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A Metagenomic Investigation of Antibiotic Resistance in Non-Clinical Environments

A Metagenomic Investigation of Antibiotic Resistance in Non-Clinical Environments

Dissertation

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by

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For my parents, who missed this adventure.

CONTENTS

SUMMARY

Antimicrobial resistance (AMR), termed a "silent pandemic" has caused 4.95 million deaths in 2019, with numbers expected to rise. AMR spans human, animal, and environmental sectors, requiring a One Health approach to address this multifaceted global challenge. This dissertation focuses on the under-represented non-clinical sectors and employs the use of metagenomic data to advance AMR research.

The primary focus in AMR research has been on clinical settings, overlooking animals and the environment and leaving data gaps in resource-limited regions. The world of AMR and metagenomic data is first introduced followed by an in-depth review of AMR in non-clinical sectors and the information metagenomic data can provide. The emphasis is on bioinformatic tools, databases, and workflows to support researchers utilising metagenomic data for AMR studies in these sectors.

Moving forward, the wastewater treatment process, including the neglected upstream and downstream freshwater systems, is examined, to assess the microbiome, resistome and mobilome at each stage. Specific differences within every wastewater treatment plant process sector and their role in AMR transmission are identified.

Inspired by the natural baseline of antibiotic resistance in soil, a comparative study of the composition of the microbiome, resistome and mobilome in different soil types, from natural to rural soils, is then further presented. Given the limited information on resistance patterns and the effects of geographical and anthropogenic factors, the influence of antibiotic resistance in different soil types is then further explored.

The swine industry, as the largest consumer of antibiotics, raises concerns about the effects of antibiotic use on the gut microbiome of animals. Antibiotics can impact animal health and promote the transmission of AMR to other non-clinical sectors and humans. How antibiotic use affects the fecal microbiome of pigs raised with and without antibiotics is examined to understand the dynamics of antibiotic resistance in the swine industry.

The burden of AMR, particularly in low- and middle-income countries, where resources for infectious disease surveillance are limited, was the inspiration to propose a method for generating metagenomic data in-field and in resource-limited settings, offering a cost-effective solution for outbreak monitoring and pathogen detection.

The main goal of this dissertation is to highlight the under-represented sectors, their significant role in AMR and to promote global inclusivity.

SAMENVATTING

Antimicrobiële resistentie (AMR), ook wel een "stille pandemie"genoemd, heeft in 2019 4,95 miljoen doden veroorzaakt, en de verwachting is dat dit aantal nog zal stijgen. AMR omvat de menselijke, dierlijke en milieusectoren, en vereist een “One Health”-benadering om deze veelzijdige wereldwijde uitdaging aan te pakken. Dit proefschrift richt zich op de ondervertegenwoordigde niet-klinische sectoren en maakt gebruik van metagenomische data om onderzoek naar AMR te bevorderen.

De primaire focus in AMR-onderzoek ligt voornamelijk in de klinische sector, waarbij dieren en het milieu over het hoofd worden gezien, en er datahiaten ontstaan in regio's met beperkte middelen. De wereld van AMR en metagenomische data worden eerst geïntroduceerd, gevolgd door een diepgaande beoordeling van AMR in niet-klinische sectoren en de informatie die metagenomische data kunnen bieden. De nadruk ligt op hulpmiddelen uit de bioinformatica, databases en workflows om onderzoekers te ondersteunen bij het gebruik van metagenomische data voor AMR-onderzoek in deze sectoren.

Hierna wordt het afvalwaterzuiveringsproces, inclusief de verwaarloosde upstream en downstream zoetwatersystemen, onderzocht om het microbioom, resistoom en mobiloom in elke fase te beoordelen. Specifieke verschillen binnen elke processector van de afvalwaterzuiveringsinstallatie en hun rol in AMR-transmissie worden hierbij geïdentificeerd.

Geïnspireerd door de natuurlijke basislijn van antibioticaresistentie in de bodem, wordt aan de hand van een vergelijkende studie van de samenstelling van het microbioom, resistoom en mobiloom in verschillende bodemtypen, van natuurlijke tot landelijke bodems gepresenteerd. Gezien de beperkte informatie over resistentiepatronen en de effecten van geografische en antropogene factoren, wordt de invloed van antibioticaresistentie in verschillende bodemtypen vervolgens verder onderzocht.

De varkensindustrie, als grootste consument van antibiotica, maakt zich zorgen over de effecten van antibioticagebruik op het darmmicrobioom van dieren. Antibiotica kunnen de gezondheid van dieren beïnvloeden en de transmissie van AMR naar andere niet-klinische sectoren en mensen bevorderen. Hoe antibioticagebruik het fecale microbioom van varkens, die met of zonder antibiotica zijn grootgebracht, beïnvloedt wordt onderzocht om de dynamiek van antibioticaresistentie in de varkensindustrie te begrijpen.

De last van AMR, met name in landen met lage en middeninkomens, waar de middelen voor het toezicht op infectieziekten beperkt zijn, was de inspiratie om een methode voor te stellen voor het genereren van metagenomische data in het veld en in omgevingen met beperkte middelen, om een kosteneffectieve oplossing te bieden voor het monitoren van uitbraken en het detecteren van pathogenen.

Het hoofddoel van dit proefschrift is om de ondervertegenwoordigde sectoren en hun belangrijke rol in AMR te bevorderen, en om wereldwijde inclusiviteit te benadrukken.

1

INTRODUCTION

1

1.1. ANTIMICROBIALS AND ANTIBIOTICS

Antimicrobials have revolutionised modern medicine by reducing infections and sepsis [1]. These antimicrobial agents kill or inhibit micro-organisms such as bacteria (antibiotics), viruses (antivirals), fungi (antifungals) and parasites (antiparasitics) and are used in clinical and non-clinical sectors [1, 2].

Antibiotics, the most well-known class of antimicrobials, was the greatest medical breakthrough of the 20th century. Since the discovery of penicillin, antibiotics played a critical role in treating common infections and making modern medical practices such as open-heart surgery and cancer treatment possible [3]. In the pre-antibiotic era, more than half of the deaths were associated with infections however, mortality rates decreased with antibiotics and infection control practices [4]. Antibiotics not only directly treat infections but also help prevent infections and their transmission between patients [4]. Due to its effectiveness and low cost, antibiotics have been used in veterinary medicine to treat and prevent infections and as a growth-promoting agent in fish farming, the food animal industry, aquaculture and agriculture [5, 6].

HOW DO ANTIBIOTICS WORK?

Antibiotics work by targeting specific features essential for the survival and reproduction of bacteria thereby inhibiting their growth or causing death (Figure

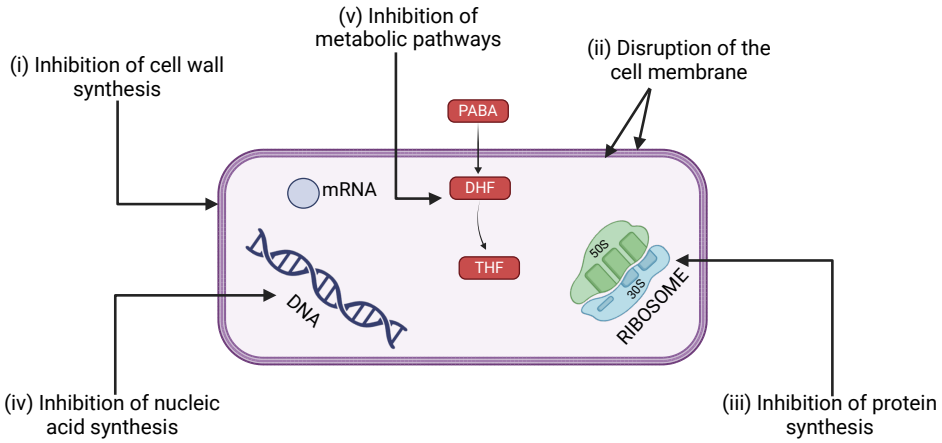

Figure 1.1: The mechanisms of action of antibiotics within the bacterial cell indicated in purple. Mechanisms include (i) the inhibition of cell wall synthesis (ii) disruption of the cell membrane (iii) inhibition of protein synthesis (iv) inhibition of nucleic acid synthesis and (v) inhibition of the metabolic pathways. (PABA = p-aminobenzoic acid; DHF = dihydrofolic acid; THF = tetrahydrofolic acid).

1.1) [7]. The mechanisms by which antibiotics work can be broadly categorised into different types: (i) the inhibition of the bacterial cell wall synthesis (beta-lactams, carbapenems and penicillin), (ii) the disruption of the cell membrane (lipopeptides), (iii) the inhibition of protein synthesis (macrolides, tetracycline and chloramphenicol), (iv) the inhibition of nucleic acid synthesis (fluoroquinolones) and (v) the inhibition of metabolic pathways (sulfonamide and trimethoprim) [7–9]. By targeting specific structures and functions within bacterial cells, antibiotics can effectively eliminate infections while minimising harm to the host.

1.2. ANTIBIOTIC RESISTANCE

As antibiotics became a mainstay of modern medicine, bacteria causing infections began to develop resistance, rendering antibiotics ineffective and leading to a global health crisis. In 2019, it was estimated that bacterial pathogens caused 7.7 million deaths globally with 1.3 million deaths directly associated with antibiotic resistance [10]. This number is predicted to increase to 10 million by 2050 and has been regarded as one of the leading causes of death worldwide, ahead of HIV/AIDS and malaria [10, 11]. This rising threat complicates infectious disease management, jeopardizes healthcare advancements, and leads to hard-to-treat infections, prolonged hospital stays, higher medical costs, and increased mortality rates [5, 12].

The increase in antibiotic resistance is attributed to many factors including the overuse and misuse of antibiotics in the clinical and non-clinical sectors [13]. In the clinical sector, the over-prescription of antibiotics, improper patient use, such as not completing prescribed courses, and the use of antibiotics when unnecessary are all factors. Additionally, in agriculture and the food animal industry, antibiotics have been employed as growth promoters, further fuelling this silent pandemic [5, 13].

1.2.1. MECHANISMS OF ANTIBIOTIC RESISTANCE

Antibiotic resistance can occur naturally, as some bacteria have antibiotic resistance genes (ARGs) embedded within their genomes (intrinsic resistance). Alternatively, bacteria can acquire ARGs through exposure to antibiotics (acquired resistance) [5, 14]. When expressed, these ARGs enable bacteria to employ various mechanisms to survive antibiotic treatment. As indicated in Figure 1.2, the most common resistance mechanisms include (i) the degradation/ modification of the antibiotic, (ii) the decreased uptake of antibiotics (iii) the use of efflux pumps to expel antibiotics out of the cell, (iv) the use of enzymes to inactive antibiotics (v) the acquisition of alternative enzymes to inhibit the effects of the antibiotics and (vi) the alteration of drug target [15–17].

INTRINSIC RESISTANCE

Natural resistance can be intrinsic, meaning the resistance genes are always expressed in the bacterial species, or it can be induced, where resistance genes naturally exist in bacteria but are only expressed following exposure to the antibiotic

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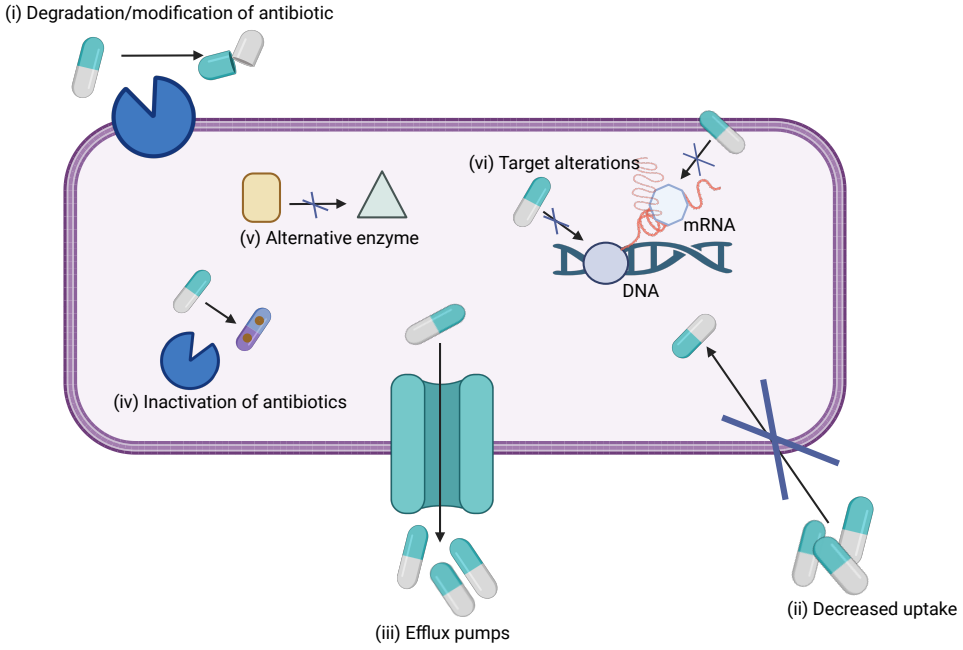

Figure 1.2: The common antibiotic resistance mechanisms in the bacterial cell (purple) include (i) the degradation/ modification of the antibiotic, (ii) the decreased uptake of antibiotics (iii) the use of efflux pumps to expel antibiotics out of the cell, (iv) the use of enzymes to inactive antibiotics (v) the acquisition of alternative enzymes to inhibit the effects of the antibiotics and (vi) the alteration of drug target.

[7, 9]. Intrinsic resistance is typically due to structural or functional characteristics of the bacteria that prevent the antibiotic from reaching its target or render the target inaccessible. This type of resistance is a part of the bacteria's normal physiology and is not acquired through genetic mutations or horizontal gene transfer [18]. It provides a baseline level of resistance that can be further enhanced through acquired resistance mechanisms [19]. Interestingly, bacteria present in soil are known to possess intrinsic resistance predating the clinical use of antibiotics [20]. While these bacteria may not pose an immediate threat to clinical or environmental health, their resistance genes could potentially be transferred to pathogenic bacteria, heightening the risk of a major health crisis [21].

ACQUIRED RESISTANCE

Acquired resistance occurs when bacteria develop the ability to withstand the effects of antibiotics through genetic changes. These changes can result from mutations in existing genes or the acquisition of new genes through horizontal gene transfer (HGT) [9, 14, 22]. Key mechanisms of acquired resistance through mutations include spontaneous mutations in the bacterial genome that alter the antibiotic's target site, thereby reducing the drug's binding affinity and effectiveness [23]. With HGT, bacteria can gain resistance genes from other bacteria via mobile genetic elements (MGEs) such as plasmids, transposons, and integrons. This transfer occurs via (i) conjugation— the transfer of plasmids containing resistance genes between bacteria through direct cell-to-cell contact; (ii) transformation—where a bacterium uptakes free DNA fragments from the environment, which may include resistance genes; and (iii) transduction— the transfer of resistance genes between bacteria via bacteriophages, which are viruses that infect bacteria (Figure 1.3) [24]. These processes enable resistance traits to spread swiftly within and across bacterial populations [17, 25]. They have been instrumental in the evolution of antibiotic resistant bacteria, significantly contributing to the global health crisis we are confronting today [7].

1.3. ANTIBIOTIC RESISTANCE AND ONE HEALTH

Antibiotic resistance is a One Health issue that recognises the health of humans, animals and the wider environment as interconnected and interdependent (Figure 1.4) [26]. In the context of antibiotic usage, one domain can be affected by another and vice versa indicating the need for integrated efforts across domains to combat antibiotic resistance. Given this interconnectedness, it is essential to consider how each domain contributes to and is affected by antibiotic resistance.

The One Health approach provides a comprehensive framework to address these challenges across human, animal, and environmental health [27]. In the clinical setting, the One Health approach advocates for the proper use of antibiotics, improved infection prevention and control measures, and robust surveillance systems to monitor and respond to antibiotic resistance trends [28–30]. Public health initiatives should aim to educate healthcare providers and the public about the responsible use of antibiotics. In the animal and agricultural sector, the One Health

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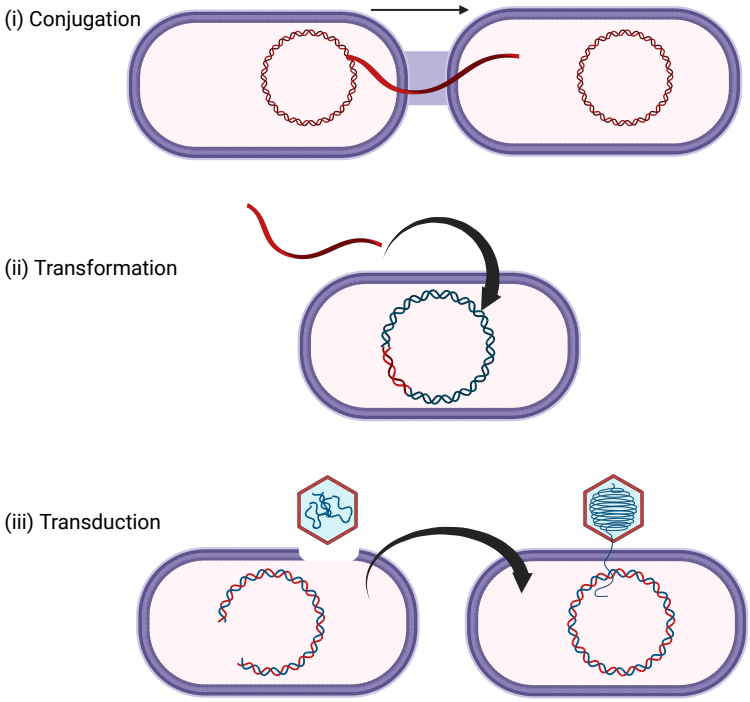

Figure 1.3: Horizontal gene transfer mechanisms ((i) conjugation: bacteria (purple) directly transfer genetic material (red) from one cell to another bacterial cell, (ii) transformation: bacteria (purple) take up DNA (red) from the environment and is incorporated into the existing genetic material and (iii) transduction: bacteriophages (brown hexagon with blue DNA inside) move genes (red and blue DNA strand) from one bacteria to another.

approach promotes the proper use of antibiotics, emphasising alternatives such as vaccination for animals, improved hygiene in farms, and bio-security measures [30]. Reducing the use of antibiotics as growth promoters is also a critical step in mitigating antibiotic resistance [29]. Overall, the One Health approach calls for better waste management practices, environmental monitoring, and policies to reduce the environmental impact of antibiotics [31].

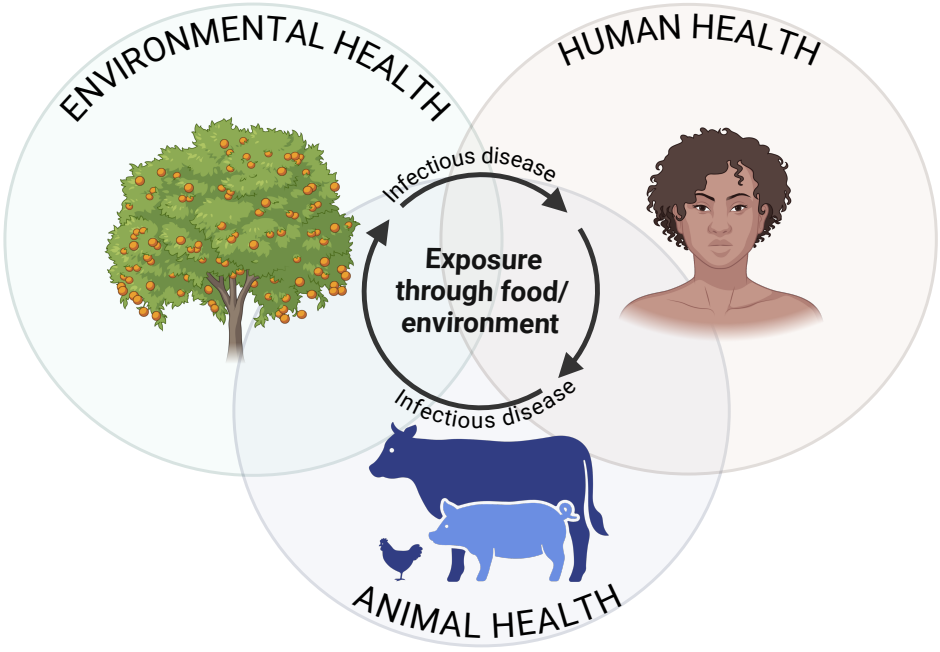

Figure 1.4: The One Health approach to antibiotic resistance. Environmental, human and animal health are interconnected spreading infectious diseases through exposure.

The interconnectedness of the human, animal, and environmental sectors is evident in how bacteria can spread across these domains, facilitating the transmission of antibiotic resistance and infectious diseases [30]. For instance, the use of antibiotics in livestock can lead to the development of resistant bacteria in animals, which can then spread to humans through direct contact, consumption of animal products, or environmental pathways such as water and soil contamination. Similarly, antibiotic resistant bacteria in the environment, originating from sources such as wastewater or agricultural run-off, can enter human and animal populations, complicating efforts to control infectious diseases [32–34]. By adopting a One Health approach, researchers and policymakers can better understand the pathways through which antibiotic resistance spreads.

1.4. ANTIBIOTIC RESISTANCE IN NON-CLINICAL SECTORS

Antibiotic resistance has become a significant concern in non-clinical environments such as agriculture, the food industry, and the broader environment [35]. These areas serve as crucial reservoirs for antibiotic resistant bacteria, ARGs and MGEs which contribute to the global crisis. The widespread use of antibiotics in livestock farming, aquaculture, and plant agriculture promotes the development and spread of resistant bacteria, which can enter the human food chain and environment through various pathways similar to those described above [36].

Non-clinical environments, including water, soil, and animals, are hotspots for the exchange of resistance genes among bacteria, facilitated by mechanisms like horizontal gene transfer [37]. This exchange allows resistance to spread across different bacterial species and even to human pathogens. Addressing antibiotic resistance requires coordinated efforts across sectors to mitigate its emergence and spread beyond clinical settings [30]. Details of the usage and effects of antibiotics in different non-clinical sectors can be found in **Chapter 2**.

1.5. USING METAGENOMICS TO STUDY ANTIBIOTIC RESISTANCE IN NON-CLINICAL SECTORS

In recent years, metagenomics has become a popular technique used for studying antibiotic resistance in different sectors. Briefly defined, it is the study of complex microbial communities directly from their natural environments, without the need for culturing individual species [38, 39]. Traditional methods often involves isolating and culturing bacteria. This process, while efficient and informative, is limited as a vast majority cannot be cultured under standard laboratory conditions [40]. Metagenomics bypasses the culturing step and directly examines the DNAs present in a particular sample of interest [39–42]. Metagenomic DNA is typically obtained by first extracting the DNA from a sample, either water, soil or animal feces, using a commercially available kit followed by sequencing using next-generation (Illumina) or third-generation (Oxford Nanopore or Pacbio) sequencing technologies [41]. By sequencing the DNA from all the bacterial micro-organisms in these environments, we gain information about the genetic diversity, type and abundance of species, the metabolic capacity and the functional potential of the micro-organisms [41]. This approach helps answer key questions such as "Who is there?" and "What are they doing?"

To interpret this data, bioinformatic tools, databases, and online platforms can be used to analyse the DNA. A detailed review of the various databases, platforms and tools as well as workflow can be found in **Chapter 2**. However, we outline a general workflow below.

Studying antibiotic resistance in this manner typically involves several key steps: sampling, DNA extraction, sequencing, and alignment (Figure 1.5) [43, 44]. Firstly, once DNA is extracted and metagenomic sequences are generated and quality controlled i.e., the removal of any low-quality reads, taxonomic classification can begin [45]. Sequences can be aligned to specific reference genomes or taxonomic databases that contain the genomes of all or most bacterial species. This identifies

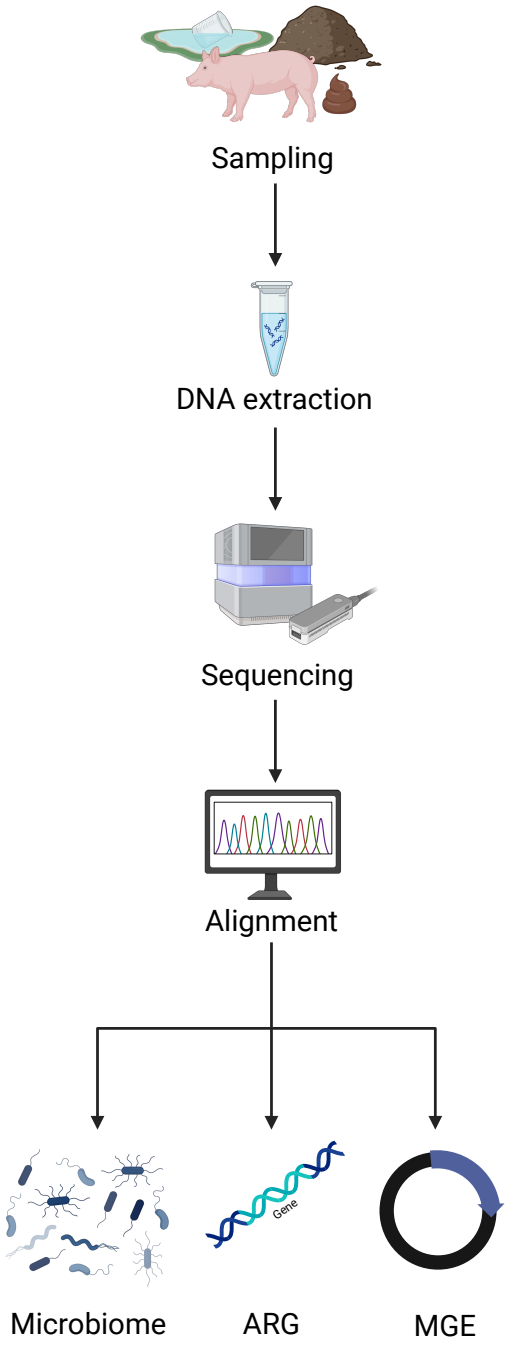

Figure 1.5: A general workflow to study antimicrobial resistance from non-clinical sectors. The key steps consist of sample collection, DNA extraction, sequencing and alignment to identify the microbiome, detect antibiotic resistance genes (ARGs) and mobile genetic elements (MGEs).

the microbial species present in a sample and determines their abundances to identify which bacteria are dominant or which can cause potential outbreaks [44, 46].

Secondly, ARG detection can be done by aligning the metagenomic sequences generated to the sequences representing different ARGs. These are found in databases or on online platforms [43, 47, 48]. By doing so, we identify which ARGs are present within a sample and quantify their numbers and abundances. This gives us information on the most prevalent ARGs and the potential effects of these ARGs in a particular environment i.e., are the ARGs identified conferring resistance to clinically or veterinary-relevant antibiotics? or are more bacteria going to become resistant to these antibiotics by HGT? [46]

Lastly, MGEs can be identified. Similarly to ARGs, metagenomic sequences that are generated can be aligned to the sequences representing either plasmids, integrons or integrative elements. Each of these can be detected with the use of their own database or online platform [49]. Identification of such MGEs can provide information on their types and their abundances giving relevant details on which type of element is prevalent. Additionally, these MGEs identified can be linked to ARGs to determine their association thereby providing insight into the spread of AMR between sectors or if bacteria can potentially gain resistance [13, 50].

To go one step further, a functional analysis step can be added to determine which genes, in our case, ARGs, are present but also how they may be expressed and impact antimicrobial resistance (AMR) [43, 46, 51]. For instance, if ARGs conferring resistance to clinically important antibiotics are expressed, we can infer that bacteria harbouring these genes will be more difficult to treat if they cause infections. This combination of gene identification, quantification, and functional analysis provides an understanding of the dynamics of AMR in non-clinical sectors, informing strategies for monitoring and controlling the spread of resistance.

1.6. CHALLENGES

Understanding AMR in non-clinical sectors presents challenges that limit our ability to fully grasp the dynamics of resistance in the environment. In this section, I highlight three major challenges encountered during this research: the under-representation of non-clinical sectors in AMR research, the scarcity of data and metadata across various sub-environments, and the limited availability of such data from low- and middle-income countries (LMICs). These challenges directly affect the reliability and comparability of metagenomic studies, which are essential for tracking AMR across diverse ecosystems. Addressing these gaps is crucial for promoting global equity in AMR research, however, LMICs are disproportionately affected by AMR yet often lack the infrastructure needed for large-scale data initiatives. These challenges also align with the core principles of the One Health approach, emphasising the need for integrated, cross-sectoral responses to AMR that are inclusive of environmental and socioeconomic contexts.

1. The overlooked non-clinical sectors in AMR research

Due to the under-representation of the environment and animals, there is a gap in knowledge regarding why and how to study AMR in non-clinical environments using metagenomics and bioinformatics. This has resulted in a limited understanding of the specific microbial communities, resistance mechanisms, and transmission of AMR within and between environments. Furthermore, applying bioinformatic tools and databases in non-clinical environments is often unclear or underdeveloped, making it challenging to design effective studies. Existing bioinformatic tools and reference databases were originally developed for clinical or model organisms, leading to poor representation of environmental taxa and ARGs. This makes it difficult to accurately identify and annotate resistance genes in metagenomic datasets from non-clinical sources, especially when dealing with unknown or poorly characterised microorganisms.

2. The lack of data and metadata in non-clinical sub-environments

The lack of sufficient data and metadata in non-clinical sectors further confirms the initial gap identified [52]. Non-clinical environments, broadly categorised into soil, water, and animals, play a critical role in AMR transmission. However, many sub-environments not traditionally considered "hotspots" are under-researched in their contributions to the spread of AMR. For example, within the water sector, sub-environments such as storm-water, agricultural run-off, aquaculture, groundwater, and upstream and downstream freshwater systems have received limited attention, while wastewater treatment plants (WWTPs) have been more extensively studied and are considered hotspots [52, 53]. Similarly, the soil is a known reservoir for AMR, but specific regions such as rural villages, deserts, saline and alkaline soils, mining regions, and marine and coastal environments remain under-explored [46, 54]. Consequently, there is insufficient information on how the different environmental factors, like climate, soil pH and human activity, affect the soil microbiome and AMR in different types of soil. In the animal sector, although the food-animal industry is continuously researched, less-studied groups, such as insects, backyard farming animals, goats, and rabbits, play a significant yet often overlooked role in AMR transmission [52, 54]. Additionally, while there is a

limited amount of metagenomic data available, there is often inconsistent metadata reporting, variations in sampling methods, and differences in sequencing protocols. These inconsistencies make it challenging to integrate and compare datasets from different environments or geographical locations. Incomplete or poorly documented data further hinder efforts to build comprehensive global datasets.

3. The lack of data from LMIC settings

An additional challenge arising from the lack of data and metadata in sub-environments and hotspots is the scarcity of data from LMICs [52, 55]. Although some metagenomic data is available, it often lacks essential metadata, and in some cases, complete metagenomic datasets are absent. This issue is particularly pronounced where limited infrastructure and resources hinder effective AMR monitoring in non-clinical environments [52]. As some LMICs generate metagenomic data, it does not represent all regions and countries. Additionally, methods commonly used in high-income countries (HICs) can be unfeasible due to resource constraints in LMICs. This lack of infrastructure hampers AMR detection and affects broader public health initiatives, such as outbreak prevention and surveillance [55, 56].

1.7. THESIS CONTRIBUTIONS

Metagenomic data in combination with various bioinformatic tools and databases can be used for AMR surveillance in the environment. **Chapter 2** expands on the value of using metagenomic data for AMR surveillance in different non-clinical environments. This chapter reviews antibiotic use across various environmental and agricultural settings, showcasing studies using metagenomic data for AMR research, and provides an overview of relevant bioinformatic tools, databases, and workflows. This chapter serves as a resource for guiding future AMR research.

As wastewater treatment plants (WWTPs), soil, and food animals are recognised as AMR hotspots, overlooked sub-environments can offer a more comprehensive understanding of AMR. In **Chapter 3**, microbial communities, resistance profiles and MGEs across different sectors of the WWTP system and overlooked freshwater environments (upstream, influent, activated sludge, effluent and downstream) are analysed. By integrating publicly available metagenomic data representing each sector of the WWTP and using a standardised pipeline, the presence of clinically relevant pathogens, including *Klebsiella pneumoniae*, *Enterobacter* sp. and various β-lactamase genes was detected throughout the WWTP process. This shows that WWTPs are not just treatment facilities but are potential sources for the ongoing circulation of resistance in the environment.

Similarly, in **Chapter 4**, a standardised pipeline was used to analyse four distinct soil types (natural, urban, rhizosphere, and rural) from nine geographically diverse locations to assess how environmental factors such as temperature, vegetation, and human activity shape microbial communities, ARGs, and MGEs. Bacteria and ARGs present in soil are highly dependent on human activity, vegetation, and environmental conditions such as soil pH and temperature. Results showed that *Bradyrhizobium* was predominant in more vegetative areas while *Pseudomonas aeruginosa* was predominant in rural soils. Additionally, ARGs were detected in

different abundances based on environmental factors. This chapter provides a deeper understanding of how different environmental conditions influence AMR dynamics in soil.

In **Chapter 5**, the impact of antibiotic use on the microbial populations, ARG profiles, and MGEs in the feces of pigs raised with and without antibiotics was studied by integrating publicly available metagenomic data and using a standardised pipeline. The findings revealed that antibiotic use significantly alters the diversity of bacterial communities and ARGs within the fecal microbiome, with implications for animal health. Importantly, this chapter underscores the role of pig fecal waste as a potential source for the environmental dissemination of AMR.

Chapter 6 presents a proof-of-concept study that tested the (cost)effectiveness of a portable, in-field lab compared to a traditional laboratory. This study assessed whether mobile technologies can support rapid environmental and foodborne pathogen surveillance. Three common sources of microbial exposure was chosen i.e., lake water, wastewater sludge and retail meat. The performance of the portable laboratory was then evaluated in DNA extraction, sequencing, microbial detection, ARG identification, and plasmid analysis. This chapter highlights the potential for scalable, real-time AMR monitoring, particularly in areas with limited access to advanced laboratory infrastructure, offering a new approach for global AMR surveillance.

Lastly, **Chapter 7** revisits the central theme of this research: that AMR is not confined to clinical settings but is intricately linked to human activity across agricultural and environmental domains. This chapter discusses the potential of metagenomic data to advance AMR research in non-clinical sectors, highlights the challenges associated with using public datasets, and addresses the limitations of cost-effective data generation. Additionally, it offers insights into how using different omics approaches can further enhance our understanding of AMR.

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2

METAGENOMIC-BASED SURVEILLANCE SYSTEMS FOR ANTIBIOTIC RESISTANCE IN NON-CLINICAL SETTINGS

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The success of antibiotics as a therapeutic agent has led to their ineffectiveness. The continuous use and misuse in clinical and non- clinical areas have led to the emergence and spread of antibiotic- resistant bacteria and its genetic determinants. This is a multi-dimensional problem that has now become a global health crisis. Antibiotic resistance research has primarily focused on the clinical healthcare sectors while overlooking the non-clinical sectors. The increasing antibiotic usage in the environment – including animals, plants, soil, and water – are drivers of antibiotic resistance and function as a transmission route for antibiotic resistant pathogens and is a source for resistance genes. These natural compartments are interconnected with each other and humans, allowing the spread of antibiotic resistance via horizontal gene transfer between commensal and pathogenic bacteria. Identifying and understanding genetic exchange within and between natural compartments can provide insight into the transmission, dissemination, and emergence mechanisms. The development of high-throughput DNA sequencing technologies has made antibiotic resistance research more accessible and feasible. In particular, the combination of metagenomics and powerful bioinformatic tools and platforms have facilitated the identification of microbial communities and has allowed access to genomic data by bypassing the need for isolating and culturing microorganisms. This review aimed to reflect on the different sequencing techniques, metagenomic approaches, and bioinformatics tools and pipelines with their respective advantages and limitations for antibiotic resistance research. These approaches can provide insight into resistance mechanisms, the microbial population, emerging pathogens, resistance genes, and their dissemination. This information can influence policies, develop preventative measures and alleviate the burden caused by antibiotic resistance.

2.1. INTRODUCTION

Antibiotic-resistant bacterial infections have contributed to 4.95 million deaths worldwide, with 1.27 million of those directly resulting from antimicrobial resistance (AMR) [1, 2]. It is the leading cause of death worldwide and affects high, low, and middle-income countries [3, 4]. Antibiotic-resistant bacterial infections are rising exponentially, making them harder to treat [5, 6]. This continuation will lead to an estimated 10 million deaths annually by 2050 if the burden of AMR is not alleviated [7].

For most of human history, bacteria have caused infectious diseases and contributed to high mortality rates [8]. To counteract this, antibiotics were routinely prescribed to treat and prevent such diseases. This saved millions of lives each year. Due to its success, antibiotics were used in animal husbandry and agricultural practices with the added benefits of growth promotion [9]. Unfortunately, this success was limited to the emergence of antibiotic-resistant bacteria (ARB) and its effects on human health and longevity [8, 10, 11].

The continuous and unwarranted use of antibiotics in humans, animals, and agricultural areas exerts selection pressure on the bacteria found in these sectors. This gives rise to bacteria resistant to multiple antibiotics classes leading to untreatable infections [12, 13].

The ability of bacteria to resist antibiotics is embedded in their evolutionary history, which has led to various phenotypic and genotypic resistance mechanisms [14, 15]. These include the inactivation, modification, degradation, and expulsion of the antibiotic or its target site, therefore protecting the bacteria [9, 16–19]. It is also possible that bacteria gain resistance through mutations, changing the nature of proteins expressed in the bacterial organism, or by horizontal gene transfer phenomena, which is the exchange of genetic material by the use of mobile genetic elements (MGEs) between bacterial strains [8, 14]. This allows bacteria to collect numerous resistant traits and ultimately become multi-drug resistant (MDR) [15, 18–20]. The ineffectiveness of antibiotics threatens the ability to treat common bacterial infections and minor injuries. Since first-line antibiotics are deemed sub-par, more expensive treatment, alternative therapy and additional care is necessary. This can result in prolonged infections and an increase in morbidity and mortality rates. This puts an economic burden on families, society, and modern medicine [15, 21–23].

For over a decade, antibiotic resistance has been studied using the traditional culture-dependent approach. This involves the use of artificial conditions to culture microorganisms. Unfortunately, some microorganisms cannot be cultured by this approach [24, 25]. Approximately 80% of bacterial species in the human gut and 99% of environmental bacterial species remain uncultured [25, 26]. Factors such as slow growth, microbial competition, specific growth requirements, and environmental stressors can affect the culturing process. This leaves limited information regarding gene transfer between these bacterial communities [24, 25, 27–29].

Whole metagenome DNA sequencing can describe the genomes of the total microbial community found in nature. This approach enables the study of culturable and non-culturable bacteria by bypassing the need for isolation and laboratory cultivation of microorganisms. DNA directly isolated from the environmental sample can broaden the understanding of the structure, gene/species richness and distribution, and the functional and metabolic potential of a microbial community [28].

Applying this approach to antibiotic resistance in different microbial communities can identify known and novel resistance genes and mobile genetic elements, i.e., plasmids, integrons, transposons, and phage's [30–33]. This information is the stepping stone to making new policies for infection and prevention control measures, thereby reducing the incidence of infection and optimizing the use of antibiotics by health professionals, in the healthcare, animal, and agricultural industries. In addition, it will provide improved awareness and understanding of antibiotic resistance as a whole.

Unfortunately, AMR monitoring systems focus on clinical and the public health sectors as a representation of AMR as a whole. Organizations such as WHO and the implementation of the Global Antimicrobial Surveillance System (GLASS) have relied on the use of traditional culturable methods for clinical AMR research. This creates a biased outlook on AMR as non-clinical sectors, and non-culturable bacteria are excluded. This review will focus on AMR in non-clinical sectors and the use of bioinformatics as an aid in this research.

2.2. DNA SEQUENCING TECHNOLOGIES USED IN ANTIBIOTIC RESISTANCE RESEARCH

Microbiomes are complex and require sequencing technologies to be comprehensive enough to capture the present representative sequences from species. Several sequencing platforms can be used to study AMR in clinical and non-clinical sectors. They are useful in detecting antibiotic resistance genes (ARGs), virulence factors, MGEs, and diversity of the microbial community [34]. The advancements in metagenomics have been driven by next-generation sequencing technologies (NGS) i.e., second and third-generation sequencing technologies (Table 2.1).

Second-generation sequencing has been the most widely used sequencing technology for microbial genomics. Second- generation sequencing technologies, i.e., Illumina and Ion Torrent, produce short reads from 100 up to 300 base pairs with high accuracy (≈0.1% error rate) [35, 36]. These short reads provide information on species population, evolutionary relationships, allelic variations, SNPs, and the detection of specific genes, i.e., ARGs [35–37]. Although short reads provide information on the genomic content of the bacterial isolates in a sample, it does not provide information on the genomic structure, i.e., which genes are present on chromosomes and MGEs such as plasmids which are crucial in understanding the dissemination of ARGs [35, 36, 38, 39].

Third-generation technologies overcome this limitation by providing a better view of the genetic structure as longer reads (on average 1–100 Kbp) span most of the repetitive sequences, linking ARGs to MGEs, e.g., plasmids [36, 37]. This long-read technology is advantageous for studying AMR in bacterial isolates, and metagenomic samples as the complexity of assembly is reduced [35]. Unfortunately, the high error rate makes it difficult to identify specific allelic variants or SNPs in chromosomal genes leading to AMR [36]. On the other hand, Pacbio and ONT are easily accessible and able to produce raw data in real-time, making them a good tool for the rapid diagnostics [40]. Long reads are also a practical method to determine the species/gene richness, distribution, and functional potential of a microbial community [27, 41].

While third-generation technologies are successful in AMR research, it is limited by the high error rate and low accuracy [36]. To overcome this, a combination of short-reads (i.e., Illumina) with long-reads (i.e., ONT) is a promising way to generate fully resolved and accurate bacterial genome assemblies which characterize antibiotic resistance genes on plasmids and genomes from environmental samples [29, 42, 43]. This combination allows for better assembly of complex genomes as long reads provide information on the structure of the genome, and short reads can be used to correct errors in long reads [35].

These sequencing technologies directly characterize the microbiomes in humans, animals, and environmental samples, providing high-throughput sequencing of either whole genomes, targeted amplicons, or whole metagenomes [44]. Whole-genome sequencing (WGS) uses DNA sequencing technologies to sequence the entire genome of isolated pure organisms to characterize genomic variants (Table 2.2) [45]. WGS overcomes the limitations of traditional culture- dependent approaches and

phenotypic tests, i.e., disk diffusion for AMR [46]. By opting to use WGS, more information on the bacterial genetic determinants conveying resistance and their association with mobile genetic elements can be established [46, 47]. This contributes to outbreak detection, infection control, and epidemiological surveillance, all of which are an essential part of the AMR surveillance [45].

Amplicon sequencing uses marker genes such as 16S (prokaryotes) or 18S rRNA (eukaryotes) genes. Both approaches require the DNA extracted directly from the microbial community and are subjected to either direct sequencing or amplification via polymerase chain reaction (PCR) (Table 2.2). In both instances, the sequences are queried against a reference database (Table 2.4). This is a reliable process to identify the composition of a microbial community down genus-level within a sample [34, 47, 48].

Metagenomic sequencing involves the fragmentation, sequencing, assembly, and annotation of the total genomic DNA isolated in a given sample [47, 49]. Similar to WGS, whole metagenome sequencing (WMS) can provide information on the entire gene content of either prokaryotic and eukaryotic organisms as well as species or strain-level identification and virulence or resistance potential [48, 50, 51]. In addition, WMS makes it possible to predict the metabolic potential of the microbial community. This comprehensive view of all gene content allows access to information on AMR that is essential for setting up control and prevention strategies in all sectors [49].

Meta-proteomics focuses on functional change in a microbiome. Assisting in the control strategies and plays a key role in identifying molecular mechanisms of bacterial pathogenesis and disease outcome determinants. It can aid in developing pathogen-specific treatment strategies that can lower the spread of AMR. Meta-proteomics is reliable for reviewing bacteria in soil, sludge, food, and the ocean [52–55]. Unfortunately, there are inconsistent protocols for sample preparation, inefficient bioinformatic tools, and challenges in measuring low-abundance proteins within a complex protein sample [56]. This technique, while reliable, should be applied with other sequencing technologies to have an in-depth analysis of microbial communities, antibiotic resistance genes, and host-pathogen interaction [57, 58].

Table 2.1: Second-generation versus third-generation sequencing technologies for antibiotic susceptibility testing

Category	**2nd Generation**	**3rd Generation**
Definition	NGS technology where nucleic acid is fragmented and amplified, followed by sequencing of short reads in parallel, where base detection is monitored by platforms like Illumina	NGS technology allowing long-read sequencing by monitoring fluorescently labeled nucleotides or electric signals as nucleic acid passes through a nanopore
Advantages	+ High reproducibility + Requires less DNA/RNA + Strain typing + Resistance determinants like SNPs + Detection of chromosomal mutations and ARGs	+ Suitable for pathogen identification + High sensitivity for microorganism detection + Longer reads detect and map mobile genetic elements in resistant strains + Provides genetic context
Limitations	- Results take days; not suitable for rapid diagnostics - High data volume and complex analysis - Cannot link ARGs to genetic context - Short reads result in fragmented assemblies	- High cost - Cannot analyze SNPs or detect chromosomal mutations - Requires high concentration of nucleic acid - Allelic variants cannot be detected
Platforms	Life Technologies (Ion Torrent, Ion Proton) Illumina (HiSeq, MiSeq)	Pacific Biosciences (PacBio RS) Oxford Nanopore (MinION, PromethION)

Table 2.2: Metagenomic sequencing vs. other techniques for the qualitative and quantitative study of antibiotic resistance

Category	Whole-genome sequencing	Meta-proteomics	Whole-metagenome sequencing	Quantitative PCR
Definition	Sequencing of an entire genome from a culture to identify species/strains, genes, and mutations associated with AMR.	Identification and quantification of proteins conferring AMR and MDR from microbial communities.	Culture-independent approach for identifying all ARGs/mutations in all organisms in complex microbial communities.	Detection, quantification, and typing of specific microbial species/strains or ARGs.
Technique	DNA extracted from a bacterial culture is sequenced to generate FastQ reads.	Peptides are generated and analyzed via high-resolution mass spectrometry to identify and quantify proteins.	DNA extracted directly from the sample is sequenced, generating reads from the microbial community.	Enzymatic reactions with fluorophores (e.g., SYBR Green, TaqMan) amplify and quantify target DNA sequences.
Advantages	+ Reproducible + High resolution + Standardized analysis + Rapid turnaround + High specificity + Read lengths of 1–50 Kb	+ Accurate prediction + Large-scale protein identification + Rapid turnaround	+ High resolution + Accurate prediction	+ Fast + High-throughput detection and quantification + Avoids cross-contamination + High sensitivity and specificity
Limitations	- Requires cultured organisms - Computationally demanding - Costly	- False positives and discovery rates in large datasets - Limited reproducibility	- Difficulty differentiating host vs. pathogen - Expensive - Computationally demanding	- Cannot distinguish live from dead cells
Outcome	· Microbial typing and tracing · Predict AMR from genomic data · Discover novel resistance genes or mutations	· Host-pathogen interactions · Virulence factors · AMR mechanisms · Identification of functions in biological pathways	· Species/strain-level identification · AMR · Virulence potential · Detect mobile genetic elements · Discover viruses	· Detect specific genes/alleles · AMR profiling · Toxin production · Typing strains and isolates

2.3. BIOINFORMATIC ANALYSIS OF METAGENOMIC SEQUENCES FOR ANTIBIOTIC RESISTANCE RESEARCH

The constant development of new computational pipelines is advantageous in providing an accurate depiction of the resistome from metagenomic sequencing data. These analyses rely on a variety of algorithms: quality control of DNA sequencing data, genome and metagenome assembly algorithms, read-mapping, variant detection, phylogenetics, taxonomic databases, and visualization [59]. AMR databases are discussed in more detail in the next section. The typical process to identify antibiotic resistance genes from sequenced metagenomes is based on one of two general techniques: (i) read-mapping and (ii) assembly. Each of these could be followed by binning or an annotation step (Table 2.3). A bioinformatics pipeline for analysing metagenomic data for antibiotic resistance research has been presented in Figure 2.2. Read mapping allows for raw read sequences to be directly aligned to reference databases (Table 2.3) using pairwise tools such as MiniMap2 [60], Bowtie2 [61], Burrows-wheeler aligner (BWA) [61], or by splitting reads into k-mers and mapping to a reference database using k-mer alignment (KMA) (Figure 2.2) [62]. This provides information on the similarity of one sequence to another by counting the number of alignments to quantify the abundance of similar sequence patterns [62].

In the last decade, read-mapping approaches were deemed superior to assembly approaches for AMR gene detection, i.e., reads are first assembled and mapped to a database using BLAST [63–65]. The success of an assembly-based approach is highly dependent on the quality of the assembly [62, 66]. An approach as such is problematic as genes of interest, i.e., ARG, s can be split over two or more contigs and will not be identified if the assembly is poor quality [62, 65].

Read-mapping based methods overcome this by using the tools above, which allow for fast mapping and alignment of raw reads against large reference genomes and entire databases [62]. This can identify genes from low abundance organisms in a complex community with speed and ease of computation. Since epidemiological databases are constantly being updated with new sequences due to natural evolution, the read-mapping approach becomes difficult as results constantly change. There is no guarantee that the read will cover a unique part of the reference sequence and will result in a tie for the best match due to the random selection [62]. Fortunately, tools like SRST2 [66] resolve the ties by pre-and post-processing of sequences for read-mapping, which can predict the presence of ARGs in a sample [65, 67].

Identification and characterization of ARGs can also be achieved using assembly-based approaches (Table 2.3 and Figure 2.2) [68]. This method allows reads to be assembled into contigs and then queried against reference databases (Table 2.4). The assembly of WMS data is complicated as there is an uneven or unknown abundance of different genomes [69]. Unrelated genomes may also contain nearly identical DNA repeats, which could represent MGEs [37]. In addition, multiple individual organisms could be from the same species but may harbour small genetic differences indicating strain variants [69]. Technical factors, e.g., library preparation, sequencing depth, and sequencing platforms, affect the accuracy in assembling WMS data into larger contigs [70]. Metagenomic assemblers such as MEGAHIT [71], MetaSPades

[72], IDBA-UD [73], and MetaVelvet [74] have been developed to overcome these challenges by normalizing sequencing depth, correcting read errors, and detecting and reporting genomic variants and repeats [69, 75]. To date, no single assembler is the best at accurately reconstructing known genomes and capturing taxonomic diversity in a metagenomic sample. Multiple assemblers are recommended to be applied to a subset of samples to determine which has the best fit [37, 69, 70].

Binning classification can predict the taxonomical composition using information contained in reads (Figure 2.2). Reads are grouped to represent an individual genome or closely related genomes (Table 2.3). Binning can be done either with reads or assembled sequences and employs two strategies to obtain taxonomic assignment; (i) sequence composition classification and (ii) sequence alignment against references. The first is based on looking at genomic signatures using k-mers to identify evolutionary conservation among species and uses software such as TETRA [76], MetaClusterTA [77], and PhylophytiaS [78]. Similarly, software like MaxBin [79] and Amphora2 [80] use k-mer signatures but also considers gene markers, GC content, and coverage information for binning on assembled sequences or reads. Another method based on reference read alignment is the Burrows-Wheeler Transform [81] which indexes like BWA [82] or Bowtie [61] and is fast and accurate in assessing species richness and abundance in WMS by aligning them to reference genomes. Genometa [83] is a software that looks at OTUs in the WMS data and groups according to genomic islands and operons. Taxonomic classification in long reads using the potential coding regions to search in annotated protein databases using BLAST [63] or Megan [84]. Binning achieved after the assembly of contigs can lead to the generation of partial genomes of unknown or uncultured organisms. This can be used to perform similarity-based binning of other metagenomic datasets. If binning is achieved before assembly, it can reduce the complexity of an assembly-based approach and may reduce the computational demands. However, caution should be taken to ensure the validity of the genome bins as there is a chance of false assignments [85, 86]. In this instance, CheckM [87] can be used to evaluate the quality of bins on metagenomic samples and assess the quality of assembled metagenomic genomes by estimation of completeness and contamination based on marker-gene validation [88].

Taxonomic assignments of reads can also be done with genome annotation. This process identifies the coding regions and their location to determine gene function. Annotation of metagenomic sequences can be done in two ways, (i) existing pipelines, e.g., RAST [89] and IMG [90], can be used for assembled genomes, and (ii) annotation can be performed on an entire community and relies on unassembled reads and short contigs. WMS data is annotated by identifying genes (feature prediction) and assigning putative gene functions and taxonomic neighbours (functional annotation) [85]. Prediction tools such as MetaGeneAnnotator [91] or MetaGeneMark use internal information to classify sequences as either coding or non-coding and are done with a low error rate of 2% [85].

Table 2.3: Various bioinformatics approaches for the prediction of AMR and MGEs.

	Read-mapping approach	**Assembly-based approach**	**Binning approach**	**Annotation**
Definition	The detection of genes without genome assembly. The direct alignment of reads to a reference database.	The identification of genes with *de novo* assembly profiling.	The evaluation of taxonomic groups by clustering assembled sequences into individual groups that represent microbial species.	The prediction of genes from either assembled or unassembled reads.
Function	Antibiotic resistance gene discovery and taxonomic identification.	Antibiotic-resistant gene discovery and taxonomic identification.	Assessment of taxonomic diversity and gene association to taxonomic groups.	Gene function and taxonomic identification.
Technique	High-quality reads are directly aligned to a reference database using pairwise alignment tools or splitting reads into k-mers and mapping to reference databases.	High-quality reads are assembled into contigs that are aligned to a reference database using BLAST or HMMscan.	The process of clustering contigs into individual genome bins by machine learning methods or comparing metagenomic sequences against a reference database of genomic sequences.	Sequences are classified by coding and non-coding regions to determine gene function.
Advantages	+ Fast + Less computationally demanding + Identify genes from low abundance organisms in complex communities + Resistome analysis of large datasets	+ Identification of protein-coding regions + Identification of known and novel resistance genes + Regulatory and mobile genetic element sequences identified	+ Easy + Reliable	+ High accuracy + High sensitivity
Limitations	- Lacks information required to study upstream and downstream factors of identified resistance genes - Large datasets may cause false-positive predictions	- Loss of information due to incomplete and poor assembly - Computationally expensive - Time-consuming - Requires a high genome coverage	- Lack of whole-genome sequences within the available databases	- Computationally demanding - Limited success for short reads - Expensive
Tools	SRST2 [66], ARIBA [64], BOWTIE2 [61], Burrows-Wheeler aligner (BWA) [82]	MEGAHIT [71], MetaSpades [72], MetaVelvet [74], Spades [92], Velvet, Abyss [93] and SOAPdenovo [94]	MetaBat2 [95], Maxbin2 [79], CONCOCT [96], GroopM [97], DASTool [98], MetaCluster [99], MEGAN [84], MGRAST [100]	RAST [89], IMG [90], MetaGeneAnnotator [91], MetaGeneMark [78]

2.4. ANTIBIOTIC RESISTANCE DATABASES

The performance of antibiotic resistance gene prediction and taxonomic identification depends on the availability of accurate databases. Two types of public databases exist, generalized AMR databases, which include a wide range of ARGs and mechanistic information, and specialized databases, which provide extensive information on specific gene families, e.g., the β-lactamase family [51].

Several bioinformatic tools and databases can be used in AMR research. They focus on fast and reliable predictions of ARB and their genetic determinants in complex communities [101]. It is important to note that the quality and type of sequence play an essential role in AMR research. The bioinformatics tool's robustness can differ when analysing sequences of low-quality [51]. Any database's value depends on maintenance, curation, and continuous updates with detailed metadata. Correctly predicting ARGs from large-scale datasets with improved accuracy depends on input parameters such as e-values, bit scores, identity, and query coverage levels [102].

2.4.1. GENERAL ANTIBIOTIC RESISTANCE DATABASES

General antibiotic resistance databases incorporate a wide variety of ARGs and mechanistic information. Established tools like ResFinder [103] accept short reads and contigs to detect the presence of acquired ARGs. This can be done for complete or partial genome sequences and uses BLAST [63], or KMA [62] approaches [103] (Table 2.4). The specificity of using BLAST to detect antibiotic resistance genes depends on the selection criteria for gene length and percentage similarity. This method requires computational expertise and could be challenging to identify multiple copies of ARGs in *de novo* assembled genomes [62, 104]. This can be solved by using longer reads which increases the cost.

In comparison, the KMA approach was developed to map the raw reads against redundant AMR databases as the traditional BLAST method remains too slow to map raw reads directly [51, 65]. This approach provides accurate bacterial genome analysis and can identify ARGs present in low abundance, which might be excluded in incomplete assemblies. There is also a chance of false positives due to sequencing errors which can be overcome by setting the minimum threshold for the number of reads needed for a positive outcome [51, 104].

The Comprehensive Antibiotic Resistance Database (CARD) [105] for the identification of resistance genes, their products, and associated phenotypes. This database allows for the identification of chromosomal mutations that confer resistance to antibiotics. Similar to ResFinder [103], the percentage coverage and identity can be adjusted to allow for the selection of new ARGs. The Resistance Gene Identifier (RGI), a tool for de novo annotation of genes, complete genomes, or genome assembled sequences for AMR, uses CARD's curated database to predict ARGs in either DNA or amino acid format. This is because some sequences cannot be mapped due to divergence in the nucleotide sequences; however, amino acid sequences in this instance can be translated to proteins to characterize the resistome [106, 107]. CARD uses two prediction models: a protein homolog model based on functional resistance homologs and a protein variant model that detects mutations conferring resistance [105, 108]. The application of ontologies such

as CARD's Antibiotic Resistance Ontology (ARO) has organized AMR information based on molecular determinants, resistance mechanisms, individual antimicrobials, antimicrobial targets, and drug classes, therefore, making this database favourable [59]. Other curated databases include; MEGARes [109], ResFams [110], NDARO [111], FARME [112], and MUSTARD [113], all of which are curated for the detection of AMR determinants in large metagenomic datasets [102, 108, 114, 115].

Interestingly, FARME, a functional antibiotic resistance metagenomic element database, focuses on AMR gene elements from environmental samples (soil, faecal matter, wastewater treatment plants, oral and aquatic biomes) rather than individual ARGs obtained from cultured clinical isolates, providing more access and analysis of non-clinical sectors. This database is compiled from publicly available DNA sequences from 30 functional metagenomic projects and their corresponding predicted proteins conferring AMR [112]. FARME contains over seven times the number of non-redundant protein sequences as compared to CARD [102, 112]. It provides information on regulatory elements, MGEs, and predicted proteins flanking ARGs, which are conserved between functional metagenomic AMR sequences from soil biomes and pathogenic clinical isolates [112, 116]. This allows for better insight into AMR in unculturable bacteria found in non-clinical settings [86, 108, 112]. ResFams is a curated database of protein families linked to their profile Hidden Markov Models (HMMs) associated with AMR function are trained using unique AMR protein sequences obtained from CARD, LaCED, and Lahey databases [102]. This platform primarily focuses on pathogen-associated ARGs and provides a comprehensive view of resistomes in the environment and the evolution of resistant pathogens [102]. ResFams were evaluated with functional metagenomic datasets and demonstrated improved sensitivity, and identified 64% more ARGs in soil and human gut microbiomes compared to BLAST-based searches of CARD and ARDB databases. This increased sensitivity is due to the HMM-based analysis. HMM, models are specific models constructed based on observed sequence variation across genes/protein families and capture possible variation for the families [106]. Although this approach is sensitive and can detect distant matches that BLAST-based approaches cannot, ResFams is computationally expensive and requires the user to provide local computational resources to run HMM-based searches [106, 107].

2.4.2. SPECIFIC ANTIBIOTIC RESISTANCE DATABASES

Specific databases are created to meet specific needs and provide extensive information about a specific gene family. So far, only specialized databases are available for β-lactamase.

The β-lactamase database (BLAD) is a catalogue of resistance patterns from all classes of β-lactamases collected from NCBI and published data with crystal structures of proteins from the Protein Data Bank (PDB) [117]. Metagenomic sequences can be queried against BLAD to obtain basic information about β-lactamases, search for the gene of interest, identify resistance patterns and search and analyse the 3D structure of the β-lactamase [118, 119].

The CBMAR β-lactamase database [120] includes detailed biochemical and molecular information to understand known and unknown β-lactamase genes. This

provides a cache of information on nucleotides, 3D protein structures, sequence alignments, and mutation profiles.

Similarly, LacED [121] is a specialized platform that contains mutational and structural data for TEM and SHV β-lactamases. This is extremely important to understand the evolution of multidrug-resistant pathogens as β-lactamase evolution, and the emergence of new enzymes affects the now available treatment for infections. Furthermore, these resistant genes are present on plasmids which help their spread to different biomes, challenging the treatment options in both clinical and non-clinical sectors [122].

Table 2.4: General and specific databases in AMR research.

General Databases	Description	Update	Website	Reference
ResFinder and ResFinderFG	A curated database that identifies acquired genes and/or finds chromosomal mutations.	Apr-21	https://cge.cbs.dtu.dk//services/ResFinder/	[103, 123]
CARD	Manually curated database and an ontology resource that provides information on ARGs and resistance mechanisms.	Jul-21	https://card.mcmaster.ca/	[105, 124–126]
SARG (v2)	ARG database derived from ARDB, CARD, and NCBI-NR databases for characterization and quantification of ARGs.	Apr-21	http://smile.hku.hk/SARGs	[127]
FARME	Curated database focused on environmentally derived metagenomes conferring resistance.	Mar-19	http://staff.washington.edu/jwallace/farme/	[112]
MEGARES	Database containing sequence data for approximately 8000 manually curated ARGs.	Oct-19	https://megares.meglab.org/	[109]
MUSTARD	Database of 6059 AMR determinants from 20 families in the human gut microbiota and curated sets of genes identified via functional metagenomics.	Sep-17	http://mgps.eu/Mustard/	[113]
RESFAMS	A profile HMM-based curated database to confirm antibiotic resistance function and organized by ontology.	Jan-15	http://www.dantaslab.org/resfams/	[110]
NDARO	Curated and collated data from multiple databases. Contains 5804 sequences.		https://www.ncbi.nlm.nih.gov/bioproject/PRJNA313047	
Specific Databases	**Description**	**Update**	**Website**	**Reference**
LacED	Curated database providing information on mutation, sequences, and structures of TEM and SHVβ-lactamases.	T:2017, S:2010	http://www.laced.uni-stuttgart.de/	[121]
CBMAR	Database identifying and characterizing novel β-lactamases based on Amber classification.	Sep-14	http://proteininformatics.org/mkumar/lactamasedb/	[120]
BLDB	A manually curated database of AR enzymes classified by class, family, and subfamily.	Jul-21	http://bldb.eu/	[119]

2.5. ANTIBIOTIC RESISTANCE IN NON-CLINICAL SECTORS

AMR is a natural phenomenon that predates the use of antibiotics. A wide array of novel and clinically characterized ARGs have been detected in environmental samples ranging from pristine environments to agricultural soil [128]. The major driving force of AMR is the mis-use of antibiotics while other factors such as poor infrastructure, i.e., hygiene and sanitation play a role in maintaining AMR [129, 130]. Approximately more than 60% of total antibiotic use occurs outside the field of human medicine [11, 131, 132]. The main non-clinical sectors that are involved in the development of AMR are animal production (food-animals and aquaculture), agriculture (plants), and the environmental (water and soil) compartment. These sectors are interconnected and facilitate the spread of pathogenic ARB within and between them and ultimately to humans. Table 2.5 summarizes the findings done on AMR in various non-clinical reservoirs through the use of metagenomics.

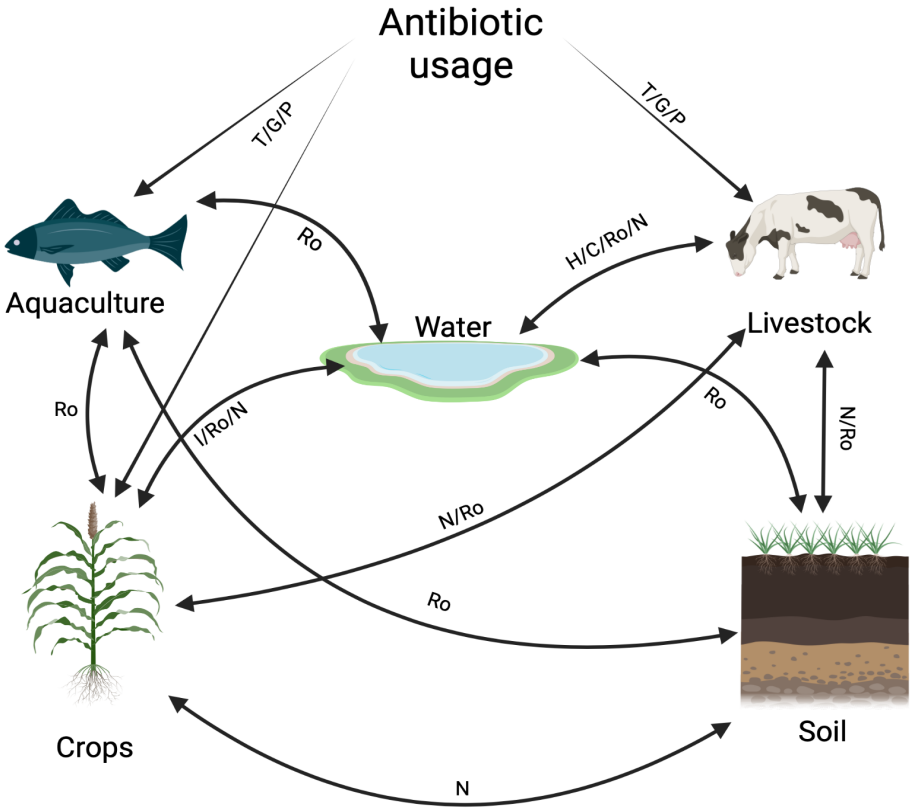

Figure 2.1: Pathway map of antibiotic agents and antibiotic resistance dissemination between livestock, agriculture, aquaculture, and water. The movement of antibiotic resistance or antibiotic agents is indicated by solid lines. The potential routes of transmission and exposure between non-clinical sectors and antibiotic usage is indicated by the abbreviations: Ro: run-off /leaching, T: treatment, P: prevention, GP: growth promoter, H: animal house, C: cleaning, N: nutrients, I: irrigation (Created with BioRender.com). Routes between non-clinical sectors (↔) and antibiotic usage in non-clinical sectors (→) have been described by the previous studies mentioned. Water ↔ crop: [133–137], water ↔ soil: [133, 135, 138–140], water ↔ livestock: [7, 141–146], water ↔ aquaculture: [138, 147–153], antibiotic use → aquaculture: [147, 149, 151, 154], antibiotic use → livestock: [124, 142, 145, 155–158], antibiotic use → crops: [159–163], livestock ↔ soil and crops: [123, 164–167].

2.5.1. FOOD-ANIMALS

Each year, half of the antibiotics produced are used for industrial farm animal production [24]. The global demand for animal protein has directly driven the use of antibiotics in livestock. The food-animal industry is a hotspot for antibiotic usage, estimated to increase by 67% in highly populated countries [7]. Antibiotics are administered as metaphylactic and prophylactic treatment for the whole flock or herd to prevent the spread of infectious diseases. Furthermore, it is used as a growth-promoting agent across the globe [7, 168, 169].

Historically, the negative effects of antibiotic usage in livestock production have been overlooked because of the drive to keep up with consumer demand [169, 170]. This has led to the prolonged use and dosage, creating an ideal condition for the emergence of ARB and ARGs [144, 155].

AMR can spread through direct and indirect contact, which was confirmed by finding the same ARBs in the poultry farm and the caretaker [171]. Antibiotics and their residues can be found in most connecting areas such as soil and plants, and animal products which can lead to health risks when consumed due to the direct toxicity, allergic reactions, carcinogenic effects, and disturbance of the beneficial microbiota in children, those who are pregnant, the elderly, and people living with immunocompromised people [172].

Areas such as the EU and United States have made efforts to ban the use of antibiotics as growth enhancers. However, they are still widely used in South Africa, Brazil, India, China, and Russia [6, 7]. This strategy is based on the assumption that susceptible strains can outnumber AMR microorganisms if the advantage of becoming resistant is decreased [173]. This was supported by a post-ban study conducted in Denmark and Norway. Interestingly, even though ARB decreased, the broilers that were studied were still colonized by ARB years after the ban came into effect. It is such that the ban on antibiotics is still up for debate as decreasing antibiotic use could increase the frequency of bacterial infections [174, 175].

The World Health Organization has recommended that farmers and the food industry stop using antibiotics for the growth and prevention of disease in healthy animals. Farms should consider the employment of proper preventive measures and farming practices to limit the risk of bacterial infections instead of the routine antibiotic dosing of animals to prevent disease. If this is done correctly, we could see the decreased use of antibiotics and the alleviation of antimicrobial resistance [23, 155, 169, 176].

2.5.2. WATER

Water represents one of the most important bacterial habitats and is a major pathway and reservoir for disseminating microorganisms and AMR. This is due to the spread of AMR by humans, and animals, or contamination of the environment by pathogens [177, 178].

GREY WATER

Wastewater treatment plants (WWTPs) collect chemical pollutants, including antibiotics, which are disposed of from households, hospitals, and factories. The WWTP aims to remove contaminants before disposing water in natural environments, e.g., streams, rivers, and lakes. While the reuse and reclamation of water through WWTPs reduce water shortage, they assist in the spread and emergence of AMR [139]. This impacts the economy and society of countries affected by AMR [136, 179]. It could be possible that proper sanitation and safe drinking water in the high-, middle-and low-income countries affect the emergence and spread of AMR. Although the removal of contaminants is successful, some ARB are not removed by the WWTP and are released with the effluent, which can spread to other natural environments [135].

Since WWTPs receive a diverse range of ARB and their genes from different sources. This ARB accumulates in the WWTP, and these high concentrations provide a selective pressure that facilitates the emergence of new ARB strains [136]. This is seen in wastewater treatment that uses biological treatment processes such as activated sludge, creating a favourable environment for ARB, ARGs, and their transmission [180]. Contradictorily, it was hypothesized that the WWTP is efficient in removing ARBs which was seen in a study of the Dutch [181]. It could be argued that AMR in WWTP decreased since the Netherlands decreased antibiotic use as a whole. While this can be observed, antibiotic residues are not fully removed from the WWTP and persist in lower concentration when released with the effluent which serves as a selection pressure for ARBs [182]. WWTPs should revise their treatment options to manage AMR concerns while also providing sanitary water to communities. Policies about sludge and biosolid disposals are needed along with better infrastructure which will help decrease the spread of AMR to other sectors [136, 183].

BLUE/GREEN WATER

Freshwater environments are among the natural environments susceptible to contamination with antibiotics. These are released through different sources such as agricultural run-off, sewage discharge, and leaching from farms [177, 184]. The combination of antibiotics with a high density of bacteria provides a favourable environment for the development of ARGs [185]. ARB carrying resistance genes can persist in freshwater and ultimately return to humans and animals by horizontal gene transfer (HGT) [186, 187]. Freshwater serves as a source of drinking water, for recreational purposes and for agricultural practices, which use 70% of all freshwater, e.g., aquifers, streams, and lakes [136]. Continuous use of freshwater encourages the spread of AMR and the emergence of new pathogens, which leads to an increased risk of infection and prolonged and untreatable infections in humans, animals, and the environment [188].

To date (Table 2.5), there is evidence showing that the consumption of contaminated water poses health risks [189–191] and that the WWTP has the potential to contribute to the dissemination of AMR in receiving rivers [140, 180]. Unfortunately, information on antibiotics in streams, lakes, beaches, pond

water, surface and groundwater, and drinking water is scarce as it is presumed that antibiotic concentrations are naturally low in these areas. However, low antibiotic concentrations still select for ARB [188]. Studies also focus on ARB and genes at discharge points, i.e., receiving river or effluent, of the WWTP. At this point, the concentration of AMR is high but declines further away from the WWTP, downstream, which could be a result of degradation through biological or non-biological processes, uptake by aquatic microorganisms through HGT, diluted, absorption onto particulate matter, or transportation into different water systems [187, 192]. More focus should be on AMR within each freshwater environment, its link to different sectors, AMR after the WWTP discharge points and in potable water to gain a more holistic view [187].

2.5.3. AQUACULTURE

Aquaculture refers to the breeding, rearing, and harvesting of aquatic organisms in different water environments. This term is commonly used to describe fish farming. Aquaculture contributes to more than half of the world's seafood consumption and production and has increased by 6% a year since 2001 [193]. The aquacultural sector is a complex interconnected system influenced by the environment, cultural, human, and economic factors [194, 195].

Like livestock production, antibiotics are used in aquaculture as a therapeutic agent to prevent and treat bacterial outbreaks. High concentrations are used due to high stocking densities, and the lack of individual treatment [196].

Antibiotics are mixed with feed before being administered to animals or can be directly applied to the aquatic environment. This leads to the dispersal and leaching of antibiotics into the environment [194]. The gut microbiota of fish is also affected by the continuous use of antibiotics which alter the benefit of host-microbiota relationships allowing the gut microbiota to become resistant to antibiotics that are administered [197]. Aquaculture is also affected by toxic materials such as silver and mercury, which co-resist and co-regulate with antibiotics. These toxic materials accumulate in fish bodies and the aquatic food web. This toxicity can be passed to humans and animals through consumption. It can affect the nervous system leading to death, as discussed by Sonone et al. [153], who reviewed the various heavy metal sources, i.e., agricultural activities, electronic waste, mining, industrial effluents, power plants, and biomedical waste and its role in degrading the aquaculture population, causing physical deformities in organisms and polluting the aquatic environment.

Several studies (Table 2.5) have shown that the excess use of antibiotics has led to the emergence of AMR. It cannot be ignored that the aquaculture industry is integrated with sewage, industrial wastewater, and land agriculture as manure and other agricultural residues are used in fish food. Areas in Western Europe and North America have banned antibiotics as a growth promoter, but it is still used as a therapeutic agent in fish food. Since 70 to 80% of antibiotics given to fish are excreted into the water, the entire body of water is exposed. This leads to leaching into different water environments and sediments, which provide long-term selection pressure in the aquatic environment, favoring horizontal gene transfer and

the spread of AMR, as discussed by Taylor et al. [198], Watts et al. [199] and Mirza et al. [187]. Such policies on aquacultural practices should be reviewed to decrease the incidence of ARB and infections [149, 151, 185, 200].

2.5.4. SOIL

Microbes in soil are of great importance as they break down organic matter, recycle nutrients, bioremediation, and produce antibiotics [201]. Variation in biotic, e.g., plants, animals and other bacteria, and abiotic, e.g., water, soil and atmosphere, conditions of soil cause the residing microbes to adapt and develop strategies for survival and successful reproduction. Antibiotic production is the most powerful adaptation strategy from soil microbes to inhibit the growth of competing microbes [201]. This strategy has led to the natural development of AMR. Antibiotics and disinfectants used in medicine, agriculture, and aquaculture have been the driver of AMR through manure/fertiliser application on soil, irrigation, and run-off. Consequences of these are the emergence of new ARGs and the spread of pathogenic ARB to other environments, humans, and animals [165, 201, 202].

Although AMR research has shown that WWTP effluents can still contain a high level of ARGs and bacteria after disinfection, there is not much research on the sludge and biosolids applied to soil and how this changes the bacterial community and resistome [188, 192]. The application of manure/fertiliser/sludge to soil should also be looked into to determine if there is any difference in soil and crop quality as they are used in agricultural and remediation practices. Studies have shown the prevalence of AMR in soil and how bioremediation and pollutants have facilitated its spread (Table 2.5).

2.5.5. PLANTS

The agricultural industry has been valued at an estimated US$ 3.2 trillion worldwide and accounts for the largest share of the GDP and employment in developing countries and underdeveloped nations. Unfortunately, the agriculture industry has been suffering due to population growth, pest resistance, and the burden of natural resources [203].

Manure is commonly used as fertilizer on vegetable farmlands to enhance crop yield. This enhances AMR as 30–90% of antibiotics consumed by animals are excreted in faeces which is used as manure. Using sewage sludge and water for irrigation contributes to rising AMR in agriculture [148, 204, 205]. Crops can take up antibiotics through water transport and passive absorption [133]. Antibiotic-resistant bacteria found in soil can reach the interior of fruits and vegetables either through germination seeds in contaminated soil or the direct transmission of bacteria from the soil to the plant via irrigation. Both these routes can cause the bacteria to colonize in the roots and the edible parts of the plant [159, 161, 206].

These edible plants are consumed by animals or sold as ready- to-eat products such as bagged salads or pre-cut vegetables, which may enhance the proliferation and survival of ARB [207–210]. This is due to many factors such as hygiene standards, irrigation water, temperature, storage, pH, soil, manure, and antimicrobial

agents. Since these products rarely go through a heating step before consumption, it increases the risk of consumers being exposed to high numbers of different pathogenic ARB [210]. This poses a threat to society as consumers who opt for ready-to-eat products are faced with the risk of acquiring foodborne illnesses which could be untreatable with antibiotics. Table 2.5 summarizes studies showing that manure, fertilizers, compost, and irrigation are all routes for the transmission and dissemination of AMR to plants.

2.6. ANTIBIOTIC RESISTANCE AND ITS TRANSMISSION

The use and misuse of antibiotics in humans, animals, and the environment have been linked to the emergence of ARB in each of these sectors. Antibiotic residues, ARB, and ARGs, are spread to different environments [178]. Most antibiotics are excreted in animal urine and faeces [211]. This is introduced to the environment directly by manure amendment, which provides nutrients for crops, or through water which is the primary link between humans, animals, and nature [178].

Since antibiotics are used in the food animal industry, ARB can spread via run-off, which can go into lakes, streams, and rivers. Water from these areas is used for irrigation for agricultural practices. This leads to the spread of ARB from food animals to water, soil, and crops (Figure 2.1). This interconnectedness facilitates the exchange of AMR and allows for the emergence of new genetic determinants. This emergence and spread can occur by HGT [24, 160, 212, 213].

Table 2.5: Studies focusing on the introduction of antibiotics and AMR in each non-clinical sector.

Reservoirs	Introduction of antibiotics	Study	References
Soil	Organic Manure/agricultural run-off	Identification of unknown tetracycline and sulphonamide resistance genes in forest and grassland soil.	[214]
	Manure application	Non-manure amended soil displayed a larger proportion of antibiotic resistance bacteria but carried fewer genes.	[164]
	Manure application /organic compost	Antibiotic resistance genes from manure amended soil accounted for 70% of total resistance genes.	[215]
	Antibiotic-producing soil bacteria	Thirteen antibiotic resistance genes and two bifunctional proteins conferring resistance to ceftazidime and β-lactamase.	[216]
	Antibiotic-producing soil bacteria	Total of eleven ARGs in soil with little human influence.	[217]
	Fertilizer/ manure application	Tetracycline-resistant bacteria in fertilized soil were three times higher than manure amended soil.	[9]
	A fertilizer with heavy metals	ARGs could potentially increase by the use of fertilizer containing heavy metals.	[218]
	Manure application	Manure-borne bacteria contribute to the increase of ARGs whereas indigenous soil bacteria prevent the dissemination of ARGs from manure to soil.	[219]
	Pollution from a petrochemical plant	The soil was more abundant in ARGs than less contaminated soil.	[125]
Plants	Manure application	ARGs on lettuce and endive were grown in manure amended soil. ARGs were detected in the endophytes and phyllosphere of the plants.	[165]
	Conventionally produced (manure application) / organic fertilizers	A higher abundance of diverse ARGs and MDR genes were present in the endophyte and phyllosphere of lettuce grown organically.	[220]
	Manure application /fertilizer	More than 50 unique ARGs were detected in the phyllosphere of maize after manure application.	[221]
	Compost/ improper sanitation/ run-off from farms	2015 to 2020, five outbreaks linked to green leafy vegetables, two linked to sprouts, and one linked a chopped salad kit in the USA.	[222]
Food-animals	Animal feed / infection prevention	Tetracycline resistant *E. coli* strain present in the gut of chickens receiving food supplemented with tetracycline.	[155]
	Animal feed/growth promoter / indirect and direct contact	ARBs were found in each processing step of the beef production chain.	[223]
	Animal feed	A total of 495 bacterial species, 50 ARGs were detected in the organic animal feed.	[224]
	Therapeutic and non-therapeutic	Antibiotics used in food animals leave antibiotic residues in eggs, milk, and meat products.	[172]
	Animal feed	Faecal samples from large-scale swine farms showed a total of 146 ARGs.	[56]
	Animal feed	Taxonomic composition analysis showed a decrease of *Proteobacteria* and an increase of *Actinobacteria* in the gut microbiome of male broilers.	[225]
Water	Effluent discharge	The abundance of pathogens and ARGs increased in the effluent after wastewater treatment.	[140]
	Chlorination	ARGs are found in high amounts in drinking water suggesting chlorination concentrates ARGs.	[190]
	Run-off / discharge	Effluent from municipal wastewater treatment plants and pharmaceutical manufacturing plants releases antibiotics into natural water bodies.	[180]
	Reuse of water / potable water	An increase in ARGs in reclaimed potable water samples and associations between 193 ARGs and plasmid-associated genes.	[226]
	Sewage run-off	The distinct taxonomic composition of bacterial species found in sewage and the sea suggests sewage is diluted in environmental freshwater.	[227]
Aquaculture	Animal feed	Fifty-one different ARGs conferring resistance against 24 different antibiotic types with MDR genes located on plasmid sequences.	[197]
	Animal feed /therapeutic	Tetracycline resistance genes were found in 81% of samples taken from a Chilean salmon farm.	[147]
	Animal feed/ therapeutic	An abundance of tetracycline, sulphonamide, and beta-lactam resistance genes which strongly correlated to silver and mercury resistance genes were found indicating co-resistance and co-regulation.	[150]

2.6.1. HORIZONTAL GENE TRANSFER

ARB exists across animal, human and environmental sectors. The evolution of AMR and the dynamics of ARGs spread across these sectors are critical for predicting emerging pathogens and controlling AMR dissemination. HGT is primarily responsible for rapid AMR spread and dissemination of ARGs between bacteria and across species [228, 229]. AMR can be established either vertically by point mutations or horizontally by acquiring MGEs such as plasmids and transposons [229].

HGT is a process that introduces variation in the bacterial genome under natural selection. It is an essential process that allows bacteria to adapt to environmental changes such as exposure to antibiotics [230]. HGT predates the production and use of antibiotics. Unfortunately, the increased usage of antibiotics has put selective pressure on bacterial strains. This has sped up the process of bacteria becoming antibiotic-resistant by various genotypic and phenotypic resistance mechanisms. This diversification can be seen in the resistance to antibiotics of different classes [15].

Genes that confer resistance can either be intrinsic or extrinsic [231]. Intrinsic resistance is the bacterium's innate ability to resist the antimicrobial agent's activity. This occurs through structural or functional characteristics. Extrinsic or acquired resistance occurs when a previously susceptible bacterium obtains the ability to resist the activity of the antimicrobial agent. This can result from the mutation of genes involved in normal physiological processes, the acquisition of foreign resistance genes, or a combination of both [17, 19, 20].

Pathogenic bacteria can acquire resistance via HGT. This has caused AMR to spread from commensal and environmental bacteria to pathogenic ones. ARGs are often carried in MGEs, e.g., plasmids, transposons, or integrons which act as vectors for transferring genetic information between bacterial cells. The three classical pathways of HGT are conjugation, transformation, and transduction. These HGT mechanisms lead to increased bacteria fitness, which is essential for survival in the presence of antibiotics [16, 18–20, 229].

Recent studies have identified other mechanisms by which DNA can transfer between hosts. Gene transfer agents are DNA-containing particles, similar to phages, but cannot carry genes for particle production [232]. Genes can also be transferred between bacteria that form intercellular connections by nanotubes or membrane fusion [230, 233]. Some bacteria can also release DNA-containing membrane-bound vesicles that carry genetic information to new hosts. Interestingly, some of these mechanisms are not under bacterial control but are controlled by semi-autonomous segments of DNA [233].

Bioinformatic approaches such as MetaCHIP [234] and WAAFLE [235] can be used to detect HGT events in metagenomic sequences. These processes involve an all-against-all BLASTN or BLASTX of genes within assembled contigs, and potential HGT events are determined based on genes with the best hits in other taxonomic groups [236]. These approaches are limited by sequencing technologies and assemblers, which often fail to assemble long regions with high sequence similarity [234]. It can be assumed that inferring HGT events will become easier as the assembly of metagenomic sequences improves [237].

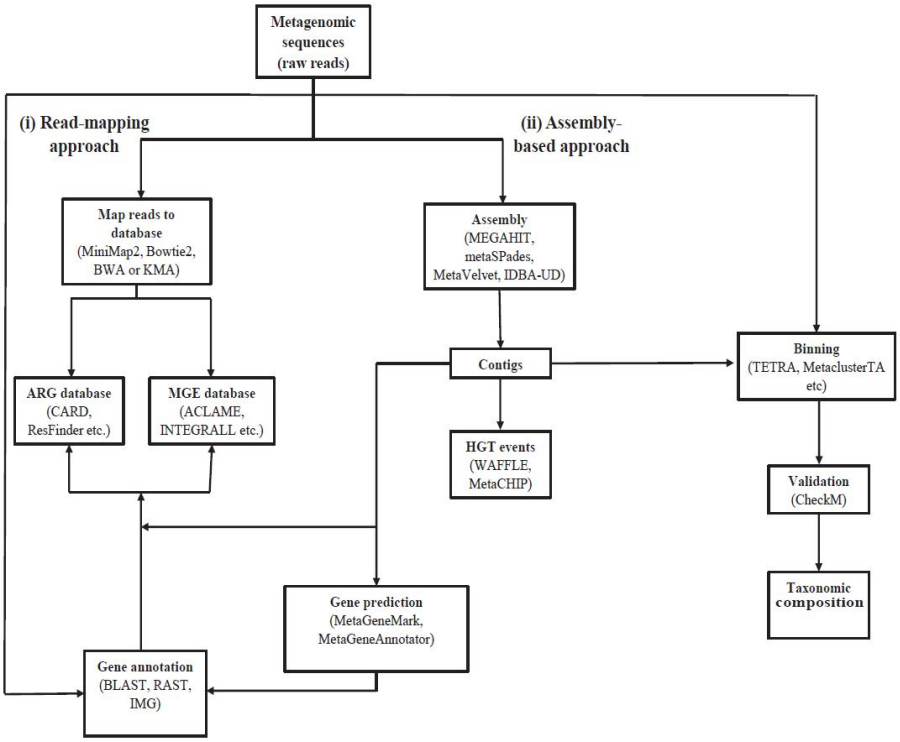

Figure 2.2: Workflow for determining antibiotic resistance genes, mobile genetic elements, horizontal gene transfer events and taxonomic composition using metagenomic data. The workflow is indicated by solid lines with example tools and databases annotated in the boxes. The complete lists are described in Tables 2.3 and 2.4.

2.7. MOBILE GENETIC ELEMENTS

Specialized MGEs mediate HGT between bacteria. These play an important role in bacterial ecology and evolution. Several factors impact HGT in bacteria, such as gene expression, protein connectivity, and biochemical properties; however, the range of genes carried on MGEs remains unknown [238]. MGEs are segments of DNA that encode enzymes and other proteins that mediate the movement of DNA within or between bacterial cells [233, 239]. MGEs carry genes other than those necessary for transfer and replication [233]. These accessory genes do not play a role in their vertical or horizontal transmission but affect the success of MGEs. The range of accessory genes encoded by MGEs and their ability to be phenotypically expressed in different genetic backgrounds are critical in its evolution [238].

The most important elements that play a role in HGT are the conjugative and mobilized elements. Mobilized elements contain all genetic information required to transfer from one bacterium to another, while conjugative elements use conjugation functions of elements such as plasmids and transposons to transfer from one host to another. These MGEs can carry multi-drug resistant (MDR) plasmids and give rise to MDR bacteria via HGT [229]. Bacteriophages also play a role in the spread of DNA by the transduction process. Some elements can translocate to new sites on a genome but cannot transfer to a new bacterial host. These include the transposons and mobile integrons [233, 240].

2.7.1. DETECTING MOBILE GENETIC ELEMENTS

The ability of MGEs containing ARGs to spread is controlled by the genetic elements and host factors. The genetic elements and host factors control the ability of MGEs containing ARG, but most important is the selective pressure in the environment. When antibiotics are present in the environment, there is strong selective pressure on the spread of resistance. Elements that promote resistance will be selected for, and those stopping the spread of mobile elements will be selected against [20, 30, 233]. To control the spread of AMR, it is important to understand MGEs and the ecology of the environments in which they spread easily [240, 241].

Several databases such as ACLAME [242, 243], ISFinder [244], INTEGRALL [245], and ICEberg [246] and web-based tools such as ISSaga2 [247], MobilomeFinder [248], oriTfinder [249], TnpPred [250] and MobileElementFinder [251] were developed to identify MGEs in metagenomic datasets [252]. These web-based tools and databases range from detecting plasmids, integrons, transposons, prophages, and insertion sequences.

Overall, there is no single solution for detecting MGEs as these databases, and web-based tools are incomplete and biased towards pathogens studied extensively. This makes it difficult and time-consuming as most web-based tools and databases need to be evaluated first and used in combination to provide an all-inclusive view on MGEs associated with AMR [253, 254]. This information can be applied to clinical and non-clinical sectors to potentially influence policies and strategies which can help control the spread of AMR [228].

2.8. OUTLOOK

Antibiotic resistance affects animals, plants, the environment and human health [255]. Monitoring and understanding the prevalence, mechanisms and spread of AMR is a priority in non-clinical sectors [37]. Since measures against AMR are focused on the clinical health sector, the environment is under-represented which allows for significant gaps in data, research and control strategies therefore failing to link the effects of AMR from one sector to the other [255]. Metagenomic sequencing allows for the detection of known and novel ARGs, HGT events, and associated MGEs in complex communities providing in-depth information on their prevalence, distribution, and transmission in non-clinical sectors such as the animal industry, agriculture, and the environment [34]. While metagenomics is promising, its implementation in non-clinical sectors is still in its early stages. Metagenomic sequencing creates a vast amount of data and requires specialized bioinformatic expertise, which makes this a costly approach [34, 47]. To date, only a few countries and laboratories have the resources and expertise to use metagenomic approaches as a surveillance system for AMR. In addition, bioinformatics methodologies need to be standardized and constantly updated and curated to allow for an accurate comparison between various samples, as the selection of bioinformatic tools and AMR databases does have a significant impact on the results [51, 102]. Nevertheless, using bioinformatic tools and databases can assist in identifying risky practices, effects of antibiotic usage, and hotspots of AMR. This can facilitate the design of new policies to control the spread of AMR between clinical and non-clinical sectors [123, 256]. Such knowledge and practices are urgent as these sectors, including the public health sector, are faced with a future of untreatable infections, increasingly costly medicinal treatment, a higher cost of living, and an increased mortality rate [256–258].

2.9. CONCLUSION

The application of metagenomic and bioinformatic approaches to AMR research can provide fast and reliable predictions of AMR and antibiotic use in various non-clinical sectors. These hold a great promise for understanding AMR molecularly, predicting outbreaks and transmission, and emerging pathogens. This information can lead to better policymaking in each sector and decrease the incidence of infections as conditions for the animal industry, agriculture, and the environmental sectors are improved.

2.10. DECLARATIONS

2.10.1. FUNDING

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3

METAGENOMIC ANALYSIS OF ANTIBIOTIC RESISTANCE ACROSS THE WASTEWATER PROCESS

This chapter has been accepted in Heliyon

Bacterial resistance to antimicrobials is a global health threat. Within the One Health context, water from regions with high antibiotic usage, such as clinical and urban areas, collects at wastewater treatment plants (WWTPs). In the WWTP, the activated sludge becomes a complex environment where various antimicrobials and microorganisms converge. While significant research has focused on the influent, activated sludge, and effluent, upstream and downstream sectors around the WWTP are often neglected. We conducted a systematic analysis using five publicly available metagenomic datasets (n=164) from different WWTP sectors and adjacent freshwater systems: upstream (n=14), influent (n=14), activated sludge (n=109), effluent (n=14), and downstream (n=13) to identify and characterise the microbiome, resistome, and mobilome. Opportunistic pathogenic bacteria, such as Pseudomonas, Aeromonas, and Acidovorax, were found in all WWTP sectors, with abundances exceeding 9% in the influent. ESKAPE pathogens, including Klebsiella pneumoniae and Enterobacter species, were identified in the effluent with abundances over 1%. We detected 230 antibiotic resistance genes (ARGs) throughout the WWTP. FTU and CKO β-lactamase gene families dominated the upstream, effluent, and downstream sectors, while the OXA β-lactamase gene family was highly abundant in the influent and activated sludge. ARGs, such as the OXA β-lactamase gene family, were linked to plasmids. Class-1 integrons, associated with the sul1 gene, a marker for anthropogenic pollution, were prevalent in the effluent and downstream sectors. Integrative elements (ICEclc, Tn4371, and PGI2), linked to ARGs, were identified in all sectors, increasing AMR dissemination. These integrative elements conferred resistance to antibiotics, including sulfonamides, tetracyclines and carbapenems. Our findings highlight the presence of ARGs and mobile genetic elements in WWTPs and nearby freshwater systems, raising concerns about AMR transmission to humans, animals, and the environment. This study emphasises the need for effective AMR monitoring and strategies in wastewater treatment to protect public and environmental health.

3.1. INTRODUCTION

Antimicrobial resistance (AMR) is responsible for at least 700,000 deaths annually worldwide. It is anticipated to rise to 10 million deaths per year by 2050, affecting all income regions [1]. The World Health Organisation has endorsed a global action plan to control antimicrobial resistance as infectious diseases caused by multidrug resistant (MDR) bacteria are one of the leading causes of death worldwide [2, 3]. The proliferation of antibiotic resistant and MDR bacteria is directly correlated to the use of antibiotics in clinical and non-clinical settings [4]. According to the One Health concept, wastewater treatment plants (WWTPs) should act as a barrier between sewage and the receiving environments as sewage contains a full spectrum of human microbiome-associated bacteria, antibiotic resistant bacteria and associated antibiotic resistance genes (ARGs) released by anthropogenic factors [4, 5].

WWTPs employ various treatment technologies and management practices. These practices can be broadly categorised into natural attenuation, advanced treatment processes and conventional practices which aim to remove solids, organic matter, nutrients and contaminants [6, 7]. Natural attenuation uses processes such as

dilution, biodegradation, and sorption, which are cost-effective and simple but inefficient in fully removing contaminants and microorganisms [7]. Advanced oxidation processes can break down antibiotics but may generate oxidation products that could introduce ARGs into microorganisms, particularly pathogenic bacteria [7–9]. Conventional treatment options, such as membrane treatments, act as robust yet costly barriers, producing brine water. In contrast, incorporating activated carbon is cost-effective but leads to sludge generation [5]. One of the most common biological processes in conventional WWTPs is the activated sludge process. This method is widely adopted for treating both municipal and industrial wastewater. However, it is concerning that despite the activated sludge process being effective in removing some ARGs, contaminants and microorganisms can persist in the treated water after the process [2, 5].

At present, WWTPs act as primary barriers against the spread of emerging contaminants such as antibacterial drugs causing antibiotic resistant bacteria [7]. However, these contaminants cannot be removed at low concentrations and removal efficiency is not satisfactory [7]. This inefficiency can result in the release of antibiotic resistant bacteria with the effluent into receiving environments e.g. rivers and lakes [5]. Moreover, WWTPs combined with activated sludge may enhance the spread of antibiotic resistant bacteria and ARGs into these overlooked environments [10, 11]. AMR surveillance has mainly focused on the influent, effluent, and activated sludge sectors, or their combinations. Che et al. [12] identified plasmids carrying ARGs conferring resistance to clinically relevant antibiotics e.g. tetracycline and macrolide-lincosamide-streptogramin in the aforementioned sectors increasing the risk of ARG-carrying bacteria spreading to rivers and lakes. Iwan et al. [13] found *E. coli* isolates increasing in resistance to antibiotic agents along the Tama River in Japan from the effluent of an urban WWTP to the downstream river. Similarly, sulfonamide levels were similar in upstream and downstream sectors of a feedlot WWTP however, the sulfonamide-resistant microorganisms and gene abundances were higher in the downstream river [14]. The presence of antibiotic agents, antibiotic resistant bacteria and ARGs present in the upstream and downstream sectors contributes to the rise of AMR and enhances horizontal gene transfer (HGT) via mobile genetic elements (MGEs) such as plasmids, integrons and transposons [15–18]. AMR surveillance should consider adjacent areas of the WWTP, such as the upstream freshwater system and downstream receiving environmental sectors, to fully address the AMR spread (Figure 3.1).

This study aims to analyse publicly available metagenomic datasets from various sectors of wastewater treatment plants (WWTP) — including upstream, influent, activated sludge, effluent, and downstream — across different geographical locations. Using a standardised pipeline, this study aims to identify bacterial populations, antibiotic resistance genes, and mobile genetic elements in each sector and examine their role in the spread of AMR into receiving environments.

3.2. METHODS AND MATERIALS

3.2.1. DATA SOURCES

164 whole-metagenome sequences representing the wastewater treatment plant were selected for this study. These included upstream (n=14), influent (n=14), activated sludge (n=109), effluent (n=14), and downstream (n=13) [19–24]. All raw reads were publicly available and obtained from NCBI-SRA (https://www.ncbi.nlm.nih.gov/sra). A combination of the following keywords was used to find whole metagenome sequences, these included "upstream", "influent", "activated sludge", "effluent", "downstream", "whole genome", "metagenome", "wastewater", "wastewater treatment plant". The identified samples were then filtered based on the sequencing technology used, specifically selecting only those generated using Illumina technology. Additionally, only samples with accompanying publications were included. Samples that did not meet these criteria were discarded.

Samples obtained were from different studies conducted in various geographical locations (Figure 3.1). Influent and effluent samples were obtained from urban and industrial sources from Portugal, Colombia, China and South Korea, upstream and downstream samples were obtained from a river source in Spain and South Korea and lastly, activated sludge samples were obtained from municipal, urban and industrial sources from China and Colombia. Samples were categorised into five sectors: upstream, influent, activated sludge, effluent and downstream based on the sample source descriptions. Upstream samples were obtained from freshwater systems over 50m away from the WWTP and in the opposite direction of water flow. Influent samples consisted of raw sewage samples taken before the treatment process whereas activated sludge samples were collected from reactors during biological treatment. The effluent samples consisted of treated water flowing out of the WWTP and the downstream samples were collected from freshwater systems more than 50m away from the WWTP, following the flow of water (Figure 3.1). Detailed information about the metagenomes retrieved from the database is included in Supplementary Table S1.

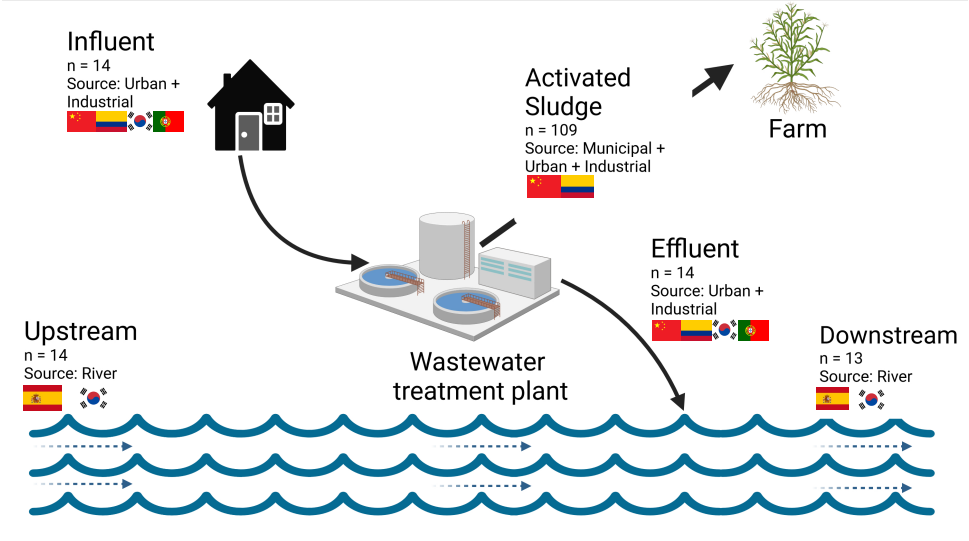

Figure 3.1: The process of the wastewater treatment plant. The upstream sector includes freshwater systems such as streams, lakes and rivers that flow toward the wastewater treatment plant (dotted arrows). The wastewater treatment plant begins with the sewage (influent) from urban and municipal areas which flow to the wastewater treatment plant (black arrows). Sewage that flows into the wastewater treatment plant can be treated by the biological treatment process i.e. activated sludge, to treat the wastewater. Treated water is released as effluent which flows downstream areas such as rivers and lakes and can be used in farming. Each flag represents the geographical location from where the samples were taken, and the sample's source is indicated above the flags. Influent and effluent samples were obtained from urban and industrial sources from Portugal, Colombia, China and South Korea, upstream and downstream samples were obtained from a river source in Spain and South Korea and lastly, activated sludge samples were obtained from municipal, urban and industrial sources from China and Colombia.

3.2.2. BIOINFORMATIC ANALYSIS

The quality of the reads was assessed using FastQC v.0.11.9 with default parameters [25]. Paired-end reads were trimmed and filtered by Trimmomatic v.0.39 [26] while specifying the ILLUMINACLIP: adapters.fa:2:30:10 LEADING:3 TRAILING:3 MINLEN:30 SLIDINGWINDOW: 4:20 parameters. The microbiome was profiled by Kraken2 v.2.1.0 [27] using the minikraken2 microbial database (2019, 8GB). Species confirmation and the estimation of abundances were done by Bracken v.2.6.2 for each sample across all studies and sectors [28]. The average relative abundances of bacterial phyla, families, genera, and species were calculated across all studies

within each sector to characterise the microbial populations present in each sector of the wastewater treatment plant (WWTP).

Average relative abundances of the bacterial species detected in each sector were used to calculate the beta-diversity between sectors of the WWTP using the R package vegan v.2.6-4. The beta-diversity of the microbiome between the different sectors of the WWTP was visualised using non-metric multidimensional scaling (NMDS) analysis with Bray-Curtis dissimilarity. Spearman's correlation analysis was used to analyse the correlation between the overall microbial communities and specifically ESKAPE pathogens found in each sector of the WWTP using the scipy.stats package in Python.

Filtered paired-end reads were *de novo* assembled using metaSPAdes v.3.15.2 [29] on meta mode with default parameters. The assembled metagenomic contigs were annotated to determine the antibiotic resistance gene families and drug classes using the Comprehensive Antimicrobial Resistance Database Resistance Gene Identifier (CARD-RGI) v.3.1.2 with default parameters including loose matches and the –low_quality –clean options [30]. Furthermore, the average relative abundances of the annotated antibiotic resistance genes in each sector were assessed. This data was normalised based on the total number of antibiotic resistance genes detected in each sector, indicating their respective proportions. Spearman's correlation analysis was used to analyse the correlation between antibiotic resistance gene families found in each sector of the WWTP using the scipy.stats package in Python.

Metagenomic contigs were aligned to detect integrons and integrative elements using the INTEGRALL database [31] and the ICEberg database [32]. Alignments were performed by BWA-mem v.0.7.10 with default parameters [33], generating a SAM file. The output SAM file was converted into a BAM file using SAMtools version v.1.9 [34]. Contigs that aligned to the databases were considered integrons and integrative elements. SAM output files containing the detected integrative elements were converted to .txt files and matched to the output .txt files generated with CARD-RGI from their respective WWTP sector. This process was conducted to determine which integrative elements carried antibiotic resistance genes. Contigs were classified as plasmid-originating using Plasclass v.0.1.1 [35]. To confirm that contigs originated from plasmids, a threshold was set with a minimum contig length of $\geq$ 1000 and probability of $\geq$ 0.75. Detected plasmids were matched to the previously generated CARD-RGI output .txt files to determine if the detected plasmids were linked to the antibiotic resistance genes previously found.

3.3. RESULTS AND DISCUSSION

Publicly available metagenomic data representing different sectors (upstream, influent, activated sludge, effluent and downstream) of the WWTP were analysed to determine the differences in the microbial communities, antibiotic resistance genes and mobile genetic elements that facilitate the spread of AMR.

3.3.1. OPPORTUNISTIC BACTERIAL PATHOGENS ARE PRESENT ACROSS THE WWTP PROCESS

We analysed the bacterial taxonomic composition in each sector of the WWTP. The main goal of the WWTP is to reduce pollutants and pathogens through chemical, biological, or physical processes, releasing treated water as effluent into downstream systems [36]. Our study classified bacterial taxa at the phyla, family, genus, and species levels to assess the microbial composition and determine the average relative abundances of environmental, pathogenic, and emerging pathogenic bacteria in each sector of the WWTP. Data on the phyla, families, genera and species can be found in Supplemental Tables S2 - S5.

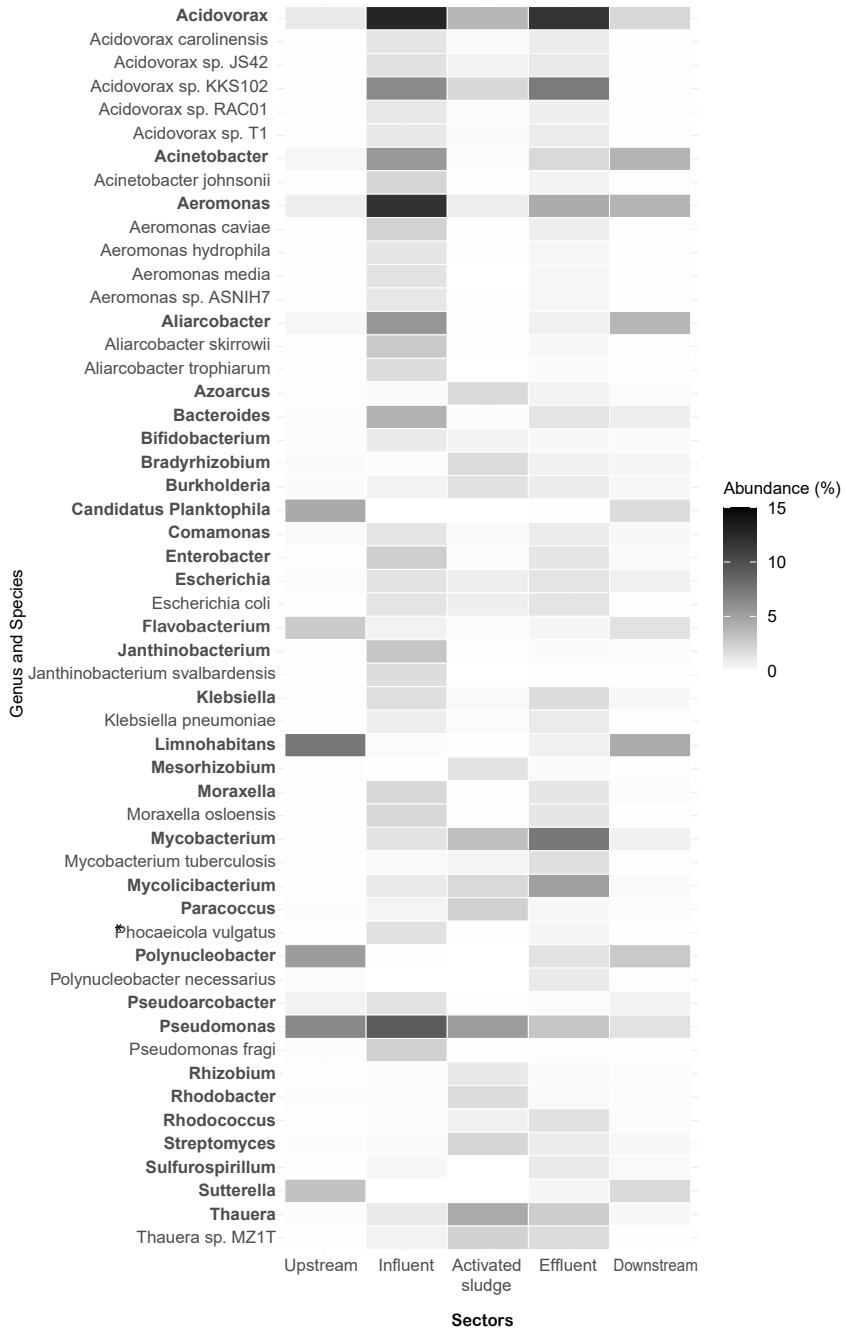

Figure 3.2: Heatmap of the relative abundances (over 1%) of all bacterial genera and species identified in each sector of the wastewater treatment plant. Bacterial genera are indicated in bold text. Respective species belonging to its genus that have an abundance of over 1% are listed below the genus in plain text. Abundances are indicated in greyscale, low = white and high = black. **Phocaeicola vulgatus* has previously been identified as *Bacteroides vulgatus* in the Bacteroides genus.

Figure 3.2 illustrates the presence of opportunistic pathogens throughout the WWTP process. In our study, the *Pseudomonas*, *Aeromonas* and *Acidovorax* genera were present in each sector of the WWTP with abundances exceeding 9% in the influent (Supplementary Table S4 and Figure 3.2). Specifically, we identified the presence of *Aeromonas caviae* (*A. caviae*) (Figure 3.2), a human pathogenic bacteria causing gastrointestinal infections, in the influent (2%), effluent (1%) and downstream (1%) sectors (Supplementary Table S5). Other pathogenic bacterial species detected included *Acinetobacter johnsonii* (*A. johnsonii*), *Aliarcobacter skirrowii* (*A. skirrowii*) and *Aliarcobacter trophiarum* (*A. trophiarum*) which had an abundance of 2% in the influent and less than 1% in the effluent and downstream sectors. Despite low abundances downstream, these bacterial species still pose a zoonotic potential as studies have linked them to meningitis and gastrointestinal infections in humans [7, 37–40].

In the activated sludge sector, we observed a blend of pathogenic and non-pathogenic bacteria, including those crucial for biological treatment. In our study, the *Pseudomonas aeruginosa* (*P. aeruginosa*) (1.61%), *Thauera sp. MZ1T* (*T. sp. MZ1T*) (3%) and *Acidovorax sp. KKS102* (*A. sp. KKS102*) (12%) species dominated the activated sludge sector (Figure 3.2). Phyla such as Proteobacteria, Bacteroides, Actinobacteria, Acidobacteria, Chloroflexi, and Planctomycetes are commonly found in activated sludge further confirming the mixture of bacteria in the activated sludge with bacteria commonly found in the influent (Supplementary Table S2) [41–44]. Given that bacteria play a vital role in biological treatment, the microbial community within the activated sludge is a key factor in evaluating WWTP efficiency. This is particularly significant as the efficiency depends on the microbial community's capacity to degrade pollutants and xenobiotic compounds like pesticides [45].

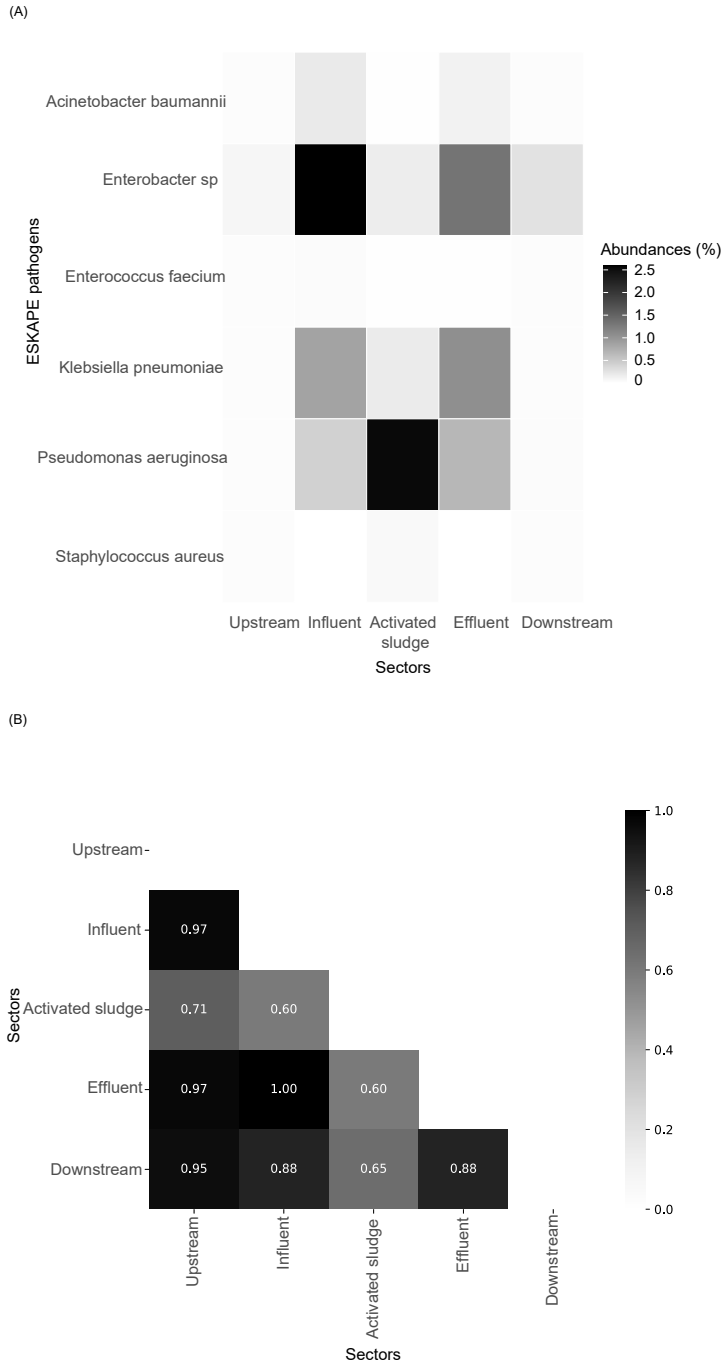

Figure 3.3: (A) Heatmap of the relative abundances of ESKAPE pathogens identified in each sector of the wastewater treatment plant. Abundances are indicated in greyscale, low = white and high = black. (B) The Spearman's correlation analysis of the ESKAPE pathogens present between each sector of the wastewater treatment plant. The correlation rank values are depicted in text and indicated by the intensity of grey.

We identified ESKAPE *(Enterococcus faecium* (*E. faecium*), *Staphylococcus aureus* (*S. aureus*), *Klebsiella pneumoniae* (*K. pneumoniae*), *Acinetobacter baumannii* (*A. baumannii*), *P. aeruginosa* , and *Enterobacter* spp.) pathogens in the different sectors of the WWTP process in Figure 3.3A. *K. pneumonia* was found at an abundance of over 1% in the effluent with *P. aeuroginosa* exceeding 1% in the activated sludge sector. *Enterobacter* spp. showed an abundance exceeding 1% in both the influent and effluent sectors with a high correlation between both sectors (Figure 3.3B). In all remaining sectors, these bacteria were present at abundances below 1%. Other ESKAPE pathogens, including *S. aureus*, *A. baumannii*, and *E. faecium*, were also present in all WWTP sectors (≤1%).

A high correlation between ESKAPE pathogens was observed across sectors of WWTP, e.g. influent and effluent (1.00), upstream and influent (0.97), and effluent and downstream (0.88), indicating that ESKAPE pathogens in the upstream and influent sectors could predict their presence in effluent and downstream sectors. Correlations of all sectors with activated sludge were lower with higher variability. Treatment seems to reduce the abundance of pathogens, but it does not eliminate them as seen in the correlation between upstream-to-effluent (0.97). Moreover, the strong effluent-to-downstream correlation highlights the environmental and public health risks posed by residual ESKAPE pathogens in the treated effluent. Despite the microbial load reduction achieved by WWTPs, previous studies have highlighted the release of ESKAPE pathogens in the effluent [46]. These bacterial pathogens can harbour multiple ARGs, conferring resistance to a broad spectrum of antibiotics, including last-line drugs such as carbapenems and glycopeptides. The potential for these pathogens to spread to other environments, animals, and humans poses a significant threat to public and environmental health [17, 47–49].

Lastly, *Escherichia coli* (*E. coli*), a key microbial indicator of water quality, was detected in both the influent and effluent (≥1%). *E. coli* may enter freshwater systems such as upstream sectors through sources such as wildlife or agricultural runoff indicating low water quality however, its presence in the influent is expected. Unfortunately, its detection in the effluent (1.42%) and downstream (≤1%) is concerning [50]. Both *E. coli* and *E. faecium*, also detected in this study, are recognised as indicators of faecal contamination [51]. Previous research suggests that *E. coli* levels in the effluent can vary depending on the geographic location of the samples. WWTPs treating hospital waste may discharge higher levels of *E. coli* compared to those handling urban or municipal effluent, as analysed in our study [52]. In untreated sewage, *E. coli* and *Enterococci* signal potential health risks due to their high concentrations in mammalian faeces [53]. Effective WWTPs should reduce such contaminants, minimising risks to human health and the environment. However, operational issues such as poor maintenance, design flaws, environmental factors, and chemical or biological conditions can enable *E. coli* to survive in the effluent [54, 55].

It should be noted that variations in bacterial communities and abundances across different sectors of the WWTP process can stem from various selection pressures or the use of public data from various studies. Environmental factors such as heavy metals, organic compounds, disinfectants, as well as the presence of diverse

bacterial types: environmental, pathogenic, non-pathogenic, human, and animal commensal bacteria can play a role in determining the bacterial communities present at different sectors [56, 57]. Additionally, data sourced from different urban and municipal WWTPs introduces variability due to factors like geographic location, climate, industrial practices, local regulations, and the distinct sample collection and processing methods used in various studies. These influences can impact microbial communities and may support the emergence of antibiotic resistant bacteria and genes, as observed with pathogens like *P. aeruginosa* and *K. pneumoniae* detected in our study.

3.3.2. SIMILAR BACTERIAL SPECIES PRESENT IN THE INFLUENT, ACTIVATED SLUDGE AND EFFLUENT

We assessed differences in microbial communities within each sector by measuring the Beta-diversity using the Bray-Curtis dissimilarity index. This index quantifies the "distance" between microbial communities based on the species abundances. Visualised with non-metric multidimensional scaling (NMDS), a smaller distance between samples within the same sector indicates greater similarity between microbial communities and their respective sectors [58]. Furthermore, we used Spearman's correlation analysis to validate the similarity in the microbial communities between each sector of the WWTP and identified the common bacterial species (Supplementary Table S6).

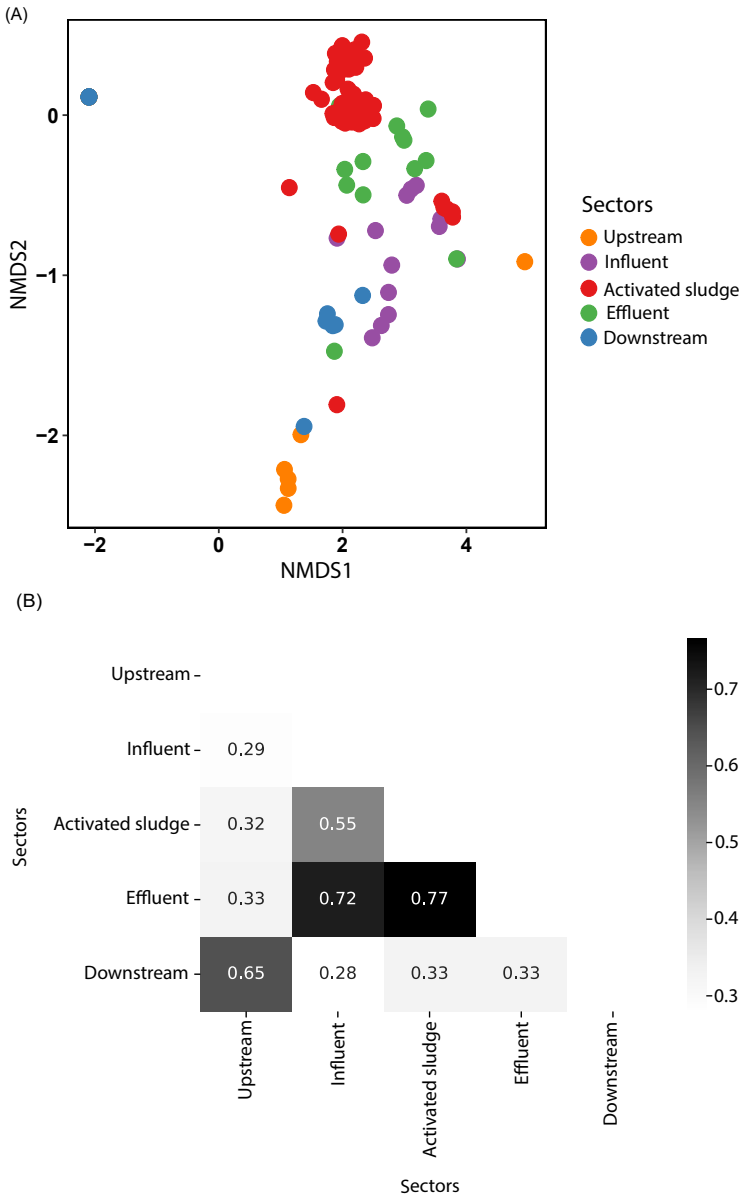

Figure 3.4: (A) Nonmetric multi-dimensional scaling ordination (NMDS) of Bray-Curtis dissimilarities of the microbial community between the different sectors of the wastewater treatment plant. The samples for each sector of the wastewater treatment plant are shown in different colours, upstream = orange, influent = purple, activated sludge = red, effluent = green, and downstream = blue. (B) Spearman's correlation analysis of the microbial communities present between each sector of the wastewater treatment plant. The correlation rank values are depicted in text and indicated by the intensity of grey.

Figure 3.4A, shows the NMDS plot representing the microbial community similarities within each WWTP sector. Each colour represents the samples from their respective WWTP sector. Samples from the influent (purple) and effluent (green) relatively cluster together while samples from the activated sludge (red) form a cluster close to the effluent.

Similar bacterial species are found in the influent, activated sludge and effluent. Firstly, the clustering of the activated sludge samples suggests its microbial community is determined by the biological treatment processes such as operational parameters of bioreactors, characteristics of the influent and the environment [59]. Additionally, the effluent (green) and influent (purple) cluster closely together with the activated sludge (red) indicating similarity between groups of bacteria in each sector. This suggests that urban and municipal areas can share common microbial communities regardless of the geographical location from which the samples are obtained. Downstream (blue) and upstream (orange) samples cluster further away from the influent, activated sludge and effluent clusters, suggesting that these microbial communities are likely shaped by the freshwater taxa and or the exposure to human activity [60].

Further analysis into the microbial communities and the WWTP sectors was done using Spearman's correlation (Figure 3.4B). A strong correlation was found between the microbial communities of the influent, activated sludge and effluent (≥0.5) indicating that these three sectors contain similar bacterial species irrespective of the geographical location and sampling times (Supplementary Table S1). Additionally, a strong correlation of 0.65 was observed between the upstream and downstream sectors which can be attributed to similar bacterial species present between the two sectors due to inefficient removal as the samples originated from the same studies (Supplementary Tables S1 and S6). We identified 42 common bacterial species with an abundance of over 0.01% in the influent, activated sludge and effluent with *A. sp. KKS102* being dominant (≥1% abundance). A list of the common bacterial species are present in Supplementary Table S6. The similar bacterial species identified in the influent, activated sludge and effluent are due to the mixture of aerobic, anaerobic, and facultative bacteria. Even in low abundances, these bacteria can be released with the effluent, contributing to the potential spread of AMR [61].

3.3.3. VARIOUS β-LACTAMASE GENE FAMILIES PRESENT IN THE WWTP

We investigated the average relative abundances of AMR gene families and identified similar AMR gene families throughout the WWTP process. These genes can exist in low abundances, even in treated water, conferring resistance against single or multiple antibiotic drug classes. We highlight two major examples observed in our data i.e., the β-lactam and efflux pump gene families (Figure 3.5 and Supplementary Table S7).

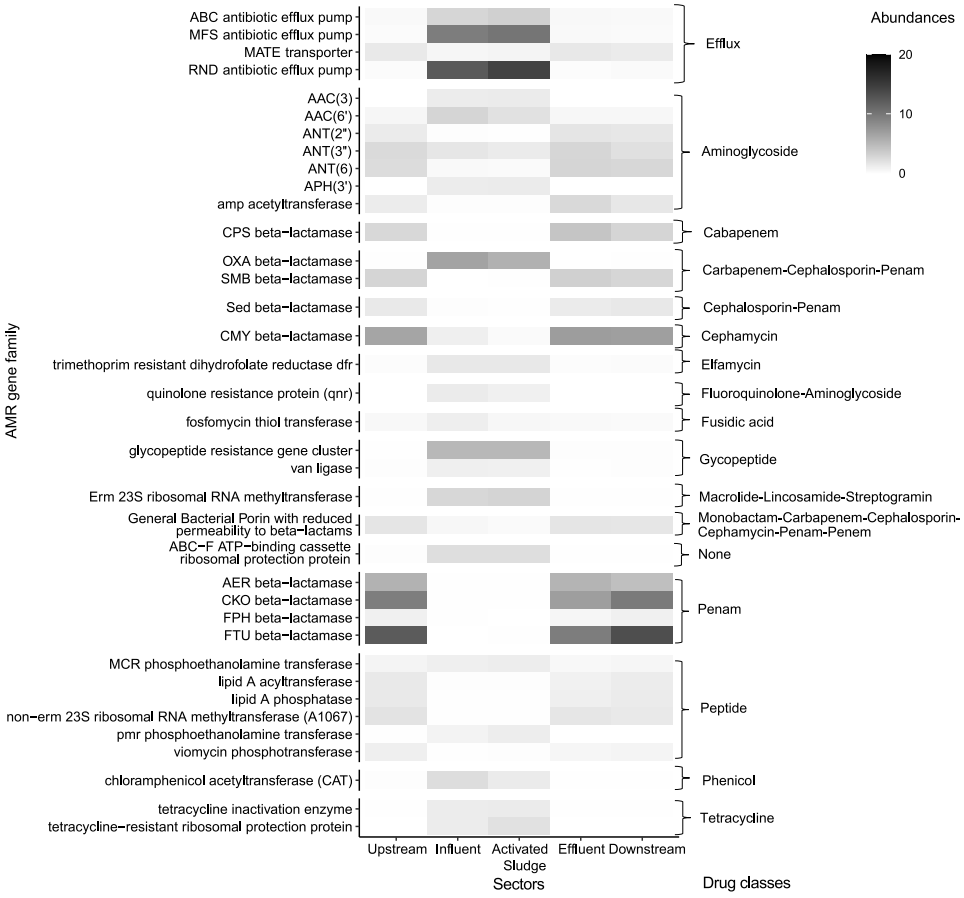

Figure 3.5: Heatmap of the abundances of antimicrobial resistance (AMR) gene families over 1% in each sector of the wastewater treatment plant (upstream, influent, activated sludge, effluent, downstream) and corresponding drug classes. Abundances are indicated by greyscale, low = white and high = black.

Firstly, β-lactam antimicrobial agents are widely used to treat bacterial infections, but their extensive use has significantly contributed to the global rise of resistance to these antibiotics in bacteria. β-lactamase gene families, including those encoding ESBLs (extended-spectrum β lactamases), contribute to this resistance by conferring single and multidrug resistance to commonly used penicillins, as well as potent antibiotics such as carbapenems, cephalosporins and penams [62].

Figure 3.5 shows several β-lactamase gene families throughout different sectors of the WWTP process. Among these, the OXA β-lactamase gene, associated with the ESBL class D group, was detected across all sectors, constituting 7% of AMR gene families in the influent and 6% in the activated sludge, but less than 0.05% in upstream, effluent, and downstream sectors. Its prevalence was notably high in 100% of upstream samples, over 95% in influent and activated sludge and over 60% in effluent and downstream samples. This variation can be attributed to several factors such as the efficiency of the WWTP in reducing the concentration of β-lactamase gene families therefore lowering its prevalence in the effluent and downstream samples. Environmental factors, such as pH and temperature, may also affect the survival and detectability of these genes. Additionally, differences in sampling methods and detection sensitivity across studies might contribute to the observed variations in the gene prevalence [54, 63]. The OXA β-lactamase gene family can be detected in non-pathogenic and environmental bacteria present in aquatic areas and may behave as a reservoir for emerging antibiotic resistance [64]. The OXA β-lactamase gene family can inactivate a variety of β-lactam antibiotics including penicillins, cephalosporins, carbapenems and extended-spectrum penicillins such as piperacillin [65, 66]. Additionally, the OXA β-lactamase gene family has been identified in various bacterial species, particularly within the Enterobacteriaceae family and non-fermenting Gram-negative bacteria such as *A. baumannii* and *P. aeruginosa* [65]. Furthermore, we detected the OXA β-lactamase gene family in the *Pseudomonas*, *Enterobacter*, *Klebsiella* and *Acinetobacter* genera in all sectors of the WWTP (Figure S2).

Conversely, the FTU and CKO β-lactamase gene families, known for conferring resistance to penam antibiotics, were detected predominantly in the upstream, effluent, and downstream sectors. The FTU β-lactamase gene family showed an abundance of over 9% in these sectors, present in more than 14% of upstream and downstream samples and 35% of effluent samples. Similarly, the CKO β-lactamase gene family was present in 45% of effluent samples and less than 14% in upstream and downstream samples. The FTU β-lactamase gene families are categorised as class A β-lactamase which has been identified in *Francisella tularensis* subsp. *holarctica* LVS (*F. tularensis subsp. holarctica* LVS) and produces the FTU-1 enzyme that functions to hydrolyse various β-lactam antibiotics (penicillins and cephalosporins) [67, 68]. Similarly, the CKO β-lactamase gene family, identified in *Citrobacter koseri* (*C. koseri*), produces the CKO-1 enzyme [69]. The presence of both FTU and CKO β-lactamase gene families allows for bacteria to survive by breaking down β-lactam antibiotics rendering them ineffective. Overall, the detection of various β-lactamase gene families across different sectors is concerning as it facilitates the dissemination of multidrug resistance, posing a threat to both clinical treatment effectiveness and

environmental health.

Secondly, efflux pump gene families, including major facilitator superfamily (MFS) and resistance-nodulation cell division (RND), play crucial roles in multidrug resistance among pathogens like *Pseudomonas* and *Streptococcus* [17, 70–72]. The presence of these gene families in the effluent can increase the incidence of bacteria selecting them. Efflux pump gene families can be located on MGEs and can efflux clinically important antibiotics such as tetracycline out of the bacterial cell wall [17, 73–75].

We identified the MFS and RND efflux pump gene family in every sector of the WWTP (Figure 3.5 and Supplementary Table S7). In our study, the MFS and RND efflux pump gene families comprised 10% - 12% of the total AMR gene families in the influent, 10% - 14% in the activated sludge and less than 0.5% of the total AMR gene families in the upstream, effluent and downstream sectors. The MFS and RND gene families were detected in all upstream samples and 90%, 98%, 70% and 60% of the influent, activated sludge, effluent and downstream samples, respectively. The MFS and RND gene families present in all sectors of the WWTP are common as these genes exist naturally in the chromosomes of bacteria. The MFS efflux pump functions to remove substrates such as antibiotics, sugars and ions out of the bacterial cell leading to cellular homeostasis and antibiotic resistance. Similarly, the RND efflux pump can expel antibiotics, dyes and detergents from the bacterial cell and aids in detoxifying the cell from harmful substances [76, 77].

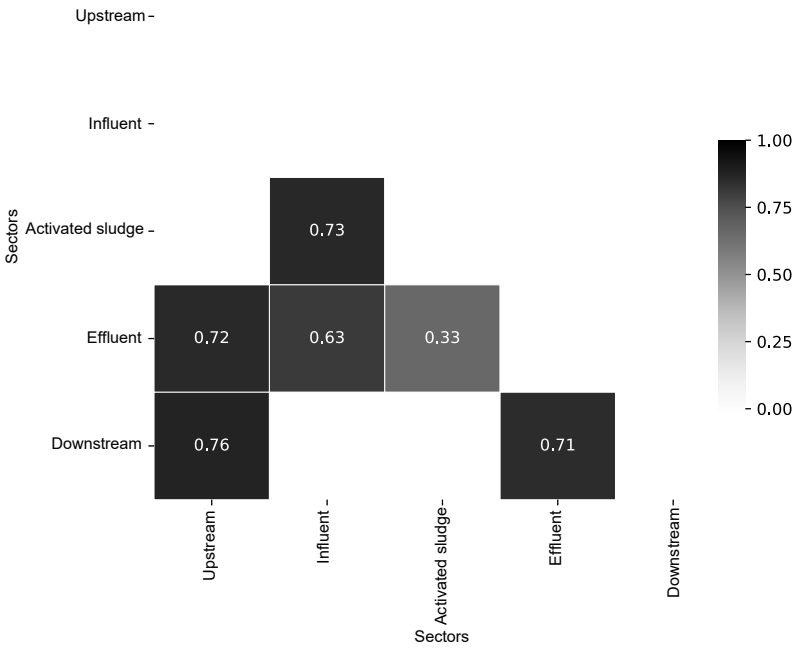

Figure 3.6: Visualisation of the Spearman's correlation analysis of the AMR gene families present between sectors that shared samples of the wastewater treatment plant. The correlation rank values are depicted in text and indicated by the intensity of grey.

Similar AMR gene families were present in the upstream, effluent and downstream sectors and in the influent and activated sludge sectors as illustrated in Figure 3.5 and 3.6. Overall, the AMR gene families identified in these sectors were positively correlated (Figure 3.6). The similarity between sectors could be due to the inefficient treatment of wastewater. However, the correlation had to be calculated within studies potentially confounding regional effects with sectorial effects. Furthermore, the presence of similar AMR gene families that can be found in environmental freshwater systems such as the CMY β-lactamase gene family, leading to high similarity between the AMR gene families found between upstream and effluent samples (0.72) [78]. A full Spearman correlation of sectors that share studies can be found in Figure S3.

A list of common AMR gene families can be found in Supplementary Table S9. Differences in the abundance and presence of AMR gene families in each sector of the WWTP can be influenced by the sector-specific microbial community and environmental factors i.e., pollutants and organic matter. Additionally, samples from various urban and municipal studies, geographical locations, and time points could have introduced differences in the AMR gene families detected within the WWTP

process sectors. However, sectors such as the influent and the activated sludge consist of a mixture of bacteria and pollutants therefore AMR gene families like the OXA β-lactamase gene family may be present in the bacterial genome. This can potentially be exchanged within the microbial population contributing to their high abundance [79]. Bacteria carrying these ARGs have the potential to survive the WWTP and spread AMR to different bacteria in different sectors [79–81].

A total of 230 AMR gene families were identified in the WWTP process, providing resistance to 25 single antibiotic drug classes and 32 combination/multiple drug classes. Additionally, the *Pseudomonas* genus harboured numerous AMR gene families including various β-lactamase gene families, yet no single ARG exceeded a 1% abundance. (Refer to Supplementary Tables S7 - S9 and Figures S1 - S2 for detailed data).

3.3.4. PLASMIDS WITH ARGS ARE RELEASED INTO THE ENVIRONMENT

We analysed metagenomic assembled contigs from different sectors of the WWTP to determine the percentage of contigs originating from plasmids and their association with AMR gene families. Plasmids, crucial for acquiring and accumulating ARGs, maintain stability, persisting even after wastewater treatment [82].

In each sector of the WWTP, 1% of contigs were classified as plasmid originating. Further analysis showed that AMR gene families were found in 0.008% - 0.15% of plasmid-contigs. More specifically, we observed a high average relative abundance of plasmid-contigs associated with the previously mentioned OXA β-lactamase gene family conferring multidrug resistance. Four other AMR gene families conferring resistance to single antibiotic drug classes were detected in a high abundance (Supplementary Table S10).

Plasmids associated with AMR gene families like the OXA β-lactamase have a greater chance of spreading AMR from the effluent to freshwater systems and capturing new ARGs. This is a potential route for transmission of multidrug resistant plasmids to other environments such as soil and animals, therefore, leading to resistance to veterinary and clinically significant antibiotics [82–84].

In our study, the OXA β-lactamase gene family represented more than 6% of the total plasmids associated with AMR gene families in every sector of the WWTP (Supplementary Table S10). Plasmids associated with the OXA β-lactamase gene were present in 76%, 71%, 72%, 100%, and 46% of samples from upstream, influent, activated sludge, effluent and downstream, respectively, suggesting that plasmids associated with the OXA β-lactamase gene are released in the effluent and can be detected downstream.

While the presence of plasmids conferring resistance to multiple drug classes is concerning, plasmids associated with a singular antibiotic drug class can spread AMR. In this study, plasmids were associated with AMR gene families of a single antibiotic drug class (tetracycline resistant ribosomal protein (*Tet(O)*), aminoglycoside resistance gene (*ANT(3")*), trimethoprim resistant dihydrofolate reductase (*dfr*) and the sulfonamide resistance gene (*sul1*) were detected. While the abundances of plasmids associated with these AMR gene families vary in each sector of the WWTP, their presence can stimulate HGT (Supplementary Table S10) [41, 85–89].

Plasmids carrying ARGs can nullify the effectiveness of commonly used antibiotics. The presence of antibiotic residues in the effluent and downstream freshwater environments further enhances the mobility and stability of plasmids. This promotes induced resistance and genetic exchange among bacterial communities through plasmids and other MGEs as documented in studies by Rahube et al. [90], Wang et al. [81] and Calderón-Franco et al. [91].

3.3.5. CLASS 1 INTEGRONS REVEAL THAT AMR POLLUTION IS PRESENT IN EACH SECTOR AROUND THE WWTP

We investigated the presence of integrons and their association with ARGs in each sector of the WWTP. Integrons are responsible for AMR dissemination via HGT, which can occur between bacteria in all environments. In this study, we searched for integrons in contigs from each sector of the WWTP and classified them as classes 1, 2, 3, unknown, and unclassified (Table 3.1).

Table 3.1: The average relative abundance (%) of the different classes of integrons in each sector of the WWTP. Integrons were classified as class 1, class 2, class 3, unknown and unclassified in the upstream, influent, activated sludge, effluent and downstream sectors of the WWTP.

Classes of integrons	**Upstream**	**Influent**	**Activated sludge**	**Effluent**	**Downstream**
Class 1	17.37%	16.94%	17.39%	19.18%	15.12%
Class 2	2.08%	2.32%	174%	1.92%	1.74%
Class 3	2.87%	3.57%	2.17%	3.92%	2.59%
Unknown	0.00%	0.06%	0.00%	0.00%	0.05%
Unclassified	77.68%	77.11%	78.71%	74.98%	80.50%

In our study, less than 1% of contigs (upstream: n = 1779, influent: n = 6858, activated sludge: n = 16137, effluent: n = 4104, downstream: n = 2010) were integrons. We classified the integrons into class 1, class 2, class 3, unknown and unclassified. Almost 80% of detected integrons remained unclassified. Class 1 and 3 integrons, commonly found in Proteobacteria across freshwater, soil, and biofilm environments, hold clinical significance, while class 2 integrons are prevalent in marine Gamma-Proteobacteria. Table 3.1 shows class 1 integrons were the most abundant, ranging from 17 - 19% in each sector of the WWTP, while class 2 and 3 integrons accounted for 2 - 4%. All classes were present in over 70% of samples in all sectors. The presence of class 1 integrons in all sectors of the WWTP is similar to the findings of Makowska et al. [92].

Class 1 integrons are associated with disinfecting agents and sulfonamide resistance [93]. We detected class 1 integrons with *qacE*-related disinfectant resistance genes throughout the WWTP sectors, whereas those carrying *sul1* and *sul3* genes for sulfonamide resistance were more abundant (Supplementary Table S11). Specifically, 31% of class 1 integrons in the upstream sector were linked to the *sul3* gene. The *sul1* gene was absent in the upstream sector but made up more than 25% of the

class 1 integrons present in the influent, activated sludge, effluent, and downstream sectors. The *sul1* and *sul3* genes, associated with class 1 integrons, were detected in over 90% of samples from the influent, activated sludge, and effluent sectors. In the downstream sector, approximately 60% of samples contained the *sul1* gene and 20% contained the *sul3* gene.

Class 1 integrons in combination with *qacE* and *sul1* act as a pollution marker and are commonly present in sewage areas like the influent [94]. In our study, sulfonamide resistance associated with class 1 integrons was high in the influent, activated sludge, effluent and downstream sectors. These sectors are prone to increased antibiotic resistance due to various factors, such as pollutants, disinfectants, organic matter, and the bacterial community present. Since sulfonamide antibiotics can persist in the environment for extended periods, the *sul1* gene will be frequently detected in the effluent, activated sludge, influent, and receiving waters (Supplementary Table S11). While we observed the presence of class 1 integrons in the downstream sector, these integrons are naturally prevalent in freshwater systems as well [93, 95, 96].

3.3.6. INTEGRATIVE ELEMENTS ARE LINKED TO ARGS IN EACH SECTOR OF THE WWTP

We identified integrative elements i.e., integrative and conjugative elements (ICEs), integrative and mobilisable elements (IMEs) and *cis*-integrative and mobilisable elements (CIMEs) in each sector of the WWTP. Since these self-transmissible elements can carry ARGs, we also determined if the integrative elements detected were linked to the ARGs previously identified in this study.

Integrative elements, comprising approximately 1% of contigs, were detected across all sectors of the WWTP, with varying average relative abundances ranging from 16.87% in activated sludge to 57.89% downstream (Table 3.2). Contrary to Calderón-Franco et al. [97], in our study, 80% of the integrative elements in activated sludge were IMEs.

ICE families linked to bacterial adaptation were identified in the WWTP. The ICE*clc* and Tn4371 families were prevalent in over 95% of all samples across WWTP sectors, with similar abundances observed in each sector (Supplementary Table S12). The ICE*clc* family facilitates bacterial adaptation by transporting and transferring ARGs between bacterial species including *Aeromonas* and *Pseudomonas*, as detected in this study. ICE*clc* utilises compounds like chlorocatechols, present in both polluted and freshwater environments to enhance bacterial proliferation, thereby promoting horizontal gene transfer [98]. Similarly, Tn4371 elements were widespread throughout the WWTP process, constituting approximately 40% of the ICE families in each sector. Tn4371 is commonly found in aquatic environments and man-made settings like sewage and industrial waste. Following processing in wastewater treatment plants, Tn4371 continues to harbour a significant load of ARGs [99, 100]. Both ICE families, prevalent in clinical and veterinary sectors, maintain the stability of accessory genes like ARGs within-host bacterial species.

IMEs were detected in every sector of the WWTP, and PGI2 was present in over 90% of samples across all sectors, showing consistent abundances (Supplementary Table S14). Like other MGEs found previously, IMEs can carry ARGs post-WWTP

processing, contributing to bacterial multidrug resistance. PGI2, a genomic island characterised in a previous study, can harbour 14 different resistance genes, highlighting its role in promoting bacterial evolution towards multidrug resistance [101].

Table 3.2: The total number of each integrative element detected in each sector of the wastewater treatment plant including the total number and percentage (%) of each integrative element carrying antibiotic resistance genes (ARGs). Integrative elements include integrative and conjugative elements (ICE), integrative and mobilizable elements (IME) and cis-integrative and mobilizable elements (CIME).

Integrative elements	Upstream	Influent	Activated sludge	Effluent	Downstream
Total ICE	4830	27796	7585	14129	6129
ICE with ARG	544 (11%)	723 (3%)	924 (12%)	597 (4%)	165 (3%)
Total IME	4101	31190	35919	15930	4313
IME with ARG	312 (8%)	871 (3%)	1645 (5%)	390 (2%)	217 (5%)
Total CIME	487	3400	1458	264	146
CIME with ARG	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)

Lastly, we assessed if the integrative elements are associated with previously detected ARGs. In the upstream and activated sludge sectors, ICEs were found to carry ARGs in 11% and 12% of detected ICEs respectively. Additionally, Table 3.2 shows that 8% of detected IMEs are linked to ARGs. Bacteria carrying both integrative elements and ARGs have a greater potential to spread infection and can survive high concentrations of antibiotics. This leads to the spread of AMR to other environments from the downstream sector, potentially causing untreatable infections when reaching animal and human communities [12, 17, 96]. Details on the specific ICE families and IMEs carrying ARGs can be found in Tables S13 and S15.

3.4. CONCLUSION

We conducted a comprehensive analysis of publicly available metagenomes representing the different sectors of the WWTP (upstream, influent, activated sludge, effluent and downstream) to identify the microbial composition, antibiotic resistance genes and mobile genetic elements. Data from different geographical locations (Portugal, China, Spain, South Korea and Colombia) were integrated to provide a more holistic view of AMR in the urban/municipal WWTP.

Our study revealed the persistent presence of environmental bacterial genera across the WWTP, identifying *Pseudomonas*, *Aeromonas*, and *Acidovorax*. These genera include (opportunistic) pathogens that pose health risks to humans and animals. Additionally, we detected the presence of all six ESKAPE pathogens across the sectors. Specifically *K. pneumoniae* and *Enterobacter* sp. was detected with a higher abundance in the effluent sector. *E. coli*, a faecal contaminant indicator, had a higher abundance in the effluent compared to other sectors.

Analysis of ARGs revealed various β-lactamase gene families throughout the sectors of the WWTP however, the OXA β-lactamase gene family is particularly prominent in the influent and activated sludge sectors while lower in the effluent and downstream suggesting that the reduction of ARGs is possible. The OXA β-lactamase gene family was also linked to more than 5% of the ARG-carrying plasmids detected indicating that ARGs can be selected for and transferred to other bacterial species. Integrons and integrative elements were identified in every sector of the WWTP process and linked to the previously detected ARGs further highlighting their role in the persistence and spread of AMR within microbial communities within the WWTP and its receiving environments, humans and animals.

This study underscores the significance of exploring the spread of MGEs and ARGs alongside associated bacterial species in non-clinical environments like WWTPs. Understanding these connections between environmental reservoirs, pollution sources, and human/animal exposure is crucial. However, the current study is limited by using public data from various WWTP studies. Although studies were based on sequencing technology, the type of WWTP, such as urban and municipal, as well as the sampling locations — upstream, influent, activated sludge, effluent, and downstream — differences in the use of public data can introduce biological variation in the types of bacteria and AMR genes detected.

Future research should take into account that upstream and downstream sectors are essential for the analysis of AMR in the WWTP and can provide information on HGT from receiving waters to other non-clinical environments. Additionally, geographical factors such as climate, local microbiome, industrial practices, and local regulations can influence the growth of microorganisms, types of AMR gene families detected, and MGEs and should be included in AMR research. Variations in influent and effluent may arise due to anthropogenic activities such as chemical and antibiotic usage, animal populations, vegetation, and pollutant discharge. Accounting for these factors is important in determining the full effect of WWTPs in managing AMR and environmental pollution [102–104].

3.5. SUPPLEMENTARY MATERIAL

Due to the extensive nature of supplementary materials associated with this chapter, they are not included in this thesis. Interested readers are encouraged to refer to the Supplementary material online
(see `https://surfdrive.surf.nl/files/index.php/s/Y27zkpPxaS4HMkq`)

3.5.1. SUPPLEMENTARY FIGURES

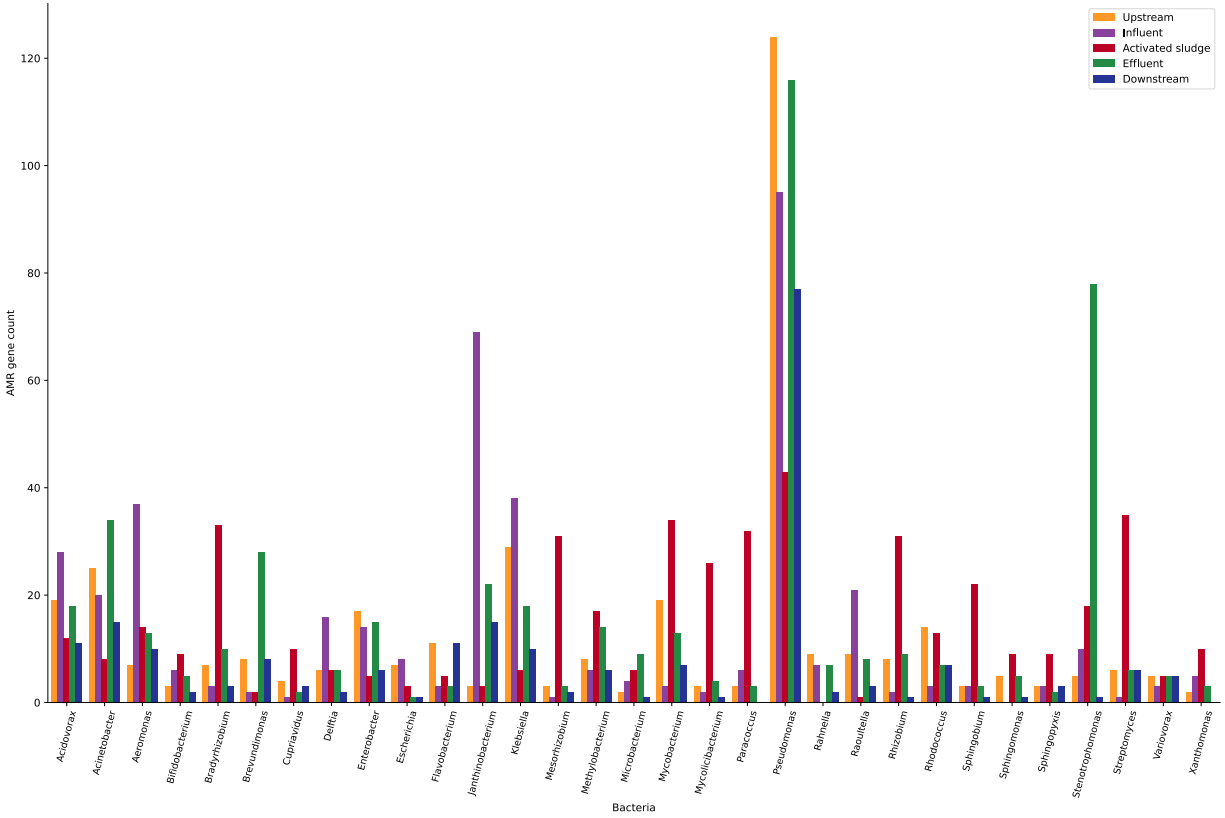

Figure S1: Barplot showing the antimicrobial resistance (AMR) gene count (≥ 20) in different bacterial genera detected in each sector of the wastewater treatment plant (WWTP). The x-axis represents the bacterial genera and the y-axis represents the AMR gene count. Sectors are colour coded (upstream – orange, influent – purple, activated sludge – red, effluent – green, downstream - blue).

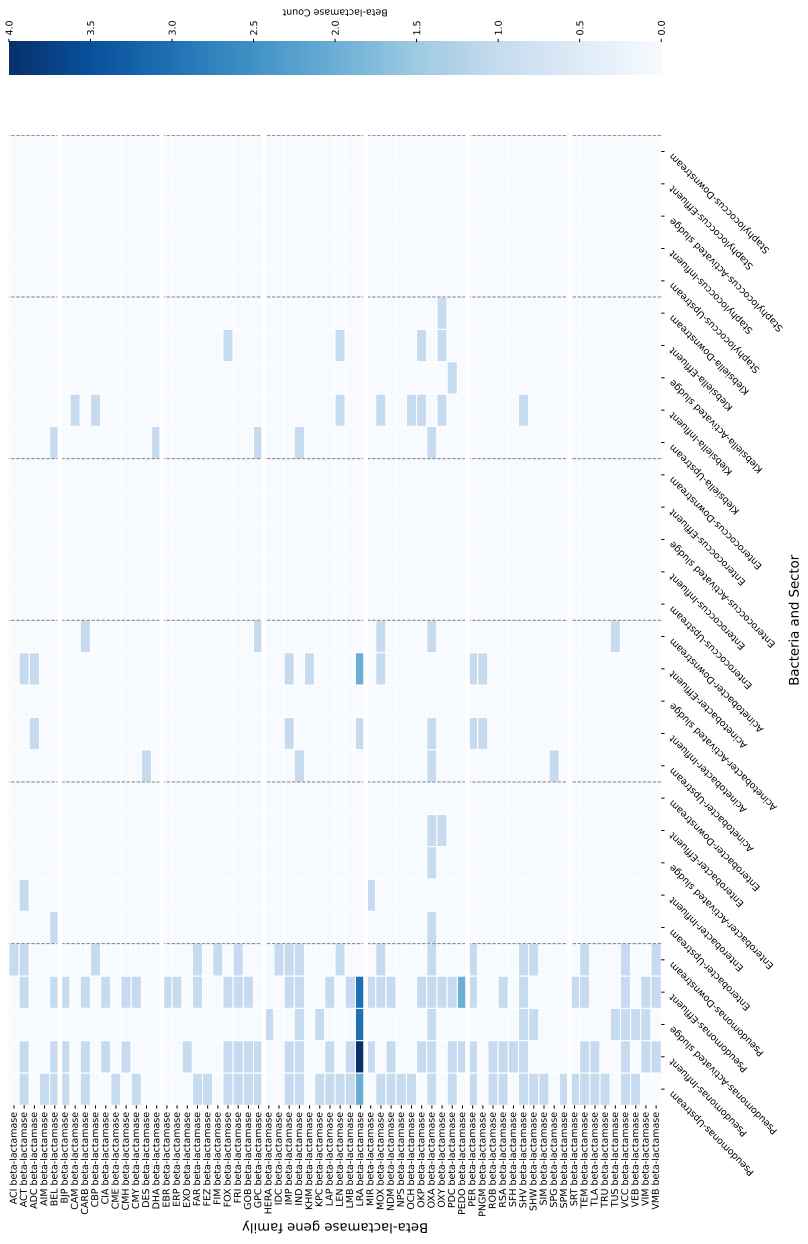

Figure S2: Heatmap showing the different Beta-lactamase gene families detected in ESKAPE pathogens in each sector of the wastewater treatment plant (WWTP). The x-axis represents the ESKAPE pathogens and the sectors of the WWTP and the y-axis represents the different Beta-lactamase gene families detected. Counts are indicated by the intensity of blue (0 = white and 4 = dark blue).

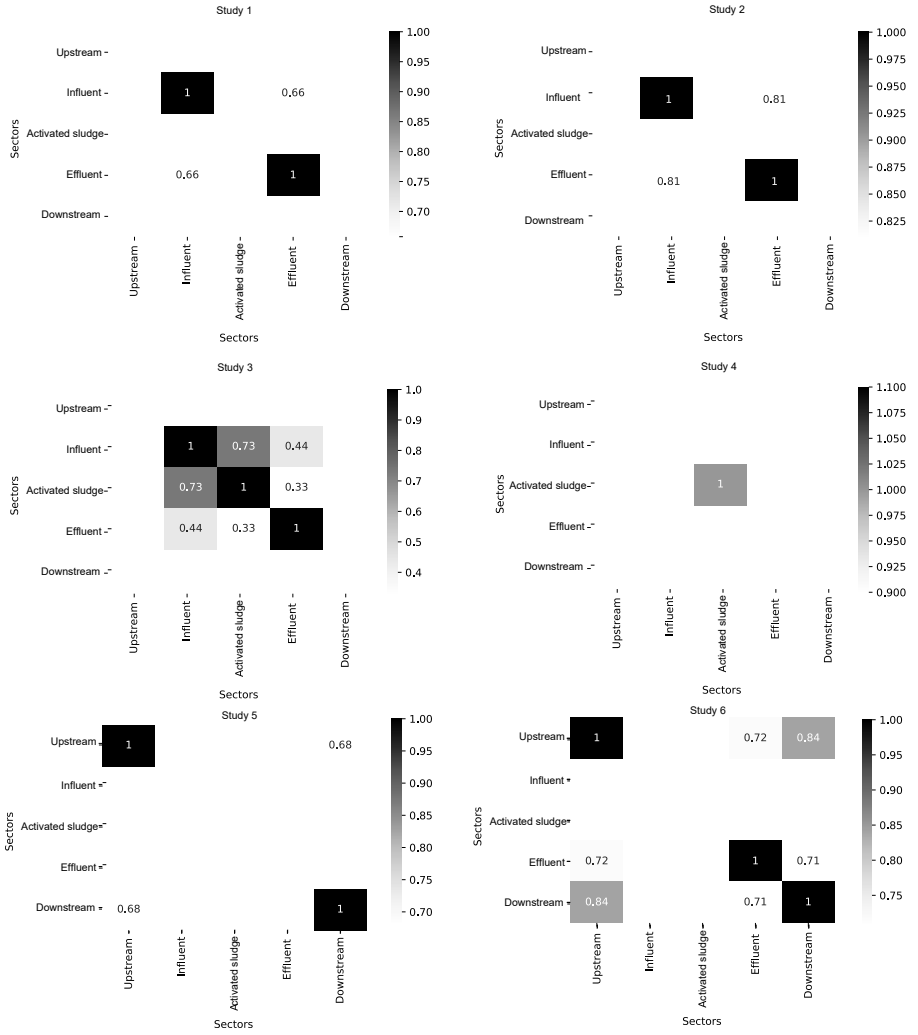

Figure S3: Visualisation of the Spearman's correlation analysis of the AMR gene families present between sectors that shared samples of the wastewater treatment plant (WWTP). Spearmans correlation was done for study 1 to study 5. The correlation rank values are depicted in text and indicated by the intensity of grey.

3.6. DECLARATIONS

3.6.1. FUNDING

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4

A METAGENOMIC STUDY OF ANTIBIOTIC RESISTANCE ACROSS DIVERSE SOIL TYPES AND GEOGRAPHICAL LOCATIONS

This chapter is under review in Frontiers in Microbiology

Soil naturally harbours antibiotic resistant bacteria and is considered to be a reservoir for antibiotic resistance. The overuse of antibiotics across human, animal, and environmental sectors has intensified this issue leading to an increased acquisition of antibiotic resistant genes by bacteria in soil. Various biogeographical factors, such as soil pH, temperature, and pollutants, play a role in the spread and emergence of antibiotic resistance in soil. In this study, we utilised publicly available metagenomic datasets from four different soil types (rhizosphere, urban, natural, and rural areas) sampled from nine distinct geographic locations to explore the patterns of antibiotic resistance in soils from different regions. Bradyrhizobium was predominant in vegetation soil types regardless of soil pH and temperature. ESKAPE pathogen Pseudomonas aeruginosa was prevalent in rural soil samples. Antibiotic resistance gene families such as 16s rRNA with mutations conferring resistance to aminoglycoside antibiotics, OXA β-lactamase, ANT(3"), and the RND and MFS efflux pump gene were identified in all soil types, with their abundances influenced by anthropogenic activities, vegetation, and climate in different geographical locations. Plasmids were more abundant in rural soils and were linked to aminoglycoside resistance. Integrons and integrative elements identified were associated with commonly used and naturally occurring antibiotics, showing similar abundances across different soil types and geographical locations. Antimicrobial resistance in soil may be driven by anthropogenic activities and biogeographical factors, increasing the risk of bacteria developing resistance and leading to higher morbidity and mortality rates in humans and animals.

4.1. INTRODUCTION

Antimicrobial resistance (AMR) has contributed to approximately 4.95 million deaths worldwide in 2019 [1]. The continuous use of antibiotics in clinical and environmental sectors such as animals, soil and water has led to the development of resistant bacteria, which can spread between humans, animals, and the environment [1]. As a result, antibiotic resistance has been identified as a major driver of mortality and one of the most critical environmental issues [2].

Soil is a large reservoir of microbial diversity and a majority of antibiotics used in clinical and non-clinical sectors have been extracted from soil microorganisms [2]. Interestingly, environmental bacteria in soil possessed antibiotic resistance genes (ARGs) pre-dating the discovery of antibiotics which ensured their survival in the natural environment [3, 4]. Despite the natural presence of ARGs in the soil microbiome, anthropogenic activity has impacted the intrinsic resistome. Fertilisation of crops, irrigation, excessive use of xenobiotics in crops, antibiotic use in livestock production and deforestation can alter the microbial community in the soil and disseminate ARGs throughout the environment [3]. This makes the soil a reservoir for intrinsic and acquired antibiotic resistance due to the mixture of ARGs from indigenous microbes and those introduced by human activities [2, 4].

Biogeographical patterns can also influence antibiotic resistant bacteria and ARGs in the soil [4]. Factors such as pH, temperature, moisture, and nutrients can affect ARG profiles and microbial composition. Soils with a neutral pH, low temperature, high moisture content and nutrient-rich will have an increased abundance and

diversity of bacteria as well as a diverse range of ARGs [5]. These factors promote the spread of antibiotic resistance by mobile genetic elements (MGEs) which enables non-pathogenic and pathogenic bacteria to acquire resistance increasing the risk of bacterial infections in humans and animals [6].

Additionally, the soil microbiome and ARGs can differ across soil types. Natural and pristine soil is largely unaffected or undisturbed by human activity or external sources, serving as a reference point for understanding microbial communities and ARGs with little anthropogenic selection pressure [7]. The rhizosphere soil surrounds the plant roots and is influenced by root secretions and agricultural practices. Urban soil encompasses anthropogenic soil near forests, parks, gardens, and residential areas, while rural soil includes non-urban areas like small villages, towns, and settlements. Soil with more human activity is expected to contain a mixture of environmental and pathogenic bacteria; and resistance genes that confer resistance to clinically relevant antibiotics, heavy metals, biocides and disinfecting agents [8]. It should be noted that similar soil types from different geographical locations will not necessarily have similar microbial communities or ARGs as agricultural activities, travel, environmental contamination, antibiotic usage, soil management practices, population and socioeconomic conditions differ between countries [9]. These factors have complex and interrelated effects on antibiotic resistance in soil.

Since soil encompasses different environmental conditions with distinct microbial environments, it is important to understand how these factors interact and contribute to AMR to develop effective mitigation strategies [5, 9]. In this study, we analysed metagenomic sequences from four different soil types: natural, urban, rhizosphere, and rural soil collected from nine different geographical locations i.e., South Africa, Singapore, China, Israel, Botswana, Chile, Germany, El Salvador, and Antarctica. We aim to determine the differences in microbial composition between each soil type sampled from these various geographical locations. Furthermore, we investigate the differences in antibiotic resistance genes and mobile genetic elements (plasmids, integrons and integrative elements) identified in each soil type with regards to geographical location.

4.2. METHODS

4.2.1. DATA SOURCES

Whole metagenomic datasets were obtained from NCBI SRA (https://www.ncbi.nlm.nih.gov/sra). These datasets represented various soil types: natural soil (NS), urban soil (US), rhizosphere soil (RHS) and rural soil (RS) from different geographical locations (South Africa, Singapore, China, Israel, Botswana, Chile, Germany, El Salvador, and Antarctica) sampled over the last 10 years. Only metagenomic sequences that were sequenced using Illumina, had a read base count over 1e+09 and an average read length over 150bp were included. Detailed information about the metagenomes retrieved from the database is included in Supplementary Table S1 and presented in summarized form in Table 4.1.

Table 4.1: Summary of included studies, respective sample source, location, and sample size. Study identifiers start with the soil abbreviation: natural soil (NS), urban soil (US), rhizosphere soil (RHS), and rural soil (RS).

Study	Sample source	Location	Sample number	Citation
NS1	Deep forest	China	3	[10]
NS2	Mountain	China	5	[11]
NS3	Desert	Israel	12	[12]
NS4	Desert	Chile	11	[13]
NS5	Antarctic	Antarctica	27	[14]
US1	Forest park, residential areas, hospital areas	China	10	[10]
US2	Road verge, park	China	10	[11]
US3	Nature reserve	Germany	8	[15]
RHS1	Greenhouse	Singapore	13	[16]
RHS2	Soybean farm	South Africa	12	[17]
RHS3	Peanut and maize farm	China	21	[18]
RS1	Rural village	El Salvador	25	[19]
RS2	Rural village	Botswana	1	[20]

4.2.2. BIOINFORMATIC ANALYSIS

Quality control of reads was conducted using FastQC v.0.11.9 [21] and filtered with Trimmomatic v.0.39 [22]. Reads were trimmed for quality and adapter contamination using Trimmomatic with the following parameters specified: ILLUMINACLIP: adapters.fa:2:30:10 LEADING:3 TRAILING:3 MINLEN:30 SLIDINGWINDOW: 4:20. The microbiome was analysed using Kraken2 v.2.1.0 [23] with the minikraken2 microbial database (2019, 8GB). Species confirmation and abundance estimation were performed with Bracken v.2.6.2 [24]. The relative abundances of bacterial genera were used to determine the microbial populations in each soil type. Statistical significance of the microbiome between studies in a soil type was assessed using the non-parametric Wilcoxon rank sum test and Friedman's ANOVA with SPSS software (IBM SPSS Statistics). Differences in the microbiome were considered statistically significant at $p \leq 0.05$. The Beta diversity of the microbiome among soil types was visualized using non-metric multidimensional scaling (NMDS) analysis with Bray-Curtis dissimilarity in the R package vegan v.2.6-4.

The filtered reads were *de novo* assembled using metaSPAdes v.3.15.2 [25] on meta mode with default parameters. The assembled metagenomes were annotated to determine the antibiotic resistance gene families and drug classes using the Comprehensive Antimicrobial Resistance Database Resistance gene identifier (CARD - RGI) v.3.1.2 with default settings including loose matches and the -low_quality -clean options [26]. The version of the database was consistent throughout the analysis. Moreover, the studies in each soil type were compared by analysing the relative

abundance of annotated antibiotic resistance genes. This comparison was based on normalizing the data using the total number of antibiotic resistance gene families in each study indicating their respective proportions. *De novo* metagenomic assembled contigs were aligned to detect integrons and integrative elements by aligning them to the INTEGRALL database [27] and the ICEberg database [28]. Alignments were done using BWA-mem v.0.7.10 with default parameters [29] which generated a SAM file. To filter out soft and hard clipped reads and undesirable alignments, the SAM files were processed using the Samclip tool with default parameters to remove alignments that could generate downstream issues. The resulting SAM file was then converted to a BAM file using SAMtools version v.1.9 [29]. Contigs that aligned with databases were considered integrons and integrative conjugative elements. Contigs were classified as plasmid-originating using Plasclass v.0.1.1 [30], with a minimum length threshold of ≥ 1000 and a probability threshold of ≥ 0.75 for contigs to be considered plasmid-originating. To determine if the detected plasmids were linked to antibiotic resistance gene families, the plasmid-originating contigs were compared to previously identified contigs carrying antibiotic resistance genes and matched to corresponding AMR drug classes.

4.3. RESULTS AND DISCUSSION

4.3.1. MICROBIAL COMMUNITIES ARE DEPENDENT ON THEIR HABITAT

To identify the bacterial communities within different types of soil from different locations (Supplementary Table S1), we performed taxonomic classification. Soil hosts a diverse range of microbial communities influenced by abiotic and biotic characteristics, microbial abundances, activity, and community composition. Consequently, there is no "typical" soil microbiome, and bacterial relative abundances vary by soil type [31].

Bacteria in natural soils are influenced by their habitat and environmental conditions (Supplementary Table S2). In Figure 4.1, *Bradyrhizobium* dominates the deep forest areas (NS1 and NS2), whereas bacteria like *Rubrobacter*, known for its thermophilic traits, are found in desert-like conditions (NS4), as confirmed by Connon et al. [32], Pajares and Bohannan [33], and Zheng et al. [10]. The microbial communities detected were statistically different between each of the natural studies indicating environmental influence ($p \geq 0.05$). Climate, pH, temperature and rainfall may all influence the abundances and types of bacteria found soil as discussed by Chase et al. [34].

Streptomyces was prevalent across all urban studies; however, the overall microbial community differed significantly between these urban studies ($p \leq 0.05$) (Figure 4.1 and Supplementary Table S3). As expected, higher abundances of *Bradyrhizobium*, *Mycobacterium*, and *Rhodopseudomonas* were observed in urban soil obtained from the nature reserve (US3). These bacteria thrive in areas with more vegetation and reduced anthropogenic activity, such as nature reserves, compared to residential and forest park areas [35–37].

Plant growth-promoting bacteria such as *Streptomyces*, *Burkholderia*, *Pseudomonas*, and *Bradyrhizobium* were found in less than 12% abundance across different rhizo-

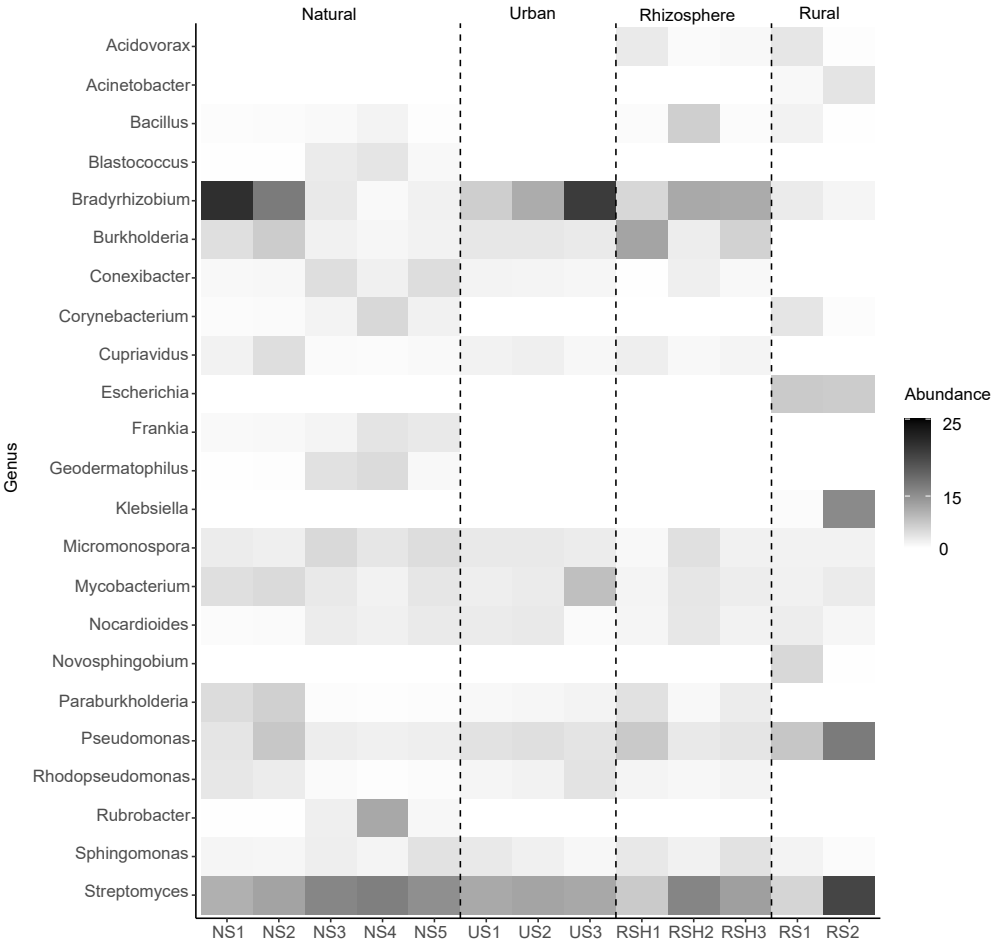

Figure 4.1: Heatmap showing the relative abundances of bacterial genera over 2% in each study from each soil type. The y-axis indicates the genera, and the x-axis represents the soil studies (Table 4.1). Each soil type is divided by the dashed black line and the abundances of bacterial genera are indicated by the intensity of grey.

sphere soil studies (Figure 4.1 and Supplementary Table S4). Overall, these microbial communities were different in each of the rhizosphere soil studies ($p \leq 0.05$). These bacteria also assist in protecting plants against pathogens by producing antibiotics [38–40]. *Bradyrhizobium*, which aids in nitrogen fixation and plant growth, is found at an 8% abundance in RHS1, 2% in RHS2 and 4% in RHS3 [31]. Soil in RHS1 and

RHS3 consisted of crops supplemented with nitrogen-phosphorus-potassium (NPK) fertiliser (Supplementary Table S1). Despite this, these studies showed a higher abundance of *Bradyrhizobium* compared to RHS2, which was not supplemented. This contradicts previous studies suggesting that *Bradyrhizobium* will be present in a low abundance when nitrogen fertilisers are used [41, 42].

Lastly, in rural soil studies ($p \leq 0.05$),*Pseudomonas, Escherichia, Streptomyces,* and *Klebsiella* were prevalent, including the ESKAPE (*Enterococcus faecium (E. faecium), Staphylococcus aureus (S. aureus), Klebsiella pneumonia (K. pneumonia), Acinetobacter baumannii (A. baumannii), Pseudomonas aeruginosa (P. aeruginosa)* , and *Enterobacter* species) pathogens *K. pneumonia* and *P. aeruginosa* (Figure 4.1 and Supplementary Tables S5 and S6 respectively). The high prevalence of these pathogens in rural areas is attributed to anthropogenic factors, such as the presence of domestic animals, open defecation, and inadequate sanitation [20]. These conditions contribute to antibiotic resistance hotspots in rural areas, as domestic animals spread faecal matter, and open sewage drains and insufficient sanitation increases the selection pressure for antibiotic resistance [43].

Overall, these results demonstrate that the bacterial composition and abundance within each soil type may be influenced by a myriad of factors, including environmental conditions, nutrients, temperature, oxygen availability, and pH types within the specific geographical location [31, 33, 39]. Understanding the composition of microbial communities is critical for comprehending soil health and potential risks associated with antibiotic resistance in various environments.

4.3.2. NATURAL, URBAN AND RHIZOSPHERE SOIL TYPES HAVE SIMILAR MICROBIAL COMMUNITIES

To assess the differences between microbial communities, present in different types of soil from different geographical locations (Table 4.1), we analysed the beta-diversity. Additionally, we aimed to determine if the geographical locations influence the microbial communities in each soil type. The beta-diversity quantifies the “distance” between microbial communities using the Bray-Curtis index and can be visualized by non-metric multidimensional scaling (NMDS). A smaller distance between samples from different countries and soil types indicates a greater similarity in microbial composition. Figure 4.2 shows the NMDS plots for each type of soil from their respective countries.

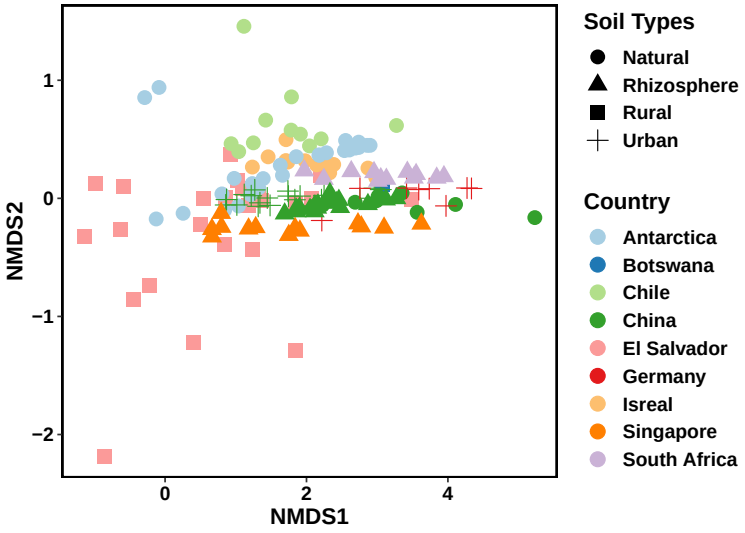

Figure 4.2: Non-metric multidimensional scaling (NMDS) visualising the dissimilarity (Beta-diversity) between microbial communities from different soil types from different countries (Table 4.1). Each soil type is represented by a different shape (natural = circle, rhizosphere = triangle, rural = square, urban = cross) and each country or region is colour-coded (Antarctica = light blue, Botswana = dark blue, Chile = light green, China = dark green, El Salvador = pink, Germany = red, Israel = salmon, Singapore = orange, South Africa = lilac). The samples that have smaller distances between them are circled in blue.

Figure 4.2 shows that most samples cluster together regardless of the soil type or geographical location. Natural, rhizosphere, urban, and many rural soil samples are closely grouped, suggesting similar microbial communities across these soil types. This similarity may be related to vegetation and varying levels of anthropogenic activity [10, 33]. However, some rural samples from El Salvador differ which could be due to human activity or soil around latrine toilets and clothes washing areas. This can limit plant-promoting bacteria and support enteric pathogens [19, 20]. Samples from specific studies like RS1 (El Salvador), NS2 (China), NS4 (Chile) and NS5 (Antarctica) are further apart, indicating variations in the microbial composition. Such differences within the same study may result from spatial variability in the soil environment, even when sampling sites are only a few centimetres apart [31].

4.3.3. ANTHROPOGENIC ACTIVITIES, VEGETATION, AND CLIMATE PLAY A ROLE IN THE SELECTION AND PREVALENCE OF ANTIBIOTIC RESISTANCE GENES

To understand the connection between AMR gene families and the different types of soil, we examined the presence and abundance of AMR gene families in the assembled metagenomes (contigs) from each study within their respective soil type (Table 4.1). While AMR gene families are naturally present in soil environments, external factors like livestock and human activities can influence their types and abundance [44].

Figure 4.3: Heatmap showing the abundance of AMR gene families present over 5% in each study within each soil type. The y-axis indicates the AMR gene families and the x-axis represents the soil studies (Table 4.1). Each soil type is divided by a dashed black line and the abundances of AMR gene families are indicated by the intensity of grey.

Overall, more than 0.001% of contigs contained AMR gene families in each study. We identified five AMR gene families that were abundant (Figure 4.3). Two of these are associated with acquired resistance: 23S rRNA, which confers resistance to macrolides, and 16S rRNA, which confers resistance to aminoglycosides. Intrinsic resistance gene families include the major facilitator superfamily (MFS) and resistance-nodulation-cell division (RND) antibiotic efflux pumps, and the OXA β-lactamase gene family, which is associated with acquired and intrinsic resistance [45, 46]. These gene families confer resistance to macrolides, and β-lactams including carbapenems, cephalosporins, and penams (Supplementary Table S7).

Figure 4.3 shows that the 23S rRNA mutation conferring resistance to macrolide antibiotics was more prevalent in urban soils as compared to the natural, rhizosphere and rural soils. Specifically, in US2 from China, 85% of the detected AMR gene families were the 23S rRNA gene with this mutation, with other urban studies like US1 had 58% abundance. In contrast, natural soil studies such as NS1 and NS2 had lower abundances of 33% and 52%, respectively. Urban soil samples were collected from areas with high human activity (forest parks, residential zones, and road verges) which have greater antibiotic pollution and selective pressures promoting the proliferation of antibiotic resistant bacteria, as detailed by Osbiston, Oxbrough, and Fernández-Martínez [47].

The MFS and RND efflux pump gene families were detected across all types of soil except for US2 and RS2 (Figure 4.3). Efflux pumps are key for intrinsic resistance, providing bacteria with a baseline level of resistance by actively expelling a broad range of antibiotics from their cells [46]. In our study, RS1 exhibited a high abundance of these efflux pump gene families, with 17% for RND and 13% for MFS (Figure 4.3 and Supplementary Table S7). The presence of these efflux pump gene families in rural soils may also be influenced by factors such as animal feedlots, domestic sewage, and human-excrement-irrigated vegetable fields [48]. Additionally, vegetation, flora and fauna influences the presence of efflux pump gene families, as discussed by Martinez et al. [49] and Pasqua et al. [50].

Lastly, we found the OXA β-lactamase gene family present in all rhizosphere and natural soil studies, except US2 and RS2, with a high abundance in US3 (7%) and RHS3 (7%) (Figure 4.3 and Supplementary Table S7). The OXA β-lactamase gene family has been shown to confer both acquired and intrinsic resistance and can be found in all soil types; from primitive natural soils with little to no anthropogenic activity to urban environments [6, 51]. Previous research also suggests that the climate affects the abundance of ARGs, with β-lactam resistance genes being more common in warmer areas [52]. However, our study found similar AMR gene families across all soil types, regardless of temperature.

4.3.4. PLASMIDS LINKED TO AMINOGLYCOSIDE RESISTANCE ARE COMMON IN ALL SOIL TYPES

To investigate the presence of plasmids and their connection to antibiotic drug classes in different soil types, we analysed the percentage of contigs classified as plasmids in each study and their association with antibiotic drug classes. Although AMR is a natural occurrence in soil, human activity has led to increased contamination with

antibiotics and ARGs in the soil. Soil plasmids can transfer these ARGs from one bacterial host to another integrating into plant and animal cells and spreading AMR [53].

Firstly, less than 1% of contigs were classified as plasmids in each study from every soil type. Further analysis linking detected plasmids to AMR drug classes revealed that, except RHS2, RHS3, RS2 and US2, all studies had more than 0.01% of plasmids associated with AMR drug classes. Notably, RS1 had 1.8% of plasmids linked to AMR. A high percentage of plasmids is common in rural communities due to unregulated access to antibiotics, limited clean water and human and animal activities. These conditions increase the risk of exposure to plasmids and ARGs from effluents, contaminated soils, and waste, enhancing plasmid accumulation and horizontal gene transfer compared to natural and urban soils [19, 20].

Secondly, we examined the association between plasmids and specific antibiotic drug classes (Supplementary Table S8). Overall, all studies from different soil types, except RHS2, RHS3, US2 and RS2, contained plasmids associated with aminoglycoside, carbapenem; cephalosporin; penam, glycopeptide and peptide drug classes. Details on the abundances of plasmids linked to AMR drug classes and the individual AMR gene families can be found in Supplementary Table S8.

Plasmids harbouring genes for the production of naturally occurring antibiotics have been discovered in both natural and urban soil studies. Among the natural soil types, NS1 showed a high abundance of plasmids associated with aminoglycosides (13%) and carbapenem; cephalosporin; penam (16%) drug classes. These high abundances were consistent with other natural studies (Supplementary Table S8). Similar to NS1, US1 had a high abundance of plasmids associated with aminoglycosides (13%), carbapenem; cephalosporin; penam (25%) along with fluoroquinolones (13%), glycopeptide (25%), macrolide (13%) and tetracycline (13%) drug classes.

Additionally, US3 had plasmids linked to similar antibiotic drug classes: aminoglycosides (16%), carbapenem; cephalosporin; penam (11%), glycopeptide (12%) and peptides (9%). In our study, the natural and urban studies, sampled from forests and deserts for natural soils and residential areas and forest parks for urban soils, showed higher abundances of plasmids associated with naturally occurring antibiotics such as aminoglycosides, glycopeptides, and β-lactams [53, 54]. These abundances may also be influenced by heavy metals and other contaminants in the soil types as noted by Zhao et al. [54].

Amended rhizosphere soil (RHS1) showed a high abundance of plasmids associated with AMR drug classes. Specifically, aminoglycoside (17%), carbapenem; cephalosporin; penam (8%), glycopeptide (10%) and sulphonamide (10%) resistance plasmids were detected in rhizosphere soils. Although plasmids were detected in RHS2 and RHS3, none were associated with previously identified ARGs. Amended soils, such as rhizosphere soils and natural soils are nutrient-rich environments that can facilitate the spread of AMR through plasmids [55, 56].

Lastly in rural soils, RS2, a single sample study, did not contain any plasmids associated with AMR. However, RS1 had a high abundance of plasmids linked to aminoglycoside (24%), carbapenem; cephalosporin; penam (9%) and sulphonamide (39%) resistance. This is consistent with a study from a rural village in India that

found resistance to carbapenems, cephalosporins, penams, sulphonamides, tetracyclines, and quinolones, with *E. coli* carrying plasmids for resistance to quinolones, cephalosporins, and colistin. This study suggested that the local antibiotic use patterns supports the persistence of plasmids with ARGs [57].

While ARGs naturally occur in soil, their transmission to different environments through plasmids is concerning, especially in environments with increased anthropogenic activity. This contributes to the spread of AMR to both pathogenic and non-pathogenic bacteria, posing a significant threat to the natural environment and human health [53].

4.3.5. AMR GENES ASSOCIATED WITH INTEGRONS THAT ARE HIGHLY ABUNDANT WERE SELECTED DUE TO THE CONSTANT USE OF ANTIBIOTICS

To identify the presence of integrons and their association with AMR gene families, we classified integrons from the metagenomic contigs present in each soil study. Furthermore, we linked the classified integrons with AMR gene families to determine the differences between soil studies. Integrons are mobile genetic elements that capture and express ARGs, particularly among gram-negative pathogenic bacteria. Integrons are present in all environments and can serve as markers for tracing sources of pollution [58]. We used the assembled metagenomic contigs to determine the percentage of classified integrons (Supplementary Table S9). In all soil types studied, less than 0.001% of contigs were identified as integrons. The integrons that were found were classified into their respective classes of 1, 2 and 3 (Supplementary Table S9).

Class 1 integrons made up a higher percentage ($\geq$ 11%) of the integrons we found between all soil types from all studies. Class 3 integrons constitutes a range of 2% to 5% of integrons found while class 2 integrons had a lower percentage of 1% to 2%. The remainder of the integrons were unclassified. These high abundances of class 1 and class 3 in all types of soil regardless of country are expected as class 1 and 3 integrons are found in Proteobacteria in soil environments whereas class 2 integrons are commonly found in marine environments. Overall, integrons are found in diverse environments which include forest soil, desert soil, Antarctic soil and plant surfaces [58].

In our study, we identified class 1, 2 and 3 integrons and their association with AMR genes (Supplementary Tables S10 and S11). We found a high abundance of class 1 integrons associated with disinfecting agents and sulphonamide resistance in all types of soil. The high abundance of the *qacE* gene with class 1 integrons can be traced back to the use of quaternary ammonium compounds which were first used as hospital disinfecting agents in 1930 and have become a part of the class 1 integron, known to be highly abundant in soils [48, 58]. Similarly, sulphonamides were the first true antibiotic to be used in the 1930s and have been selected for and can be commonly found in class 1 integrons [58].

A high abundance of class 2 integrons was associated with aminoglycoside resistance (*aadB*) which ranged from 15% in the RS1 to 75% in NS3 and NS4, and 8% in

RHS3 and US1 (Supplementary Tables S10 and S11). Aminoglycoside antibiotics were originally isolated from soil bacteria and commonly used in agriculture, increasing their presence in soil environments, and potentially affecting plant-soil microbial communities. They also enter soils through human waste, municipal wastewater systems, and clinical use. These direct and indirect exposures contributes to the detection of aminoglycoside resistance genes in various soil types, often associated with class 1 and class 2 integrons [59, 60].

Previous studies have shown that the *aadB* aminoglycoside resistance gene is frequently associated with class 1, 2, and 3 integrons and is present in *P. aeruginosa, Salmonella spp., A. baumannii, Klebsiella spp.,* and *Escherichia coli* (*E. coli*) which are present in our study [61]. Additionally, Jones et al. [62] showed that the *aadB* gene, associated with class 1 integrons, had a close association with the *blaSHV* gene conferring resistance to penicillin and cephalosporins. This association was not seen in our study. Lastly, class 3 integrons were predominantly associated with the *qacE* gene, which provides resistance to disinfectants. Over 55% of class 3 integrons in all soil studies were linked to the *qacE* gene, with 100% in NS4, 97% in RHS2, and 97% in US3. Similarly to class 1 integrons, the *qacE* gene is commonly found in class 3 integrons as it is considered to be a conserved segment and had been selected for in the past [61].

4.3.6. INTEGRATIVE AND MOBILIZABLE ELEMENTS WERE IDENTIFIED IN EACH TYPE OF SOIL

Finally, we used the assembled metagenomic contigs to determine the percentage of integrative elements. Integrative elements consist of integrative and conjugative elements (ICE), integrative and mobilizable elements (IME) and cis-integrative and mobilizable elements (CIME) that are self-transmittable and can carry and spread ARGs to other bacterial hosts [63]. We found that all the studies across all soil types had less than 0.1% of contigs classified as integrative elements. ICE elements were the most prevalent integrative element compared to IMEs and CIMEs. In the RS2 study, 100% of the integrative elements were CIMEs, while other studies had less than 20% CIMEs. The abundance of ICEs in all studies across all soil types was over 70%, with no ICE elements found in RS2.

The most prominent ICE families among all studies in all soil types were Tn4371 (≥12%), SXT/R391 (≥5%) and ICE*clc* (≥8%) (Supplementary Table S12). These ICE families are commonly found in soil and aid in the degradation of pollutants [64–66]. Additionally, we identified different SGI1 IME families present in the different studies within the different types of soil (Supplementary Table S13). SGI1 has been characterized as a Salmonella genomic island that can spread multi-drug resistance to human and animal pathogens [67]. Bacteria carrying these integrative elements have a higher potential for spreading infections thus facilitating the spread of AMR to other environments [63]. This underscores the importance of continuously monitoring mobile genetic elements in different soil types.

4.4. CONCLUSION

We analysed publicly available metagenomic data to investigate the microbial population, antibiotic resistance genes and mobile genetic elements in rhizosphere, urban, natural, and rural soils sampled from South Africa, Singapore, China, Israel, Botswana, Chile, Germany, El Salvador, and Antarctica to understand the spread of antibiotic resistance in different soil environments.

Our study revealed that plant-promoting bacteria such as *Bradyrhizobium*, dominate soil rich in vegetation and nutrients such as the rhizosphere and natural soil regardless of geographic location. Urban soils contain a mix of plant-promoting bacteria and pollutant-degrading bacteria, such as *Arthrobacter*, while rural village soils harbour a combination of environmental bacteria and opportunistic pathogens like *E. coli*. Bacteria in the soil are dependent on physiological (temperature, mixing of soil profiles, water change, soil compaction), chemical (heavy metal, soil pH, polyaromatic hydrocarbons) and biological (artificial vegetation, faecal contamination) factors. As such, more pathogenic bacteria especially those on the ESKAPE pathogen list are dominant in areas with high human activity. These bacteria can carry antibiotic resistant genes that may spread to domestic and food animals, vegetation, water sources and humans leading to untreatable infections in all sectors.

Antibiotic resistant gene families detected in this study i.e., 23S rRNA with mutation conferring resistance to macrolide antibiotics, major facilitator superfamily (MFS) antibiotic efflux pump, resistance-nodulation-cell division (RND) antibiotic efflux pump, OXA β-lactamase, ANT(3") and 16s rRNA with mutation conferring resistance to aminoglycoside antibiotics in soil. These genes favour the development of multidrug resistance, posing a threat to antibiotic effectiveness. While antibiotic resistant gene families such as the RND and MFS efflux pump gene families occur naturally in soil, anthropogenic activity, vegetation, and climate may play a role in the emergence of AMR and the acquisition of ARGs. Additionally, plasmids associated with AMR gene families like OXA β-lactamase facilitate the spread of resistance from soil to various environments like water, humans or animals.

Integrons detected in this study were linked to commonly used antibiotics in all soil types, suggesting that the overuse of antibiotics contributes to the spread and emergence of antibiotic resistant bacteria. These bacteria may also possess adaptive strategies provided by integrative elements, SXT/R391, Tn4371, ICE*clc* and IMEs which can potentially carry accessory genes i.e., ARGs. Integrative elements allow bacteria in the soil to stabilise and adapt to environmental conditions and human activity as well as favouring gene transfer and the spread of AMR. To fully understand the emergence and spread of AMR in the environment, factors such as geographical location, biogeographical patterns, and soil type must be considered. By analysing MGEs and ARGs in diverse soil types from different countries, we can gain insights into how AMR spreads and adopt strategies to manage it effectively. Future research should focus on collecting more rural soil data and considering additional factors such as antibiotic usage, residues, industrial practices, and local regulations to address AMR and environmental pollution.

4.5. SUPPLEMENTARY MATERIAL

Due to the extensive nature of supplementary materials associated with this chapter, they are not included in this thesis. Interested readers are encouraged to refer to the Supplementary material online (see `https://surfdrive.surf.nl/files/index.php/s/Y27zkpPxaS4HMkq`)

4.6. DECLARATIONS

4.6.1. FUNDING

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5

A COMPARATIVE METAGENOMIC ANALYSIS OF ANTIBIOTIC RESISTANCE IN THE FECAL MICROBIOME OF PIGS RAISED WITH AND WITHOUT ANTIBIOTICS

This chapter is under review in BMC Animal Microbiome

The use of antibiotics in swine production has raised concerns about its impact on animal health, microbial ecology, and the emergence of antibiotic resistance. The gut microbiome, a critical component of animal health, is susceptible to disruptions caused by antibiotic use. The fecal microbiome can provide a snapshot of changes in the gut microbiome and provide information on the spread of antibiotic resistance from pig feces to the environment. We investigated the differences between the fecal microbiome of pigs raised with (AB+) and without (AB-) antibiotics to assess how antibiotic exposure influenced the microbial community, antibiotic resistance genes (ARGs), and mobile genetic elements (MGEs). Our findings revealed that the use of antibiotics combined with study-specific conditions alters the fecal microbiome. Antibiotic usage in pigs explains 20% of the variation in the diversity of bacterial species detected between AB+ and AB- pigs. Similarly, variations in ARGs were observed in the two groups. The RND efflux pump gene family was abundant in both groups. The OXA β-lactamase gene and associated plasmids were more prevalent in the AB- group. We also identified integrons linked to chloramphenicol resistance and transposon-associated integrative elements that can facilitate the spread of antibiotic resistance genes within and between bacterial species. These findings highlight that antibiotics influence both the microbial community and the antibiotic resistance gene repertoire, regardless of study-specific or environmental conditions. This emphasises the potential for fecal matter to contribute to the spread of antibiotic resistance in the environment, underscoring the need for more research to fully understand these dynamics in agricultural settings.

5.1. INTRODUCTION

Antimicrobial resistance (AMR), resulting from excessive antibiotic use in humans, animals, and the environment, poses a significant global health risk. By 2030, antibiotic usage is projected to increase by 67%, with over half attributed to animals raised for food production [1, 2].

Antibiotics have been integral to the growth and development of food animals for more than half a century, particularly in swine production. Antibiotics are routinely used to treat, control and prevent diseases, and to increase productivity. Sub-therapeutic doses are used to control symptomatic infections between animals in close contact, to prevent disease at points of high risk, e.g. when the animal is stressed during extreme weather, post-vaccination or moving [3, 4]. This practice has turned the food animal industry into a hotspot for antibiotic resistant dissemination [2].

Among various food animals (cattle, poultry, and pigs), the pig industry is the most prolific user of antibiotics [5]. In pigs, antibiotics are given to whole groups by mixing antibiotics into feed or adding antibiotic powder or solution into drinking water [3]. Previous studies have shown that antibiotics, such as beta-lactams, tetracyclines, sulfonamides, lincosamides, macrolides, and quinolones, are typically used in swine production [6]. However, the choice of antibiotics is influenced by age-specific diseases, common pathogens, market availability, and cost [7]. Farm management practices also play a role in antibiotic use. Larger farms, due to their higher risk of

pathogen transmission within herds, are more likely to use medicated feed compared to smaller farms. However, it has been proposed that while costly, good farm biosecurity practices and vaccine administration reduce disease incidence and antibiotic use [8].

In recent years, the connection between antibiotic use in livestock and the emergence and spread of antibiotic resistance has been established. This has led to the European Union banning the "non-therapeutic" use of antimicrobials as growth promoters since 2006. Restrictions on the use of antibiotics followed in the United States since 2017 and China since 2020 [9]. Unfortunately, countries with large livestock production, such as China and Brazil, still remain the largest producer and consumer of antibiotics with 52% being used in animal production [7, 9]. The primary concern is that the antibiotics used in livestock sectors are also critically important for human health, including cephalosporins and fluoroquinolones. This can lead to bacteria becoming resistant to the last line of defence antibiotics and causing untreatable infections in humans and animals [10].

The gut microbiome of pigs plays a significant role in nutrition, metabolism and health. Alteration of the gut microbiome using probiotics or vaccines can prevent diseases leading to feeding efficiency, resistance to diarrhoea, meat quality, and immune function [11, 12]. Antibiotic usage influences the gut microbiome of pigs amongst other factors such as age, host genes, gender and the castration of male pigs. The gut microbiome harbours a diverse microbial community which supports the health of the animal, helping to exclude pathogens [9]. A highly diverse gut microbiome can become less diverse after antibiotic treatment causing lasting changes in the composition of the microbiome of pigs promoting antibiotic resistant bacteria and increasing the prevalence of antibiotic resistance genes (ARGs) [9]. Antibiotic resistant bacteria and ARGs can be excreted with fecal matter as a significant amount (30-80%) of antibiotics are excreted due to incomplete metabolism. Animal feces are commonly used as manure in agricultural practices in surrounding farms which can promote the spread of antibiotic resistance. Additionally, unconsumed feed, containing these antibiotics, can contaminate soil directly or indirectly affecting aquatic and farming systems through run-off [1].

Since it has been assumed that the fecal microbiome is a subset of the gut microbiome in pigs, it can be used to understand the effects of antibiotic usage on the gut microbiome, monitor the spread of antibiotic resistance to other environments and mitigate the risks by influencing farm policies [13]. We aim to use publicly available metagenomic data obtained from the fecal microbiome of pigs raised with and without antibiotics to identify and characterise the differences in the microbial community, ARGs and mobile genetic elements. Through this, we aim to establish the impact of antibiotics on the gut microbiome of pigs to broaden our understanding of antimicrobial resistance in food animals.

METHODS AND MATERIALS

5.1.1. DATA SOURCES

103 publicly available metagenomic sequencing sets were obtained from studies that raised pigs with (AB+) and without antibiotics (AB-) (Table 5.1). Studies that had pigs raised without antibiotics and those that received antibiotics for illness only were categorised as "without antibiotics" (n = 56). Pigs raised with antibiotics were categorised as such (n = 47). Data was obtained from NCBI SRA (https://www.ncbi.nlm.nih.gov/sra) and the EBI MGNIFY database (https://www.ebi.ac.uk/metagenomics). A combination of keywords was used on NCBI SRA, these included "pigs", "sus scrofra", "feces", "metagenomes" and "fecal". On the EBI MGNIFY database, keywords included: biome = "fecal", text = "fecal", "metagenome", "fecal animal", "pigs". A summary of the studies used is provided in Table 5.1. Detailed information about the metagenomes retrieved from the databases is included in Supplementary Table S1.

Table 5.1: Summary of included studies, sample number for each group of pigs (AB+/AB-), antibiotic usage (usage), treatment and antibiotic type (type) used in the study.

Pigs	Study	Sample no.	Usage	Treatment	Type	Citation
AB+	1	10	Yes	Raised	Tetracycline	[14]
AB-	2	27	No	-		[15]
AB+	3	11	Yes	Raised	Antifolates, tetracycline, beta-lactams	[16]
AB-		9	Yes	Illness	Antifolates, chloramphenicol, tetracycline, beta-lactams	[16]
AB-	4	7	No	-		[17]
AB+	5	26	Yes	Raised	Oxytetracycline	[18]
AB-		13	No	-		[18]

5.1.2. BIOINFORMATIC ANALYSIS

To ensure that good quality reads were used throughout the study, quality control of reads was done using FastQC v.0.11.9 [19], and filtered using Trimmomatic v.0.39 [20]. Reads were trimmed for quality and adaptor contamination using Trimmomatic [20] while specifying ILLUMINACLIP: adapters.fa:2:30:10 LEADING:8 TRAILING:8 MINLEN:30 SLIDINGWINDOW: 4:20 parameters. The microbiome of pigs AB- and AB+ groups were profiled by Kraken2 v.2.1.0 [21] using the minikraken2

microbial database (2019, 8GB). Thereafter, species confirmation and the estimation of abundance were done by Bracken v.2.6.2 [22]. The relative abundances of bacterial genera and species were used to determine the composition of the microbial population. The average relative abundances of the bacterial species were used to calculate the alpha and beta-diversity between the groups of pigs raised with and without antibiotics.

Principal component analysis (PCA) was performed in R v.2.6-4 using the prcomp function to determine the differences between fecal samples obtained from pigs raised with and without antibiotics. Scores for each principal component (PC1 and PC2) of the microbiome were plotted on the x-axis and y-axis. The level of correlation was observed at the species level.

For the alpha-diversity, the Shannon diversity index was used to determine the diversity of the microbiome and resistome using the PAST v.4.03 software [23]. The Kolmogorov-Smirnov test was used to assess whether there were significant differences in the Shannon diversity indices between the AB+ and AB- groups using the scipy.stats package in Python. A p-value greater than 0.05 ($p \geq 0.05$) indicated significant differences in the microbiome and resistome.

The beta-diversity with the Bray-Curtis dissimilarity was used to assess the difference between the fecal microbiome and resistome of pigs raised with and without antibiotics. The beta-diversity was visualised using Non-Metric Multidimensional Scaling (NMDS) and was conducted using the vegan package in R v.2.6-4. The goodness of fit for the NMDS ordination was assessed with 999 permutations to determine the significance of the clustering patterns using the envfit function in the vegan package in R v.2.6-4. Differences in the beta-diversity between pigs AB+ and AB- were considered significant at $p \leq 0.05$.

The filtered reads were *de novo* assembled using MegaHit v.1.2.9 [24] with default parameters. Assembled metagenomes were annotated to determine the antibiotic resistance gene families and their corresponding drug classes using the Comprehensive Antimicrobial Resistance Database Resistance Gene Identifier (CARD-RGI) v.3.1.2 with default settings including loose matches and the –low_quality –clean options [25]. The version of the database was kept consistent throughout the analysis. The relative abundances of the annotated antibiotic resistance genes in each group (AB+ and AB-) were assessed. This data was normalised based on the total number of antibiotic resistance genes detected in each group, indicating their respective proportions. The covariance between the bacterial species and antibiotic resistance genes was calculated in Python v.3.7 using numpy v.1.23.4 to determine if antibiotic usage is responsible for variation.

De novo metagenomic contigs were aligned for the detection of integrons and integrative elements by alignment to the INTEGRALL database [26] and the ICEberg database [27]. BWA-mem v.0.7.10 with default parameters was used to perform this alignment [28], generating a SAM file. To filter soft and hard clipped reads and undesirable alignments, the SAM files were filtered by the Samclip tool with default parameters. The output SAM file was converted into a BAM file using SAMtools v.1.9 [29]. Contigs that aligned to the databases were considered integrons and integrative conjugative elements.

PlasClass v.0.1.1 [30] was used to classify if plasmid-originating contigs. To ensure that contigs were truly plasmid-originating, a threshold of length ≥ 1000 and probability ≥ 0.75 was used. To determine if the detected plasmids were associated with the found antibiotic resistance genes, the plasmid-originating contigs were matched to the previously determined contigs carrying antibiotic resistance genes.

5.2. RESULTS AND DISCUSSION

In this study, publicly available metagenomic data representing the fecal microbiome of pigs raised with (AB+) and without (AB-) antibiotics were analysed to identify and characterise the microbiome, resistome and mobilome.

5.2.1. THE FECAL MICROBIOME IS INFLUENCED BY STUDY-SPECIFIC CONDITIONS AND ANTIBIOTIC USAGE

To investigate the effects of antibiotic usage on the taxonomic composition of the fecal microbiome between pigs raised with (AB+) and without (AB-) antibiotics, we conducted a PCA analysis using the average relative species abundances. Previous studies have shown that antibiotic administration in pigs alters the gut microbiome, consequently affecting the fecal microbiome of these animals [31].

In our study, five metagenomic datasets were used and categorised based on antibiotic usage (Table 5.1). In Figure 5.1 samples were colour-coded according to their studies with shapes representing antibiotic usage (Table 5.1). A smaller distance between samples on the PCA plot indicates a greater similarity between samples.

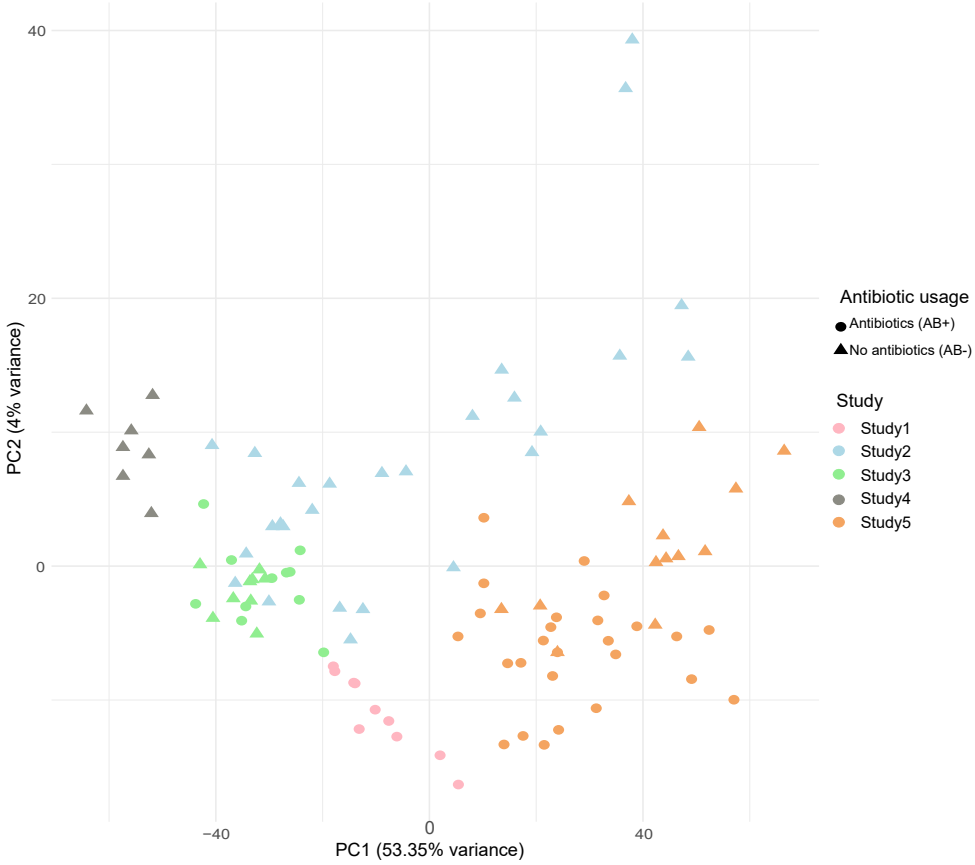

Figure 5.1: Visualisation of the PCA analysis using the average relative abundance of the bacterial species detected in fecal microbiome of pigs raised with (AB+) and without antibiotics (AB-) with the explained variance in brackets. Each colour represents a sample in a study (study 1= pink, study 2 = blue, study 3 = green, study 4 = grey, study 5 = orange) and shapes represent antibiotic usage (antibiotics or AB+ = circle and no antibiotics or AB- = triangle).

Antibiotic usage affects the fecal microbiome in combination with the study's origin. Figure 5.1 shows the grouping of samples according to their respective studies. This grouping indicates that the microbial populations in each study are influenced by study-specific conditions. Specifically, samples grouped as AB+ and AB- obtained from study 3 and study 5 overlap within their respective studies. Study 3 sampled from Canada while Study 5 sampled from Austria. Unfortunately, due to the lack of metadata we cannot make inferences based on the study-specific conditions;

however, these can include geographical location, pig diet and management practices. These factors can affect the composition of the fecal microbiome. When samples are labelled by antibiotic usage, as shown in Figure 5.1, samples group accordingly, indicating an impact on the composition of antibiotics on the fecal microbiome combined with study-specific conditions. Overall, pigs raised with and without antibiotic usage can have zoonotic pathogens such as *Clostridioides difficile (C. difficile)* in the feces, as detected in our study (Figure 5.2) [32]. This can potentially spread to other environments such as soil, water, and other animals [33, 34].

5.2.2. PROBIOTIC BACTERIA ARE DOMINANT IN THE FECAL MICROBIOME OF PIGS RAISED WITH AND WITHOUT ANTIBIOTICS

To assess the differences in the microbial composition in the fecal microbiome of pigs AB+ and AB-, we performed taxonomic classification. Additionally, we investigated the similarities between the microbial communities to assess if antibiotic usage influences the bacterial species found and their abundances. Antibiotic administration has previously been shown to affect the indigenous microbes in animal feces leading to changes in the microbial community structure and resistance [35].

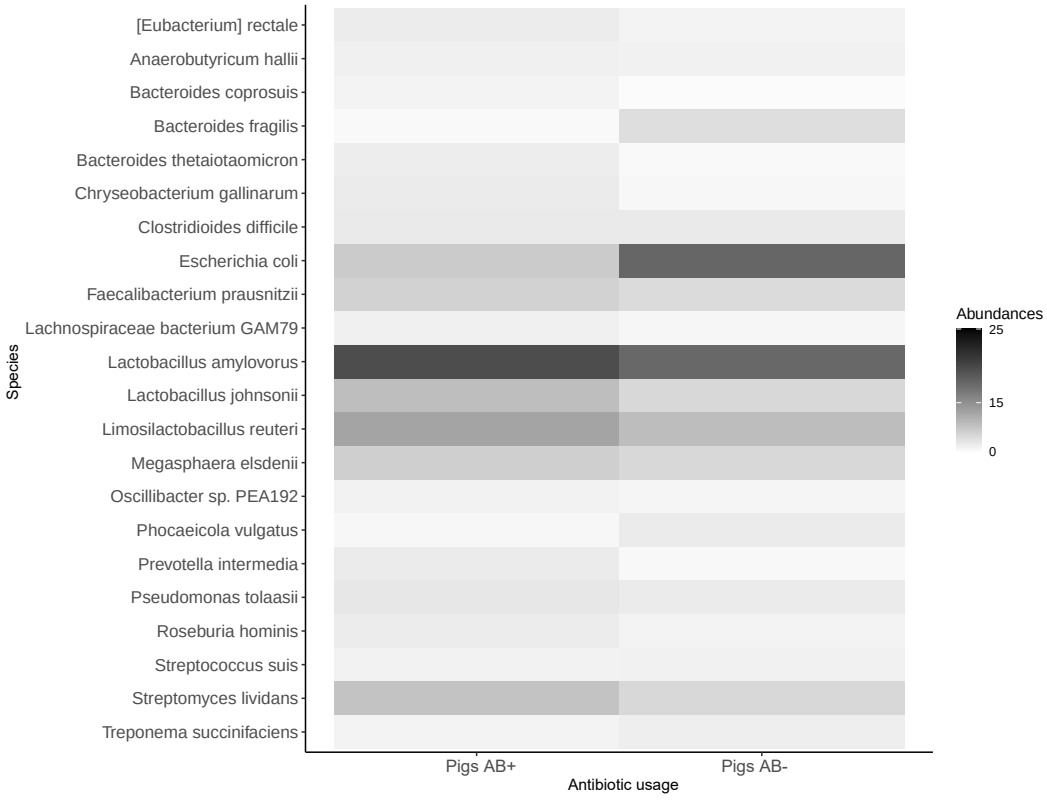

Figure 5.2: Heatmap showing the average relative abundance of bacterial species over 1% in the fecal microbiome, comparing pigs raised with (AB+) and without antibiotics (AB-). Higher abundances are indicated by the intensity of grey in the heatmap.

Based on the normalised data, a higher number of genera and species were present in the fecal microbiome of pigs raised without antibiotics (AB-). Specifically, we identified a total of 998 genera and 2580 species across the AB+ pigs studies

compared to a total of 1024 genera and 2845 species across the AB- pigs studies. This increased number of species in the AB- group suggests that the absence of antibiotics allows for a more complex and varied microbial community (Supplementary Tables S2-S3). It should be noted that the samples included were obtained from different studies therefore sample processing and sequencing depth can influence these results.

The higher number of bacterial species in pigs AB- may contribute to a more resilient gut ecosystem, potentially enhancing overall health and disease resistance [36]. 14 species that had an abundance of over 1% were in common between the two groups (Supplementary Table S5). This is supported by the Jaccard similarity of 0.685 indicating that 68% of the total bacteria present were shared between pigs AB+ and AB-. Additionally, the Shannon_H diversity index was 4.018 for pigs AB+ compared to 4.007 for pigs AB- suggesting that pigs raised with antibiotics (AB+) have a more diverse microbiome as compared to pigs AB- ($p \geq 0.05$). Details on the abundances of the bacterial genera, species, unique species and commonly identified species can be found in Supplementary Tables S2 - S5.

Probiotic bacteria are dominant in the fecal microbiome of pigs AB+ and AB- (Figure 5.2). We detected a higher average relative abundance of *Lactobacillus* genera (Supplementary Table S2), specifically *Lactobacillus amylovorus (L. amylovorus)* in the fecal microbiome of pigs AB+ and AB-. The *Lactobacilli* species is one of the dominant bacterial groups establishing a stable population in the intestinal tract of piglets [37]. *Lactobacillus* is also a probiotic candidate known to have antibacterial properties. In animal husbandry, *L. amylovorus* is a feed additive which assists in the daily weight gain of animals and resistance against enteric pathogens such as *Salmonella enterica (S. enterica)* and *Escherichia coli (E. coli)* [38–40].

Combining probiotic bacteria with antibiotics may suppress opportunistic pathogens (Figure 5.2). In our study, we observed the *Escherichia* genera are abundant in the fecal microbiome of pigs AB- with most of the species attributed to the enteric opportunistic pathogen, *E. coli*. Contrastingly, this abundance is low in pigs AB+ indicating that the use of antibiotics in the growth of pigs may suppress the proliferation of *E. coli*. Previous studies have shown that while *E. coli* isolates are found in the feces of pigs before antibiotic administration, the use of antibiotics can suppress these opportunistic pathogens leading to changes in the abundances [41, 42].

Furthermore, we identified the unique bacterial species present in pigs AB+ and AB- (Supplementary Table S4). While we see a mixture of pathogenic bacteria and those that support gut health, pathogenic bacteria such as *Legionella pneumophila (L. pneumophila)*, *Yersinia enterocolitica (Y. enterocolitica)*, and *Vibrio cholerae (V. cholerae)* were identified in the feces of pigs AB+ and not in AB-. On the other hand, beneficial gut-supporting bacteria such as *Bifidobacterium* were identified in pigs AB- indicating a healthier gut microbiome which can lead to decreased diarrhoea incidence and the inhibition of pathogens [43].

As stated by Rochegue et al. [44], antibiotics usage may reduce microbial diversity however, this was not evident in our study even though pigs AB+ and AB- exhibited differences in the microbial community. The use of antibiotics can influence the microbial community and potentially promote the selection of antibiotic resistant bacteria.

VARIATION IN THE MICROBIAL COMMUNITY OF AB- PIGS

To determine the differences in microbial communities found between the fecal microbiome of pigs AB+ and AB-, we measured the beta-diversity using the Bray-Curtis dissimilarity index. This index quantifies the distance between microbial communities based on species abundances. Visualised with non-metric multidimensional scaling (NMDS), a smaller distance between samples within a group i.e., AB+ or AB-, indicates a greater similarity between these microbial communities [45].

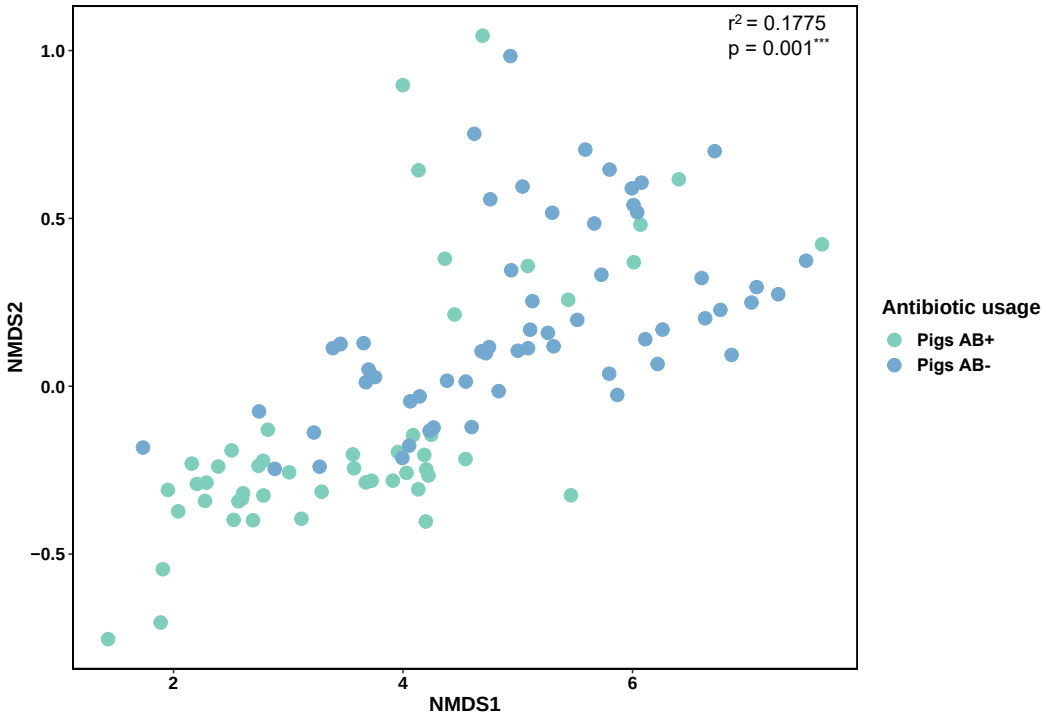

Figure 5.3: Visualisation of the beta-diversity analysis of the microbial community by Non-metric Multi-dimensional Scaling Ordination (NMDS) using Bray-Curtis dissimilarities between the fecal microbiome of pigs raised with and without antibiotics. Samples for each antibiotic usage group are indicated in different colours (Pigs AB+ = light blue, Pigs AB- = dark blue). Variation (r^2) and p-value, calculated using the goodness of fit, is indicated in the top right corner with *** denoting the statistical significance of the difference between pigs AB+ and AB-.

Figure 5.3 illustrates the Non-Metric Multidimensional Scaling (NMDS) analysis of the fecal microbiome, highlighting differences in the microbial populations between pigs raised with antibiotics (AB+) and those without antibiotics (AB-). Figure 5.3 shows the AB+ samples are more tightly grouped with less distance between them, indicating a high similarity in their microbial communities.

In contrast, the AB- samples are more dispersed as shown by Figure 5.3, reflecting greater variability in their microbial communities. This implies that the microbiome of untreated pigs may be more influenced by external factors such as diet, environmental conditions, and other study-specific variables. The diversity in the AB- group indicates a more heterogeneous microbial population, potentially contributing to different health and functional outcomes such as a balanced immune system and better digestion [46]. Statistical analysis confirms the effect of antibiotics on microbial

diversity, with antibiotics explaining 17.75% of the variation in microbial communities (r^2 = 0.1775, p = 0.001). Previous research has shown that less diversity in the gut microbiome of pigs is an indication of poor health. As stated previously, external factors can influence the gut microbiome however, microbial infections and the constant use of antibiotics lead to pigs being in an unhealthy state [47].

5.2.3. SIMILAR AMR GENE FAMILIES ARE PRESENT IN PIGS RAISED WITH AND WITHOUT ANTIBIOTICS

To investigate the effects of antibiotic usage on antibiotic resistance genes (ARGs) in the fecal microbiome of pigs AB+ and AB-, we identified the AMR gene families as bacteria that are constantly exposed to antibiotics may carry different ARGs [48]. Furthermore, we determined the differences in the AMR gene family composition by conducting a beta-diversity analysis visualised by NMDS. Similar to our analysis of the microbial composition, a smaller distance between samples of the AB+ or AB- groups indicates a greater similarity in AMR gene family composition. Additionally, we calculated the Shannon_H diversity and Jaccard similarity index to assess the diversity within a group and if there are similar AMR gene families found between the pigs AB+ and AB- groups. Details can be found in Supplementary Tables S6 - S8.

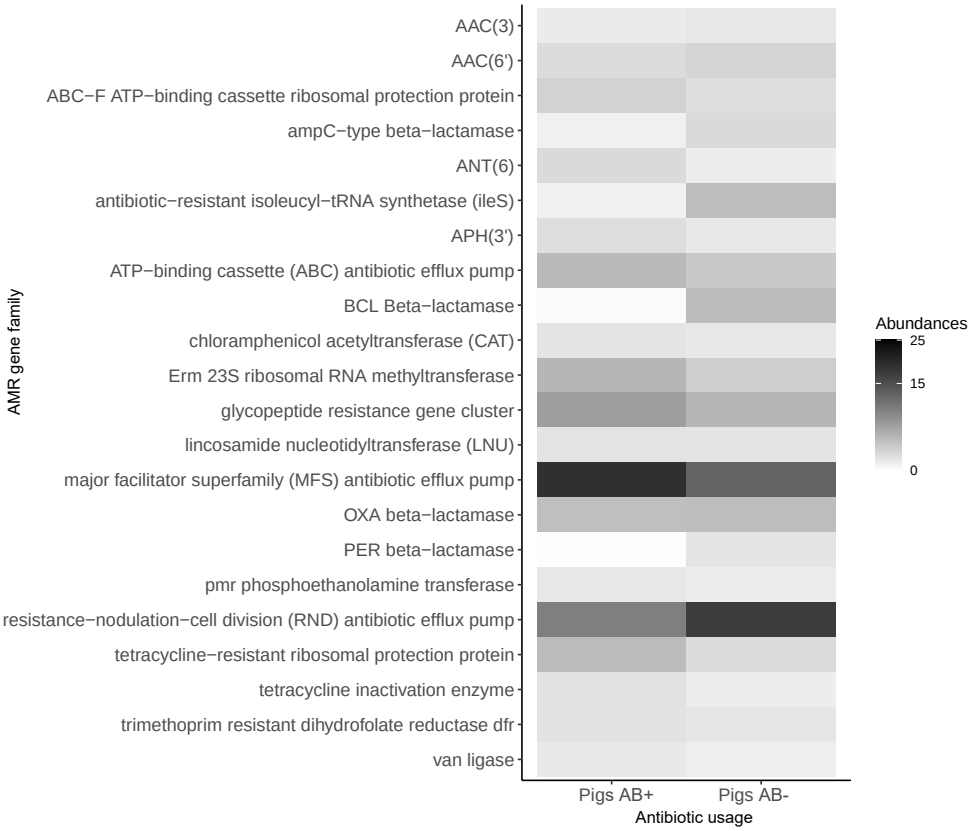

Figure 5.4: Heatmap showing the average relative abundance of AMR gene families over 1% in the fecal microbiome, comparing pigs raised with (AB+) and without antibiotics (AB-). Higher abundances are indicated by the intensity of grey in the heatmap.

Figure 5.4 shows the AMR gene families identified with an average relative abundance of over 1% in the fecal microbiome of pigs AB+ and AB-. The resistance nodulation cell division superfamily (RND) efflux pump, contributing to multi-drug resistance by expelling antibiotics from the bacterial cell wall, comprised 11% abundance of the AMR gene families detected in pigs AB- and 15% in pigs AB+ (Supplementary Table S6) [49]. Previous studies have shown the presence of RND efflux pump gene families is common in pig raised with and without antibiotics. Additionally, RND efflux pumps are commonly found in gram-negative bacteria e.g. *E. coli* and can export β-lactams out of the cell membrane [16, 50–53]. Interestingly, in our study, the presence of *E .coli* combined with the use of β–lactam antibiotics in feed (AB+) and to treat illness (AB-) may have contributed to the high abundance

of the RND efflux pump gene family observed.

Additionally, the OXA β-lactamase gene family had similar average relative abundance among the AMR gene families detected in the fecal microbiome of AB- pigs (3%) as compared to AB+ (3.5%) (Figure 5.4 and Supplementary Table S6). Since β-lactams are the most sold antibiotics used for food-producing animals i.e., pigs, it was expected that there would be a higher abundance of the OXA β-lactamase gene family present in AB+ samples. However, pigs raised without antibiotics or organically can be more exposed to environmental sources, pathogens, and ARGs [54]. The abundance of the OXA β-lactamase gene family in pigs AB- could also be attributed to either the administration of β-lactams to treat illnesses or injuries, horizontal gene transfer, management practices in farms or environmental differences [55, 56].

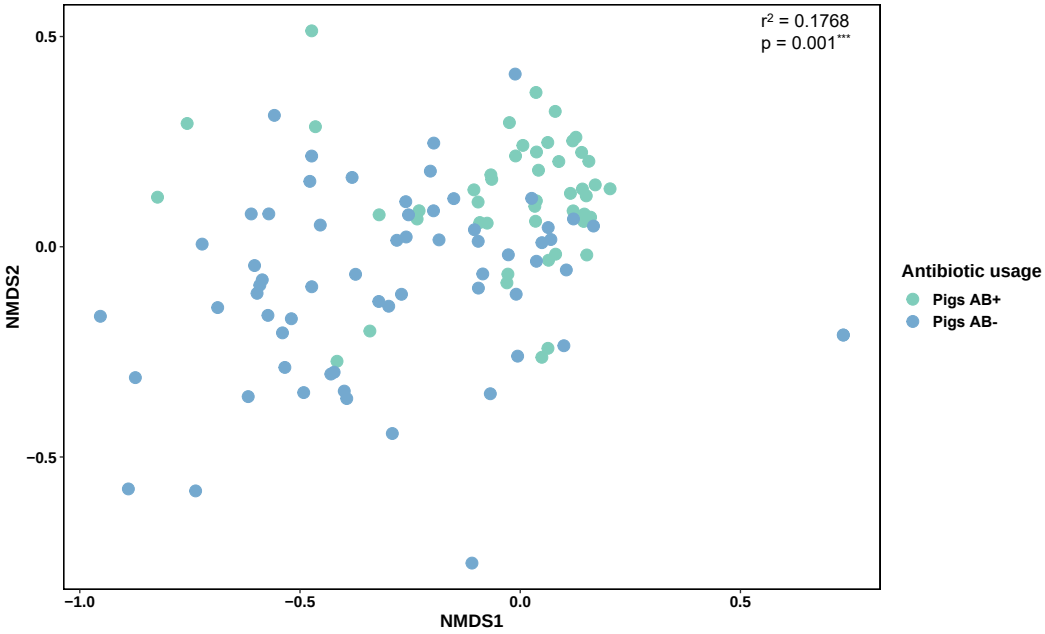

Figure 5.5: Visualisation of the beta-diversity of the AMR gene families in the fecal microbiome of pigs raised with and without antibiotics by Non-Metric Multidimensional Scaling (NMDS). Samples are colour-coded based on antibiotic usage (Pigs AB+ = light blue, Pigs AB- = dark blue). Variation (r^2) and p-value are indicated in the top right corner with *** denoting the statistical significance of the difference between pigs AB+ and AB-

Antibiotic usage causes variation in AMR gene families detected. Figure 5.5 illustrates the beta-diversity of AMR gene families present in pigs treated with antibiotics (AB+) and those not treated with antibiotics (AB-). Samples within the AB+ group are tightly clustered together whereas samples from the AB- group have

larger distances between them indicating variation in the resistome between both groups. This is further supported by the Shannon_H diversity indices of 3.968 for AB+ and 4.052 for AB- ($p \geq 0.05$). This variation in AMR gene families can be caused by antibiotic usage ($r^2 = 0.1768$, $p = 0.001$). Interestingly, variations in AMR gene families are similar to the variation reported previously with the microbial communities ($r^2 = 0.1775$, $p = 0.001$) however, covariation analysis has shown that minimal co-variation occurs between the bacteria and the AMR gene families identified suggesting that antibiotic usage is responsible for differences in AMR gene families and microbiome composition (Supplementary Table S7).

Additionally, the Jaccard similarity index of 0.744 suggests that only 74% of the AMR gene families are shared between the AB+ and AB- groups, indicating an overlap in the AMR gene family composition between both groups. This can be seen in Figure 5.5, as some samples from the AB+ and AB- are close together while others are dispersed indicative of variation of AMR gene family composition. 11 AMR gene families present at over 1% were common between both groups (Supplementary Table S8). Differences in AMR gene families may be influenced by environmental factors, housing, or farm management practices, though more research is needed to fully understand the role of animal husbandry in shaping ARG distributions [56–58].

5.2.4. PLASMIDS LINKED TO COMMONLY USED ANTIBIOTICS

To investigate the presence of plasmids and their connection to antibiotic resistance in the fecal microbiome of pigs AB+ and AB-, we determined the percentage of contigs classified as plasmids. Furthermore, we examined their association with AMR gene families. Plasmids play a significant role in the spread of ARGs and AMR as bacteria and antibiotic residues present in feces can enhance horizontal gene transfer [9, 59, 60].

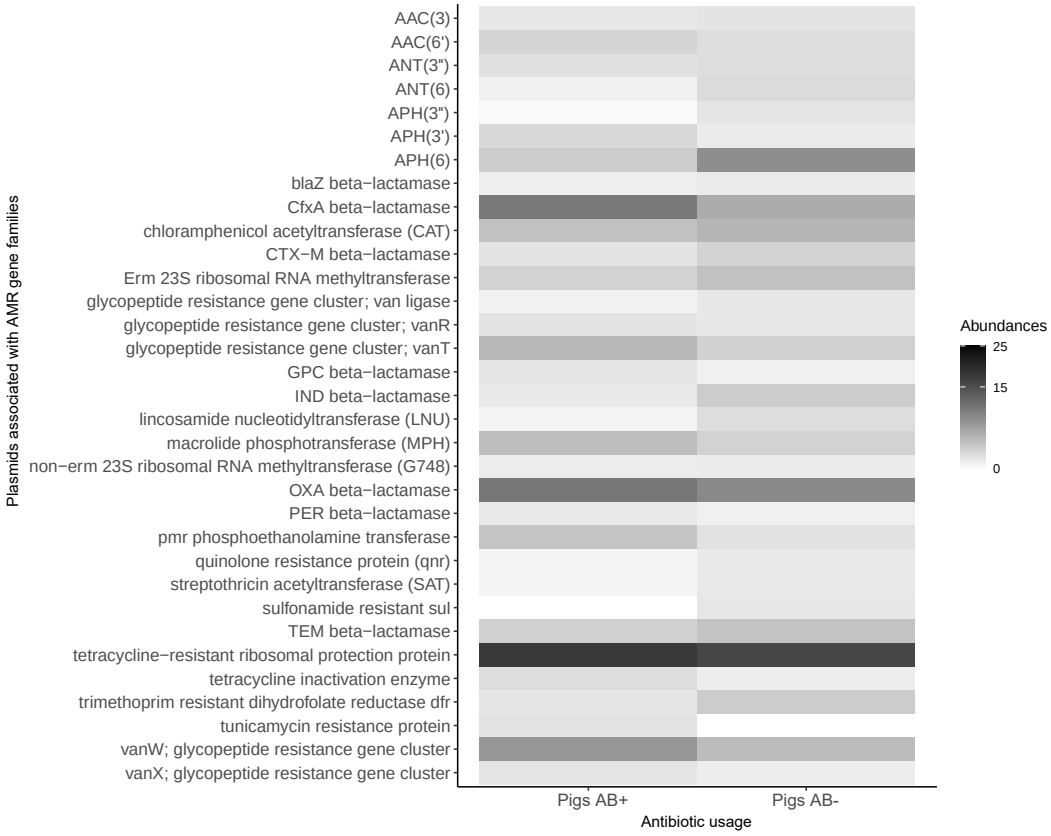

Figure 5.6: Heatmap showing the average relative abundances of plasmids associated with AMR gene families over 1% in the fecal microbiome, comparing pigs raised with (AB+) and without antibiotics (AB-). Higher abundances are indicated by the intensity of grey in the heatmap.

Over 1% of contigs were classified as plasmids in both groups. Further analysis showed that the AMR gene families were associated with 0.1% of the classified plasmids. Plasmids associated with AMR gene families were detected in both pigs AB+ and AB- (Figure 5.6 and Supplementary Table S9). In Figure 5.6 we observed a high average relative abundance of plasmids associated with the tetracycline-resistant ribosomal protection protein conferring resistance to the tetracycline antibiotic and the OXA β-lactamase gene family conferring multi-drug resistance to carbapenem; cephalosporin; penam.

Plasmids linked to tetracycline resistance were detected in the fecal microbiome of pigs AB+ and AB- (11%). Tetracycline has been commonly used in animal feed and for the treatment of illnesses, including this study (Table 5.1 and Figure 5.6)

[61]. Previous studies have discussed the continuous usage of tetracycline antibiotics selecting for plasmids associated with tetracycline resistance [8]. Furthermore, tetracycline resistance genes have been detected in soil that has been amended with animal manure indicating the transfer of genes via plasmids from one environment to another [62–65].

Secondly, plasmids associated with the OXA β-lactamase gene family were present at an abundance of 6-7% of the AMR-associated plasmids detected in pigs AB+ and AB- (Figure 5.6 and Supplementary Table S9). The OXA β-lactamase gene family confers multi-drug resistance to carbapenem; cephalosporin and penams. Similar to the use of tetracycline, β-lactams were used in both groups as feed additives or for the treatment of illnesses [18]. Plasmids associated with β-lactams are a public health concern as these can be transmitted through environmental sources such as manure, soil, air, wildlife and humans, especially farm workers [66, 67].

The detection of plasmids associated with tetracycline resistance and OXA β-lactams is not surprising as previous research has indicated that piglets can exhibit a pre-existing resistance pattern from birth that reflects the environment [68]. This aligns with the data used in our study, as antibiotics such as tetracycline, oxytetracycline, and β-lactams were used in pigs raised with antibiotics. The use of antibiotics even for treatment can enhance the selection for ARGs and the horizontal transfer of these genes by plasmids. While the use of antibiotics is a common practice for therapeutic purposes, the low dosages can contribute to the emergence of antibiotic resistant bacteria thereby spreading to animals and humans [18, 31].

5.2.5. CLASS 1 INTEGRONS LINKED TO CHLORAMPHENICOL RESISTANCE

To investigate the presence of integrons in the fecal microbiome of pigs AB+ and AB-, we classified contigs as integrons. Integrons are responsible for the spread of AMR via horizontal gene transfer between pathogenic and non-pathogenic bacteria found in all environments [69]. In our study, we determined the number of integrons present in the contigs from pigs AB+ and AB-. We then classified them into classes 1, 2, and 3 to determine their abundances. Furthermore, we linked the detected integrons to ARGs to assess their role in AMR dissemination (Supplementary Table S10).

Table 5.2: The average relative abundances of integrons in their respective classes detected in the fecal microbiome of pigs raised with (AB+) and without antibiotics (AB-).

Classes of Integrons	Pigs AB+	Pigs AB-
Class 1	18%	14%
Class 2	5%	3%
Class 3	1%	1%

Firstly, less than 0.01% of contigs were classified as integrons. From the detected integrons we classified them into their respective groups of classes 1, 2 and 3. Table 5.2 shows that class 1 integrons were more abundant as compared to classes 2 and 3. Class 1 and 3 integrons are commonly found in freshwater and soil environments whereas class 2 integrons are found in marine environments [69]. Additionally, bacteria bearing class 1 integrons have been reported in environmental reservoirs such as farms, agricultural land and wastewater systems [70, 71]. It has been stated that the abundance of class 1 integrons can be identified in millions to billions of copies in a single gram of feces from agricultural animals [72].

Class 1 integrons associated with the *cmxA* antibiotic resistance gene was detected in a higher abundance in pigs AB+ (30%) (Supplementary Table S10). This abundance was lower in AB- (15%). Chloramphenicol has been used to treat illness in our study, however, we have no information on the use of chloramphenicol in pigs raised with antibiotics. Previous research has shown that *cmxA* gene is linked to chloramphenicol resistance and has been detected in pig feces and in amended agricultural soils [70, 71, 73, 74]. This indicates that these ARGs can spread from livestock farms and animal feces to pathogens in soils via horizontal gene transfer i.e. integrons [73, 74].

A similar trend was observed with class 3 integrons as 71% of the class 3 integrons detected in pigs AB+ were associated with *blaOXA-2* gene. This was lower in the pigs AB- (39%) (Supplementary Table S9). This trend could be indicative of the selective pressure exerted by antibiotic use, where the presence of antibiotics may encourage the proliferation of resistant genes like *blaOXA-2* within integrons. Class 3 integrons are known for their role in antibiotic resistance and can capture and express genes conferring resistance to various antibiotics such as β-lactams [75, 76].

The prevalence of such genes associated with integrons in livestock is a concern for public health, as it may contribute to the spread of antimicrobial resistance from animals to humans through the food chain or other means of zoonotic transfer. It's important to monitor these trends and implement strategies to mitigate the spread of resistance genes, ensuring both animal health and public safety [77].

5.2.6. INTEGRATIVE ELEMENTS DETECTED IN SIMILAR ABUNDANCES

To identify the presence of integrative elements in the fecal microbiome of pigs AB+ and AB-, we determined the proportion (%) of integrative elements present in the contigs. Integrative elements are comprised of integrative and conjugative elements (ICEs), integrative and mobilisable elements (IMEs) and *cis*-integrative and mobilisable elements (CIMEs).

Integrative elements were detected in less than 0.0001% of contigs in both pigs AB+ and AB-. From those detected, approximately 50% of the integrative elements were ICEs, similar to a previous study [78]. ICE families were identified in both antibiotic groups, ie, AB+ and AB- (Supplementary Table S11). Tn916 made up 2-3% of the detected ICE families and has been classified as a conjugative transposon which can carry ARGs. Tn916 has also been linked to pathogenic bacteria such as *Enterococcus fecalis (E. fecalis)* and *Staphylococcus aureus (S. aureus)* [79]. This highlights the potential risk of AMR spreading within and between bacterial populations in livestock which can have implications for animal health and food safety.

The high abundance of various transposons among the identified IME families further emphasises the dynamic nature of genetic elements within these bacterial communities (Supplementary Table S12). Transposons are mobile genetic elements that can move within and between genomes, contributing to genetic diversity and adaptability [80]. Their prevalence in both AB+ and AB- groups suggests a widespread potential for genetic exchange and the evolution of bacterial populations in response to environmental pressures, such as the use of antibiotics.

Overall, these findings underscore the importance of monitoring integrative elements and their associated families, such as ICEs and transposons, to better understand their impact on bacterial communities within livestock and the broader implications for antibiotic resistance management.

5.3. CONCLUSION

We conducted a comprehensive analysis of the fecal microbiome of pigs raised with (AB+) and without (AB-) antibiotics to assess how antibiotic exposure influences the microbial community, antibiotic resistance genes (ARGs), and mobile genetic elements (MGEs).

Our study demonstrated that antibiotic usage influences the fecal microbiome of pigs together with the farm and environmental conditions specific to the studies used. Notably, while both pigs AB+ and AB- exhibited a high average relative abundance of the probiotic bacteria *Lactobacilli*, there was a difference in the diversity of bacterial species between the two groups. The use of antibiotics contributes to the variation in the fecal microbiome of pigs, however, it may not strongly influence the overall microbial community structure.

Similarly, a diverse set of antibiotic resistance genes was observed in the AB- group, underscoring the impact of antibiotic use on the gut microbiome. However, this impact does not create a huge variation between the resistome of AB+ and AB- as indicated by the Shannon_diversity index. Unfortunately, this study is limited in sample size and metadata to confirm the influence of the antibiotics on the microbial community, resistome and mobilome.

We also identified the widespread presence of the RND efflux pump gene family in both groups, although the OXA β-lactamase gene was more prevalent in the AB- group. Additionally, plasmids associated with tetracycline and OXA β-lactamase resistance were detected. Integrons carrying chloramphenicol resistance genes and integrative elements linked to transposons were also identified, further illustrating the complexity of ARG dissemination in these environments.

Understanding the impact of antibiotic usage on the gut microbiome of pigs is critical for comprehending the spread of ARGs, especially since a significant portion of ARGs and antibiotic-resistant bacteria are excreted in feces. Although our study provides valuable insights into how antibiotic use affects the fecal microbiome, it is limited by the sample size, the absence of metadata and the fact that fecal samples may not fully represent the entire gut microbiome. Nevertheless, these findings highlight the potential for fecal matter to influence environmental microbial communities and contribute to the broader dissemination of antibiotic resistance.

Future research should focus on the broader sampling of the gut microbiome to better understand the full impact of antibiotic use in agricultural settings.

4. SUPPLEMENTARY MATERIAL

Due to the extensive nature of supplementary materials associated with this chapter, they are not included in this thesis. Interested readers are encouraged to refer to the Supplementary material online (see `https://surfdrive.surf.nl/files/index.php/s/Y27zkpPxaS4HMkq`)

5. DECLARATIONS

5.1. FUNDING

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6

PORTABLE IN-FIELD DNA SEQUENCING FOR RAPID DETECTION OF PATHOGENS AND ANTIMICROBIAL RESISTANCE: A PROOF-OF-CONCEPT STUDY

This chapter is under review in BMC Microbiology

Infectious diseases represent a major global health threat due to the rapid spread of pathogens, leading to widespread outbreaks. Concurrently, antimicrobial resistance (AMR) is increasing, making standard treatments less effective and complicating infection management. Effective surveillance systems are essential to address these challenges. We conducted a proof-of-concept study to evaluate a portable in-field microbial lab against a traditional molecular lab for DNA isolation, sequencing, microbial detection, antibiotic resistance gene identification, and plasmid classification. Samples from lake water, wastewater treatment plant sludge, and retail meat were selected to reflect relevant surveillance vectors. This approach provides valuable data for environmental monitoring, public health, and food safety, aiding in outbreak preparedness. We compared results using five metrics: DNA yield and purity, read N50, taxonomic classification, antibiotic resistance gene identification (ARGs), and plasmid classification. Our study found that metagenomic bacterial DNA isolation from environmental and food sources is feasible with portable lab technology, producing ≥800ng of DNA, suitable for Nanopore sequencing. DNA from retail meat, lake, and sludge samples resulted in similar read numbers and read N50 values. Taxonomic classification was achieved at the genus and species levels. A Jaccard similarity of over 50% was observed in the top 20 most abundant species between chicken samples, and lake samples. ESKAPE pathogens were detected in chicken and lake samples. ARGs and plasmids were also identified in both retail meat and lake samples.

6.1. INTRODUCTION

Infectious diseases are one of the leading causes of death worldwide. Pathogens rapidly evolve and spread disease, leading to more virulent forms, including antimicrobial resistant bacteria [1]. The World Health Organisation (WHO) has named antimicrobial resistance (AMR) as one of the top ten global health threats among air pollution, non-communicable diseases, influenza and Ebola [2]. In 2019, infectious diseases caused an estimated 13.7 million deaths, including 4.95 million AMR-associated deaths. This number is projected to rise to 10 million deaths annually by 2050 [3, 4]. Organisations such as the Food and Agriculture Organization of the United Nations (FAO), the World Organisation for Animal Health (WOAH), and WHO have been working together to implement global action plans to tackle the burden caused by AMR [4]. This involves the strengthening of knowledge and evidence through research and surveillance systems as well as the monitoring of AMR across clinical and non-clinical sectors in all countries [5, 6].

Over the past decade, infectious diseases and AMR have been studied using traditional culture-dependent approaches and other molecular diagnostic techniques such as polymerase chain reaction (PCR), electrophoresis, multi-locus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE) [6–8]. These methods, while effective, provide limited information on only specific pathogens, can fail to detect pathogens, only detect culturable bacteria, are time-consuming, require trained personnel and dedicated laboratories and can be expensive [1, 6, 7, 9]. Unfortunately, due to the rapid spread and evolution of pathogens and

microorganisms, new rapid detection techniques are needed [1, 7].

The COVID-19 pandemic highlighted the need for rapid detection methods as the surge in testing overwhelmed the local capacities in different countries [10]. Conventional diagnostic workflows for COVID-19 detection, relying on specialised equipment and supplies, faced shortages, creating an urgent need for cost-effective solutions [10–13].

In response, platforms utilising Third Generation Sequencing (TGS) were developed to facilitate real-time surveillance of viral pathogens. Examples of such platforms employed long-read Oxford Nanopore Technologies (ONT) sequencing, integrated with Isothermal Rapid Viral Amplification for Near Real-time Analysis (NIRVANA), CalmBelt and sequence variation analysis to facilitate rapid COVID-19 genome characterisation for outbreak tracking and diagnostics [12, 14, 15]. Such technologies offer the added benefit of portability, making them suitable for deployment in various locations, including wastewater monitoring [12, 16]. Additionally, a study comparing ONT sequencing with Illumina sequencing for COVID-19 detection found that ONT was not only faster but also more cost-effective, reducing both sequencing time and costs. This efficiency was further enhanced by using the rapid sequencing kit (SQK-RBK), which significantly reduced library preparation time [17].

The utility of ONT sequencing extends beyond COVID-19, proving valuable in antimicrobial resistance research. For example, a study by Martin et al. [18], utilised 16S rDNA amplicon sequencing with the ONT MinION and a portable Bento Bio Pro lab for detecting antibiotic-resistant *Campylobacter* in chickens in less than 5 hours. Similarly, Serpa et al. [19] employed ONT sequencing for detecting bacteria causing lower respiratory infections using CRISPR/cas9 techniques. ONT metagenomic sequencing has also demonstrated its capability to identify, characterise, and trace pathogens across diverse environments, including clinical, agricultural, environmental, and food safety contexts [9, 18–22]. As discussed by Bloemen et al. [9], ONT sequencing has been successfully applied in remote locations such as the ice caps in Iceland, sea ice in Allen Bay, Canada, and desert areas in Spain. These studies, utilising portable laboratory equipment and the ONT MinION device, have demonstrated that in-field sample processing can be performed effectively without issues related to sample transportation or preservation, thus ensuring reliable taxonomic classification.

Despite its advantages, the ONT MinION sequencer, though portable, still requires pre-sequencing procedures, such as DNA isolation and library preparation, to be conducted in a traditional laboratory setting. The development of portable devices like the Bento lab [23] offers a solution by enabling genomics experiments to be conducted in various locations without the need for the resources typically found in molecular labs. Such equipment is cost-effective as it consists of a thermocycler, heating block, gel electrophoresis, transilluminator and centrifuge. It is also available in a cheaper, entry-level model and the more expensive pro-level model, both of which can be connected to a battery to conduct in-field genomics experiments [24–26]. Additionally, the Flongle adaptor can be used with the MinION sequencing device. While it does enable the use of lower throughput, the Flongle flow cells may offer a cost-effective approach to long-read sequencing which has been suitable

for bacterial identification in clinical environments [27]. Such a portable laboratory device combined with a MinION and Flongle adaptor can be useful for antimicrobial resistance research, surveillance and in-field microbial detection.

In this proof-of-concept study, we first developed a modified DNA isolation protocol that can be used with the portable Bento lab (Pro-level) to yield high-quality DNA. Secondly, we assessed the differences between DNA isolated using a Bento lab (BL) and a traditional molecular lab (TL). We evaluated differences based on the following criteria: (i) DNA yield and purity, (ii) total nucleotide volume generated (Kb) and read N50 (Kb), (iii) similarity in taxonomic composition, (iv) identification of antibiotic resistance genes (ARG) and (v) classification of plasmids.

6.2. METHODS AND MATERIALS

6.2.1. SAMPLE COLLECTION AND PROCESSING

Three different samples were collected to assess their environmental and food safety impacts; lake water (n=1), sludge (n=1) and retail meat product (n=1). 2L of lake water was collected in a sterile bottle from the surface (5cm - 10cm) of a recreational lake in Delft, The Netherlands (52°01'18.2"N 4°22'59.3"E). Retail poultry (whole carcass) was purchased from a supermarket in Delft, The Netherlands and washed with 1L of distilled water. The carcass rinsate from the retail poultry bag was transferred into a sterile bottle for further processing. Lastly, 1.8mL of sludge was obtained at a wastewater treatment plant in Amsterdam West, The Netherlands. All samples were collected and stored on ice until DNA isolation was done on the same day.

6.2.2. DNA ISOLATION

To assess the differences between the portable pro-level Bento lab (BL) (Bento Bioworks, UK) and a traditional molecular lab (TL), DNA was isolated from all samples using both these lab types (n=6) and concentration was assessed using a Qubit 3 (Thermo Fisher Scientific, Wilmington, DE, USA). DNA was isolated from the sludge and carcass rinsate samples using the DNeasy Ultraclean Microbial extraction kit (Qiagen Inc., Valencia, CA) and from the lake water using the DNeasy PowerWater DNA kit (Qiagen Inc., Valencia, CA). Before isolation, 300mL of lake water was filtered using a vacuum pump with a 0.2mm cellulose filter (Merck Millipore, Ireland). DNA isolation was done following the manufacturers' instructions in the traditional molecular lab.

With regards to the Bento lab, we chose the pro-level model which has a varying centrifugation speed (max 13 500 RPM). The centrifuge capacity is also limited to 2ml Eppendorf tubes which can be overcome by increasing centrifugation times during experiments. Modifications to the DNA isolation protocols were made for the Bento lab as follows:

Modifications to the DNeasy PowerWater DNA extraction kit:
Step 8: Transfer the supernatant to a clean 2ml collection tube → transfer to 2ml Eppendorf tube.
Step 9: 13 000 x g for 1 min → 8000 x g for 3 min.
Step 10: Transfer the supernatant to a 2ml collection tube → transfer to 2ml Eppendorf tube.
Step 12: 13 000 x g for 1 min → 8000 x g for 3 min.
Step 13: Transfer the supernatant to a 2ml collection tube → transfer to 2ml Eppendorf tube.
Step 15-20: Load 650μl of supernatant onto an MB spin column → place MB spin column in a 1.5ml Eppendorf tube with cap cut off (modified collection tube).
Centrifuge at 13 000 x g for 1 min → 8000 x g for 3 min.
Step 22: 13 000 x g for 1 min → 8000 x g for 3 min.

Modifications to the DNeasy Ultraclean Microbial kit:
Step 1: Add 1.8ml of microbial (bacteria, yeast) culture to a 2ml collection tube → 1.8ml carcass rinsate / sludge in a 2ml Eppendorf tube.
Centrifuge at 10 000 x g for 1 min → 8000 x g for 2 min.
Decant the supernatant.
Centrifuge at 10 000 x g for 1 min → 8000 x g for 2 min.
Step 2: Transfer resuspended cells to a PowerBead Tube → PowerBeads from the PowerBead Tube were added to a 2ml Eppendorf tube.
Step 5: Centrifuge the tubes at a maximum of 10 000 x g for 30 s → 8000 x g for 2 min,
Step 6: Transfer the supernatant to a clean 2 ml Collection Tube → transfer to 2ml Eppendorf tube.
Step 8: Centrifuge the tubes at 10 000 x g for 1 min → 8000 x g for 3 min.
Step 9: Transfer the entire volume of supernatant to a clean 2 ml Collection Tube → transfer to 2ml Eppendorf tube.
Step 11: Load 700μl of supernatant onto an MB spin column → place MB spin column in a 1.5ml Eppendorf tube with cap cut off (modified collection tube) and load 700μl of supernatant to spin column.
Centrifuge at 10 000 x g for 30s → 8000 x g for 3 min.
Discard the flowthrough and add the remaining supernatant to the MB Spin Column → place the MB spin column in a 1.5ml Eppendorf tube with cap cut off (modified collection tube).
Centrifuge again at 10 000 x g for 30s → 8000 x g for 3 min.
Step 12: Add 300μl of Solution CB and centrifuge at 10 000 x g for 30s → 8000 x g for 3 min.
Step 13: Discard the flow-through. Centrifuge at 10 000 x g for 1 min → 8000 x g for 3 min.
Step 14: Place the MB Spin Column in a new 2 ml Collection Tube → place MB spin column in a new 1.5ml Eppendorf tube with cap cut off (modified collection tube).
Step 16: Centrifuge at 10 000 x g for 30s → 8000 x g for 3 min.
Step 17: Discard the MB Spin Column. The DNA is now ready for downstream applications → Store the flowthrough in a 1.5ml or 2ml Eppendorf tube.

6.2.3. ONT NANOPORE SEQUENCING

DNA libraries were prepared using only the Bento lab and the Rapid Sequencing Kit V.14 (SQK RAD114) (Oxford Nanopore Technologies, USA) as per the manufacturer's instructions for the Flongle application with Flongle flow cells (R10.4.1. FLO-MIN114). 75ng of DNA isolated using both lab types (n=6) and the Zymobiomics microbial community standard was used as a control (n=1) (Zymo Research Corporation, Irvine, CA, USA). Sequencing of all samples was run for 24 hours. The MinKNOW GUI (ONT, v.19.05.0) was used for setting appropriate sequencing parameters (analysis protocol, flow cell type, run duration, basecalling and for following the progression of the sequencing runs in real-time (number of reads, read length distribution and read quality, and pore activity status).

6.2.4. BIOINFORMATIC ANALYSIS

Raw .Pod5 files were basecalled using Dorado v.0.4.3 with the (–model dna_r10.4.1_e8.2_400bps_sup@v4.2.0). Basecalled bam files were converted to FASTQ files using Samtools v.1.9 and further processed using NanoFilt v.3 [28]. Taxonomic classification on the filtered reads representing each sample (n=7) was done using a modified protocol provided by (Taxonomic-Profiling-Minimap-Megan) at:
(https://github.com/PacificBiosciences/pb-metagenomics-tools). The NCBI nt database was downloaded (11-06-2024) and indexed according to Portik et al. [29].

Minimap2 v.2.26 [30] was used to align the raw reads to the NCBI nt database using the -ac map-ont –sam-hit-only –secondary=no –split-prefix command to generate a sam file. Sam files were converted to rma files using MEGAN v.6.25.9 [31] with the sam2rma function, megan-nucl-Feb2022.db and -lg -ram option to generate rma files with assigned read counts. The MEGAN6 community edition v.6.19 was used to visualise the rma files and export the taxonomic classifications with assigned read counts.

Antibiotic resistance genes were detected using BLAST v.2.11.0 [32] against the Comprehensive Antibiotic Resistance Database (CARD) v.4.0.2 [33] with an 80% identity and 60% query coverage to obtain the number of antibiotic resistance genes identified, the antibiotic resistance gene families and corresponding drug class. Reads were classified as plasmids by Plasclass v.0.1.1 [34] and were considered plasmid originating when a probability of over 0.75 was assigned to a read. Plasmid-assigned reads were cross-referenced with the identified antibiotic resistance genes to ascertain the presence of antibiotic resistance genes within the plasmids.

6.3. RESULTS

In this proof-of-concept study, we first aimed to determine if a modified DNA isolation protocol can be used with a portable lab (Bento lab) to yield high-quality DNA. Secondly, we compared the results generated from using a Bento lab to a traditional laboratory across various metrics: reads generated, read N50, total

data produced, microbial classification, detection of antibiotic resistance genes, and plasmid classification.

6.3.1. DNA ISOLATION IS FEASIBLE WITH MODIFIED PROTOCOL AND BENTO LAB

To assess the differences between the DNA isolated from different samples obtained by the Bento lab (BL) and the traditional lab (TL), we measured the DNA yield and purity. The prolevel Bento lab faces limitations due to its centrifugation speed and the size of Eppendorf tubes that can be used, which are critical factors in DNA isolation protocols. To address these constraints, a modified protocol is required that can still deliver high-quality DNA with good purity. DNA yield and purity are crucial in sequencing as they can directly impact the quality of the results. A high DNA yield ensures that there is enough DNA to be sequenced while purity ensures that no contaminants are affecting the sequencing process [35].

Table 6.1: Table showing the DNA yield (ng) and DNA purity (A260/A280) in the Chicken, Lake, and Sludge DNA samples isolated using the Bento lab (BL) and traditional lab (TL).

DNA yield (ng) produced		
Sample type	Bento lab (BL)	Traditional lab (TL)
Chicken	800	1780
Lake	2340	1630
Sludge	11750	7580
Purity of the DNA (A260/A280) samples		
Sample type	Bento lab (BL)	Traditional lab (TL)
Chicken	1.990	1.976
Lake	1.628	1.838
Sludge	1.900	1.897

We isolated metagenomic bacterial DNA with sufficient yield from each sample using both the modified DNA isolation protocol with the Bento lab and the unmodified protocol with the traditional lab. We measured the DNA yield and purity of each DNA sample isolated from the Bento lab (n=3) and the traditional lab (n=3). Table 6.1 shows the DNA yield (ng) isolated from the lake, sludge and the retail meat product. The DNA yield was sufficient for downstream sequencing for all sample types. The purity of the DNA samples varied, with measurements taken at A260/A280. As indicated in Table 6.1, all samples, excluding Lake_BL, had a DNA purity ranging from 1.8 - 1.9 which can be considered pure according to past metagenomic research [35–37]. Interestingly, the DNA isolated from the sludge and the retail meat product had similar values of 1.9 which could be attributed to the use of the same DNA isolation kit. However, we expected to see differences between

the Bento lab samples and the traditional lab samples due to the modifications in the DNA isolation protocol.

The purity recorded for Lake_BL was 1.6, indicating the presence of phenol, protein, or other contaminants [38, 39]. This suggests that the modified DNA isolation protocol (DNeasy PowerWater DNA extraction kit) and the use of the Bento lab for the lake sample results in lower purity compared to the unmodified protocol used in the traditional lab. The Bento lab is restricted by centrifugation speed and the size of Eppendorf tubes that can fit into the centrifuge rotor. The lower purity suggests that these restrictions may impact the purity of DNA. Nevertheless, it has been stated that low phenol contamination in DNA samples has minimal effects on the rapid sequencing kit during the library preparation step and has no impact on the sequencing process [40, 41].

6.3.2. SEQUENCING SAMPLES ISOLATED USING THE BENTO LAB AND TRADITIONAL LAB YIELDS SIMILAR RESULTS

To compare the differences between the reads generated, read N50 and total data produced between metagenomic DNA samples obtained using the Bento lab and the traditional lab, we sequenced each sample using the MinION with the Flongle adaptor. The number of reads generated during sequencing can influence taxonomic classification and the identification of ARGs and plasmids as a higher number of reads will lead to more accurate classifications [42]. A full summary can be found in Supplementary Table S1.

Table 6.2: Summary of MinION sequencing results for metagenomic bacterial DNA isolated from control, lake, sludge, and chicken samples using both the Bento lab (BL) and traditional lab (TL).

Sample type	Reads generated (K)	Read N50 (Kb)	Total data produced (Mb)
Control	46.57	5.94	2780
Chicken BL	16.950	9.84	265.93
Chicken TL	14.432	7.24	230
Lake BL	21.515	11.8	964.35
Lake TL	20.111	5.63	1330
Sludge BL	5.678	102.42	93.75
Sludge TL	6.823	46.67	188.43

Table 6.2 shows that the reads generated, read N50, and data volume produced from the same sample types isolated using both lab technologies were within a similar range. Firstly, the Zymobiomics microbial community was used as a control to ensure that the rapid sequencing kit and the MinION with the Flongle worked correctly, as library preparation was done only using the Bento lab. According to Oxford Nanopore Technologies, the Flongle can produce up to 2.6Gb data [43]. We produced 46Kb reads and 2.7Gb of data from sequencing the control.

According to Table 6.2, the same sample types, regardless of their DNA isolation protocol or technology, generated a similar number of reads. While the rapid

sequencing kit suggests a DNA input of 100ng - 150ng, we used 75ng of DNA during the library preparation step. This lower amount was chosen to optimise resource use and minimise costs when working with limited amounts of sample in the field. Additionally, using 75ng allowed us to test the sensitivity of the sequencing protocol and assess its performance with reduced input, ensuring that the library preparation and sequencing could still yield high-quality data. Unfortunately, the amount of DNA used in library preparation does affects the data that is produced as a lower amount of DNA will yield low data output and shorter length reads which is evident in our study. The rapid sequencing kit, while requiring fewer manipulations in the library preparation steps, does not include a clean-up step. This can impact the sequencing results, as residual contaminants may remain in the sample [44].

We hypothesised that the varying pore availability and differences in DNA fragmentation could impact the read count and read N50 values observed during sequencing. While the Flongle flow cell has a maximum of 126 channels, the manufacturer guarantees 60 available pores for sequencing [43]. In our study, we had 40 to 70 pores available during the sequencing runs of all samples (Supplementary Table S1). While the lake and retail meat product samples generated a high number of reads, the DNA isolated from the sludge samples yielded a low number of much longer reads (5K reads, read N50 of 46Kb, 90Mb data). Due to the length of the DNA being sequenced, the pores within the flow cell can become blocked or lost i.e., inactive and stop operating, resulting in a low read count with a high read N50 [43]. We propose that a higher number of reads with a lower read N50 can be achieved by increasing the fragmentation time in the rapid sequencing kit protocol [45]. Currently, the fragmentation step (step 6) in the rapid sequencing kit states incubation should be done at 30°C for 1 minute and then 80°C for 1 minute however, we propose only increasing the incubation at 80°C to 2-4 minutes. Further analysis should be done on the effects of the fragmentation time on the read N50 and the reads generation by sequentially increasing the time at 80°C and observing the read N50 and reads generated during sequencing. Similar to our study, Maguire et al. [42] stated that the rapid sequencing kit, similar to the one used in our study, results in poor Nanopore sequencing reactions and low output of reads (0.3–1.7M reads) in agricultural water. However, a low number of reads can be sufficient for genus and species-level identification.

6.3.3. A HIGH DEGREE OF SIMILARITY IS SEEN IN THE TOP 20 MOST ABUNDANT SPECIES BETWEEN BENTO LAB SAMPLES AND TRADITIONAL LAB SAMPLES

To identify the microbial community at genus and species-level between the DNA isolated from the lake, sludge and retail meat product processed by the Bento lab and traditional lab, we performed taxonomic classification. Furthermore, we assessed similarities between the microbial communities found in the Bento lab and traditional lab samples. Details on the relative abundances of the bacterial genera, species, common bacterial genera, species, and similarity are in Supplementary Tables S2 -S5.

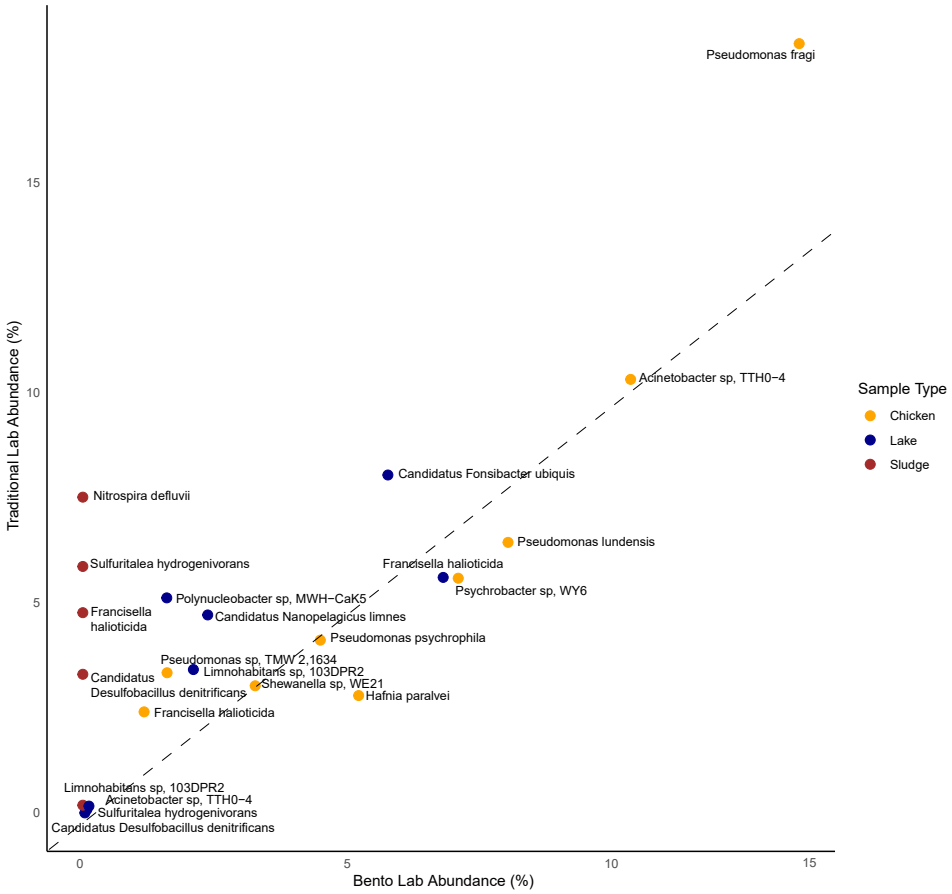

Figure 6.1: The abundances of bacterial species present in the chicken, lake and sludge samples processed by the Bento lab and the traditional lab. The x-axis represents the abundance of bacterial species detected from Bento lab samples and the y-axis represents the abundance of bacterial species detected from the traditional lab samples. All bacterial species shown in this figure have abundances of over 3%. Dots in the figure are bacterial species found within a specific sample type and are colour-coded accordingly (Chicken = orange, Lake = blue and Sludge = brown.)

Species with a higher number of assigned reads were common between the same sample types regardless of the lab technologies. Firstly, food spoilage bacteria were present in both retail meat product (chicken) samples. The *Pseudomonas* genus was dominant in both chicken samples each of which was isolated using both lab technologies (Supplementary Table S2). On a genus level, we identified the same number of genera in the DNA isolated using both lab types (Figure 6.1). Only 8

of these genera (≥ 1%) were common between the Chicken_BL and Chicken_TL, resulting in a Jaccard similarity index of 0.43 (Supplementary Tables S4 & S5), indicating a moderate similarity in the bacterial communities identified between the two chicken samples. The Jaccard similarity index increased to 0.54 when focusing on the top 20 most abundant genera, suggesting a higher degree of similarity in the most dominant bacteria identified between the two samples (Supplementary Table S5). Figure 6.1 shows the species-level identification revealing that *Pseudomonas fragi (P. fragi)* was the dominant species present in both Chicken_BL and Chicken_TL (Supplementary Table S3). *P. fragi* is commonly found on retail meat products and contributes to the spoilage of meat which can potentially be a health hazard [46, 47]. In total, 237 bacterial species were identified between the chicken samples however, only 19 species were common (≥ 1%) and had a Jaccard similarity of 0.4449 (Supplementary Tables S4 & S5). This similarity increased to 0.9047 when focusing on the top 20 abundant species, indicating a high level of consistency in identifying the most abundant species between the two samples regardless of the adapted DNA protocol.

Figure 6.1 shows environmental freshwater bacteria were detected in both lake samples. The top three genera identified between lake samples were the *Flavobacterium*, *Limnohabitans* and *Polynucleobacter* bacteria (Supplementary Table S2). While a higher number of genera were detected in Lake_BL (413) compared to Lake_TL (276), 15 similar bacterial genera (≥ 1%) were detected between the two lake samples (Supplementary Table S2). A Jaccard similarity of 42% was calculated indicating that only 42% of the bacterial genera identified are common between Lake_BL and Lake_TL (Supplementary Table S5). Similar to the chicken samples, the similarity increased when only focussing on the top 20 abundant genera (66%). On the species level, we identified a total of 1039 different species with 11 species in common between the lake samples (≥ 1%) (Supplementary Tables S3 & S4). The Jaccard similarity index of the top 20 abundant species in Lake_BL and Lake_TL was 0.666 indicating a moderate level of overlap between the two samples, with approximately 66.6% of the top 20 abundant species being common to both lake samples (Supplementary Table S5).

The Jaccard similarity may be influenced by the number of reads assigned to taxa during the taxonomic classification step. In this study, approximately 8000 reads in the Lake_BL sample were classified compared to 1900 reads in the Lake_TL sample. These differences indicate that more bacterial species were detected in the Lake_BL sample, contributing to a greater number of unique genera and, consequently, a lower overlap between the two samples (Supplementary Table S1).

Regarding the DNA isolated from sludge, we could only perform taxonomic classification on the Sludge_TL sample as shown in Figure 6.1 (Supplementary Table S3). This was due to the insufficient number of reads generated when the Sludge_BL was sequenced (Table 6.2). We identified a total of 159 genera and 266 species in Sludge_TL with the predominant species being *Nitrospira defluvii (N. defluvii)* which is commonly detected in sludge (Supplementary Tables S2 & S3) [48–50].

Overall, our study demonstrates that while there are differences in the observed microbial communities between the DNA isolated using the Bento lab and the

traditional lab, there is a moderate to high level of similarity between the most abundant species. This suggests that both methods are effective for microbial community detection, although the use of traditional labs with no modified DNA isolation protocol may yield more comprehensive results in some cases, such as with sludge samples. Additionally, the use of the Flongle can enhance the capabilities of portable labs like the Bento lab for in-field microbial detection. However, the reduced data volume of the Flongle provides a more limited view of the microbial community, primarily detecting the most abundant species compared to the MinION [47, 51]. While this provides valuable insight into the dominant microbial populations in environmental and food sources, it may not capture the full diversity of less abundant species present in the microbial community.

6.3.4. ESKAPE PATHOGENS CAN BE DETECTED IN LOW ABUNDANCES

To determine the presence of the ESKAPE (*Enterococcus faecium (E. faecium), Staphylococcus aureus (S. aureus), Klebsiella pneumonia (K. pneumonia), Acinetobacter baumannii (A. baumannii), Pseudomonas aeruginosa (P. aeruginosa)*, and *Enterobacter* spp.) pathogens in the lake, sludge and retail meat product (chicken) samples and assess the similarities between samples processed using the Bento lab (BL) and the traditional lab (TL), we investigated the reads assigned during taxonomic classification.

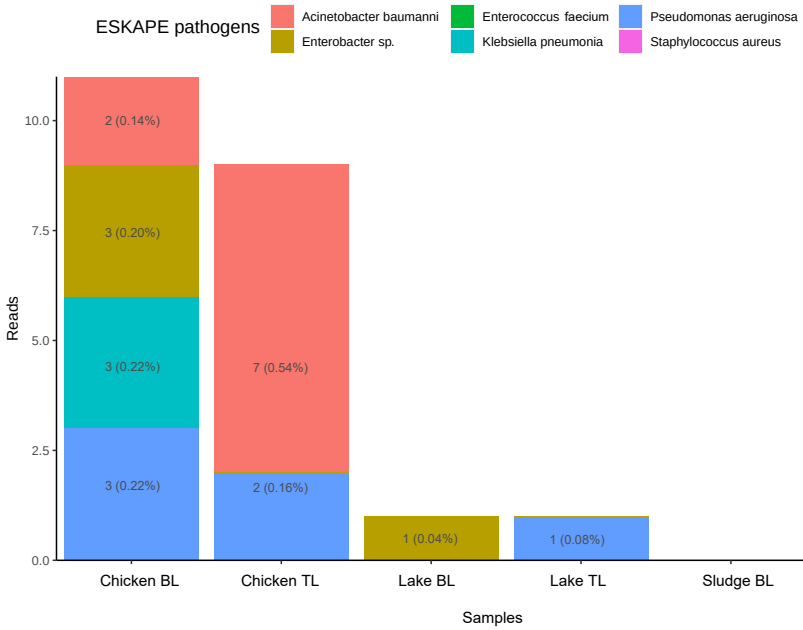

Figure 6.2: The number of reads assigned to ESKAPE pathogens detected in each DNA sample isolated using the Bento lab and the traditional lab. The x-axis indicates the different samples and the y-axis indicates the number of reads assigned to the ESKAPE pathogen. The number of reads and corresponding abundances in percentages for each ESKAPE pathogen are indicated in text within each bar. Each ESKAPE pathogen is indicated in different colours.

Figure 6.2 shows the presence of ESKAPE pathogens in the lake, sludge and chicken DNA samples. We identified *P. aeruginosa* and *A. baumanni* in both the chicken samples (≤1%) with *K. pneumonia* and *Enterobacter* sp. detected in the Chicken_BL (≤1%) (Supplementary Table S6). Additionally, we detected *P. aeruginosa* and *Enterobacter* in the lake samples (Supplementary Table S6). The presence of ESKAPE pathogens in the lake and chicken samples may be concerning. However, ESKAPE pathogens are not generally pathogenic, as they are natural colonisers of humans and animals [52, 53]. Additionally, these microorganisms can be commonly found in soil and aquatic environments [54, 55]. Further analysis on confirming pathogenic strains should be done either via conducting polymerase chain reaction (PCR), mass spectrometry (MS), Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) or via the use of computational tools such as StrainGE [56, 57].

Lastly, due to the lack of data in Sludge_BL, we were unable to detect ESKAPE

pathogens however, we did detect *Enterococcus faecalis (E. faecalis)* and *P. aeruginosa* in Sludge_TL. It is evident by the read count that there is difficulty in the detection of pathogens and other bacteria as after the pre-processing steps, the Sludge_BL sample had only 184 reads compared to the 10K reads of Sludge_TL.

The variation in the reads assigned to the ESKAPE pathogens between samples processed by the Bento lab and the traditional lab can largely be attributed to differences in read counts generated during the sequencing run. Higher read counts typically result in more comprehensive and accurate microbial identification. This variation highlights the importance of optimising the pre-sequencing and sequencing protocols to ensure consistent and reliable detection of microbial communities, particularly for environmental and food safety applications.

6.3.5. FAMILY LEVEL SIMILARITY FOR AMR GENES

To determine the similarities between antibiotic resistance genes (ARGs) in the lake, sludge and retail meat product (chicken) DNA samples that were isolated using the Bento lab and traditional lab, we investigated the presence of ARGs. Identifying the presence of ARGs is crucial for public health as bacteria carrying these genes can potentially cause infections. Additionally, the identification of ARGs in environmental and food sources can assist in mitigating the spread of AMR to other environmental and clinical sectors [6].

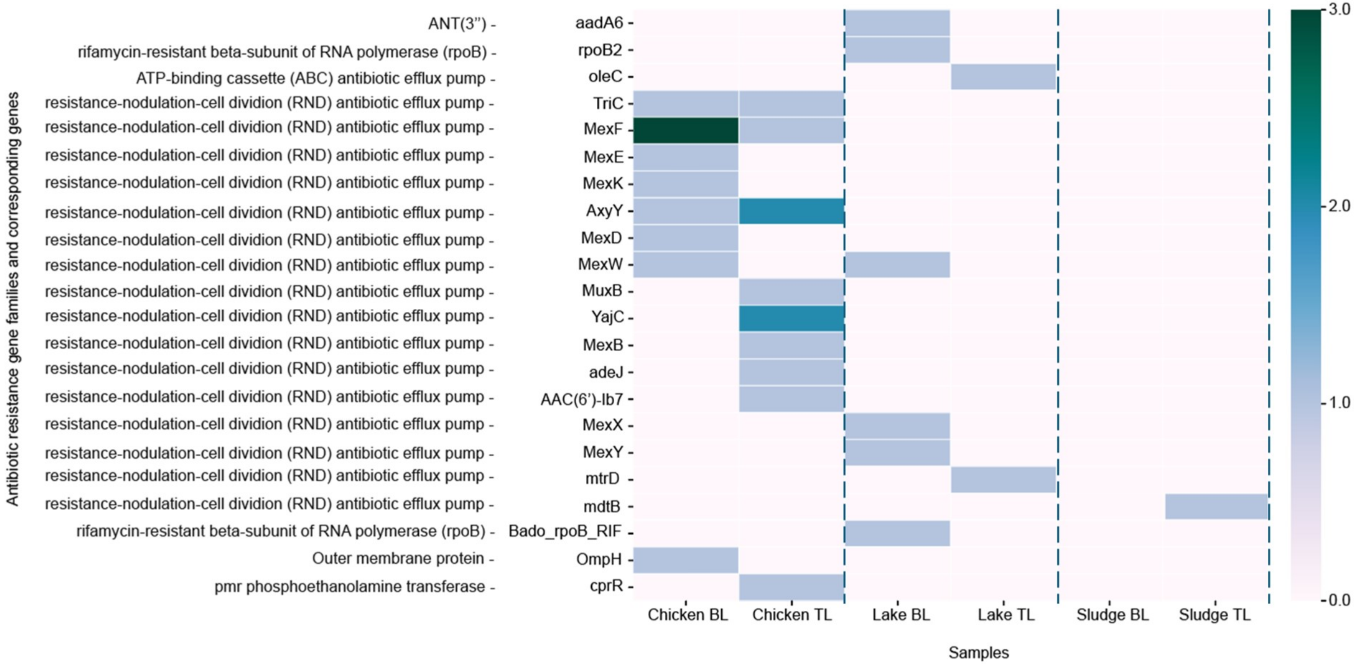

Figure 6.3: Heatmap showing the number of antibiotic resistance genes and corresponding gene families detected in each DNA sample isolated using the Bento lab and the traditional lab. The x-axis indicates the samples, the y-axis indicates the ARGs and AMR gene families and the colour intensity corresponds to the respective number of ARGs identified within their specific sample. The numbers are indicated by the colour intensity with low = pink and high = green and the dashed line indicates the separation between sample types

6

Figure 6.3 shows the identification and number of ARGs with corresponding AMR gene families present in the different lake, sludge and chicken samples processed by the Bento lab and traditional lab. The RND efflux pump gene family was commonly detected. We identified 10 different ARGs in Chicken_BL and 11 different ARGs in Chicken_TL with only 3 ARGs in common (Supplementary Table S7). However, 90% of the ARGs detected in both chicken samples were from the RND efflux gene pump family. On the gene level, 3 *MexF* genes were detected in Chicken_BL conferring resistance to cephalosporin, macrolides, aminoglycosides, and fluoroquinolone while 2 *YajC* and 2 *AxyY* genes were predominant in the Chicken_TL sample, conferring resistance to multiple antibiotic drug classes: tetracyclines, disinfecting agents and antiseptics, rifamycin, glycylcycline, cephalosporin, penam, phenicol, oxazolidinone, fluoroquinolone, glycopeptide, macrolide and aminoglycosides (Supplementary Table S7). This is similar to a study conducted by Heir et al. [58] who detected the RND efflux pump gene family in retail products from Norwegian slaughterhouses. The RND efflux pump gene family and the ARGs comprising the family are commonly associated with *Escherichia*, *Salmonella* and *Pseudomonas*, all of which were detected in our chicken samples [58–61].

More variation in the detected ARGs was observed between the lake samples. Figure 6.3 shows that 50% of the ARGs identified in the Lake_BL and TL samples belong to the RND efflux pump gene family. We also detected ARGs conferring to aminoglycosides, rifamycin in Lake_BL and macrolides in Lake_TL with no common ARGs detected (Supplementary Table S7). The detected AMR gene families align with the findings of Filipic et al. [62], who concluded that the RND efflux pump gene family was prevalent in a recreational lake. The detection of RND efflux pump gene families in both retail meat products and lake water may be a biosafety and food-safety hazard as these gene families can confer antibiotic resistance and multi-drug resistance in bacteria. They can be found in both pathogenic, non-pathogenic and opportunistic bacteria such as *Escherichia coli (E. coli), K. pneumonia, Enterobacter spp, P. aeruginosa, A. baumanni* and emerging pathogens such as *Strenotrophomos maltophila (S. maltophila)* [63–65]. The RND efflux pump is naturally found in the chromosome of bacteria which can spread to humans and animals potentially spreading antibiotic resistance and outbreaks.

While we see differences in the ARGs detected within similar sample types, there is a similarity in the AMR gene family level. Differences in the detection of ARGs can be attributed to the low number of reads or the percentage identity used during the alignment against the CARD database however, our study used an 80% identity, which previous studies have shown to be a strict cut-off [66]. Even though we did detect ARGs, the low number observed is likely due to the limited data volume generated, influenced by factors such as the DNA isolation process, library preparation, or the smaller output of the Flongle, which produces only 2.6Gb of data compared to the MinION's 48Gb. Unfortunately, the number of reads to identify and confirm the presence of ARGs has not been defined yet as some studies have suggested one read is relevant due to the low threshold for long-read sequencing while others suggest that there should be at least 10 reads [67].

6.3.6. A HIGHER NUMBER OF PLASMIDS DETECTED IN THE CHICKEN AND LAKE BENTO LAB SAMPLES

To determine the presence of plasmids between each sample type, we classified reads as plasmids. Furthermore, we investigated if the plasmids detected were carrying ARGs. Plasmids are important vehicles for rapid adaptation of bacterial populations changing to their environmental conditions. Plasmids can carry accessory genes such as ARGs and transfer these genes to pathogenic and non-pathogenic bacteria. An understanding of plasmids and the ARGs they carry is imperative in understanding the spread of antibiotic resistance and the role they play in emerging resistance and infections [68].

Table 6.3: The number of reads classified as plasmids and the number of reads associated with ARGs.

	Chicken BL	**Chicken TL**	**Lake BL**	**Lake TL**	**Sludge BL**	**Sludge TL**
Plasmids	544	496	2114	1944	8	324
Plasmids associated with ARGs	-	1 AAC(6')-ib7	1 aadA6	-	-	-

Table 6.3 shows the number of reads classified as plasmids in the metagenomic bacterial DNA isolated from the lake, chicken and sludge samples processed using the Bento lab and the traditional lab. We detected plasmids in all DNA samples isolated using the Bento lab and the traditional lab (Table 6.3). As expected the number of reads classified as plasmids within a sample type differed according to the number of reads within the dataset. A higher number of reads were generated from the Bento lab DNA samples, therefore a higher number of reads were classified as plasmid derived. The chicken samples had a difference of 9.16%, while the lake samples exhibited an 8.20% difference. In both instances, the Bento lab samples revealed a higher plasmid count. Interestingly, the Sludge_BL sample contained the lowest number of reads that could still be used to detect plasmids. This is likely because the reads in the Sludge_BL samples have longer read lengths (N50) which could make them more suitable for plasmid classification by PlasClass [34].

Secondly, Table 6.3 shows two reads classified as plasmids that are associated with the previously detected ARGs. Both plasmids were associated with aminoglycoside resistance genes, *AAC(6')-ib7* and *aadA6*, and detected in Chicken_TL and Lake_BL samples, respectively.

The differences in the number of plasmids identified and those associated with ARGs between the Bento lab and traditional lab samples may be attributed to differences in read numbers and read lengths. The higher number of reads from the Bento lab samples may have allowed for the detection of more plasmids, while the longer read lengths in the traditional lab samples may have facilitated the classification of plasmids even with fewer reads. This highlights the trade-offs between read numbers and read lengths in metagenomic analyses. As stated previously, the Flongle provides limited reads which may restrict the detection of plasmids especially those less abundant or present in low copy numbers.

6.4. CONCLUSION

Our proof-of-concept study has shown that the pre-sequencing steps can be achieved by using a portable pro-level Bento lab. We successfully isolated metagenomic bacterial DNA from environmental and food sources using a modified DNA isolation protocol and portable lab. We isolated DNA with sufficient yield needed for sequencing using both lab technologies and generated reads for each sample type using the MinION and Flongle.

We identified bacteria down to the species level from the DNA samples that were isolated using the Bento lab and the traditional lab. DNA isolated from the same samples showed bacterial similarities of over 40% however this increased between more abundant genera and species. We also identified ESKAPE pathogens in the lake, sludge and retail meat product (chicken) samples with an abundance of less than 1%. Furthermore, we detected ARGs in the lake, sludge and retail meat product (chicken) samples from both lab types. The RND efflux pump gene family was common between the same sample types. Lastly, we identified a similar number of plasmids in all samples with two plasmids associated with aminoglycoside resistance.

Since this study is a proof-of-concept, the study is limited by the number of samples and the limited amount of reads generated by the Flongle. To overcome this, future research should benchmark the modified DNA isolation protocol with the Bento lab and the unmodified DNA isolation protocol with the traditional molecular lab using a higher number of samples from various sources. This will aid in investigating how results from DNA concentration, purity, read counts, read N50 and identification differ between the Bento lab and the traditional lab. Furthermore, optimisation of pre-sequencing steps using portable lab equipment such as the Bento lab needs to be conducted to improve DNA quality and data generation.

In this study, the Bento lab equipment was used during the library preparation step with the rapid sequencing kit which required fewer resources and less equipment, eliminating the need for modifications during the library preparation step. However, this streamlined process may not apply to other sequencing kits due to the limitations of the Bento lab setup. Additional experiments using various sequencing kits and adapting library preparation protocols with diverse samples to gather more comprehensive data, thereby supporting in-field research efforts is needed. Additionally, research into in-field microbial detection and AMR should also take into account and compare the data generated by the Flongle versus the MinION.

We believe that this proof-of-concept study can be the stepping stone into future research aiming at portability and real-time data generation essential for antibiotic resistance surveillance in different types of environments.

6.5. SUPPLEMENTARY MATERIAL

Due to the extensive nature of supplementary materials associated with this chapter, they are not included in this thesis. Interested readers are encouraged to refer to the Supplementary material online (see `https://surfdrive.surf.nl/files/index.php/s/Y27zkpPxaS4HMkq`)

6.6. DECLARATIONS

6.6.1. FUNDING

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6.6.2. DATA AVAILABILITY

The sequence data has been submitted to the Sequence Read Archive (SRA) under Bioproject PRJNA1168083.

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7

DISCUSSION

Microbes, among the earliest life forms on Earth, have existed for over 3 billion years, showcasing remarkable diversity and resilience that enable them to inhabit nearly every environment, from extreme temperatures and volcanic landscapes to the human body [1]. These organisms play vital roles in shaping ecosystems and influencing nutrient cycles, climate, and the health of plants, animals, and humans [2]. Despite their ancient lineage and ubiquity, 99% of bacterial and archaeal species remain unidentified, highlighting a significant gap in our understanding of the microbial world [3].

Traditional methods for studying microbes have relied on culture-dependent approaches, yet less than 2% of environmental microbial species can be cultured in laboratories [4]. This leaves a vast amount of uncultivable "microbial dark matter" unexplored. However, advancements in DNA sequencing and computational technologies have made it feasible to study this dark matter [5]. Metagenomics has emerged as a powerful tool for identifying microbial species in complex ecosystems without the need for cultivation, especially in less-studied or previously inhospitable environments [5]. A deeper understanding of microbial diversity and function has the potential to drive breakthroughs in fields such as medicine, environmental science, and agriculture. Importantly, this limited understanding directly impacts global health. In the context of antimicrobial resistance (AMR), human intervention through the overuse of antibiotics has accelerated the emergence of multidrug-resistant pathogens, contributing to what is now recognised as a "silent pandemic" [6]. This dissertation has focussed on illuminating the microbial dark matter in non-clinical environments by studying AMR using metagenomics.

7.1. BREAKTHROUGHS IN AMR REQUIRE COLLABORATION AND INTEGRATION

While AMR is widely acknowledged as a One Health issue, non-clinical environments remain under-represented [7]. Additionally, obtaining metagenomic data across diverse non-clinical sectors within a single study presents considerable challenges, particularly in a field where most available information comes from clinical and public health datasets. This imbalance raises critical questions: *Why is research in non-clinical sectors limited?* and *How can AMR in these sectors be effectively studied using metagenomic data?*

These questions guided the second chapter of this thesis, where the groundwork for exploring non-clinical sectors through metagenomics and bioinformatics is presented. This chapter comprehensively reviews antibiotic use across water, aquaculture, soil, plants, and food-animal sectors, highlighting how metagenomics and bioinformatic tools can advance research in these areas. This review underscores the contributions of these non-clinical sectors to AMR and the urgent need to integrate them into future AMR research alongside clinical sectors. A critical limitation of AMR research is the persistent view of humans, animals, and the environment as separate entities, even though the One Health approach emphasises their interconnectedness. While this interdependence is frequently acknowledged in the literature, it remains neglected in research practices, including this thesis. To truly address AMR, research must prioritise collaboration between clinical and environmental sectors by incorporating data from all sectors.

Another challenge affecting the One Health approach to AMR research is the lack of both data and metadata in non-clinical sectors and sub-environments. Although a limited amount of metagenomic data is available, it is often accompanied by insufficient or incomplete metadata. This further complicates the use of public datasets. Reusing public data for AMR research can be a powerful way to draw new conclusions and improve monitoring strategies; however, the absence of metagenomic data and metadata makes it difficult to draw definitive conclusions. Various obstacles can be faced when working with public data from different studies and countries, such as variability in sample processing and data quality, lack of standardised data and metadata, restricted access to raw or processed files and study/country-specific differences. Obstacles as such are common due to the various types of infrastructure present in different laboratories across the globe. Unfortunately, these differences can compromise the reproducibility, interoperability and cross-study comparisons that are essential in One Health contexts [8]. Although the FAIR (Findability, Accessibility, Interoperability, and Reusability) data principles have been proposed as a framework to improve data stewardship and usability, implementation is still inconsistent across data repositories and studies. For example, missing metadata (e.g., sampling conditions, sequencing platforms, or geographical context) frequently limits dataset utility in environmental AMR studies [9].

To overcome these limitations and gain a broader perspective on AMR trends across various non-clinical sectors, publicly available metagenomic datasets can be integrated and analysed using a standardised bioinformatic pipeline. Although this approach may appear limited due to data variability, it can enable valuable preliminary insights into resistance patterns and the distribution of resistant bacteria. These initial findings can inform hypothesis generation, guide targeted sampling strategies, and help prioritise surveillance efforts. Moreover, integrating public datasets across studies allows for the identification of overarching trends in microbial diversity and the prevalence of ARGs across diverse environments and geographical regions.

7.2. AMPLIFICATION OF AMR ACROSS ENVIRONMENTAL AND HUMAN-IMPACTED CONTEXTS

One trend consistently observed was the substantial impact of human actions and environmental conditions on AMR patterns. It is often a misconception that humans or the clinical sector are separate from the environment, yet the findings in this thesis reinforce that anthropogenic activity exerts a significant influence. Chapter 3, for example, examines the wastewater treatment plant process, revealing how environmental bacteria and ARGs prevalent in nature become amplified by human activity. This is highlighted by the presence of pathogenic bacteria and ARGs conferring resistance to clinically relevant antibiotics in the influent, originating from urban and municipal areas. Contrastingly, environmental bacteria and ARGs are present in upstream freshwater systems that are less affected by human activity. It can be argued that the WWTP process is efficient in reducing the microbial load and ARGs present; however, the persistence and spread of bacteria carrying these ARGs within the WWTP and to receiving environments, humans and animals is possible.

Furthermore, in Chapter 4, microbial communities in soil are shaped by environmental and anthropogenic factors, leading to notable shifts in both community structure and ARG profiles. While core microbiota are shared across soil types, the presence of human activity correlates with an increased detection of opportunistic pathogens and ARGs associated with clinically relevant antibiotics. The influence of human activity, geography, climate, and pollution on the soil resistome shows the need for integrated AMR monitoring in environmental settings.

In the food-animal industry, antibiotic use satisfies the farmers, the economy and society [10]. However, findings presented in Chapter 5 challenge this perception by revealing the detrimental effects of antibiotic use on animal health. Pigs raised without antibiotics exhibited microbial communities and genetic profiles that promote natural resilience and overall health, in contrast to antibiotic-treated pigs. For instance, beneficial gut-associated bacteria were prevalent in antibiotic-free pigs, whereas treated pigs harboured both probiotic species and opportunistic pathogens. Moreover, the detection of ARGs in both treated and untreated pigs shows the influence of environmental factors and site-specific conditions on the resistome. Human intervention, particularly through antibiotic use, significantly alters microbial communities in the food-animal industry, with lasting implications for animal health and antimicrobial resistance.

7.3. STANDARDISATION, COST-EFFECTIVENESS AND EASY-TO-USE TECHNOLOGIES IS THE WAY TO PREPARE FOR PANDEMICS

Understanding and mitigating AMR is essential to pandemic preparedness, as antimicrobial resistant bacterial infections can complicate responses to disease outbreaks and strain healthcare systems [11]. The COVID-19 pandemic highlighted how global interconnectedness accelerates pathogen spread and magnifies health impacts—a reality that holds equally for AMR. The presence of resistant pathogens could significantly affect future pandemic and outbreak responses, making it essential to standardise prepared-

ness methods and outbreak detection. This generates high-quality and comparable data across different regions [12, 13]. However, limited resources and infrastructure in low- and middle-income countries present a major barrier. As a result, metagenomic data from these regions is sparse, and few bioinformatic platforms can compensate for infrastructural limitations.

One potential solution to overcoming the challenges of AMR surveillance in resource-limited settings is the establishment of adaptable, standardised methodologies that can be implemented across different income levels, regardless of resource availability. The COVID-19 pandemic accelerated the shift towards in-field detection methods for surveillance, but this approach requires foundational protocols not solely dictated by resource-rich settings [13]. This insight guided Chapter 6 of this thesis, which focused on portability and in-field microbial detection for AMR research. This proof-of-concept study demonstrated that commercially available DNA extraction protocols can be adapted for use with portable laboratory setups and Oxford Nanopore Sequencing Technologies, such as the MinION, in field settings. These results showed that field-based setups could produce DNA of comparable quality to that obtained in traditional lab environments, offering a promising step toward accessible AMR surveillance methods globally.

However, this approach has limitations, including the high cost of portable lab setups, MinION devices, flow cells, and the storage of long-read sequencing data. Additionally, the need for bioinformatics platforms can pose challenges, particularly in low- and middle-income countries. Despite these challenges, the primary focus of this research is to generate high-quality metagenomic DNA from environmental and food sources in the field. Overcoming these limitations, particularly in resource-limited settings, remains a crucial goal. Although these challenges persist, the growing trend in AMR surveillance is shifting towards in-field microbial detection for outbreak monitoring [13].

One way to make this transition possible in low-resource settings is to develop user-friendly platforms that can be easily accessed and operated in remote locations. These platforms would classify microbial taxonomy and identify resistance genes without needing advanced laboratory facilities or extensive bioinformatics expertise. By enabling in-field AMR surveillance, such platforms could facilitate the rapid collection of critical data on microbial communities and resistance profiles, even in areas with limited infrastructure. This capability would empower researchers and healthcare workers in remote or resource-limited settings to respond more quickly to emerging AMR threats, contributing to a more comprehensive global understanding of AMR patterns. Moreover, it would enhance collaborative efforts across countries, creating a unified, more effective approach to tackling AMR on a worldwide scale.

7.4. EFFECTIVE COLLABORATION AND METHODOLOGICAL INTEGRATION ARE KEY TO GAINING DEEPER INSIGHTS INTO AMR

While bioinformatic platforms and tools exist for microbial detection and essentially AMR research, the collaboration between wet lab and computational research is crucial in leveraging the power of -omics data for AMR studies. Metagenomics and other

high-throughput techniques generate vast datasets that require sophisticated bioinformatics for effective analysis [5]. However, interpreting these results often demands an in-depth understanding of microbial ecosystems and experimental verification. Additionally, expanding AMR studies using other -omics approaches such as proteomics, transcriptomics, and functional metagenomics, can further enrich our understanding of how resistance mechanisms operate and how AMR spreads within microbial communities [14]. These approaches offer unique insights into AMR that metagenomics alone may not capture. For example, while metagenomics provides valuable data on the presence and diversity of AMR genes within an environment, proteomics and transcriptomics help elucidate the expression and regulation of these genes under varying conditions [15]. Functional metagenomics, in particular, enables researchers to explore the activity of resistance genes within different microbial ecosystems, revealing not just their presence but also their roles in conferring resistance under environmental pressures. Integrating these techniques can transform our understanding of AMR by connecting genetic information with functional evidence, helping us better predict the behaviour of resistant microbes across diverse settings [16].

Ultimately, tackling AMR requires more than technological progress, it necessitates a paradigm shift towards holistic, One Health frameworks that unify clinical, environmental, and agricultural sectors. By fostering equitable resource distribution and global collaboration, we can develop adaptive and accessible methodologies to safeguard public health. These efforts will not only deepen our understanding of AMR but also strengthen our preparedness for future pandemics, ensuring a healthier and more resilient world.

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LIST OF PUBLICATIONS

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