes, demonstrating the value of linking phenotypic and genotypic analysis. Four studies applied both phenotypic and genotypic methods. For instance, Li et al. (2019) stated that metals, such as copper, zinc and silver, at sub-lethal concentrations led to an increase in AMR via genetic mutations. This study was conducted by analysing the MIC of antibiotics and metals in a 96-well microplate, and the genetic profile of the resistant mutants through whole genome sequencing.

The majority of the articles that suggested that heavy metals promote the dissemination of MGEs and/or ARGs applied only genotypic methods to detect AMR and HMR. The use of genotypic methods allows the detection of new resistance variants, with an accurate and rapid approach. Through HT-qPCR and metagenomic analysis, Wang et al. (2021b) demonstrated the role of heavy metals, such as arsenic, copper, zinc and lead, on the co-selection of ARGs, especially at high metal concentrations, and asserted that ARGs abundance and profile could be altered by the presence of heavy metals. Exclusively phenotypic methods, such as antibiotic susceptibility test by disc diffusion and minimum inhibitory concentration of heavy metals, were performed in the study conducted by Safari Sinegani and Younessi (2017) which identified heavy metals as one of factors responsible for the high abundance of AMR in agricultural soil. The use of phenotypic methods is constructive to establish the prevalence of AMR in bacterial populations,

to evaluate how resistance profiles have changed over time, and to identify new resistance phenotypes.

Further investigations established a positive association between heavy metals and AMR using phenotypic methods or a combination of phenotypic and genotypic approaches. A strong correlation between cadmium and gentamycin resistance was detected in one study, which evaluated AMR by agar dilution test, and the HMR by MIC in animal faeces samples, and suggested a future genomic investigation (Marazzato *et al.*, 2020). Back et al. (2020) identified the important role of co-selection of tetracycline resistance and zinc resistance in CC398 livestock-associated (LA)-methicillin-resistant *Staphylococcus aureus* (MRSA) in Korea's swine population using both phenotypic and genotypic methods to analyse AMR and HMR. It would be of interest to investigate the relationship between AMR phenotypes and genotypes in order to determine if there is a match between them, and to identify the more accurate and fastest approach to conduct such comparative analysis.

It has been demonstrated that within the same study phenotypic and genotypic results on AMR and HMR analysis can be mismatched, indicating there is not always concordance between the phenotypic results and resistance genes profile (Schwan et al., 2021, Neuert et al., 2018, Do Nascimento et al., 2017); therefore, a combination of culture-based analysis and genotypic methods may produce results with a more complete overview. The extensive diversity of methods used makes the comparison of studies difficult; it would be preferable to establish a harmonised procedure for the detection of AMR and HMR in the environment, and measuring heavy metal concentrations, to enable more informative comparative analysis.

2.4.3 Heavy metals influence the abundance and spread of mobile genetic elements

The presence of MGEs, such as integrons, plasmids, transposons and insertion sequences, has been detected in almost 70% (n=51) of the studies included in this review. A significant number of these investigations (n=32)

established the role of heavy metals, such as copper, zinc, arsenic, cadmium and lead, in affecting the distribution of ARGs, and the abundance and spread of MGEs in the environment (n=22); so that heavy metal content positively related with ARG abundance, in samples which included agricultural soil, urban soil, soil from livestock, water and animal faeces.

Several studies demonstrated that heavy metals can influence ARG dissemination by facilitating the spread of MGEs. It was shown that cadmium at sub-inhibitory concentrations enhanced conjugative transfer of the RP4 plasmid from *P. putida* KT2442 to the microbial community in fresh water (Pu *et al*., 2021a). Cadmium treatment has been shown to lead to an increase in cell membrane permeability, and affected the mRNA expression levels of genes involved in conjugation, repressing some genes that downregulate the expression of the genes required for conjugation (Pu *et al*., 2021a). Wang et al. (2020) observed that heavy metals, including copper and zinc, promoted conjugative transfer of the multi-resistance plasmid RP4 through bacterial cell membrane damage. A further study provided evidence of the ability of heavy metals, including copper and zinc, to promote ARG transfer through conjugation in the water environment. In this case, heavy metals were involved in generation of reactive oxygen species, alteration of expression of conjugation related genes, and an increase of in cell membrane permeability (Zhang *et al.*, 2018c). Otinov et al. (2020) highlighted that some metal oxide nanoparticles (MONPs), such as zinc-oxide (ZnO) and boehmite (AlOOH), can enhance the efficacy of transformation and conjugation, respectively, while others, such as titanium dioxide (TiO_2) can inhibit transfer mechanisms.

Furthermore, other studies established that exposure to heavy metals can result in an increase in the abundance of MGEs such as integrons, insertion sequences and transposons, which are related to the transfer mechanisms of ARGs among bacteria. For example, cadmium was shown to increase the abundance of ARGs and *int*I1 in oxytetracycline polluted soil and lettuce tissue (Guo *et al.*, 2021). Tongyi et al. (2020) observed that the abundance of *int*I1, insertion sequences, and ARGs increased, along with the gradient of

zinc concentration in the soil, indicating that zinc can affect ARG mobility. In urban soils heavy metals, such as arsenic, cadmium, cobalt, chromium, copper, mercury, nickel and zinc, can alter MGE abundances (Zhao et al., 2019b).

In contrast to these studies that consider MGEs as a main factor influencing ARG dissemination, Qiao et al. (2021) proposed that MGEs have only an indirect effect on AMR dissemination by influencing the microbial community composition and that, instead, was considered the main driver of ARG spread.

Overall, the role of heavy metals on the dissemination of MGEs, and consequently ARGs, is evident. Through the regulation of gene expression, the production of reactive oxygen species and the alteration of cell permeability, heavy metals can affect MGE spread, and thus even AMR dissemination.

2.5 Conclusion

The results obtained in this review demonstrate a link between heavy metals and AMR in the primary food production environment. Although the studies included differed in their objectives, sample types, sampling locations and methods applied, all asserted the important role of heavy metals on AMR dissemination and persistence. Some studies demonstrated that heavy metals can apply selective pressure on AMR; others that heavy metals can promote the dissemination of MGEs and/or ARGs in the environment; and others that MGEs play an important role in ARGs and MRGs dissemination. However, a better understanding of how heavy metals can influence AMR and ARGs in the primary food production environment is needed to inform the development of effective mitigation measures. Limited investigations regarding heavy metals and AMR dissemination in the environment have been conducted to date in many regions of the world. Therefore more research should be undertaken to better understand the mechanisms through which heavy metals impact on AMR, particularly in the context of feed and food production. Harmonising the approaches to AMR and HMR

analysis and measurement of heavy metal concentration is critical. The increased availability of metagenomic analysis provide the opportunity of obtaining more in-depth information on AMR profiles in microbial communities, linked with physiochemical properties such as heavy metal concentrations, in different ecological niches.

2.6 Appendix A: Supplementary material

The supplementary material for Chapter Two "Evaluating the impact of heavy metals on antimicrobial resistance in the primary food production environment: A scoping review" is available at DOI of the reference below.

Anedda E., Farrell M.L., Morris D., Burgess C.M. 2023. Evaluating the impact of heavy metals on antimicrobial resistance in the primary food production environment: A scoping review. *Environmental Pollution*, 320, p.121035. DOI: https://doi.org/10.1016/j.envpol.2023.121035

Chapter Three

Characterization of antimicrobial resistant Enterobacterales isolated from spinach and soil following zinc amendment.

Published in Environmental Pollution

Anedda, E., Ekhlas, D., Alexa, E., Farrell, M. L., Gaffney, M. T., Madigan, G., Morris, D. & Burgess, C. M. 2024. Characterization of antimicrobial resistant Enterobacterales isolated from spinach and soil following zinc amendment. *Environmental Pollution, 361***,** p.124774. DOI:

https://doi.org/10.1016/j.envpol.2024.124774.

Abstract:

Antimicrobial resistant bacteria can occur in the primary food production environment. The emergence and dissemination of antimicrobial resistance (AMR) in the environment can be influenced by several factors, including the presence of heavy metals. The aim of this study was to examine the presence and characteristics of antimicrobial resistant Enterobacterales in soils and spinach grown in soils with and without zinc amendment. A total of 160 samples (92 soil and 68 spinach) were collected from two locations, in which some plots had been amended with zinc. Samples were cultured on selective agars for detection of extended-spectrum beta-lactamase-producing Enterobacterales (ESBL), carbapenem-resistant Enterobacterales and ciprofloxacin-resistant Enterobacterales. Samples were also cultured for enumeration of total Enterobacterales. Isolates were identified by MALDI-TOF. Antimicrobial susceptibility testing was carried out in accordance with EUCAST (2021) and CLSI (2020) criteria. The whole genome sequence (WGS) of selected isolates was determined. Inductively Coupled Plasma Atomic Emission Spectrometer was performed on soil samples in order to measure the concentration of zinc. In total 20 antimicrobial resistant Enterobacterales were isolated from the soil (n = 8) and spinach samples (n = 12). In both sample types, *Serratia fonticola* (n= 16) was the dominant species, followed by *Escherichia coli* (n=1), *Citrobacter freundii* (n=1) and *Morganella morganii* (n=1) detected in spinach samples, and *Enterobacter cloacae* (n=1) detected in a soil sample. The WGS identified genes conferring resistance to different antimicrobials in agreement with the phenotypic results, 14 *S. fonticola* isolates were confirmed as ESBL producers and harboured the bla_{FONA} gene. Genes that encoded for zinc resistance and multidrug efflux pumps, transporters that can target both antimicrobials and heavy metals, were also identified. Overall, the findings of this study suggest the presence of zinc did not influence the antimicrobial resistant Enterobacterales in soil or spinach samples.

3.1 Introduction

Antimicrobial resistance (AMR) is a major public health concern globally. Although AMR is an ancient natural phenomenon in the environment (D'Costa et al., 2011), the increasing use of antimicrobials in humans, animals and agriculture has played a role as a selective pressure for AMR dissemination in the environment (Koutsoumanis et al., 2021). Anthropogenic activities, such as the use of antimicrobials to prevent and treat diseases in agriculture, have the potential to influence the composition and dissemination of antimicrobial resistance genes (ARGs) present in the environment (Roca et al., 2015, Farrell et al., 2021, Stanton et al., 2022).

The carbapenems and third-generation cephalosporins are recognised as critically important antimicrobials by the World Health Organisation (WHO) (WHO, 2019). Resistance to third-generation cephalosporins can be mediated by extended-spectrum beta-lactamase (ESBL) enzymes (Blaak et al., 2014). ESBL-producing Enterobacterales (ESBL-PE) and carbapenem-resistant Enterobacterales (CRE) can enter the environment through human and animal waste, and can contaminate the natural environment and food production settings (EMA, 2018). Following ingestion, these antimicrobial resistant bacteria (ARB) may colonize the human gastrointestinal tract and transfer ARGs into the human microbiota via mobile genetic elements (MGEs) (Carlet, 2012, Meurs et al., 2020). Resistant Enterobacterales can also be associated with infection, such as urinary tract infections and bloodstream infections (Yousefipour et al., 2019, Meurs et al., 2020).

Bacteria can be naturally resistant to antimicrobials (intrinsic resistance), or can develop resistance after acquisition of ARGs via MGEs (e.g. plasmids and transposons) or a chromosomal mutation which can be induced by various selective pressures (acquired resistance) (Munita and Arias, 2016). For example, the use of antimicrobials, biocides and heavy metals in the food production environment can have an impact on AMR occurrence and dissemination (Koutsoumanis et al., 2021). Specifically, heavy metals can influence AMR through different mechanisms: co-resistance, cross-resistance, co-regulation and biofilm formation (Yu et al., 2017, Baker-Austin

et al., 2006, Chapman, 2003, Koutsoumanis et al., 2021). Co-resistance occurs when ARGs and metal resistance genes (MRGs) are located on the same MGEs; therefore, the presence of heavy metals, such as zinc and copper, can increase the abundance and spread of MGEs, and consequently facilitate ARG dissemination (Li et al., 2022, Wang et al., 2022, Tongyi et al., 2020). Thus, the link between AMR and the presence of heavy metals is evident (Anedda et al., 2023), although its influence in different food production settings is less clear.

Although many heavy metals are vital elements for organisms, they can be toxic if heavy metal concentrations are in excess. Zinc has both structural and catalytic functions in proteins (Hood and Skaar, 2012, Huang et al., 2017, Lee et al., 2005). It is a component of DNA-binding proteins and a membrane stabilizer (Choudhury and Srivastava, 2001), and is necessary for the expression of some genes, for example genes that encode for flagella in *Salmonella* Typhimurium (Ammendola et al., 2016). However, zinc can be toxic when present in high concentrations, as it can interfere with respiratory electron transport systems (Choudhury and Srivastava, 2001). Therefore, bacteria may carry genes that confer resistance to zinc, such as the *czc* operon, or ZntA P-type ATPase which encode for transporters and efflux pumps, in order to maintain cellular zinc homeostasis (Choudhury and Srivastava, 2001, Huang et al., 2017).

Zinc is a naturally occurring element, and its concentration varies depending on the geology of the region. However, zinc can also be released into the environment through anthropogenic activities. For example, zinc in animal faeces can be transferred into soils after animal manure application, it can be used as a preventative measure against fungal disease in cattle and sheep, and it can be used as a fertilizer to optimize plant growth (Alloway, 2008, Heydari et al., 2022). Although, zinc is a vital micronutrient for plant growth, an excess or a deficiency of it in agricultural soils may have severe consequences. Zinc deficiency can lead to a decrease in crop production and leaf photosynthetic capacity, while zinc toxicity can be responsible for

inhibiting root growth and causing chlorosis in young leaves (Barman et al., 2018).

As zinc is commonly used in primary food production to support plant production, the aim of this study was to investigate the impact of zinc application on the presence of ARB (specifically ESBL-PE and CRE) in soils and spinach grown in these soils.

3.2 Materials and methods

3.2.1 Sampling collection

Spinach and soil samples were collected in May, June and October 2021 from two geographically distinctive locations, A and B, 40 km away from each other, in Ireland. Location A and B were based in the East of Ireland and were non-commercial research sites. The sites were initially inversion ploughed and then rotovated twice more before 1.6m wide beds were formed with a 1.8m width bedformer. At each site, two different areas were divided into plots of 2.75m x 1.8m, and fertiliser was applied as 105:35:170 N:P:K (Nitrogen, Phosphorus and Potassium) as per soil test and recommended rates. Spinach seeds were then direct drilled into the beds at 500,000 seed per acre. At both sites, twelve plots were amended with zinc (Zn) in 2019 and 2020 (Supplementary Material, Appendix B - Figure 1). For zinc amendment, 45 kg/ha of zinc sulphate, $Zn(SO_4)$, was dispensed as a water suspension onto the plots. This rate was selected as a maximum application rate from both an agronomic and economic perspective. At each site, an additional twelve plots were randomly chosen as controls (C) where zinc was not added. The soil from both locations was taxonomically classified as haplic gleysol, in accordance to the international standard for soil classification system World Reference Base for Soil Resources (WRB).

In total 160 samples were collected from the two locations. Sixty-eight spinach samples were collected over two sampling trips in June and October 2021 (Location A: 17 Zn and 17 C; Location B: 17 Zn and 17 C). Ninety-two soil samples were collected over two sampling trips in May and October

2021 (Location A: 23 Zn and 23 C; Location B: 23 Zn and 23 C). Approximately 300g soil samples, which fell into the loam category, were collected in triplicate from three random points in each sample plot and stored in sterile bags. Approximately 50g of spinach leaves from across the sample plot were harvested into sterile bags. The samples were then transferred to the laboratory in a cooler box, and processed within 24 hours.

3.2.2 Isolation and identification of resistant Enterobacterales

The total number of Enterobacterales in each sample was determined as described in ISO-21528- 2:2017), with dilutions cultured in duplicate on Violet Red Bile Glucose Agar (VRBGA), and incubated for 22±2 hours at 37°C.

For each sample 10g was enriched in 90 mL of Buffered Peptone Water (BPW) for 24 hours at 37°C, and 150µL of this enrichment was cultured on Brilliance ESBL agar (Oxoid), COLOREX mSuperCARBA (E&O Labs), and MacConkey agar (Oxoid) plus a 5 µg ciprofloxacin disc (Oxoid) applied to the centre of the plate, for detection of ESBL-PE, CRE and CIP-RE respectively. All agar plates were incubated at 37°C for 22±2 hours. Colonies were selected based on their colour and morphology, and a single colony per colour/morphology was selected per plate.

Matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry (Bruker Microflex) was used for the identification of presumptive Enterobacterales in accordance with the manufacturer's instructions and as described in the Ekhlas et al. (2023b) study.

3.2.3 Antimicrobial susceptibility testing

Enterobacterales isolated from selective agars were tested for susceptibility to six antimicrobial classes by disc diffusion in accordance with EUCAST (EUCAST version 11.0, 2021) and CLSI (CLSI version 30, 2020) criteria (European Committee on Antimicrobial Susceptibility Testing (EUCAST), 2021, Clinical and Laboratory Standards Institute (CLSI), 2020). The antimicrobial classes tested were: aminoglycosides (gentamicin (10 µg) (CN), kanamycin (30 µg) (K), streptomycin (10 µg) (S)), beta-lactams (ampicillin (10

µg) (AMP), cefotaxime (5 µg) (CTX), cefoxitin (30 µg) (FOX), cefpodoxime (10 µg) (CPD), cefpodoxime / clavulanic acid (10 µg) (CPD/CV), ceftazidime (10 µg) (CAZ), ertapenem (10 µg) (ETP), meropenem (10 µg) (MEM)), phenicols (chloramphenicol (30 µg) (C)), quinolones (ciprofloxacin (5 µg) (CIP), nalidixic acid (30 µg) (NA)), tetracyclines (tetracycline (30 µg) (TET)), and trimethoprim (5 µg) (W). *Escherichia coli* ATCC 25922 was used as a quality control strain. Cefpodoxime (10 µg) and cefpodoxime / clavulanic acid (10 µg) discs were used to confirm extended spectrum beta-lactamase production.

3.2.4 Soil chemical analysis

The following soil properties were assessed: pH, concentration of cadmium (Cd), chromium (Cr), copper (Cu), lead (Pb), mercury (Hg), nickel (Ni), phosphorus (P) potassium (K) and zinc (Zn). Specifically, Cd, Cr, Cu, Zn, Pb, K, P and Ni were determined through Inductively Coupled Plasma Atomic Emission Spectrometer (ICP-OES); while, Hg was measured by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (Khan et al., 2022, Wilschefski and Baxter, 2019). This analysis was conducted at Southern Scientific Services Ltd., Farranfore, Co. Kerry, Ireland.

3.2.5 Whole genome sequencing analysis of ESBL-PE/CRE isolates

The DNA of all isolates identified as ESBL-PE (n = 19) and CRE (n = 1) was extracted using the DNeasy UltraClean Microbial Kit (Qiagen, United Kingdom) following the manufacturer's instructions. Whole genome sequencing was performed using Illumina NextSeq 2000. DNA libraries were prepared with the Illumina DNA prep kit as per the manufacturer's instructions, 2 x 150 bp sequencing was performed on the Illumina Nextseq2000 platform using a P2 flow cell. The quality of the sequencing results was assessed using FASTQC (v0.11.8) and MultiQC (v1.9) (Ewels et al., 2016, Andrews, 2010), and the reads were trimmed using Trimmomatic (v0.38) (Bolger et al., 2014). The reads that passed the quality control step (phred ≥ 33) were assembled to contigs using SPAdes (v3.15.3) (Prjibelski et

al., 2020). The resistance genes and plasmids were identified via ABRicate (v1.0.1) (https://github.com/tseemann/abricate), using the databases ResFinder and CARD for antimicrobial resistance genes, PlasmidFinder for plasmids, and MeGARes for heavy metal resistance genes. Moreover, the BacMet2 database was employed for further analysis of antimicrobial resistance genes and heavy metal resistance genes (Pal et al., 2014). In order to assess plasmids, the Platon database (v1.6) (Schwengers et al., 2020) was used to separate assembled scaffolds into chromosomal and plasmidic scaffolds, in which resistance genes were analysed using ResFinder. The serotype of the *E. coli* isolate was determined using the EcOH database (Ingle et al., 2016), and its phylotype was determined using the EzClermont tool (Waters et al., 2020). *E.coli* virulence genes were identified using VFDB database (Chen et al., 2005). Pangenome analysis and average nucleotide identity (ANI) were utilised for genomic comparison of all *Serratia fonticola* isolates using anvi'o (v7.1) (Eren et al., 2021) and ANIclustermap (v. 1.2.0) (https://github.com/moshi4/ANIclustermap).

3.2.6 Statistical methods and visualization

Statistical analysis of soil chemical analyses was performed using the two sample paired Wilcoxon test, for which the p-value was adjusted through Bonferroni correction with significance established at $p < 0.05$. The results' analysis and data visualization were carried out using RStudio (Version 1.3.1093), with the corresponding rstatix, ggplot2, and RColorBrewer packages.

3.2.7 Data availability

The sequences data of the isolates analysed in this study are publicly available at BioProject: PRJNA1132929.

3.3 Results

3.3.1 Enumeration of Enterobacterales in soil and spinach samples

Enterobacterales were enumerated in all samples collected (92 soil and 68 spinach samples) (Figure 3.1). A value of equal to or greater than 2 log CFU/g of samples was found in 83 soil samples (90%) and in 59 spinach samples (86%). The Enterobacterales average concentration was 3.58 log CFU/g in soil samples, while in spinach samples was 3.67 log CFU/g. The highest values obtained were, 6.3 log CFU/g and 6.24 log CFU/g, in soil and spinach samples respectively. In spinach samples, values equal to or greater than 5 log CFU/g were present in 13 samples, while this level was observed in four soil samples.

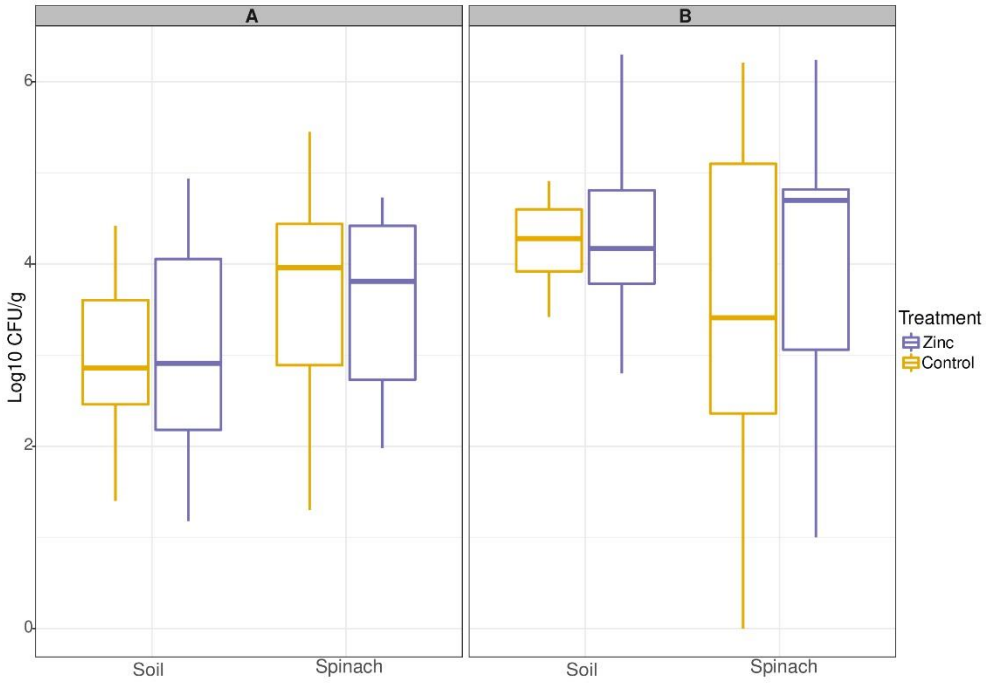

Figure 3.1 Enumeration of Enterobacterales in zinc amended plots (Zinc = purple) and control plots (Control = gold) in soil and spinach samples from two different locations (A and B).

3.3.2 Detection of antimicrobial resistant Enterobacterales

Eleven ESBL-PE isolates and one CRE isolate were identified in spinach samples, and eight ESBL-PE were isolated from soil samples. No CIP-RE were

isolated. Among the Enterobacterales isolates identified from individual samples, *S. fonticola* was the predominant species (n= 16) in both sample types, followed by *Escherichia coli* (n= 1), *Enterobacter cloacae* (n= 1), *Citrobacter freundii* (n= 1) and *Morganella morganii* (n= 1) (Figure 3.2). Specifically, *S. fonticola* was isolated at location A from five soil samples, three of which were collected from zinc amended plots and two control plots, and from nine spinach samples, three of which were collected from zinc amended plots and six from control plots. The remaining two *S. fonticola* isolates were collected from soil samples at location B (one from a zinc amended plot and one from a control plot). The *C. freundii* and *M. morganii* isolates were both isolated from spinach samples from a control plot at location A and a zinc amended plot at location B, respectively. The *E. cloacae* was isolated from one soil sample collected from a zinc amended plot at location B. The *E. coli* isolate was recovered from a spinach sample from a zinc amended plot at location B.

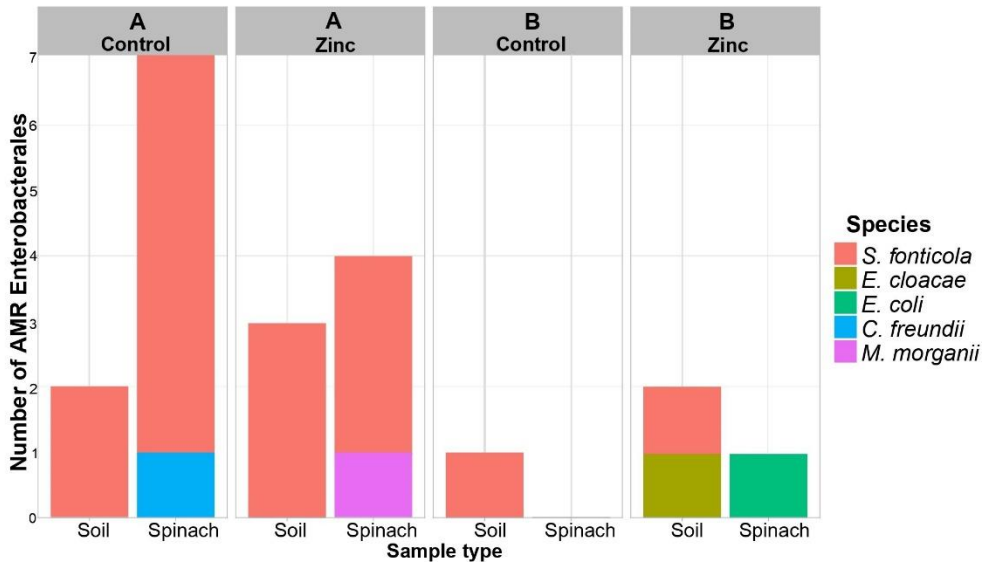

Figure 3.2 Number of antimicrobial resistant Enterobacterales isolated from soil and spinach samples, collected from plots with and without zinc amendment (Zn and C plots) from two different locations (A and B).

3.3.3 Antimicrobial resistance profile of Enterobacterales in soil and spinach samples

The phenotypic antimicrobial resistance profiles of the resistant Enterobacterales are illustrated in Figure 3.3. All isolates were resistant to AMP and CPD antimicrobials. The *E. coli* and *M. morganii* isolates were also resistant to trimethoprim and tetracyclines, respectively. Overall, 14/16 *S. fonticola* isolates were confirmed as ESBL-producers.

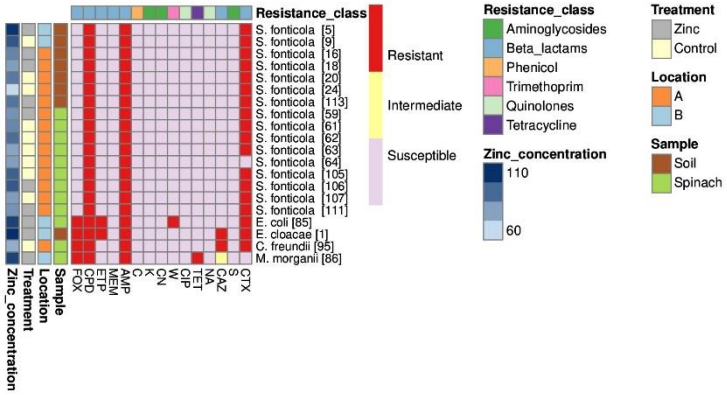

Figure 3.3 Heatmap showing the antimicrobial resistance profiles of Enterobacterales isolated from soil and spinach, according to the EUCAST (v.11, 2021) and CLSI (v. 30, 2020) breakpoints interpretation of zone diameters.

3.3.4 Detection of antimicrobial resistance genes, heavy metal resistance genes and plasmids.

WGS analyses revealed that all *S. fonticola* isolates harboured the beta-lactam resistance gene *bla*$_{\text{FONA}}$ (Figure 3.4). Seven isolates harboured *bla*$_{\text{FONA-6}}$, four isolates harboured *bla*$_{\text{FONA-2}}$, three isolates *bla*$_{\text{FONA-4}}$, one isolate *bla*$_{\text{FONA-3}}$ and one harboured *bla*$_{\text{FONA-5}}$. These findings correlated with the AST results, where all the *S. fonticola* isolates were phenotypically resistant to ampicillin and cefpodoxime. *M. morganii* harboured *bla*$_{\text{DHA-12}}$, *catII* and *tet*(D) (Figure 3.4), which confer resistance to beta-lactams, chloramphenicols and tetracyclines, respectively. However, *M. morganii* was phenotypically resistant to beta-lactams and tetracyclines, but not to chloramphenicol. The *E. cloacae* isolate harboured *bla*$_{\text{act-25}}$ and *bla*$_{\text{act-16}}$,

fosA2 and *bacA* (Figure 3.4), which confer resistance to beta-lactams, fosfomycin and bacitracin respectively. Additionally, the *oqxA* and *oqxB* genes were identified in the *E. cloacae* isolate. These genes encode for multidrug efflux pumps, which can confer low-level resistance to quinolones. *C. freundii* harboured *bla*$_{\text{CMY-41}}$, *bla*$_{\text{CMY-152}}$ and *ampH*, and the bacitracin resistance gene *bacA* (Figure 3.4). The *E. coli* isolate harboured *bla*$_{\text{EC}}$ and *ampH*, which confer resistance to beta-lactams, and *bacA* and *mphB*, which confer resistance to bacitracin and macrolide (Figure 3.4).

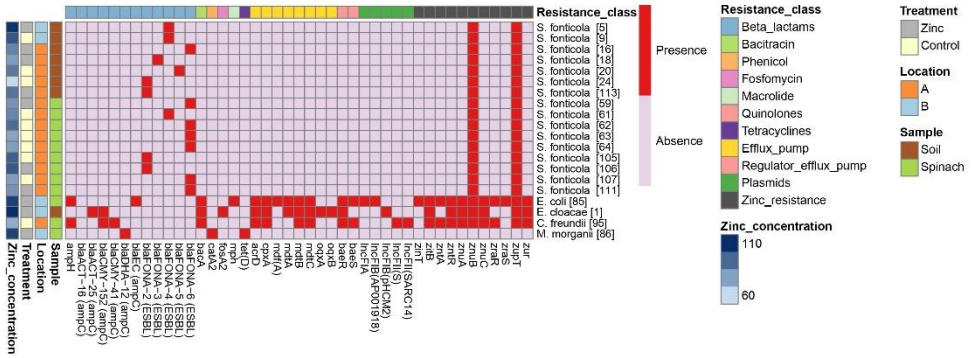

Figure 3.4 Heatmap showing the genotypic profiles of Enterobacterales, including the antimicrobial resistance genes, efflux pump genes, plasmids and zinc resistance associated genes.

The pangenomes of the *S. fonticola* were compared (Figure 3.5). Overall 8,357 gene clusters with 84,757 genes identified using NCBI blastp. The core genome of the isolates consisted of 3,766 gene clusters with 62,578 genes. Additionally, ANI analysis showed high similarity among some *S. fonticola* isolates (Supplementary Material, Appendix B – Figure 2). Specifically, *S. fonticola* [62], *S. fonticola* [63] and *S. fonticola* [59], which were isolated from individual spinach samples collected from different plots at location A in June 2021, have closely related genomes with 99.9% nucleotide identity and share the same phenotypic and genotypic profiles. Similarly, *S. fonticola* [111] and *S. fonticola* [24], which were isolated from two different sample types (*S. fonticola* [111] from spinach and *S. fonticola* [24] from soil) of

different plots at location A in May and October 2021, have 99.6% nucleotide identity (Supplementary Material, Appendix B – Figure 2). However, these two isolates harbour different *bla*$_{FONA}$ genes, the *S. fonticola* [111] harboured the *bla*$_{FONA-6}$, the *S. fonticola* [24] *bla*$_{FONA-2}$.

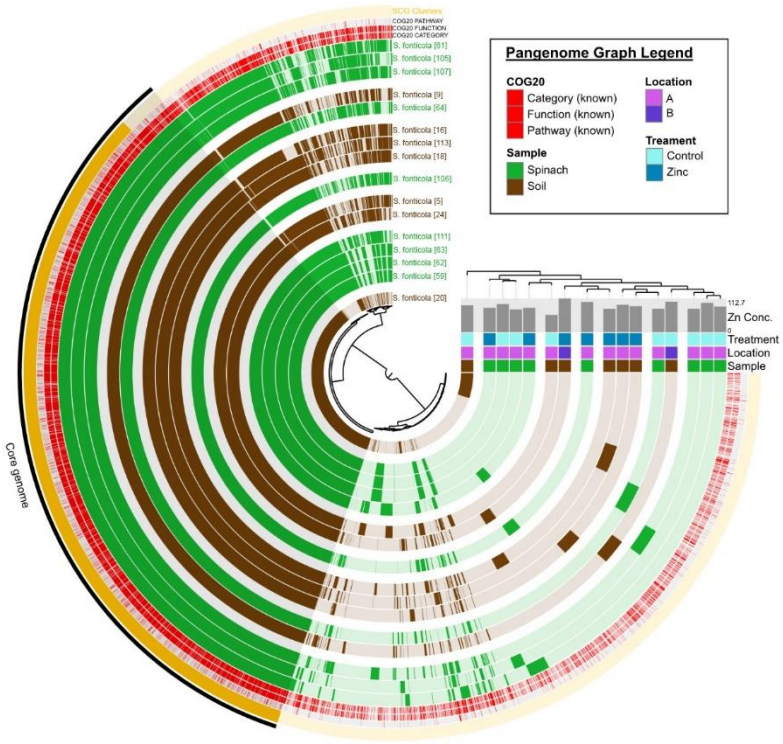

Figure 3.5 Pangenome visualization of sixteen Serratia fonticola strains. Gene clusters are indicated as opaque coloured elements, and ordered based on their presence and absence, using the Euclidean distance and Ward clustering (inner dendrogram). Samples are hierarchically clustered based on the frequency of gene clusters (outer dendrogram).

Zinc resistance associated genes were identified in all isolates, except for *M. morganii* (Figure 3.4). Specifically, *S. fonticola* isolates harboured two genes, *znuB* and *zupT*, which encode for zinc transporter proteins; *E. coli*, *E. cloacae* and *C. freundii* harboured several zinc resistance associated genes, including *zitB* and *zntA*. Moreover, the genes *baeR*, *baeS* and *cpxA*, which can be activated by copper or zinc and can induce the expression of the multidrug efflux pumps genes *mdtABC* and *acrD* (Nishino et al., 2021, Nishino et al., 2007, Wang and Fierke, 2013) were present in *E. cloacae*, in *C. freundii* and *E. coli* (Figure 3.4). Further genes encoding for multidrug efflux pumps, such

as EmrAB, MdtEF and AcrAB-TolC, were identified in *E. coli*, *E. cloacae* and *M. morganii*, with more details shown in Supplementary Material (Appendix B - Table 1).

No plasmids were detected in *M. morganii* or in any of the *S. fonticola* isolates (Figure 3.4). However, *IncF* plasmid replicons, which are the most common plasmid groups detected in Enterobacterales (Yang et al., 2015), were identified in all of the remaining isolates. Specifically, *E. coli* carried IncFIA and IncFIB(AP001918), *E. cloacae* carried IncFIB(pHCM2), *C. freundii* carried IncFII(S) and IncFII(SARC14). No ARGs were predicted to be plasmid borne using Platon database.

The *E. coli* isolate was identified as O179:H8. This serotype has been reported as a Shiga toxin-producing *E. coli* (STEC) in sheep and cattle (Beutin and Strauch, 2007). However, in this study no STEC virulence genes were detected, and the *E. coli* isolate was identified as the B1 phylotype group.

3.3.5 Chemical properties of soil

A significant difference (P value < 0.05) in zinc concentration was detected between the two plot types (zinc and control) at each location (Figure 3.6). The concentration of the other metals tested did not differ considerably between the zinc amended plots and the control plots.

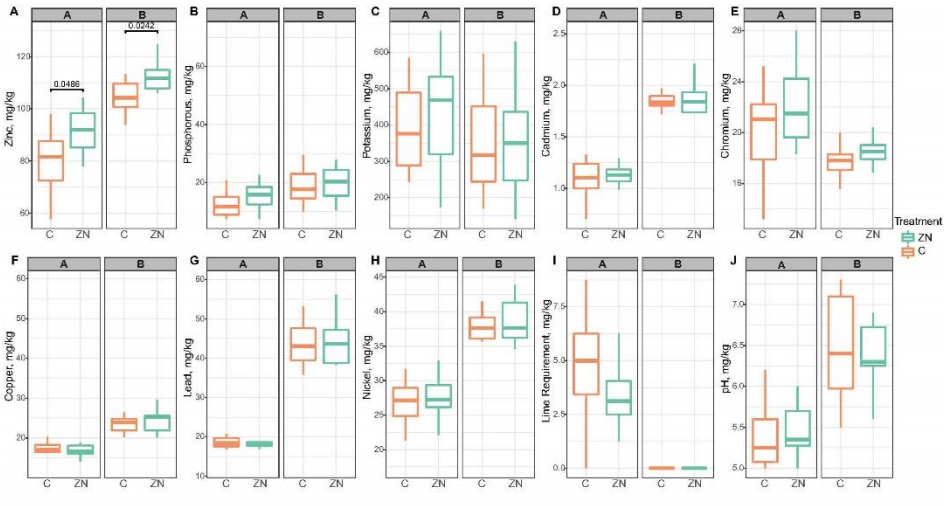

Figure 3.6 Physiochemical properties of soil samples, including zinc (A), phosphorous (B), potassium (C), cadmium (D), chromium (E), copper (F), lead (G) and nickel (H) concentrations, lime requirement (I) and pH (J), distinguished according to the treatment (ZN or C) in two different location (A or B).

3.4 Discussion

The natural environment, including soil, water and vegetation, can be a reservoir of ARB and ARGs. The transmission of ARB to humans and animals can occur through the feed and food chain. External factors, such as heavy metals, can impact on the resistome characteristics (Zhuang et al., 2021, Anedda et al., 2023). This study examined the impact of zinc amendment on the presence of antimicrobial resistant Enterobacterales in soil and spinach samples.

Enterobacterales naturally occur in the environment and can be influenced by growth practices. Reports have described the occurrence of ESBL-PE and CRE on soil and vegetable due to processes such as manure application on fields and/or contaminated water irrigation (Montero et al., 2021, Amador et al., 2019). Therefore, fresh vegetables, which are often consumed raw, can be a vehicle of ARB (Moon et al., 2022).

In this study, the concentration of Enterobacterales in both soil and spinach samples was similar to findings of other studies. Pintor-Cora et al. (2021) reported Enterobacterales average counts of 3.3 log CFU/g in soil samples,

similarly in the present study the average count measured in soil samples was 3.58 log CFU/g.

An average count of 3.67 log CFU/g was measured in spinach samples in the current study, with 86% of Enterobacterales counts greater than 2.0 log CFU/g. Pintor-Cora et al. (2021) reported 82.9% of Enterobacterales counts greater than 2.0 log CFU/g in vegetable samples, while other studies reported an average Enterobacterales count slightly higher than the current study. For example, a mean of 5.23 log CFU/g was reported in spinach samples from markets (Colosi et al., 2020), a mean of 6.0 log CFU/g in fresh-cut spinach (Abadias et al., 2008), and a variation from 2.0 to 5.0 log CFU/g along the shelf-life of ready-to-eat spinach (Uhlig et al., 2022) have been reported. This may be due to different agricultural practices (Igbinosa et al., 2023) or the processes that spinach undergoes to enter the retail chain, such as handling, cutting, or slicing, and which can cause an increase in contamination (Abadias et al., 2008).

Antimicrobial resistant isolates of *S. fonticola, M. morganii*, *E. cloacae*, *C. freundii* and *E. coli* identified on spinach and other vegetables have been reported in previous studies, along with other Enterobacterales species, such as *Rahnella aquatilis*, *Klebsiella pneumonia* and *Salmonella* spp. (van Hoek et al., 2015, Pintor-Cora et al., 2021, Moon et al., 2022, Richter et al., 2021, Richter et al., 2020, Chelaghma et al., 2021). These findings suggest that fresh produce are a potential route for dissemination of ARB in the environment and transmission to retail food.

In a previous review focused on the role of vegetables and fruit as reservoirs of AMR bacteria, the most common ARGs identified in spinach samples, detected in *E. coli*, *E. cloacae*, *M. morganii* and *S. fonticola*, were: $bla_{\text{CTX-M}}$, bla_{SHV}, bla_{TEM}, bla_{OXA}, bla_{KPC} and bla_{FONA} (Chelaghma et al., 2021). None of these ARGs were detected in the current study, with the exception of bla_{FONA}, which was identified in all *S. fonticola* isolates. The bla_{FONA} is reported as an ESBL gene encoded on the chromosome of *S. fonticola* (Naas et al., 2008, Tanimoto et al., 2021); however, in *S. fonticola* [18] and *S. fonticola* [24] the production of ESBLs was not confirmed phenotypically,

although they were resistant to AMP,CPD and CTX. This phenotype was observed also in another study (Rybak et al., 2022), where 4 out of 16 *S. fonticola* isolates were not ESBL producers and were resistant to AMP and CTX. It has been reported that FONA enzyme of *S. fonticola* has high amino acid sequence similarity with the ESBL enzyme SFO-1, encoded by a plasmid on *E. cloacae* (Matsumoto and Inoue, 1999, Tanimoto et al., 2021), suggesting that SFO-1 may have derived from the FONA enzyme (Pintor-Cora et al., 2021). Although *S. fonticola* is an environmental bacterium, it has been associated with human infections, such as skin infections, urinary tract infections and blood infections (Aljorayid et al., 2016, Raphael and Riley, 2017). *S. fonticola* harbouring *bla*$_{FONA}$ genes have also been detected in chicken meat in Japan (Tanimoto et al., 2021) and in fresh pork meat in Germany (Schill et al., 2017). These findings indicate that *S. fonticola* has the ability to spread in the food production environment. Therefore, it would be of interest to further investigate the link between the environment, farming practices, and animals to better understand the dissemination of potential *bla*$_{FONA}$-carrying *S. fonticola*.

Furthermore, additional ARGs identified in the current study have also been reported in meat samples in previous studies. For instance, the macrolide resistance gene *mph(B)* detected in the *E. coli* isolate and the *bla*$_{CMY}$ gene detected in *C. freundii* have been identified in *E. coli* from retail veal in the USA (Tate et al., 2021).

A number of Gram negative bacteria, including *E. cloacae*, *C. freundii* and *M. morganii*, harbour chromosomally encoded ampC genes (Tamma et al., 2019). In this study, the genes *bla*$_{DHA}$, *bla*$_{ACT}$ and *bla*$_{CMY}$, which are reported as plasmid-mediated ampC beta-lactamase, were identified in *M. morganii, E. cloacae* and *C. freundii*, respectively. These genes have been previously identified in *E. cloacae* from fresh vegetables and ready-to-eat salads (Iseppi et al., 2018), from fresh produce (Colosi et al., 2020) and from human clinical samples (Wu et al., 2018).

The detection of genes that encode for multidrug efflux pumps, such as AcrAB-TolC, may indicate AMR phenotype due to the efflux pumps' capacity

to remove multiple classes of antimicrobials from the bacterial cells (cross-resistance), causing the reduction of available therapeutic options (Chowdhury et al., 2019, Muntean et al., 2022, Nishino et al., 2021). Interestingly, efflux pumps can target different substrates, such as antimicrobials and heavy metals (Nishino et al., 2007), and heavy metals can contribute to efflux pump expression. It has been demonstrated that copper and zinc can activate *baeSR* expression in *E. coli* and *S. enterica*; consequently, the expression of efflux pumps, such as AcrD and MdtABC, which confer resistance to beta-lactams, novobiocin and deoxycholate, is induced (Nishino et al., 2021, Wang and Fierke, 2013). However, *baeSR* is overexpressed in response to high zinc stress (300 μM $ZnSO_4$) (Wang and Fierke, 2013). In this study the zinc concentration in the zinc amended plots varied from 77.9 to 124.6 mg/Kg, which may not exert such a selective pressure to induce the overexpression of *baeSR*. The distribution of zinc resistance associated genes may also not be influenced by the zinc concentration in the zinc amended plots. The zinc resistance genotypic profiles of *S. fonticola* did not differ among the isolates, which harboured the same zinc associated genes regardless the origin of the isolates from control or amended plots. *E. coli* and *C. freundii* carried genes such as *zitB* and *zntA*, which are involved in zinc export, rending the bacteria resistant to zinc. However, *E. coli* was isolated from a zinc amended plot, while *C. freundii* from a control plot. *M. morganii*, originated from a zinc amended plot, did not harbour any zinc resistance associated genes. Therefore, the concentration of zinc may not have affected the zinc resistance gene profile. Plasmids play an important role in AMR dissemination, as they can carry genes that confer resistance to multiple drugs, such as antimicrobials and heavy metals. The IncF group is the most commonly detected plasmid group among Enterobacterales, which often harbour genes that confer resistance to a broad range of antimicrobials, such as beta-lactams, aminoglycosides, tetracyclines, chloramphenicols and quinolones (Rozwandowicz et al., 2018, Yang et al., 2015). For example, the genes *fosA3* and *oqxAB*, which confer resistance to fosfomycin and quinolone, have been identified on IncF

plasmids in studies by Lv et al. (2020) and Wong et al. (2016); while the study by Khezri et al. (2020) identified the genes *bla*$_{\text{TEM-1B}}$, *aph*, *sul*, *tetA*, and *dfr* on IncF plasmids which confer resistance to beta-lactams, aminoglycosides, sulfonamides, tetracyclines and trimethoprim. Pal et al. (2015) reported the co-occurrence on the same plasmids of the resistance gene *cadD*, which confer resistance to cadmium and zinc, and resistance genes that confer resistance to aminoglycosides and macrolides. This phenomenon called co-resistance represents a mechanism through which heavy metals can impact AMR and promote ARGs dissemination. However, the co-occurrence of MRGs and ARGs in plasmids was more commonly found in clinical isolates than in environmental isolates.

Although plasmids, especially the IncF group, were detected in the isolates in this study, none of the ARGs nor MRGs detected were predicted to be plasmid borne. In future studies, long read sequencing would be beneficial to elucidate the role of plasmids on ARG carriage in these isolates.

Metal concentration, metal availability and different chemical forms of heavy metals can affect metal toxicity (Seiler and Berendonk, 2012, Yu et al., 2017); and can have an impact on bacteria in soil. Several studies have demonstrated that high levels of zinc can affect the composition of bacterial communities in soil, the antimicrobial resistance profile and the MGEs (Li et al., 2022, Tongyi et al., 2020, Urra et al., 2019). Tongyi et al. (2020) set three levels of zinc: zero for the control, 200 mg/kg (Zn200) and 800 mg/kg (Zn800); Li et al. (2022) used 600 mg/kg for low zinc level (LZn) and 7,200 mg/kg (HZn) for high zinc levels to be added in soil pots. In both these studies, the zinc availability was lower than the concentration, about 93 mg/kg for Zn200 and 417 mg/kg for Zn800 (Tongyi et al., 2020), and around 90 mg/kg in LZn and 347 mg/kg in HZn (Li et al., 2022). Moreover, the studies mentioned above were controlled experiments in pots. The current study was a field trial which may have resulted in the zinc penetrating into the soil at different depths, influencing the zinc availability. Additionally, the zinc concentration varied from 77.9 to 124.6 mg/Kg in zinc amended plots, and from 57.7 to 113.3 mg/Kg in control plots. Compared to Tongyi et al. (2020)

and Li et al. (2022) studies, the diverse zinc concentration measured and the different methodology may account for the similarity in AMR profiles of the Enterobacterales isolated from zinc amended and control plots in the current study. Nonetheless, this study focused on a specific group of organisms and only a relatively small number of isolates resulted resistant to antimicrobials. Therefore, it would be of interest to conduct a soil resistome analysis to understand whether zinc amendment can impact the wider soil AMR bacterial communities.

3.5 Conclusion

This study provides the first data on the presence of antimicrobial resistant Enterobacterales in Irish crop production settings. Although various studies have shown that heavy metals, including zinc, can influence the bacterial composition in the environment, the AMR profile and the MGE abundance findings reveal that the influence of zinc might not have been discernible due to the relatively low zinc concentration employed in our field trial. Furthermore, the real-world conditions of a field trial may have mitigated the manifestation of zinc's effects. This study demonstrated that fresh produce and the primary food production environment can harbour Enterobacterales with resistance phenotypes and genotypes of clinical relevance. Therefore, soil and vegetables can potentially act as vehicles of clinically important ARB, potentially fostering the transmission of ARB to animals and humans through the food chain, underscoring the need for continued research and surveillance.

3.6 Appendix B: Supplementary Material

The supplementary material for Chapter Three “Characterization of antimicrobial resistant Enterobacterales isolated from spinach and soil following zinc amendment” is available at DOI of the reference below.

Anedda, E., Ekhlas, D., Alexa, E., Farrell, M. L., Gaffney, M. T., Madigan, G., Morris, D. & Burgess, C. M. 2024. Characterization of antimicrobial resistant Enterobacterales isolated from spinach and soil following zinc amendment. *Environmental Pollution*, 361, p.124774.

DOI: https://doi.org/10.1016/j.envpol.2024.124774

Chapter Four

Comparison of antimicrobial resistant Enterobacterales isolates from the dairy production environment in low and high zinc containing regions.

Published in Science of the Total Environment

Anedda, E., Alexa, E. A., Farrell, M. L., Croffie, M., Madigan, G., Morris, D. & Burgess, C. M. 2024. Comparison of antimicrobial resistant Enterobacterales isolates from the dairy production environment in low and high zinc containing regions. *Science of the Total Environment*, *953,* p.175905 DOI:

https://doi.org/10.1016/j.scitotenv.2024.175905

Abstract:

Heavy metals occur naturally in the environment, and their concentration varies in soil across different regions. However, the presence of heavy metals may influence the antimicrobial resistance (AMR) in bacterial populations. Therefore, the objective of this study was to investigate and characterise the antimicrobial resistance profiles of Enterobacterales in soil and bovine milk filters from high and low zinc-containing regions in Ireland. In total, 50 soil samples and 29 milk filters were collected from two geographic locations with varying soil zinc concentrations. Samples were cultured for the enumeration and detection of Enterobacterales. Specifically, extended-spectrum beta-lactamase-producing Enterobacterales, carbapenem-resistant Enterobacterales and ciprofloxacin-resistant Enterobacterales were isolated using selective media. Species identification was performed using MALDI-TOF. The phenotypic resistance profiles of selected Enterobacterales were determined by disk diffusion testing, following EUCAST and CLSI criteria; while, the genotypic resistance profiles of the same isolates were determined by whole genome sequencing (WGS). Heavy metal concentrations were also measured for all soil samples. A total of 40 antimicrobial resistant Enterobacterales were identified in soil (n= 31) and milk filters (n= 9). The predominant species detected in the high zinc-containing region was *Escherichia coli* in both sample types (soil n=10, milk filters n=2), while in the low zinc-containing region *Serratia fonticola* was predominant in soil samples (n=8) and *E. coli* in milk filters (n=4). Ten *E. coli* isolates identified from soil samples in the high zinc-containing region were multidrug resistant, showing resistance to all the antimicrobials tested, except for carbapenems. The WGS findings confirmed the phenotypic resistance results. Moreover, zinc resistance-associated genes and genes encoding for efflux pumps were identified. The current study revealed distinct phenotypic resistance profiles of Enterobacterales in low and high zinc-containing regions, and highlighted the benefit of utilising milk filters for AMR surveillance in dairy production.

4.1 Introduction

Antimicrobial resistance (AMR) is recognised as one of the greatest threats to public health we currently face. The World Health Organization (WHO) emphasised significant concerns in relation to the spread of carbapenemase-producing Enterobacterales and third-generation cephalosporin-resistant *Escherichia coli* (*E. coli*) in the 2022 Global Antimicrobial Resistance and Use Surveillance System (GLASS) report (WHO, 2022b). Third-generation cephalosporins and carbapenems have been identified as critically important antimicrobials for human health by the WHO, indicating their use in circumstances where alternative therapies are limited and when infections are caused by resistant bacteria from non-human sources, which can be transmitted to humans (WHO, 2019). Third-generation cephalosporin-resistant *E. coli* was reported as being responsible for a median of 42% of bloodstream infections across 76 countries in 2020 (WHO, 2022b). Carbapenems serve as last-resort antimicrobials; hence, resistance to carbapenems may lead to increased frequency of treatment failure (Elshamy and Aboshanab, 2020). Although third-generation cephalosporins can be used in Europe to treat animal infections subject to specific restrictions, for example as last resort antimicrobials, the EU legislation strictly prohibits use of carbapenems in animals (EMA, 2023). Nevertheless, antimicrobial resistant bacteria (ARB), such as third-generation cephalosporin-resistant and carbapenem-resistant Enterobacterales, have been detected in several livestock species, including pigs, cattle, sheep and chicken (Haulisah et al., 2021, AbuOun et al., 2021, Bonardi and Pitino, 2019). The occurrence of ARB in the livestock sector and in the associated environment poses a threat for the dissemination of ARB through the food chain (Koutsoumanis et al., 2021). Dairy cattle, for example, can be vehicles of extended spectrum beta-lactamase-producing Enterobacterales (ESBL-PE) (Dantas Palmeira and Ferreira, 2020), and contaminated milk can be responsible for ARB dissemination through the food chain (Gelalcha and Kerro Dego, 2022).

The abuse and misuse of antimicrobials in humans and animals has led to an increase of AMR dissemination, but other compounds, including heavy metals, can also impact AMR in the environment (Anedda et al., 2023). The occurrence of heavy metals in soil arises from two phenomena: natural processes and anthropogenic influence (Su et al., 2022). The natural presence of heavy metals in soil is due to geogenic factors, such as the original material from which soil develops and weathering (Wuana and Okieimen, 2011). However, anthropogenic activities also contribute to the heavy metal concentration in soil. These activities include industrial operations, such as mining, smelting, burning of fossil fuels, disposal of industrial waste, and agricultural activities, such as the use of fertilizers, pesticides, manure application or land irrigation with contaminated water (Rashid et al., 2023, Li et al., 2024).

In Ireland, metal distribution in the landscape is mostly due to glacial and fluvio-glacial deposits (Mcgrath and Fleming, 2007). However, industrial and agricultural practices may also affect the content and distribution of heavy metals in the Irish soil. For example, it has been noted that a greater concentration of heavy metals is present in urban areas with historical industries, such as coal burning, textile manufacture and drink production (Glennon et al., 2014). In the agricultural sector, the spread of biosolids in lands can also increase the heavy metal content (Topal and Elitok, 2018, Fijalkowski et al., 2017) and according to the Environmental Protection Agency in Ireland (EPA) almost 80% of biosolids are applied on Irish lands (Nag et al., 2022). The heavy metal content and distribution in Ireland are surveyed and documented by the Geological Survey Ireland (GSI) (https://www.gsi.ie/en-ie/Pages/default.aspx). Key information on heavy metals, including zinc, was reported in the Tellus Geochemical Survey (Browne et al., 2021), where a substantial difference in the zinc distribution in soil was evident across different areas.

Ireland is a global leader in dairy production. The temperate climate, with recurring rainfall and fertile soils, ensure that grassland pasture is the main

feedstock source (Moscovici Joubran et al., 2021). A pasture-based dairy production system provide nutritional benefits in milk products (Timlin et al., 2023), and reduced costs associated with supplemental feed (Moscovici Joubran et al., 2021). Given the diverse distribution of zinc concentration and the importance of dairy production in Ireland, this study aimed to investigate the occurrence and characteristics of antimicrobial resistant Enterobacterales in soil and milk filter samples obtained from high and low zinc-containing regions of Ireland.

4.2 Materials and methods

4.2.1 Sample collection

In total 50 soil samples and 29 bovine milk filters were collected in 2022 and 2023 from dairy farms located in regions with low and high zinc level across Ireland. The identification of suitable regions, based on soil zinc content, and recruitment of appropriate farms was facilitated through collaboration with GSI (Geological Survey Ireland) and the Teagasc Advisory Service. Twenty-five soil samples and seven milk filters were collected in October 2022 from five farms in the low zinc region (location B), with an additional eight milk filters collected from the same farms in August 2023. Twenty-five soil samples and five milk filters were collected in March 2023 from five farms in the high zinc region (location A), plus nine milk filters from the same farms in September 2023. The soil samples on each farm were collected from five different spots across each field in a W pattern, the day after the cows grazed in the field. A soil core with a depth of 10 cm was used for the collection of approximately 100 g soil for each spot. The soil collected from region A was fine loam type, classified as *Luvisol* according to the World Reference Base for Soil Resources (WRB), while in region B it was coarse loam type, classified as Typical Brown Earths in accordance to WRB. Used milk filter socks were collected directly from the milking machine. Soil samples and milk filters were placed into sterile bags and transported to the laboratory, where they were processed within 24 hours.

4.2.2 Chemical analysis of soil samples

The concentration of 16 chemical compounds, including zinc (Zn), magnesium (Mg), aluminium (Al), phosphorus (P), sulphur (S), potassium (K), calcium (Ca), vanadium (V), chromium (Cr), manganese (Mn), iron (Fe), cobalt (Co), nickel (Ni), copper (Cu), arsenic (As) and lead (Pb), was measured in soil samples. Energy-dispersive X-ray fluorescence spectrometry (EDXRF) was employed, as previously described by Croffie et al. (2020).

4.2.3 Soil and bovine milk filter samples processing

Enumeration of Enterobacterales and isolation of resistant Enterobacterales was undertaken on soil samples and milk filters. Specifically, the enumeration of Enterobacterales was conducted using Violet Red Bile Glucose Agar (VRBGA). For the target microorganisms, soil samples and milk filters were enriched in Buffered Peptone Water (BPW) at 37°C for 24 hours. The enrichment were plated on Brilliance ESBL media (Oxoid), COLOREX mSuperCARBA (E&O Labs), and MacConkey media (Oxoid) plus a 5 µg ciprofloxacin disk (Oxoid), for the detection of ESBL-PE, carbapenem-resistant Enterobacterales (CRE) and ciprofloxacin-resistant Enterobacterales (CIP-RE), respectively. Colonies from selective media were chosen based on their colour and morphology, and identified using Matrix-assisted laser desorption ionization time of flight (MALDI-TOF) (Clark et al., 2013). The Enterobacterales isolated were analysed for susceptibility to 16 antimicrobials by disk diffusion testing in accordance with EUCAST and CLSI guidelines, using *E. coli* ATCC 25922 as a quality control strain (Clinical and Laboratory Standards Institute (CLSI), 2020, European Committee on Antimicrobial Susceptibility Testing (EUCAST), 2021). Specifically, the antimicrobials tested were: ampicillin (AMP-10 µg), cefoxitin (FOX-30 µg), ceftazidime (CAZ-10 µg), cefotaxime (CTX-5 µg), cefpodoxime (CPD-10 µg), cefpodoxime/clavulanic acid (CPD/CV-10 µg/1 µg), ertapenem (ETP-10 µg), meropenem (MEM-10 µg), ciprofloxacin (CIP-5 µg), nalidixic acid (NA-30 µg), streptomycin (S-10 µg), gentamicin (CN-10 µg), kanamycin (K-30 µg), tetracycline (TET-30 µg), chloramphenicol (C-30 µg) and trimethoprim (W-5

µg). The combination disk method of cefpodoxime (10 µg) and cefpodoxime/clavulanic acid (10 µg/1 µg) was applied to verify phenotypic ESBL production.

4.2.4 Whole genome sequencing analysis of Enterobacterales

The DNA of 40 isolates obtained from the selective media previously described was extracted using the DNeasy UltraClean Microbial Kit (Qiagen, United Kingdom) following the manufacturer's instructions. DNA concentration and quality were measured using the Qubit 4.0 Fluorometer (Invitrogen, ThermoFisher Scientific, United Kingdom) and Nanodrop™ 1000 Spectrophotometer. An Illumina NextSeq 2000 was employed to perform the whole genome sequencing. The raw sequencing reads were then analysed using different bioinformatic tools. FASTQC (v0.11.8) and MultiQC (v1.9) (Ewels et al., 2016, Andrews, 2010) were used for quality assessment of the sequences, and adapter trimming was done using Trimmomatic (v0.38) (Bolger et al., 2014). Read assembly and taxonomic sequence classification were performed using SPAdes (v3.15.3) and Kraken 2 (v2.1.1), respectively (Prjibelski et al., 2020, Wood et al., 2019). The presence of antimicrobial resistance genes (ARGs), heavy metal resistance genes (MRGs) and plasmids were assessed using ABRicate (v1.0.1) (https://github.com/tseemann/abricate) with reference to ResFinder (v4.1) and CARD (v3.2.4) databases for ARGs, Megares database for MRGs, and Plasmidfinder database for plasmids. The BacMet2 database was also employed for zinc resistance associated genes screening (Pal et al., 2014). Plasmid content was assessed using the Platon database (v1.6) (Schwengers et al., 2020), where plasmidic and chromosomal scaffolds were distinguished and resistance genes were screened using ABRicate. The *E. coli* isolates were tested for their phylotype and serotype using the EzClermont and EcOH databases respectively (Ingle et al., 2016, Waters et al., 2020), and for virulence genes using the VFDB database (Chen et al., 2005).

4.2.5 Statistical methods and visualization

Statistical evaluation of chemical compounds in soils was conducted using the paired samples Wilcoxon test. The significance level was set at $p < 0.05$, for which the Bonferroni correction was applied. R version 4.2.1 (https://cran.r-project.org/) and RStudio 1.3.1093 (https://posit.co/products/open-source/rstudio/) were used by employing the *rstatix* and *ggplot2* packages (Kassambara, 2023, Wickham, 2016).

4.2.6 Data availability

The sequences analysed in this study are publicly available at National Center for Biotechnology Information (NCBI) under BioProject: PRJNA1133711**.**

4.3 Results

4.3.1 Chemical characteristics of soil samples

The zinc concentration of soils examined differed significantly between regions A and B (P value = 2.1×10^{-9}) (Figure 4.1). There was a median zinc concentration of 193 mg/Kg across the five farms in region A, while a median of 48.65 mg/Kg was measured at the five sites in region B. In addition, the concentrations of arsenic, iron, vanadium, cobalt, manganese, nickel, lead and magnesium, which were higher in region A, showed highly significant differences between the two locations, with P values up to 3.16×10^{-14}. Concentrations of potassium, chromium, copper, aluminium and sulphur also differed significantly between locations A and B. There was no significant difference in the concentration of calcium and phosphorous between the two locations.

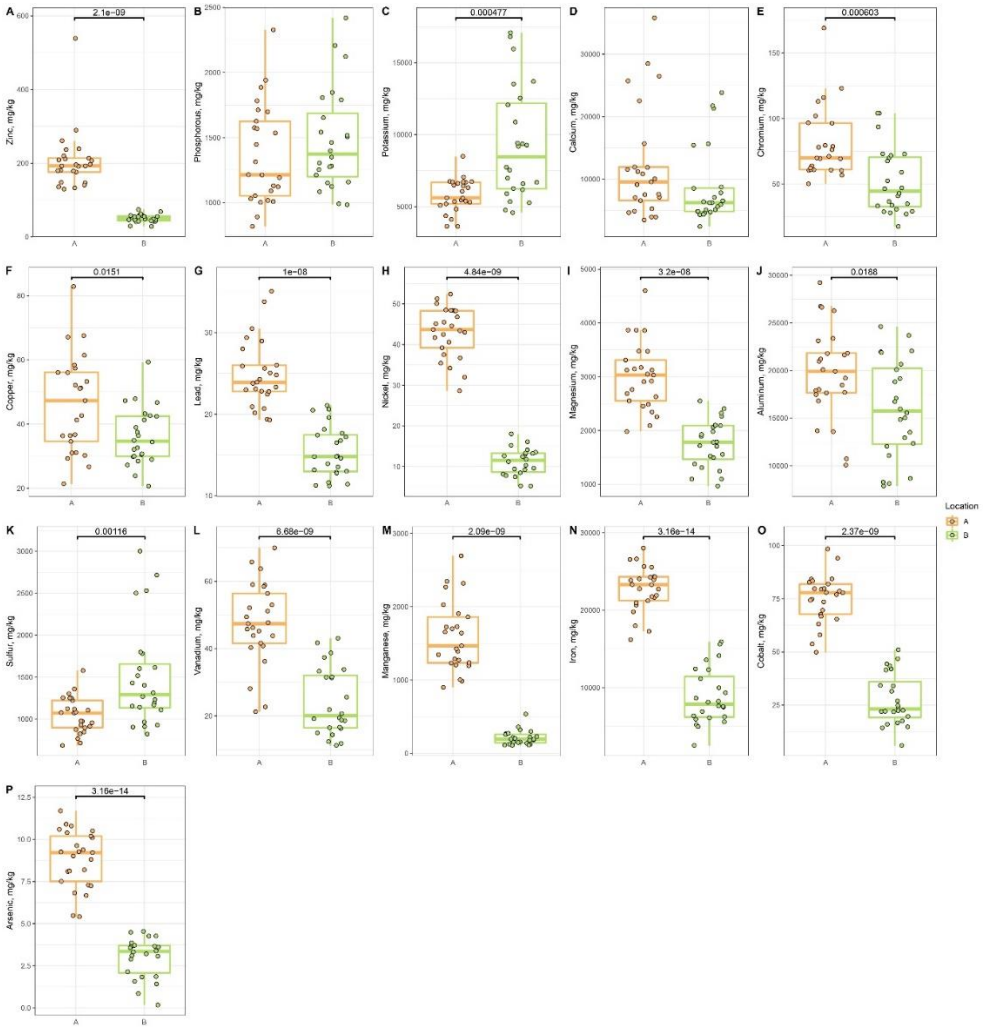

Figure 4.1 Difference of zinc (A), phosphorus (B), potassium (C), calcium (D), chromium (E), copper (F), lead (G), nickel (H), magnesium (I), aluminium (J), sulphur (K), vanadium (L), manganese (M), iron (N), cobalt (O) and arsenic (P) concentrations in soil samples between locations A and B. Coloured dots inside each boxplot correspond to the samples collected on each farm.

4.3.2 Identification of antimicrobial resistant Enterobacterales from soil and bovine milk filters

An average concentration of Enterobacterales of 2.2 log CFU/g was present in the 50 soil samples. A statistically significant difference was observed between the two regions, with an average of 1.58 log CFU/g present in samples from region A and 2.82 log CFU/g in region B (Figure 4.2).

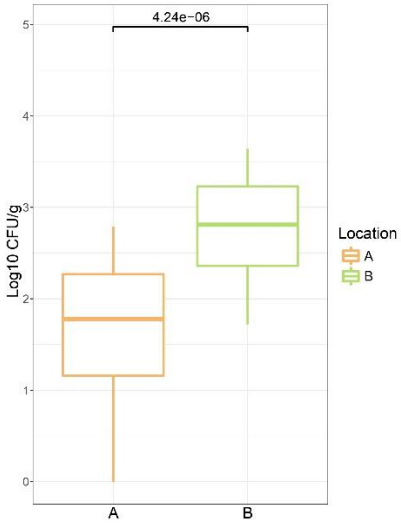

Figure 4.2 Distribution of Enterobacterales in soil samples collected from two different areas (A and B) where the concentration of zinc is high (A) and low (B).

A total of 40 antimicrobial resistant Enterobacterales isolates were identified in soil (n= 31; A=14, B=17) and bovine milk filters (n= 9; A=3, B=6) (Figure 4.3). In soil samples from region A, the predominant species was *E. coli* (n= 10), followed by *Serratia fonticola* (n= 3) and *Proteus terrae* (n= 1); while in soil samples from region B, *S. fonticola* (n= 8) was the species mostly commonly identified, followed by *Citrobacter freundii* (n= 6) and *E. coli* (n= 3). In bovine milk filters, *E. coli* was the predominant species in both regions (n= 2 in region A; n= 4 in region B), followed by *Morganella morganii* (n= 1) in region A, and *Proteus vulgaris* (n= 1) and *C. freundii* (n= 1) in region B.

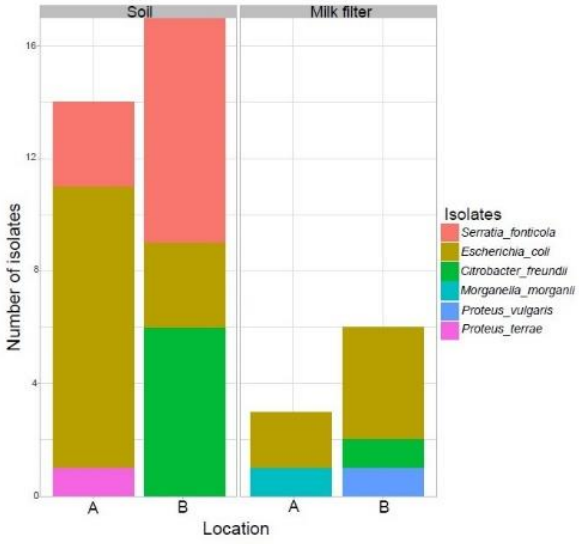

Figure 4.3 Number of antimicrobial resistant Enterobacterales isolated from soil and bovine milk filter samples, from regions A and B.

4.3.3 Antimicrobial resistance phenotypic profiles of Enterobacterales from soil and bovine milk filters

The antimicrobial susceptibility test (AST) revealed the detection of ten multidrug resistant *E. coli* isolates from individual soil samples from five different farms in region A; these isolates were resistant to all antimicrobials tested except for ETP and MEM (Figure 4.4). The *P. terrae* [D4A], from a soil sample from location A, was also resistant to three antimicrobial classes. Additionally, 10 ESBL-PE were isolated from soil samples from region A (n= 2) and B (n= 8), all *S. fonticola*. Among the isolates collected from milk filters, seven were resistant to three or more antimicrobial classes. Specifically, four *E. coli* isolates [M1F2], [M2Fa2], [M3F2a] and [M4F2a] were identified from region B, and one *E. coli* [D2F2] from region A; plus one *C. freundii* [M3F1] and one *P. vulgaris* [M2Fb1] from location B. No ESBL-PE were isolated from milk filters.

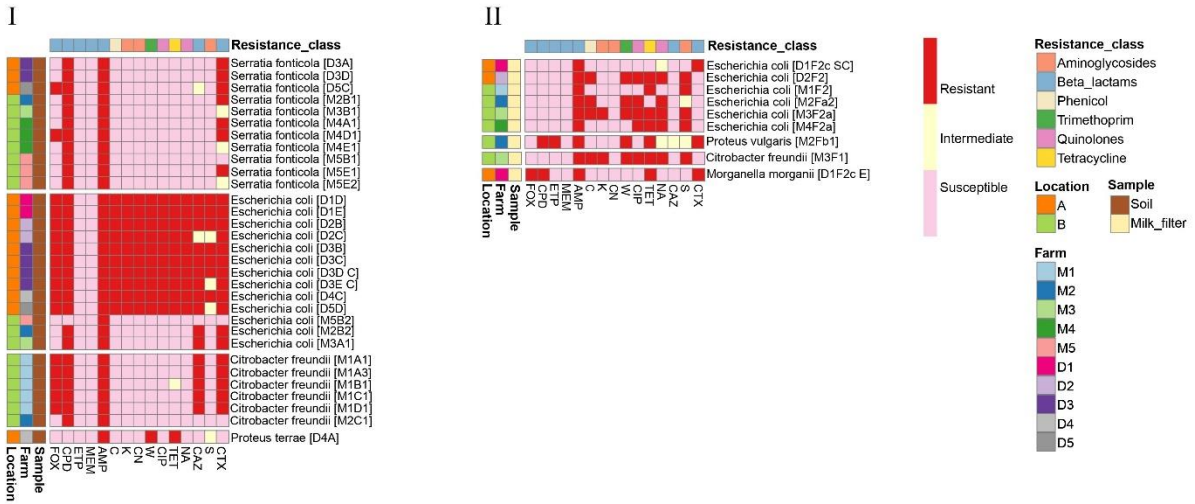

Figure 4.4 Heatmaps showing the antimicrobial resistance profiles of Enterobacterales isolated from soil (I) and bovine milk filters (II) from regions A and B, which had high and low soil zinc concentrations, respectively.

4.3.4 Antimicrobial and heavy metal resistance genotypic profiles of Enterobacterales

WGS correlated with phenotypic results for all the antimicrobials tested in *E. coli*, *Proteus* and *Citrobacter* isolates (Figure 4.5). All the *E. coli* isolates from both sample types carried the *mdf(A)* gene, which encode for a multidrug resistance protein, the *mphB* gene that confer resistance to macrolides and the *bacA* gene, which confers resistance to bacitracin. Moreover, all the soil multidrug resistant (MDR) *E. coli* isolates that were resistant to three or more antimicrobial classes, harboured *sul* genes that encode for sulphonamide resistance. Macrolides, bacitracin and sulphonamides were not part of the antimicrobial panel tested however. Similarly, *C. freundii* isolates harboured the *bacA* gene, and the MDR *C. freundii* [M3F1] from milk filter carried the *sul-1* gene. The *C. freundii* [M3F1] isolate from a milk filter differed from the other *C. freundii* isolates from soil samples. It carried the gene $bla_{\text{TEM-1}}$, along with other genes, thereby exhibiting resistance to more than three antimicrobial classes while, the *C. freundii* isolates from soil samples harboured the $bla_{\text{CMY-101}}$, $bla_{\text{CMY-104}}$ or $bla_{\text{CMY-82}}$ genes, and were not MDR. Similarly to the *E. coli* isolates, the *S. fonticola* [M4E1] isolate carried the *mdf(A)* and the *mphB* genes. Despite possessing the chromosome-encoded ESBL resistance gene bla_{FONA}, the *S. fonticola* [D5C] isolate did not exhibit an ESBL phenotype. The *M. morganii* [D1F2c E] isolate harboured the bla_{DHA} and *tet*(D) genes which conferred resistance to AMP, FOX, CPD and CTX, and tetracyclines; while, the *catA2* gene detected was not correlated phenotypically with chloramphenicol resistance.

Based on WGS data, from one to seven plasmid replicons were detected in 26 of the 40 isolates, with Inc and Col the major families identified (Figure 4.5). Specifically, Col156 and IncFIB(AP001918)_1 were the most predominant plasmids detected (n=14 for each), with eight isolates harbouring both IncFIB(AP001918)_1 and Col156. Additionally, the

quinolone resistance gene *qnrD1* was identified on Col3M_1 in *P. vulgaris* [M2Fb1] and *C. freundii* [M3F1] isolates.

The presence of zinc resistance genes was similar between the two locations regardless of zinc concentration, with a total of 14 genes identified among all the isolates, except *Proteus* (Figure 4.5). Most of these genes were detected in the *E. coli* and *C. freundii* isolates, including *zntA* and *zitB*, while the *zraP* gene was identified in four out of seven *C. freundii* isolates. Moreover, several genes encoding for efflux pumps, including the resistance-nodulation-cell division (RND) efflux pump AcrAB-TolC and MdtABC, were detected in *E. coli*, *C. freundii* and *S. fonticola* [M4E1] (Supplementary Material, Appendix C – A). Other MRGs were also identified among the various isolates (Supplementary Material, Appendix C – A). Of particular interest were genes identified exclusively in isolates from the high zinc containing region (Figure 4.5). Specifically, *pcoA*, *pcoB*, *pcoC*, *pcoD* and *pcoS,* which confer copper resistance, were harboured in the MDR *E. coli* isolates from soil samples of region A. These isolates also carried *ybtP* and *ybtQ,* which encode for ATP binding cassette that is involved in iron transport. These copper and iron resistance genes were not detected in the *E. coli* isolates from soil samples from the low zinc containing region.

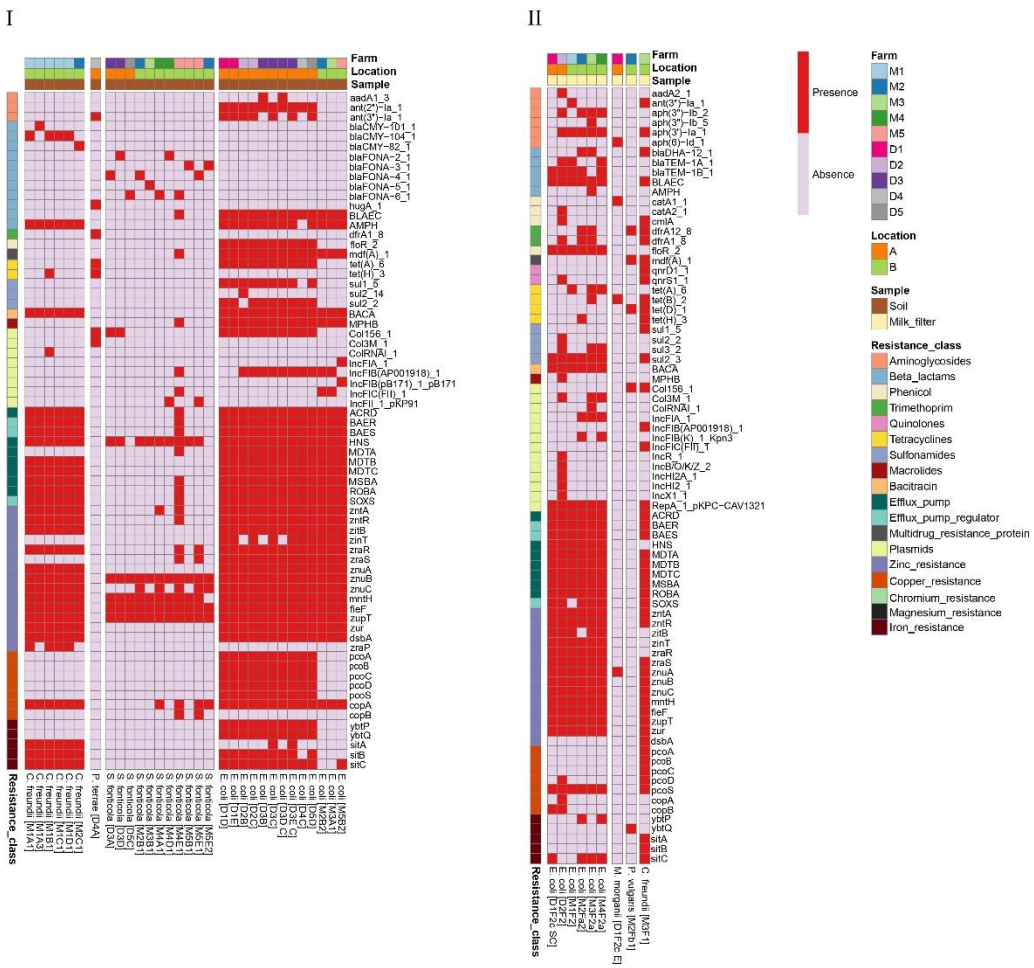

Figure 4.5 Heatmaps showing the genes identified in Enterobacterales isolated from soil (I) and bovine milk filters (II) from regions A and B, with high and low zinc concentration soil, respectively.

The *E. coli* phylotypes and serotypes identified differed between locations and sample types (Supplementary Material, Appendix C – B). All the *E. coli* isolates harboured genes that encode for several virulence factors (Supplementary Material, Appendix C – B). Genes encoding for fimbriae type 1 were identified in *E. coli* from region B, while type 4 fimbriae were found in those from region A. The *csgB, csgD, csgF* and *csgG* genes, which are involved in biofilm formation, and the *espL1, espL4, espP, espR1, espR4, espX4, espX5* and *espY1* genes, commonly found in enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC), were detected in all *E. coli* isolates. Moreover, in the *E. coli* isolates from soil samples of region A adhesin-encoding genes, such as *afaA-VIII*, *afaB-VIII*, *afaC-VIII, afaD-VIII, afaE-VIII* and *afaF-VII*, and the *astA* gene, which encodes for an enteroaggregative heat-stable enterotoxin (EAST1), were identified. The shiga-toxin genes *stx2A* and *stx2B* were detected in one *E. coli* isolate, [M2B2].

4.4 Discussion

The occurrence of heavy metals in soil can impact the composition of the soil bacterial community, resulting in a decrease of biodiversity and abundance of soil bacteria (Sazykin et al., 2023). In our study, a lower concentration of Enterobacterales was observed in the higher metal containing area. Rabow et al. (2023) observed a reduction of soil bacterial growth rate in a high heavy metal concentration area, where the highest copper concentration was 41.4 mg/Kg. Specifically, Rabow et al. (2023) evaluated the impact of heavy metals on AMR in the natural environment, focusing on copper in a heavy metal contaminated boreal forest. In this setting, an increase of tetracycline resistance was observed, along with the gradual increase of copper concentration. However, this positive correlation between heavy metal and AMR was not confirmed in a short-term laboratory experiment conducted in the same study (Rabow et al., 2023). In contrast, several microcosm experimental studies have demonstrated that AMR increases in high heavy metal containing environments. For example,

a significant increase of tetracycline resistance was observed in agricultural soil microcosms amended with 333 mg/kg of copper and 500 mg/kg of zinc, environmentally realistic heavy metal concentrations (Song et al., 2017). The Song et al. (2017) study demonstrated the role of zinc on the co-selection of AMR in soil samples. In the current study, while similar numbers of isolates were analysed from both locations, MDR was more common in the area with higher zinc concentration. However, the role of zinc as a potential selective pressure for AMR was not observed, with no link observed between the presence of zinc resistance genes and ARGs, or region. This might be due to the fact that the study was conducted in soils containing naturally varying heavy metal concentrations. Working in field soil presents several challenges compared to the controlled environments such as laboratory experiments. Varied and complex soil bacterial communities, interactions among microorganisms, and variable environmental factors, such as pH, climatic conditions and antimicrobial residues, may differentially impact AMR and interfere with the AMR and heavy metal correlation (Goswami et al., 2023, Cycoń et al., 2019, Islam et al., 2020). Despite these challenges, working in field soil represents the real-world scenario.

The presence of high heavy metal concentrations in soil can exert a selective pressure on bacterial populations, leading to the subsequent development of resistance to antimicrobials. The current study highlighted a significant difference between the antimicrobial resistance profiles of the isolates identified in soil from the higher heavy metal containing region and the isolates collected from the lower heavy metal region. Similarly, a higher incidence of resistance to multiple antimicrobial classes was observed in Gram negative bacteria isolated from copper contaminated soil plots compared to the same bacterial species isolated from the control plots (Berg et al., 2005). In another study resistance to heavy metals and to a wider variety of antimicrobials was also detected in Gram negative bacteria (*E. coli*) isolated from lead–zinc tailing soil samples compared to the Gram positive bacteria (*B. cereus*) identified in the same samples (Hu et al., 2007). In a

laboratory based experiment, a notable increase of ARGs' and mobile genetic elements' (MGEs) abundance was detected in soil amended with cadmium and/or sulfadiazine (Fu et al., 2023). These findings underscore the impact heavy metals may have on AMR; however, further research is needed to elucidate the reasons for the differences in resistance profiles observed in our study. Among the zinc resistance genes identified, *zraP, zntA* and *zitB* encode for zinc resistance-associated protein and zinc efflux transporters, rendering bacteria resistant to zinc (Pal et al., 2014). However, these genes occurred in isolates from both regions regardless of zinc concentration, suggesting that different concentrations of zinc may not have influenced the metal resistance gene distribution in the current study. MRGs are often found grouped in clusters within the genome (Lan et al., 2023). For example, the *czc* cluster provides resistance to zinc, copper and cadmium, and it can be induced following zinc and copper exposure (Ramnarine et al., 2024), or gene mutations (Perron et al., 2004). However, the *czc* cluster was not detected in this study. Due to a significant difference in the concentration of a number of metals between regions A and B (Figure 4.1), other MRGs, in addition to zinc resistance genes, were investigated. Particularly relevant is the presence of the operon *pco*ABCD in the MDR *E. coli* isolates from soil samples in region A. The *pco*ABCD is a plasmid-borne determinant that confers resistance to copper, and it has been identified in several Enterobacterales genera, such as *Escherichia*, *Citrobacter* and *Salmonella* (Chalmers et al., 2018). Although often plasmid-borne, it is frequently flanked by Tn7-like transposon, which can integrate in the bacterial chromosome (Rensing et al., 2018). The Tn7-like transposon carrying the *pco* operon has been identified within IncHI plasmids, including IncHI2, and its role in transferring *pco* among plasmids within the Enterobacterales has been established (Fang et al., 2016). Interestingly, the *pco* operon can co-exist with other antimicrobial resistance genes, including $bla_{\text{CTX-M}}$, $bla_{\text{TEM-1}}$, *oqxAB*, *floR*, *sul*, *dfrA* and *tet*, on plasmids in several Enterobacterales species isolated from food production environment (Fang et al., 2016, Rebelo et al., 2023). In this study, the *pco* operon was not predicted to be

located on a plasmid using the Platon database; however, Tn7-like transposons carrying the *pco* genes can be integrated into the bacterial chromosome (Moreno-Switt et al., 2012). Our findings highlighted the co-occurrence of the *pco* operon and MDR phenotypes in *E. coli* isolates. The same *E. coli* isolates that harboured the *pco* operon, carried also the genes *ybtP* and *ybtQ* which encode for an ATP binding cassette involved in iron(III)-yersiniabactin transport in *Yersinia pestis* (Koh et al., 2016). The genes *ybtP* and *ybtQ* are part of the *Yersinia* high-pathogenicity island (HPI), a virulence strategy commonly found in *E. coli* isolates associated with urinary tract infections (Koh et al., 2016).

The efflux pumps identified, which are transporters able to expel substrates from inside the cell to the external environment, may play an important role in the interaction between heavy metal exposure and AMR occurrence (Nguyen et al., 2023). Perron et al. (2004) elucidated the impact of heavy metal exposure on efflux pump expression, specifically in *P. aeruginosa* the zinc-induced efflux pump CzrCBA conferred resistance to heavy metals and carbapenems. Similarly, in *E. coli* the *soxS* gene, which encodes for the regulator of the multidrug efflux pump AcrAB, was overexpressed in response to $Cr_2O_7^{2-}$ and Cu^{2+} exposure (Harrison et al., 2009). In *Salmonella enterica* and *E. coli*, zinc and copper can activate the regulators *baeR* and *baeS*, which induce *mdtABC* and *acrD*, multidrug efflux pumps that confer resistance to heavy metals and antimicrobials, including beta-lactams (Nishino et al., 2021, Pletzer and Weingart, 2014). Genes, including *acrD*, *mdtA*, *baeR*, *baeS* and *soxS*, were identified in several isolates in this study, from both low and high zinc-containing regions. Moreover, the detection of *emrA* and *emrB*, which encode for the efflux pump EmrAB that confer resistance to quinolones, may explain the phenotypic resistance to quinolones in *E. coli* and *Proteus* isolates (Puértolas-Balint et al., 2019).

Several studies that have demonstrated the ability to detect bacterial pathogens in milk filters (Del Collo et al., 2017). Milk filters are also used to remove particles and debris present in milk; however, they are useful for

bacterial identification and prevalence in herds (Van Kessel et al., 2008). Most of the studies on milk filters have focused on foodborne pathogens, such as *Listeria monocytogenes* (Van Kessel et al., 2011) and *Campylobacter* spp. (Del Collo et al., 2017), due to the recognition that the detection of pathogens in milk filter serves as an indicator of potential milk contamination (Giacometti et al., 2012). However, the focus of this study was on the antimicrobial resistance profiles of Enterobacterales isolated from milk filters, similar to the Van Kessel et al. (2013) study that investigated the prevalence of antimicrobial resistant *Salmonella enterica* in milk filters and bulk milk. In their study, a low but significant incidence of antimicrobial resistant *Salmonella* was observed in milk filters; therefore, it was suggested to include the monitoring of milk filters for the surveillance of AMR in dairy herds (Van Kessel et al., 2013). In the present study, the majority of Enterobacterales isolates (77.8%) detected in milk filters were resistant to three or more antimicrobial classes (MDR). In contrast, Dell'Orco et al. (2019) did not identify MDR *E. coli* isolates on milk filters, while Van Kessel et al. (2013) reported that 11.4% of *S. enterica* isolates identified in bulk milk and milk filters were MDR. None of the genera identified in the milk filters of the present study overlapped with the Gram negative bacteria reported in the Plassard et al. (2021) study despite of employing similar methodologies to monitor ESBL-producing and Carbapenem-producing Gram negative bacteria. Plassard et al. (2021) predominantly detected *Pseudomonas* spp. in milk filter samples, although none exhibited multidrug resistance. Conversely, although the sample size was limited, most of the isolates in this study exhibited resistance to three or more antimicrobial classes, indicating a substantial level of multidrug resistance on the milk filters examined. No antimicrobial use was reported on the farms at the time of sampling. The milk filters harboured different species with varying phenotypic and genotypic profiles. Although antimicrobial use and feed types can influence this, in this study the cows were not been subjected to any antimicrobial treatment before the sample collection, and they were not fed with any additional feed besides pasture grazing. The variation of

antimicrobial resistant Enterobacterales observed may instead be due to environmental contamination and/or farm management practices, such as the storage of slurry, cubicle bedding materials, teat management practices and milking system (automated or traditional milking parlour) (McLaughlin et al., 2022).

The genotypic profiles of the isolates identified in this study matched the phenotypic profiles for the most part. Only few exceptions were found, such as the presence of the gene *catA2* in *M. morganii* [D1F2c E], which was phenotypically sensitive to chloramphenicol, and the ESBL gene bla_{FONA} in *S. fonticola* without showing an ESBL phenotype. The first phenomenon might be due to the alteration or deletions of the gene promoter region resulting in the silencing of *catA2* gene (Deekshit and Srikumar, 2022). The presence of bla_{FONA} in *S. fonticola* without showing an ESBL phenotype has also been observed in previous studies (Rybak et al., 2022, Rybak et al., 2021). Although resistance to β-lactams, such as AMP, CPD and CTX was phenotypically observed, some *S. fonticola* isolates lacked ESBL activity using the double-disk synergy assay (Rybak et al., 2022; Rybak et al., 2021). WGS also predicted the presence of the quinolone resistance gene *qnrD1* on Col3M_1 plasmid in *P. vulgaris* [M2Fb1] and *C. freundii* [M3F1] isolates. Resistance to quinolones is often plasmid mediated, and *qnrD*-bearing plasmids can be often found in *Proteus* species (Jacoby et al., 2014). This agrees with the findings of our study and of others, such as Peng et al. (2023) who identified *qnrD1* on Col3M_1 plasmid in *P. mirabilis* and *P. terrae* from chicken and pig faeces. Although *E. coli*, *C. freundii* and *S. fonticola* are commonly found in the environment, including soil (Aljorayid et al., 2016, van Elsas et al., 2011, Jabeen et al., 2023), their prevalence may be impacted by other factors. Specifically, biofilm formation, stress resistance and/or nutrient utilisation can influence the ability of these bacteria to survive in the environment (Jabeen et al., 2023, Hasson et al., 2018, van Elsas et al., 2011). In this study, genes encoding for the adherence, biofilm formation, motility and nutritional factors were mostly found in *E. coli* isolates

(Supplementary Material, Appendix C – B). Only a few genes that encode for the regulation of virulence factors, such as *ompD*, *rpos*, *phoB* and *phoR*, were identified in *C. freundii*, while the *smaI* gene involved in biofilm formation in *S. fonticola* observed in Hasson et al. (2018) study was not detected (Supplementary Material, Appendix C – A).

It has been demonstrated that *E. coli* isolates of different phylotype groups have different phenotypic and genotypic features, as well as diverse potential to cause disease (Clermont et al., 2013). The *E. coli* belonging to phylotype B2 and D are mostly associated with extra-intestinal infections in humans (Fulham et al., 2022), while A and B1 phylotypes are mainly identified as commensal *E. coli* isolates (Carlos et al., 2010). Phylotype B1 is commonly found in the environment and animal faeces, including cow, goat and sheep faeces (Bhowmik et al., 2023, Carlos et al., 2010). The detection of *E. coli* belonging to phylotype B1 in milk filter samples and three soil samples may be due to faecal contamination. Phylotype C is closely related to group B1, but it is less commonly found than the main phylotypes, such as A, B1, B2 and D (Escobar-Páramo et al., 2004). However, in the current study the *E. coli* isolates identified in soil samples harbouring a MDR profile were all phylotype C. Similarly, Bhowmik et al. (2023) identified a high percentage of MDR *E. coli* of phylotype C; however, in their study phylotype C was associated with *E. coli* isolates originated from animal faeces and not from soil. Phylotype C was the rarest phylotype reported in the Lagerstrom and Hadly (2023) study. The apparent rarity of this phylotype may be due to the fact that it results as phylotype A using the triplex PCR method (Moissenet et al., 2010), resulting in underreporting in studies that use this method for classification. Although reports of *E. coli* of phylotype C are somewhat rare, the potential for gut colonization and virulence of these isolates was demonstrated in Moissenet et al. (2010) study on newborn meningitis. The detection of *E. coli* isolates with phylotype C from region A may suggest a possible common ancestor. This finding raises interesting questions about the origin and potential dissemination of these MDR *E. coli*

between the farms, despite the isolates being isolated from different farms. Further investigation is needed to elucidate the specific transmission pathways of the MDR *E. coli* of phylotype C within the farm environment.

4.5 Conclusion

The findings of this study align with several field-based studies results, and clear differences were observed in the profile of AMR isolates obtained from the different heavy metal containing regions. Although the mechanisms through which zinc might impact AMR dissemination remains unclear, this study underscores an interesting detection of Enterobacterales with resistance to all the antimicrobials tested (except for carbapenems) in the area with higher heavy metal concentration compared to the low heavy metal-containing area. The occurrence of zinc resistance genes was similar across both locations; therefore, zinc concentration may not have directly influenced zinc resistance in Enterobacterales in this study. Interestingly, the *pco*ABCD, associated with copper resistance, was only identified in region A, where copper concentrations were significantly higher than region B. Additionally, the detection of MDR *E. coli* isolates originating from different farms in region A suggests a shared origin. Therefore, understanding how these resistant isolates emerged and spread between the farms is crucial. This study also highlights the benefit of using milk filters to investigate AMR in dairy production. With nine resistant Enterobacterales isolates found in eight out of 29 milk filter samples, showing resistance to three or more antimicrobial classes, this study indicates the potential for these bacteria to disseminate in food production, and emphasizes the importance of monitoring milk filters for AMR dissemination in dairy herds. Further research is necessary to understand the dynamics of AMR dissemination in both soil and milk filters across farms considering its implication in dairy production and food safety.

4.6 Appendix C: Supplementary Material

The supplementary material for Chapter Four “Comparison of antimicrobial resistant Enterobacterales isolates from the dairy production environment in low and high zinc containing regions.” is available at DOI of the reference below.

Anedda, E., Alexa, E. A., Farrell, M. L., Croffie, M., Madigan, G., Morris, D. & Burgess, C. M. 2024. Comparison of antimicrobial resistant Enterobacterales isolates from the dairy production environment in low and high zinc containing regions. *Science of the Total Environment*, 953, p.175905

DOI: https://doi.org/10.1016/j.scitotenv.2024.175905

Chapter Five

Examination of the microbial communities and associated resistomes in dairy production in regions of differing heavy metal concentrations.

5.1 Introduction

Antimicrobial resistance (AMR) is one of the most severe threats for human, animal and environmental health. Despite the introduction of strict restrictions on the use of antimicrobials worldwide, antimicrobial resistant bacteria (ARB) are commonly present in the environment (Larsson and Flach, 2022). The use of antimicrobials in livestock production, as well as agricultural and aquacultural practices, are key drivers of AMR in the environment, including the primary food production environment (Kelbrick et al., 2023, Seiler and Berendonk, 2012). However, other factors, such as heavy metals and biocides, have been reported as selective pressures for AMR occurrence in the environment (Koutsoumanis et al., 2021). Several studies have demonstrated co-selection between heavy metals and AMR, where different mechanisms can be involved, such as co-resistance, cross-resistance, co-regulation and biofilm formation (Baker-Austin et al., 2006, Chapman, 2003). Mobile genetic elements (MGEs) play a crucial role in the association between heavy metals and AMR by transferring antimicrobial resistance genes (ARGs) and heavy metal resistance genes (MRGs) among different microorganisms (Murray et al., 2024). The implementation of next-generation sequencing (NGS) methods can provide deeper insights into the association between heavy metals and AMR, leading to an in-depth comprehension of the entire microbial community, the abundance and diversity of resistance genes (Bianconi et al., 2023, Yamin et al., 2023). Among NGS techniques, shotgun metagenomics results in sequencing of all genomes present in a sample (Edwin et al., 2024). The simultaneous identification and characterization of all microorganisms makes this technique unbiased, leading to the detection of all species including the unculturable ones, without the need for isolation, cultivation or enrichment, which can be time-consuming (Kumar et al., 2024, Ekhlas et al., 2023a). Moreover, the metagenomic approach provides large amounts of data to explore the microbial communities and the reservoir of ARGs (resistome) in complex samples (Zaheer et al., 2018). However, there are challenges and limitations with this technique. The high cost of this technique limits its use,

and impacts the sample size, which can consequently affect the statistical analysis (Ekhlas et al., 2023a). It requires bioinformatics and technical expertise, and high-performance computational tools to analyse and store the large amount of data generated (Billington et al., 2022). Additionally, for some complex samples, such as blood, saliva, and milk, a large quantity of host genome DNA needs to be removed (Pereira-Marques et al., 2019). Nonetheless, metagenomic techniques applied in food production settings can be utilised to help understand and manage microbial risks and lower the occurrence of foodborne illness (Billington et al., 2022). In the current study, a shotgun metagenomic method was employed to investigate the bacterial community composition and resistome in soil and bovine milk filter samples, collected from two different geographical regions with low and high zinc concentrations in Ireland, complementing the research detailed in Chapter Four. The objective was to assess the microbial communities and resistomes in relation to the heavy metal concentrations, in order to achieve a more comprehensive picture of the AMR and heavy metal occurrence in the dairy production environment.

5.2 Materials and methods

5.2.1 Sampling procedure

Fifty soil and 18 bovine milk filter samples were collected from two distinct geographical regions in Ireland, characterized by low (location B) and high (location A) zinc concentrations, as previously described in Chapter Four. Five dairy farms were selected within each region. From each farm, five soil samples were collected and subsequently pooled after DNA extraction, resulting in a total of 10 composite soil samples (five from each region) for metagenomic analysis. Additionally, a total of 18 milk filter samples (nine from each region) were collected for metagenomic analysis. All samples were transported to the laboratory under cool conditions and stored at - 80 °C immediately upon arrival to preserve sample integrity.

5.2.2 DNA extraction and sequencing

DNA was extracted from 0.25 g of each soil sample and milk filter. The extraction was conducted using the DNeasy PowerSoil Pro Kit (Qiagen, Germany) for both sample types in accordance to the manufacturer's instructions. DNA concentration was measured using Qubit 3.0 Fluorometer (Invitrogen, ThermoFisher Scientific, United Kingdom). The DNA extracted from five samples of each farm was pooled together, resulting in one DNA sample for each farm, for a total of 10 DNA soil samples. Both soil and milk filter DNA samples were then processed for shotgun metagenomic sequencing. The libraries were prepared using Nextera XT Library Preparation Kit (Illumina Inc., San Diego, CA) and, after size assessment using Agilent Bioanalyzer, libraries were pooled equimolarly. The sequencing was conducted using the Illumina NovaSeq 6000 (2 x 150bp) in Teagasc Food Research Centre Moorepark. The sequencing depth of the soil samples ranged from 6.3 million to 33.7 million reads (average of 24.3 million reads); while, milk filter samples had a sequencing depth from 58.1 thousand to 26.2 million reads (average of 12.3 million reads).

5.2.3 Data analysis

The raw metagenomic reads were quality trimmed and the adapters removed using TrimGalore (v 0.6.1), a wrapper for Cutadapt and FastQC (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). The host genome *Bos Taurus* was removed from milk filter sequences using Bowtie2 (v 2.4.4) (Langmead and Salzberg, 2012). SAMtools (v 1.10) was then used to filter the unmapped paired-end sequences and convert them from SAM format to BAM (Li et al., 2009). BAM files were further converted to FASTQ format employing Bedtools (v 2.27.1) (Quinlan and Hall, 2010). The taxonomy profiles of soil samples and milk filters without host genome were determined using Kraken2 (v 2.1.1) (Wood and Salzberg, 2014) and refined using Bracken2 (v 2.2) (Lu et al., 2017). MetaSPAdes (v 3.15.3) was used to assemble the trimmed sequences into contigs (Nurk et al., 2017). Different databases were employed for the identification of the resistome.

Specifically, AMRFinder (v. 3.10.23) and RGI (v. 6.0.3) were used for the detection of ARGs, MRGs, stress (biocide and heat) resistance genes and virulence genes, with a minimum identity and coverage of 60% (Feldgarden et al., 2019, Alcock et al., 2022). Further analysis were conducted for the identification of MRGs using BacMet2 (Pal et al., 2014).

The relative abundance of bacterial phyla and genera was analysed using dplyr and tidyverse packages in R version 4.2.1 (https://cran.r-project.org/) and RStudio 1.3.1093 (https://posit.co/products/open-source/rstudio/). Principal coordinate analysis (PCoA) was conducted using Bray-Curtis dissimilarity using vegan and ggplot2 packages in RStudio, in order to visualize clustering patterns between microbial communities of each sample based on taxonomic abundance data.

5.3 Results

5.3.1 Bacterial community composition

A total of 177 phyla, 3,689 genera and 25,954 species were identified among soil and milk filter samples. Figure 5.1 shows the relative abundance of bacterial phyla and genera in soil samples. Proteobacteria, Actinobacteria, Acidobacteria and Verrucomicrobia were the most abundant phyla in soil samples from region A; while, in region B, Actinobacteria was slightly more abundant than Proteobacteria, followed by Acidobacteria and Verrucomicrobia. At the genus level, soil samples from region A were dominated by *Bradyrhizobium, Streptomyces, Candidatus Actinomarina* and *Mycobacterium*; similarly, soil samples from region B where dominated by the same genera except that the third most abundant genus was *Nocardioides*.

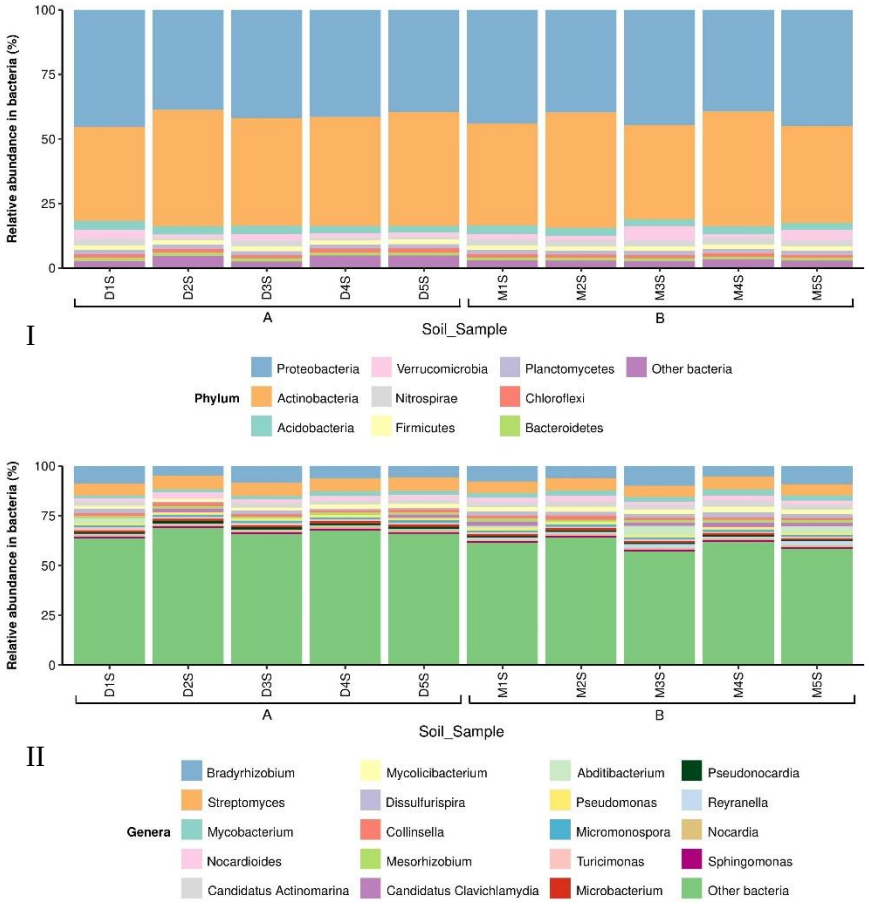

Figure 5.1 Relative abundance of bacterial (I) phyla and (II) genera in soil samples from regions A and B.

Figure 5.2 shows the relative abundance of bacterial phyla and genera in milk filter samples. The most abundant phyla from both regions was Proteobacteria, followed by Actinobacteria, Fermicutes and Bacteroidetes in region A, and Actinobacteria, Bacteroidetes and Fermicutes in region B. The most abundant genera in milk filter samples differed between samples and the two regions. In region A, the most predominant were *Pseudomonas, Acinetobacter, Corynebacterium and Microbacterium*; while in region B, *Pseudomonas, Corynebacterium, Acinetobacter* and *Collinsella* were the most abundant.

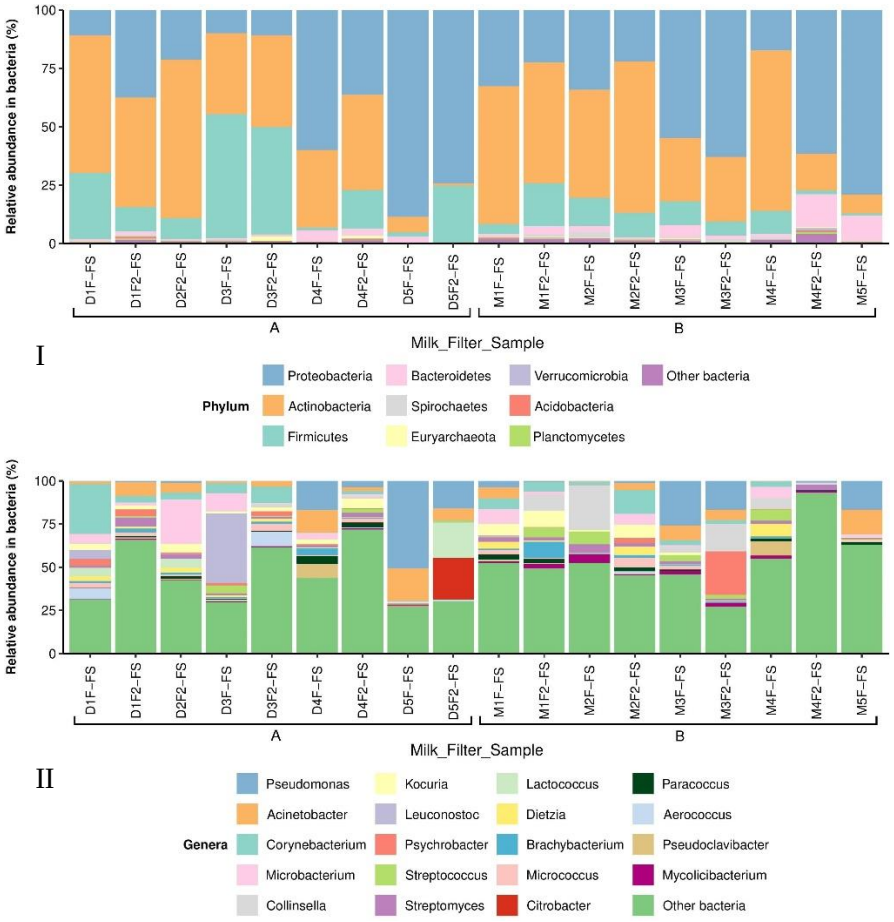

Figure 5.2 Relative abundance of bacterial (I) phyla and (II) genera in milk filters from regions A and B.

In regard to the species present in soil and milk filter samples, Figure 5.3 and Figure 5.4 illustrate the richness, the Simpson's diversity and the Principal Coordinate analysis (PCoA) identified in soil (Figure 5.3) and milk filters (Figure 5.4) in regions A and B. In soil samples, the average of the number of species in region A and B was similar, around 25,000 species, with lower values in two samples from region A. The species diversity, which includes the number of species and the abundance of individuals, was higher in region A. The PCoA analysis identified clustering of samples within the regions but the two regions clustered separately.

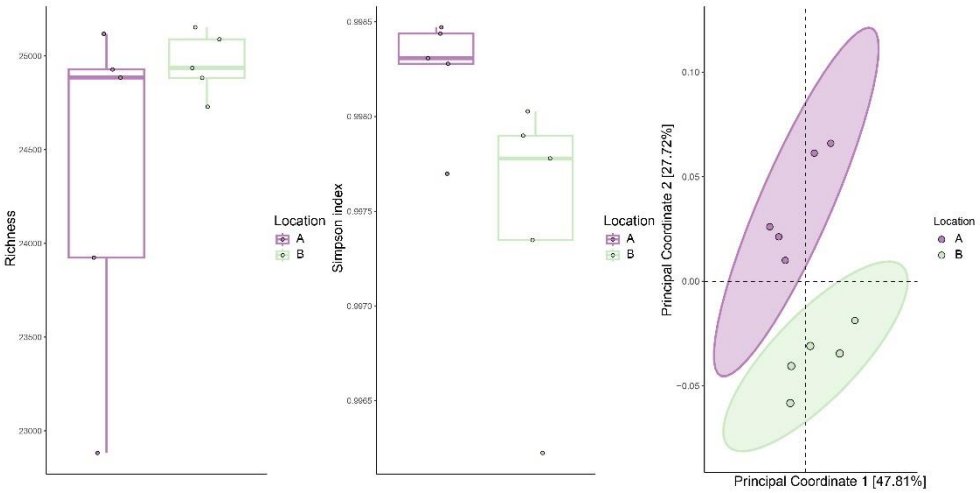

Figure 5.3 Richness, Simpson's diversity and Principal Coordinate analysis (PCoA) of microbial species identified in soil samples from regions A and B.

In milk filter samples, the number of species was different between regions A and B, with a higher average number of 20,000 species detected in region A and a lower average of around 7,000 identified in region B. However, the Simpson diversity was high in both regions. The PCoA shows that the samples from the same region clustered together, and overlapped with samples from the other region (Figure 5.4).

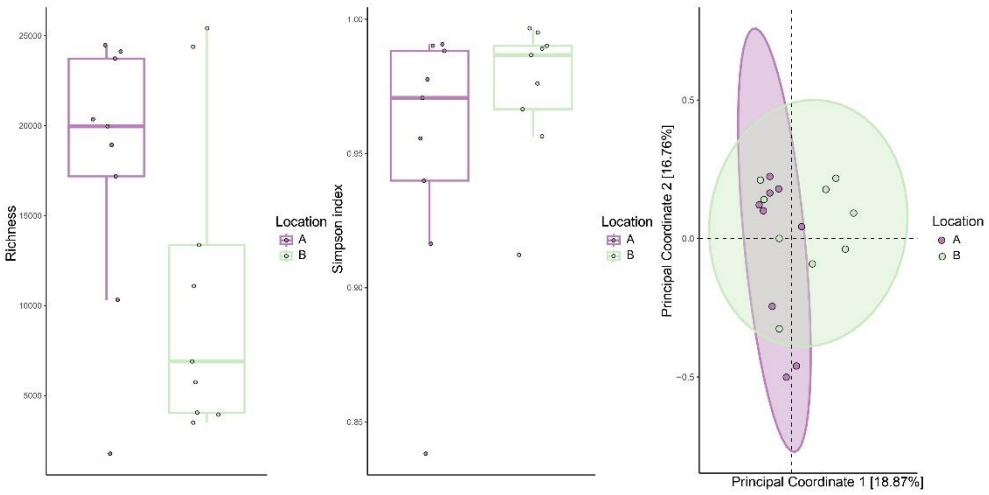

Figure 5.4 Richness, Simpson's diversity and Principal Coordinate analysis (PCoA) of microbial species identified in milk filters from region A and B.

5.3.2 Resistome analysis

The ARGs identified in soil samples belonged to several antimicrobial classes, including resistance to aminoglycosides, glycopeptides, beta-lactams and rifamycins, and were heterogenously distributed between samples from both regions A and B (Figure 5.5). It is evident that some ARGs were present in both regions, whilst some were only detected in one region or the other. Genes encoding for efflux pumps and genes conferring resistance to stress factors, such as heat and biocides, were also present in both regions (Supplementary Material, Appendix D – Figure 5.9). The MRGs identified in soil samples were heterogeneously distributed between samples from regions A and B, with copper and arsenic resistance genes most prevalent (Figure 5.6).

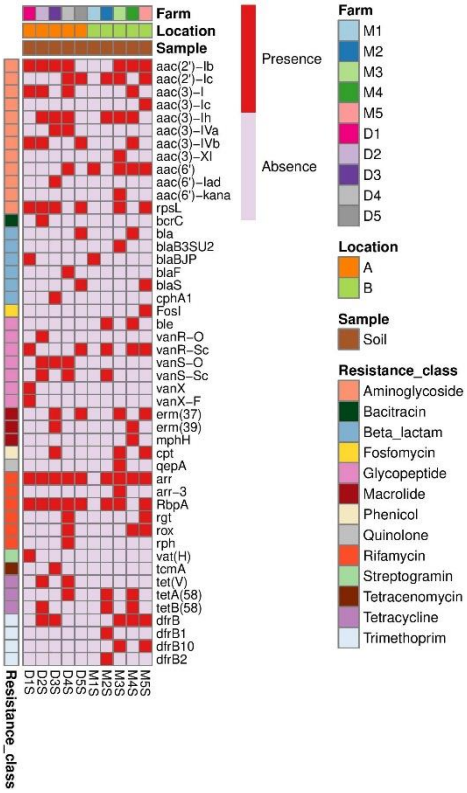

Figure 5.5 Heatmap showing the antimicrobial resistance genes identified in soil samples collected from regions A and B.

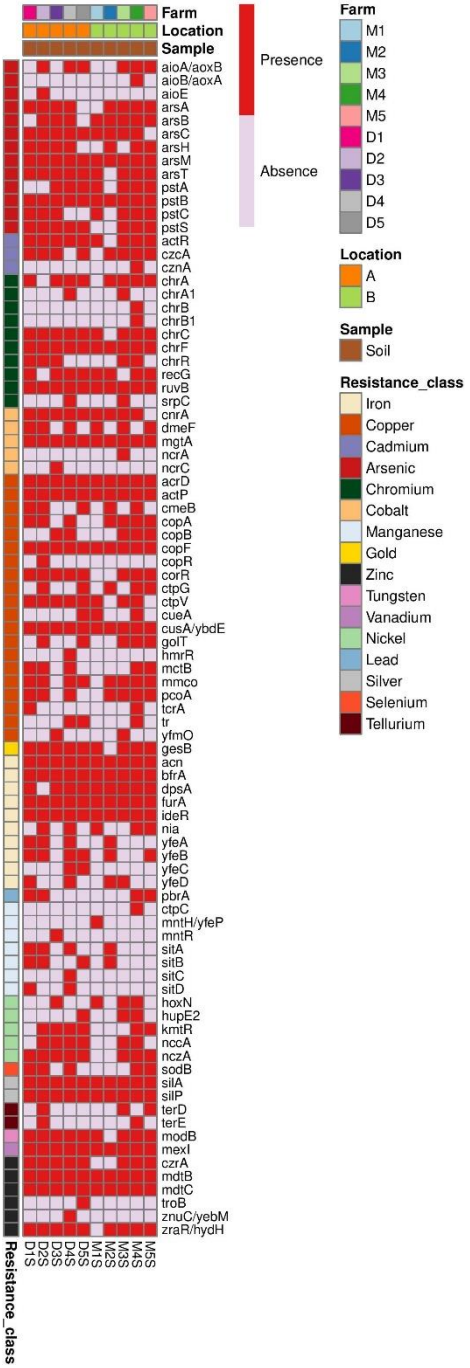

Figure 5.6 Heatmap showing the metal resistance genes identified in soil samples collected from regions A and B.

The ARGs and MRGs detected in milk filters were mostly present in filters from region B (Figure 5.7 and Figure 5.8). Of the samples collected, the largest proportion of ARGs were detected in samples M2F-FS, D4F2-FS and M5F-FS (Figure 5.7), while the highest number of MRGs were detected in samples M5F-FS, M2F2-FS, M2F-FS and D5F2-FS (Figure 5.8).

The most prevalent ARGs confer resistance to aminoglycosides, beta-lactams, macrolide and tetracyclines, while the most prevalent MRGs conferred resistance to copper, zinc and iron. Additionally, efflux pump genes, virulence genes and genes conferring resistance to stress factors were mostly identified in milk filters from region B (Supplementary Material, Appendix D – Figure 5.10).

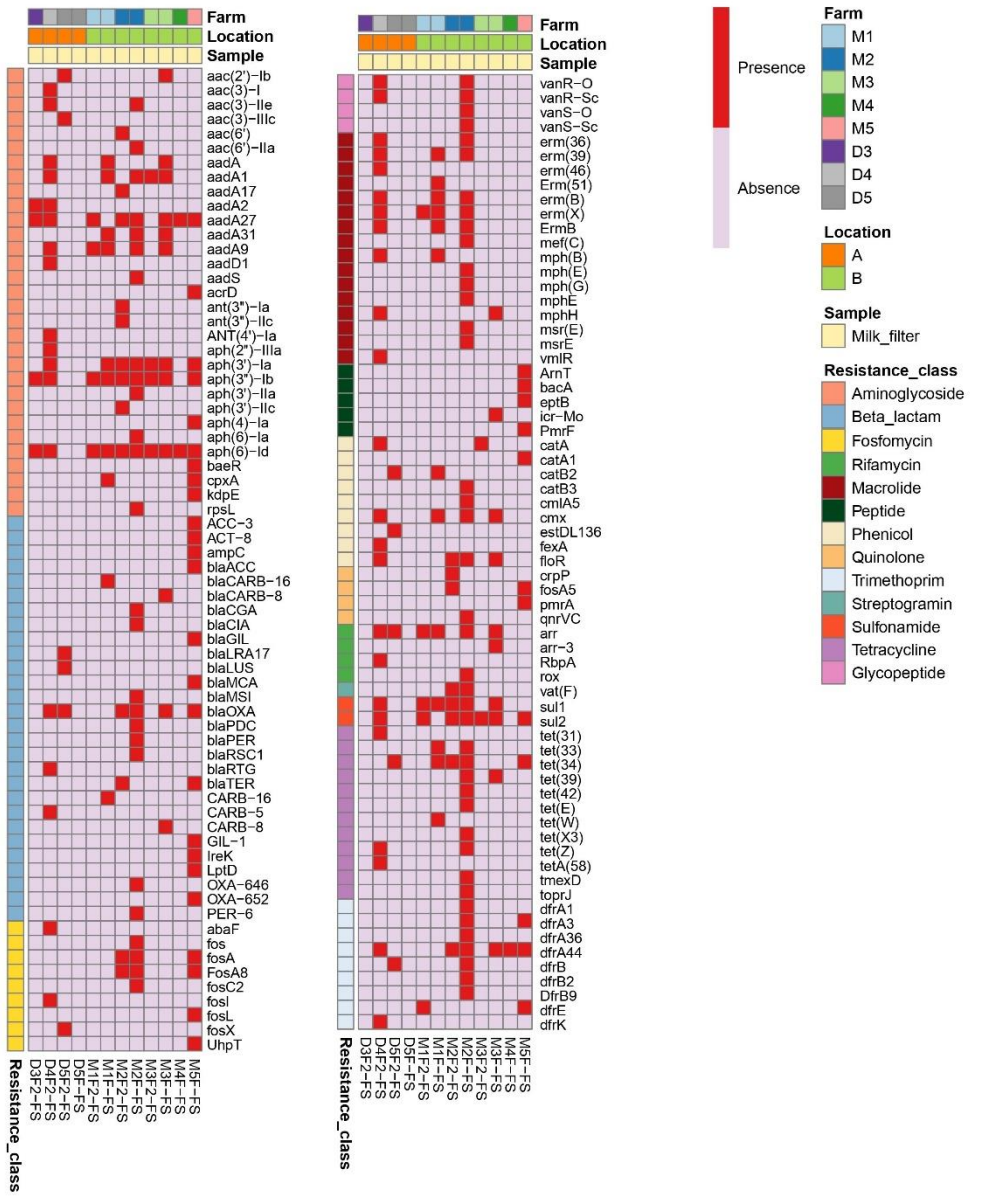

Figure 5.7 Heatmap showing the antimicrobial resistance genes identified in milk filter samples collected from regions A and B. The heatmap is presented in two segments to aid readability.

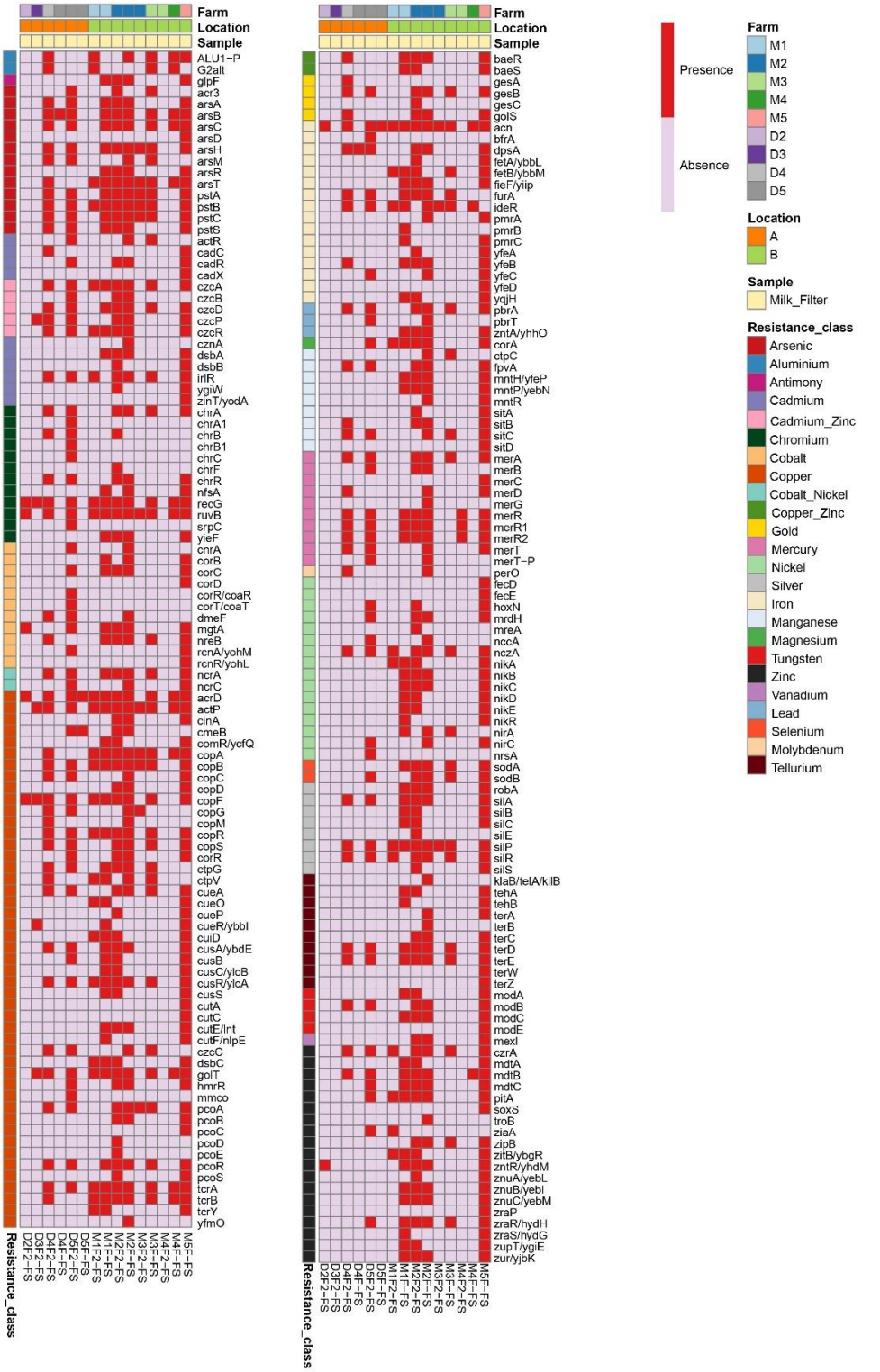

Figure 5.8 Heatmap showing the metal resistance genes identified in milk filter samples collected from regions A and B. The heatmap is presented in two segments to aid readability.

5.4 Discussion and conclusion

The objective of this study was to elucidate the bacterial community structure and resistome in dairy pasture soil and milk filters in regions of high and low zinc concentration. In both regions Proteobacteria, Actinobacteria, Acidobacteria were the dominant phyla, although there was variation in the order of abundance between the regions. Similarly, Qiao et al. (2021) identified Proteobacteria and Actinobacteria as the dominant phyla in farmlands in their study.

The findings of this study, revealed a heterogenous distribution of ARGs and MRGs in soil samples between regions A and B, suggesting that heavy metals, despite their higher concentrations in region A, may not play a significant role in influencing AMR in these soils. This contrasts with previous research, such as Qiao et al. (2021), who found that heavy metals like arsenic, lead, and cadmium in farmlands surrounding metal tailings strongly influenced the microbial community. Tongyi et al. (2020) demonstrated that increasing zinc levels in soils in microcosm experiments led to higher ARG abundance and diversity. However, our findings do not show such a correlation between zinc concentration and ARG distribution, indicating that zinc may not exert the same selective pressure in our study as seen in other environments. Additionally, Zhou et al. (2016) observed a strong relationship between heavy metals and ARG on dairy farms, but the lack of such a pattern in our study suggests that other environmental factors or microbial community dynamics may mitigate zinc's impact on AMR. This indicates that while heavy metals like zinc have been shown to contribute to AMR in certain contexts, this effect may not be universal across different ecosystems or metal concentrations. The microbial community composition in milk filters aligns with prior studies, where Proteobacteria and Actinobacteria were the predominant phyla and *Pseudomonas*, *Acinetobacter* and *Corynebacterium* were the genera most abundant in milk filters (Rubiola et al., 2022). The detection of a substantial number ARGs and MRGs in milk filters indicates their presence in raw milk and indicates the value of milk filters as a means to monitor AMR in dairy production settings.

Overall, our findings demonstrate that the dairy production environment is a reservoir for AMR, with potential implications for the dissemination of ARGs and MRGs within the agricultural environment and in food production. The presence of varying zinc levels in the pasture land did not appear to influence the ARG or MRG profiles in either sample type examined.

5.5 Appendix D: Supplementary Material

Supplementary material for Chapter Five "Metagenomic study on antimicrobial resistance and heavy metals in the Irish dairy production environment."

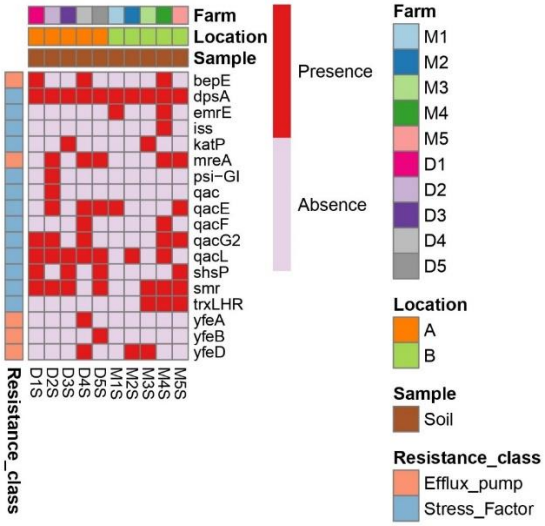

Figure 5.9 Heatmap showing genes conferring resistance to stress factors, such as heat and biocides, and genes encoding for efflux pumps identified in soil samples from regions A and B.

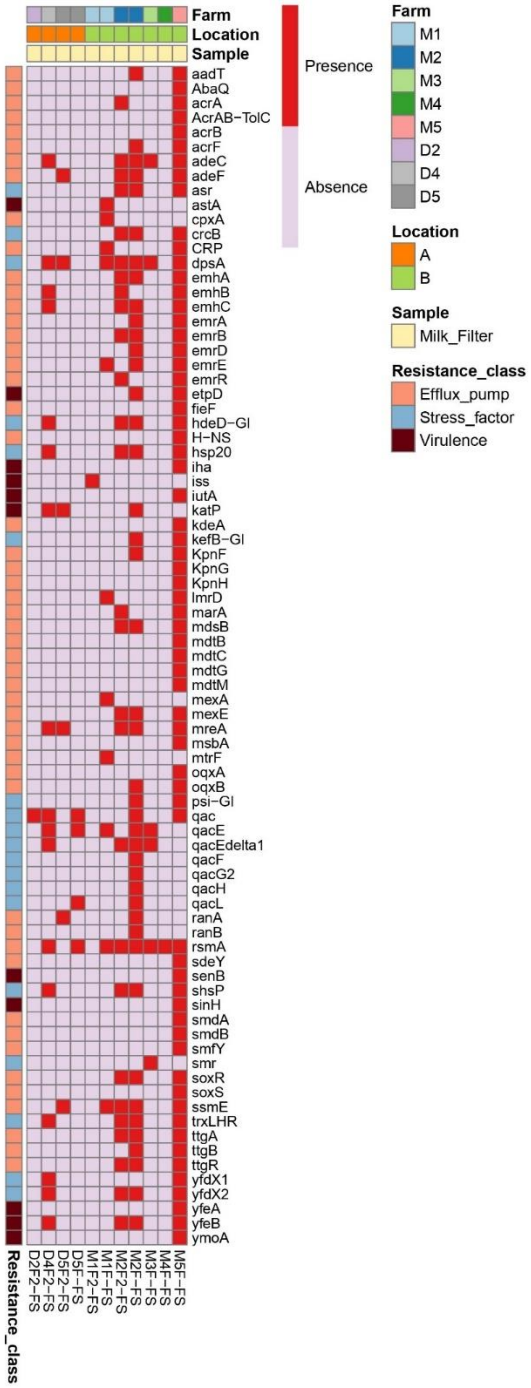

Figure 5.10 Heatmap showing genes conferring resistance to stress factors, such as heat and biocides, virulence genes and genes encoding for efflux pumps identified in milk filters from regions A and B.

Chapter Six: Discussion, Limitations and Future Work

6.1 Discussion

This thesis sought to investigate whether heavy metals, specifically zinc, influence AMR dissemination and the AMR profile of Enterobacterales in the primary food production environment. The food chain acts as a key pathway for the dissemination of ARB among humans, animals and the environment; therefore, it is important to better understand the link between heavy metals and AMR in the primary food production environment. Several studies have demonstrated the role heavy metals can play as a selective pressure for AMR dissemination (Cao et al., 2020, Komijani et al., 2021, Silva et al., 2021). However, the findings of this thesis did not demonstrate a notable correlation between the presence of zinc in soil and AMR, and various factors may have influenced the current thesis observations.

6.1.1 Influences of heavy metal characteristics and environmental factors on AMR development.

As outlined in Chapter Three, microbial heavy metal toxicity is caused by a combination of factors, including metal concentration, availability, and chemical form. Several studies that recognised heavy metals as drivers of AMR focused on high heavy metal concentrations (Berg et al., 2010, Tongyi et al., 2020, Li et al., 2022). The concentrations of zinc measured in this thesis differed from concentrations identified in other studies. Berg et al. (2010) observed a higher resistance to tetracycline and vancomycin in soil bacterial communities collected from soil with copper concentrations of 3,172 mg/Kg. Li et al. (2022) demonstrated the role of zinc on AMR transfer in soil bacterial communities using a zinc concentration of 7,200 mg/kg in soil. While concentration plays a significant role, it is not the sole determinant of metal toxicity. In fact, metal concentration does not necessarily correlate with metal bioavailability. Furthermore, bioavailability considers the total heavy metals available for absorption, and it is influenced by environmental conditions, such as pH and concentration of organic matter. Low pH increases the solubility of metals such as zinc, while a high concentration of organic matter can decrease the solubility and bioavailability of zinc (Schulz-

Zunkel and Krueger, 2009). In Chapters Three and Chapter Four, metal bioavailability, soil pH, or organic matter concentration were not measured. Therefore, while metal concentrations were measured, the lack of data on these additional variables may have influenced the results, potentially explaining differences with previous studies. The environmental conditions can also alter the chemical form of metals, affecting the metal toxicity and impacting the role of metals in the development of AMR. For example, the chromium chemical form Cr^{3+} is less toxic to bacteria compared to chromium associated with oxygen, such as $Cr_2O_7^{2-}$ (Baldiris et al., 2018). Ions like $Cr_2O_7^{2-}$ and Cu^{2+} can be responsible for oxidative stress. This condition can cause upregulation of genes, such as *soxS*, which are involved in the regulation of efflux pumps, like the AcrAB efflux pump in *E. coli* (Harrison et al., 2009). Consequently, the activation of efflux pumps can lead to an increase of AMR (Seiler and Berendonk, 2012).

6.1.2 Variation in experimental design and soil composition

The experimental setting may have contributed to the contrasting findings observed in this study when compared with others. Chapter Three and Chapter Four were based on field trials which represented a real-world scenario. This means that soil properties may vary more widely than in pot experiments. Sui et al. (2019) reported heavy metals as one of the key factors responsible for ARGs' increase in soil bacterial communities. However, their study, which focused on the impact of swine slurry on soil, was conducted using experimental pots with controlled characteristics. Similarly, Li et al. (2022) used glass microcosm jars to conduct their study that identified a greater influence of zinc and copper on ARGs' transfer than the doxycycline antimicrobial in agricultural soil. Unlike experimental pots, where the soil is confined to a specific area, in field trials soil conditions can often fluctuate, and the penetration of heavy metals applied into the soil may not be uniform. Moreover, the vicinity to heavy metal contamination sources, such as industries and mines, may affect the results. Several studies investigated the role of heavy metals on AMR occurrence in soil collected

from areas close to mines or industries. For example, Cheng et al. (2021) assessed the ARGs' abundance and diversity in farmland soil collected at various distances from a phosphorus chemical industry. Similarly, Chen et al. (2019) examined the occurrence and relationship of MRGs and ARGs in a copper mine area in the South of China, focusing on four different sampling sites used as farmland and plantation. In these studies, where industries and mines are sources of heavy metals, the period of heavy metal exposure in the environment plays a crucial role, resulting mostly in a long-term field exposure. For instance, in the study previously mentioned the tailing dam had been used for 47 years before the research study (Chen et al., 2019). Wu et al. (2020) identified an increase in the relative abundance of ARGs in soil used for vegetable production following long-term manure application over 10 years. In Chapter Three, the zinc sulphate was applied to the soil one and two years before the study commenced; therefore, it was not possible employ a temporal approach to study the impact of zinc over the time.

Moreover, varied use of land may affect the soil microbial community composition and characterization. A clear difference in the predominant antimicrobial resistant Enterobacterales between Chapter Three and Chapter Four is evident. In Chapter Three the most prevalent antimicrobial resistant Enterobacterales species detected was *S. fonticola*, while in Chapter Four most of the isolates belonged to *E. coli* species. This observation may be due to the land use in the two chapters; specifically, in Chapter Three both sites used for crop production, while in Chapter Four the soil was from ten farms used for livestock grazing. This may explain why the environmental species *S. fonticola* was predominant in Chapter Three, and *E. coli*, which is commonly found in faeces, was predominant in Chapter Four. This aligns with findings from previous studies, where *S. fonticola* was one of the most frequently identified ESBL-producing Enterobacterales in vegetables and the agricultural environment, following *Rahnella aquatilis*, while *E. coli* was commonly detected in livestock production environments (Blaak et al., 2014, Peng et al., 2021a).

6.1.3 Potential role of copper on AMR occurrence

Although in Chapter Four the correlation between zinc and AMR was not observed, an interesting association between copper and MDR resistance profiles of *E. coli* isolates from the high copper and zinc concentration region was identified. This difference between zinc and copper effects may be due to different levels of selective pressure exerted by the two metals, and the ability of the resistance genes to move among bacteria. The copper resistance *pcoABCD* system identified in the MDR *E. coli* isolates in Chapter Four has been reported to be commonly linked with ARGs on MGEs which can be integrated in the chromosome (Rebelo et al., 2023). In contrast, the zinc resistance genes identified in Chapter Four are mostly involved in the zinc transportation and homeostasis (Hara et al., 2017); while, the zinc resistance *czc* system, which has been found linked with ARGs (Baker-Austin et al., 2006, Perron et al., 2004), was not detected in the current thesis. The elevated level of copper may have contributed to the predominance of MDR *E. coli* isolates encoding the *pcoBCD* system from the high heavy metal containing region in Chapter Four.

6.1.4 Focus on Enterobacterales order and methods employed

Another characteristic that distinguishes this thesis is the focus on a specific bacterial order, the Enterobacterales, as outlined in Chapters Three and Chapter Four. Several studies have demonstrated the relationship between heavy metals and AMR using molecular methods that do not rely on bacterial culture (Furlan et al., 2020, Zou et al., 2021, Duan et al., 2019, Liu et al., 2021). While the focus on Enterobacterales has its advantages, using only molecular methods, such as PCR targeting specific resistance genes, provides an understanding of the genomic characteristics in relation to AMR without focusing on a specific species. This approach allows for the detection of ARGs and MGEs present in the microbial community, expanding the investigation beyond the Enterobacterales-related ARGs/MGEs. This may reveal the role of MGEs in transferring ARGs and MRGs among microorganisms. However, by focusing on Enterobacterales and employing

both culture-based and genomic methods, it was possible evaluate both phenotypic and genotypic profiles, ensuring a comprehensive analysis within this bacterial group while still addressing broader environmental AMR dynamics. Although the role of MGEs in the dissemination of ARGs and MRGs has been widely demonstrated (Wang et al., 2020, Wang et al., 2018, Pu et al., 2021a), in Chapter Three and Chapter Four the MGEs examined did not appear to be linked with ARGs. The co-occurrence of ARGs and MRGs on MGEs has been reported to be more prevalent in clinical settings than in environmental ones (Wang et al., 2024, Li et al., 2017).

A metagenomic approach enables the characterization of the total DNA present in a sample, including the DNA of uncultivable organisms. The literature review in Chapter Two demonstrated that recent studies examined the impact of heavy metals on AMR employing a metagenomic approach. Most of these studies emphasized that heavy metals can alter microbial community composition and they can play a critical role in the co-selection and dissemination of ARGs in the environment (Zhao et al., 2021, Wang et al., 2021b, Peng et al., 2021b). However, in Chapter Five it was demonstrated the distribution of ARGs and MRGs did not greatly differ between the soils in the two regions with different heavy metal concentrations. However, additional research is needed to investigate the abundance and functional activity of the genes detected, the presence of MGEs, and the role of horizontal gene transfer in the two regions.

This research produced novel and valuable findings related to the isolation and characterization of antimicrobial resistant Enterobacterales in Ireland's primary food production environment. As outlined in Chapter Two, according to the inclusion criteria applied for conducting the scoping review, most of the studies about the impact of heavy metals on AMR in the primary food production environment were undertaken in China. The need for broader geographical representativeness was evident. Therefore, this thesis sought to provide new data on this regard, with particular focus on Ireland. Ireland is a global producer of zinc, and due to the widespread use of this

metal in agriculture, the analysis of zinc's role as a selective pressure on AMR in the Irish environment was particularly relevant. Thus, this thesis filled a gap on data in the Irish setting and contributed to a broader understanding of global AMR trends related to heavy metal exposure in the primary food production environment.

Furthermore, this thesis was based on real-world scenarios, allowing for the investigation of AMR in the actual conditions of the primary food production environment. This approach facilitated the detection of realistic data on the occurrence and spread of ARB, as well as insights into the environmental factors that may influence AMR development. Additionally, in a real-world setting, findings can be directly used to develop new measures to tackle AMR.

To the author's knowledge, this thesis provided the first description of the antimicrobial resistant Enterobacterales in the Irish crop and dairy production environment. It demonstrated that fresh produce, milk filters and primary food production environment can harbour clinically relevant antimicrobial resistant Enterobacterales. Therefore, the potential role of these sources as carriers of ARB is significant, warranting further investigation to better understand their impact on the transmission of AMR through the food chain. Monitoring antimicrobial resistant Enterobacterales in the primary food production environment has been demonstrated to be crucial for ensuring food safety and preventing the spread of ARB through the food chain, playing an integral role in understanding AMR from a One Health perspective.

6.2 Limitations

One of the major limitations of this thesis is the relatively small sample sizes in Chapters Three and Chapter Four, which was principally due to sample availability, as spinach is harvested and cattle graze only during certain months of the year, financial constraints, and the time needed to process and analyse the samples. In Chapter Three, a total of 160 samples of soil (n = 92) and spinach (n = 68) were collected, and in Chapter Four the number

of samples collected were 50 soil and 29 milk filters. For the metagenomic analysis in Chapter 5 28 samples were analysed. The sample sizes may have limited the possibility to optimally detect the effects of zinc on AMR within Enterobacterales species or the wider bacterial community, reducing the chances of identifying statistically significant differences between zinc-amended and control plots (Chapter Three) as well as high and low heavy metal regions (Chapters Four and Five). Therefore, a larger sample size may have led to a better understanding of the distribution and relationships between heavy metals and antimicrobial resistant Enterobacterales in the primary food production environment.

Another potential limitation is the lack of measurement of the environmental conditions that may influence the heavy metal impact. As outlined in Chapter One, metal toxicity is not only due to metal concentrations, but it depends on several factors. Specifically, pH, redox state and concentration of organic matter can influence the metal valency and the metal bioavailability, thereby affecting the heavy metal toxicity (Seiler and Berendonk, 2012). For example, a high level of organic matter can limit the mobility and bioavailability of some heavy metals, like zinc and chromium, which can bind to organic matter; while, the solubility of metals, such as zinc, lead and cadmium is higher when low level of pH occur (Seiler and Berendonk, 2012). Therefore, the absence of these environmental measurements in Chapter Three and Chapter Four may have limited the ability to accurately evaluate the heavy metal toxicity, and to understand the heavy metal behaviour and impact on the link with AMR.

6.3 Future Work

The completion of work outlined in Chapter Five is underway, providing a comprehensive investigation of the microbial communities in the dairy environment. Following on from the taxonomy profiling, the assessment of the genera and species abundances, and the detection of ARGs and MRGs, further analysis on the abundances of these genes, the correlation among them and the investigation of other genetic factors, with particular interest

on MGEs, is crucial. This will allow a better understanding of the genetic mechanisms of AMR and heavy metal resistance in bacterial populations, including the role of horizontal gene transfer and its contribution to the spread of resistance.

Considering the findings in Chapter Four, where a potential association between copper and the multidrug resistance profiles of *E. coli* isolates was identified, future work may investigate the specific role of copper as a selective pressure for AMR in the primary food production environment. The detection of the *pcoABCD* copper resistance system, which is commonly linked with ARGs on MGEs, highlights the possibility to explore the genetic mechanisms facilitating co-resistance between copper and antimicrobials. To achieve this, further genomic analysis of isolates from regions with high copper concentrations could be performed to assess the co-localization of copper resistance genes and ARGs. Additionally, in Chapter Five, metagenomic sequencing data from soil and milk filter samples could be used to quantify the abundance of copper resistance genes across microbial communities and correlate their presence with the profiles of ARGs and MGEs. These further investigations may provide a more comprehensive understanding of the relationship between heavy metals and AMR in the primary food production environment. Understanding the impact of heavy metals on AMR is critical for multiple stakeholders, including public health authorities, policymakers, farmers, and scientists, contributing to global efforts to tackle AMR through a One Health approach.

Chapter seven: Bibliography

Abadias, M., Usall, J., Anguera, M., Solsona, C. & Viñas, I. 2008. Microbiological quality of fresh, minimally-processed fruit and vegetables, and sprouts from retail establishments. *International journal of food microbiology,* 123 (1)**,** p. 121-129, https://doi.org/10.1016/j.ijfoodmicro.2007.12.013.

Abdallah, H. M., Al Naiemi, N., Elsohaby, I., Mahmoud, A. F. A., Salem, G. A. & Vandenbroucke-Grauls, C. M. J. E. 2022. Prevalence of extended-spectrum β-lactamase-producing Enterobacterales in retail sheep meat from Zagazig city, Egypt. *BMC Veterinary Research,* 18 (1)**,** p. 191, https://doi.org/10.1186/s12917-022-03294-5.

Abuoun, M., Jones, H., Stubberfield, E., Gilson, D., Shaw, L. P., Hubbard, A. T. M., Chau, K. K., Sebra, R., Peto, T. E. A., Crook, D. W., Read, D. S., Gweon, H. S., Walker, A. S., Stoesser, N., Smith, R. P., Anjum, M. F. & On Behalf of the Rehab, C. 2021. A genomic epidemiological study shows that prevalence of antimicrobial resistance in Enterobacterales is associated with the livestock host, as well as antimicrobial usage. *Microbial Genomics,* 7 (10)**,** p. 000630, https://doi.org//10.1099/mgen.0.000630.

Acharya, K. P. & Wilson, R. T. 2019. Antimicrobial Resistance in Nepal. *Frontiers in Medicine,* 6**,** p. 105, https://doi.org/10.3389/fmed.2019.00105.

Acolatse, J. E. E., Portal, E. a. R., Boostrom, I., Akafity, G., Dakroah, M. P., Chalker, V. J., Sands, K. & Spiller, O. B. 2022. Environmental surveillance of ESBL and carbapenemase-producing gram-negative bacteria in a Ghanaian Tertiary Hospital. *Antimicrobial Resistance & Infection Control,* 11 (1)**,** p. 49, https://doi.org/10.1186/s13756-022-01090-2.

Afzal, A. & Mahreen, N. 2024. Emerging insights into the impacts of heavy metals exposure on health, reproductive and productive performance of livestock. *Frontiers in Pharmacology,* 15**,** p. 1375137, https://doi.org/10.3389/fphar.2024.1375137.

Akhtar, A., Fatima, N. & Khan, H. M. 2022. Beta-Lactamases and Their Classification: An Overview. *Beta-Lactam Resistance in Gram-Negative Bacteria: Threats and Challenges.* Singapore: Springer Nature Singapore**,** p. 25-33. https://doi.org/10.1007/978-981-16-9097-6_3.

Alawi, M., Smyth, C., Drissner, D., Zimmerer, A., Leupold, D., Müller, D., Do, T. T., Velasco-Torrijos, T. & Walsh, F. 2024. Private and well drinking water are reservoirs for antimicrobial resistant bacteria. *npj Antimicrobials and Resistance,* 2 (1)**,** p. 7, https://doi.org/10.1038/s44259-024-00024-9.

Alcock, B. P., Huynh, W., Chalil, R., Smith, K. W., Raphenya, Amogelang r., Wlodarski, M. A., Edalatmand, A., Petkau, A., Syed, S. A., Tsang, K. K., Baker, S. J. C., Dave, M., Mccarthy, Madeline c., Mukiri, K. M., Nasir, J. A., Golbon, B., Imtiaz, H., Jiang, X., Kaur, K., Kwong, M., Liang, Z. C., Niu, K. C., Shan, P., Yang, J. Y. J., Gray, Kristen l., Hoad, G. R., Jia, B., Bhando, T., Carfrae, Lindsey a., Farha, Maya a., French, S., Gordzevich, R., Rachwalski, K., Tu, Megan m., Bordeleau, E., Dooley, D., Griffiths, E., Zubyk, H. L., Brown, E. D., Maguire, F., Beiko, Robert g., Hsiao, W. W. L., Brinkman, F. S. L., Van domselaar, G. & Mcarthur, A. G. 2022. CARD 2023: expanded curation, support for machine learning, and resistome prediction at the Comprehensive Antibiotic Resistance Database. *Nucleic Acids Research,* 51 (D1)**,** p. D690-D699, https://doi.org/10.1093/nar/gkac920.

Aljorayid, A., Viau, R., Castellino, L. & Jump, R. L. P. 2016. Serratia fonticola, pathogen or bystander? A case series and review of the literature. *IDCases,* 5**,** p. 6-8, https://doi.org/10.1016/j.idcr.2016.05.003.

ALLOWAY, B. J. 2008. Zinc in soils and crop nutrition. 2nd ed. 16 *International Zinc Association and International Fertilizer Industry Association*. Available: https://www.topsoils.co.nz/wp-content/uploads/2014/09/Zinc-in-Soils-and-Crop-Nutrition-Brian-J.-Alloway.pdf

Amador, P., Fernandes, R., Prudêncio, C. & Duarte, I. 2019. Prevalence of Antibiotic Resistance Genes in Multidrug-Resistant Enterobacteriaceae on Portuguese Livestock Manure. *Antibiotics,* 8 (1)**,** p. 23, https://doi.org/10.3390/antibiotics8010023.

Ambler, R. P. 1980. The structure of beta-lactamases. *Philos Trans R Soc Lond B Biol Sci,* 289 (1036)**,** p. 321-31, https://doi.org/10.1098/rstb.1980.0049.

Ammendola, S., D'amico, Y., Chirullo, B., Drumo, R., Ciavardelli, D., Pasquali, P. & Battistoni, A. 2016. Zinc is required to ensure the expression of flagella and the ability to form biofilms in *Salmonella enterica* sv Typhimurium. *Metallomics,* 8 (10)**,** p. 1131-1140, https://doi.org/10.1039/C6MT00108D.

Andreoletti, O., Budka, H., Buncic, S., Colin, P., Collins, J. D., De Koeijer, A., Griffin, J., Havelaar, A., Hope, J., Klein, G., Kruse, H., Magnino, S., Martinez López, A., Mclauchlin, J., Nguyen-Thé, C., Noeckler, K., Noerrung, B., Prieto Maradona, M., Roberts, T., Vågsholm, I. & Emmanuel, V. 2008. Foodborne antimicrobial resistance as a biological hazard - Scientific Opinion of the Panel on Biological Hazards. *EFSA Journal,* 6 (8)**,** p. 765, https://doi.org/10.2903/j.efsa.2008.765.

ANDREWS, S. 2010. *FastQC: a quality control tool for high throughput sequence data* [Online]. Available: https://www.bioinformatics.babraham.ac.uk/projects/fastqc/ [Accessed 20 Sept 2022].

Anedda, E., Farrell, M. L., Morris, D. & Burgess, C. M. 2023. Evaluating the impact of heavy metals on antimicrobial resistance in the primary food production environment: A scoping review. *Environmental pollution* 320**,** p. 121035-121035, https://doi.org/10.1016/j.envpol.2023.121035.

Anju, V. T., Siddhardha, B. & Dyavaiah, M. 2020. Enterobacter Infections and Antimicrobial Drug Resistance. *Model Organisms for Microbial Pathogenesis, Biofilm Formation and Antimicrobial Drug Discovery.* Singapore: Springer Singapore**,** p. 175-194. https://doi.org/10.1007/978-981-15-1695-5_11.

Anjum, M. F., Zankari, E. & Hasman, H. 2018. Molecular Methods for Detection of Antimicrobial Resistance. *Antimicrobial Resistance in Bacteria from Livestock and Companion Animals.* p. 33-50. https://doi.org/10.1128/microbiolspec.ARBA-0011-2017.

Anjum, R., Grohmann, E. & Malik, A. 2011. Molecular characterization of conjugative plasmids in pesticide tolerant and multi-resistant bacterial isolates from contaminated alluvial soil. *Chemosphere,* 84 (1)**,** p. 175-181, https://doi.org/10.1016/j.chemosphere.2011.02.002.

Anokyewaa Appau, A. A. & Ofori, L. A. 2024. Antibiotic Resistance Profile of *E. coli* Isolates from Lettuce, Poultry Manure, Irrigation Water, and Soil in Kumasi, Ghana. *Int J Microbiol,* 2024**,** p. 6681311, https://doi.org/10.1155/2024/6681311.

Ardakani, Z., Canali, M., Aragrande, M., Tomassone, L., Simoes, M., Balzani, A. & Beber, C. L. 2023. Evaluating the contribution of antimicrobial use in farmed animals to global antimicrobial resistance in humans. *One Health,* 17**,** p. 100647, https://doi.org/10.1016/j.onehlt.2023.100647.

Aronson, J. K. 2016. Chloramphenicol. *Meyler's Side Effects of Drugs (Sixteenth Edition).* Oxford: Elsevier**,** p. 229-236. https://doi.org/10.1016/B978-0-444-53717-1.00472-8.

Arya, S., Williams, A., Reina, S. V., Knapp, C. W., Kreft, J. U., Hobman, J. L. & Stekel, D. J. 2021. Towards a general model for predicting minimal metal concentrations co-selecting for antibiotic resistance plasmids. *Environmental Pollution,* 275, https://doi.org/10.1016/j.envpol.2021.116602.

Aurilio, C., Sansone, P., Barbarisi, M., Pota, V., Giaccari, L. G., Coppolino, F., Barbarisi, A., Passavanti, M. B. & Pace, M. C. 2022. Mechanisms of Action of Carbapenem Resistance. *Antibiotics,* 11 (3)**,** p. 421, https://doi.org/10.3390/antibiotics11030421.

Babakhani, S. & Oloomi, M. 2018. Transposons: the agents of antibiotic resistance in bacteria. *Journal of Basic Microbiology,* 58 (11)**,** p. 905-917, https://doi.org/10.1002/jobm.201800204.

Back, S. H., Eom, H. S., Lee, H. H., Lee, G. Y., Park, K. T. & Yang, S. J. 2020. Livestock-associated methicillin-resistant *Staphylococcus aureus* in Korea: Antimicrobial resistance and molecular characteristics of LA-MRSA strains isolated from pigs, pig farmers, and farm environment. *Journal of Veterinary Science,* 21 (1), https://doi.org/10.4142/jvs.2020.21.e2.

Baker-Austin, C., Wright, M. S., Stepanauskas, R. & Mcarthur, J. V. 2006. Co-selection of antibiotic and metal resistance. *Trends in Microbiology,* 14 (4)**,** p. 176-182, https://doi.org/10.1016/j.tim.2006.02.006.

Baldiris, R., Acosta-Tapia, N., Montes, A., Hernández, J. & Vivas-Reyes, R. 2018. Reduction of Hexavalent Chromium and Detection of Chromate Reductase (ChrR) in *Stenotrophomonas maltophilia*. *Molecules,* 23 (2), https://doi.org/10.3390/molecules23020406.

Barman, H., Das, S. & Roy, A. 2018. Zinc in soil environment for plant health and management strategy. *Univers. J. Agric. Res,* 6 (5)**,** p. 149-154, https://doi.org/10.13189/ujar.2018.060501

Benjamin, A. B., Stunkard, L. M., Ling, J., Nice, J. N. & Lohman, J. R. 2023. Structures of chloramphenicol acetyltransferase III and *Escherichia coli* β-ketoacylsynthase III co-crystallized with partially hydrolysed acetyl-oxa (dethia) CoA. *Acta Crystallographica Section F: Structural Biology Communications,* 79 (3)**,** p. 61-69, https://doi.org/10.1107/S2053230X23001206.

Berg, J., Thorsen, M. K., Holm, P. E., Jensen, J., Nybroe, O. & Brandt, K. K. 2010. Cu exposure under field conditions coselects for antibiotic resistance as determined by a novel cultivation-independent bacterial community tolerance assay. *Environmental Science and Technology,* 44 (22)**,** p. 8724-8728, https://doi.org/10.1021/es101798r.

Berg, J., Tom-Petersen, A. & Nybroe, O. 2005. Copper amendment of agricultural soil selects for bacterial antibiotic resistance in the field. *Letters in Applied Microbiology,* 40 (2)**,** p. 146-151, https://doi.org/10.1111/j.1472-765X.2004.01650.x.

Beutin, L. & Strauch, E. 2007. Identification of Sequence Diversity in the *Escherichia coli* fliC Genes Encoding Flagellar Types H8 and H40 and Its Use in Typing of Shiga Toxin-Producing E. coli O8, O22, O111, O174, and O179 Strains. *Journal of Clinical Microbiology,* 45 (2)**,** p. 333-339, https://doi.org/10.1128/JCM.01627-06.

Bhat, B. A., Mir, R. A., Qadri, H., Dhiman, R., Almilaibary, A., Alkhanani, M. & Mir, M. A. 2023. Integrons in the development of antimicrobial resistance: critical review and perspectives. *Frontiers in Microbiology,* 14**,** p. 1231938, https://doi.org/10.3389/fmicb.2023.1231938.

Bhowmik, A., Shah, S. M. T., Goswami, S., Sirajee, A. S. & Ahsan, S. 2023. Predominance of Multidrug Resistant *Escherichia coli* of Environmental Phylotype in Different Environments of Dhaka, Bangladesh. *Trop Med Infect Dis,* 8 (4), https://doi.org/10.3390/tropicalmed8040226.

Bianconi, I., Aschbacher, R. & Pagani, E. 2023. Current Uses and Future Perspectives of Genomic Technologies in Clinical Microbiology. *Antibiotics,* 12 (11)**,** p. 1580, https://doi.org/10.3390/antibiotics12111580.

Bibi, M., Samiullah, Behlil, F., Afzal, S. & Sahifa 2023. Essential and non-essential heavy metals sources and impacts on human health and plants. *Pure and Applied Biology (PAB),* (2)**,** p. 835-847, http://dx.doi.org/10.19045/bspab.2023.120083.

Billington, C., Kingsbury, J. M. & Rivas, L. 2022. Metagenomics Approaches for Improving Food Safety: A Review. *J Food Prot,* 85 (3)**,** p. 448-464, https://doi.org/10.4315/jfp-21-301.

Blaak, H., Van Hoek, A. H. a. M., Veenman, C., Docters Van Leeuwen, A. E., Lynch, G., Van Overbeek, W. M. & De Roda Husman, A. M. 2014. Extended spectrum ß-lactamase- and constitutively AmpC-producing Enterobacteriaceae on fresh produce and in the agricultural environment. *International journal of food microbiology,* 168-169**,** p. 8-16, https://doi.org/10.1016/j.ijfoodmicro.2013.10.006.

Blair, J. M. A., Webber, M. A., Baylay, A. J., Ogbolu, D. O. & Piddock, L. J. V. 2015. Molecular mechanisms of antibiotic resistance. *Nature Reviews Microbiology,* 13 (1)**,** p. 42-51, https://doi.org/10.1038/nrmicro3380.

Bo, G. 2000. Giuseppe Brotzu and the discovery of cephalosporins. *Clinical Microbiology and Infection,* 6**,** p. 6-8, https://doi.org/10.1111/j.1469-0691.2000.tb02032.x.

Bolger, A. M., Lohse, M. & Usadel, B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics (Oxford, England),* 30 (15)**,** p. 2114-2120, https://doi.org/10.1093/bioinformatics/btu170.

Bonardi, S. & Pitino, R. 2019. Carbapenemase-producing bacteria in food-producing animals, wildlife and environment: A challenge for human health. *Ital J Food Saf,* 8 (2)**,** p. 7956, https://doi.org/10.4081/ijfs.2019.7956.

Boolchandani, M., D'souza, A. W. & Dantas, G. 2019. Sequencing-based methods and resources to study antimicrobial resistance. *Nature Reviews Genetics,* 20 (6)**,** p. 356-370, https://doi.org/10.1038/s41576-019-0108-4.

Boyd, S. E., Livermore, D. M., Hooper, D. C. & Hope, W. W. 2020. Metallo-β-Lactamases: Structure, Function, Epidemiology, Treatment Options, and the Development Pipeline. *Antimicrob Agents Chemother,* 64 (10)**,** p. 10-1128, https://doi.org/10.1128/aac.00397-20.

Brooks, B. D. & Brooks, A. E. 2014. Therapeutic strategies to combat antibiotic resistance. *Advanced Drug Delivery Reviews,* 78**,** p. 14-27, https://doi.org/10.1016/j.addr.2014.10.027.

BROWNE, M. A., SZPAK, M. & GALLAGHER, V. 2021. Tellus Geochemical Survey: deeper topsoil multi-element maps for the northern half of Ireland. *Geological Survey of Ireland*. https://gsi.geodata.gov.ie/downloads/Geochemistry/Reports/Tellus_S_Geochemistry_Soils_ICPar_50percent_Map_Booklet.pdf.

Bush, K. 2012. Antimicrobial agents targeting bacterial cell walls and cell membranes. *Rev Sci Tech,* 31 (1)**,** p. 43-56, https://doi.org/10.20506/rst.31.1.2096.

Bush, K. 2018. Past and Present Perspectives on β-Lactamases. *Antimicrob Agents Chemother,* 62 (10)**,** p. 10-1128, https://doi.org/10.1128/aac.01076-18.

Bush, K. 2023. Classification for β-lactamases: historical perspectives. *Expert Review of Anti-infective Therapy,* 21 (5)**,** p. 513-522, https://doi.org/10.1080/14787210.2023.2194633.

Bush, K. & Jacoby, G. A. 2010. Updated functional classification of beta-lactamases. *Antimicrob Agents Chemother,* 54 (3)**,** p. 969-76, https://doi.org/10.1128/aac.01009-09.

Buta, M., Korzeniewska, E., Harnisz, M., Hubeny, J., Zieliński, W., Rolbiecki, D., Bajkacz, S., Felis, E. & Kokoszka, K. 2021. Microbial and chemical pollutants on the manure-crops pathway in the perspective of "One Health" holistic approach. *Science of the Total Environment,* 785, 147411 https://doi.org/10.1016/j.scitotenv.2021.147411.

Cao, J., Yang, G., Mai, Q., Zhuang, Z. & Zhuang, L. 2020. Co-selection of antibiotic-resistant bacteria in a paddy soil exposed to As(III) contamination with an emphasis on potential pathogens. *Science of the Total Environment,* 725, 138367 https://doi.org/10.1016/j.scitotenv.2020.138367.

Carlet, J. 2012. The gut is the epicentre of antibiotic resistance. *Antimicrobial Resistance and Infection Control,* 1 (1)**,** p. 39, https://doi.org/10.1186/2047-2994-1-39.

Carlos, C., Pires, M. M., Stoppe, N. C., Hachich, E. M., Sato, M. I., Gomes, T. A., Amaral, L. A. & Ottoboni, L. M. 2010. *Escherichia coli* phylogenetic group determination and its application in the identification of the major animal source of fecal contamination. *BMC Microbiol,* 10**,** p. 1-10, https://doi.org/10.1186/1471-2180-10-161.

Carr, V. R., Pissis, S. P., Mullany, P., Shoaie, S., Gomez-Cabrero, D. & Moyes, D. L. 2023. Palidis: fast discovery of novel insertion sequences. *Microbial Genomics,* 9 (3)**,** p. 000917, https://doi.org/10.1099/mgen.0.000917.

Cella, E., Giovanetti, M., Benedetti, F., Scarpa, F., Johnston, C., Borsetti, A., Ceccarelli, G., Azarian, T., Zella, D. & Ciccozzi, M. 2023. Joining Forces against Antibiotic Resistance: The One Health Solution. *Pathogens,* 12 (9)**,** p. 1074, https://doi.org/10.3390/pathogens12091074.

Chalmers, G., Rozas, K. M., Amachawadi, R. G., Scott, H. M., Norman, K. N., Nagaraja, T. G., Tokach, M. D. & Boerlin, P. 2018. Distribution of the pco Gene Cluster and Associated Genetic Determinants among Swine Escherichia coli from a Controlled Feeding Trial. *Genes (Basel),* 9 (10)**,** p. 504, https://doi.org/10.3390/genes9100504.

Chapman, J. S. 2003. Disinfectant resistance mechanisms, cross-resistance, and co-resistance. *International Biodeterioration & Biodegradation,* 51 (4)**,** p. 271-276, https://doi.org/10.1016/S0964-8305(03)00044-1.

Chelaghma, W., Loucif, L., Bendahou, M. & Rolain, J.-M. 2021. Vegetables and Fruit as a Reservoir of β-Lactam and Colistin-Resistant Gram-Negative Bacteria: A Review. *Microorganisms (Basel),* 9 (12)**,** p. 2534, https://doi.org/10.3390/microorganisms9122534.

Chen, J., Li, J., Zhang, H., Shi, W. & Liu, Y. 2019. Bacterial Heavy-Metal and Antibiotic Resistance Genes in a Copper Tailing Dam Area in Northern China. *Front Microbiol,* 10**,** p. 1916, https://doi.org/10.3389/fmicb.2019.01916.

Chen, L., Yang, J., Yu, J., Yao, Z., Sun, L., Shen, Y. & Jin, Q. 2005. VFDB: a reference database for bacterial virulence factors. *Nucleic Acids Res,* 33**,** p. D325-8, https://doi.org/10.1093/nar/gki008.

Chen, R., Li, C., Ge, H., Qiao, J., Fang, L., Liu, C., Gou, J. & Guo, X. 2024. Difference analysis and characteristics of incompatibility group plasmid replicons in gram-negative bacteria with different antimicrobial phenotypes in Henan, China. *BMC Microbiology,* 24 (1)**,** p. 64, https://doi.org/10.1186/s12866-024-03212-9.

Chen, S., Li, X., Sun, G., Zhang, Y., Su, J. & Ye, J. 2015. Heavy metal induced antibiotic resistance in bacterium LSJC7. *International Journal of Molecular Sciences,* 16 (10)**,** p. 23390-23404, https://doi.org/10.3390/ijms161023390.

Cheng, J. H., Tang, X. Y., Guan, Z. & Liu, C. 2021. Occurrence of antibiotic resistome in farmland soils near phosphorus chemical industrial area. *Science of the Total Environment,* 796, 149053 https://doi.org/10.1016/j.scitotenv.2021.149053.

Chique, C., Hynds, P. D., Andrade, L., Burke, L., Morris, D., Ryan, M. P. & O'dwyer, J. 2020. Cryptosporidium spp. in groundwater supplies intended for human consumption – A descriptive review of global prevalence, risk factors and knowledge gaps. *Water Research,* 176**,** p. 115726, https://doi.org/10.1016/j.watres.2020.115726.

Choudhury, R. & Srivastava, S. 2001. Zinc resistance mechanisms in bacteria. *Current science (Bangalore),* 81 (7)**,** p. 768-775, https://www.jstor.org/stable/24106396.

Chowdhury, N., Suhani, S., Purkaystha, A., Begum, M. K., Raihan, T., Alam, M. J., Islam, K. & Azad, A. K. 2019. Identification of AcrAB-TolC Efflux Pump Genes and Detection of Mutation in Efflux Repressor AcrR from Omeprazole Responsive Multidrug-Resistant *Escherichia coli* Isolates Causing Urinary Tract Infections. *Microbiol Insights,* 12**,** p. 1178636119889629, https://doi.org/10.1177/1178636119889629.

Clark, A. E., Kaleta, E. J., Arora, A. & Wolk, D. M. 2013. Matrix-assisted laser desorption ionization-time of flight mass spectrometry: a fundamental shift in the routine practice of clinical microbiology. *Clin Microbiol Rev,* 26 (3)**,** p. 547-603, https://doi.org/10.1128/cmr.00072-12.

Clermont, O., Christenson, J. K., Denamur, E. & Gordon, D. M. 2013. The Clermont *Escherichia coli* phylo-typing method revisited: improvement of specificity and detection of new phylo-groups. *Environmental Microbiology Reports,* 5 (1)**,** p. 58-65, https://doi.org/10.1111/1758-2229.12019.

CLINICAL AND LABORATORY STANDARDS INSTITUTE (CLSI). 2020. Performance Standards for Antimicrobial Susceptibility Testing. 30th ed. CLSI supplement M100 Wayne, PA ClSI.

Colosi, I. A., Baciu, A. M., Opriș, R. V., Peca, L., Gudat, T., Simon, L. M., Colosi, H. A. & Costache, C. 2020. Prevalence of ESBL, AmpC and Carbapenemase-Producing Enterobacterales Isolated from Raw Vegetables Retailed in Romania. *Foods,* 9 (12)**,** p. 1726, https://doi.org/10.3390/foods9121726.

Committee to Study the Human Health Effects of Subtherapeutic Antibiotic Use in Animal Feeds 1980. *The Effects on Human Health of Subtherapeutic Use of Antimicrobials in Animal Feeds, 3044*, National Academies Press https://doi.org/10.17226/21.

Cottagnoud, P., Cottagnoud, M., Acosta, F. & Stucki, A. 2013. Efficacy of Ceftaroline Fosamil against Penicillin-Sensitive and -Resistant Streptococcus pneumoniae in an Experimental Rabbit Meningitis Model. *Antimicrobial*

Agents and Chemotherapy, 57 (10)**,** p. 4653-4655, https://doi.org/10.1128/aac.00286-13.

Croffie, M. E., Williams, P. N., Fenton, O., Fenelon, A., Metzger, K. & Daly, K. 2020. Optimising sample preparation and calibrations in EDXRF for quantitative soil analysis. *Agronomy,* 10 (9)**,** p. 1309, https://doi.org/10.3390/agronomy10091309.

Cui, X., Zhang, H. & Du, H. 2019. Carbapenemases in Enterobacteriaceae: Detection and Antimicrobial Therapy. *Frontiers in Microbiology,* 10**,** p. 1823, https://doi.org/10.3389/fmicb.2019.01823.

Cycoń, M., Mrozik, A. & Piotrowska-Seget, Z. 2019. Antibiotics in the soil environment degradation and their impact on microbial activity and diversity. *Frontiers in microbiology,* 10**,** p. 412419, https://doi.org/10.3389/fmicb.2019.00338.

D'costa, V. M., King, C. E., Kalan, L., Morar, M., Sung, W. W. L., Schwarz, C., Froese, D., Zazula, G., Calmels, F., Debruyne, R., Golding, G. B., Poinar, H. N. & Wright, G. D. 2011. Antibiotic resistance is ancient. *Nature,* 477 (7365)**,** p. 457-461, https://doi.org/10.1038/nature10388.

Dantas Palmeira, J. & Ferreira, H. M. N. 2020. Extended-spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae in cattle production a threat around the world. *Heliyon,* 6 (1)**,** p. e03206, https://doi.org/10.1016/j.heliyon.2020.e03206.

Darby, E. M., Trampari, E., Siasat, P., Gaya, M. S., Alav, I., Webber, M. A. & Blair, J. M. A. 2023. Molecular mechanisms of antibiotic resistance revisited. *Nature Reviews Microbiology,* 21 (5)**,** p. 280-295, https://doi.org/10.1038/s41579-022-00820-y.

Das, M., Ojha, A. K., Dolma, K. G., Majumdar, T., Sarmah, P., Hazarika, S., Modi, D., Gogoi, D., Das, S. & Ramamurthy, T. 2024. Monitoring the potential dissemination of antimicrobial resistance in foods, environment, and clinical samples: a one health prospective. *Food Science and Biotechnology***,** p. 1-11, https://doi.org/10.1007/s10068-024-01676-z.

Deekshit, V. K. & Srikumar, S. 2022. 'To be, or not to be'—The dilemma of 'silent' antimicrobial resistance genes in bacteria. *Journal of Applied Microbiology,* 133 (5)**,** p. 2902-2914, https://doi.org/10.1111/jam.15738.

Del Collo, L. P., Karns, J. S., Biswas, D., Lombard, J. E., Haley, B. J., Kristensen, R. C., Kopral, C. A., Fossler, C. P. & Van Kessel, J. a. S. 2017. Prevalence, antimicrobial resistance, and molecular characterization of Campylobacter spp. in bulk tank milk and milk filters from US dairies. *J Dairy Sci,* 100 (5)**,** p. 3470-3479, https://doi.org/10.3168/jds.2016-12084.

Delavat, F., Miyazaki, R., Carraro, N., Pradervand, N. & Van Der Meer, J. R. 2017. The hidden life of integrative and conjugative elements. *FEMS Microbiology Reviews,* 41 (4)**,** p. 512-537, https://doi.org/10.1093/femsre/fux008.

Dell'orco, F., Gusmara, C., Loiacono, M., Gugliotta, T., Albonico, F., Mortarino, M. & Zecconi, A. 2019. Evaluation of virulence factors profiles and antimicrobials resistance of *Escherichia coli* isolated from bulk tank milk and raw milk filters. *Res Vet Sci,* 123**,** p. 77-83, https://doi.org/10.1016/j.rvsc.2018.12.011.

Dewi, R. R., Hassan, L., Daud, H. M., Matori, M. F., Nordin, F., Ahmad, N. I. & Zakaria, Z. 2022. Prevalence and Antimicrobial Resistance of *Escherichia coli*, *Salmonella* and *Vibrio* Derived from Farm-Raised Red Hybrid Tilapia (Oreochromis spp.) and Asian Sea Bass (Lates calcarifer, Bloch 1970) on the

West Coast of Peninsular Malaysia. *Antibiotics (Basel),* 11 (2)**,** p. 136, https://doi.org/10.3390/antibiotics11020136.

Dhingra, S., Rahman, N. a. A., Peile, E., Rahman, M., Sartelli, M., Hassali, M. A., Islam, T., Islam, S. & Haque, M. 2020. Microbial Resistance Movements: An Overview of Global Public Health Threats Posed by Antimicrobial Resistance, and How Best to Counter. *Front Public Health,* 8**,** p. 535668, https://doi.org/10.3389/fpubh.2020.535668.

Di Cesare, A., Eckert, E. M. & Corno, G. 2016. Co-selection of antibiotic and heavy metal resistance in freshwater bacteria. *Journal of Limnology,* 75 (2S)**,** p. 59-66, https://doi.org/10.4081/jlimnol.2016.1198.

Di Martino, P. 2022. Antimicrobial agents and microbial ecology. *AIMS Microbiol,* 8 (1)**,** p. 1-4, https://doi.org/10.3934/microbiol.2022001.

Directive 86/278/EEC of 12 June 1986 on the protection of the environment, and in particular of the soil, when sewage sludge is used in agriculture. 1986. European Parliament. Available: http://data.europa.eu/eli/dir/1986/278/2022-01-01

Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed. 2002. European Parliament. Available: http://data.europa.eu/eli/dir/2002/32/2019-11-28

Directive 2009/48/EC of the european parliament and of the council of 18 June 2009 on the safety of toys. 2009. European Parliament. Available: https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:02009L0048-20181126

Do Nascimento, V., Day, M. R., Doumith, M., Hopkins, K. L., Woodford, N., Godbole, G. & Jenkins, C. 2017. Comparison of phenotypic and WGS-derived antimicrobial resistance profiles of enteroaggregative Escherichia coli isolated from cases of diarrhoeal disease in England, 2015-16. *J Antimicrob Chemother,* 72 (12)**,** p. 3288-3297, https://doi.org/10.1093/jac/dkx301.

Dorman, C. J. 2019. DNA supercoiling and transcription in bacteria: a two-way street. *BMC Mol Cell Biol,* 20 (1)**,** p. 26, https://doi.org/10.1186/s12860-019-0211-6.

Duan, M., Gu, J., Wang, X., Li, Y., Zhang, R., Hu, T. & Zhou, B. 2019. Factors that affect the occurrence and distribution of antibiotic resistance genes in soils from livestock and poultry farms. *Ecotoxicology and Environmental Safety,* 180**,** p. 114-122, https://doi.org/10.1016/j.ecoenv.2019.05.005.

Dumancas, G. G., Hikkaduwa Koralege, R. S., Mojica, E. R. E., Murdianti, B. S. & Pham, P. J. 2014. Penicillins. *In:* Wexler, P. (ed.) *Encyclopedia of Toxicology (Third Edition).* Oxford: Academic Press**,** p. 768-772. https://doi.org/10.1016/B978-0-12-386454-3.00764-8.

Duse, A., Persson Waller, K., Emanuelson, U., Ericsson Unnerstad, H., Persson, Y. & Bengtsson, B. 2016. Occurrence and Spread of Quinolone-Resistant *Escherichia coli* on Dairy Farms. *Appl Environ Microbiol,* 82 (13)**,** p. 3765-3773, https://doi.org/10.1128/aem.03061-15.

ECDC. 2022. Health burden of infections with antibioticresistant bacteria in the European Union and the European Economic Area, 2016-2020. *European Centre for Disease Prevention and Control* Stockholm: ECDC. Available: https://www.ecdc.europa.eu/en/publications-data/health-burden-infections-antibioticresistant-bacteria-2016-2020.

ECDC. 2023. Antimicrobial resistance in the EU/EEA (EARS-Net) - Annual Epidemiological Report 2022. *European Centre for Disease Prevention and Control.* Stockholm: ECDC. Available:

https://www.ecdc.europa.eu/en/about-us/networks/disease-networks-and-laboratory-networks/ears-net-data.

Edwin, N. R., Fitzpatrick, A. H., Brennan, F., Abram, F. & O'sullivan, O. 2024. An in-depth evaluation of metagenomic classifiers for soil microbiomes. *Environmental Microbiome,* 19 (1)**,** p. 19, https://doi.org/10.1186/s40793-024-00561-w.

Ekhlas, D., Cobo Díaz, J. F., Cabrera-Rubio, R., Alexa, E., Ortiz Sanjuán, J. M., Garcia Manzanilla, E., Crispie, F., Cotter, P. D., Leonard, F. C., Argüello, H. & Burgess, C. M. 2023a. Metagenomic comparison of the faecal and environmental resistome on Irish commercial pig farms with and without zinc oxide and antimicrobial usage. *Animal Microbiome,* 5 (1)**,** p. 62, https://doi.org/10.1186/s42523-023-00283-3.

Ekhlas, D., Sanjuán, J. M. O., Manzanilla, E. G., Leonard, F. C., Argüello, H. & Burgess, C. M. 2023b. Comparison of antimicrobial resistant *Escherichia coli* isolated from Irish commercial pig farms with and without zinc oxide and antimicrobial usage. *Gut pathogens,* 15 (1)**,** p. 8-8, https://doi.org/10.1186/s13099-023-00534-3.

Elshamy, A. A. & Aboshanab, K. M. 2020. A review on bacterial resistance to carbapenems: epidemiology, detection and treatment options. *Future Sci OA,* 6 (3)**,** p. Fso438, https://doi.org/10.2144/fsoa-2019-0098.

EMA. 2017. Questions and answers on veterinary medicinal products containing zinc oxide to be administered orally to food-producing species. *European Medicines Agency London, UK*. Available: https://www.ema.europa.eu/en/documents/referral/zinc-oxide-article-35-referral-questions-answers-veterinary-medicinal-products-containing-zinc-oxide_en.pdf

EMA. 2018. Reflection Paper on Antimicrobial Resistance in the Environment: Considerations for Current and Future Risk Assessment of Veterinary Medicinal Products Draft. Available: https://www.ema.europa.eu/en/documents/scientific-guideline/draft-reflection-paper-antimicrobial-resistance-environment-considerations-current-and-future-risk-assessment-veterinary-medicinal-products-first-version_en.pdf.

EMA. 2023. Scientific advice under Article 107(6) of Regulation (EU) 2019/6 for the establishment of a list of antimicrobials which shall not be used in accordance with Articles 112, 113 and 114 of the same Regulation or which shall only be used in accordance with these articles subject to certain conditions. *European Medicines Agency*. Available: https://www.ema.europa.eu/system/files/documents/regulatory-procedural-guideline/vet_reg_cascade_list_report_en.pdf.

Eren, A. M., Kiefl, E., Shaiber, A., Veseli, I., Miller, S. E., Schechter, M. S., Fink, I., Pan, J. N., Yousef, M. & Fogarty, E. C. 2021. Community-led, integrated, reproducible multi-omics with anvi'o. *Nature microbiology,* 6 (1)**,** p. 3-6, https://doi.org/10.1038/s41564-020-00834-3.

Escobar-PáRamo, P., Clermont, O., Blanc-Potard, A.-B. A., Bui, H., Le BouguéNec, C. & Denamur, E. 2004. A Specific Genetic Background Is Required for Acquisition and Expression of Virulence Factors in Escherichia coli. *Molecular Biology and Evolution,* 21 (6)**,** p. 1085-1094, https://doi.org/10.1093/molbev/msh118.

EUROPEAN COMMITTEE ON ANTIMICROBIAL SUSCEPTIBILITY TESTING (EUCAST). 2021. Breakpoint tables for interpretation of MICs and zone diameters. Version 11.0.

Ewels, P., Magnusson, M., Lundin, S. & Käller, M. 2016. MultiQC: summarize analysis results for multiple tools and samples in a single report. *Bioinformatics (Oxford, England),* 32 (19)**,** p. 3047-3048, https://doi.org/10.1093/bioinformatics/btw354.

Fàbrega, A., Madurga, S., Giralt, E. & Vila, J. 2009. Mechanism of action of and resistance to quinolones. *Microb Biotechnol,* 2 (1)**,** p. 40-61, https://doi.org/10.1111/j.1751-7915.2008.00063.x.

Fang, L., Li, X., Li, L., Li, S., Liao, X., Sun, J. & Liu, Y. 2016. Co-spread of metal and antibiotic resistance within ST3-IncHI2 plasmids from *E. coli* isolates of food-producing animals. *Scientific Reports,* 6 (1)**,** p. 25312, https://doi.org/10.1038/srep25312.

Farrell, M. L., Chueiri, A., O'connor, L., Duane, S., Maguire, M., Miliotis, G., Cormican, M., Hooban, B., Leonard, A., Gaze, W. H., Devane, G., Tuohy, A., Burke, L. P. & Morris, D. 2023. Assessing the impact of recreational water use on carriage of antimicrobial resistant organisms. *Science of The Total Environment,* 888**,** p. 164201, https://doi.org/10.1016/j.scitotenv.2023.164201.

Farrell, M. L., Joyce, A., Duane, S., Fitzhenry, K., Hooban, B., Burke, L. P. & Morris, D. 2021. Evaluating the potential for exposure to organisms of public health concern in naturally occurring bathing waters in Europe: A scoping review. *Water Research,* 206**,** p. 117711, https://doi.org/10.1016/j.watres.2021.117711.

Feldgarden, M., Brover, V., Haft Daniel, H., Prasad Arjun, B., Slotta Douglas, J., Tolstoy, I., Tyson Gregory, H., Zhao, S., Hsu, C.-H., Mcdermott Patrick, F., Tadesse Daniel, A., Morales, C., Simmons, M., Tillman, G., Wasilenko, J., Folster Jason, P. & Klimke, W. 2019. Validating the AMRFinder Tool and Resistance Gene Database by Using Antimicrobial Resistance Genotype-Phenotype Correlations in a Collection of Isolates. *Antimicrobial Agents and Chemotherapy,* 63 (11)**,** p. 10-1128, https://doi.org/10.1128/aac.00483-19.

Figueiredo, R., Card, R. M., Nunez-Garcia, J., Mendonça, N., Da Silva, G. J. & Anjum, M. F. 2019. Multidrug-resistant *Salmonella enterica* isolated from food animal and foodstuff may also be less susceptible to heavy metals. *Foodborne Pathogens and Disease,* 16 (3)**,** p. 166-172, https://doi.org/10.1089/fpd.2017.2418.

Fijalkowski, K., Rorat, A., Grobelak, A. & Kacprzak, M. J. 2017. The presence of contaminations in sewage sludge – The current situation. *Journal of Environmental Management,* 203**,** p. 1126-1136, https://doi.org/10.1016/j.jenvman.2017.05.068.

Fisher, J. F. & Mobashery, S. 2014. The sentinel role of peptidoglycan recycling in the β-lactam resistance of the Gram-negative Enterobacteriaceae and Pseudomonas aeruginosa. *Bioorg Chem,* 56**,** p. 41-8, https://doi.org/10.1016/j.bioorg.2014.05.011.

Flemming, H.-C. & Wingender, J. 2010. The biofilm matrix. *Nature Reviews Microbiology,* 8 (9)**,** p. 623-633, https://doi.org/10.1038/nrmicro2415.

Frost, L. S., Leplae, R., Summers, A. O. & Toussaint, A. 2005. Mobile genetic elements: the agents of open source evolution. *Nature Reviews Microbiology,* 3 (9)**,** p. 722-732, https://doi.org/10.1038/nrmicro1235.

Fu, Y., Zhu, Y., Dong, H., Li, J., Zhang, W., Shao, Y. & Shao, Y. 2023. Effects of heavy metals and antibiotics on antibiotic resistance genes and microbial communities in soil. *Process Safety and Environmental Protection,* 169**,** p. 418-427, https://doi.org/10.1016/j.psep.2022.11.020.

Fulham, M., Webster, B., Power, M. & Gray, R. 2022. Implications of *Escherichia col*i community diversity in free-ranging Australian pinniped pups. *Infection, Genetics and Evolution,* 104**,** p. 105351, https://doi.org/10.1016/j.meegid.2022.105351.

Furlan, J. P. R., Dos Santos, L. D. R., Moretto, J. a. S., Ramos, M. S., Gallo, I. F. L., Alves, G. D. a. D., Paulelli, A. C., Rocha, C. C. D. S., Cesila, C. A., Gallimberti, M., Devóz, P. P., Júnior, F. B. & Stehling, E. G. 2020. Occurrence and abundance of clinically relevant antimicrobial resistance genes in environmental samples after the Brumadinho dam disaster, Brazil. *Science of the Total Environment,* 726, 138100 https://doi.org/10.1016/j.scitotenv.2020.138100.

Gallo, I. F. L., Furlan, J. P. R., Sanchez, D. G. & Stehling, E. G. 2019. Heavy metal resistance genes and plasmid-mediated quinolone resistance genes in *Arthrobacter sp*. isolated from Brazilian soils. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology,* 112 (10)**,** p. 1553-1558, https://doi.org/10.1007/s10482-019-01281-9.

Gelalcha, B. D. & Kerro Dego, O. 2022. Extended-Spectrum Beta-Lactamases Producing Enterobacteriaceae in the USA Dairy Cattle Farms and Implications for Public Health. *Antibiotics (Basel),* 11 (10)**,** p. 1313, https://doi.org/10.3390/antibiotics11101313.

Giacometti, F., Serraino, A., Finazzi, G., Daminelli, P., Losio, M. N., Bonilauri, P., Arrigoni, N., Garigliani, A., Mattioli, R., Alonso, S., Piva, S., Florio, D., Riu, R. & Zanoni, R. G. 2012. Foodborne pathogens in in-line milk filters and associated on-farm risk factors in dairy farms authorized to produce and sell raw milk in northern Italy. *J Food Prot,* 75 (7)**,** p. 1263-9, https://doi.org/10.4315/0362-028x.Jfp-12-028.

Gillings, M. R., Gaze, W. H., Pruden, A., Smalla, K., Tiedje, J. M. & Zhu, Y. G. 2015. Using the class 1 integron-integrase gene as a proxy for anthropogenic pollution. *ISME Journal,* 9 (6)**,** p. 1269-1279, https://doi.org/10.1038/ismej.2014.226.

Glennon, M. M., Harris, P., Ottesen, R. T., Scanlon, R. P. & O'connor, P. J. 2014. The Dublin SURGE Project: geochemical baseline for heavy metals in topsoils and spatial correlation with historical industry in Dublin, Ireland. *Environ Geochem Health,* 36 (2)**,** p. 235-54, https://doi.org/10.1007/s10653-013-9561-8.

Glibota, N., Grande Burgos, M. J., Gálvez, A. & Ortega, E. 2019. Copper tolerance and antibiotic resistance in soil bacteria from olive tree agricultural fields routinely treated with copper compounds. *Journal of the Science of Food and Agriculture,* 99 (10)**,** p. 4677-4685, https://doi.org/10.1002/jsfa.9708.

Goswami, A., Adkins-Jablonsky, S. J., Barreto Filho, M. M., Shilling, M. D., Dawson, A., Heiser, S., O'connor, A., Walker, M., Roberts, Q. & Morris, J. J. 2023. Heavy Metal Pollution Impacts Soil Bacterial Community Structure and Antimicrobial Resistance at the Birmingham 35th Avenue Superfund Site. *Microbiol Spectr,* 11 (2)**,** p. e0242622, https://doi.org/10.1128/spectrum.02426-22.

Gothwal, R. & Thatikonda, S. 2017. Role of environmental pollution in prevalence of antibiotic resistant bacteria in aquatic environment of river: case of Musi

river, South India. *Water and Environment Journal,* 31 (4)**,** p. 456-462, https://doi.org/10.1111/wej.12263.

Gothwal, R. & Thatikonda, S. 2021. Modeling Fluoroquinolone Resistance in Polluted Aquatic Environment of a River. *Journal of Hazardous, Toxic, and Radioactive Waste,* 25 (2), 04020080 https://doi.org/10.1061/(ASCE)HZ.2153-5515.0000591.

Government of Ireland (2021). Ireland's One Health National Action Plan on Antimicrobial Resistance 2021–2025 (iNAP2). Available at: https://www.gov.ie/en/publication/d72f1-joint-action-on-antimicrobial-resistance

Grossman, T. H. 2016. Tetracycline Antibiotics and Resistance. *Cold Spring Harb Perspect Med,* 6 (4)**,** p. a025387, https://doi.org/10.1101/cshperspect.a025387.

Guo, H., Xue, S., Nasir, M., Gu, J. & Lv, J. 2021. Impacts of cadmium addition on the alteration of microbial community and transport of antibiotic resistance genes in oxytetracycline contaminated soil. *Journal of Environmental Sciences,* 99**,** p. 51-58, https://doi.org/10.1016/j.jes.2020.04.015.

Habib, S. S., Batool, A. I., Rehman, M. F. U. & Naz, S. 2023. Evaluation and Association of Heavy Metals in Commonly Used Fish Feed with Metals Concentration in Some Tissues of O. niloticus Cultured in Biofloc Technology and Earthen Pond System. *Biological Trace Element Research,* 201 (6)**,** p. 3006-3016, https://doi.org/10.1007/s12011-022-03379-0.

Halema, A. A., El-Beltagi, H. S., Al-Dossary, O., Alsubaie, B., Henawy, A. R., Rezk, A. A., Almutairi, H. H., Mohamed, A. A., Elarabi, N. I. & Abdelhadi, A. A. 2024. Omics technology draws a comprehensive heavy metal resistance strategy in bacteria. *World Journal of Microbiology and Biotechnology,* 40 (6)**,** p. 193, https://doi.org/10.1007/s11274-024-04005-y.

Hao, Y. L., Li, G., Xiao, Z. F., Liu, N., Azeem, M., Zhao, Y., Xu, Y. Y. & Yu, X. W. 2021. Distribution and influence on the microbial ecological relationship of antibiotic resistance genes in soil at a watershed scale. *Sustainability (Switzerland),* 13 (17), 9748 https://doi.org/10.3390/su13179748.

Hara, T., Takeda, T.-A., Takagishi, T., Fukue, K., Kambe, T. & Fukada, T. 2017. Physiological roles of zinc transporters: molecular and genetic importance in zinc homeostasis. *The Journal of Physiological Sciences,* 67 (2)**,** p. 283-301, https://doi.org/10.1007/s12576-017-0521-4.

Harrison, J. J., Tremaroli, V., Stan, M. A., Chan, C. S., Vacchi-Suzzi, C., Heyne, B. J., Parsek, M. R., Ceri, H. & Turner, R. J. 2009. Chromosomal antioxidant genes have metal ion-specific roles as determinants of bacterial metal tolerance. *Environmental Microbiology,* 11 (10)**,** p. 2491-2509, https://doi.org/10.1111/j.1462-2920.2009.01973.x.

Hasegawa, H., Suzuki, E. & Maeda, S. 2018. Horizontal Plasmid Transfer by Transformation in Escherichia coli: Environmental Factors and Possible Mechanisms. *Front Microbiol,* 9**,** p. 2365, https://doi.org/10.3389/fmicb.2018.02365.

Hasman, H. & Aarestrup, F. M. 2002. tcrB, a gene conferring transferable copper resistance in Enterococcus faecium: occurrence, transferability, and linkage to macrolide and glycopeptide resistance. *Antimicrob Agents Chemother,* 46 (5)**,** p. 1410-6, https://doi.org/10.1128/aac.46.5.1410-1416.2002.

Hasson, S. O., Al-Hamadani, A. H. & Al-Azawi, I. H. 2018. Occurrence of Biofilm Formation in Serratia fonticola and Pantoea sp. Isolates among Urinary

Catheterized Patients. *Nano Biomedicine and Engineering,* 10 (3)**,** p. 295-304, https://doi.org/10.5101/nbe.v10i3.p295-304.

Haulisah, N. A., Hassan, L., Bejo, S. K., Jajere, S. M. & Ahmad, N. I. 2021. High Levels of Antibiotic Resistance in Isolates From Diseased Livestock. *Frontiers in Veterinary Science,* 8**,** p. 652351, https://doi.org/10.3389/fvets.2021.652351.

HAYNES, E., RAMWELL, C., GRIFFITHS, T., WALKER, D., SMITH, J. 2020. Review of Antibiotic Use in Crops, Associated Risk of Antimicrobial Resistance and Research Gaps. . Prepared for Department for Environment, Food and Rural Affairs (Defra) & The Food Standards Agency (FSA). https://doi.org/10.46756/sci.fsa.vnq132.

He, X., Xu, Y., Chen, J., Ling, J., Li, Y., Huang, L., Zhou, X., Zheng, L. & Xie, G. 2017. Evolution of corresponding resistance genes in the water of fish tanks with multiple stresses of antibiotics and heavy metals. *Water Research,* 124**,** p. 39-48, https://doi.org/10.1016/j.watres.2017.07.048.

Health Protection Surveillance Centre (HPSC) (2023). Antimicrobial Resistance (EARS-Net) Data in Ireland – 2023. Available at: https://www.hpsc.ie/a-z/microbiologyantimicrobialresistance/europeanantimicrobialresistancesurveillancesystemearss/ears-netdataandreports/EARS-Net%20Report%202023.pdf.

Helgesen, E., Sætre, F. & Skarstad, K. 2021. Topoisomerase IV tracks behind the replication fork and the SeqA complex during DNA replication in *Escherichia coli*. *Scientific Reports,* 11 (1)**,** p. 474, https://doi.org/10.1038/s41598-020-80043-4.

Heydari, A., Kim, N. D., Horswell, J., Gielen, G., Siggins, A., Taylor, M., Bromhead, C. & Palmer, B. R. 2022. Co-Selection of Heavy Metal and Antibiotic Resistance in Soil Bacteria from Agricultural Soils in New Zealand. *Sustainability (Basel, Switzerland),* 14 (3)**,** p. 1790, https://doi.org/10.3390/su14031790.

Ho, M. X., Hudson, B. P., Das, K., Arnold, E. & Ebright, R. H. 2009. Structures of RNA polymerase-antibiotic complexes. *Curr Opin Struct Biol,* 19 (6)**,** p. 715-23, https://doi.org/10.1016/j.sbi.2009.10.010.

Holden, E. R. & Webber, M. A. 2020. MarA, RamA, and SoxS as Mediators of the Stress Response: Survival at a Cost. *Front Microbiol,* 11**,** p. 828, https://doi.org/10.3389/fmicb.2020.00828.

Hölzel, C. S., Tetens, J. L. & Schwaiger, K. 2018. Unraveling the Role of Vegetables in Spreading Antimicrobial-Resistant Bacteria: A Need for Quantitative Risk Assessment. *Foodborne Pathog Dis,* 15 (11)**,** p. 671-688, https://doi.org/10.1089/fpd.2018.2501.

Hooban, B., Fitzhenry, K., Cahill, N., Joyce, A., O' Connor, L., Bray, J. E., Brisse, S., Passet, V., Abbas Syed, R., Cormican, M. & Morris, D. 2021. A Point Prevalence Survey of Antibiotic Resistance in the Irish Environment, 2018–2019. *Environment International,* 152**,** p. 106466, https://doi.org/10.1016/j.envint.2021.106466.

Hooban, B., Joyce, A., Fitzhenry, K., Chique, C. & Morris, D. 2020. The role of the natural aquatic environment in the dissemination of extended spectrum beta-lactamase and carbapenemase encoding genes: A scoping review. https://doi.org/10.1016/j.watres.2020.115880.

Hood, M. I. & Skaar, E. P. 2012. Nutritional immunity: transition metals at the pathogen-host interface. *Nature reviews. Microbiology,* 10 (8)**,** p. 525-537, https://doi.org/10.1038/nrmicro2836.

Hu, H. W., Wang, J. T., Li, J., Li, J. J., Ma, Y. B., Chen, D. & He, J. Z. 2016. Field-based evidence for copper contamination induced changes of antibiotic resistance in agricultural soils. *Environmental Microbiology,* 18 (11)**,** p. 3896-3909, https://doi.org/10.1111/1462-2920.13370.

Hu, H. W., Wang, J. T., Li, J., Shi, X. Z., Ma, Y. B., Chen, D. & He, J. Z. 2017. Long-term nickel contamination increases the occurrence of antibiotic resistance genes in agricultural soils. *Environmental Science and Technology,* 51 (2)**,** p. 790-800, https://doi.org/10.1021/acs.est.6b03383.

Hu, Q., Dou, M.-N., Qi, H.-Y., Xie, X.-M., Zhuang, G.-Q. & Yang, M. 2007. Detection, isolation, and identification of cadmium-resistant bacteria based on PCR-DGGE. *Journal of Environmental Sciences,* 19 (9)**,** p. 1114-1119, https://doi.org/10.1016/S1001-0742(07)60181-8.

Huang, K., Wang, D., Frederiksen, R. F., Rensing, C., Olsen, J. E. & Fresno, A. H. 2017. Investigation of the Role of Genes Encoding Zinc Exporters zntA , zitB , and fieF during Salmonella Typhimurium Infection. *Frontiers in microbiology,* 8**,** p. 2656-2656, https://doi.org/10.3389/fmicb.2017.02656.

Huang, L., Wu, C., Gao, H., Xu, C., Dai, M., Huang, L., Hao, H., Wang, X. & Cheng, G. 2022. Bacterial Multidrug Efflux Pumps at the Frontline of Antimicrobial Resistance: An Overview. *Antibiotics (Basel),* 11 (4)**,** p. 520, https://doi.org/10.3390/antibiotics11040520.

Hubeny, J., Harnisz, M., Korzeniewska, E., Buta, M., Zieliński, W., Rolbiecki, D., Giebułtowicz, J., Nałęcz-Jawecki, G. & Płaza, G. 2021. Industrialization as a source of heavy metals and antibiotics which can enhance the antibiotic resistance in wastewater, sewage sludge and river water. *PLoS ONE,* 16 (6 June), e0252691 https://doi.org/10.1371/journal.pone.0252691.

Hudcová, H., Vymazal, J. & Rozkošný, M. 2019. Present restrictions of sewage sludge application in agriculture within the European Union. *Soil and Water Research,* 14 (2)**,** p. 104-120, https://doi.org/10.17221/36/2018-SWR.

Hülter, N., Ilhan, J., Wein, T., Kadibalban, A. S., Hammerschmidt, K. & Dagan, T. 2017. An evolutionary perspective on plasmid lifestyle modes. *Current Opinion in Microbiology,* 38**,** p. 74-80, https://doi.org/10.1016/j.mib.2017.05.001.

Igbinosa, E. O., Beshiru, A., Igbinosa, I. H., Cho, G. S. & Franz, C. 2023. Multidrug-resistant extended spectrum β-lactamase (ESBL)-producing *Escherichia coli* from farm produce and agricultural environments in Edo State, Nigeria. *PLoS One,* 18 (3)**,** p. e0282835, https://doi.org/10.1371/journal.pone.0282835.

Ingle, D. J., Valcanis, M., Kuzevski, A., Tauschek, M., Inouye, M., Stinear, T., Levine, M. M., Robins-Browne, R. M. & Holt, K. E. 2016. In silico serotyping of *E. coli* from short read data identifies limited novel O-loci but extensive diversity of O:H serotype combinations within and between pathogenic lineages. *Microb Genom,* 2 (7)**,** p. e000064, https://doi.org/10.1099/mgen.0.000064.

Iseppi, R., De Niederhäusern, S., Bondi, M., Messi, P. & Sabia, C. 2018. Extended-spectrum β-lactamase, AmpC, and MBL-producing gram-negative bacteria on fresh vegetables and ready-to-eat salads sold in local markets. *Microbial Drug Resistance,* 24 (8)**,** p. 1156-1164, https://doi.org/10.1089/mdr.2017.0198.

Islam, W., Noman, A., Naveed, H., Huang, Z. & Chen, H. Y. H. 2020. Role of environmental factors in shaping the soil microbiome. *Environmental*

Science and Pollution Research, 27 (33)**,** p. 41225-41247, https://doi.org/10.1007/s11356-020-10471-2.

Jabeen, I., Islam, S., Hassan, A. K. M. I., Tasnim, Z. & Shuvo, S. R. 2023. A brief insight into *Citrobacter* species - a growing threat to public health. *Frontiers in Antibiotics,* 2**,** p. 1276982, https://doi.org/10.3389/frabi.2023.1276982.

Jacoby, G. A. 2009. AmpC beta-lactamases. *Clin Microbiol Rev,* 22 (1)**,** p. 161-82, https://doi.org/10.1128/cmr.00036-08.

Jacoby, G. A., Strahilevitz, J. & Hooper, D. C. 2014. Plasmid-mediated quinolone resistance. *Microbiol Spectr,* 2 (5)**,** p. 475-503, https://doi.org/10.1128/microbiolspec.PLAS-0006-2013.

Jin, M., Yuan, H., Liu, B., Peng, J., Xu, L. & Yang, D. 2020. Review of the distribution and detection methods of heavy metals in the environment. *Analytical methods,* 12 (48)**,** p. 5747-5766, https://doi.org/10.1039/d0ay01577f.

Jones, I. A. & Joshi, L. T. 2021. Biocide Use in the Antimicrobial Era: A Review. *Molecules,* 26 (8)**,** p. 2276, https://doi.org/10.3390/molecules26082276.

Kaderabkova, N., Bharathwaj, M., Furniss, R. C. D., Gonzalez, D., Palmer, T. & Mavridou, D. a. I. 2022. The biogenesis of β-lactamase enzymes. *Microbiology,* 168 (8)**,** p. 001217, https://doi.org/10.1099/mic.0.001217.

Kampouris, I. D., Klümper, U., Kramer, L., Sorum, H., Wedekind, H. & Berendonk, T. U. 2022. Dissemination of antibiotic resistance in antibiotic-free recirculating aquaculture systems. *Journal of Hazardous Materials Advances,* 8**,** p. 100201, https://doi.org/10.1016/j.hazadv.2022.100201.

Kassambara, A. 2023. rstatix: Pipe-Friendly Framework for Basic Statistical Tests., https://doi.org/10.32614/CRAN.package.rstatix.

Kelbrick, M., Hesse, E. & S, O. B. 2023. Cultivating antimicrobial resistance: how intensive agriculture ploughs the way for antibiotic resistance. *Microbiology (Reading),* 169 (8)**,** p. 001384, https://doi.org/10.1099/mic.0.001384.

Kester, M., Karpa, K. D. & Vrana, K. E. 2012. Treatment of Infectious Diseases. *Elsevier's Integrated Review Pharmacology (Second Edition).* Philadelphia: W.B. Saunders**,** p. 41-78. https://doi.org/10.1016/B978-0-323-07445-2.00004-5.

Khan, S. R., Sharma, B., Chawla, P. A. & Bhatia, R. 2022. Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES): a Powerful Analytical Technique for Elemental Analysis. *Food Analytical Methods,* 15 (3)**,** p. 666-688, https://doi.org/10.1007/s12161-021-02148-4.

Khezri, A., Avershina, E. & Ahmad, R. 2020. Plasmid Identification and Plasmid-Mediated Antimicrobial Gene Detection in Norwegian Isolates. *Microorganisms (Basel),* 9 (1)**,** p. 52, https://doi.org/10.3390/microorganisms9010052.

Koh, E. I., Hung, C. S. & Henderson, J. P. 2016. The Yersiniabactin-Associated ATP Binding Cassette Proteins YbtP and YbtQ Enhance *Escherichia coli* Fitness during High-Titer Cystitis. *Infect Immun,* 84 (5)**,** p. 1312-1319, https://doi.org/10.1128/iai.01211-15.

Komijani, M., Shamabadi, N. S., Shahin, K., Eghbalpour, F., Tahsili, M. R. & Bahram, M. 2021. Heavy metal pollution promotes antibiotic resistance potential in the aquatic environment. *Environmental Pollution,* 274**,** p. 116569, https://doi.org/10.1016/j.envpol.2021.116569.

Koutsoumanis, K., Allende, A., Avelino, Á. O., Bolton, D., Sara, B. C., Chemaly, M., Davies, R., De Cesare, A., Herman, L., Hilbert, F., Lindqvist, R., Nauta, M., Ru, G., Simmons, M., Skandamis, P., Suffredini, E., Argüello, H., Berendonk,

T., Cavaco, L. M., Gaze, W., Schmitt, H., Topp, E., Guerra, B., Liébana, E., Stella, P. & Peixe, L. 2021. Role played by the environment in the emergence and spread of antimicrobial resistance (AMR) through the food chain. *EFSA Journal,* 19 (6), p. e06651, https://doi.org/10.2903/j.efsa.2021.6651.

Kumar, B., Lorusso, E., Fosso, B. & Pesole, G. 2024. A comprehensive overview of microbiome data in the light of machine learning applications: categorization, accessibility, and future directions. *Frontiers in Microbiology,* 15, p. 1343572, https://doi.org/10.3389/fmicb.2024.1343572.

Kumar, P. 2017. 33 - Pharmacology of Specific Drug Groups: Antibiotic Therapy. *Pharmacology and Therapeutics for Dentistry (Seventh Edition).* Mosby, p. 457-487. https://doi.org/10.1016/B978-0-323-39307-2.00033-3.

Laffite, A., Al Salah, D. M. M., Slaveykova, V. I., Otamonga, J.-P. & Pote, J. 2020. Impact of anthropogenic activities on the occurrence and distribution of toxic metals, extending-spectra beta-lactamases and carbapenem resistance in sub-Saharan African urban rivers. *Science of the Total Environment,* 727, p. 138129, https://doi.org/10.1016/j.scitotenv.2020.138129.

Lagerstrom, K. M. & Hadly, E. A. 2023. Under-Appreciated Phylogroup Diversity of *Escherichia coli* within and between Animals at the Urban-Wildland Interface. *Applied and Environmental Microbiology,* 89 (6), p. e00142-23, https://doi.org/10.1128/aem.00142-23.

Lan, Y., Liu, M., Song, Y., Cao, Y., Li, F., Luo, D. & Qiao, D. 2023. Distribution, characterization, and evolution of heavy metal resistance genes and Tn7-like associated heavy metal resistance Gene Island of Burkholderia. *Front Microbiol,* 14, p. 1252127, https://doi.org/10.3389/fmicb.2023.1252127.

Langmead, B. & Salzberg, S. L. 2012. Fast gapped-read alignment with Bowtie 2. *Nature Methods,* 9 (4), p. 357-359, https://doi.org/10.1038/nmeth.1923.

Larsson, D. G. J. & Flach, C.-F. 2022. Antibiotic resistance in the environment. *Nature Reviews Microbiology,* 20 (5), p. 257-269, https://doi.org/10.1038/s41579-021-00649-x.

Lee, L. J., Barrett, J. A. & Poole, R. K. 2005. Genome-Wide Transcriptional Response of Chemostat-Cultured *Escherichia coli* to Zinc. *Journal of Bacteriology,* 187 (3), p. 1124-1134, https://doi.org/10.1128/JB.187.3.1124-1134.2005.

Li, C., Li, M., Zeng, J., Yuan, S., Luo, X., Wu, C. & Xue, S. 2024. Migration and distribution characteristics of soil heavy metal(loid)s at a lead smelting site. *Journal of Environmental Sciences,* 135, p. 600-609, https://doi.org/10.1016/j.jes.2023.02.007.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G. & Durbin, R. 2009. The Sequence Alignment/Map format and SAMtools. *Bioinformatics,* 25 (16), p. 2078-9, https://doi.org/10.1093/bioinformatics/btp352.

Li, L. G., Xia, Y. & Zhang, T. 2017. Co-occurrence of antibiotic and metal resistance genes revealed in complete genome collection. *The Isme Journal,* 11 (3), p. 651-662, https://doi.org/10.1038/ismej.2016.155.

Li, M. C., Lu, J., Lu, Y., Xiao, T. Y., Liu, H. C., Lin, S. Q., Xu, D., Li, G. L., Zhao, X. Q., Liu, Z. G., Zhao, L. L. & Wan, K. L. 2021. rpoB Mutations and Effects on Rifampin Resistance in Mycobacterium tuberculosis. *Infect Drug Resist,* 14, p. 4119-4128, https://doi.org/10.2147/idr.S333433.

Li, N., Chen, J., Liu, C., Yang, J., Zhu, C. & Li, H. 2022. Cu and Zn exert a greater influence on antibiotic resistance and its transfer than doxycycline in agricultural soils. *Journal of hazardous materials,* 423 (Pt B)**,** p. 127042-127042, https://doi.org/10.1016/j.jhazmat.2021.127042.

Li, X., Gu, A. Z., Zhang, Y., Xie, B., Li, D. & Chen, J. 2019. Sub-lethal concentrations of heavy metals induce antibiotic resistance via mutagenesis. *Journal of Hazardous Materials,* 369**,** p. 9-16, https://doi.org/10.1016/j.jhazmat.2019.02.006.

Liakopoulos, A., Mevius, D. & Ceccarelli, D. 2016. A Review of SHV Extended-Spectrum β-Lactamases: Neglected Yet Ubiquitous. *Front Microbiol,* 7**,** p. 1374, https://doi.org/10.3389/fmicb.2016.01374.

Lin, X. & Kück, U. 2022. Cephalosporins as key lead generation beta-lactam antibiotics. *Applied Microbiology and Biotechnology,* 106 (24)**,** p. 8007-8020, https://doi.org/10.1007/s00253-022-12272-8.

Liu, C., Li, X., Zheng, S., Kai, Z., Jin, T., Shi, R., Huang, H. & Zheng, X. 2021. Effects of wastewater treatment and manure application on the dissemination of antimicrobial resistance around swine feedlots. *Journal of Cleaner Production,* 280, 123794 https://doi.org/10.1016/j.jclepro.2020.123794.

López-Jacome, L. E., Mercado-Casillas, Y. M., Méndez-Sotelo, B. J., Jiménez-Cortes, J. G., Tovar-García, A., Estrada-Velasco, A. Y., Almeida-Villegas, J. A., Martínez, J. D. P. & García-Contreras, R. 2022. Anti-Bacterial Agents. *In:* Rezaei, N. (ed.) *Encyclopedia of Infection and Immunity.* Oxford: Elsevier**,** p. 494-509. https://doi.org/10.1016/B978-0-12-818731-9.00186-5.

Lu, J., Breitwieser, F. P., Thielen, P. & Salzberg, S. L. 2017. Bracken: estimating species abundance in metagenomics data. *PeerJ Computer Science,* 3**,** p. e104, https://doi.org/10.7717/peerj-cs.104.

Lu, X. M. & Liu, X. P. 2021. Distribution of Metal Resistance Genes in Estuarine Sediments and Associated Key Impact Factors. *Microbial Ecology***,** p. 1-10, https://doi.org/10.1007/s00248-021-01699-7.

Lu, X. M. & Lu, P. Z. 2019. Synergistic effects of key parameters on the fate of antibiotic resistance genes during swine manure composting. *Environmental Pollution,* 252**,** p. 1277-1287, https://doi.org/10.1016/j.envpol.2019.06.073.

Lv, L., Huang, X., Wang, J., Huang, Y., Gao, X., Liu, Y., Zhou, Q., Zhang, Q., Yang, J., Guo, J. Y. & Liu, J. H. 2020. Multiple Plasmid Vectors Mediate the Spread of fosA3 in Extended-Spectrum-β-Lactamase-Producing Enterobacterales Isolates from Retail Vegetables in China. *mSphere,* 5 (4)**,** p. 10-1128, https://doi.org/10.1128/mSphere.00507-20.

Ma, F., Xu, S., Tang, Z., Li, Z. & Zhang, L. 2021. Use of antimicrobials in food animals and impact of transmission of antimicrobial resistance on humans. *Biosafety and Health,* 3 (1)**,** p. 32-38, https://doi.org/10.1016/j.bsheal.2020.09.004.

Maillard, J.-Y. & Pascoe, M. 2024. Disinfectants and antiseptics: mechanisms of action and resistance. *Nature Reviews Microbiology,* 22 (1)**,** p. 4-17, https://doi.org/10.1038/s41579-023-00958-3.

Majiduddin, F. K., Materon, I. C. & Palzkill, T. G. 2002. Molecular analysis of beta-lactamase structure and function. *Int J Med Microbiol,* 292 (2)**,** p. 127-37, https://doi.org/10.1078/1438-4221-00198.

Marazzato, M., Aleandri, M., Massaro, M. R., Vitanza, L., Conte, A. L., Conte, M. P., Nicoletti, M., Comanducci, A., Goldoni, P., Maurizi, L., Zagaglia, C. & Longhi, C. 2020. *Escherichia coli* strains of chicken and human origin:

Characterization of antibiotic and heavy-metal resistance profiles, phylogenetic grouping, and presence of virulence genetic markers. *Research in Veterinary Science,* 132**,** p. 150-155, https://doi.org/10.1016/j.rvsc.2020.06.012.

Marutescu, L. G., Jaga, M., Postolache, C., Barbuceanu, F., Milita, N. M., Romascu, L. M., Schmitt, H., De Roda Husman, A. M., Sefeedpari, P., Glaeser, S., Kämpfer, P., Boerlin, P., Topp, E., Gradisteanu Pircalabioru, G., Chifiriuc, M. C. & Popa, M. 2022. Insights into the impact of manure on the environmental antibiotic residues and resistance pool. *Front Microbiol,* 13**,** p. 965132, https://doi.org/10.3389/fmicb.2022.965132.

Mata, M. T., Baquero, F. & Pérez-Díaz, J. C. 2000. A multidrug efflux transporter in *Listeria monocytogenes*. *FEMS Microbiol Lett,* 187 (2)**,** p. 185-8, https://doi.org/10.1111/j.1574-6968.2000.tb09158.x.

Matsumoto, Y. & Inoue, M. 1999. Characterization of SFO-1, a Plasmid-Mediated Inducible Class A β-Lactamase from *Enterobacter cloacae*. *Antimicrobial Agents and Chemotherapy,* 43 (2)**,** p. 307-313, https://doi.org/10.1128/AAC.43.2.307.

Mazhar, S. H., Li, X., Rashid, A., Su, J., Xu, J., Brejnrod, A. D., Su, J. Q., Wu, Y., Zhu, Y. G., Zhou, S. G., Feng, R. & Rensing, C. 2021. Co-selection of antibiotic resistance genes, and mobile genetic elements in the presence of heavy metals in poultry farm environments. *Science of the Total Environment,* 755, 142702 https://doi.org/10.1016/j.scitotenv.2020.142702.

Mcgrath, D. & Fleming, G. A. 2007. *Trace Elements and Heavy Metals In Irish Soils,* Teagasc https://api.semanticscholar.org/CorpusID:130955650.

Mclaughlin, D., Bradley, A., Dottorini, T., Giebel, K., Leach, K., Hyde, R. & Green, M. 2022. Identifying associations between management practices and antimicrobial resistances of sentinel bacteria recovered from bulk tank milk on dairy farms. *Preventive Veterinary Medicine,* 204**,** p. 105666, https://doi.org/10.1016/j.prevetmed.2022.105666.

Meurs, L., Lempp, F. S., Lippmann, N., Trawinski, H., Rodloff, A. C., Eckardt, M., Klingeberg, A., Eckmanns, T., Walter, J., Lübbert, C., Sin, M. A., Antão, E.-M., Behnke, M., Bleidorn, J., Boklage, E., Eckmanns, T., Forstner, C., Gastmeier, P., Gensichen, J., Gropmann, A., Hagel, S., Hanke, R., Hanke, W., Klingeberg, A., Klimmek, L., Kraft, U., Kramer, T., Lehmkuhl, M., Ludwig, N., Lübke-Becker, A., Makarewicz, O., Moeser, A., Petruschke, I., Pletz, M. W., Remschmidt, C., Salm, F., Schmücker, K., Schneider, S., Schröder, C., Schwab, F., Trebbe, J., Vincze, S., Vollmar, H. C., Walter, J., Weis, S., Wetzker, W. & Wieler, L. H. 2020. Intestinal colonization with extended-spectrum beta-lactamase producing Enterobacterales (ESBL-PE) during long distance travel: A cohort study in a German travel clinic (2016–2017). *Travel medicine and infectious disease,* 33**,** p. 101521-101521, https://doi.org/10.1016/j.tmaid.2019.101521.

Mohapatra, S. S., Dwibedy, S. K. & Padhy, I. 2021. Polymyxins, the last-resort antibiotics: Mode of action, resistance emergence, and potential solutions. *J Biosci,* 46 (3)**,** p. 85, https://doi.org/10.1007/s12038-021-00209-8.

Moissenet, D., Salauze, B., Clermont, O., Bingen, E., Arlet, G., Denamur, E., Mérens, A., Mitanchez, D. & Vu-Thien, H. 2010. Meningitis caused by Escherichia coli producing TEM-52 extended-spectrum beta-lactamase within an extensive outbreak in a neonatal ward: epidemiological investigation and characterization of the strain. *J Clin Microbiol,* 48 (7)**,** p. 2459-63, https://doi.org/10.1128/jcm.00529-10.

Montero, L., Irazabal, J., Cardenas, P., Graham, J. P. & Trueba, G. 2021. Extended-Spectrum Beta-Lactamase Producing-*Escherichia coli* Isolated From Irrigation Waters and Produce in Ecuador. *Front Microbiol,* 12**,** p. 709418, https://doi.org/10.3389/fmicb.2021.709418.

Moon, S. H., Udaondo, Z., Abram, K. Z., Li, X., Yang, X., Dicaprio, E. L., Jun, S.-R. & Huang, E. 2022. Isolation of AmpC- and extended spectrum β-lactamase-producing Enterobacterales from fresh vegetables in the United States. *Food control,* 132**,** p. 108559, https://doi.org/10.1016/j.foodcont.2021.108559.

Moreno-Switt, A. I., Den Bakker, H. C., Cummings, C. A., Rodriguez-Rivera, L. D., Govoni, G., Raneiri, M. L., Degoricija, L., Brown, S., Hoelzer, K., Peters, J. E., Bolchacova, E., Furtado, M. R. & Wiedmann, M. 2012. Identification and characterization of novel *Salmonella* mobile elements involved in the dissemination of genes linked to virulence and transmission. *PLoS One,* 7 (7)**,** p. e41247, https://doi.org/10.1371/journal.pone.0041247.

Moscovici Joubran, A., Pierce, K. M., Garvey, N., Shalloo, L. & O'callaghan, T. F. 2021. Invited review: A 2020 perspective on pasture-based dairy systems and products. *Journal of Dairy Science,* 104 (7)**,** p. 7364-7382, https://doi.org/10.3168/jds.2020-19776.

Munita, J. M. & Arias, C. A. 2016. Mechanisms of Antibiotic Resistance. *Microbiol Spectr,* 4 (2)**,** p. 481-511, https://doi.org/10.1128/microbiolspec.VMBF-0016-2015.

Muntean, M., Muntean, A.-A., Preda, M., Manolescu, L., Dragomirescu, C., Popa, M.-I. & Popa, G. 2022. Phenotypic and genotypic detection methods for antimicrobial resistance in ESKAPE pathogens (Review). *Experimental and therapeutic medicine,* 24 (2)**,** p. 1-12, https://doi.org/10.3892/etm.2022.11435.

Murray, C. J., Ikuta, K. S., Sharara, F., Swetschinski, L., Aguilar, G. R., Gray, A., Han, C., Bisignano, C., Rao, P. & Wool, E. 2022. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. *The lancet,* 399 (10325)**,** p. 629-655, https://doi.org/10.1016/S0140-6736(21)02724-0.

Murray, L. M., Hayes, A., Snape, J., Kasprzyk-Hordern, B., Gaze, W. H. & Murray, A. K. 2024. Co-selection for antibiotic resistance by environmental contaminants. *npj Antimicrobials and Resistance,* 2 (1)**,** p. 9, https://doi.org/10.1038/s44259-024-00026-7.

Mustafa, G. R., Zhao, K., He, X., Chen, S., Liu, S., Mustafa, A., He, L., Yang, Y., Yu, X., Penttinen, P., Ao, X., Liu, A., Shabbir, M. Z., Xu, X. & Zou, L. 2021. Heavy Metal Resistance in *Salmonella* Typhimurium and Its Association With Disinfectant and Antibiotic Resistance. *Frontiers in Microbiology,* 12**,** p. 702725, https://doi.org/10.3389/fmicb.2021.702725.

Naas, T., Poirel, L. & Nordmann, P. 2008. Minor extended-spectrum β-lactamases. *Clinical Microbiology and Infection,* 14**,** p. 42-52, https://doi.org/10.1111/j.1469-0691.2007.01861.x.

Nag, R., O'rourke, S. M. & Cummins, E. 2022. A GIS study to rank Irish agricultural lands with background and anthropogenic concentrations of metal(loid)s in soil. *Chemosphere,* 286**,** p. 131928, https://doi.org/10.1016/j.chemosphere.2021.131928.

Neuert, S., Nair, S., Day, M. R., Doumith, M., Ashton, P. M., Mellor, K. C., Jenkins, C., Hopkins, K. L., Woodford, N., De Pinna, E., Godbole, G. & Dallman, T. J. 2018. Prediction of Phenotypic Antimicrobial Resistance Profiles From

Whole Genome Sequences of Non-typhoidal *Salmonella enterica*. *Frontiers in Microbiology,* 9**,** p. 592, https://doi.org/10.3389/fmicb.2018.00592.

Nguyen, T. H. T., Nguyen, H. D., Le, M. H., Nguyen, T. T. H., Nguyen, T. D., Nguyen, D. L., Nguyen, Q. H., Nguyen, T. K. O., Michalet, S., Dijoux-Franca, M. G. & Pham, H. N. 2023. Efflux Pump Inhibitors in Controlling Antibiotic Resistance: Outlook under a Heavy Metal Contamination Context. *Molecules,* 28 (7)**,** p. 2912, https://doi.org/10.3390/molecules28072912.

Nishino, K., Nikaido, E. & Yamaguchi, A. 2007. Regulation of Multidrug Efflux Systems Involved in Multidrug and Metal Resistance of *Salmonella enterica* Serovar Typhimurium. *Journal of Bacteriology,* 189 (24)**,** p. 9066-9075, https://doi.org/10.1128/JB.01045-07.

Nishino, K., Yamasaki, S., Nakashima, R., Zwama, M. & Hayashi-Nishino, M. 2021. Function and Inhibitory Mechanisms of Multidrug Efflux Pumps. *Frontiers in microbiology,* 12**,** p. 737288-737288, https://doi.org/10.3389/fmicb.2021.737288.

Nurk, S., Meleshko, D., Korobeynikov, A. & Pevzner, P. A. 2017. metaSPAdes: a new versatile metagenomic assembler. *Genome Res,* 27 (5)**,** p. 824-834, https://doi.org/10.1101/gr.213959.116.

O'neill, J. 2016. Tackling drug-resistant infections globally: final report and recommendations. https://doi.org/APO-63983.

Otinov, G. D., Lokteva, A. V., Petrova, A. D., Zinchenko, I. V., Isaeva, M. V., Kovtunov, E. A. & Koshel, E. I. 2020. Positive and negative effects of metal oxide nanoparticles on antibiotic resistance genes transfer. *Antibiotics,* 9 (11)**,** p. 1-9, 742 https://doi.org/10.3390/antibiotics9110742.

Ovung, A. & Bhattacharyya, J. 2021. Sulfonamide drugs: structure, antibacterial property, toxicity, and biophysical interactions. *Biophys Rev,* 13 (2)**,** p. 259-272, https://doi.org/10.1007/s12551-021-00795-9.

Pal, C., Asiani, K., Arya, S., Rensing, C., Stekel, D. J., Larsson, D. G. J. & Hobman, J. L. 2017. Chapter Seven - Metal Resistance and Its Association With Antibiotic Resistance. *Advances in Microbial Physiology.* Academic Press**,** p. 261-313. https://doi.org/10.1016/bs.ampbs.2017.02.001.

Pal, C., Bengtsson-Palme, J., Kristiansson, E. & Larsson, D. G. 2015. Co-occurrence of resistance genes to antibiotics, biocides and metals reveals novel insights into their co-selection potential. *BMC Genomics,* 16**,** p. 964, https://doi.org/10.1186/s12864-015-2153-5.

Pal, C., Bengtsson-Palme, J., Rensing, C., Kristiansson, E. & Larsson, D. G. J. 2014. BacMet: antibacterial biocide and metal resistance genes database. *Nucleic acids research,* 42**,** p. D737-D743, https://doi.org/10.1093/nar/gkt1252.

Pana, Z. D. & Zaoutis, T. 2018. Treatment of extended-spectrum β-lactamase-producing Enterobacteriaceae (ESBLs) infections: what have we learned until now? *F1000Res,* 7, https://doi.org/10.12688/f1000research.14822.1.

Papp-Wallace, K. M., Endimiani, A., Taracila, M. A. & Bonomo, R. A. 2011. Carbapenems: past, present, and future. *Antimicrob Agents Chemother,* 55 (11)**,** p. 4943-60, https://doi.org/10.1128/aac.00296-11.

Patel, N. P. & Malangoni, M. A. 2009. Antimicrobial Agents for Surgical Infections. *Surgical Clinics of North America,* 89 (2)**,** p. 327-347, https://doi.org/10.1016/j.suc.2008.09.005.

Peng, J.-J., Balasubramanian, B., Ming, Y.-Y., Niu, J.-L., Yi, C.-M., Ma, Y. & Liu, W.-C. 2021a. Identification of antimicrobial resistance genes and drug resistance analysis of *Escherichia coli* in the animal farm environment. *Journal of*

Infection and Public Health, 14 (12), p. 1788-1795, https://doi.org/10.1016/j.jiph.2021.10.025.

Peng, K., Li, Y., Wang, Q., Yang, P., Wang, Z. & Li, R. 2023. Integrative conjugative elements mediate the high prevalence of tmexCD3-toprJ1b in Proteus spp. of animal source. *mSystems,* 8 (5), p. e0042923, https://doi.org/10.1128/msystems.00429-23.

Peng, S., Zheng, H., Herrero-Fresno, A., Olsen, J. E., Dalsgaard, A. & Ding, Z. 2021b. Co-occurrence of antimicrobial and metal resistance genes in pig feces and agricultural fields fertilized with slurry. *Sci Total Environ,* 792, p. 148259, https://doi.org/10.1016/j.scitotenv.2021.148259.

Pereira-Marques, J., Hout, A., Ferreira, R. M., Weber, M., Pinto-Ribeiro, I., Van Doorn, L. J., Knetsch, C. W. & Figueiredo, C. 2019. Impact of Host DNA and Sequencing Depth on the Taxonomic Resolution of Whole Metagenome Sequencing for Microbiome Analysis. *Front Microbiol,* 10, p. 1277, https://doi.org/10.3389/fmicb.2019.01277.

Perron, K., Caille, O., Rossier, C., Van Delden, C., Dumas, J.-L. & Köhler, T. 2004. CzcR-CzcS, a Two-component System Involved in Heavy Metal and Carbapenem Resistance in *Pseudomonas aeruginosa**. *Journal of Biological Chemistry,* 279 (10), p. 8761-8768, https://doi.org/10.1074/jbc.M312080200.

Pintor-Cora, A., Álvaro-Llorente, L., Otero, A., Rodríguez-Calleja, J. M. & Santos, J. A. 2021. Extended-Spectrum Beta-Lactamase-Producing Enterobacteriaceae in Fresh Produce. *Foods,* 10 (11), p. 2609, https://doi.org/10.3390/foods10112609.

Plassard, V., Gisbert, P., Granier, S. A. & Millemann, Y. 2021. Surveillance of Extended-Spectrum β-Lactamase-, Cephalosporinase- and Carbapenemase-Producing Gram-Negative Bacteria in Raw Milk Filters and Healthy Dairy Cattle in Three Farms in Île-de-France, France. *Frontiers in Veterinary Science,* 8, p. 633598, https://doi.org/10.3389/fvets.2021.633598.

Pletzer, D. & Weingart, H. 2014. Characterization and regulation of the Resistance-Nodulation-Cell Division-type multidrug efflux pumps MdtABC and MdtUVW from the fire blight pathogen Erwinia amylovora. *BMC Microbiology,* 14 (1), p. 185, https://doi.org/10.1186/1471-2180-14-185.

Pokharel, S., Shrestha, P. & Adhikari, B. 2020. Antimicrobial use in food animals and human health: time to implement 'One Health' approach. *Antimicrob Resist Infect Control,* 9 (1), p. 181, https://doi.org/10.1186/s13756-020-00847-x.

Prjibelski, A., Antipov, D., Meleshko, D., Lapidus, A. & Korobeynikov, A. 2020. Using SPAdes de novo assembler. *Current protocols in bioinformatics,* 70 (1), p. e102, https://doi.org/10.1002/cpbi.102.

Pu, Q., Fan, X. T., Li, H., An, X. L., Lassen, S. B. & Su, J. Q. 2021a. Cadmium enhances conjugative plasmid transfer to a fresh water microbial community. *Environmental Pollution,* 268, 115903 https://doi.org/10.1016/j.envpol.2020.115903.

Pu, Q., Fan, X. T., Sun, A. Q., Pan, T., Li, H., Bo Lassen, S., An, X. L. & Su, J. Q. 2021b. Co-effect of cadmium and iron oxide nanoparticles on plasmid-mediated conjugative transfer of antibiotic resistance genes. *Environ Int,* 152, p. 106453, https://doi.org/10.1016/j.envint.2021.106453.

Puértolas-Balint, F., Warsi, O., Linkevicius, M., Tang, P.-C. & Andersson, D. I. 2019. Mutations that increase expression of the EmrAB-TolC efflux pump confer increased resistance to nitroxoline in *Escherichia coli*. *Journal of*

Antimicrobial Chemotherapy, 75 (2)**,** p. 300-308, https://doi.org/10.1093/jac/dkz434.

Qiao, L., Liu, X., Zhang, S., Zhang, L., Li, X., Hu, X., Zhao, Q., Wang, Q. & Yu, C. 2021. Distribution of the microbial community and antibiotic resistance genes in farmland surrounding gold tailings: A metagenomics approach. *Science of the Total Environment,* 779, https://doi.org/10.1016/j.scitotenv.2021.146502.

Quinlan, A. R. & Hall, I. M. 2010. BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics,* 26 (6)**,** p. 841-2, https://doi.org/10.1093/bioinformatics/btq033.

Rabow, S., Soares, M. & Rousk, J. 2023. Can heavy metal pollution induce soil bacterial community resistance to antibiotics in boreal forests? *Journal of Applied Ecology,* 60 (2)**,** p. 237-250, https://doi.org/10.1111/1365-2664.14322.

Ramatla, T., Mafokwane, T., Lekota, K., Monyama, M., Khasapane, G., Serage, N., Nkhebenyane, J., Bezuidenhout, C. & Thekisoe, O. 2023. "One Health" perspective on prevalence of co-existing extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* and *Klebsiella pneumoniae*: a comprehensive systematic review and meta-analysis. *Annals of Clinical Microbiology and Antimicrobials,* 22 (1)**,** p. 88, https://doi.org/10.1186/s12941-023-00638-3.

Ramnarine, S. D. B., Ali, O., Jayaraman, J. & Ramsubhag, A. 2024. Early transcriptional changes of heavy metal resistance and multiple efflux genes in *Xanthomonas campestris pv. campestris* under copper and heavy metal ion stress. *BMC Microbiology,* 24 (1)**,** p. 81, https://doi.org/10.1186/s12866-024-03206-7.

Raphael, E. & Riley, L. W. 2017. Infections Caused by Antimicrobial Drug-Resistant Saprophytic Gram-Negative Bacteria in the Environment. *Frontiers in Medicine,* 4**,** p. 183, https://doi.org/10.3389/fmed.2017.00183.

Rashid, A., Schutte, B. J., Ulery, A., Deyholos, M. K., Sanogo, S., Lehnhoff, E. A. & Beck, L. 2023. Heavy Metal Contamination in Agricultural Soil: Environmental Pollutants Affecting Crop Health. *Agronomy,* 13 (6)**,** p. 1521, https://doi.org/10.3390/agronomy13061521.

Rebelo, A., Almeida, A., Peixe, L., Antunes, P. & Novais, C. 2023. Unraveling the Role of Metals and Organic Acids in Bacterial Antimicrobial Resistance in the Food Chain. *Antibiotics,* 12 (9)**,** p. 1474, https://doi.org/10.3390/antibiotics12091474.

Regulation (EC) No 1223/2009 of the European Parliament and of the council of 30 November 2009 on cosmetic products. 2009. Council, E. P. A. Available: https://health.ec.europa.eu/system/files/2016-11/cosmetic_1223_2009_regulation_en_0.pdf

Regulation (EU) 2019/1009 of the European Parliament and of the Council of 5 June 2019 laying down rules on the making available on the market of EU fertilising products. 2019. Available: http://data.europa.eu/eli/reg/2019/1009/oj

Commission Regulation (EU) 2023/915 of 25 April 2023 on maximum levels for certain contaminants in food and repealing Regulation (EC) No 1881/2006. 2023. Available: http://data.europa.eu/eli/reg/2023/915/oj

Rensing, C., Moodley, A., Cavaco, L. M. & Mcdevitt, S. F. 2018. Resistance to Metals Used in Agricultural Production. *Antimicrobial Resistance in Bacteria from*

Livestock and Companion Animals. p. 83-107. https://doi.org/10.1128/9781555819804.ch5.

Reygaert, W. C. 2018. An overview of the antimicrobial resistance mechanisms of bacteria. *AIMS Microbiol,* 4 (3)**,** p. 482-501, https://doi.org/10.3934/microbiol.2018.3.482.

Richter, L., Du Plessis, E. M., Duvenage, S., Allam, M., Ismail, A. & Korsten, L. 2021. Whole Genome Sequencing of Extended-Spectrum- and AmpC- β-Lactamase-Positive Enterobacterales Isolated From Spinach Production in Gauteng Province, South Africa. *Frontiers in microbiology,* 12**,** p. 734649-734649, https://doi.org/10.3389/fmicb.2021.734649.

Richter, L., Du Plessis, E. M., Duvenage, S. & Korsten, L. 2020. Occurrence, Phenotypic and Molecular Characterization of Extended-Spectrum- and AmpC- β-Lactamase Producing Enterobacteriaceae Isolated From Selected Commercial Spinach Supply Chains in South Africa. *Frontiers in microbiology,* 11**,** p. 638-638, https://doi.org/10.3389/fmicb.2020.00638.

Roca, I., Akova, M., Baquero, F., Carlet, J., Cavaleri, M., Coenen, S., Cohen, J., Findlay, D., Gyssens, I., Heure, O. E., Kahlmeter, G., Kruse, H., Laxminarayan, R., Liébana, E., López-Cerero, L., Macgowan, A., Martins, M., Rodríguez-Baño, J., Rolain, J. M., Segovia, C., Sigauque, B., Tacconelli, E., Wellington, E. & Vila, J. 2015. The global threat of antimicrobial resistance: science for intervention. *New Microbes and New Infections,* 6**,** p. 22-29, https://doi.org/10.1016/j.nmni.2015.02.007.

Rout, G. R. & Das, P. 2009. Effect of metal toxicity on plant growth and metabolism: I. Zinc. *Sustainable agriculture***,** p. 873-884, https://doi.org/10.1051/agro:2002073

Rozwandowicz, M., Brouwer, M. S. M., Fischer, J., Wagenaar, J. A., Gonzalez-Zorn, B., Guerra, B., Mevius, D. J., Hordijk, J., Di, I, I., I, Wagenaar, L. S. K. O., Ls Iras Eepi, E. & Afdeling, I. 2018. Plasmids carrying antimicrobial resistance genes in Enterobacteriaceae. *Journal of antimicrobial chemotherapy,* 73 (5)**,** p. 1121-1137, https://doi.org/10.1093/jac/dkx488.

Rubiola, S., Macori, G., Chiesa, F., Panebianco, F., Moretti, R., Fanning, S. & Civera, T. 2022. Shotgun metagenomic sequencing of bulk tank milk filters reveals the role of Moraxellaceae and Enterobacteriaceae as carriers of antimicrobial resistance genes. *Food Res Int,* 158**,** p. 111579, https://doi.org/10.1016/j.foodres.2022.111579.

Ruiz, J. 2019. Transferable Mechanisms of Quinolone Resistance from 1998 Onward. *Clin Microbiol Rev,* 32 (4)**,** p. 10-1128, https://doi.org/10.1128/cmr.00007-19.

Rybak, B., Potrykus, M., Plenis, A. & Wolska, L. 2022. Raw Meat Contaminated with Cephalosporin-Resistant Enterobacterales as a Potential Source of Human Home Exposure to Multidrug-Resistant Bacteria. *Molecules,* 27 (13)**,** p. 4151, https://doi.org/10.3390/molecules27134151

Rybak, B., Wawrzyniak, N., Wolska, L. & Potrykus, M. 2021. *Escherichia coli* and *Serratia fonticola* ESBLs as a potential source of antibiotics resistance dissemination in the Tricity water reservoirs. *Acta Biochim Pol,* 68 (3)**,** p. 437-448, https://doi.org/10.18388/abp.2020_5740.

Safari Sinegani, A. A. & Younessi, N. 2017. Antibiotic resistance of bacteria isolated from heavy metal-polluted soils with different land uses. *Journal of Global Antimicrobial Resistance,* 10**,** p. 247-255, https://doi.org/10.1016/j.jgar.2017.05.012.

Samreen, Ahmad, I., Malak, H. A. & Abulreesh, H. H. 2021. Environmental antimicrobial resistance and its drivers: a potential threat to public health. *Journal of Global Antimicrobial Resistance,* 27**,** p. 101-111, https://doi.org/10.1016/j.jgar.2021.08.001.

Samtiya, M., Matthews, K. R., Dhewa, T. & Puniya, A. K. 2022. Antimicrobial Resistance in the Food Chain: Trends, Mechanisms, Pathways, and Possible Regulation Strategies. *Foods,* 11 (19)**,** p. 2966, https://doi.org/10.3390/foods11192966.

Sargeant, J. M. & O'connor, A. M. 2020. Scoping Reviews, Systematic Reviews, and Meta-Analysis: Applications in Veterinary Medicine. *Frontiers in Veterinary Science,* 7**,** p. 11, https://doi.org/10.3389/fvets.2020.00011.

Sauberan, J. B., Neely, M. N. & Bradley, J. S. 2023. Antibacterial Agents. *In:* Long, S. S. (ed.) *Principles and Practice of Pediatric Infectious Diseases (Sixth Edition).* Philadelphia: Elsevier**,** p. 1537-1563.e4. https://doi.org/10.1016/B978-0-323-75608-2.00292-5.

Sawa, T., Kooguchi, K. & Moriyama, K. 2020. Molecular diversity of extended-spectrum β-lactamases and carbapenemases, and antimicrobial resistance. *Journal of Intensive Care,* 8 (1)**,** p. 13, https://doi.org/10.1186/s40560-020-0429-6.

Sazykin, I., Khmelevtsova, L., Azhogina, T. & Sazykina, M. 2023. Heavy Metals Influence on the Bacterial Community of Soils: A Review. *Agriculture,* 13 (3)**,** p. 653, https://doi.org/10.3390/agriculture13030653

Schill, F., Abdulmawjood, A., Klein, G. & Reich, F. 2017. Prevalence and characterization of extended-spectrum β-lactamase (ESBL) and AmpC β-lactamase producing Enterobacteriaceae in fresh pork meat at processing level in Germany. *International journal of food microbiology,* 257**,** p. 58-66, https://doi.org/10.1016/j.ijfoodmicro.2017.06.010.

Schulz-Zunkel, C. & Krueger, F. 2009. Trace metal dynamics in floodplain soils of the river Elbe: a review. *J Environ Qual,* 38 (4)**,** p. 1349-62, https://doi.org/10.2134/jeq2008.0299.

Schwan, C. L., Lomonaco, S., Bastos, L. M., Cook, P. W., Maher, J., Trinetta, V., Bhullar, M., Phebus, R. K., Gragg, S., Kastner, J. & Vipham, J. L. 2021. Genotypic and Phenotypic Characterization of Antimicrobial Resistance Profiles in Non-typhoidal *Salmonella enterica* Strains Isolated From Cambodian Informal Markets. *Frontiers in Microbiology,* 12**,** p. 711472, https://doi.org/10.3389/fmicb.2021.711472.

Schwengers, O., Barth, P., Falgenhauer, L., Hain, T., Chakraborty, T. & Goesmann, A. 2020. Platon: identification and characterization of bacterial plasmid contigs in short-read draft assemblies exploiting protein sequence-based replicon distribution scores. *Microbial genomics,* 6 (10)**,** p. e000398, https://doi.org/10.1099/mgen.0.000398.

Seiler, C. & Berendonk, T. U. 2012. Heavy metal driven co-selection of antibiotic resistance in soil and water bodies impacted by agriculture and aquaculture. *Frontiers in Microbiology,* 3**,** p. 399, https://doi.org/10.3389/fmicb.2012.00399.

Sethuvel, D. P. M., Bakthavatchalam, Y. D., Karthik, M., Irulappan, M., Shrivastava, R., Periasamy, H. & Veeraraghavan, B. 2023. β-Lactam Resistance in ESKAPE Pathogens Mediated Through Modifications in Penicillin-Binding Proteins: An Overview. *Infect Dis Ther,* 12 (3)**,** p. 829-841, https://doi.org/10.1007/s40121-023-00771-8.

Shanmugakani, R. K., Srinivasan, B., Glesby, M. J., Westblade, L. F., Cárdenas, W. B., Raj, T., Erickson, D. & Mehta, S. 2020. Current state of the art in rapid diagnostics for antimicrobial resistance. *Lab on a Chip,* 20 (15)**,** p. 2607-2625, https://doi.org/10.1039/D0LC00034E.

Sharma, E., Chen, Y., Kelso, C., Sivakumar, M. & Jiang, G. 2024. Navigating the environmental impacts and analytical methods of last-resort antibiotics: Colistin and carbapenems. *Soil & Environmental Health,* 2 (1)**,** p. 100058, https://doi.org/10.1016/j.seh.2024.100058.

Shifaw, E. 2018. Review of Heavy Metals Pollution in China in Agricultural and Urban Soils. *Journal of health & pollution,* 8 (18)**,** p. 180607-180607, https://doi.org/10.5696/2156-9614-8.18.180607.

Siddiqui, M. T., Mondal, A. H., Gogry, F. A., Husain, F. M., Alsalme, A. & Rizwanul Haq, Q. M. 2020. Plasmid-mediated ampicillin, quinolone, and heavy metal co-resistance among esbl-producing isolates from the Yamuna River, New Delhi, India. *Antibiotics,* 9 (11)**,** p. 1-17, https://doi.org/10.3390/antibiotics9110826.

Siguier, P., Gourbeyre, E. & Chandler, M. 2014. Bacterial insertion sequences: their genomic impact and diversity. *FEMS Microbiol Rev,* 38 (5)**,** p. 865-91, https://doi.org/10.1111/1574-6976.12067.

Silva, I., Tacão, M. & Henriques, I. 2021. Selection of antibiotic resistance by metals in a riverine bacterial community. *Chemosphere,* 263, 127936 https://doi.org/10.1016/j.chemosphere.2020.127936.

Silver, S. & Phung, L. T. 1996. Bacterial heavy metal resistance: new surprises. *Annu Rev Microbiol,* 50**,** p. 753-89, https://doi.org/10.1146/annurev.micro.50.1.753.

Singh, A. S., Nayak, B. B. & Kumar, S. H. 2020. High Prevalence of Multiple Antibiotic-Resistant, Extended-Spectrum β-Lactamase (ESBL)-Producing *Escherichia coli* in Fresh Seafood Sold in Retail Markets of Mumbai, India. *Veterinary Sciences,* 7 (2)**,** p. 46, https://doi.org/10.3390/vetsci7020046.

Singh, C. K., Sodhi, K. K., Shree, P. & Nitin, V. 2024. Heavy Metals as Catalysts in the Evolution of Antimicrobial Resistance and the Mechanisms Underpinning Co-selection. *Current Microbiology,* 81 (6)**,** p. 148, https://doi.org/10.1007/s00284-024-03648-2.

Song, J., Rensing, C., Holm, P. E., Virta, M. & Brandt, K. K. 2017. Comparison of Metals and Tetracycline as Selective Agents for Development of Tetracycline Resistant Bacterial Communities in Agricultural Soil. *Environ Sci Technol,* 51 (5)**,** p. 3040-3047, https://doi.org/10.1021/acs.est.6b05342.

Spencer, A. C. & Panda, S. S. 2023. DNA Gyrase as a Target for Quinolones. *Biomedicines,* 11 (2)**,** p. 371, https://doi.org/10.3390/biomedicines11020371.

Squadrone, S. 2020. Water environments: metal-tolerant and antibiotic-resistant bacteria. *Environmental Monitoring and Assessment,* 192 (4)**,** p. 238, https://doi.org/10.1007/s10661-020-8191-8.

Srivastava, V., Sarkar, A., Singh, S., Singh, P., Ademir, S. F. D. A. & Singh, R. P. 2017. Agroecological Responses of Heavy Metal Pollution with Special Emphasis on Soil Health and Plant Performances. *Frontiers in Environmental Science,* 5**,** p. 74, https://doi.org/10.3389/fenvs.2017.00064.

Stanton, I. C., Bethel, A., Leonard, A. F. C., Gaze, W. H. & Garside, R. 2022. Existing evidence on antibiotic resistance exposure and transmission to humans

from the environment: a systematic map. *Environmental evidence,* 11 (1), p. 8-8, https://doi.org/10.1186/s13750-022-00262-2.

Strateva, T. & Yordanov, D. 2009. *Pseudomonas aeruginosa* – a phenomenon of bacterial resistance. *Journal of Medical Microbiology,* 58 (9), p. 1133-1148, https://doi.org/10.1099/jmm.0.009142-0.

Su, C., Meng, J., Zhou, Y., Bi, R., Chen, Z., Diao, J., Huang, Z., Kan, Z. & Wang, T. 2022. Heavy Metals in Soils From Intense Industrial Areas in South China: Spatial Distribution, Source Apportionment, and Risk Assessment. *Frontiers in environmental science,* 10, p. 820536, https://doi.org/10.3389/fenvs.2022.820536.

Sui, Q., Zhang, J., Chen, M., Wang, R., Wang, Y. & Wei, Y. 2019. Fate of microbial pollutants and evolution of antibiotic resistance in three types of soil amended with swine slurry. *Environmental Pollution,* 245, p. 353-362, https://doi.org/10.1016/j.envpol.2018.11.003.

Tamma, P. D., Doi, Y., Bonomo, R. A., Johnson, J. K. & Simner, P. J. 2019. A Primer on AmpC β-Lactamases: Necessary Knowledge for an Increasingly Multidrug-resistant World. *Clin Infect Dis,* 69 (8), p. 1446-1455, https://doi.org/10.1093/cid/ciz173.

Tanimoto, K., Nomura, T., Hashimoto, Y., Hirakawa, H., Watanabe, H. & Tomita, H. 2021. Isolation of Serratia fonticola Producing FONA, a Minor Extended-Spectrum β-Lactamase (ESBL), from Imported Chicken Meat in Japan. *Japanese journal of infectious diseases,* 74 (1), p. 79-81, https://doi.org/10.7883/yoken.JJID.2020.114.

Tate, H., Li, C., Nyirabahizi, E., Tyson, G. H., Zhao, S., Rice-Trujillo, C., Jones, S. B., Ayers, S., M'ikanatha, N. M., Hanna, S., Ruesch, L., Cavanaugh, M. E., Laksanalamai, P., Mingle, L., Matzinger, S. R. & Mcdermott, P. F. 2021. A National Antimicrobial Resistance Monitoring System Survey of Antimicrobial-Resistant Foodborne Bacteria Isolated from Retail Veal in the United States. *Journal of food protection,* 84 (10), p. 1749-1759, https://doi.org/10.4315/JFP-21-005.

Tchounwou, P. B., Yedjou, C. G., Patlolla, A. K. & Sutton, D. J. 2012. Heavy metal toxicity and the environment. *Exp Suppl,* 101, p. 133-64, https://doi.org/10.1007/978-3-7643-8340-4_6.

Thomsen, P. T., Sørensen, J. T. & Ersbøll, A. K. 2008. Evaluation of Three Commercial Hoof-Care Products Used in Footbaths in Danish Dairy Herds. *Journal of Dairy Science,* 91 (4), p. 1361-1365, https://doi.org/10.3168/jds.2007-0820.

Tiimub, B. M., Zhou, Z. C., Zhu, L., Liu, Y., Shuai, X. Y., Xu, L., Niyungeko, C., Meng, L. X., Sun, Y. J. & Chen, H. 2021. Characteristics of bacterial community and ARGs profile in engineered goldfish tanks with stresses of sulfanilamide and copper. *Environmental Science & Pollution Research,* 19, p. 19, https://doi.org/10.1007/s11356-021-13239-4.

Timlin, M., Fitzpatrick, E., Mccarthy, K., Tobin, J. T., Murphy, E. G., Pierce, K. M., Murphy, J. P., Hennessy, D., O'donovan, M., Harbourne, N., Brodkorb, A. & O'callaghan, T. F. 2023. Impact of varying levels of pasture allowance on the nutritional quality and functionality of milk throughout lactation. *Journal of Dairy Science,* 106 (10), p. 6597-6622, https://doi.org/10.3168/jds.2022-22921.

Tongyi, Y., Yanpeng, L., Xingang, W., Fen, Y., Jun, L. & Yubin, T. 2020. Co-selection for antibiotic resistance genes is induced in a soil amended with zinc. *Soil*

Use and Management, 36 (2), p. 328-337, https://doi.org/10.1111/sum.12545.

Tooke, C. L., Hinchliffe, P., Bragginton, E. C., Colenso, C. K., Hirvonen, V. H. A., Takebayashi, Y. & Spencer, J. 2019. β-Lactamases and β-Lactamase Inhibitors in the 21st Century. *J Mol Biol,* 431 (18), p. 3472-3500, https://doi.org/10.1016/j.jmb.2019.04.002.

Topal, E. I. A. & Elitok, Z. 2018. Seasonal monitoring of Cu and Zn in the sewage sludge of malatya advanced biological wastewater treatment plant. *International Journal of Pure and Applied Sciences,* 4 (1), p. 51-60, https://doi.org/10.29132/ijpas.365425.

Tuo, X., Gu, J., Wang, X., Sun, Y., Duan, M., Sun, W., Yin, Y., Guo, A. & Zhang, L. 2018. Prevalence of quinolone resistance genes, copper resistance genes, and the bacterial communities in a soil-ryegrass system co-polluted with copper and ciprofloxacin. *Chemosphere,* 197, p. 643-650, https://doi.org/10.1016/j.chemosphere.2018.01.033.

Turner, R. J., Huang, L. N., Viti, C. & Mengoni, A. 2020. Metal-Resistance in Bacteria: Why Care? *Genes (Basel),* 11 (12), p. 1470, https://doi.org/10.3390/genes11121470.

Uhlig, E., Kjellström, A., Oscarsson, E., Nurminen, N., Nabila, Y., Paulsson, J., Lupan, T., Velpuri, N. S. B. P., Molin, G. & Håkansson, Å. 2022. The live bacterial load and microbiota composition of prepacked “ready-to-eat” leafy greens during household conditions, with special reference to E. coli. *International journal of food microbiology,* 377, p. 109786-109786, https://doi.org/10.1016/j.ijfoodmicro.2022.109786.

Urra, J., Alkorta, I., Mijangos, I., Epelde, L. & Garbisu, C. 2019. Application of sewage sludge to agricultural soil increases the abundance of antibiotic resistance genes without altering the composition of prokaryotic communities. *The Science of the total environment,* 647, p. 1410-1420, https://doi.org/10.1016/j.scitotenv.2018.08.092.

Van Elsas, J. D., Semenov, A. V., Costa, R. & Trevors, J. T. 2011. Survival of *Escherichia coli* in the environment: fundamental and public health aspects. *The ISME Journal,* 5 (2), p. 173-183, https://doi.org/10.1038/ismej.2010.80.

Van Hoek, A. H. a. M., Veenman, C., Van Overbeek, W. M., Lynch, G., De Roda Husman, A. M. & Blaak, H. 2015. Prevalence and characterization of ESBL- and AmpC-producing Enterobacteriaceae on retail vegetables. *International journal of food microbiology,* 204, p. 1-8, https://doi.org/10.1016/j.ijfoodmicro.2015.03.014.

Van Kessel, J. a. S., Karns, J. S., Lombard, J. E. & Kopral, C. A. 2011. Prevalence of *Salmonella enterica*, *Listeria monocytogenes*, and *Escherichia coli* Virulence Factors in Bulk Tank Milk and In-Line Filters from U.S. Dairies†. *Journal of Food Protection,* 74 (5), p. 759-768, https://doi.org/10.4315/0362-028X.JFP-10-423.

Van Kessel, J. S., Karns, J. S., Wolfgang, D. R., Hovingh, E., Jayarao, B. M., Van Tassell, C. P. & Schukken, Y. H. 2008. Environmental sampling to predict fecal prevalence of *Salmonella* in an intensively monitored dairy herd. *J Food Prot,* 71 (10), p. 1967-73, https://doi.org/10.4315/0362-028x-71.10.1967.

Van Kessel, J. S., Sonnier, J., Zhao, S. & Karns, J. S. 2013. Antimicrobial resistance of *Salmonella enterica* isolates from bulk tank milk and milk filters in the United States. *J Food Prot,* 76 (1), p. 18-25, https://doi.org/10.4315/0362-028x.Jfp-12-263.

Vandorf, S., Shah, P. & Yost, C. N. 2024. Ertapenem Versus Meropenem for the Treatment of Extended Spectrum Beta-Lactamase-Producing Enterobacterales Bacteremia in Critically Ill Patients. *Annals of Pharmacotherapy,* 58 (7)**,** p. 690-697, https://doi.org/10.1177/10600280231205219.

Vats, P., Kaur, U. J. & Rishi, P. 2022. Heavy metal-induced selection and proliferation of antibiotic resistance: A review. *J Appl Microbiol,* 132 (6)**,** p. 4058-4076, https://doi.org/10.1111/jam.15492.

Velez, G. 2009. Inductively coupled plasma: The future of heavy metals testing. *Life Science. Technical Bulletin,* 7**,** p. 1-2.

Verhaegen, M., Bergot, T., Liebana, E., Stancanelli, G., Streissl, F., Mingeot-Leclercq, M.-P., Mahillon, J. & Bragard, C. 2023. On the use of antibiotics to control plant pathogenic bacteria: a genetic and genomic perspective. *Frontiers in Microbiology,* 14, https://doi.org/10.3389/fmicb.2023.1221478.

Wang, D. & Fierke, C. A. 2013. The BaeSR regulon is involved in defense against zinc toxicity in E. coli. *Metallomics,* 5 (4)**,** p. 372-383, https://doi.org/10.1039/c3mt20217h.

Wang, F., Zhang, W. & Niu, D. 2021a. Foodborne Enterobacteriaceae of Animal Origin. *Frontiers in Cellular and Infection Microbiology,* 11**,** p. 11:772359, https://doi.org/10.3389/fcimb.2021.772359.

Wang, Q., He, X., Xiong, H., Chen, Y. & Huang, L. 2022. Structure, mechanism, and toxicity in antibiotics metal complexation: Recent advances and perspectives. *Science of The Total Environment,* 848**,** p. 157778, https://doi.org/10.1016/j.scitotenv.2022.157778.

Wang, Q., Liu, L., Hou, Z., Wang, L., Ma, D., Yang, G., Guo, S., Luo, J., Qi, L. & Luo, Y. 2020. Heavy metal copper accelerates the conjugative transfer of antibiotic resistance genes in freshwater microcosms. *Science of the Total Environment,* 717, 137055 https://doi.org/10.1016/j.scitotenv.2020.137055.

Wang, X., Lan, B., Fei, H., Wang, S. & Zhu, G. 2021b. Heavy metal could drive co-selection of antibiotic resistance in terrestrial subsurface soils. *Journal of Hazardous Materials,* 411, 124848 https://doi.org/10.1016/j.jhazmat.2020.124848.

Wang, X., Yang, F., Zhao, J., Xu, Y., Mao, D., Zhu, X., Luo, Y. & Alvarez, P. J. J. 2018. Bacterial exposure to ZnO nanoparticles facilitates horizontal transfer of antibiotic resistance genes. *NanoImpact,* 10**,** p. 61-67, https://doi.org/10.1016/j.impact.2017.11.006.

Wang, X., Zhang, H., Yu, S., Li, D., Gillings, M. R., Ren, H., Mao, D., Guo, J. & Luo, Y. 2024. Inter-plasmid transfer of antibiotic resistance genes accelerates antibiotic resistance in bacterial pathogens. *Isme j,* 18 (1)**,** p. wrad032, https://doi.org/10.1093/ismejo/wrad032.

Waters, N. R., Abram, F., Brennan, F., Holmes, A. & Pritchard, L. 2020. Easy phylotyping of Escherichia coli via the EzClermont web app and command-line tool. *Access Microbiol,* 2 (9)**,** p. acmi000143, https://doi.org/10.1099/acmi.0.000143.

WHO. 2014a. Antimicrobial Resistance. Global Report on Surveillance : Global Report on Surveillance. *World Health Organization* Geneva, Switzerland. Available: http://ebookcentral.proquest.com/lib/nuig/detail.action?docID=1741841 https://apps.who.int/iris/handle/10665/112642.

WHO. 2014b. China: National action plan to contain antimicrobial resistance 2016-2020 (Chinese & English). *World Health Organization*. Available: https://www.who.int/publications/m/item/china-national-action-plan-to-contain-antimicrobial-resistance-(2016-2020).

WHO. 2015. Global action plan on antimicrobial resistance. *World Health Organization* Geneva. Available: https://www.who.int/publications/i/item/9789241509763.

WHO. 2019. Critically important antimicrobials for human medicine. *World Health Organization*. Available: https://www.who.int/publications/i/item/9789241515528.

WHO. 2020. Antimicrobial resistance. *World Health Organization*. Available: https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance.

WHO. 2022a. Antibiotics resistance. *World Health Organization*. Available: https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance.

WHO. 2022b. Global Antimicrobial Resistance and Use Surveillance System (GLASS) Report 2022. *World Health Organization*. https://www.who.int/publications/i/item/9789240062702.

WHO. 2024. Bacterial Priority Pathogens List, 2024: bacterial pathogens of public health importance to guide research, development and strategies to prevent and control antimicrobial resistance. *World Health Organization*. Available: https://www.who.int/publications/i/item/9789240093461.

Wickham, H. 2016. ggplot2: Elegant Graphics for Data Analysis. https://ggplot2.tidyverse.org.

Wilschefski, S. C. & Baxter, M. R. 2019. Inductively Coupled Plasma Mass Spectrometry: Introduction to Analytical Aspects. *Clin Biochem Rev,* 40 (3)**,** p. 115-133, https://doi.org/10.33176/aacb-19-00024.

Wilson, A., Fox, E. M., Fegan, N. & Ípek Kurtböke, D. 2019. Comparative Genomics and Phenotypic Investigations into Antibiotic, Heavy Metal, and Disinfectant Susceptibilities of Salmonella enterica Strains Isolated in Australia. *Frontiers in Microbiology,* 10 (JULY), 1620 https://doi.org/10.3389/fmicb.2019.01620.

Wong, M. H., Xie, M., Xie, L., Lin, D., Li, R., Zhou, Y., Chan, E. W. & Chen, S. 2016. Complete Sequence of a F33:A-:B- Conjugative Plasmid Carrying the oqxAB, fosA3, and bla(CTX-M-55) Elements from a Foodborne *Escherichia coli* Strain. *Front Microbiol,* 7**,** p. 1729, https://doi.org/10.3389/fmicb.2016.01729.

Wood, D. E., Lu, J. & Langmead, B. 2019. Improved metagenomic analysis with Kraken 2. *Genome Biology,* 20 (1)**,** p. 257, https://doi.org/10.1186/s13059-019-1891-0.

Wood, D. E. & Salzberg, S. L. 2014. Kraken: ultrafast metagenomic sequence classification using exact alignments. *Genome Biology,* 15 (3)**,** p. R46, https://doi.org/10.1186/gb-2014-15-3-r46.

Wu, C., Lin, C., Zhu, X., Liu, H., Zhou, W., Lu, J., Zhu, L., Bao, Q., Cheng, C. & Hu, Y. 2018. The β-Lactamase Gene Profile and a Plasmid-Carrying Multiple Heavy Metal Resistance Genes of *Enterobacter cloacae*. *International journal of genomics,* 2018**,** p. 1-12, https://doi.org/10.1155/2018/4989602.

Wu, G., Cao, W., Liu, L. & Wang, F. 2017. Water pollution management in China: recent incidents and proposed improvements. *Water Supply,* 18 (2)**,** p. 603-611, https://doi.org/10.2166/ws.2017.139.

Wu, N., Zhang, W., Xie, S., Zeng, M., Liu, H., Yang, J., Liu, X. & Yang, F. 2020. Increasing prevalence of antibiotic resistance genes in manured agricultural soils in northern China. *Frontiers of Environmental Science and Engineering,* 14 (1), 1 https://doi.org/10.1007/s11783-019-1180-x.

Wu, Y., Li, X., Yu, L., Wang, T., Wang, J. & Liu, T. 2022. Review of soil heavy metal pollution in China: Spatial distribution, primary sources, and remediation alternatives. *Resources, conservation and recycling,* 181**,** p. 106261, https://doi.org/10.1016/j.resconrec.2022.106261.

Wuana, R. A. & Okieimen, F. E. 2011. Heavy metals in contaminated soils: a review of sources, chemistry, risks and best available strategies for remediation. *International Scholarly Research Notices,* 1**,** p. 402647, https://doi.org/10.5402/2011/402647.

Xu, C., Kong, L., Liao, Y., Tian, Y., Wu, Q., Liu, H. & Wang, X. 2022. Mini-Review: Antibiotic-Resistant *Escherichia coli* from Farm Animal-Associated Sources. *Antibiotics (Basel),* 11 (11)**,** p. 1535, https://doi.org/10.3390/antibiotics11111535.

Xu, Y., Xu, J., Mao, D. & Luo, Y. 2017. Effect of the selective pressure of sub-lethal level of heavy metals on the fate and distribution of ARGs in the catchment scale. *Environmental Pollution,* 220**,** p. 900-908, https://doi.org/10.1016/j.envpol.2016.10.074.

Yamin, D., Uskoković, V., Wakil, A. M., Goni, M. D., Shamsuddin, S. H., Mustafa, F. H., Alfouzan, W. A., Alissa, M., Alshengeti, A., Almaghrabi, R. H., Fares, M. a. A., Garout, M., Al Kaabi, N. A., Alshehri, A. A., Ali, H. M., Rabaan, A. A., Aldubisi, F. A., Yean, C. Y. & Yusof, N. Y. 2023. Current and Future Technologies for the Detection of Antibiotic-Resistant Bacteria. *Diagnostics (Basel),* 13 (20)**,** p. 3246, https://doi.org/10.3390/diagnostics13203246.

Yang, Q., Sun, J., Li, L., Deng, H., Liu, B., Fang, L., Liao, X. & Liu, Y. 2015. IncF plasmid diversity in multi-drug resistant *Escherichia coli* strains from animals in China. *Frontiers in Microbiology,* 6**,** p. 964, https://doi.org/10.3389/fmicb.2015.00964.

Yassine, I., Rafei, R., Osman, M., Mallat, H., Dabboussi, F. & Hamze, M. 2019. Plasmid-mediated quinolone resistance: Mechanisms, detection, and epidemiology in the Arab countries. *Infection, Genetics and Evolution,* 76**,** p. 104020, https://doi.org/10.1016/j.meegid.2019.104020.

Yazdankhah, S., Skjerve, E. & Wasteson, Y. 2018. Antimicrobial resistance due to the content of potentially toxic metals in soil and fertilizing products. *Microbial Ecology in Health and Disease,* 29 (1)**,** p. 1548248, https://doi.org/10.1080/16512235.2018.1548248.

Younessi, N., Safari Sinegani, A. A. & Khodakaramian, G. 2020. Comparison of antibiotic resistance of coliforms and *Escherichia coli* strains in industrial and antimicrobial-free poultry manure. *Archives of Agronomy and Soil Science,* 68 (2)**,** p. 257-272, https://doi.org/10.1080/03650340.2020.1831692.

Yousefipour, M., Rasoulinejad, M., Hadadi, A., Esmailpour, N., Abdollahi, A., Jafari, S. & Khorsand, A. 2019. Bacteria Producing Extended Spectrum β-lactamases (ESBLs) in Hospitalized Patients: Prevalence, Antimicrobial Resistance Pattern and its Main Determinants. *Iran J Pathol,* 14 (1)**,** p. 61-67, https://doi.org/10.30699/ijp.14.1.61.

Yu, Z., Gunn, L., Wall, P. & Fanning, S. 2017. Antimicrobial resistance and its association with tolerance to heavy metals in agriculture production. *Food Microbiol,* 64**,** p. 23-32, https://doi.org/10.1016/j.fm.2016.12.009.

Yue, Z., Zhang, J., Zhou, Z., Ding, C., Wan, L., Liu, J., Chen, L. & Wang, X. 2021. Pollution characteristics of livestock faeces and the key driver of the spread of antibiotic resistance genes. *Journal of Hazardous Materials,* 409, 124957 https://doi.org/10.1016/j.jhazmat.2020.124957.

Zaheer, R., Noyes, N., Ortega Polo, R., Cook, S. R., Marinier, E., Van Domselaar, G., Belk, K. E., Morley, P. S. & Mcallister, T. A. 2018. Impact of sequencing depth on the characterization of the microbiome and resistome. *Scientific Reports,* 8 (1)**,** p. 5890, https://doi.org/10.1038/s41598-018-24280-8.

Zhang, B., Wang, M. M., Wang, B., Xin, Y., Gao, J. & Liu, H. 2018a. The effects of bio-available copper on macrolide antibiotic resistance genes and mobile elements during tylosin fermentation dregs co-composting. *Bioresource Technology,* 251**,** p. 230-237, https://doi.org/10.1016/j.biortech.2017.12.051.

Zhang, F., Zhao, X., Li, Q., Liu, J., Ding, J., Wu, H., Zhao, Z., Ba, Y., Cheng, X., Cui, L., Li, H. & Zhu, J. 2018b. Bacterial community structure and abundances of antibiotic resistance genes in heavy metals contaminated agricultural soil. *Environmental Science and Pollution Research,* 25 (10)**,** p. 9547-9555, https://doi.org/10.1007/s11356-018-1251-8.

Zhang, N., Juneau, P., Huang, R., He, Z., Sun, B., Zhou, J. & Liang, Y. 2021. Coexistence between antibiotic resistance genes and metal resistance genes in manure-fertilized soils. *Geoderma,* 382, 114760 https://doi.org/10.1016/j.geoderma.2020.114760.

Zhang, Y., Gu, A. Z., Cen, T., Li, X., He, M., Li, D. & Chen, J. 2018c. Sub-inhibitory concentrations of heavy metals facilitate the horizontal transfer of plasmid-mediated antibiotic resistance genes in water environment. *Environmental Pollution,* 237**,** p. 74-82, https://doi.org/10.1016/j.envpol.2018.01.032.

Zhao, X., Shen, J. P., Zhang, L. M., Du, S., Hu, H. W. & He, J. Z. 2020. Arsenic and cadmium as predominant factors shaping the distribution patterns of antibiotic resistance genes in polluted paddy soils. *Journal of Hazardous Materials,* 389, 121838 https://doi.org/10.1016/j.jhazmat.2019.121838.

Zhao, X., Wang, J., Zhu, L. & Wang, J. 2019a. Field-based evidence for enrichment of antibiotic resistance genes and mobile genetic elements in manure-amended vegetable soils. *Science of the Total Environment,* 654**,** p. 906-913, https://doi.org/10.1016/j.scitotenv.2018.10.446.

Zhao, Y., Cocerva, T., Cox, S., Tardif, S., Su, J. Q., Zhu, Y. G. & Brandt, K. K. 2019b. Evidence for co-selection of antibiotic resistance genes and mobile genetic elements in metal polluted urban soils. *Science of the Total Environment,* 656**,** p. 512-520, https://doi.org/10.1016/j.scitotenv.2018.11.372.

Zhao, Y., Hu, H.-W., Su, J.-Q., Hao, X., Guo, H., Liu, Y.-R. & Zhu, Y.-G. 2021. Influence of Legacy Mercury on Antibiotic Resistomes: Evidence from Agricultural Soils with Different Cropping Systems. *Environmental Science & Technology,* 55 (20)**,** p. 13913-13922, https://doi.org/10.1021/acs.est.1c04030.

Zhou, B., Wang, C., Zhao, Q., Wang, Y., Huo, M., Wang, J. & Wang, S. 2016. Prevalence and dissemination of antibiotic resistance genes and coselection of heavy metals in Chinese dairy farms. *Journal of Hazardous Materials,* 320**,** p. 10-17, https://doi.org/10.1016/j.jhazmat.2016.08.007.

Zhou, Y., Niu, L., Zhu, S., Lu, H. & Liu, W. 2017. Occurrence, abundance, and distribution of sulfonamide and tetracycline resistance genes in agricultural soils across China. *Science of the Total Environment,* 599-600**,** p. 1977-1983, https://doi.org/10.1016/j.scitotenv.2017.05.152.

Zhuang, M., Achmon, Y., Cao, Y., Liang, X., Chen, L., Wang, H., Siame, B. A. & Leung, K. Y. 2021. Distribution of antibiotic resistance genes in the environment. *Environmental pollution (1987),* 285**,** p. 117402-117402, https://doi.org/10.1016/j.envpol.2021.117402.

Zou, H. Y., He, L. Y., Gao, F. Z., Zhang, M., Chen, S., Wu, D. L., Liu, Y. S., He, L. X., Bai, H. & Ying, G. G. 2021. Antibiotic resistance genes in surface water and groundwater from mining affected environments. *Science of the Total Environment,* 772, 145516 https://doi.org/10.1016/j.scitotenv.2021.145516.

Dissemination of research

- Anedda, E., Morris, D., Burgess, C.M. A scoping review to evaluate the impact of heavy metals in the agri-food environment as selective pressure for the mobilisation of antimicrobial resistance. *OHEJP ASM 2021*. Copenhagen, Denmark. 9-11 June 2021.
- Anedda, E. Three Minutes Thesis. *OHEJP ASM 2021*. Copenhagen, Denmark. 9-11 June 2021.
- Anedda, E., Madigan, G., Morris, D., Burgess, C.M. Assessment of the presence of antimicrobial resistant bacteria in spinach and its production environment after zinc application. *OHEJP ASM 2022*. Orvieto, Italy. 11-13 April 2022
- Anedda, E. Three Minutes Thesis. *OHEJP ASM 2022*. Orvieto, Italy. 11-13 April 2022
- Anedda, E. Roundtable discussion on One Health EJP PhD projects. *OHEJP ASM 2022*. Orvieto, Italy. 11-13 April 2022
- Anedda, E., Madigan, G., Morris, D., & Burgess, C. Evaluating the impact of zinc application on the presence of antimicrobial resistance in spinach and its production environment. *ONE Conference 2022*. Brussels, Belgium. 21-24 June 2022
- Anedda, E. Teagasc Festival of Farming and Food. Teagasc Food Research Centre Ashtown, Dublin. 15 November 2022.
- Anedda, E. BTYSTE-BT Young Scientist & Technology Exhibition. Dublin, 12 January 2023.
- Anedda, E., Ekhlas, D., Alexa, E., Gaffney, M., Madigan, G., Burgess, C.M., Morris, D. Occurrence of AMR Enterobacterales in soil and spinach in the presence and absence of zinc amendment. *ENVIRON2023*. Letterkenny, Ireland. 3-4-5 April 2023

- Anedda, E. Symposium on *Listeria monocytogenes*. Teagasc Food Research Centre, Ashtown, Ireland. 24-25 May 2023.
- Anedda, E., Madigan, G., Croffie, M., Morris, D., Burgess, C.M. Assessment of antimicrobial resistant Enterobacterales from the dairy production environment in low and high zinc containing areas. *Antimicrobial resistance: a challenge for public health, animal health and the environment*. *AMCRA*. 27 June 2023.
- Anedda, E., Madigan, G., Croffie, M., Morris, D., Burgess, C.M. Assessment of antimicrobial resistant Enterobacterales from the dairy production environment in low and high zinc containing areas. *Antimicrobial Resistance in Animals and the Environment*, *ARAE 2023*. Tours, France. 3-5 July 2023.
- Anedda, E. Food Microbiology Conference. Trinity, Dublin, Ireland. 20 July 2023.
- Anedda, E. Last Meeting OHEJP. Paris, France. 11-12 September 2023.
- Anedda, E., Madigan, G., Croffie, M., Morris, D., Burgess, C.M. Assessment of antimicrobial resistant Enterobacterales from the dairy production environment in low and high zinc containing areas. *One Health Annual Conference*. Galway, Ireland. 12 October 2023
- Anedda, E. Teagasc Festival of Farming and Food. Teagasc Food Research Centre Ashtown, Dublin, 16 November 2023.
- Anedda, E., Madigan, G., Croffie, M., Morris, D., Burgess, C.M. Detection and characterisation of antimicrobial resistant Enterobacterales on dairy farms in low and high zinc containing areas. *World Antimicrobial Awareness Week Conference*. Maynooth, Ireland. 23 November 2023.

Awards received during this Ph.D. thesis

- Best Water Related Oral Presentation at Environ 2023.

Anedda, E., Ekhlas, D., Alexa, E., Gaffney, M., Madigan, G., Burgess, C.M., Morris, D. Occurrence of AMR Enterobacterales in soil and spinach in the presence and absence of zinc amendment. *ENVIRON2023*. Letterkenny, Ireland. 3-4-5 April 2023