Antimicrobial resistance persistence and dissemination in environmental and animal microbiomes through plasmid transfer

Cian Smyth B.Sc.(Hons.)

Maynooth University
National University of Ireland Maynooth

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Supervisor:	Head of Department:
Prof. Fiona Walsh	Prof. Paul Moynagh

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Declaration of Authorship

This thesis has not been submitted in whole or in part to this or any other university for any degree, and is original work of the author except where otherwise stated.

Signed: _____

Date: _____

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Supplementary Data

Supplementary data for the following thesis is available from the link below.

https://drive.google.com/drive/folders/1m4G1uw6hp43LbdslpT4xsSm1EpedKtBg?usp=share_link

Abstract

` The following PhD thesis examines the crucial role of Wastewater Treatment Plants (WWTPs) and grasslands in the spread and maintenance of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) via a One Health context. Through an in-depth investigation of two urban WWTPs in Ireland, the research sheds light on the prevalence and mechanisms of antibiotic resistance among faecal coliforms. Results revealed that more than 90% of isolated faecal coliforms exhibited resistance to common antibiotics like amoxicillin and ampicillin, with a significant proportion being multidrug-resistant. Notably, the study discovered a substantial presence of extended-spectrum β-lactamase (ESBL) producing isolates, indicating a high potential for horizontal gene transfer, which could contribute to the spread of antibiotic resistance.

Complementing this research, the study provides a novel exploration of the global scale of antimicrobial resistance (AMR) plasmids. These plasmids isolated from chicken caecum, containing combinations of known AMR genes, metal resistance genes, virulence factors, and replicon types, were detected across 63 countries in diverse environments and species, including humans, animals, and the environment. This compelling evidence underscores the urgent need for expanded global surveillance and sequencing of AMR plasmids to better understand and combat the spread of antibiotic resistance.

Furthermore, the research delves into the often-overlooked role of grasslands within the environmental-animal nexus of AMR. Contrary to common focus areas such as

soil or vegetables, our data suggests grasslands hold a more diverse and abundant reservoir of ARGs and mobile genetic elements (MGEs). These findings have critical implications for human and animal health, particularly in the context of grazing food animals.

Lastly, through the characterisation of 173 plasmids isolated from the two Irish WWTPs, the research unveils a globally interconnected evolutionary history and an extensive prevalence of multidrug-resistant (MDR) plasmids. The results further confirm the significant role WWTPs play in the distribution and persistence of AMR, highlighting the importance of these environments in the development of strategic AMR control and the necessity for a comprehensive, globally-linked surveillance system.

Chapter 1

Introduction

1.1 Antibiotic Resistance Emergence and Impact

The World Health Organisation (WHO), Food and Agricultural Organisation of the United Nations (FAO) and World Organisation for Animal Health (OIE) identified antimicrobial resistance (AMR) as a critically important One Health problem. In 2017 the WHO identified 12 global priority pathogens classified as containing important AMR, including plasmid-mediated carbapenem-resistant, extended spectrum beta-lactamase (ESBL)-producing or fluoroquinolone-resistant Gram-negative pathogens [1]. The United Nations declaration on AMR which all 193 member states signed and the WHO declaration of priority pathogens, highlights the importance of the AMR problem [2]. Each year, AMR results in approximately 1 million disability-adjusted life years (DALYs) lost in EU/EEA countries [3]. Antimicrobial resistance can also have an impact on the economy of nations, in particular those that have a lower spending on necessary health infrastructure, and thus can be a contributor to poverty [4]. It is therefore important to view the impact of AMR through the broad lens of both human health and economic impact when assessing its threat to humanity.

Resistant bacteria and genes can be transferred from the environment, food, animals and humans reciprocally either in or as pathogens or commensals. The persistent use of antimicrobials in livestock farming has created an intensive selective pressure that encourages the selection of AMR gene mechanisms as well as the persistent dissemination of these genes throughout the environment [5]. The impact that this form of agriculture has on AMR spread can be seen through the temporal change that occurs when regulations are implemented such as the notable decrease in the faecal carriage of vancomycin-resistant *Enterococcus faecium* in chickens after an EU avoparcin ban

[6]. The anthropogenic contamination of the environment with antimicrobial resistant bacteria (ARB), antimicrobial resistance genes, and antibiotics due to industry, medical and human waste has led to unique hotspots of antimicrobial resistance mechanism selection, antimicrobial resistant bacteria spread and the possibility of horizontal gene transfer (HGT) with environmental bacteria [7]. Wastewater treatment plants (WWTPs) have also been shown to be a significant reservoir of both (ARB) and antimicrobial resistant genes (ARG) [8] and possible niches for AMR (HGT) proliferation [9,10]. This and the specific interaction between animal and environmental AMR sources that can occur in WWTPs and the subsequent possible interaction with human populations create a need to develop further understanding of ARB, ARG and HGT in WWTPs.

Many different classes of antimicrobials currently used to treat infections in humans and animals were discovered and developed between the 1940s and 1980s. However, since 1990 only three novel classes of antimicrobials have been launched: pleuromutilins, lipoglycopeptides, and oxazolidinones. These new classes have limited or no activity against the gram-negative pathogens such as *Escherichia coli* or *Klebsiella pneumoniae*, which are on the WHO's priority list [11]. Therefore, we need to preserve our current arsenal of antimicrobials. Despite the current lack of a consistent pipeline for the development of novel antibiotics, in-silico computational methodologies have allowed for a new approach to this problem with progress being made through the use of deep neural networks to predict the possible antimicrobial activity in molecules structurally different to those currently in use [12]. This finding elucidates the substantial impact that in-silico computational analysis can have on the field in the future. One mode of action is to limit the transfer of AMR genes and ARB

from the environment to animals and humans via the food chain. However, we need to understand the resistome and microbiome of animal food sources, and WWTPs before we can limit the transfer.

1.2 Antimicrobial Resistance in Grass and Soil

The phyllosphere (aerial surface of plants) has been estimated previously to be one of the largest microbial habitats on earth [13] and despite numerous studies focussing on soil, the microbiome and resistomes of grass phyllospheres has rarely been studied [14,15]. The interlinked relationship of soil and grasslands in particular is not well understood particularly when focused on the introduction of manure to grasslands despite covering more than a third of European agricultural area [16].

Grass and soil relationships are of importance within the One Health framework due to their interactions with disparate environmental biomes and by being persistent reservoirs of ARB and ARGs that can subsequently impact related agricultural and clinical environments.

1.3 Wastewater Treatment Plants and AMR

The dissemination of AMR within bacterial communities and the selection of new resistance mechanisms are due to the large-scale use of antibiotics in agricultural, veterinary and human clinical environments.[17–20]. The emergence of antibiotic resistant bacteria (ARB) is a major public health issue which poses a serious therapeutic challenge worldwide [18]. Wastewater treatment plants (WWTPs) are

considered potential sources of ARB and antibiotic resistance genes (ARGs), which play an important role in the spread of antibiotic resistance into the environment [21,22]. Urban WWTPs receive wastewater from human communities, which contains high concentrations of chemical matter, including antibiotics and microorganisms, including ARB. It has been shown previously that wastewater from hospital waste as well as farm wastewater and runoff can be major sources of AMR in aquatic biomes [23] . This is of importance due to the particularly high presence of ARB and ARGs in hospital wastewater with several studies in Europe and Asia showing that the concentrations of ARB and ARG in hospital wastewater were 2-9 orders of magnitude higher than in municipal wastewater [24–26]. Within urban watersheds, municipal wastewater has also been shown to contain levels of antibiotics and ARBs that could create potential for AMR spread [27]. Therefore, WWTPs are favourable environments with optimal conditions for the development and spread of ARB and ARGs of clinical importance [28,29].

Both ARB and ARGs were detected in wastewater samples globally [30–34]. However, little is known of the fate of these bacteria; and the role of WWTPs in releasing ABR and ARGs into the environment through treated effluent [35]. A recent report by Flach et al. shows no evidence for the selection of antibiotic resistance in WWTPs [36]; however, large amounts of resistant bacteria were identified throughout the wastewater treatment process [37,38]. The conventional wastewater treatment process can remove some ARB [39,40], but ARB were still found in large proportions in the effluent [41,42]. In some cases, ARB were detected at higher rates in WWTP effluent than in the influent [43,44]. This finding has been further elucidated with [45] determining that multi drug resistance (MDR) was found to increase significantly in sites downstream of a

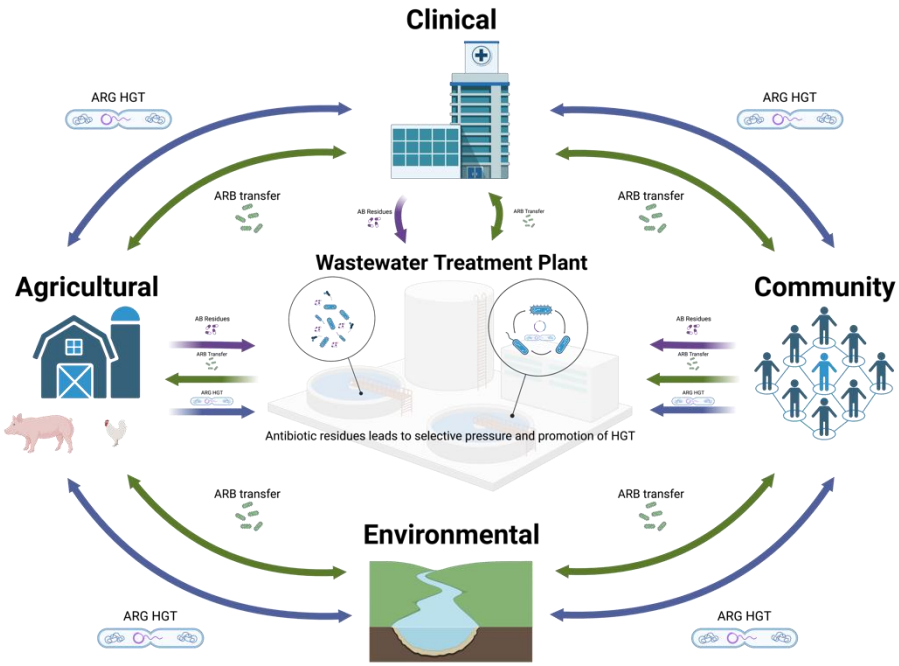
WWTP. This persistence of ARGs and associated MDR within these water systems has also been associated with mobile genetic elements (MGE) such as novel ARG-bearing plasmids that have been found to be highly enriched in WWTP effluent [46] .

When discussing possible dissemination of AMR within WWTP communities it is important to understand the distinct microbiome that this microbial ecosystem contains as well as the variation that can be seen depending on the specific treatment methods used, climate present and geographic location. Examination of global diversity within activated-sludge WWTPs revealed that despite a large diversity within such ecosystems, there is a clear core community that consists of 30 taxa with most of the core community belonging to Pseudomonadota [47]. This coincides with the finding that plasmids found in Pseudomonadota can form significantly broad HGT linkages [48] and with four of six ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter* species) pathogens found within this phylum WWTPs can be seen as a distinctly important reservoir of ARGs due to possible dissemination and genomic interchange pathways.

The threat of AMR can be viewed through the context of One Health as it has been linked to factors related to human, environmental and animal health. Within this framework, it has been shown that WWTPs can harbour significant reservoirs of ARB and ARG. [49] [50] [51] [52] These localised concentrations of AMR are thus considered potential hotspots for the dissemination of ARG between bacterial populations via HGT through ARGs contained on MGEs. [53] Amongst MGEs, plasmids play a consistent role in the dissemination of ARGs within bacterial populations. [54] These mobile resistance genes are of great importance due to their ability to propagate AMR

within bacterial populations and between distinct biomes which justifies their importance to the One Health approach. [55]

Previous WWTP studies have focused on the detection and classification of ARGs and ARB using traditional gene detection methods such as PCR [56,57] and while metagenomic analysis has been able to characterise plasmids in WWTP previously [58] the short read only methods used does not allow for the best possible characterisation and resolutions of plasmids and their functional composition. [59]



1.4 Antimicrobial resistance (AMR)

Antimicrobial resistant genes have been detected in all environments including clinical, natural and engineered habitats and despite arising from the pre-antibiotic age [60] through the evolutionary pressure of resource competition, anthropogenic factors have been considered one of the main drivers of dissemination [61]. When discussing ARGs it is important to take into consideration, the distinction between natural and acquired resistance. Natural resistance can be classified into two categories; Intrinsic and acquired. Intrinsic is defined by the perpetual expression of the resistance trait within a species and induced, naturally occurring genes in a species that express after exposure to antibiotics [62]. Acquired resistance is resistance conferred through HGT or chromosomal mutation and can be temporary or permanent. Plasmid mediated resistance is the most common amongst this group. The concept of resistance dissemination encompasses such acquired resistance and the common nature of plasmid mediated resistance calls for the surveillance, classification, and analysis of such resistance transfer systems.

AmpC cephalosporinases and extended-spectrum β-lactamases (ESBLs) are some of the most clinically important antibiotic resistance mechanisms [63]. The dissemination of AmpC or ESBL producing *Escherichia coli* were identified in different types of aquatic environments, particularly in wastewater [64–67]. The prevalence of AmpC or ESBL producing bacteria pose a global health problem due to limitations of therapeutic options for the treatment of infections caused by these bacteria [68]. The ESBL genes are frequently located on mobile genetic elements [69]. Among more than 300 subtypes of ESBL genes, *bla*$_{\text{TEM}}$ and *bla*$_{\text{SHV}}$ groups were the most common ESBL genes identified in human pathogens until the late 1990s. These groups were replaced by *bla*$_{\text{CTX-M}}$ genes since the beginning of the 2000s and *Escherichia coli* became the

most prevalent ESBL producing bacteria among clinical *Enterobacteriaceae* [70]. The prevalence of ARGs within relevant biomes must be given an associated context to determine their clinical relevance and importance [71]. One key characteristic that has been noted as a possible identifier of this importance is the ability of the ARG to be mobilised through MGE mechanisms that allow for HGT, such as plasmids [72]

The monitoring of antibiotic resistance from WWTPs provides the information required to track the dissemination of ARB and ARGs into the environment [73,74]. Moreover, the analysis of ARB and ARGs in urban WWTPs is considered as an alternative method for the indirect study of antibiotic resistance in human populations from which the WWTPs receive wastewater [75]. Indeed, the resistance rates of indicator bacteria in wastewater may give useful information to identify the changes in resistance in human populations [76].

1.5 Plasmids and Horizontal Gene Transfer (HGT)

Antimicrobial resistance dissemination through HGT can involve a number of different pathways such as conjugation, transformation, transduction and gene transfer agents (GTAs). Conjugation (the multi-step process requiring cell to cell contact) encoded by plasmid bound conjugation genes or chromosomal integrative conjugation elements (ICEs) is a key factor in AMR persistence and is considered a prevalent steward of ARG transfer when compared to other mobile genetic element (MGE) HGT mechanisms due to its features of protection and transfer efficiency [77]. Plasmids in particular are considered important vectors of AMR within bacterial communities and can be transferred via conjugation, transformation and transduction, in some cases

across large taxonomic distances [77]. They can vary in size and copy number, from large low-copy number plasmids that can reach hundreds of kilobases in length and are frequently conjugative, to small high-copy number plasmids that although lacking function conjugative mechanisms, can mobilise via other plasmids [78]. This extended spectrum of possible HGT pathways that plasmids use creates a prescient need to develop insight into the plasmids involved in AMR and persistence within the environment.

The impact plasmid evolution has on AMR must also be noted as the plastic nature of plasmids allows for increased evolutionary variability that is not seen at a prokaryotic chromosomal level. Mutation and recombination, the evolutionary drivers of selection have a distinct difference when comparing plasmids to their hosts chromosome. As mutation rates can increase linearly with respect to gene copy number, the high-copy nature of some plasmids can thus lead to higher rates of mutation of plasmid bound genes [78]. This along with the higher rate of recombination due to repeated regions such as transposable elements found within plasmids [79,80] suggests that plasmid bound genes, such as resistance genes can evolve at a higher rate than their chromosomal counterparts. This is further bolstered by their apparent mosaicism as substantial plasmid fragments can be carried on disparate plasmids across prokaryotic taxa and subsequently elucidate the relative importance they play in the dissemination and variation of ARGs [81]. Due to the random nature of plasmid allele segregation, daughter cells often inherit a divergent allelic composition to that of their progenitor cell. This encompasses a significant factor in plasmids evolutionary role in the form of segregational drift [82]. The interplay of segregational drift and plasmid interference (the competition of establishment between two beneficial mutations in a plasmid pool in a

single cell line [83]) leads to a distinct difference in the fixations times of plasmid and chromosomally bound mutations and therefore reinforces the standing genetic variation that can allow for a faster evolutionary response to environmental stressors such as antibiotics when compared to chromosomal de-novo mutation [78].

Plasmid mediated resistance has been shown to be a key driver of AMR in many microbiomes including environmental, animal and clinical [84–86] and therefore it is of the utmost importance to identify, analyse and elucidate their nature in order to provide useful insight into possible plasmid based AMR reduction frameworks. There is a dearth of information on the AMR plasmids contained within pathogenic or non-pathogenic bacteria within WWTP effluent. To date, plasmid data have mainly been limited to plasmids present in culturable pathogens. Plasmids enable AMR gene (ARG) transfer across bacteria and biomes and through the food chain. Thus it is these plasmids that are the risk to the further spread of AMR across One Health to and from the environment. The contribution of specific plasmids to the dissemination of ARGs across One Health is unknown. Analysis of AMR plasmids conferring AMR or multi-drug resistance (MDR) may enable us to track and understand the dynamics of the spread of these mechanisms of resistance across One Health. Through comparison of the entire plasmids we will be able to track and trace their movements across One Health and identify their risk to One Health.

The investigation of AMR in plasmids is not without its weaknesses. Traditional molecular experimentation was physically intensive, and did not lend to the high-throughput of samples required to dive into the complex networks involved in plasmid AMR evolution, dissemination and persistence. Thus, plasmid studies focused on the

extraction and characterisation of plasmids through molecular means with pulse field gel electrophoresis, replicon typing PCR, and ARG PCR. This fundamental work was vital in the formation of initial plasmid understanding and subsequent plasmid AMR insights. With the advent of next generation sequencing technologies (NGS), great strides were taken in the development of bacterial analysis pipelines that could allow for the mass sampling and sequencing of selected isolates. Despite this, issues still remained with the predominant short-read sequencing technologies struggle to assemble plasmids due to their repetitive nature and the common fragmentation of short-read bacterial assemblies [87,88]. The introduction of long-read sequencing allowed for the mitigation of previous plasmid resolution obstacles and provided a new hybrid-analysis framework for the investigation of AMR plasmids in a variety of settings [89].

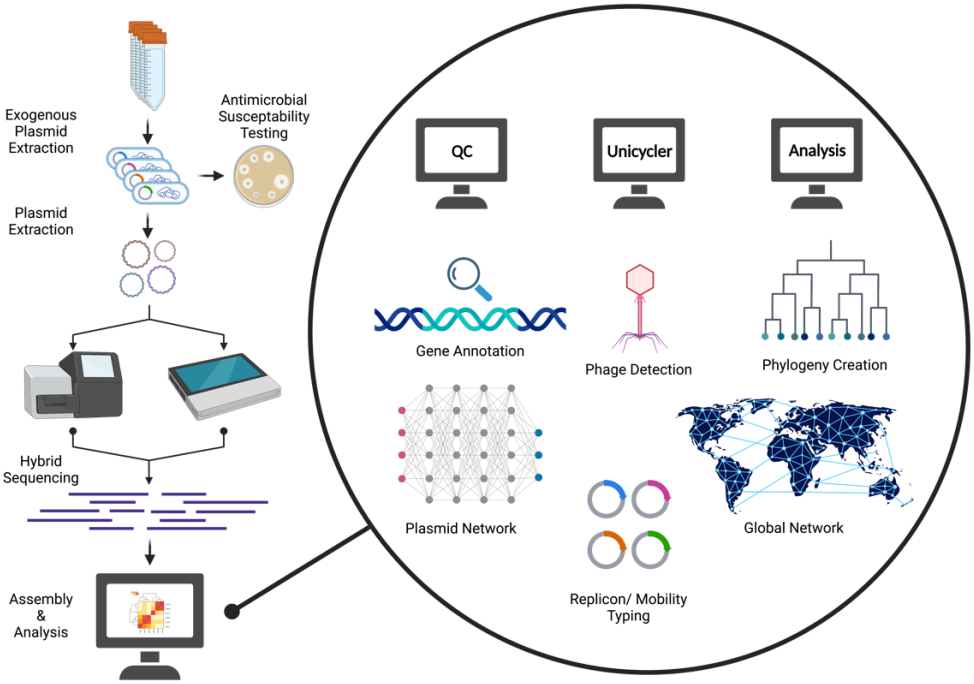
1.6 Computational Plasmidomic Methodologies

Comparative genomic studies have allowed for the confirmation and quantification of ARGs in diverse metagenomes as well as elucidating potentially coevolving gene groups such as metal resistant genes (MRGs) and biofilm forming virulence factors. [90] [91] [92] As mentioned previously a combination of short and long-read sequencing technologies via hybrid-assembly has been shown to produce high quality, complete genomes from complex samples with the ability to resolve complete circularised plasmids [93] [94].This procedural development has allowed for the improved enquiry into the genomic structure of plasmids and the possible mobile ARG present as well as the co-occurrence of other gene groups. This lays the foundation for an improved experimental approach that incorporates both molecular and in-silico methodologies

to develop a vital understanding of plasmid based ARGs, plasmid dissemination and evolution.

In-silico detection and characterisation methods for plasmids have been utilised for many years [95] and despite their significant impact on plasmid studies, the ever-evolving nature of computational analysis and NGS has led to new paths in plasmid determination and description [96–98]. The improvement in such tools has increased the accuracy and volume of plasmid data and thus has expanded the possibilities for subsequent analysis via networking and statistical techniques.

The incorporation of clustering and network visualisation in plasmidome studies has provided a novel way to further understand the interlinked dissemination pathways, co-evolving genes, and possible evolutionary conduits present within large plasmid datasets [99,100]. Networks based on sequence similarity provide an enhanced option for visualising plasmid evolution that cannot be easily described in phylogenetic trees due to recombination and insertion events [101]. This approach uses minhashing and the Jaccard similarity coefficient to rapidly estimate sequence similarity while negating the computational power required by other similarity estimation methods.


Exogenous Plasmid Extraction
Antimicrobial Susceptability Testing
Plasmid Extraction
Hybrid Sequencing
Assembly & Analysis
QC
Unicycler
Analysis
Gene Annotation
Phage Detection
Phylogeny Creation
Plasmid Network
Global Network
Replicon/ Mobility Typing

Jaccard Similarity Coefficient

The size of the intersection divided by the size of the union of two label sets.

$$J(A,B)= \frac{|A \cap B|}{|A \cup B|} \approx \frac{|S(A \cup B) \cap S(A) \cap S(B)|}{|S(A \cup B)|}$$

Eq.1: J = Jaccard Index, A = K-mer Hash set 1, B = K-mer Hash set 2, S = Randomised subset, ∪ = Union.

The utilisation of this rapid method allows for clustering of extremely large datasets and thus the possibility of developing an insight into possible plasmid similarity patterns globally [102]. Subsequent hypergeometric distribution, inference-based enrichment analysis can further expand on this data by determining significant associations between metadata linked plasmids and de-novo assemblies.

Hypergeometric Distribution

A discrete probability distribution that calculates the likelihood an event happens k times in n trials when you are sampling from a small population without replacement.

$$\rho_x(k)= Pr(X=k)= \frac{\binom{K}{k}\binom{N-K}{n-k}}{\binom{N}{n}}$$

Probability mass function

Eq.2: N = Population size, K = Number of success states in population, n = quantity drawn in each trial, k = Number of observed successes, $\binom{a}{b}$ = Binomial coefficient

1.7 Thesis Aims

When viewing the PhD thesis aims from a meta perspective it could be seen that there were three clear aims.

i. To develop a further understanding of possible coliform ARB and their associated resistance mechanisms found within Irish WWTP effluent.
ii. Determine the range of ARM within disparate environmental and animal biomes from both an MGE and metagenomic perspective.
iii. Utilise novel methods to create a new framework for the analysis of mobile AMR in distinct environmental niches.

Each of these aims has been addressed and completed in the following thesis chapters.

Chapter 2

Antibiotic resistant and extended-spectrum β-lactamase producing faecal coliforms in wastewater treatment plant effluent

Cian Smyth[1], Aidan O'Flaherty[1], Fiona Walsh[1], Thi Thuy Do[1]*

[1]Antimicrobial Resistance & Microbiome Research Group, Department of Biology, Maynooth University, Maynooth, Co. Kildare, Ireland

*Correspondence: Thi Thuy Do, Thithuy.do@mu.ie

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2.1 Abstract

Wastewater treatment plants (WWTPs) provide optimal conditions for the maintenance and spread of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs). In this work we describe the occurrence of antibiotic resistant faecal coliforms and their mechanisms of antibiotic resistance in the effluent of two urban WWTPs in Ireland. This information is critical to identifying the role of WWTPs in the dissemination of ARB and ARGs into the environment. Effluent samples were collected from two WWTPs in Spring and Autumn of 2015 and 2016. The bacterial susceptibility patterns to 13 antibiotics were determined. The phenotypic tests were carried out to identify AmpC or extended-spectrum β-lactamase (ESBL) producers. The presence of ESBL genes were detected by PCR. Plasmids carrying ESBL genes were transformed into *Escherichia coli* DH5α recipient and underwent plasmid replicon typing to identify incompatibility groups. More than 90% of isolated faecal coliforms were resistant to amoxicillin and ampicillin, followed by tetracycline (up to 39.82%), ciprofloxacin (up to 31.42%) and trimethoprim (up to 37.61%). Faecal coliforms resistant to colistin (up to 31.62%) and imipenem (up to 15.93%) were detected in all effluent samples. Up to 53.98% of isolated faecal coliforms expressed a multi-drug resistance (MRD) phenotype. AmpC production was confirmed in 5.22% of isolates. The ESBL genes were confirmed for 11.84% of isolates (9.2% of isolates carried blaTEM, 1.4% blaSHV-12, 0.2% blaCTX-M-1 and 1% blaCTX-M-15). Plasmids extracted from 52 ESBL isolates were successfully transformed into recipient *E. coli*. The detected plasmid incompatibility groups included the IncF group, IncI1, IncHI1/2 and IncA/C. These results provide evidence that treated wastewater is

polluted with ARB and MDR faecal coliforms and are sources of ESBL-producing, carbapenem and colistin resistant Enterobacteriaceae.

2.2 Introduction

The dissemination of AMR within bacterial communities and the selection of new resistance mechanisms are due to the large-scale use of antibiotics in agricultural, veterinary and human clinical applications [1–4]. The emergence of antibiotic resistant bacteria (ARB) is a major public health issue which poses a serious therapeutic challenge worldwide [2]. Wastewater treatment plants (WWTPs) are considered potential sources of ARB and antibiotic resistance genes (ARGs), which play an important role in the spread of antibiotic resistance into the environment [5,6]. The urban WWTPs receive wastewater from human communities, which contains high concentrations of chemical matter, including antibiotics and microorganisms, including ARB. Therefore, WWTPs are favourable environments with optimal conditions for the development and spread of ARB and ARGs [7,8]. Both ARB and ARGs were detected in wastewater samples globally [9–13]. However, little is known of the fate of these bacteria; and the role of WWTPs in releasing ABR and ARGs into the environment through treated effluent [14]. A recent report by Flach et al. shows no evidence for the selection of antibiotic resistance in WWTPs [15]; however, large amounts of resistant bacteria were identified throughout the wastewater treatment process [16,17]. The conventional wastewater treatment process can remove some ARB [18,19], but ARB were still found in large proportions in the effluent [20,21]. In some cases, ARB were detected at higher rates in WWTP effluent than in the influent [22,23].

AmpC cephalosporinases and extended-spectrum β-lactamases (ESBLs) are some of the most clinically important antibiotic resistance mechanisms [24]. The dissemination of AmpC or ESBL producing *Escherichia coli* were identified in different types of

aquatic environments, particularly in wastewater [23,25–27]. The prevalence of AmpC or ESBL producing bacteria pose a global health problem due to limitations of therapeutic options for the treatment of infections caused by these bacteria [28]. The ESBL genes are frequently located on mobile genetic elements [29]. Among more than 300 subtypes of ESBL genes, blaTEM and blaSHV groups were the most common ESBL genes identified in human pathogens until the late 1990s. These groups were replaced by blaCTX-M genes since the beginning of the 2000s and *Escherichia coli* became the most prevalent ESBL producing bacteria among clinical *Enterobacteriaceae* [30].

The monitoring of antibiotic resistance from WWTPs provides the information required to track the dissemination of ARB and ARGs into the environment [31,32]. Moreover, the analysis of ARB and ARGs in urban WWTPs is considered as an alternative method for the indirect study of antibiotic resistance in human populations from which the WWTPs receive wastewater [33]. Indeed, the resistance rates of indicator bacteria in wastewater may give useful information to identify the changes in resistance in the human populations [34]. The objectives of this study were to characterise the faecal coliforms resistome leaving urban WWTPs via the effluent. This was achieved through i) assessment of the prevalence of antibiotic resistant faecal coliforms in the effluent from two Irish urban WWTPs, ii) characterisation of the antibiotic resistance profiles of these bacteria, iii) identification of the occurrence of AmpC or ESBL producing faecal coliforms and iv) identification of the resistance mechanisms and their potential mobility.

2.3 Materials & Methods

2.3.1 Isolation of total faecal coliforms

Final effluent samples were collected from two urban WWTPs in Ireland during early Spring and late Autumn in 2015 and 2016. These WWTPs were representative of medium sized WWTPs with 100% urban agglomerations, include tertiary treatment, and the distance between them was less than 100 km. Faecal coliforms were isolated using the membrane filtration method (Novo and Manaia, 2010). The effluent samples (1 mL and 10 mL) were filtered through nitrocellulose membranes (Sigma Aldrich/Merck). The filters were then incubated on membrane faecal coliform (m-FC) agar at 37 °C for 24 h in the presence or absence of antibiotics: amoxicillin (32 mg/L), ciprofloxacin (1 mg/L) or tetracycline (16 mg/L). All procedures were performed in triplicate.

2.3.2 Antibiotic susceptibility test using agar dilution and disk diffusion methods

Bacterial isolates were subjected to antibiotic susceptibility testing using the agar dilution method following the CLSI recommendations [35]. The antibiotics tested were tetracycline, amoxicillin, ampicillin, ciprofloxacin, kanamycin, gentamicin, colistin, chloramphenicol and trimethoprim. The imipenem (10 μg), meropenem (10 μg), cefotaxime (30 μg) and ceftazidime (30 μg) disks were used in the disk diffusion method [35] to determine the susceptibility of these antibiotics. The minimum inhibitory concentration (MIC) breakpoints for Enterobacteriaceae in the CLSI guidelines [35] were used to identify ARB. The EUCAST MIC breakpoint for colistin was used [36].

Bacterial isolates resistant to three or more different antibiotic classes of antibiotics were defined as multidrug resistant. The resistance percentages of bacteria were calculated as: percentage (%) = [(Number of resistant faecal coliforms to an antibiotic/total number of tested faecal coliforms) X 100].

2.3.3 Phenotypic identification of the production of Metallo-beta-lactamase (MBL), ESBL and AmpC enzymes

Bacteria resistant to imipenem and/or meropenem were subjected to the Imipenem-EDTA double-disk synergy test as described previously [37]. The overnight culture of tested bacteria were adjusted to 0.5 McFarland standard and plated on Mueller Hinton (MH) agar following the standard disk diffusion procedure (CLSI). A 10 µg imipenem disk and a blank filter paper disks were placed on the plates at the distance of 10 mm (edge to edge). On the blank disk, 10 µL of a 0.5M EDTA solution was added. The plates were incubated overnight at 37 °C. When the strain showed a synergistic inhibition zone was considered as MBL positive.

Isolates resistant to cefotaxime and/or ceftazidime were subjected to ESBL testing following the CLSI guidelines [35]. The bacteria were adjusted to 0.5 McFarland standard concentration and were plated on MH agar. The cefotaxime (30 µg) and/or ceftazidime (30 µg) and cefotaxime and/or ceftazidime in combination with clavulanate (30 µg/10 µg) disks were used placed onto the surface of the agar containing the bacteria. An increase of ≥5 mm in the inhibition zone for either tested antibiotic in combination with clavulanate comparing with the antibiotic itself was considered as ESBL positive.

The production of AmpC was determined using the phenylboronic acid disk test [38]. Briefly, bacterial cultures were plated on MH agar. The cefoxitin (30 μg) and cefoxitin disk (30 μg) in combination with phenylboronic acid (300 μg) were placed on the inoculated surface of the MH agar. After overnight incubation at 37 °C, the bacterial strains that produced a defined increase (≥5 mm) in the inhibition zone with added phenylboronic acid were considered to be AmpC producers.

2.3.4 Identification of antibiotic resistance genes and bacterial species

Putative MBL producing carbapenem resistant isolates (identified using the imipenem-EDTA double-disk synergy test) were subjected to multiplex PCR to identify the carbapenem resistance genes. The primer sets included blaGES, blaGIM, blaIMI, blaIMP, blaKPC, blaNDM, blaOXA-23, blaOXA-40, blaOXA-48, blaOXA-51, blaOXA-58, blaVIM (Table 1) [39]. Isolates displaying a positive ESBL phenotype and were phenotypically negative for AmpC production were further analysed using the ESBL multiplex-PCR. The primer sets were used to detect the blaTEM, blaSHV, and blaCTX-M-group genes 1, 2, 8, 9, and 25 (Table 1) [40,41].

Bacterial isolates resistant to colistin were further analysed by for the presence of the plasmid mediated colistin resistance mechanisms. Five primer sets were used to screen for the presence of mcr-1, 2, 3, 4 and 5, as recommended by the EU reference laboratory-antimicrobial resistance (Table 1) [42]. The bacterial species of isolates carrying ESBL genes was identified by Sanger sequencing of the V3 and V4 region of bacterial 16S rRNA using forward primer: 5′-

TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCA G-3′ and reverse primer: 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATC TAATCC-3' [43].

2.3.5 Plasmid transfer by transformation and replicon typing

Plasmids were extracted from ESBL positive isolates carrying the blaTEM, blaSHV, and blaCTX-M genes using the Macherey-Nagel NucleoSpin plasmid isolation kit. The plasmids were transformed into *Escherichia coli* DH5α using heat-shock transformation [44,45]. The transformants were selected on LB agar supplemented with ampicillin (32 mg/L). The presence of blaTEM, blaSHV, and blaCTX-M genes in transformants were confirmed by PCR. All transformants were subjected to antimicrobial susceptibility testing against imipenem, meropenem, ertapenem, ciprofloxacin, chloramphenicol, tetracycline, amikacin, gentamycin, kanamycin, trimethoprim and colistin. Replicon typing via PCR were performed on ESBL transformants with 18 pairs of primers recognizing FIA, FIB, FIC, HI1, HI2, I1-Iγ, L/M, N, P, W, T, A/C, K, B/O, X, Y, F, and FIIA in 3 multiplex panels [46].

2.4 Results

2.4.1 Antibiotic susceptibility patterns

In total, 498 faecal coliforms were isolated from all WWTP effluent samples, comprising 226 isolates from WWTP A and 272 from WWTP B. These isolates were subjected to antibiotic susceptibility testing. Among the tested β-lactam antibiotics, more than 90% of bacteria isolated from the two WWTPs were resistant to amoxicillin and ampicillin (Table 2, Fig. 1); greater than 20% were resistant to cefotaxime or ceftazidime. All ceftazidime resistant isolates were also resistant to cefotaxime. Carbapenem resistance was detected at relatively lower levels (Table 2). Colistin resistance was found at a higher percentage in WWTP B effluent than in WWTP A. We also identified that there were no differences in the identification of colistin resistant isolates in antibiotic susceptibility testing by the agar dilution method compared to the microbroth dilution method. Multi-drug resistant (MDR) faecal coliforms were detected at approximately 50% of the total isolates tested (Table 2). The resistance prevalence to other antibiotics were found at similar levels between the two WWTPs.

2.4.2 Detection of antibiotic resistance genes and speciation

Faecal coliforms with resistance to cefotaxime and ceftazidime were considered putative ESBL-producers (n = 157). AmpC production was confirmed in 26 of these isolates (13 from WWTP A and 13 from WWTP B). Of the 131 remaining isolates, 89 (39 from WWTP A and 50 from WWTP B) were identified as phenotypic ESBL

producers. The ESBL multiplex PCR revealed the presence of *bla*$_{TEM}$, *bla*$_{SHV-12}$ and *bla*$_{CTX-M}$ group 1 genes in 62 isolates (Table 3). Almost all ESBL producers were *E. coli* (46 carrying *bla*$_{TEM}$, 2 carrying *bla*$_{SHV-12,}$ 1 carrying *bla*$_{CTX-M-1}$ and 2 carrying *bla*$_{CTX-M-15}$). In addition, 7 isolates were *Klebsiella* spp. (5 carrying *bla*$_{SHV}$, 2 carrying *bla*$_{CTX-M-15}$) and 1 *bla*$_{CTX-M-15}$ positive *Enterobacter* spp.

In total, 79 isolates (36 from WWTP A and 43 from WWTP B) resistant to imipenem were screened for MBL production. Metallo-beta-lactamase production was identified in 36 isolates. However, all isolates were negative for known MBL genes, using the MBL multiplex PCR. More than 100 colistin resistant isolates were detected. However, none of these isolates were positive for the *mcr*-genes using the *mcr*-targeted PCR analysis.

2.4.3 Plasmid transformation, plasmid resistance profile and replicon typing

The plasmids were extracted from 62 ESBL faecal coliform isolates (30 from WWTP A and 32 from WWTP B). Plasmids extracted from 52 ESBL isolates (46 plasmids carried *bla*$_{TEM}$, 2 plasmids *bla*$_{CTX-M15}$, 1 *bla*$_{CTX-M-1}$ and 3 with *bla*$_{SHV-12}$) were successfully transferred into *E. coli* Dh5α recipients. The plasmids extracted from the 10 ESBL isolates (3 with *bla*$_{TEM}$, 3 with *bla*$_{CTX-M15}$ and 4 with *bla*$_{SHV-12}$) could not be transferred, suggesting that the ESBL genes identified in these isolates are located on the bacterial chromosomes.

All transformants were sensitive to the tested carbapenem antibiotics (imipenem, meropenem and ertapenem) and gentamicin (Table 4). The chloramphenicol and

or tetracycline resistance was found at the highest number among all transformants, followed by trimethoprim. Eight transformants show a multidrug resistance phenotype. The plasmids from 21 transformants could be typed using PCR replicon typing (Table 5). The IncF group of replicons was the most prevalent across plasmids from both WWTPs. The IncI1 type was detected only in bacteria isolated from the WWTP B effluent samples.

2.5 Discussion

Our work presents the antibiotic resistance patterns of faecal coliforms in the effluent of two WWTPs in Ireland. Wastewaters with faecal contamination are considered reservoirs for ARB and ARGs in the environment [47,48]. Among all tested antibiotics, resistance to amoxicillin and ampicillin was most prevalent, followed by tetracycline and, cefotaxime and ciprofloxacin. High levels of β-lactam resistance were previously detected in Enterobacteriaceae in an urban WWTP [49], and resistance to tetracycline and fluoroquinolones was found at lower rates. The higher resistance rate of *E. coli* to ampicillin and tetracycline as well as lower rates of resistance to ciprofloxacin were detected in WWTPs in Portugal [50]. A study of raw sewage in Brazil identified 100% sensitivity of *E. coli* to ciprofloxacin and amoxicillin and tetracycline resistance levels in the range of 50–75% [51]. The proportions of ciprofloxacin resistant faecal coliforms were 31.42% in WWTP A effluent and 26.47% in WWTP B. This resistance rate is higher in comparison to reported levels of ciprofloxacin resistance in the *E. coli* isolated from other WWTPs [49,52,53]. In general, differences in antibiotic resistance percentages were observed between the two WWTPs, particularly for colistin, trimethoprim and kanamycin (Fig. 1). As these two WWTPs are using the same treatment process, this difference may be associated with their location or other external factors.

The presence of colistin resistant and carbapenem resistant isolates in tested WWTP effluents raises the possibility of transferability or risk to human health. These antibiotics are known as ‘last resort’ antibiotics to treat MDR bacteria. Previous studies on colistin resistant Enterobacteriaceae focus mainly on food, human and

animal samples [54–56]. To date, there are only a few studies conducted on the identification of *mcr* genes in waterborne bacteria[57–61]. 114 faecal coliform isolates resistant to colistin (28 from WWTP A and 86 from WWTP B) were detected in our work, none of them were positive for the *mcr* genes. Among colistin resistant isolates, the plasmids extracted from 43 isolates (4 from WWTP A and 39 from WWTP B) were successfully transferred to the recipient *E. coli* DH5α in the transformation studies. The proportion of colistin resistant coliforms in our study was lower than those in the studies of [58], where they found approximately 60% of isolates with resistance to colistin. Carbapenem resistant Enterobacteriaceae were studied in hospital wastewater and in WWTPs previously [62–65]. The percentage of carbapenem resistance phenotypes in our work were considered high in comparison to previous studies in wastewater [27,66]. However, there were no known carbapenem resistance genes detected in the carbapenem resistant coliforms. This suggests that other resistance mechanisms are responsible for the resistance phenotypes and thus require further study to characterise these novel mechanisms.

Multi-drug resistant faecal coliforms were retrieved at high rates from all effluent samples. In the study of Lefkowitz and Durán [67], 60% of *E. coli* in WWTP effluent were resistant to two or more antibiotics and 25% to four or more antibiotics. The study of [68] in WWTP effluent showed no more than 12% of *E. coli* were resistant to two antibiotics and less than 10% to three or more antibiotics. *Escherichia coli* (34.3%) were found to be resistant to two or more antibiotics and 8.8% to four or more antibiotics in treated wastewater in Portugal [49]. The MDR faecal coliforms in our study were found in the same range of the study of Lefkowitz and Durán, but at a higher percentage than in others.

The ESBL or AmpC producing faecal coliforms were recovered from all WWTP effluent samples. The rate of ESBL producing *Enterobacteriacea* in our work were within the range of previous studies. It was considerably high in comparison to some studies of WWTP effluent (0.5–9.8%) [23,69–72]. However it was lower than those studies in wastewater (45–100%) [73,74]. The high load of bacteria and rich nutrient environment in WWTPs facilitates the transfer of ARGs among bacteria [7,8]. These may explain the relatively high rates of ESBL producers in WWTP effluent.

The *bla*$_{\text{TEM}}$ were the most prevalent beta-lactamase in this study, which is similar to a study on hospital wastewater in Brazil [74]. However, this is in contrast with other findings with *bla*$_{\text{CTX-M}}$ being the most frequent ESBL genes from various samples including in hospital effluent, surface water and WWTPs [23,30,75]. This result was confirmed by qPCR data performed in another study in the framework of the StARE project which showed the detection of *bla*$_{\text{TEM}}$ genes, where *bla*$_{\text{SHV}}$ and *bla*$_{\text{CTX-M}}$ group 1 were not detected [76]. Most of ESBL genes were found in *E. coli*, others were carried by *Klebsiella* spp. in our work. These results are in an agreement with previous findings which indicated that *E. coli* were the most common ESBL-producers among Enterobacteriaceae [30].

Transformation of plasmids carrying ESBL genes were successful for 88% of ESBL donor isolates in this work. Different replicon variants were found in the ESBL plasmids. The most prevalent replicon was IncF group which were also reported in other studies of plasmid replicon typing in *Enterobacteriaceae* [77–79]. These replicons have a narrow host range and can be transfer easily among *E. coli* species [80]. The

cross-resistance of ESBL producers to other antibiotic causes of great concern as ESBL genes are frequently located on conjugative plasmids carrying other ARGs [81]. In this work eight of 52 transferable plasmids carrying ESBL genes expressed a MDR phenotype.

2.6 Conclusion

Effluent samples from two WWTPs demonstrated the presence of ARB and MDR and, of particular importance, a source of a relatively high proportion of ESBL-producing, carbapenem and colistin resistant Enterobacteriaceae. Although the bacteria were phenotypically resistant to colistin or carbapenems no known mobile resistance mechanisms were detected, despite the ability to transfer the resistance phenotype via transformation. Thus, faecal coliforms from WWTP effluent are sources of novel ARGs conferring resistance to antibiotics of last line of defence. The ability of these bacteria to survive in water has been demonstrated for many years. The significance of this study is the identification of the role of WWTPs as a potential control point to reduce or stop the movement of resistant bacteria and genes into the environment from further upstream sources, such as human or animal waste. In addition, this enables the use of additional treatment technologies to be added to WWTPs to stop or reduce such ARB and ARGs entering the water environments globally.

Author Contributions: Cian Smyth: Methodology, Validation, Investigation, Resources, Formal analysis, Data curation. **Aidan O'Flaherty:** Investigation. **Fiona Walsh:** Supervision, Funding acquisition, Writing - review & editing. **Thi Thuy Do:** Supervision, Project administration, Conceptualization, Methodology, Validation, Investigation, Resources, Writing - original draft, Visualization.

Declaration of competing interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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2.7 Tables

Table 1: Primers used in multiplex PCRs to detect ESBL [40,41], MBL [39] and *mcr*-genes [42].

Gene Target	Primers	PCR Product (bp)	Amplification Conditions
*bla*CTX-M	Group 1-(F) AAA AAT CAC TGC GCC AGT TC	415	Initial denaturation step of 5 min at 94 °C
	Group 1-(R) AGC TTA TTC ATC GCC ACG TT		30 cycles
	Group 2-(F) CGA CGC TAC CCC TGC TAT T	552	25 s at 94 °C
	Group 2-(R) CCA GCG TCA GAT TTT TCA GG		40 s at 52 °C
	Group 9-(F) CAA AGA GAG TGC AAC GGATG	205	50 s at 72 °C
	Group 9-(R) ATT GGA AAG CGT TCA TCA CC		Final extension for 6 min at 72 °C
	Group 8-(F) TCG CGT TAA GCG GAT GAT GC	666	
	Group 8-(R) AAC CCA CGA TGT GGG TAG C		

Gene Target	Primers	PCR Product (bp)	Amplification Conditions
	Group 25-(F) GCA CGA TGA CAT TCG GG	327	
	Group 25-(R) AAC CCA CGA TGT GGG TAG C		

Gene Target	Primers	PCR Product (bp)	Amplification Conditions
*bla*TEM	*bla*TEM-(F) CATTTCCGTGTCGCCCTTATTC	800	Initial denaturation step of 10 min at 94 °C
	*bla*TEM-(R) CGTTCATCCATAGTTGCCTGAC		30 cycles
*bla*SHV	*bla*SHV- (F) AGCCGCTTGAGCAAATTAAAC	713	40 s at 94 °C
	*bla*SHV- (R) ATCCCGCAGATAAATCACCAC		40 s at 60 °C
			1 min at 72 °C
			Final extension for 7 min at 72 °C.

Gene Target	Primers	PCR Product (bp)	Amplification Conditions
MBL-multiplex 1	*bla*VIM-(F) GATGGTGTTTGGTCGCATATC	202	Initial denaturation step of 3 min at 94 °C
	*bla*VIM-(R) CGTCATGAAAGTGCGTGGAG		30 cycles
	*bla*KPC-(F) CGCCAATTTGTTGCTGAAGG	312	30 s at 94 °C
	*bla*KPC-(R) CAGGTTCCGGTTTTGTCTCC		15 s at 58 °C
	*bla*OXA-40-(F) AGTTTCTCTCAGTGCATGTTCA	413	1 min at 72 °C
	*bla*OXA-40-(R) CCCGCTTTACTTCTTTCTGCA		Final extension for 5 min at 72 °C.
	*bla*NDM-(F) GTTTGATCGTCAGGGATGGC	517	
	*bla*NDM-(R) CTCATCACGATCATGCTGGC		
	*bla*OXA-48-(F) GGTAGCAAAGGAATGGCAAGAA	611	
	*bla*OXA-48-(R) CGACCCACCAGCCAATCTTA		
	*bla*OXA-23-(F) TCTGGTTGTACGGTTCAGCA	718	
	*bla*OXA-23-(R) GCATTTCTGACCGCATTTCC		

Gene Target	Primers	PCR Product (bp)	Amplification Conditions
MBL-multiplex 2	*bla*IMI-(F) AGACTCGATCGTTGGGAGTT	206	Initial denaturation step of 3 min at 94 °C
	*bla*IMI-(R) CAATCGCTTGGTACGCTAGC		30 cycles
	*bla*OXA-58-(F) ATCAAGAATTGGCACGTCGT	303	30 s at 94 °C
	*bla*OXA-58-(R) CCACATACCAACCCACTTGC		15 s at 58 °C
	*bla*GES-(F) CTCAGATCGGTGTTGCGATC	416	1 min at 72 °C
	*bla*GES-(R) TGTATCTCTGAGGTCGCCAG		Final extension for 5 min at 72 °C
	*bla*GIM-(F) TTATCCTGGGCGACTGACAG	508	
	*bla*GIM-(R) CAGCGGTCGGTTGCATTAAT		
	*bla*IMP-(F) GAAGGCGTTTATGTTCATAC	587	
	*bla*IMP-(R) GTACGTTTCAAGAGTGATGC		
	*bla*OXA-51-(F) TGTGGTAAGCACTTGATGGG	704	
	*bla*OXA-51-(R) ATTGCCATAACCAACACGCT		

Gene Target	Primers	PCR Product (bp)	Amplification Conditions
mcr-multiplex	*mcr-1*-(F) AGTCCGTTTGTTCTTGTGGC	320	Initial denaturation step of 15 min at 94 °C
	mcr-1-(R) AGATCCTTGGTCTCGGCTTG		25 cycles
	mcr-2-(F) CAAGTGTGTTGGTCGCAGTT	715	30 s at 94 °C
	mcr-2-(R) TCTAGCCCGACAAGCATACC		90 s at 58 °C
	mcr-3-(F) AAATAAAAATTGTTCCGCTTATG	929	1 min at 72 °C
	mcr-3-(R) AATGGAGATCCCCGTTTTT		Final extension for 10 min at 72 °C
	mcr-4-(F) TCACTTTCATCACTGCGTTG	1116	
	mcr-4-(R) TTGGTCCATGACTACCAATG		
	mcr-5-(F) ATGCGGTTGTCTGCATTTATC	1644	
	mcr-5-(R) TCATTGTGGTTGTCCTTTTCTG		

Table 2: Prevalence of antibiotic resistance phenotypes in faecal coliforms isolated from WWTP effluent samples.

CIP: Ciprofloxacin, AMX: Amoxicillin, AMP: Ampicillin, CTX: Cefotaxime, CAZ: Ceftazidime, IPM: Imipenem, MEM: Meropenem, TET: Tetracycline, KAN: Kanamycin, GEN: Gentamicin, CST: Colistin, CHL: Chloramphenicol, TMP: Trimethoprim, MDR: multidrug resistant.

Antibiotic	**Antibiotic-resistant faecal coliforms in WWTP effluent samples**			
	WWTP effluent A (Total number of isolates = 226)		**WWTP effluent B (Total number of isolates = 272)**	
	Number of resistant isolates	**Percentage resistant (%)**	**Number of resistant isolates**	**Percentage resistant (%)**
CIP	71	31.42	72	26.47
AMX	205	90.71	259	95.22
AMP	207	91.59	253	93.01
CTX	77	34.07	80	29.41
CAZ	49	21.68	62	22.79
IPM	36	15.93	43	15.81
MEM	13	5.75	14	5.15

Antibiotic	Antibiotic-resistant faecal coliforms in WWTP effluent samples			
	WWTP effluent A (Total number of isolates = 226)		WWTP effluent B (Total number of isolates = 272)	
	Number of resistant isolates	Percentage resistant (%)	Number of resistant isolates	Percentage resistant (%)
TET	90	39.82	103	37.87
KAN	28	12.39	12	4.41
GEN	32	14.16	26	9.56
CST	28	12.39	86	31.62
CHL	19	8.41	25	9.19
TMP	85	37.61	61	22.43
MDR	122	53.98	131	48.16

Table 3: Extended-spectrum β-lactamase genes identified in faecal coliforms isolated from WWTP effluent samples.

ESBL gene	WWTP A Number of ESBL isolates	WWTP B Number of ESBL isolates
*bla*TEM-116	6	5
*bla*TEM-12	3	2
*bla*TEM-1-like (>99% identical to *bla*TEM-143 or *bla*TEM-164)	16	17
*bla*CTX-M-1	0	1
*bla*CTX-M-15	2	3
*bla*SHV-12	3	4

Table 4: Number of resistant transformants obtained from ESBL donor faecal coliforms.

CIP: Ciprofloxacin, IPM: Imipenem, MEM: Meropenem, ERT: Ertapenem, TET: Tetracycline, AMK: Amikacin, KAN: Kanamycin, GEN: Gentamicin, CST: Colistin, CHL: Chloramphenicol, TMP: Trimethoprim, MDR: multidrug resistant.

Resistance phenotype	**No. of resistant transformants**	
	WWTP A (Total No. of transformants = 24)	**WWTP B (Total No. of transformants = 28)**
CIP	1	0
IPM	0	0
MEM	0	0
ERT	0	0
TET	5	11
AMK	1	0
KAN	1	1
GEN	0	0

Resistance phenotype	**No. of resistant transformants**	
	WWTP A (Total No. of transformants = 24)	**WWTP B (Total No. of transformants = 28)**
CST	0	3
CHL	12	4
TMP	1	9
MDR	3	5

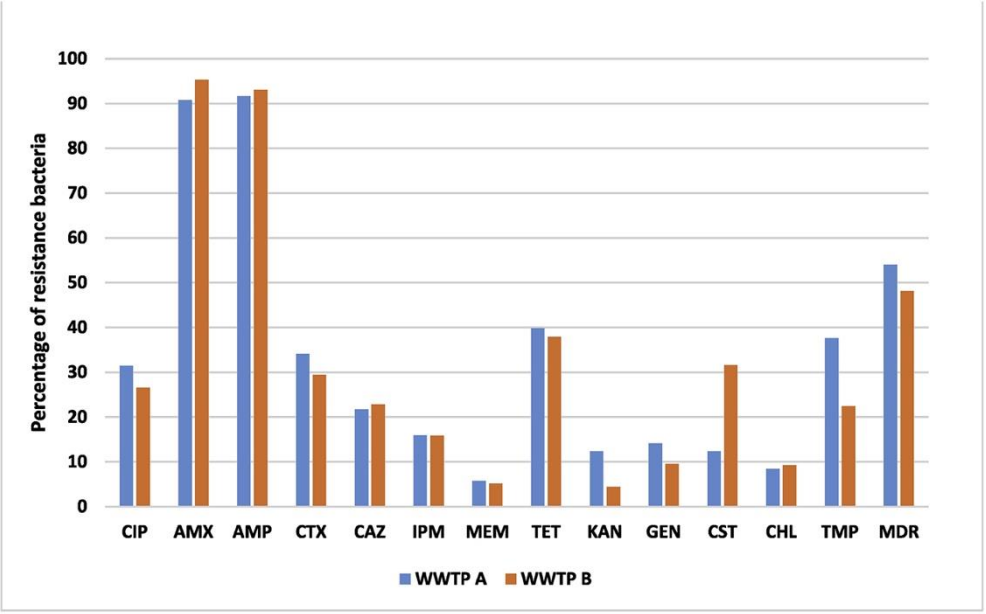
Table 5: Plasmid replicon types among 21 transformants obtained from ESBL donor faecal coliforms.

Transformant	**Source**	**Replicon(s)**	**ESBL gene**
A1	WWTP-A	HI2	*bla*TEM-1-like
A2	WWTP-A	HI1	*bla*TEM-12
A3	WWTP-A	FIIA	*bla*TEM-12
A4	WWTP-A	F	*bla*CTX-M-15
A5	WWTP-A	F	*bla*TEM-1-like
A6	WWTP-A	FIB	*bla*TEM-1-like
B1	WWTP-B	I1	*bla*TEM-1-like
B2	WWTP-B	I1	*bla*TEM-1-like
B3	WWTP-B	FI	*bla*TEM-116
B4	WWTP-B	I1	*bla*TEM-1-like
B5	WWTP-B	F, FIA	*bla*CTX-M-15

Transformant	Source	Replicon(s)	ESBL gene
B6	WWTP-B	A/C	*bla*TEM-12
B7	WWTP-B	F	*bla*TEM-1-like
B8	WWTP-B	HI2	*bla*TEM-12
B9	WWTP-B	HI1	*bla*TEM-1-like
B10	WWTP-B	I1	*bla*CTX-M-1
B11	WWTP-B	F	*bla*TEM-1-like
B12	WWTP-B	I1	*bla*SHV-12
B13	WWTP-B	FIA	*bla*TEM-1-like
B14	WWTP-B	FIA	*bla*TEM-1-like

2.8 Figures

Figure 1: Percentage of faecal coliforms isolated from WWTP effluent samples identified with antibiotic resistance phenotypes. nCIP: Ciprofloxacin, AMX: Amoxicillin, AMP: Ampicillin, CTX: Cefotaxime, CAZ: Ceftazidime, IPM: Imipenem, MEM: Meropenem, TET: Tetracycline, KAN: Kanamycin, GEN: Gentamicin, CST: Colistin, CHL: Chloramphenicol, TMP: Trimethoprim, MDR: multidrug resistant.



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Chapter 3

Shooting hoops: globetrotting plasmids spreading more than just antimicrobial resistance genes across One Health

Cian Smyth[1]†, Robert J. Leigh[1]†, Sarah Delaney[1], Richard A. Murphy[2] and Fiona Walsh[1]*

[1]Antimicrobial Resistance & Microbiome Research Group, Department of Biology, Maynooth University, Maynooth, Co. Kildare, Ireland

[2]Alltech European Bioscience Centre, Dunboyne, Co. Meath, Ireland.

†These authors also contributed equally to this work

*Correspondence: Fiona Walsh, fiona.walsh@mu.ie

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3.1 Abstract

Our study provides novel insights into the global nature of antimicrobial resistance (AMR) plasmids across the food chain. We provide compelling evidence of the globetrotting nature of AMR plasmids and the need for surveillance to sequence plasmids with a template of analyses for others to expand these data. The AMR plasmids analysed were detected in 63 countries and in samples from humans, animals and the environment. They contained a combination of known and novel AMR genes, metal resistance genes, virulence factors, phage and replicon types.

3.2 Introduction

The World Health Organisation (WHO), Food and Agricultural Organisation of the United Nations (FAO) and World Organisation for Animal Health (OIE) identified antimicrobial resistance (AMR) as a critically important One Health problem. In 2017 WHO identified 12 global priority pathogens classified as containing important AMR, including plasmid- mediated carbapenem-resistant, extended spectrum beta-lactamase (ESBL)-producing or fluoroquinolone-resistant Gram-negative pathogens [1]. The European Union summary report on AMR in zoonotic and indicator bacteria from animals and food in 2018/19 frequently identified ampicillin-, sulfamethoxazole-, trimethoprim- and tetracycline-resistant *Escherichia coli* across pig, poultry and bovine populations [2]. *E. coli* with 'very high' resistance to ciprofloxacin and chloramphenicol were identified in poultry [2]. Due to the focus of many studies on *E. coli* we know much less about AMR in non-pathogenic and other zoonotic bacteria present in food- producing animals. There is a dearth of information on the AMR plasmids contained within the non- pathogenic or zoonotic bacteria within food-producing animals. To date, plasmid data have mainly been limited to plasmids present in culturable pathogens.

Plasmids enable AMR gene (ARG) transfer across bacteria and biomes and through the food chain. Thus it is these plasmids that are the risk to the further spread of AMR across One Health from animals to humans or the environment and vice versa. The contribution of specific plasmids to the dissemination of ARGs across One Health is unknown. Analysis of AMR plasmids conferring AMR or multi-drug resistance (MDR) may enable us to track and understand the dynamics of the spread of these

mechanisms of resistance across One Health. Through comparison of the entire plasmids will we be able to track and trace their movements across One Health and identify their risk to One Health. Our study aimed to characterize the AMR plasmids isolated from poultry that could be expressed in *E. coli* in the context of other resistance and virulence genes and identify their global nature by comparison with all sequenced plasmids across One Health of human, animal and the environment.

3.3 Materials & Methods

3.3.1 Sample Collections

Broiler caecal samples (n=34) were obtained from a commercial poultry production unit in the UK. The poultry were all fed the same diet. No antimicrobials were supplemented in their diet. All animals were taken from a commercial hatchery and transported to the commercial sheds on the day of hatching. Approximately 10 000 birds were mirror imaged from the hatchery into the production sheds. The birds were raised and fed under typical commercial production conditions, receiving feed and water ad libitum. All other conditions were kept uniform for all sheds. At days 21 (D21) and 34 (D34) post- hatch, the intact caecal pouches of randomly caught birds were removed immediately after euthanization. The entire contents of each caecal pouch were removed, lyophilized and stored at −80 °C until analysis.

3.3.2 Exogenous Plasmid Isolation

Plasmids harbouring ARGs were isolated from each of the 34 caecal samples using the exogenous plasmid isolation method, as previously described [3]. Briefly, the total transferable plasmid populations from each of the 'donor' caecal samples (n=34) were individually transferred to the 'recipient' rifampicin-resistant *E. coli* DH5α via biparental mating. Exogenous transconjugants were selected on Eosin Methylene Blue (EMB) agar (Sigma) with rifampicin (100 mgl^{-1}) and ampicillin, tetracycline, gentamicin, colistin, cefotaxime or ciprofloxacin at breakpoint concentrations according to CLSI guidelines [4]. From each antimicrobial selective plate with growth

after exogenous isolation, a transconjugant was selected at random. If the same plate appeared to have bacteria with different features (colour, morphology), representatives of each colony type were selected.

3.3.3 Antimicrobial Susceptibility Analysis

Antimicrobial susceptibility testing was performed on the exogenous transconjugants via the disc diffusion method according to CLSI guidelines for ampicillin, tetracycline, kanamycin, cefotaxime, ciprofloxacin, gentamicin, trimethoprim, imipenem and chloramphenicol antimicrobials [4]. The presence of ESBL enzymes was investigated using the CLSI confirmatory combination disc test method [4]. The test was considered positive when an increase in the growth- inhibitory zone around the cefotaxime disc with clavulanic acid was 5 mm or greater than the diameter around the disc containing cefotaxime alone.

3.3.4 Plasmid Sequencing

Plasmids from ESBL- positive transconjugants across D21 and D35 were selected for plasmid sequencing (n=9 exogenous transconjugant *E. coli*). The plasmids were extracted from the transconjugants using the Macherey-Nagel NucleoSpin Plasmid kit following the low-copy number protocol according to the manufacturer's guidelines. Each transconjugant contained several plasmids, and thus 22 plasmids were sequenced to closed circular plasmids.

3.3.5 Sequencing

Extracted plasmids were sequenced using the SQK- LSK- 109 Ligation Sequencing kit [Oxford Nanopore Technologies (ONT)] with the NBD- 104 Barcoding kit used for multiplexing. No departures from standard kit protocols were made. Completed libraries were run on an ONT MinION (R9).

3.3.6 Pre-assembly Quality Control (QC)

DNA outputs were basecalled and demultiplexed using ONT- Guppy v.1.1.alpha17-6- g5cecf99, and this process was also used to remove any attached barcodes and adaptors. Filtlong v.0.2.0 was used to filter for any excessively short reads as well as filtering for any reads of low quality based on qQ-score [5].

3.3.7 Assembly

Reads were de novo assembled using Unicycler v.0.4.9 long- read only [6]. This package was run with default parameters.

3.3.8 Post-assembly QC

Visual assessment of assembled contigs was performed with Bandage v.0.8.1 [7]. This allowed for an easy inspection of circularized contigs and their size/depth.

3.3.9 Plasmid Annotation

In total, 22 plasmids were observed to be circular using Bandage. One other plasmid-derived sequence was observed to be both long (41 468 bp) and of high coverage. Each plasmid sequence was annotated using Prokka v.1.14.6 using the RNA profiling flag (--rfam) and, otherwise, default settings [8]. Due to the high level of hypothetical genes arising from Prokka annotation, each plasmid sequence was further annotated using BAKTA v.1.2.1 with default settings [9]. Annotations derived from BAKTA are provided as supplementary data [10]. Plasmids were visualized using in- house software. The sample name indicates the transconjugant and the second number the specific plasmid; for example, F4_7 is transconjugant 4 with plasmid number 7.

3.3.10 Resistome, Virulome and Secondary Metabolism Profiling

The total plasmid DNA of each sample and each circularized plasmid was analysed for the presence of ARGs using ABRicate v.1.0.1 for AMR using the Comprehensive Antimicrobial Resistance Database (CARD) v.3.09 [11,12]. Each circular plasmid was profiled in addition for metal and biocide resistance using BacMet v.2.0, and for virulence factors using the Virulence Factor Database (VFDB) v.5 [10,13]. As plasmids often display rapid evolutionary rates, the minimum percentage identity (--minid) flag was reduced to 50 % (from the default 80%). As ABRicate requires a nucleotide input and as BACMET is only provided in amino acid format, the amino acid database was back translated (using translation table 11) prior to annotation [14]. As resistance may arise via point mutation, PointFinder v.3.1.1 was used with all available bacterial profiles (*Campylobacter spp., Enterococcus faecalis, Enterococcus faecium,*

Escherichia coli, Helicobacter pylori, Klebsiella spp., Mycobacterium tuberculosis, Neisseria gonorrhoeae, Salmonella spp. and Staphylococcus aureus) to assess for resistance arising in chromosomally translocated genes [15]. Despite exhaustive searching, no resistance arising via point mutation was detected.

3.3.11 Plasmid Clustering and Global Relatedness

Plasmid clustering and global relatedness Each circular plasmid was searched against each other plasmid sequenced for this study using the distance function (dist) in Mash v.2.2.2[16]. Instances where both the distance score (D) and P-value (P)≤0.1 were considered homologous. The 0.1 score filters were chosen to replicate the cut- offs used during the construction of PLSDB v.0.1.7[17]. Each circular plasmid analysed in this study was assessed against all plasmids in PLSDB v.0.1.7 using the distance Mash dist algorithm. Again, instances where both the distance score (D) and P-value (P)≤0.1 were retained for further inspection. Each retained hit was further annotated using the metadata provided by PLSDB. The MASH distance score is highly correlated to the average nucleotide identity (ANI; a pairwise measure of genomic similarity between two genome's coding regions subtracted from 1 (1−ANI) and a distance score of ≤0.05 equates to a ≥95 % ANI [16,18]. A distance score of 0.1 is assumed to equate to ≥90 % ANI.

3.3.12 Geographical Mapping

Geocoordinates for each mapped plasmid were extracted from PLSDB metadata (where available) and converted to a representative country using the

'Nomatim().reverse' function in the GeoPy v.2.2.0 python library. The list of countries associated with each plasmid was used to annotate a world atlas to visualize the extent of spread.

3.3.13 Temporal Analysis of Plasmid Character State Evolution

Where available, dates- of- isolation were extracted for each mapped plasmid from PLSDB. Each date- of- isolation was represented in year format to standardize the data, and plasmids without dates- of- isolation were excluded from further analyses. Drug resistance, metal resistance and virulence factor profiles were established for each plasmid using ABRicate as above, and metaprofiles were constructed for each plasmid group and ranked based on year (Supplementary Data, available with the online version of this article). As F5_3 (suspected phage- plasmid) only matched two other plasmids (and none was observed to contain a resistance gene or virulence factor), these plasmids were not subjected to further temporal analysis.

3.3.14 Prophage Detection

Each circular plasmid was assessed for prophages using Phigaro v.2.3.0 under default settings [19]. Detected prophages were individually annotated using BAKTA and assessed for resistance and virulence profiles using ABRicate with the CARD, BACMET and VFDB databases as above. A database of International Committee on the Taxonomy of Viruses (ICTV) exemplar virus genomes was downloaded from NCBI Assembly [20]. Each prophage was searched against the ICTV database using the Mash screen algorithm using instances where both identity ≥0.5 (50 %) and P≤0.1 are

reported; hits from non- phage genomes were removed. The identity stringency score was lowered to 50 % to allow for detection of rapidly degrading or mutating phage fragments.

3.4 Results

3.4.1 Antimicrobial Resistance Profiles

Nine ESBL- positive transconjugant samples (Table 1) were resistant to ampicillin, cefotaxime and tetracycline. Transconjugants 3, 4, 5, 8 and 9 were also resistant to aminoglycosides (3, 8, 9), trimethoprim (4, 8, 9), ciprofloxacin (5, 9) or imipenem (8), or a combination.

3.4.2 Plasmid Descriptions

Both the total sequenced DNA (circularized and non- circularized) plasmid data and the circularized plasmids were analysed. The AMR genes detected across the total DNA (Tables 1 and 2, Supplementary data 1) conferred resistance to seven antimicrobial classes and quaternary ammonium compounds (QACs). However, some of the phenotypes and genotypes did not match. While *blaTEM* and *blaSHV* beta-lactamase genes were detected across all samples, only three contained the ESBL *blaSHV-2* gene. Others contained *blaSHV* genes, which are not classified as beta-lactamases or ESBLs [21]. Two samples only contained the beta- lactamase *blaTEM- 1* genes. No mobile quinolone resistance genes were detected in the ciprofloxacin-resistant samples and nor were mutations in their quinolone resistance regions (QRDRs). Sample 6 contained no tetracycline resistance genes and sample 8 no imipenem or aminoglycoside resistance genes. Sample 4 was resistant to trimethoprim but contained no known resistance gene. Thus, as the phenotype was present, these isolates must contain novel resistance genes hidden within their plasmid DNA.

3.4.3 Circularized Plasmid Descriptions

We resolved 22 circularized plasmids and one linear plasmid with a very high coverage but which could not be circularized (sample 8). The circularized plasmid sizes were highly variable (3.365–190.3 kb); five plasmids were less than 10 kb and 12 exceeded 100 kb (Supplementary Data 2). Coding proportions were stable across all samples except for plasmid F4_7, which displayed a considerably lower coding proportion (0.585). Variation in GC% variation was observed (0.448–0.552). Sixteen plasmids contained an *oriC*, and both F2_2 and F3_6 contained three *oriC* copies. Comparatively, 19 plasmids contained an *oriT*. The plasmids F2_1, F5_3 and F9_2 lacked both an *oriT* and *oriC*.

3.4.4 Plasmid Clustering of Circularised Plasmids

Circularized plasmids (n=22) plus plasmid 8_22 clustered into one of four groups (A–D) or were singletons (Table 2, Supplementary Data 2). Group A comprised five plasmids (F1_2, F4_2, F6_1, F7_2 and F9_3); Group B four plasmids (F1_17, F4_7, F7_6 and F9_5); Group C three plasmids (F3_4, F5_2 and F8_22); and Group D five plasmids (F1_1, F2_2, F4_1, F5_1 and F9_1). All other plasmids were singletons (n=5). A range of replicons were present across the plasmids (Supplementary Data 3). Replicon types were generally consistant within cluster groups: Group A: IncB/O/K/Z_2, Group B: Col440II_1 and ColRNAI_1, Group C: IncN_1 and Group D: IncFIB – within this group plasmid F2_2 also contained IncFIA and IncFIC replicons. The remaining six plasmids contained Col440II_1 (F1_18), IncFII and IncI1 (F1_3), IncFIA, IncHIA and IncHIB (F2_1), IncX1 and IncX3 (F3_6), no replicon

type (F5_3), and FIB (F9_2). Col440II_1 and ColRNAI_1 plasmids were less than 7 kb. The plasmids between 40 and 50 kb were either IncN or IncW and those greater than 100 kb were IncB/O/K/2, IncF or IncH. This demonstrates the wide range of plasmid types identified across the samples and with similar AMR profiles. There was no correlation between replicon type and resistance phenotype or genotype.

3.4.5 Resistance Profiling of Circularized Plasmids

Beta- lactam resistance genes were identified in 15 of 23 plasmids, ten of which (all Group D, C, and singleton plasmids F1_3, F3_4 and F3_6) contained the penicillinase *blaTEM*, and five (all Group A) contained ESBL *blaSHV-2* (Supplementary Data 4). No plasmid contained both *blaTEM* and *blaSHV*. Tetracycline-resistant genotypes were observed in plasmids F5_1 via *tetA* alone, both *tetA* and *tetB* in F1_1, and in F2_1, F4_1 and F9_1 via *tetB* and *tetC*. The tetracycline resistance repressor gene *tetR(B)* and the *tetC* genes were detected beside the *tetB* genes and the *tetR(A)* gene beside the *tetA* gene in F5_1. Plasmid F1_1 contained only the *tetR(B)* gene. Plasmids F5_2 and F8_22 contained the *tet(C)* and *tetR(A)* genes and F3_4 contained only the *tetR(A)* repressor. Aminoglycoside resistance genes *aph(3'')-Ib* and *aph(6)-Id* conferring streptomycin resistance were observed together (F1_1, F4_1, F5_1, F9_1). Plasmid F2_1 contained *aadA22* and F5_1 also contained *aadA1*, both of which confer resistance to streptomycin and spectinomycin. Three singleton plasmids displayed trimethoprim resistance genotypes: F3_6 via *dfrA8*, F5_1 via *dfrA1* and F9_2 via *dfrD*. However, neither of the *dfrA*-containing transconjugants displayed resistance to trimethoprim. Fourteen sequential point mutations were observed between nucleotides 133 and 146, followed by an 11 amino acid deletion event from sites 147–157 in

dfrA1. These mutations are at the C terminus of the Dfr Pfam domain (PF00186) and, while the active site is at the N terminus of this domain, the mutations may limit binding potential. No point mutations were detected in dfrA8. Transposon- mediated lincosamide resistance (*lnuG*) was observed in F2_1. Sulphonamide resistance sul1 genes were observed in plasmids: F2_1 (singleton) and F5_1 (Group D). Both were adjacent to the QACs efflux SMR transporter *qacEΔ1*. No plasmid- mediated carbapenemase or colistin resistance genes were detected, and thus these plasmids did not confer resistance to the last line of defence antimicrobials. A multidrug (aminoglycoside, β-lactam and tetracycline) resistance genotype (via *aph(3'')-Ib, aph(6)-Id*, *blaTEM* and *tetA*) was observed in four Group D plasmids. The exception was plasmid F2_2, which contained only *blaTEM*. Plasmid F5_1 (Group D) additionally contained sulphonamide and trimethoprim resistance genes. Group B plasmids, F1_17, F4_7, F7_6, F9_5 and singletons F1_18 and F5_3 contained no AMR, metal resistance nor virulence genes.

3.4.6 Metal resistance and Virulence Genes

Metal resistance gene (MRG) abundances varied across plasmids from zero (n=17) to two (F2_1), three (Group D F2_2), four (Group D F1_1, F4_1, F9_1) or 11 (Group D F5_1) (Supplementary Data 5 and 6). Each plasmid with MRGs also contained ARGs. The Group D plasmids comprised the *sitABC* MRGs conferring resistance towards manganese, iron and hydrogen peroxide with the addition of the *merR* gene or, in the case of plasmid F5_1, all eight mercury resistance genes (*merABCDEPRT*) and qacEΔ1. F2_1 contained *corA*, which confers nickel, cobalt and manganese resistance, and *qacEΔ1*.

The MRG- positive plasmids also contained virulence factor genes: Group D plasmids (F1_1, F2_2, F4_1 F5_1 and F9_1) and the singleton F2_1. Plasmid F2_1 only contained the *astA* gene, the genotype for heat- stable enterotoxin 1 production. The Group D plasmids all contained the siderophore (salmochelin) production genotypes via the *iroBCDEN* cassette and displayed mammalian cell autophagy and phagosome escape protein *icsA*/*sopA*. The *iss* gene was identified across all Group D plasmids and the *cvaC* gene (colicin V) was detected in F2_2 and F5_1. The *iss* gene confers increased serum survival, which is important in protecting against phagocytosis. Seventeen plasmids contained no virulence factor genes.

3.4.7 Phage Identification

An unidentifiable yet transposable prophage was detected on three Group D plasmids (F1_1, F2_2 and F4_1) (Supplementary Data 7 and 8). Three *Myoviridiae* phages were observed on three singleton plasmids (F2_1, F5_3 and F9_2). Plasmid F9_2 also contained two individual *Siphoviridiae* prophages. Containment analyses suggested that all prophages were related to *Escherichia–Shigella–Salmonella* (ESS) clade phages (Supplementary Data 9), specifically an stx-converting phage. Further inspection of the unidentifiable phages revealed they contained no structural component genes and consisted almost entirely of transposition elements and regulatory machinery, which may indicate a novel intragenomic phage lifecycle (similar to a permanent lysogenic lifecycle without ever entering into the lytic cycle). The phage on F2_2 contained the macrolide resistance genes *macA* and *macB*. Further inspection of the *Myoviridae* prophages in plasmid F5_3 suggest intact prophages with full lytic enzymatic profiles (presence of holin, endolysin and spanin). The Myoviridae

phage in F5_3 encompasses 84.38 % of the entire plasmid, suggesting that this plasmid may, in fact, be a circular phage genome or a newly described phage-plasmid [22].

3.4.8 Global Plasmid Relatedness

The circularized plasmids were compared with all plasmids in PLSDB v.0.1.7 (n=34 513 plasmids) PLSDB[23]. Most hits were observed to be within *Enterobacteriaceae* (Supplementary Data 10). *E. coli* was the most commonly associated host species, except for plasmid F1_18, where *Salmonella enterica* (n=29) was most prevalent but *E. coli* was next most prevalent (n=26). When metadata was complete, 15 plasmids (encompassing all of Groups A, C and D, and plasmids F5_3 and F9_2) were most commonly associated with plasmids isolated from human samples (Supplementary Data 10). Seven plasmids (encompassing all of Group B and plasmids F1_18 and F9_5) were most often associated with faecal samples from farm floors, five with pig faeces (F1_17, F1_18, F3_6, F4_7, F7_6), and one each with cow faeces (F7_6) and sheep faeces (F9_5). One plasmid was most commonly associated with river samples (F1_3). All plasmids, except plasmid F5_3, were associated with bird samples (Supplementary Data 10). Plasmid F5_3 was only associated with two human samples, a *blaNDM-5* plasmid from *E. coli* (NZ_AP023208.1), isolated from a Japanese patient with no history of foreign travel, and the other from an *Escherichia albertii* isolated in the USA (NZ_CP024288) [24].

The list of countries associated with each mapped plasmid extracted from PLSDB metadata was used to annotate a world atlas to visualize the extent of spread (Fig. 1a-e). In total, 63 countries were represented across all non- Antarctic continents. Each

plasmid was observed in both the USA and Japan. All plasmids except F5_3 were observed in Canada, China and the UK. These data, as with all genomic data, are biased to include only the countries with data and metadata submitted to online databases. Thus, a lack of observation in a specific country does not necessarily equate to a lack of plasmid. We suggest that this is the first sighting of plasmid F5_3 in Europe and the global dissemination of the other plasmids.

3.4.9 Temporal Analysis of Plasmid Character State Evolution

Drug resistance genotypes seem to be temporally linked, with most prominently used antibiotics correlating with resistance genotypes for given years [25]. Plasmids isolated between 1960 and 1979 were observed to confer resistance to aminoglycosides [*ANT(3'')-IIa*, *APH(3'')-Ib*, *APH(3')-Ia* and *APH(3'')-IIa*], β-lactams (*blaOXA-2*, *blaTEM-1*, *blaTEM-150*), phenicols (via *catI*), sulfonamides (*sul1* and *sul2*) and tetracyclines [*tet(A)* and *tet(B)*]. Comparatively, non-OXA-mediated ESBL resistance (*blaCTX-M*) was not observed until 2001, mirroring previous resistance profile reports. The mercury resistance *mer* operon was the most prevalent MRG present across time. Resistance to silver (*sil* operon), to tellurite (*ter* operon) and to QACs were also well represented. Resistance to these heavy metals may also confer secondary oxidative stress mitigation [26].

3.5 Discussion

AMR surveillance to date has predominantly focused on culturable pathogens, monitoring their phenotypic resistance to a range of antimicrobials and the identification of the genes. Plasmids mediate AMR and MDR gene transfer across bacteria, commensals or pathogenic bacteria of humans, animals and the environment [27]. However, due to difficulties in analysing plasmids most genomic studies have focused on genes or whole genome sequences rather than resolving the plasmid sequences. Our study identified a definitive spread of AMR plasmids across One Health as they all mapped to plasmids identified from human, animal or environmental samples. The lowest coverage of the One Health triangle identified was the environment. However, the number of complete plasmids sequenced from environmental samples is relatively low. This is a large gap in our knowledge as there are few data on the AMR plasmid content of water, soil or plant samples.

Our study demonstrates that the same plasmids have already transferred globally across 63 different countries, as demonstrated by the mapping of the plasmids based on the metadata. This study demonstrates that it is not only the AMR genes or the host pathogens moving across One Health but it is entire plasmids. There was no one specific biomarker that could group the isolated plasmids. There were different AMR genes, different replicon types, and variation across metal resistance and virulence factors. Further studies of plasmids with differing resistance profiles can use our template to explore the global and One Health nature of sequenced plasmids. This will provide novel insight into the global nature of every sequenced plasmid in the future.

The plasmids analysed in this study provide novel insight into the AMR, virulence and metal resistance plasmids present. While the AMR phenotype of the transconjugants included resistance to ciprofloxacin and imipenem, no known ARGs were detected. Thus, these plasmids are a reservoir of novel fluoroquinolone and carbapenem resistance genes. Sequenced plasmids contain a large number of uncharacterized hypothetical genes. Genes conferring resistance to a range of antimicrobials were also identified on the same plasmid. Additionally, multiple plasmids conferring resistance to one or two antimicrobials were detected in the same sample. There are many studies of the ESBL profiles and known ARGs conferring resistance in *E. coli* globally across One Health but relatively little information about the plasmids conferring ESBL resistance or other AMR in food animals and their link to those identified in humans, other animals or the environment [2]. Comparison of our plasmid sequences with those globally identified their presence in *E. coli* and other pathogens isolated from humans, other food animals and the environment across time. We identified plasmids lacking AMR, virulence genes or MRGs, which were all colicin E- type plasmids, suggesting the colicin E plasmids were transferred with AMR plasmids. Therefore, the use of antimicrobials may be also selecting colicin plasmids. Colicin has long been known as a plasmid- borne bacteriocin that kills other *E. coli* cells lacking the same plasmid [28].

Metal resistance and virulence genes associated with human extraintestinal pathogenic *E. coli* (ExPEC) and avian pathogenic *E. coli* (APEC) were present in Group D plasmids and sample F2_1 (*astA*). The virulence factors of APEC include *hlyE*, *cvaC*, *iss*, *fimC*, *tsh*, *lucC* and *sitA* [29]. However, there is no definitive list of virulence genes common to APEC and while it is the agent of coliobacilliosis it is also a commensal

of the poultry gut. The plasmids identified contained only *iss*, *sitA* and *cvaC* (n=2), from the APEC virulence genes. Both *iss* and *cvaC* are important virulence factors in neonatal meningitis *E. coli* (NMEC), which are not present in uropathogenic *E. coli* but are present in APEC. However, they contained the core ExPEC genes present in NMEC: *iroBCDEN*, *icsA*/*sopA*, *sitABCD* and *hlyF* (n=4), one contained *iucABCD* and *iutA*, but none contained *ompT* nor *bor*. While the virulence factors do not definitively describe the plasmid as conferring virulence in a specific ExPEC, the genes present display the potential to confer virulence to hosts including poultry and humans.

Temporal data sampling was strongly biased towards the past decade, probably due to falling sequencing costs allowing for more frequent analyses, and strongly biased towards *Enterobacteriaceae*, specifically the genera *Escherichia*, *Salmonella* and *Shigella*. A general trend was observed across all plasmid groups whereby different resistance genes and virulence factors were transiently incorporated and lost over time, suggesting differential evolutionary pressures; however, as the plasmid network is non- transitively retained, these results suggest a highly successful, yet malleable and dynamic, plasmid backbone (Supplementary Data 13–20). Due to the aforementioned bias, the diversity and malleability of these plasmids is most noticeable is samples isolated in the past decade. Despite the global persistence of most plasmid groups, neither resistance nor virulent genotypes seem to be largely geographically enriched.

Three of four plasmid groups (Groups A, C and D) were associated with clinical isolates, and Group B was most commonly associated with agriculture, although all plasmids (except F5_3) were also associated with food products. We detected β-lactam and tetracycline resistance across all samples. These are commonly observed in

agricultural isolates so this result is consistent with current knowledge [30]. The presence of Ig domain-like genes within the F9_2 *Siphoviridae* phage suggests an important role in virulence. These proteins function as invasins and adhesins in both enteropathogenic *E. coli* (EPEC) and enterohaemorrhagic *E. coli* (EHEC) promoting the development of diarrhoeal disease [31].

3.6 Conclusions

Our study provides novel insights into the global nature of resistance, virulence and colicin plasmids across the food chain and time. We have provided compelling evidence of the globetrotting nature of AMR plasmids and the need for surveillance to sequence plasmids using our template of analyses for others to expand these data. The AMR plasmids analysed may contain novel ARGs, which remain to be characterized. The analysis of AMR needs to include the investigation of plasmids globally to truly identify the risk to animal, human and environmental health from AMR and potential for co-selection by the use of QACs or metals.

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Conflicts of Interest: The authors declare no conflicts of interest.

Ethical Statement: The birds were raised and fed under typical commercial production conditions, receiving feed and water ad libitum. Animals were euthanized in accordance with humane killing protocols as set forth in European Union Council Regulation (EC) 1099/2009.

3.7 Tables

Table 1: Antimicrobial resistance profiles and number of AMR genes within each ESBL-positive transconjugants.

AMP, ampicillin; CIP, ciprofloxacin; CN, gentamicin; CTX, cefotaxime; I, intermediate; IMP, imipenem; KAN, kanamycin; QACs, quaternary ammonium compounds; R, resistant; S, susceptible; according to CLSI guidelines (2018).; TET, tetracycline; W, trimethoprim.

Sample	AMR Profile	Aminoglycosides	Bacitricin	Beta-lactamases	Lincosamides	QACs	Sulphonamides	Tetracyclines	Trimethoprim	Total
1	AMP, CTX, TET	2	0	6	0	0	0	3	0	11
2	AMP, CTX, TET	0	0	1	2	1	1	2	0	7
3	AMP, CTX, KAN, CN, TET	0	2	3	0	0	0	2	2	9
4	AMP, CTX, TET, W	2	0	3	0	0	0	2	0	7
5	AMP, CTX, TET, CIP	3	0	1	0	2	1	1	1	9

Sample	AMR Profile	Aminoglycosides	Bacitricin	Beta-lactamases	Lincosamides	QACs	Sulphonamides	Tetracyclines	Trimethoprim	Total
6	AMP, CTX, TET	0	2	2	0	0	0	0	0	4
7	AMP, CTX, TET	2	0	4	0	0	0	2	0	8
8	AMP, CTX, KAN, TET, W, IMP	0	2	2	0	0	0	0	0	4
9	AMP, CTX, KAN, TET, CIP, W	3	0	5	0	0	2	3	3	16

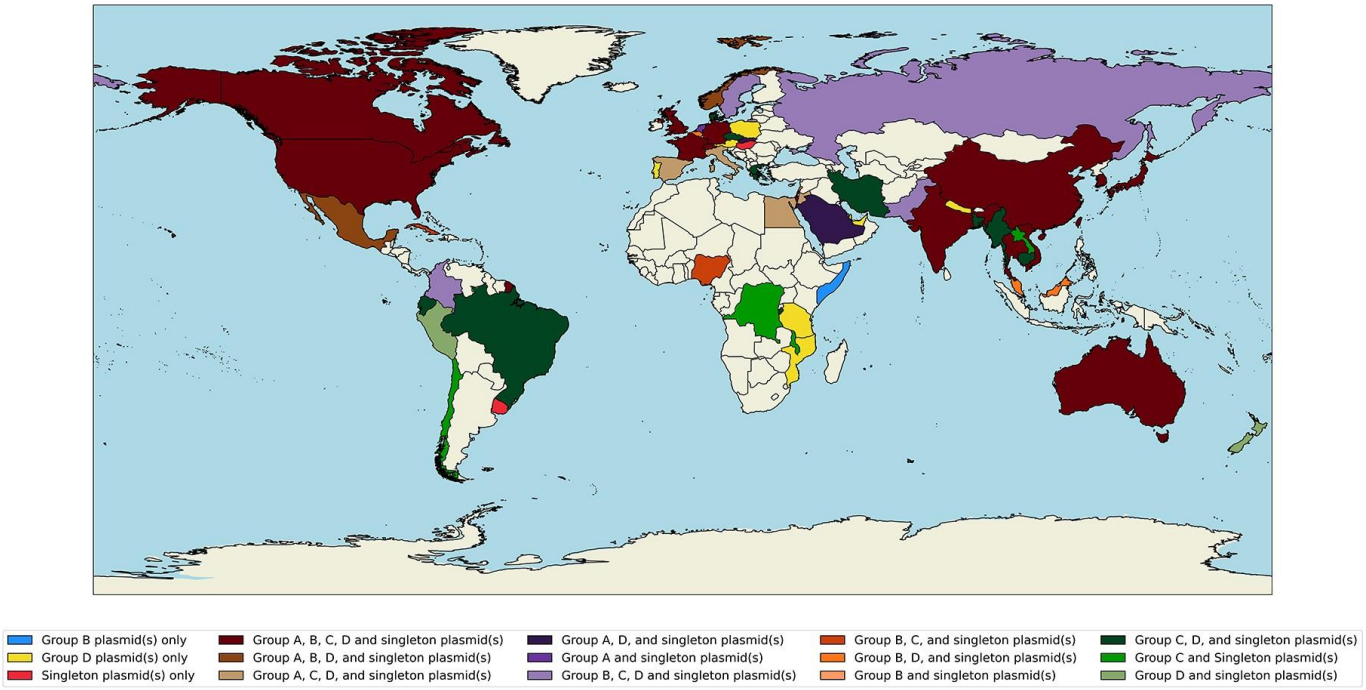
Table 2: Clustering groups, replicon types and ARGs detected in each circularized plasmid.

Plasmid ID	Cluster ID	Replicon Type	Total number of ARGs	*ANT(3'')-IIa*	*APH(3'')-Ib*	*APH(6)-Id*	*SHV-2*	*TEM-1*	*dfrA1*	*dfrA8*	*lnuG*	*sul1*	*tet(A)*	*tet(B)*	*dfrD*
F1_2	A	IncB/O/K/Z_2	1	.	.	.	Y	.	.	.	.	.	.	.	.
F4_2	A	IncB/O/K/Z_2	1	.	.	.	Y	.	.	.	.	.	.	.	.
F6_1	A	IncB/O/K/Z_2	1	.	.	.	Y	.	.	.	.	.	.	.	.
F7_2	A	IncB/O/K/Z_2	1	.	.	.	Y	.	.	.	.	.	.	.	Y
F9_3	A	IncB/O/K/Z_2	1	.	.	.	Y	.	.	.	.	.	.	.	.
F1_17	B	Col440II_1 and ColRNAI_1	0	.	.	.	.	.	.	.	.	.	.	.	.
F4_7	B	Col440II_1 and ColRNAI_1	0	.	.	.	.	.	.	.	.	.	.	.	.
F7_6	B	Col440II_1 and ColRNAI_1	0	.	.	.	.	.	.	.	.	.	.	.	.
F9_5	B	Col440II_1 and ColRNAI_1	0	.	.	.	.	.	.	.	.	.	.	.	.
F3_4	C	IncN_1	2	.	.	.	.	Y	.	.	.	.	Y	.	.
F5_2	C	IncN_1	2	.	.	.	.	Y	.	.	.	.	Y	.	.
F8_22	C	IncN_1	2	.	.	.	Y	.	.	.	.	.	Y	.	.
F1_1	D	IncFIB_1	4	.	Y	Y	.	Y	.	.	.	.	.	Y	.
F2_2	D	IncFIA_1, IncFIB_1, IncFIC(FII)_1	1	.	.	.	.	Y	.	.	.	.	.	.	.
F4_1	D	IncFIB_1	4	.	Y	Y	.	Y	.	.	.	.	.	Y	.
F5_1	D	IncFIB_1	7	Y	Y	Y	.	Y	Y	.	.	Y	Y	.	.

Plasmid ID	Cluster ID	Replicon Type	Total number of ARGs	*ANT(3'')-IIa*	*APH(3'')-Ib*	*APH(6)-Id*	*SHV-2*	*TEM-1*	*dfrA1*	*dfrA8*	*lnuG*	*sul1*	*tet(A)*	*tet(B)*	*dfrD*
F9_1	D	IncFIB_1	4	.	Y	Y	.	Y	.	.	.	.	.	Y	.
F1_18	Singleton	Col440II_1	0	.	.	.	.	.	.	.	.	.	.	.	.
F1_3	Singleton	IncFII(pECLA)_1, IncI1_1_Alpha	1	.	.	.	.	Y	.	.	.	.	.	.	.
F2_1	Singleton	IncFIA(HI1)_1_HI1, IncHI1A_1, IncHI1B(R27)_1_R27	4	Y	.	.	.	.	.	.	Y	Y	.	Y	.
F3_6	Singleton	IncX1_1, IncX3_1	2	.	.	.	.	Y	.	Y	.	.	.	.	.
F5_3	Singleton	None	0	.	.	.	.	.	.	.	.	.	.	.	.
F9_2	Singleton	IncFIB(pHCM2)_1_pHCM2	0	.	.	.	.	.	.	.	.	.	.	.	.

3.8 Figures

Figure 1: Global distribution world map of countries containing a plasmid from PLSDB associated with each plasmid extracted from the chicken samples.


Group B plasmid(s) only
Group D plasmid(s) only
Singleton plasmid(s) only
Group A, B, C, D and singleton plasmid(s)
Group A, B, D, and singleton plasmid(s)
Group A, C, D, and singleton plasmid(s)
Group A, D, and singleton plasmid(s)
Group A and singleton plasmid(s)
Group B, C, D and singleton plasmid(s)
Group B, C, and singleton plasmid(s)
Group B, D, and singleton plasmid(s)
Group B and singleton plasmid(s)
Group C, D, and singleton plasmid(s)
Group C and Singleton plasmid(s)
Group D and singleton plasmid(s)

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Chapter 4

Comparison of soil and grass microbiomes and resistomes reveals grass as a greater antimicrobial resistance reservoir than soil

Thi Thuy Do [a l], Cian Smyth [a], Fiona Crispie [b], Catherine Burgess [c], Fiona Brennan [d], Fiona Walsh [a e].

[a]Antimicrobial Resistance & Microbiome Research Group, Department of Biology, Maynooth University, Maynooth, Co. Kildare, Ireland

[b]Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork P61 C996, Ireland

[c]Teagasc Food Research Centre, Ashtown, Dublin D15 KN3K, Ireland

[d]Teagasc, Crops, Environment and Land-Use Programme, Johnstown Castle, Co. Wexford Y35 Y521, Ireland

[e]Kathleen Lonsdale Institute for Human Health Research, Maynooth University, Maynooth, Co. Kildare, Ireland

*Correspondence: fiona.walsh@mu.ie

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4.1 Abstract

Grasslands cover a large proportion of global agricultural landmass used to feed herbivores and ruminants and link the environment to the food chain via animals onto humans. However, most scientific studies of antimicrobial resistance and microbiomes at the environmental – animal nexus have focused on soil or vegetables rather than grasslands. Based on previous microbiome phyllosphere-soil studies we hypothesised that the microbiome and resistomes across soil and grass would have a core of shared taxa and antimicrobial resistance genes (ARGs), but that in addition each would also have a minority of unique signatures. Our data indicated grass contained a wider variety and higher relative abundance of ARGs and mobile genetic elements (MGEs) than soil with or without slurry amendments. The microbiomes of soil and grass were similar in content but varied in the composition proportionality. While there were commonalities across many of the ARGs present in soil and on grass their correlations with MGEs and bacteria differed, suggesting a source other than soil is also relevant for the resistome of grass. The variations in the relative abundances of ARGs in soil and on grass also indicated that either the MGEs or the bacteria carrying the ARGs comprised a higher relative abundance on grass than in soil. We conclude that while soil may be a source of some of these genes it cannot be the source for all ARGs and MGEs. Our data identifies grass as a more diverse and abundant reservoir of ARGs and MGEs in the environment than soil, which is significant to human and animal health when viewed in the context of grazing food animals.

4.2 Introduction

The United Nations declaration on antimicrobial resistance (AMR), which all 193 member states signed and the WHO declaration of priority pathogens, highlights the importance of the AMR problem [1,2]. Each year, AMR results in approximately 1 million disability-adjusted life years (DALYs) lost in EU/EEA countries [3]. Antimicrobial resistant bacteria and genes can be transferred from the environment to food animals and via the food chain to humans either in or as food-borne pathogens or commensals. Many different classes of antimicrobials currently used to treat infections in humans and animals were discovered and developed between the 1940s and 1980s. However, since 1990 only three novel classes of antimicrobials have been launched: pleuromutilins, lipoglycopeptides, and oxazolidinones. These new classes have limited or no activity against the gram-negative pathogens such as *Escherichia coli* or *Klebsiella pneumoniae*, which are on the WHO's priority list [4]. Therefore, we need to preserve our current arsenal of antimicrobials. One mode of action is to limit the transfer of AMR genes and bacteria from the environment to animals and humans via the food chain. However, we need to understand the resistome and microbiome of the animal food source before we can limit the transfer.

The phyllosphere (aerial surface of plants) is estimated to cover over 10^9 km^2 and contain 10^{26} bacterial cells, making it one of the largest microbial habitats on earth [5]. In Europe, grasslands covering more than a third of the European agricultural area are used to feed herbivores and ruminants and provide important ecosystem services [6]. Grass in the field is in a constant interactive relationship with the soil in its rhizosphere. The soil likewise is within a constant relationship with the grass.

Therefore, neither exists in isolation but in a symbiotic relationship. However, they are rarely studied together. The microbiome and resistome on grasslands are rarely studied [7,8]. To ensure sustainable agriculture and healthy ecosystems we need to understand the grassland microbiomes and resistomes and how these (both soil and grass) change with the addition of slurry [9,10]. Studies to date analysing the impact of manure on the microbiome and resistome of grassland have focused solely on the soil and have not included the grass [11–15].

Overlaps of microbiomes and resistomes have been detected in the plant-soil ecosystem, suggesting the possibility of dissemination of microbes and antimicrobial resistance genes (ARGs) between soil and plants [16]. Fresh produce contaminated with enteric pathogens have been frequently reported to originate from environmental sources with wild animals or agricultural activities [17]. The microbiome of soil has been found to overlap with those in the leaves and flowers of grape vines and between lettuce roots and soil amended with poultry litter [18,19]. However, the provenance of phyllosphere microorganisms is not yet established [8]. A recent study estimated that at least 25 % of the *Arabidopsis thaliana* phyllosphere bacteria reached the phyllosphere from the soil [8]. These bacteria represented 40 % of the bacterial taxa detected. However, the microbiomes did not converge between soil and leaves/flowers where the phyllosphere was not in contact with the soil. In contrast a study of two perennial grasses (switchgrass and miscanthus) identified soil as a major reservoir of leaf microorganisms [7]. These studies however did not investigate the shared resistomes across soil and phyllosphere. Previous phyllosphere studies investigated either the microbiome or targeted antimicrobial resistant bacteria or genes on vegetables [19]. Based on previous phyllosphere-soil microbiome studies we hypothesised that the

microbiome and resistomes of soil under the grass and grass would have a core of shared taxa and resistance genes, but that in addition each would also have a minority of unique signatures. We introduced pig slurry treated and compared with untreated to understand if the application of the pig slurries would alter the common microbiome and resistome of the soil and grass or the individual elements of each in the short term. This is a unique study, as previous studies have focused on soil and not included grass in relation to resistomes, or have compared only microbiomes across soil and phyllosphere.

4.3 Materials and Methods

4.3.1 Field side, plot preparation, and slurry application

The field trial was conducted from August to October 2019 in Teagasc Research Facilities in Johnstown Castle, Wexford, Co. Wexford, Ireland. No farm animals were present on this land for the seven months prior to the field trial. The pig slurry samples were described previously (19). Briefly, pig slurry was collected from an Irish pig farm in agreement with the farm owner. The slurry was treated with three methods, storage for 4 months, compost for 8 weeks, and AD for 90 days. Products of slurry treatments and the fresh collected raw slurry were spread on field plots based on the phosphorous content following the EU regulations. One hundred 1 m wide x 1 m long plots were randomly established in the field for control without slurry application and slurry application. Each slurry type was spread on 20 plots, which were designed for sample collection for 5 timepoints with 4 replicates each.

4.3.2 Soil and Grass Sampling

Soil cores were collected from four random plots on the field prior to the slurry application for the soil background analysis (T-1). A standard agronomic corer was used to collect 10 cores at the depth of 10 cm along a W-shaped path. These cores were mixed well in a clean plastic sampling bag to make a composite sample.

During the field trial, soil cores were collected from control (no slurry applied) (4 plots) and slurry applied plots (4 plots per treatment) fortnightly for the first 3

timepoints, and at 2.5 months post-T0 for the last time points. Grass samples were randomly collected in the field before trimming to 5 cm for slurry spreading (T-1). During the field trial, grass was harvested to get approximately 200 g from control (no slurry applied) (4 plots) and slurry applied plots (4 plots per treatment) fortnightly for the first 3 timepoints, and at 2.5 months post-T0 for the last time points.

Soil and grass samples were transferred to the laboratory immediately and were processed within 24 h. Soil samples were stored at −80 °C for further molecular analysis. Grass samples were rinsed with PBS buffer (Oxoid) as previously described for leaf washes [20] The resulting buffer was used for microbial testing and to extract microbial DNA for further molecular analysis.

4.3.3 DNA Extraction

Total DNA was extracted from 0.25 g of each soil sample replicate using the DNeasy PowerSoil Kit (Qiagen). The PBS washes of 50 g of each grass sample replicate were centrifuged at 3000g for 15 min. The resulting pellets were used for DNA extractions using the DNeasy PowerSoil kit (Qiagen). The quality and quantity of extracted DNA were examined using a DeNovix DS-11 spectrophotometer and Invitrogen Qubit Fluorometer (dsDNA high-sensitivity assay kit) (Waltham, MA). DNA was extracted in triplicate from each sample and extracts were pooled to obtain a single DNA sample per experimental unit at each time point.

4.3.4 Metagenomic Sequencing and HT-qPCR arrays

Extracted DNA from soil and grass samples were prepared using Illumina TruSeq DNA library preparation kits before sequencing on the Illumina NextSeq 500 platform (paired-end, 2 × 150 bp sequencing) in Teagasc Next Generation DNA Sequencing Facility. DNA samples were also used for HT-qPCR arrays. The HT-qPCR arrays were performed using the SmartChip™ Real-Time PCR system (TakaraBio, CA, USA) by Resistomap Oy (Helsinki, Finland). The mix of DNA samples with primer sets and the qPCR reagents were loaded in each 100 uL reaction well of the SmartChip™ with 5182 wells. A primer set of 216 pairs of primers targeted 186 ARGs conferring resistance to major antibiotic classes, 6 integrons, 22 MGEs, and total bacterial genes 16S rRNA was used in the qPCR array. The melting curves and Ct values were analysed using default parameters of the SmartChip™ qPCR software. The qPCR was conducted in three technical replicates for each DNA sample.

4.3.5 Data Analyses

4.3.5.1 Metagenomics

The adapter sequences were trimmed from shotgun sequencing raw reads in the fastq format using Cutadaprt (V2.10). We used Sickle (v1.33) with the minimum window of quality score of 20 to remove the low-quality reads with the length <20 bp. The FastQC (v 0.11.9) was used to examine the quality of filtered reads before assembly with Megahit (v1.2.6, −-kmin-1pass –presets meta-large). The assembled contigs were subjected to Kaiju taxonomic classifier (v1.2.6, parameters: –kmin-1pass –presets

meta-large) to assign the taxonomy profile for each sample. The microbial communities were analysed in the MicrobiomeAnalyst online platform [21].

The microbial genome annotation on filtered reads was carried out using Prokka (v 1.14.6, default settings) [22]. The protein FASTA files resulted from the Prokka software were used to identify the KEGG Orthologs (KOs) by Kofamscan (v 1.3.0) [23]. The KEGG pathways were assigned by MinPath software (v 1.5) based on the Ko's lists [24]. Data were analysed and visualised using Calypso online [25].

4.3.5.2 qPCR

The qPCR data of the samples were filtered based on the following criteria: (1) a gene was detected in at least two technical replicates; (2) the Ct values ≤27; and (3) the amplification efficiency was in the range of (1.8–2.2). The relative gene copy number was calculated in Eq. (1) in the work of Chen et al., 2016 [10]. The gene relative abundance was identified by dividing the relative copy numbers by the 16S rRNA gene copy number. The data was then visualised in the MicrobiomeAnalyst online platform [21]

Eq. (1) - Relative gene copy number = $10^{(27 - Ct)/(10/3)}$

4.3.5.3 Correlation Analysis

The interaction between (1) ARGs and MGEs; and (2) microbial communities and ARGs were analysed through Spearman's correlation analysis with the SciPy package (Virtanen et al., 2020). The correlation between ARGs and MGEs was considered strong and significant when Spearman's rank value $|r| > 0.85$ and $p < 0.05$ and $|r| > 0.5$ and $p < 0.05$ between microbial communities and ARGs. The Cytoscape software (v3.8.2) was implemented to build the network based on strong and significant Spearman's correlations [26]. The ARG and MGE genes showed high interaction between genes, so we set the threshold $|r| > 0.85$ to build a network with appropriate nodes and edges to manage these properly. However, the ARGs vs Phyla did not build a formative network if |r| was set >0.85 (network with very few nodes and edges). Therefore, the correlation network in soil was formed based on Spearman's correlation $r > 0.5$ and the network in grass based on Spearman's correlation $r > 0.6$. Higher ARG, MGE and phyla abundance were detected in grass samples than in soil, thus bigger, more interactive networks were built in grass than soil. The correlation values are Spearman's rank correlation coefficient (Spearman's correlation: R, rho). These values measure the interaction/association (strength and direction of association) between two variables on at least an ordinal scale. The interaction/association between two variables can be positive (increase together) or negative (one increase, another decrease) in the range $[-1,1]$. The interaction/association is stronger when the absolute value |r| is higher, close to 1.For the current research, an absolute value of the correlation coefficient ≥ 0.3 was estimated as the appropriate threshold. In our case, depending on the interaction, we set the strong correlation at different coefficient values.

4.4 Results

4.4.1 Soil and Grass Microbiomes

We detected 6944 OTUs in soil and 6774 OTUs in grass. Almost all genera present on grass (98.7 %) were also detected in the soil microbiomes. However, the relative abundances of the genera varied across grass and soil.

4.4.2 Microbial Composition of Soil and grass over Time and Slurry Treatments

After quality trimming and assembling, metagenomic sequencing reads were assigned to Bacteria, Archaea, Virus and other unclassified organisms with Kaiju (Dataset S1). The most abundant phyla and genera were assigned and compared (Figs. S1, S2).

4.4.3 Alpha Diversity: Chao1 and Shannon PCoA of Soil vs Grass

The alpha diversities of the microbiomes were compared using the Chao 1 (richness: number of taxonomic groups) and Shannon indexes (evenness: distribution of abundances of the groups) (Fig. 1). Krustall-Wallis was used to determine statistical significance across all samples. Chao 1 richness values varied but were not significantly different across all samples ($p = 0.22249$). The Shannon indexes of evenness levels were significantly higher in soil samples compared with those in grass samples ($p = 0.00032885$). The composition of the microbiomes was also analysed through principal coordinate analysis (PCoA) based on Bray-Curtis dissimilarity (Fig. 2, PERMANOVA test, $p < 0.001$). The soil control (without slurry application) and

soil treatment (with slurry application) formed absolute overlapping clusters. The grass control and treatment samples also overlapped with each other. However, the soil and grass clusters were completely separated with no overlap.

Grass and soil samples were divided into 5 groups: Control (Soil-C and Grass-C) contained soil and grass samples collected from field plots without pig slurry application; Storage (Soil-St and Grass-St): samples collected from field plots with application of pig slurry product of storage treatment, Fresh (Soil-F and Grass-F) samples collected from field plots with application of raw pig slurry without treatment, Compost (Soil-Cp and Grass-Cp) samples collected from field plots with application of pig slurry compost, and AD (Soil-AD and Grass-AD) samples collected from filed plots with application of AD digestate of pig slurry.

Soil and Grass controls contained soil and grass samples collected from the field plots without pig slurry application. Soil and Grass treatments contained soil and grass samples collected from the field plots where different treated products of pig slurry (storage, compost, and AD) were spread onto.

4.4.4 Beta-diversity

The composition of the microbial communities was assessed at the phylum level for all samples (Fig. S1). The top three most abundant phyla across the soil and grass sample groups with and without slurry were consistent in taxa but varied in relative abundances across soil and grass; the phyla Proteobacteria and Actinobacteria were dominant in all samples. However, the abundance profiles of the remaining phyla

differed. Soil samples were represented by the two most abundant phyla: Proteobacteria and Actinobacteria, while other phyla in the top ten were observed at lower (<6 %) relative abundances. Grass samples were characterised mainly by 3 most abundant phyla: Proteobacteria, Actinobacteria, and Bacteroidetes, other phyla were found at very low abundance levels (<2 %) (Dataset S2). The main phyla on grass were the same as on *Galium album* [27] and perennial grasses switchgrass (*Panicum virgatum L.*) and miscanthus (*Miscanthus* x *giganteus*): Proteobacteria and Bacteroidetes [7]. These also agree with several other phyllosphere communities [5,28–32]. The soil phyla were also similar to those previously determined [33,34]. The application of different treated slurry did not impact the bacterial compositions of soil or grass.

The relative abundance profiles at genus level were highly similar between slurry treated and untreated samples in both soil and grass across the timepoints (Fig. S2) . The abundance profile was notably different between soil and grass samples. In contrast to the abundance order at the phylum level, soil samples were predominated by the genus Bradyrhizobium, while other genera were detected at a very low relative abundance level. Among grass samples, the two most abundant genera were Pseudomonas and Sphingomonas, other genera in the top ten were found at the lower relative abundances but at values greater than in soil. The predominance of Pseudomonas and Sphingomonas was consistent with findings of other phyllosphere microbiomes [5,7,28–32]

4.4.5 Characteristics of the Resitomes and Mobile Genetic Elements (MGEs) of Soil and Grass over Time and under Slurry Treatments

A total of 140 different ARGs and 24 different MGEs were detected across all samples (Dataset S3). The detected ARGs were divided into 11 main antibiotic classes to which they conferred resistance: aminoglycoside, beta-lactam, multi-drug resistance (MDR), macrolide-lincosamide-streptogramin B (MLSB), colistin, phenicol, quinolone, sulfonamides, tetracycline, trimethoprim, and vancomycin. Four MGE groups were identified comprising integrons, transposons, insertional sequences, and plasmid-associated genes. In total 116 genes (ARG and MGE) were detected across the soil and within at least one soil sample and 158 genes across the grass and within at least one grass sample. The relative abundance of total ARGs in grass samples was consistently higher in all samples than in soil samples (Fig. S3). Soil at time 0 prior to the application of composted slurry had an unusually high ARG relative abundance, not consistent across the other soil samples. All ARGs and MGEs present in soil were detected in grass samples. Those absent from soil but present in grass comprised aminoglycoside, beta-lactam, carbapenem, chloramphenicol, quinolone, tetracycline, and vancomycin resistance genes, in addition several MGEs were detected only in grass. As grass contained a wider variety and higher relative abundance of ARGs and MGEs we conclude that while soil may be a source of some of these genes it cannot be the source for all ARGs and MGEs as some are absent from the soil.

Clinically important plasmid mediated resistance genes detected included several *blaCTX-M* genes (in soil and grass), carbapenem resistance genes (*blaNDM, blaIMP, blaVIM* and *blaOXA-48* in both soil and grass, and *blaKPC, blaOXA-51* in grass), colistin resistance genes *mcr1* in soil and grass and *mcr4* in one grass sample type and the quinolone resistance genes *qepA* in all soil and grass and *qnrB* in all grass samples. At the threshold of over 20 % prevalence and over 0.01 relative abundance, 13 genes were found in core resistome shared across all soil and grass samples (Fig. S4). Among them, only three genes (*tetG, qepA,* and intI1_1) were found at 100 % prevalence in all samples. The five most abundant ARGs conferred resistance to aminoglycosides, beta-lactams, quinolones, tetracyclines, or vancomycin. The total relative abundances of the detected ARGs in soil and grass samples did not significantly increase due to slurry application (Fig. S3). The highest relative abundance within the grass samples, but not the soil samples occurred at timepoint 4 (ten weeks following slurry application). The control samples also contained this increase, suggesting a factor other than slurry is the contributing factor(s).

The alpha diversities of the resistomes and the MGEs were compared separately using the Chao 1 and Shannon indexes (Fig. 3). Krustall-Wallis was used to determine statistical significance across all samples. Richness values for ARGs and MGEs were significantly different across all samples ($p = 3.0132e-05$ and 9.7868e-05, respectively), with higher values for the grass samples than the soil. The evenness levels for ARGs but not MGEs were significantly higher in grass samples compared with those in soil samples ($p = 4.6731e-05$). The composition of resistomes and MGEs was analysed through principal coordinate analysis (PCoA) based on Bray-Curtis dissimilarity (Fig. S5, PERMANOVA test, $p < 0.001$). The ARGs and MGEs detected

in control soils (without slurry application) and treated (with slurry application) formed overlapping clusters. The ARGs and MGEs grass control and treatment samples also overlapped with each other. This indicates a similar resistance gene profile in soil with/without treatments, as well as in grass with/without treatment. The majority of soil clusters were separated from grass clusters for ARGs with overlaps in four grass treated samples and five soil treated samples (total sample n = 50). Further separation and overlap (n = 15 soil with 15 grass samples) was observed in relation to the MGEs across soil and grass samples.

The MGE alpha diversity (c and d). Grass sample groups have a significantly higher richness (c) (Chao 1) of MGE than soil groups. The evenness (d) is also higher in grass compared with soil; however, the differences are not significant.

Grass and soil samples were divided into 5 groups: Control (Soil-C and Grass-C) contained soil and grass samples collected from field plots without pig slurry application; Storage (Soil-St and Grass-St): samples collected from field plots with application of pig slurry product of storage treatment, Fresh (Soil-F and Grass-F) samples collected from field plots with application of raw pig slurry without treatment, Compost (Soil-Cp and Grass-Cp) samples collected from field plots with application of pig slurry compost, and AD (Soil-AD and Grass-AD) samples collected from filed plots with application of AD digestate of pig slurry.

4.4.6 Network Analysis

4.4.6.1 Association between ARG and MGEs

The network analysis based on strong and significant Spearman's correlations ($r > 0.85$ and $p < 0.05$) between ARGs and MGEs was employed to understand the co-occurrence of ARGs and MGEs across all the grass and soil samples (Fig. 4, Dataset S4). There were only positive interactions found (i.e. no negative correlations) when analysing the network between ARGs and MGEs in soil and grass samples. These results indicate the co-location or co-association of these ARGs with MGEs and the potential for dissemination of these genes via horizontal gene transfer within soil and grass microbial communities. The larger the size of the nodes the greater the degree of the interactions. Within the soil networks six different clusters were identified comprising one large and five smaller clusters. The ARGs of greatest clinical significance were contained within the large cluster with the MGEs (predominantly repA, IncP, IncW, tnpA, orf37-IS26) acting as the central linking components. The grass networks formed two clusters, one large and one small. Similarly, to the soil the MGEs (repA, IncW, IncN, tnpA, IncF, IncQ and int1) act as the main linkers of the ARGs, but some of the MGEs differed from the soil (repA, IncW, tnpA). While some ARGs were present across all samples they were not always clustered with the same MGE e.g., qepA correlated with intI3_2, IncW_trwAB, IncN_rep, tnpA_3, IncQ_oriT, intI1_4 and repA in the grass and IS1111, repA, tnpA_3 and orf37-IS26 in the soil samples. Thus, while the ARG was detected across all samples it may be moving either on the same MGEs (repA, tnpA) or on different MGEs across the samples, indicating

multiple potential modes of mobility for the same genes within and across different samples.

4.4.6.2 Correlation analysis between microbial taxa and ARGs

The relationship and interaction between microbial phyla and ARGs were investigated in the network based on Spearman's correlation analysis (Fig. 4B). The network in soil comprised 44 nodes (from 14 microbial phyla and 30 ARGs), and 42 edges (built from 14 negative and 28 positive correlations). A positive correlation indicates the presence of both items together, a negative correlation indicates the presence of one item but the absence of the other. The network in grass consisted of 70 nodes (27 phyla and 43 ARGs), and 179 edges (built from 15 negative and 164 positive correlations). Thus, the soil networks were simpler than the grass networks. In soil Fusobacteria and Proteobacteria had the most positive interactions with ARGs. These results indicated their role as primary ARG hosts. Actinobacteria had positive correlations with tetM and negative interactions with blaKPC_2. These phyla did not produce the same results in the grass samples. The grass networks were more complex and included many more ARGs and phyla interactions, most of which were positive. In contrast to soil, Proteobacteria were relatively low in interactions with ARGs, their only interaction with ARGs was a negative interaction with dfrA12. The relative abundances of Proteobacteria in soil and grass samples was not significantly different (Fig. S1). The main grass phyla with ARG networks were Spirochaetes and Aquificae, neither of which were correlated with ARGs in the pig slurry (Do et al., 2022). Therefore, the slurry was not the source of these bacteria.

ARG and MGE network based on the interaction of ARGs and MGEs, (a) in soil samples, (b) in grass samples. There were only positive interactions found when analysing the network between ARGs and MEGs in soil and grass samples with Spearman's correlation $r > 0.85$ and $p < 0.05$. These results indicate the location of these genes on MGEs →the dissemination of these genes via HGT within soil and grass microbial communities.

ARGs and microbial phyla network built on the interactions between microbial phyla and ARGs in soil (c) and grass (d) samples. Network in soil was formed based on Spearman's correlation $r > 0.5$, $p < 0.05$. Network in grass, Spearman's correlation $r > 0.6$, $p < 0.05$. Red edges: positive interaction, blue edges: negative interactions.

4.4 Discussion

While studies have suggested that soil is an important source of bacterial transfer to the phyllosphere [7,8,18,28,32,35–37], no studies have analysed the commonality of the total resistome between soil and the phyllosphere and we have yet to identify any studies that have analysed the total resistome of grass. This study showed that the grass phyllosphere contained both a wider array of ARGs and MGEs and a larger relative abundance of these genes than the soil. Soil is a recognised and well-studied reservoir and source of a wide range and relative abundance of ARGs [38–40]. While the classes of antimicrobials to which the ARGs conferred resistance were present both on grass and in soil there were unique many ARGs only present in the grass samples. There was also significantly higher evenness and richness in the grass resistomes than the soil resistomes. The grass phyllosphere contained ARGs and MGEs not detected in soil e.g. qnrB and IncF, respectively, and multiple versions of the same ARG types e.g. blaCTX-M 1–6 in grass relative to blaCTX-M 1–4 in soil. Network analysis identified that where the same ARGs and MGE genes were detected in soil and grass samples the correlation between these ARGs and MGEs did not occur in both grass and soil. Thus, while some ARGs were present across grass and soil the specific mobile elements capable of moving the ARGs between different bacteria were significantly different. These data suggests that the ARGs either did not move from soil or that they moved into new MGEs once present on grass. The former is more likely due to the number of movements required across the wide range of ARGs. In addition, the correlation between ARGs or MGEs and bacteria were different in soil and grass, indicating that the common ARGs and MGEs either moved from the bacteria in soil to different bacteria when on grass or that the common ARGs and MGEs were

mobilised by bacteria from sources other than soil. Our data indicates that while there are commonalities across many of the ARGs present in soil and on grass their modes of movement, correlations with MGEs and bacteria differ, suggesting a source other than soil is also relevant for the resistome of grass. The variations in the relative abundances of ARGs in soil and on grass also indicate that either the MGEs or the bacteria carrying the ARGs comprise a higher relative abundance on grass than in soil.

Our data identifies grass as a more diverse and more abundant reservoir of ARGs and MGEs in the environment than soil. As the microbiome of the grass samples was consistent over time and with other studies we suggest that these resistome findings may also be representative of grass globally [7,27,41–44] but at least the resistome is not due to a unique grass microbiome. However, this requires further verification. In Europe, grasslands covering more than a third of the European agricultural area are used to feed herbivores and ruminants. Should our findings be reproduced across grasslands then this represents a very large reservoir of ARGs that is connected directly to the food chain.

Our study has identified commonalities across the phyla of soil and grass, which were not significantly impacted by slurry. A number of studies have demonstrated that slurry-derived ARB and their ARGs may persist in soil for a few weeks and up to years and that these ARGs can be horizontally transferred into native soil bacteria [12,13,45–51]. Previous studies have examined the impact of slurry application on the levels of ARGs in the phyllosphere of leafy vegetables [52,53], or changes in ARGs abundance and dissemination [54–56]. Mobile genetic elements such as intI1 and genes encoding transposase have been detected in leaf endophytes, as well as in the phyllosphere of

lettuce [57,58], maize [52], Brassica chinensis L [59], and Coriandrum sativum L [60] Slurry-amended soils have been associated with increased detection of ARB and ARGs on lettuce and root vegetables; however, this has not been associated with all crops or ARGs [54,57,61]. Our results agree with the latter studies, although many ARGs were detected. The results contrast with our own previous studies on soil and lettuce [62]. This raises the question of whether this is unique to grass rhizosphere and phyllosphere.

The microbiome compositions, the evenness and the PCoA were significantly different between soil and grass. This finding is in agreement with Vorholt et al., [32] who found that the communities on leaves not in contact with the soil did not converge with the soil communities. However, other environmental factors (but not slurry) may have contributed to these significant differences. Few studies have compared the microbiomes of soil and grass phyllosphere. Yan et al., 2020 compared soil and grass across Australian urban and national park environments, but not agricultural use [63] They identified an overlap of 87.6 % in the total genera identified in the grass phyllosphere with the soil genera. Our study identified a 98.7 % overlap in genera across grass and soil. The Shannon index and the PCoA of Yan et al., demonstrated statistically significant differences between both, which agrees with the data in this study [63] While there were overlaps in the genera detected across grass and soil in this study the relative abundances varied considerably. This suggests that while genera are shared across soil and grass, the phyllosphere provides a selective environment for the relative abundances of genera different to the soil to flourish and that filtering of the composition occurs on the grass. Bradyrhizobium (soil genera high, grass low) are an example of a large proportion of the soil genera composition, which was not detected on grass. They are nitrogen-fixing symbiotes, with a niche in the roots of plants. Thus,

survival on plant surfaces is unlikely. The major grass genera included Pseudomonas and Sphingomonas, which have been previously identified as major genera on plants and grass specifically. Acinetobacter spp. was also detected on grass in the top 10 abundances; these are potential opportunistic pathogens of humans. Other genera detected in relatively high abundances on grass by Shade et al., e.g. Methylbacterium or Bacillus were not detected in the ten most abundant genera in this study [7].

4.6 Conclusions

While scientists have focused much of the environmental resistome analysis on soil as a resistome reservoir and link between the animal and environment this study identified the importance of grass as a reservoir of ARGs in addition to the soil resistome. The data identifies grass as a more expansive, diverse and persistent reservoir and source of ARGs, MGEs and differential abundance of microbiomes to soil. Grass and plants must be included as they directly link the environment back to the animal or human eating the plant and we have demonstrated the wide array of ARGs linked to MGEs present on grass, which is greater than in soil.

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Data and materials availability: The sequencing data have been submitted in the NCBI Sequencing Read Archive (SRA) under the bio-project PRJNA773121. Relative abundances of microbial taxa, ARGs, MGEs; and correlation analysis data as well as HT-qPCR array data are available as supplementary material (Supplementary Data).

Author contributions: TTD and FW: Design and/or interpretation of the reported experiments, acquisition and/or analysis of data, Drafting and revising the manuscript, Administrative, technical, or supervisory support. CS: Acquisition and/or analysis of data. FC: Acquisition and/or analysis of data. CB and FB: Drafting and revising the manuscript, Administrative, technical or supervisory support.

Declaration of competing interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability: The location of the raw data is described in the manuscript. The sequencing data have been submitted in the NCBI Sequencing Read Archive (SRA) under the bio-project PRJNA773121.

4.7 Figures

Figure 1: Microbial Diversity a) Chao 1 (richness) values vary but are not significantly different across all samples; b) The Shannon indexes (evenness) were significantly higher in soil samples compared with those in grass samples.

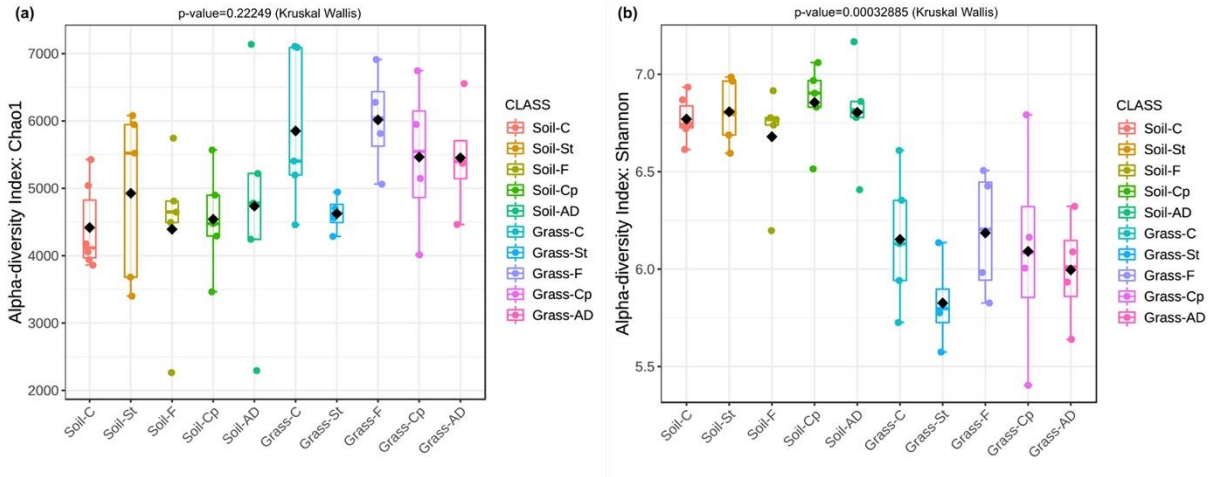


Figure 2: PERMANOVA PCoA: The soil control (without slurry application) and soil treatment (with slurry application) form absolute overlapping clusters. The grass control and treatment samples also absolutely overlap with each other. However, soil clusters are completely separated from grass clusters.

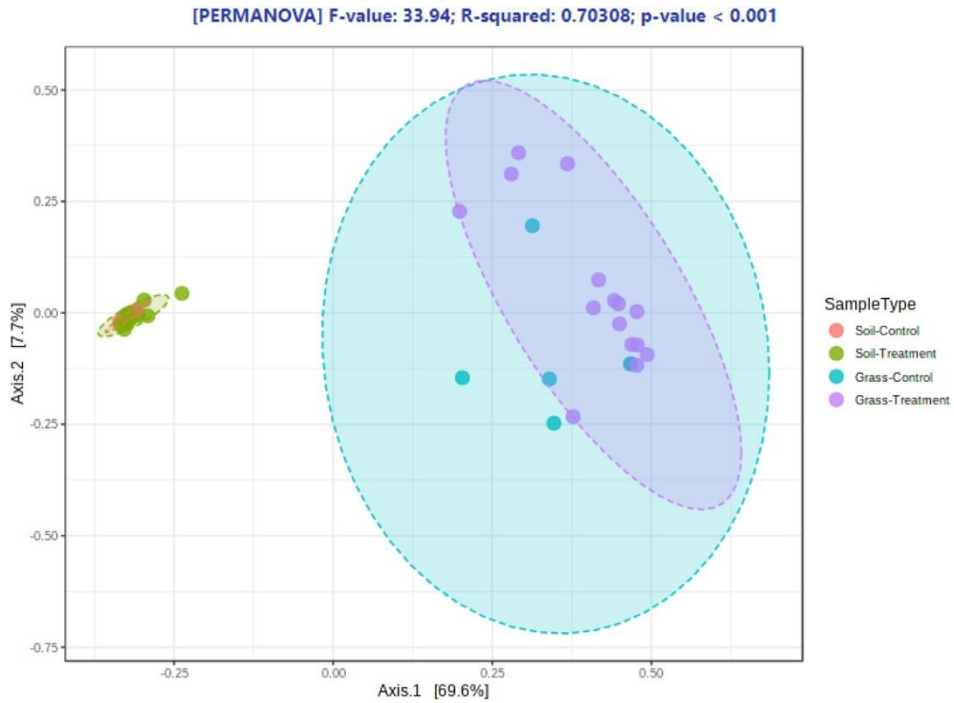


Figure S1: Microbial community composition at phylum level. Top 10 abundance phyla in soil and grass samples.

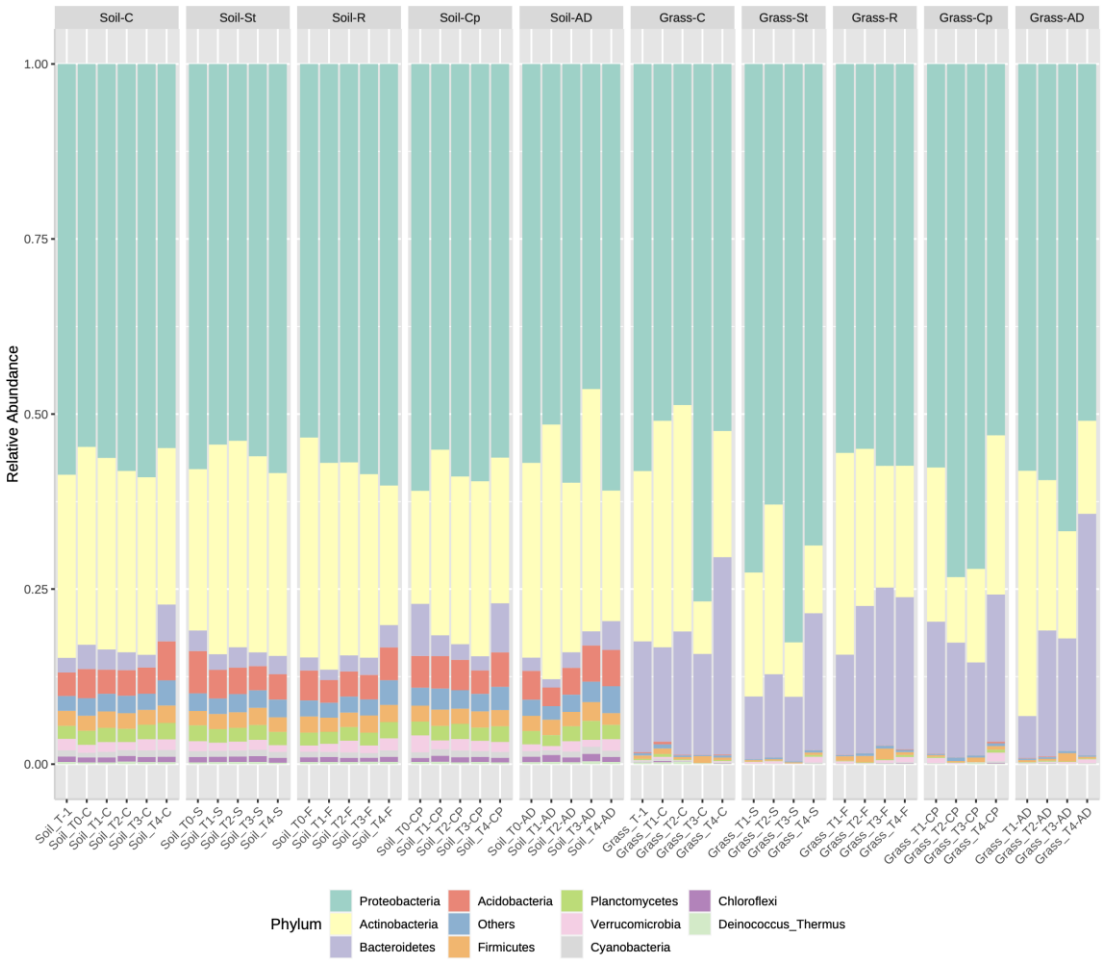


Figure S2: Microbial community composition at genera level. Top 10 abundance genera in soil and grass samples.

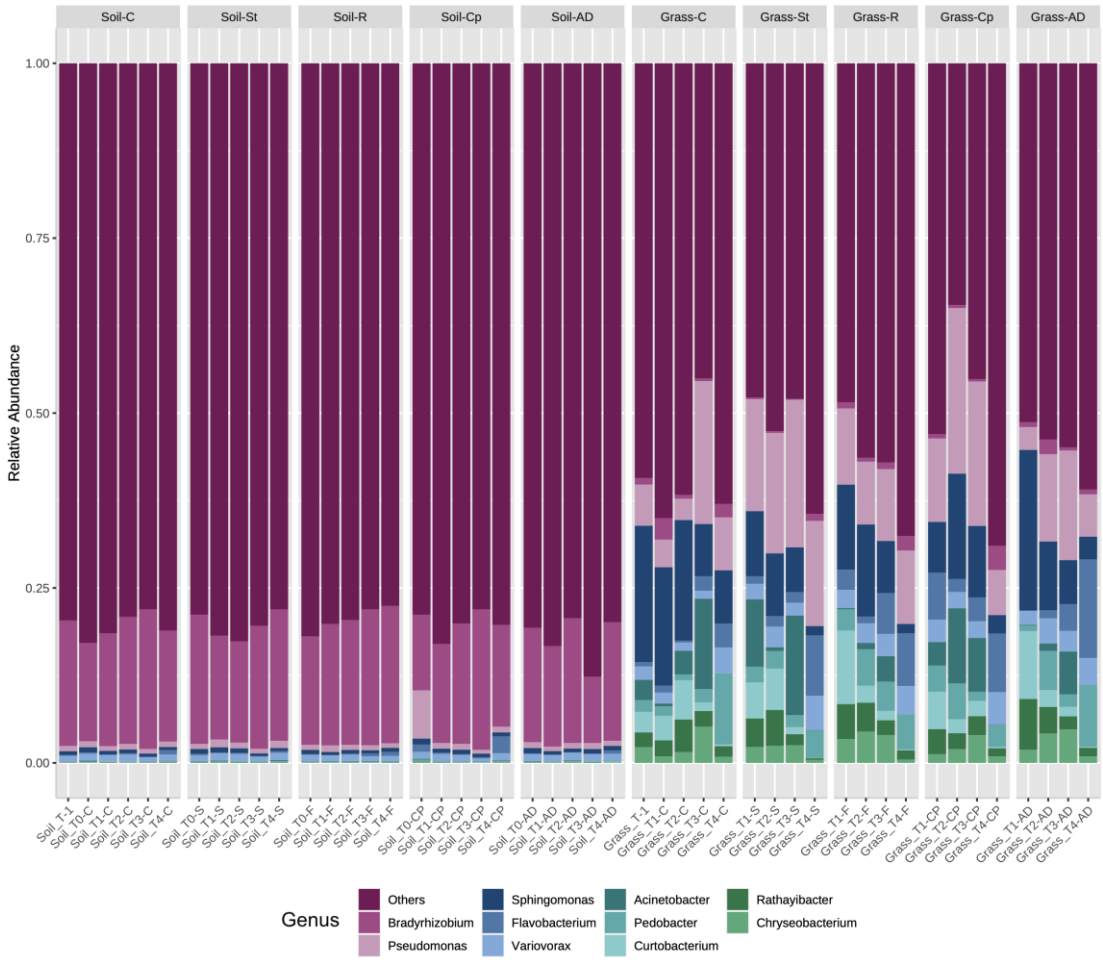


Figure S3: Relative abundances of the resistome genes detected in soil and grass across treatment and time.

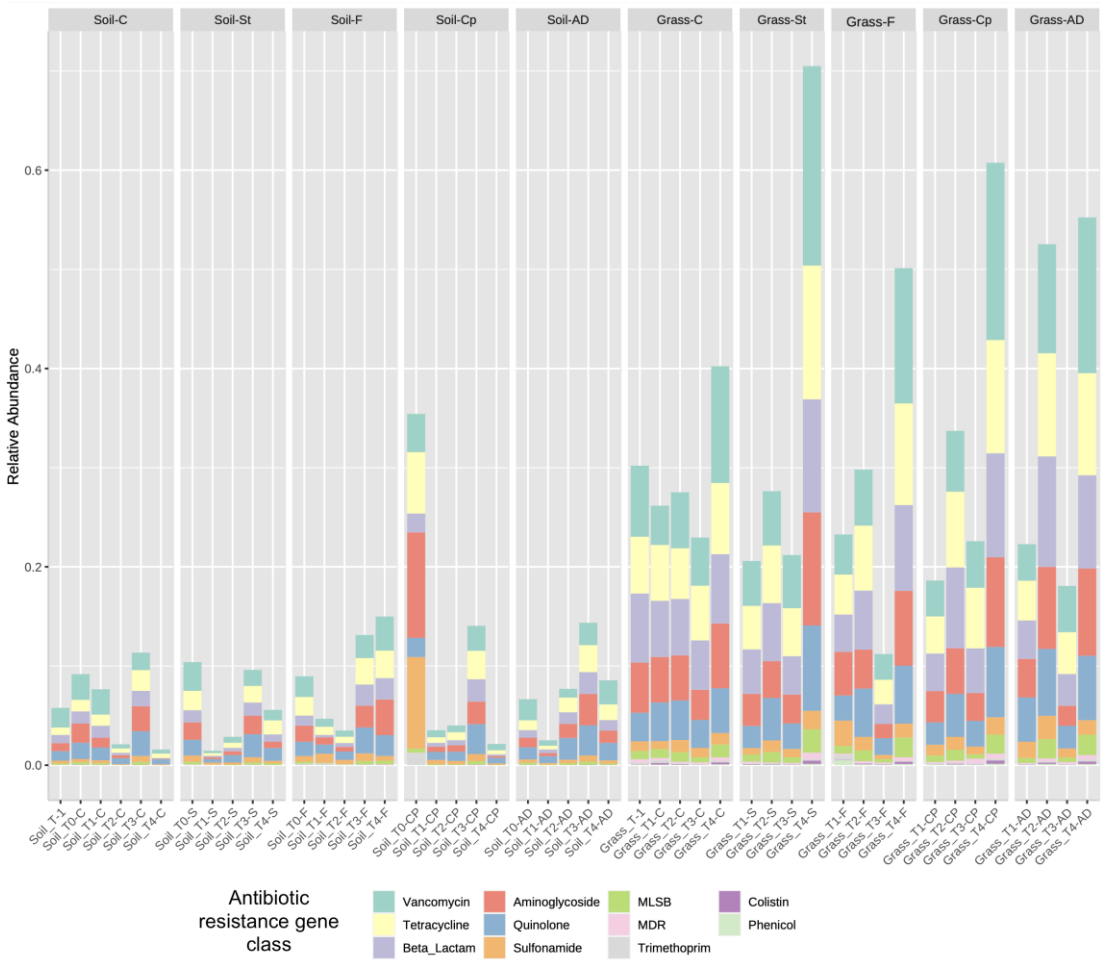


Figure S4: Core resistome observed at > 20% of gene prevalence and > 0.01 gene relative abundance. The prevalence value indicates the presence of genes across all samples considering the relative abundance of genes ≥ 0.01 (1%), e.g. when this value is 1, the gene is present in all samples.

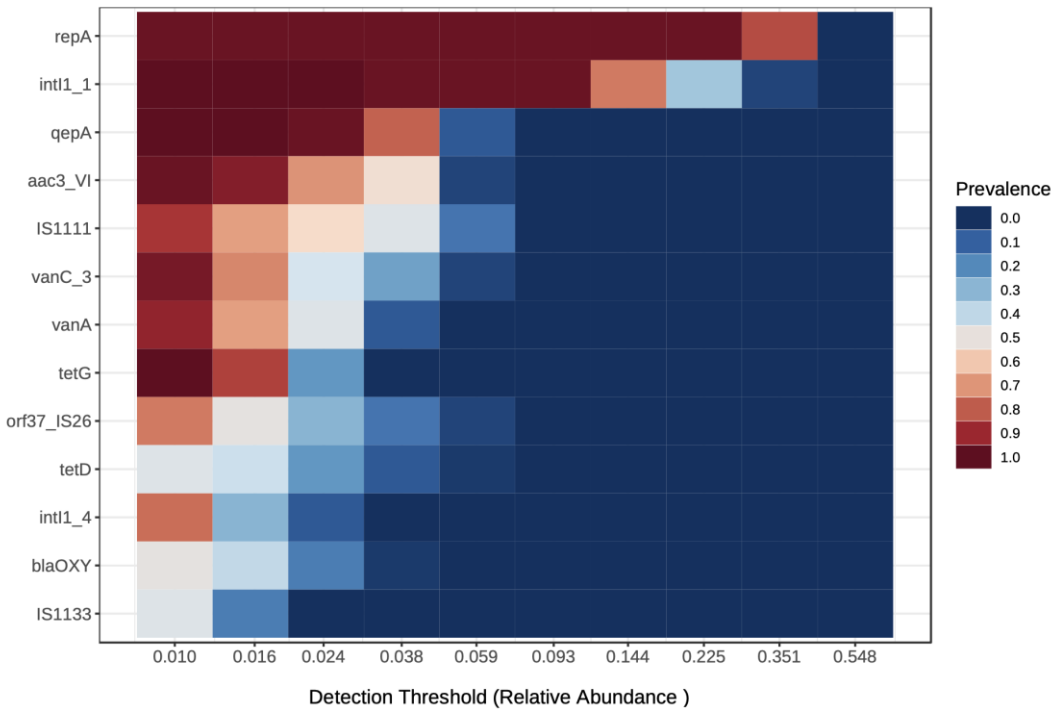
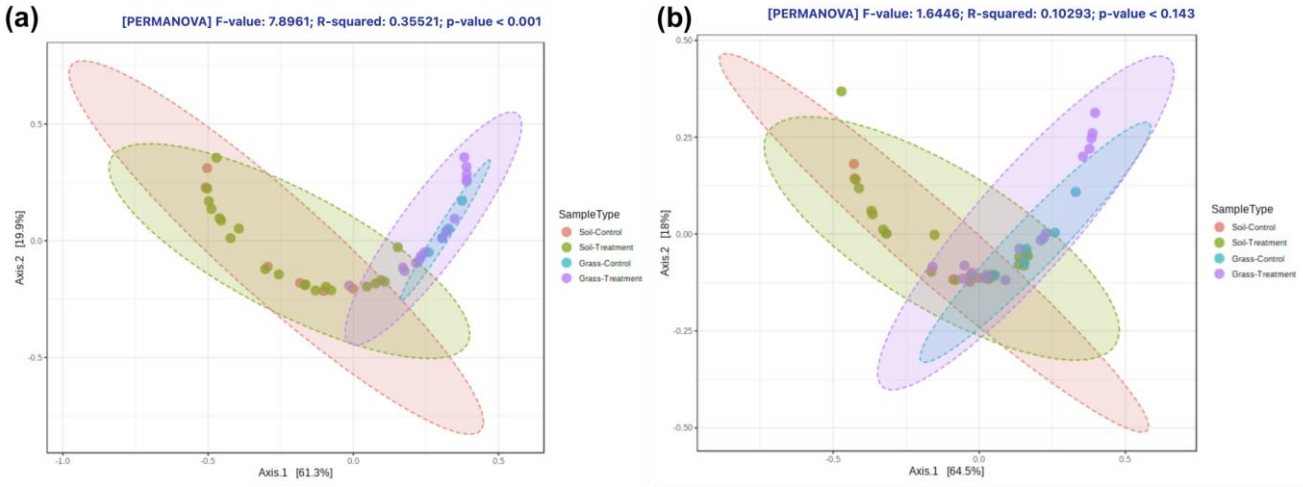


Figure S5: Principal Component Analysis (PCoA) of the resistome and mobilome of soil and grass.

(a) Resistome PCoA: A match overlap was found within soil samples as well as within grass samples, indicating a similar resistance gene profile in soil with/ without treatments as well as in grass with/without treatment (the abundance figure showed the same trend). A less overlap was found between soil and grass clusters.

(b) MGE PCoA: A match overlap was found within soil samples as well as within grass samples, indicating a similar MGE gene profile in soil with/ without treatments as well as in grass with/without treatment. A lower overlap was found between soil and grass clusters, but higher than for the resistome.



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Chapter 5

An investigation into plasmid bound antimicrobial resistance in Irish wastewater treatment plant effluent

Cian Smyth[1], Robert J Leigh[1], Thi Thiy Do[1], Fiona Walsh[1]

[1]Antimicrobial Resistance & Microbiome Research Group, Department of Biology, Maynooth University, Maynooth, Co. Kildare, Ireland.

5.1 Abstract

Wastewater treatment plants (WWTPs) have been shown previously to be environmental hotspots for antibiotic resistant bacteria (ARB) and provide optimal conditions for the maintenance and dissemination of and antibiotic resistance genes (ARGs). Mobile genetic elements (MGE) play a crucial role in the spread of such ARG throughout the environment via horizontal gene-transfer (HGT) with plasmids holding a distinct importance due to their ability to transfer variable resistance across bacterial species.

In this work, we identify 173 circularised plasmids isolated from two Irish WWTPs and subsequently characterise their ARGs, metal resistance genes (MG), virulence factors (VF), toxin-antitoxin systems (TAS) and related replicon types. The possible global evolutionary pathway of identified plasmids was also determined via a comparative-based cluster analysis.

The resulting detailed characterisation and analysis identified several key insights into the plasmids present in Irish WWTP effluent and their involvement in the dissemination and persistence of antimicrobial resistance (AMR) within the environment, agriculture, and human populations. Multiple multidrug resistant (MDR) plasmids were identified within this population with a significant number of plasmids being classified as megaplasmids greater than 100kbp. The plasmids isolated have a globally interlinked evolutionary history with clear patterns of conserved gene association.

This study confirms previous evidence of the important role WWTP environments have in the distribution and persistence of AMR as well as the globally linked nature of WWTP plasmids.

5.2 Introduction

The rising threat of AMR has been shown to be of critical importance for worldwide health with the World Health Organisation predicting the current trajectory of AMR dissemination to lead to a post-antibiotic era within this century. [1] The threat of AMR can be viewed through context of One Health as it has been linked to factors related to human, environmental and animal health. Within this framework, it has been shown that wastewater treatment plants (WWTPs) can harbour significant reservoirs of antimicrobial resistant bacteria (ARB) and antimicrobial resistance genes (ARG). [2, 3, 4, 5] These localised concentrations of AMR are thus considered potential hotspots for the dissemination of ARG between bacterial populations via horizontal gene transfer (HGT) through ARG contained on (MGEs) [6]. Amongst MGEs, plasmids play a consistent role in the dissemination of ARGs within bacterial populations [7]. These mobile resistance genes are of great importance due to their ability to propagate AR within bacterial populations and between distinct biomes which justifies their importance to the One Health approach [8] .

Previous WWTP studies have focused on the detection and classification of ARG and ARB using traditional gene detection methods such as PCR [9,10] and while metagenomic analysis has been able to characterise plasmids in WWTP previously [11] the short-read only methods used does not allow for the best possible characterisation and resolutions of plasmids and their functional composition [12] . This study aimed to extend the current understanding of mobile resistance in WWTP effluent while utilizing hybrid sequencing methods that mitigate previous impediments to plasmid resolution.

Comparative genomic studies have allowed for the confirmation and quantification of ARGs in diverse metagenomes as well as elucidating potentially coevolving gene groups such as metal resistant genes (MRGs) and biofilm forming virulence factors. [13] [14] [15]A combination of short and long-read sequencing technologies *via* hybrid-assembly has been shown to produce high quality, complete genomes from complex samples with the ability to resolve complete, closed circularised plasmids. [16] [17] This procedural development thus allows for the improved enquiry into the genomic structure of plasmids and the possible mobile ARG present as well as the co-occurrence of other gene groups. The utilisation of these methods also allowed for clustering and subsequent enrichment analysis between isolated plasmids and PLSDB [18] which provided a novel insight into the possible transmission path and origin of identified plasmids within the WWTP effluent biome.

Our study aimed to resolve and fully sequence and close the sequences of the AMR plasmids identified within WWTP effluent and subsequently determine the prevalence of mobile ARGs, MRGs, virulence factors, and conjugative machinery. The use of graph-based data analyses was also used to contextualise these completely closed circular sequenced plasmid characterisations in comparison to plasmids globally by evaluating similarity with plasmids across human, animal, and environmental biomes.

5.3 Materials and Methods

5.3.1 Sample Collection

Previously exogenously extracted plasmids that were transferred to *E. coli* from WWTP effluent samples were utilised in this study ($n_{samples}$=47)

5.3.2 Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing (AST) was performed on each isolate using the disk diffusion method according to CLSI guidelines[20] for ampicillin, tetracycline, amikacin, kanamycin, cefotaxime, ciprofloxacin, ceftazidime, trimethoprim, ertapenem, meropenem, imipenem and chloramphenicol antimicrobials with no notable deviation. Colistin resistance was determined using broth-microdilution (Table 1)

5.3.3 Plasmid Extraction

Plasmid extractions were performed using the Macherey-Nagel NucleoSpin Plasmid kit following the low-copy number protocol according to the manufacturer's guidelines with no notable deviation giving n=173 plasmids.

5.3.4 Short-Read Sequencing

Illumina short-read sequencing was initiated with an extractions quality assessment using NanoDrop and Qubit as per the sequencing centre (Novogene) guidelines. Extracted DNA was sequenced by Novogene using an Illumina NovaSeq 6000 with PE150 and Q30 ≥80%. This provided far greater than100× coverage for each plasmid.

5.3.5 Long-Read Sequencing

Long-read sequencing was performed in-house using the Oxford Nanopore Technologies (ONT) MinION. Ligation library preparation was performed using the SQK-LSK-109 Ligation Sequencing kit with no noticeable deviation from protocols. Multiplexing was performed with the NBD-104 Barcoding kit.

5.3.6 Pre-assembly Quality Control (QC)

Raw short reads were filtered and trimmed using Cutadapt *v*.3.0[21]. Raw long reads were processed using Filtlong *v*.2.0[22] for size and quality, with demultiplexing steps and adapter removal utilising Guppy v.6.1.2 [23]

5.3.7 Hybrid-Assembly

Hybrid assembly was performed using Unicycler *v*.0.5.0[24] with default settings.

5.3.8 Post-assembly QC

Visual assessment of assembled contigs was performed with Bandage *v*.0.8.1[25]. This allowed for an easy inspection of circularised contigs.

5.3.9 Plasmid Identification

Plasmid identification among consensus sequences was performed using Platon v.1.5.0 [26] utilising default settings. In total, 173 circularised plasmids were identified.

5.3.10 Plasmid Annotation

Each circularised plasmid was annotated using BAKTA *v*.1.2.2 using default settings. BAKTA utilises the following packages for analysis tRNAscan-SE 2.0 [27], ARAGORN [28], Infernal [29], PILER-CR [30], Pyrodigal [31], HMMER3 [32], AMRFinderPlus [33], Blast [34] and DeepSig [35].

5.3.11 Resistome, metalome, virulome and replicon profiling

Each circularised plasmid was analysed for the presence of ARGs using ABRicate *v*.1.0.1 [36] for AMR using the Comprehensive Antimicrobial Resistance Database (CARD) *v*.3.09 [37]. Each circular plasmid was profiled for metal and biocide resistance with BacMet *v*.2.0 [38] (using a previously published back translated dataset [39]) and for

virulence factors using the Virulence Factor Database (VFDB) *v*.0.5[40], plasmid replicon type was determined using PlasmidFinder *v*.2.1[41] . As ABRicate requires a nucleotide input and as BACMET is only provided in amino acid format, the amino acid database was back translated (using translation table 11) prior to annotation (Table SI).

5.3.12 Toxin-antitoxin System Determination

Toxin-antitoxin systems (TAS) were characterised using the TASER analysis pipeline provided by the TASmania TAS database [42] (Table SI).

5.3.13 Mobilisation Determination

Predicted plasmid mobilisation was determined using the MOBSuite, MOBTyper *v*3.0.3[43] with default settings. (Table SI).

5.3.14 Prophage Detection

Each circular plasmid was assessed for prophages using Phigaro *v*.2.3.0[44] under default settings (Table SI).

5.3.15 Gene Co-occurrence Analysis

Coinfinder *v.*1.1[45] was utilised to determine possible positive gene associations or dissociations. Coinfinder uses a binomial test and while the authors advise the use of

a Bonferroni–Dunn correction, this was not implemented so all coincidences could be explored and instances where (P)≤0.005 were considered statistically significant.

5.3.16 Plasmid clustering and global relatedness

Each plasmid was examined reciprocally against each other using the distance function in Mash *v*.2.2.2 [46] Instances where both the distance score (D) and P-value (P)≤0.1 were considered homologous. Each circular plasmid analysed in this study was assessed against all plasmids in PLSDB *v*.0.1.7 [47] using the distance Mash distance algorithm. Again, instances where both the distance score (D) and P-value (P) ≤ 0.1 were retained for further inspection. (Table SI).

5.3.17 Enrichment Analysis

Enrichment analysis was performed using a hypergeometric distribution model with Python with SciPy v1.0[48] (Table SI).

5.3.18 Plasmid Feature Correlation Analysis

Plasmid size (bp) GC content (%), gene density, tetra nucleotide frequencies and plasmids Shannon's entropy was determined, and correlation identified utilising an in-house Python 3.7 pipeline utilising Biopython *v*1.8.1[49], pandas *v*.2.0[50], SciPy v1.0[48] and plotly *v*.5.14.1[51].

5.4 Results

5.4.1 Antibiotic Susceptibility

Antibiotic susceptibility testing was performed on 47 *Escherichia coli* containing the exogenously extracted plasmids (Table 1). Among these isolates, 100% were resistant to both Tetracycline and Kanamycin 89% to Trimethoprim while 38% were resistant to either Ceftazidime or Cefotaxime. Ciprofloxacin resistance occurred in 87% of isolates while 72% of isolates showed resistance to Ampicillin. No resistance was detected to any of the three Carbapenem antibiotics tested (Ertapenem, Meropenem, Imipenem) nor to Amikacin. Resistance to Chloramphenicol was found in 43% of isolates. Resistance to Colistin was present in 29% of isolates. All isolates were multi-drug resistant (resistant to three or more classes of antibiotics).

5.4.2 Sizes of 173 circularised plasmid-derived contigs

A total of 173 circular plasmid were obtained through a hybrid assembly of Illumina and ONT Minion reads using Unicycler with default settings. The circular plasmid sequence sizes ranged from 2337bp to 292404bp in length. Mega-plasmids with a length of over 100kb encompassed 63/173 (36%) sequences. (Figure 1)

5.4.3 Determination of antibiotic and metal resistance genes present in assembled plasmids

All plasmids (*n* = 173) were screened for the presence of antibiotic resistance genes (ARG). Plasmids harbouring at least one ARG covered 73/173 (42%) with a total of 436 gene instances found within this group (Figure 2). Multidrug-resistant (MDR) plasmids accounted for 57/73 (73%) of the resistant group. Plasmids containing seven ARGs were the most prevalent and 12 ARGs were the maximum number detected on any one plasmid. Antimicrobial resistance genes conferring resistance to 12 antibiotic classes were found amongst the MDR plasmid group.

The ARGs conferring resistance to the aminoglycosides was the most prevalent and varied ARGs present. Aminoglycoside resistance genes were detected 122 times across 56 plasmids. The plasmids most frequently contained two aminoglycoside resistance genes concurrently, *AAC(3)-IIe* and *ANT(3'')-IIa* or *ANT(3'')-IIa* and *aadA2*. However, plasmids also contained one aminoglycoside resistance gene: *ANT(3'')-IIa* or *APH(3')-Ia* or *aadA16* or four genes: *AAC(3)-IIb*, *AAC(6')-IIc*, *APH(3'')-Ib* and *APH(6)-Id*. Antimicrobial resistance genes associated with resistance to sulphonamides were the 2nd most prevalent group with 73 instances across 37 plasmids. The *sul1* gene was detected on 58 plasmids with 21 having 2 copies. The *sul3 gene* had 15 instances across 15 plasmids. The *sul1* and *sul3* genes were never detected on the same plasmid. Antimicrobial resistance genes conferring resistance to the beta-lactam drug class was the 3rd most prevalent and varied with 63 instances derived from five distinct genes; Beta-lactamase ARGs were the predominant group in this class with TEM-1, TEM-122 and TEM-150 having 24, 12 and two instances, respectively. The extended-spectrum beta-lactamase genes SHV-134 and TEM-12

were also detected with 22 and three instances respectively. Detection of SHV-134 and TEM-1 on the same plasmid were the only beta-lactamase gene co-occurrences.

Tetracycline resistance ARGs were the 4th most prevalent drug class represented with 55 instances across three distinct genes. The *tetA* gene was present on 46 plasmids while *tetB* and *tetD* were present on two and 7 plasmids, respectively. There were no co-occurrences of tetracycline resistance genes.

Diaminopyrimidine antibiotic resistance genes were present in *drfA1*, *drfA19* and *dfrA27* which were detected on 14, 7 and 16 plasmids, respectively with no co-occurrences.

Fluoroquinolone ARGs were detected on 21 different plasmids. The *aac6'Ib-cr* and *qnrB* genes were co-located on the same plasmids (n = 16) and the *qnrA* gene on five plasmids.

ARGs conferring resistance to phenicol and rifampin drug classes were both found in 16 instances. Two distinct phenicol ARGs were detected with *cmlA1* and *catI* having 15 and one instance respectively. *arr-3* accounted for the 16 rifampin ARG instances. Resistance to macrolide antibiotics was represented with three distinct ARGs. *ereA2* had the highest number of instances with seven while *mphE* and *msrE* both had two instances. Instances of resistance to the drug of last resort colistin had the lowest of any group, with 7 found for the plasmid associated MCR-9 gene.

Plasmids that were found to contain ARGs but no metal resistance genes (MRGs) included 17/173 (10%).

5.4.4 Metal resistance Genes

Of the 173 plasmids, 77 (45%) were found to contain at least one MRG. Plasmids containing both ARGs and MRGs were present in 56/173 (32%). Plasmids found to contain only MRGs were present at 21/173 (12 %). (Figure 3)

The maximum number of distinct MRGs on one plasmid was 24. MRGs conferred resistance to eight different metal classes. Mercury resistance was present at the highest frequency with 210 genes found across 30/77 (39%) plasmids. Within this group *merA*, *merC*, *merD*, *merE*, *merP* and *merT* (*mer*CDEPT operon) were detected in all 30 plasmids and either *merR1* or *merR2* were co-located with the mercury resistance operon. The seven MRGs (*terA*, *terB*, *terC*, *terD*, *terE*, *terW* and *terZ*) conferring resistance to tellurium were co-located across 21/77 (27%). Iron resistance genes (*sitA*, *sitB* and *sitC*) were detected together across 14 plasmids. Three MRGs (*QacEdelta1, qacF, qacE*) conferring resistance to antiseptics/ammonium compounds were identified with 70 instances across 54/77 (70%) plasmids, thus were the most widespread MRG class. *QacEdelta1* was the most prevalent MRG within this group with 53 instances found across 37/77 (48%) of plasmids with 16/77 (21%) having two instances present. *qacF* and *qacE* were found in 15/77 (19%) and 2/77 (3%) plasmids, respectively. The *qacH* gene, which confers resistance to disinfectants was detected on 15 plasmids.

Arsenic resistance genes (*arsB*, *arsC* and *arsH*) were co-located across 7/77 (9%) of plasmids. Metal resistance genes conferring resistance to copper, lead and nickel/cobalt were identified in 7/77 (9%) plasmids with two distinct genes conferring

resistance in each group: *ncrC* and *rcnR/yohL* for nickel/cobalt, *pbrA* and *pbrC/PbrB* for lead and *pcoE* and *pcoS* for copper. Multi-metal resistance was detected in 21 (27%) of plasmids and 7 (9%) plasmids harboured resistance to every class except iron. Multi-metal resistance plasmids conferring resistance to mercury, antiseptics/ammonium compounds and tellurium were the most prevalent (14/77 (18%)). Of the plasmids conferring resistance to one metal class, antiseptics/ammonium compounds were the most prevalent at 33/77 (43%) while iron and mercury were found in 14/77 (18%) and 9/77 (12%), respectively.

5.4.5 Virulence Factors

Virulence factors (VFs) were detected in 16/173 (9%) of plasmids across 4 VF classes. Fourteen plasmids contained the VF genes *icsP/sopA, iroB, iroC, iroD, iroE, iroN, iucA, iucB, iucC*, *iucD, iutA* and *senB*.

5.4.6 Replicon Typing

Plasmid replicon types (RT) were detected in 94% of plasmids. Single instance RT were present in 97/173 (56%) of plasmids while 65/173 (38%) of plasmids contained more than one RT with 42/173 (24%) having two instances per plasmid and 23/173 (13%) having 3 instances. Nine distinct RT families were represented. (Figure 5)

No specific RT dominated across the plasmids. However, IncF RT families were present in the highest proportion with 53/173 (31%) of plasmids containing at least one IncF RT and 71 total instances. Of this group, IncFII_1 was the most prevalent across 9% of the RT positive plasmids. IncFIB, IncFIC and IncFII were the next most prevalent with 14/173 (8%) instances each. IncFII, IncFIA_1, IncFII and IncFIB were also represented with seven, two, two, and one instances respectively. Within this group, plasmids containing both two and three RTs were detected, with 2/173 (1%) plasmids containing IncFIA_1, IncFIB and IncFII and 14/173 (8%) plasmids containing both IncFIB and IncFIC. IncI RTs were associated with the IncF family. All 14/173 (8%) IncI1 RTs were present with IncFII.

The Col family plasmids (five distinct Col RTs) were the next most represented, 52 (30%) plasmids contained at least one Col RT across 67 instances. Within this RT family, Col(Ye4449) was the most prevalent, 18/173 (10%) of plasmids contained this RT. ColRNAI and Col440II were the next most prevalent with 15/173 (9%) and 14/173 (8%) respectively. Each plasmid with an instance of Col440II also contained ColRNAI with one plasmid containing a single ColRNAI. ColE10 and Col440I were also represented amongst this family with 13/173 (8%) and 7/173 (4%) respectively with both being the sole RT on each respective plasmid.

IncH family RTs were also present with 21/173 (12%) plasmids containing both IncHI2A and IncHI2. The RepA was also found in every plasmid containing both IncH RTs 21/173 (12%). IncN and IncP6 were the only representatives of their respective families detected, with both the only RT present in 16/173 (9%). IncA/C2 and IncX5 were the least prevalent RTs with 2/173 (1%) and 1/173 (0.6%), respectively.

5.4.7 Phage

Phage and prophage were determined using Phigaro in default settings. 15/173 (9%) plasmids contained at least one Phigaro instance with one plasmid containing three distinct instances. Two distinct taxonomies were present, Myoviridae and Siphoviridae, with both found in 1/17 (6%) plasmid each and the rest of plasmids 15/17 (88%) remaining unclassified (Figure 6).

5.4.8 Co-occurrence Analysis

Co-occurrence analysis of plasmid genes and replicon types and subsequent modularity determination revealed six distinct co-occurrence clustering groups. No dissociation between any genes was found (Figure 7).

Group A and B were both isolated, with group A containing the Col440II_1 and ColRNAI_1 replicons and group B containing all VF genes apart from *senB* determined to associate together along with IncFIC(FII)_1 and IncFIB(AP001918)_1.

Group C contained *bla*TEM-1, IncFII(pECLA)_1_pECLA and Inc1_1_Alpha with *bla*TEM-1 having the only association with any other group node (Group F: *merR2*).

Groups D, E and F were interconnected. However, despite group D and E containing mostly ARGs only *tetA* held associative connections between each group. Group D contained associative connections between several ARGs (*aadA2, cmlA1, bla*SHV-

134*, sul3*), *qacH, qacF* and IncFII. Group E held more associative connections to group F than any other group. It contained a single replicon (IncN_1), numerous ARGs (*aadA16, dfrA27, qnrB17, sul1, arr-3,tet(A), AAC(6")-Ib-cr*) and *qacEdelta1*. Group F was the largest due to the presence of the *ter* and *mer* operons while also containing the replicons RepA_1_pKPC-CAV1321 and IncHI2_1 and the ARGs *bla*TEM-122, *AAC(3)-IIe*, *ANT(3")-IIa* and *dfrA1* (Figure 8).

5.4.9 Toxin-antitoxin systems

Detection of toxin-antitoxin system (TAS) related features identified 32 distinct genes. These genes were located across the entire plasmid group with larger plasmids containing a more diverse collection. Some smaller plasmids that were classed as non-resistant were identified to contain complete TAS such as the *ccdA* and *ccdB* system. There were also several instances were plasmids contained a single TAS feature or no TAS genes at all (Figure 9).

5.4.10 Plasmid Mobility

Plasmid mobility typing revealed 93/173 (54%) of identified plasmids were classified as conjugative, 71/173 (41%) as mobilisable and 9/173 (5%) as non-mobilisable (Figure 10).

5.4.11 Relatedness Clustering

Relatedness clustering of all 173 plasmids determined that there were 17 groups present with 5 of these being singletons. (Figure 11) Plasmids where clustered together using instances where both the distance score (D) and P-value (*P*)≤0.1 were considered homologous, replicating the creation of the PLSDB dataset. Plasmids with no resistance genes (AMR or MRG) and no virulence factors grouped together. Groups zero, one, three, four, nine, 15 and 16 contained no instances of ARG or MRG. When comparing groups containing only non-resistant (NR) plasmids to those that contain MRG or ARG using a brunner-munzel test, the resistant groups were found to be significantly larger, of which 55/86 (64%) were classified as mega plasmids (>100kbp). There GC content of groups containing only NR plasmids was shown to be significantly higher than those that contained only resistant plasmids. There were no groups that contained both resistant and NR plasmids (Figure 12).

The analysis of NR plasmids determined that Col family replicons were most prevalent at 52/79 (66%). Group zero, one and four contained Col(Ye4449), Col440I and ColE10 respectively, while group three plasmids all contained Col440II and ColRNAI replicons. NR plasmids from group 16 contained the IncP6 replicon while the singletons, group nine and group 15 contained IncFIB and IncX5 replicons. Group two plasmids had no associated replicon type. Group nine, a singleton was found to contain three instances of phage comprising the *Myoviridae* and *Siphoviridae* viral families and one of unknown origin. The total genomic content of these groups was analysed due to their lack of MRG, ARG and virulence factor genes. The majority (34%) of

annotations were hypothetical genes. Plasmids in this group that were found to be of Col replicon types contained no colicin genes. Mobilisation and maintenance were the next most prominent gene classes found within the NR plasmids.

Antimicrobial or metal resistance plasmids were present in 54% of the plasmid cohort. These plasmids were separated into groups five, six, seven, eight, ten, eleven, twelve, thirteen and fourteen, grouping based on their genetic similarity. Their average plasmid size was higher than those of the NR groups while the GC content was lower.

Group 14 was the largest and most varied of the groups (n = 31 plasmids). Antimicrobial resistance genes were present across 17 of these plasmids. Their plasmid sizes ranged from 100 to 144 kb, which means that every plasmid is a mega plasmid (>100kb). All plasmids within this group where of the IncF replicon type and this group was the only group to contain virulence factors. This group consisted of three sub-groups. The largest subgroup comprised 15/31 (48%) plasmids. Every plasmid in this subgroup contained an IncFII replicon type, genes conferring resistance to aminoglycoside, beta-lactam, sulfonamide, and tetracycline drug classes as well as chloramphenicol, *qacF* was the only MR present. No virulence factor or phage gene were present. The second largest subgroup contained 14/31 (45%) of plasmids. Every plasmid in his subgroup contained the two replicons IncFIC(FII) and IncFIB. The *sitABC* operon were the only MR gene detected. Virulence factor presence was confirmed in this subgroup with the *iroBCDEN* gene cluster, *iucABCD* and *iutA* genes as well as the *icsP/sopA* gene identified. No ARG or phage genes were detected.
The smallest subgroup of group 14 contained 2/31 (6%) plasmids. This subgroup was the only plasmid grouping to contain ARG, MR genes and virulence factor genes.

Multiple replicons were found on each plasmid: Inc_FIA, IncFIB and IncFII. The ARGs *APH(3')-Ia*, TEM-1 and *tet(B),* conferring resistance to aminoglycosides, beta-lactams and tetracyclines, respectively were present. The MR *mer* operon was identified within this subgroup along with the virulence factor, enterotoxin-coding *senB*. No phage genes were detected.

Group seven contained 21/173 (12%) of all plasmids analysed. Plasmids in this group were all greater than 200kb (mega plasmids). Every plasmid shared the same multi replicon type with IncHI2A, IncHI2 and RepA detected. Group 7 plasmids were sub-grouped into two distinct units based on their genotype with some variation existing within each subgroup. The larger subgroup contained 14/21 (67%) plasmids. Within this subgroup ARG's conferring resistance to aminoglycosides (*AAC(3)-IIe, ANT(3'')-IIa*), diaminopyrimidines (*dfrA1*), tetracyclines (*tetA*) beta-lactams were present. Variation was noticed within the beta-lactam resistance genes with 2/14 (14%) plasmids being found to contain *TEM-150* rather than *TEM-122.* All plasmids in this subgroup contained mercury (*mer* operon) and tellurium (*ter operon)* metal resistance genes and the *qacEdelta1* gene. No virulence factors genes were present while phage genes of an unknown origin were detected. The smaller subgroup within group seven held 7/21 (33%) of plasmids. Each plasmid contained the same ARG's for aminoglycoside (*AAC(3)-IIb, AAC(6')-IIc, APH(3'')-Ib, APH(6)-Id*), colistin (*MCR-9*), macrolide (*EreA2*), beta-lactam (*TEM-1,SHV-134*), diaminopyrimidine (*dfrA19*), sulfonamide (*sul1*) tetracycline (tetD) with five plasmids containing a fluoroquinolones resistance gene (*QnrA1*), some variation e.g. xyz was present. Two of the seven plasmids differed in their absence of *qnrA1* and only having a single copy of the *sul1* gene. All plasmids harbored resistance genes to arsenic, mercury, tellurium,

nickel, lead, copper/silver as well as the *rcnR* repressor and *qacEdelta1* efflux genes. No virulence factors or phage genes were present. This subgroup comprised the largest plasmids and resistance variety for both AR and MR of the entire dataset.

Group five contained 16/173 (9%) plasmids with sizes ranging from 54 to 56kb. All plasmids contained in this group had the IncN replicon, ARG to the classes aminoglycoside (*AAC(6')-Ib-cr, aadA16*), fluoroquinolone (*AAC(6')-Ib-cr, QnrB17*), rifampin (*arr-3*), diaminopyrimidine (*drfA27*), sulfonamide (*sul1*) and tetracycline (*tetA*) and the *qacEdelta1* gene. No virulence factor nor phage genes were identified. Group 10 contained 14/173 (8%) plasmids (96-97kb) with no variation found among identified replicon types (IncFII and IncI1) or ARG class (beta-lactam). No virulence factor nor phage genes were identified. Group six contained 7/173 (4%) plasmids with the same replicon type (IncFII). No ARGs, virulence factors or phage genes were present. MR genes associated with mercury resistance were identified (*mer* operon). All plasmids in this group were 140kb in length. Group 13 contained two plasmids (177kb) both of which contained the IncA RT, ARG to classes (aminoglycoside, macrolide and beta-lactam - genes) and the MRF *qacE*. No virulence factor nor phage genes were present. Group eight, 11 and 12 represented singleton plasmids with only group eight having an identified replicon (ColRNA). Group eight had ARGs conferring resistance to chloramphenicol and beta-lactam classes, while group 11 and 12 had genes conferring resistance to beta-lactam and tetracycline classes, respectively. No virulence factors or phage was found in these groups. Groups eight and 12 were some of the smallest plasmids identified at 5.5Kb. The group 11 plasmid (size kb) is classified as a mega plasmid.

5.4.12 Global Relatedness and Enrichment Analysis

The entire PLSDB plasmid dataset was clustered a mash score of 0.1 with the 173 plasmids within this dataset. Enrichment analysis was performed using associated PLSDB metadata to determine possible significant enrichment or reduction across five categories location, species, sample source, sample host, mega plasmids.

5.4.12.1 Location clusters

Plasmids from this study significantly *P* (<0.005) clustered to a greater degree with PLSDB plasmid that associated with 10 countries including Bangladesh, Canada, Guadeloupe, Hong Kong, Israel, Myanmar, Nepal, Switzerland, United Kingdom and Vietnam. Plasmids from this dataset also clustered to a significant *P*(<0.005) degree less with PLSDB plasmid associated with Chile, Denmark, Germany, Malaysia, Mexico, The Netherlands, Poland, Russia, South Korea and Spain. (Figure 13)

5.4.12.2 Taxa clusters

Species level taxonomic association was also determined between this dataset and PLSDB plasmids with 17 significant results. Plasmids were found to cluster significantly more with PLSDB plasmids associated *Klebsiella pneumoniae* and *E. coli.* Significantly *P*(<0.005) less clustering was determined with PLSDB plasmids

associated with *P. aeruginosa, P. putida, A. baumannii, P. mirabilis, Y. enterocolitica, A. salmonicida, A. caviae, V. parahaemolyticus, S. aureus, S. haemolyticus, B. cereus, B. subtilis, C. sakazakii,* uncultured bacterium and *E. roggenkampii.*

5.4.12.3 Sample source clusters

The plasmids in this dataset were found to cluster more significantly $P(<0.005)$ with PLSDB plasmids associated with bovine faeces, cattle, ground turkey, pig farm pigs and human stool and urine samples while clustering significantly $P(<0.005)$ less with PLSDB plasmids associated with human blood and rectal swabs, food, sewage, soil, and water (Table SI).

5.4.12.4 Sample host clusters

Sample host data from PLSDB elucidated two significant P (<0.005) results, with the plasmids being more likely to cluster with PLSDB plasmids associated with chickens while being less likely to cluster with those of human origin (Table SI).

5.4.12.5 Megaplasmids

When looking at mega plasmids, it was found that the dataset had a significantly $P(<0.005)$ greater proportion of mega plasmids than would be statistically likely (Table SI).

5.5 Discussion

The surveillance of AMR has often been discussed through the use of clinical short-read WGS studies that while providing vital insights into the genetic context of AMR found in such settings, does not offer insights into the transfer, persistence and evolution of plasmid AMR outside of the patient and pathogen. Hybrid plasmid sequencing allows the transfer, persistence and evolution of plasmid mediated AMR and MDR to be further scrutinised due to the capacity to generate closed plasmids rather than plasmid fragments, as is frequently the case with short-read WGS. In this study we generated 173 closed circular plasmids from Irish WWTP effluent, an environmental biome that has been shown to be a hotspot of AMR [52]. The plasmids clustered phylogenetically into two groups, one contained a diverse range of AMR genes while also harbouring a multitude of metal resistance and virulence genes and an array of different replicons and the other group contained no AMR, metal resistance nor virulence genes while containing replicons.

The characterisation of 173 fully circularised plasmids allowed for the development of insights into the variety of ARGs, metal resistance and virulence genes and the different replicon types, their cooccurrence on single plasmids as well as plasmid functional composition and size characterisation. WWTP effluent has previously been found to be a possible reservoir of clinically relevant ARGs [53] and this dataset confirmed that there is a wide variety of ARGs present within Irish WWTP effluent. Further scrutiny of these results determined that the plasmids isolated within this study did not harbour ARGs conferring resistance to multiple drugs of last resort, with *MCR-9* being the only ARG in this category identified.

The prominence of plasmids lacking ARGs, MGs and VFs is of particular note within this study. The classification of 46% of isolated plasmids within Irish WWTP effluent that appear to persist despite having no apparent selective advantage nor confer a selective marker has not been previously noted. Despite some TA-systems being identified which may explain their presence, most of these non-resistant plasmids do not contain any genes which could be found to account for their persistence. Most of these plasmids could be classified as mobilisable which in association with their smaller size could lead to persistence via co-transfer with the larger conjugative mega plasmids that were co-transferred. The presence of currently unidentified genes that could pertain to their ability to survive is also possible due to the large number of hypothetical genes that were found within this group. The ancestry of these plasmids also showed that they form distinct groups unrelated to those that contain known drivers of persistence such as ARGs. This suggests that the plasmids have not likely previously held ARGs and instead have evolved separately and consequently must have their own factors for persistence and survival. This observation leads to the possibility that these non-resistant plasmids are more common than expected and could have been inadvertently ignored in previous studies due to the studies focus on ARG/MG determination and short-read exclusive sequencings inability to fully resolve circularised plasmids thus leading to their characterisation as fragmented assemblies [12].

The quantity of plasmids that could be classified as mega plasmids was of particular importance due to the elevated levels of ARGs, MGs and VFs that were found to cooccur within them. This dataset was shown to be statistically enriched for mega

plasmids when compared to PLSDB and therefore questions arise to the reasons for their presence and ability to persist despite their hypothesised metabolic cost to their host. Irish WWTP effluent has been shown to contain some of the highest antibiotic concentrations in Europe [54] and this could provide a setting where the collective selective pressure against multiple antibiotic classes allows for the development and perseverance of MDR megaplasmids. Coincidentally, the plasmids also contain a large number of MR and VFs which could possibly bring about survival through effective mutualism with their host, when present in pathogenic bacteria. Some however, contained TA-systems that would suggest a more parasitic persistence coevolutionary synergy[55]. The plasmids large size, multiple interspersed incompatibility groups, and polarised compositional distances between otherwise unrelated plasmids could also suggest that these megaplasmids are the result of the fusion of smaller plasmids coexisting in the WWTP environment.

This dataset of WWTP effluent plasmids also provided some key insights into possible plasmid transmission pathways. Significant enrichment for both animal and human waste plasmids was noticed while those relating to environmental samples were decreased. This could suggest that the presence of such plasmids in the WWTP effluent environment could be due to animal waste entering the water supply which would coincide with the specific animal related resistance profiles discussed previously which could suggest a potential greater need of surveillance to mitigate against potential public health concerns.

5.6 Conclusions

Our study offers a novel understanding into the risk that WWTP effluent plasmids hold due to their contained resistance and virulence. The evidence provided suggests that the current knowledge of megaplasmids in particular should be expanded through the use of next generation sequencing technologies that fully elucidate their ARGs, MGs, VFs and genetic composition. Steps should be taken to fully understand the possible transmission pathways and origin of plasmids within these biomes through the investigation of plasmids in a global context and thus allowing for a stronger direction in the development of AMR mitigation strategies in the future.

5.7 Tables

Table 1: Phenotypic resistance profiles for 47 exogenous plasmid extraction samples. R: Resistant, I: Intermediate, S: Susceptible.

Sample	Trimethoprim	Amikacin	Cefotaxime	Ceftazidime	Ciprofloxacin	Ertapenem	Meropenem	Imipenem	Kanamycin	Chloramphenicol	Ampicillin	Tetracycline	Colistin
1	R	S	S	S	R	S	S	S	R	R	R	R	S
2	R	S	S	S	R	S	S	S	R	R	R	R	S
3	R	S	S	S	R	S	S	S	R	R	R	R	S
4	R	S	S	S	R	S	S	S	R	R	R	R	R
5	R	S	S	S	R	S	S	S	R	R	R	R	R
6	R	S	S	S	R	S	S	S	R	R	R	R	S
7	R	S	S	S	R	S	S	S	R	R	R	R	S
8	R	S	S	S	R	S	S	S	R	R	R	R	S
9	R	S	S	R	R	S	S	S	R	R	R	R	S
10	R	S	S	S	R	S	S	S	R	R	S	R	R
11	R	S	S	S	R	S	S	S	R	S	S	R	R
12	R	S	S	S	R	S	S	S	R	R	R	R	R
13	R	S	S	S	R	S	S	S	R	S	S	R	R
14	R	S	S	S	R	S	S	S	R	R	R	R	R
15	R	S	S	S	S	S	S	S	R	S	S	R	S
16	R	S	S	S	R	S	S	S	R	S	S	R	S
17	R	S	S	S	R	S	S	S	R	S	S	R	S
18	R	S	S	S	R	S	S	S	R	S	S	R	S
19	R	S	S	S	R	S	S	S	R	S	S	R	S

Sample	Trimethoprim	Amikacin	Cefotaxime	Ceftazidime	Ciprofloxacin	Ertapenem	Meropenem	Imipenem	Kanamycin	Chloramphenicol	Ampicillin	Tetracycline	Colistin
20	R	S	S	S	R	S	S	S	R	S	S	R	R
21	R	S	S	S	R	S	S	S	R	S	S	R	S
22	R	S	S	S	R	S	S	S	R	S	S	R	R
23	R	S	S	S	R	S	S	S	R	S	S	R	S
24	R	S	S	S	R	S	S	S	R	S	S	R	S
25	R	S	R	R	R	S	S	S	R	S	R	R	R
26	R	S	R	R	R	S	S	S	R	S	R	R	S
27	R	S	R	R	R	S	S	S	R	S	R	R	S
28	R	S	R	R	R	S	S	S	R	S	R	R	S
29	R	S	S	R	R	S	S	S	R	S	R	R	S
30	R	S	R	R	R	S	S	S	R	S	R	R	S
31	R	S	R	R	R	S	S	S	R	S	R	R	S
32	R	S	R	R	R	S	S	S	R	S	R	R	S
33	R	S	R	S	R	S	S	S	R	S	R	R	S
34	S	S	S	S	S	S	S	S	R	S	R	R	S
35	S	S	S	S	S	S	S	S	R	S	R	R	S
36	S	S	S	S	R	S	S	S	R	S	R	R	S
37	S	S	S	S	S	S	S	S	R	S	R	R	R
38	S	S	S	S	S	S	S	S	R	S	R	R	S
39	R	S	R	R	R	S	S	S	R	S	R	R	R
40	R	S	R	R	R	S	S	S	R	R	R	R	I
41	R	S	R	R	R	S	S	S	R	R	R	R	R
42	R	S	R	R	R	S	S	S	R	R	R	R	S
43	R	S	R	R	R	S	S	S	R	R	R	R	S

Sample	Trimethoprim	Amikacin	Cefotaxime	Ceftazidime	Ciprofloxacin	Ertapenem	Meropenem	Imipenem	Kanamycin	Chloramphenicol	Ampicillin	Tetracycline	Colistin
44	R	S	R	R	R	S	S	S	R	R	R	R	S
45	R	S	R	R	R	S	S	S	R	R	R	R	R
46	R	S	R	R	R	S	S	S	R	R	R	R	S
47	R	S	R	R	R	S	S	S	R	R	R	R	I

5.8 Figures

Figure 1: Plasmid Length (kbp) and GC (%).

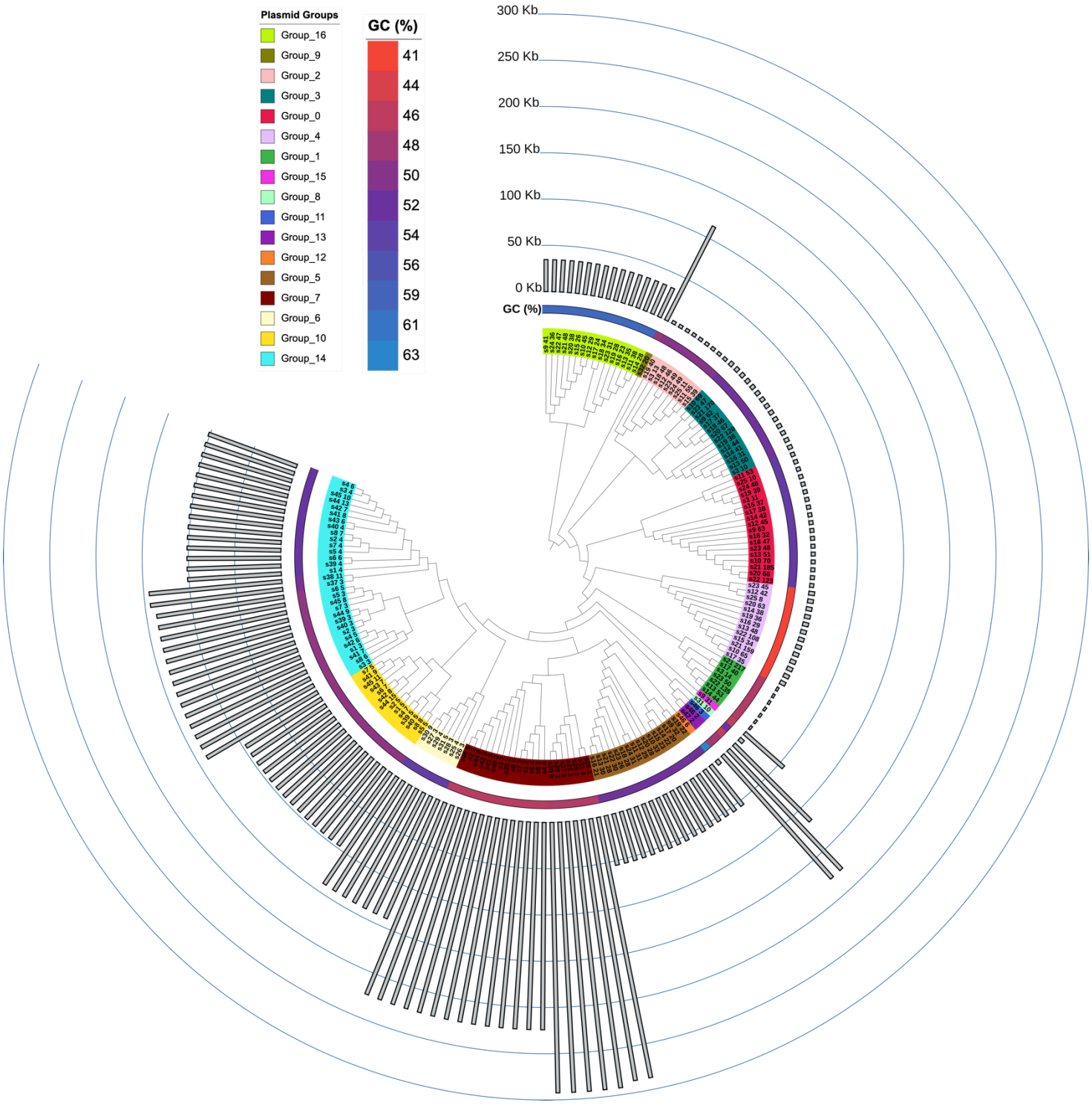


Figure 2: Antimicrobial resistance gene presence. Clades that do not contain ARG have been collapsed without effecting topology of ARG containing plasmids.

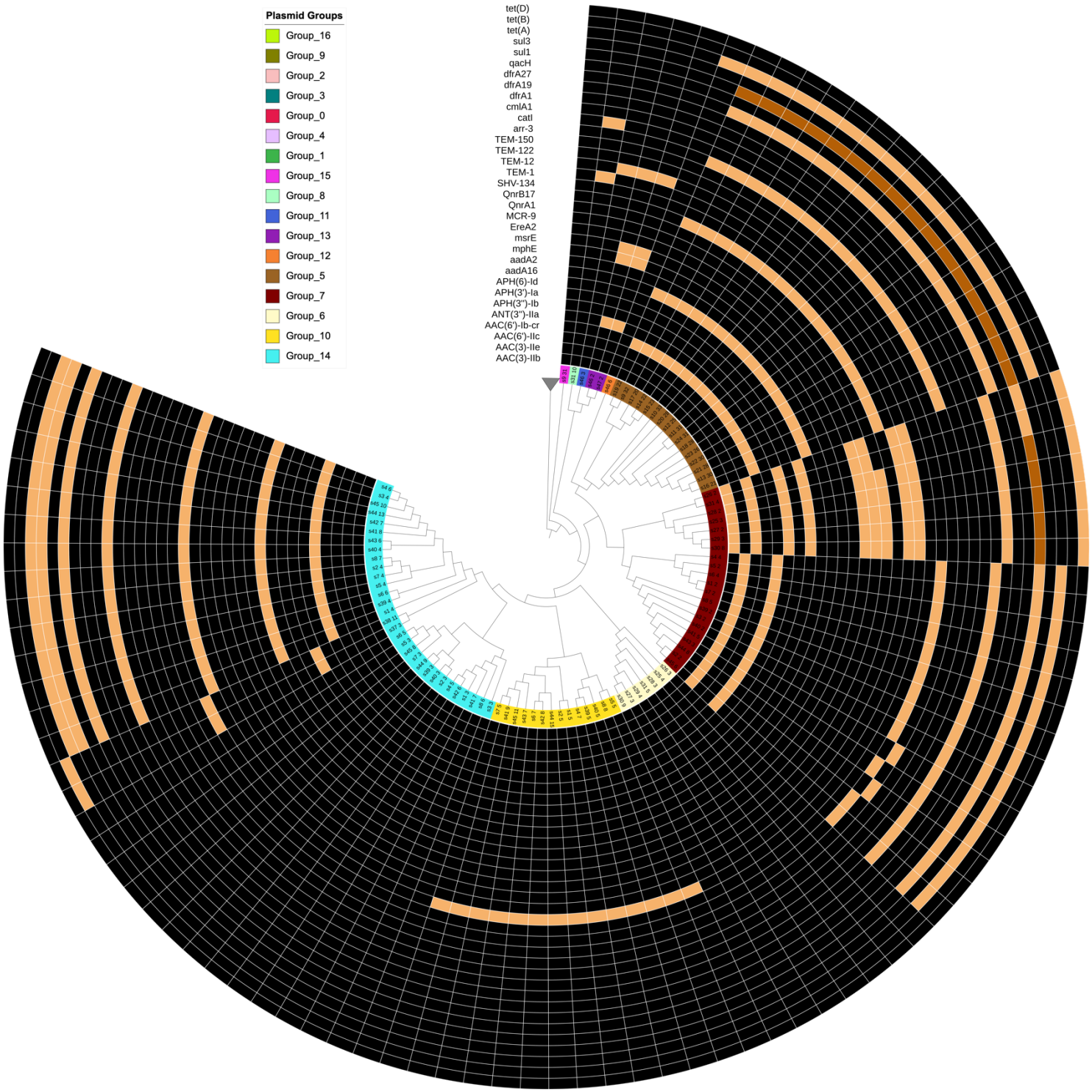


Figure 3: Metal resistance gene presence. Clades that do not contain MG have been collapsed without effecting topology of MG containing plasmids.

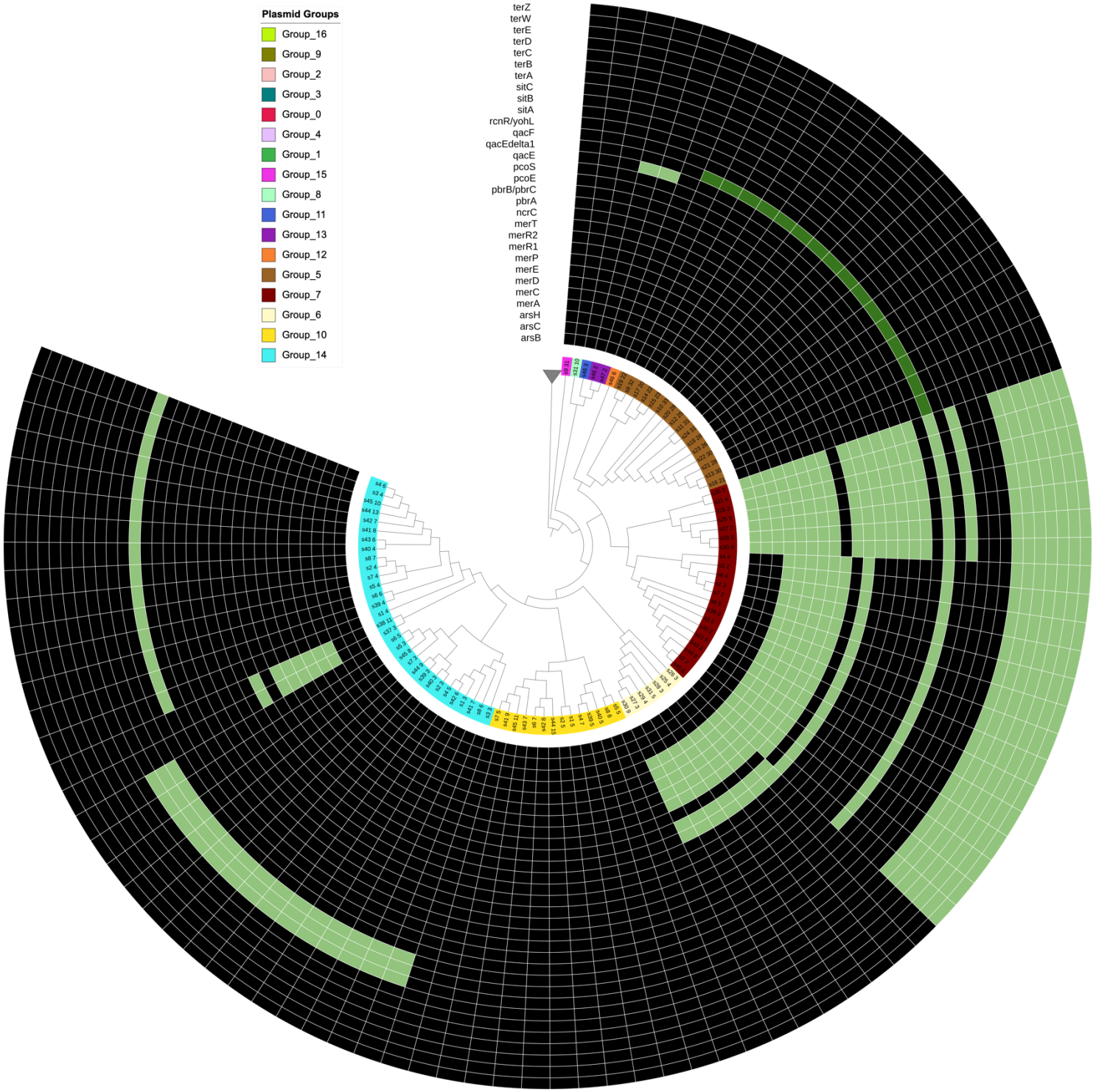


Figure 4: Virulence factors identified.

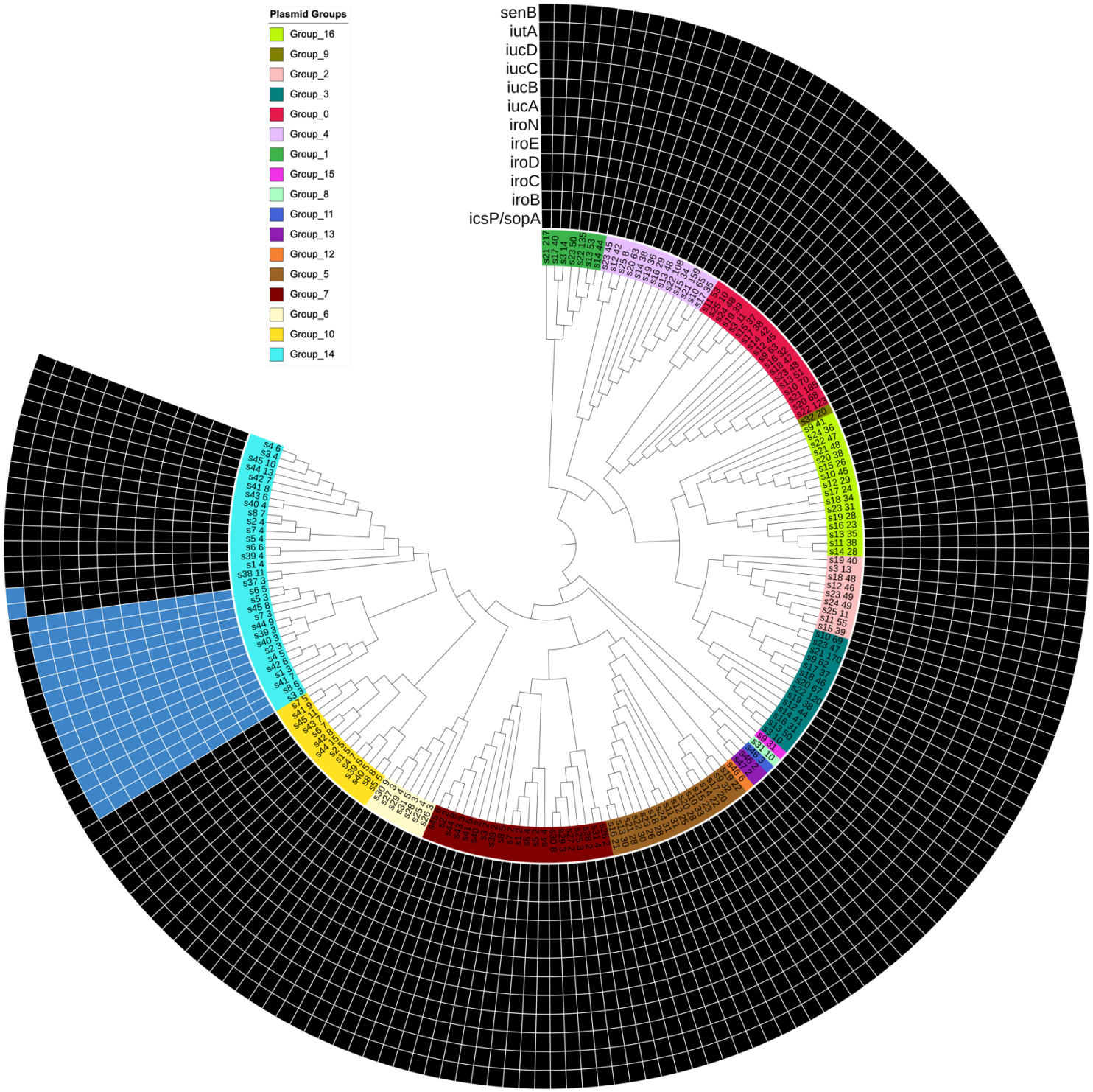


Figure 5: Replicon types identified.

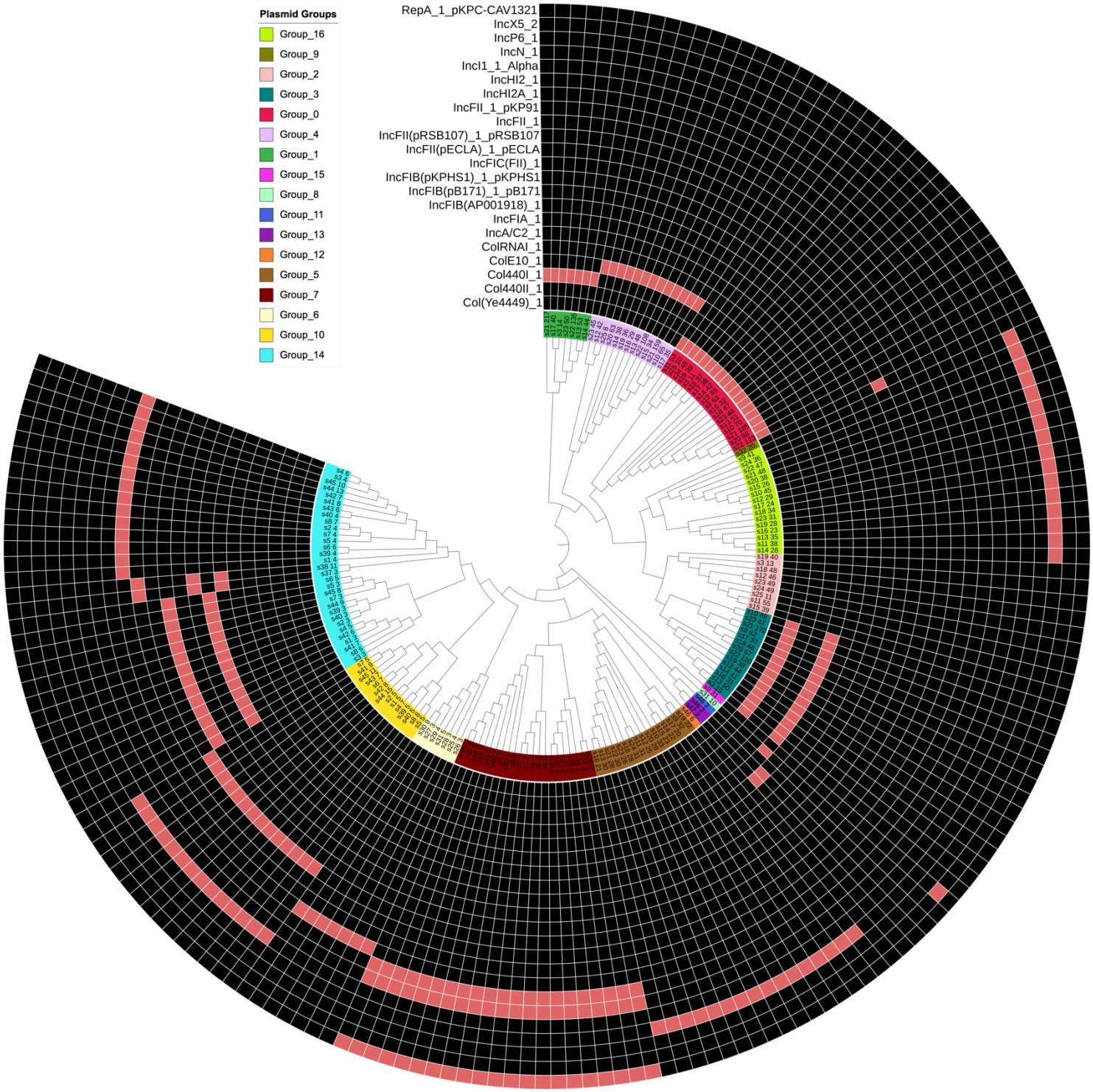


Figure 6: Plasmid phage detected.

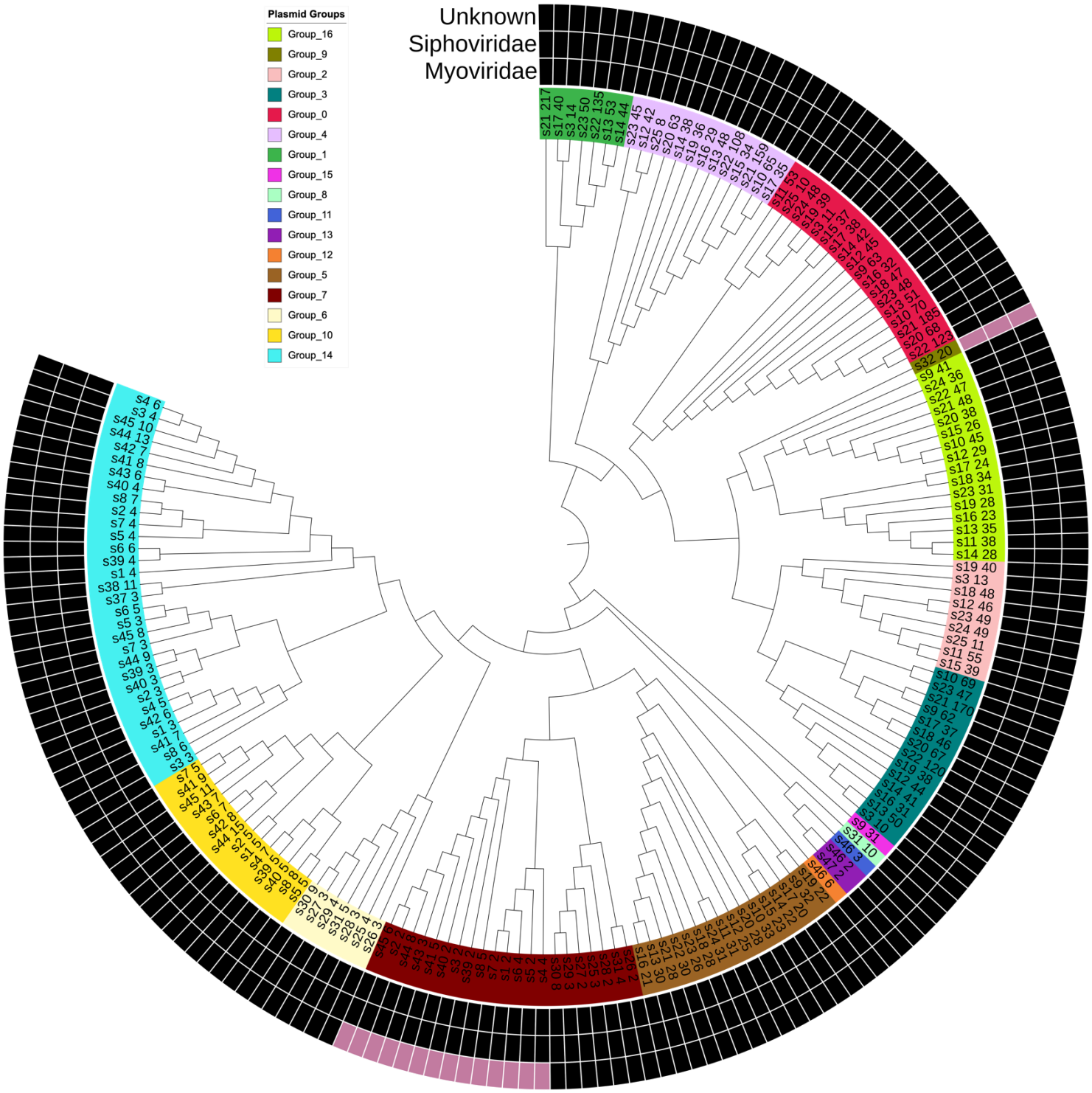


Figure 7: Co-occurrence analysis.

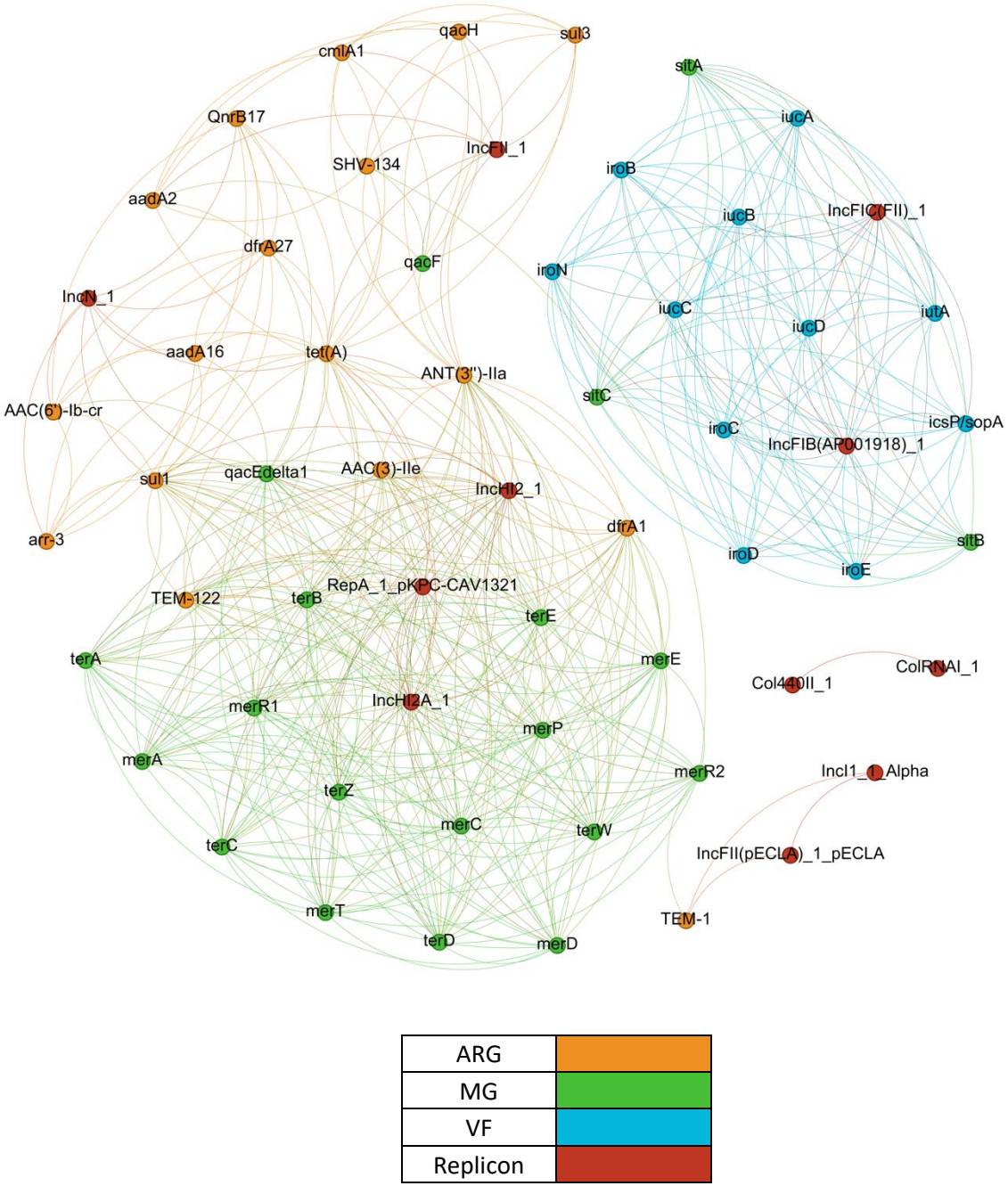
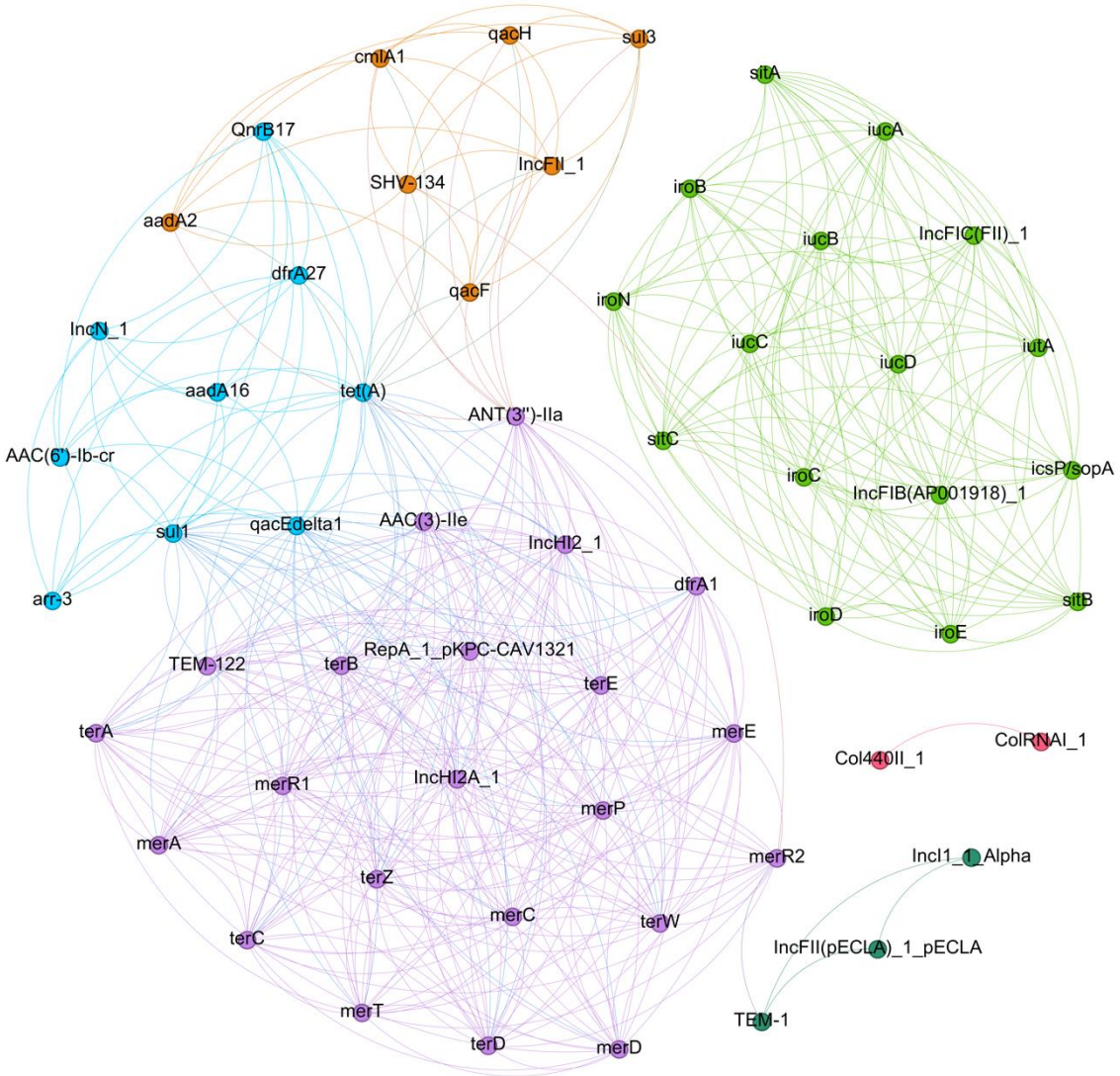


Figure 8: Co-occurrence modularity groups.



Group	
A	
B	
C	
D	
E	
F	

Figure 9: Toxin-antitoxin systems.

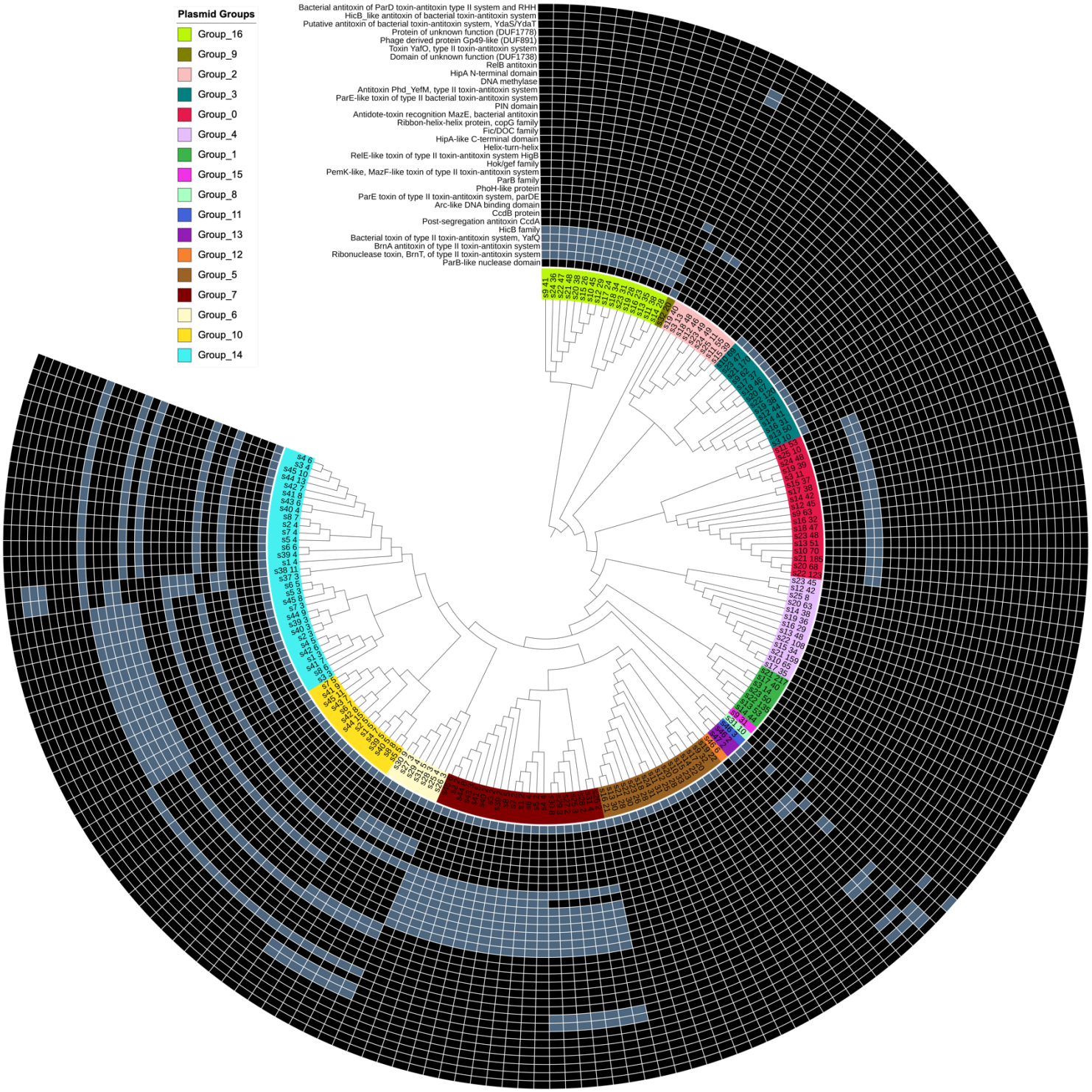


Figure 10: Plasmid Mobility

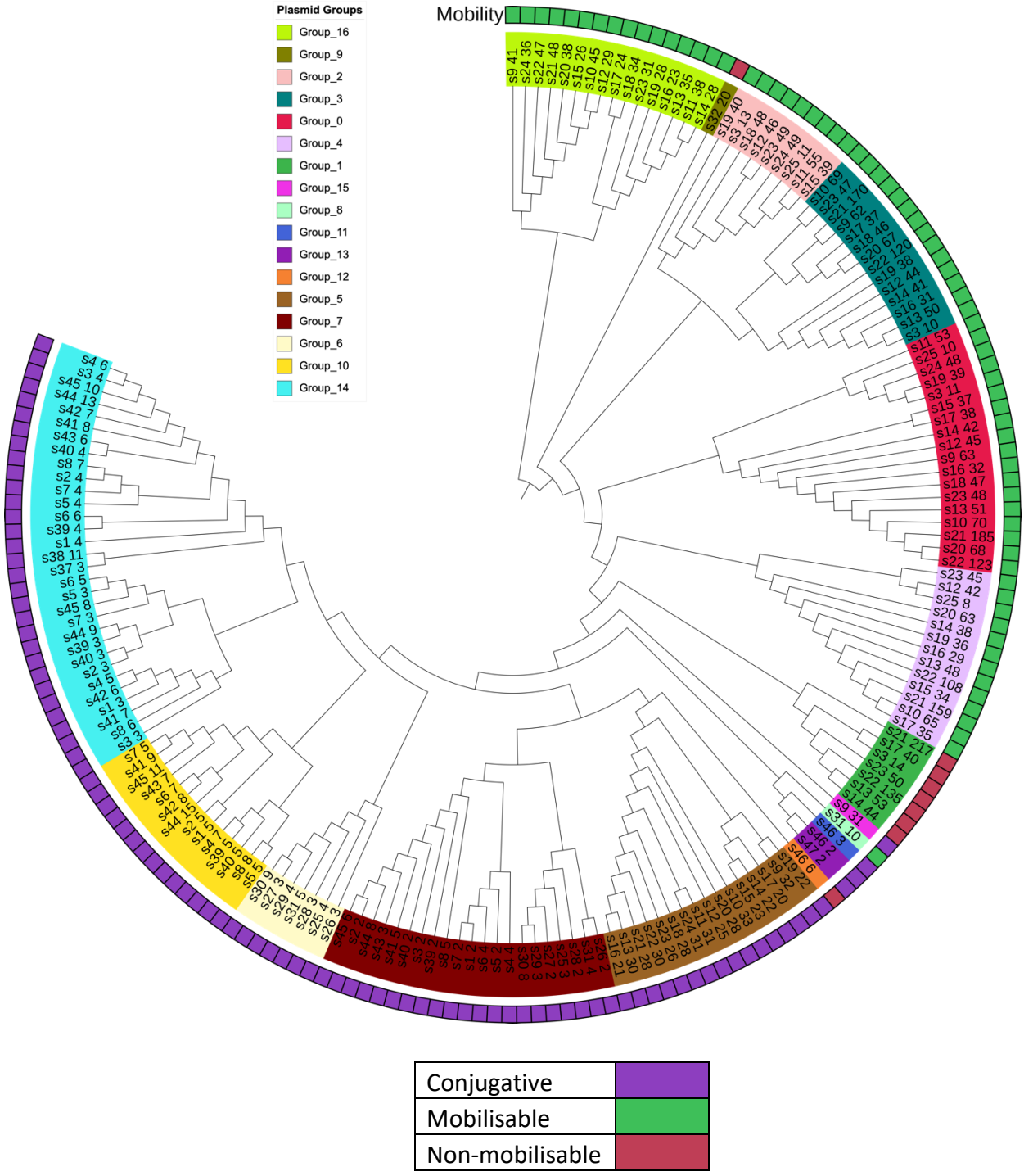


Figure 11: Interplasmid relatedness.

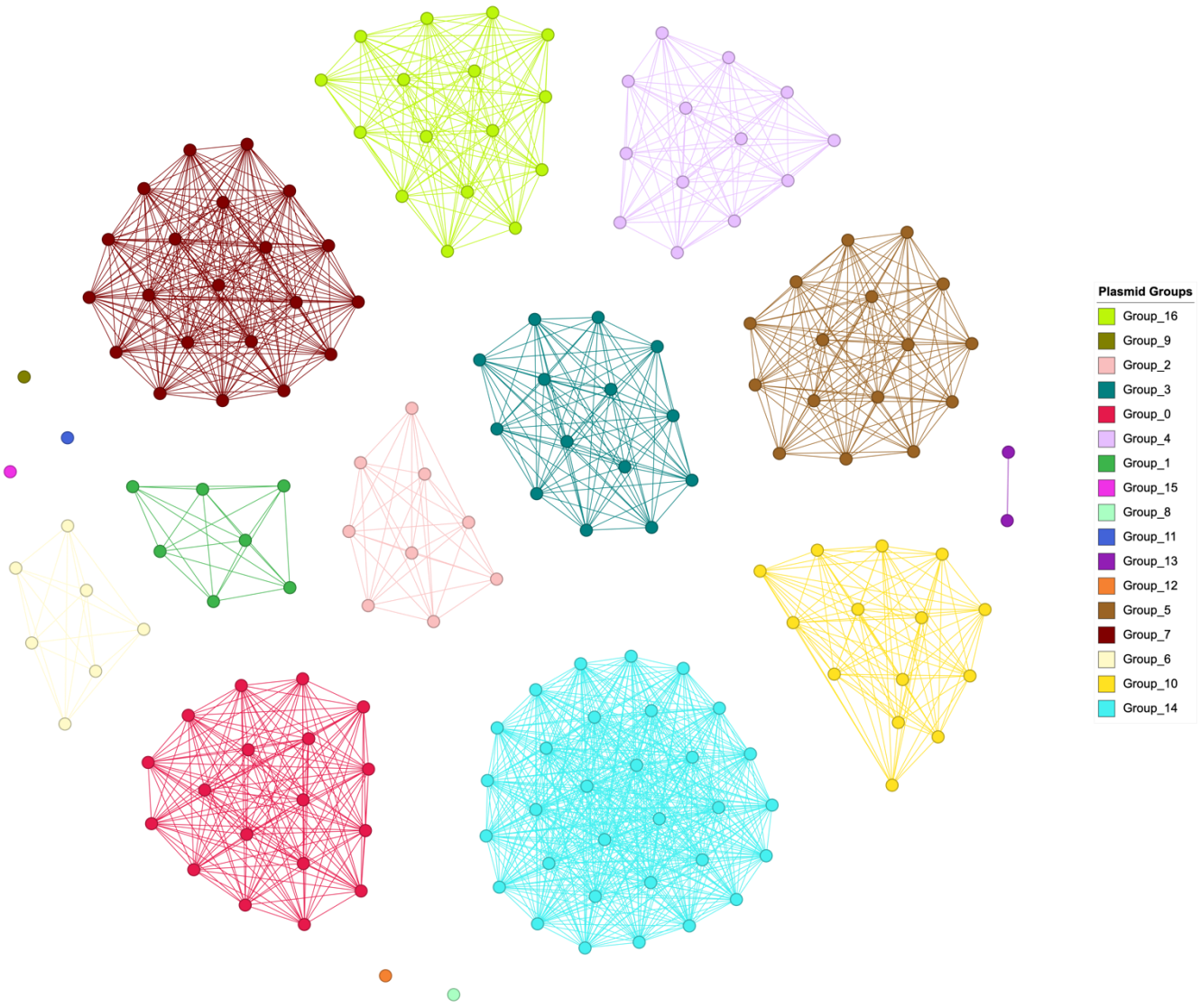


Figure 12: Non-resistant (NR) group.

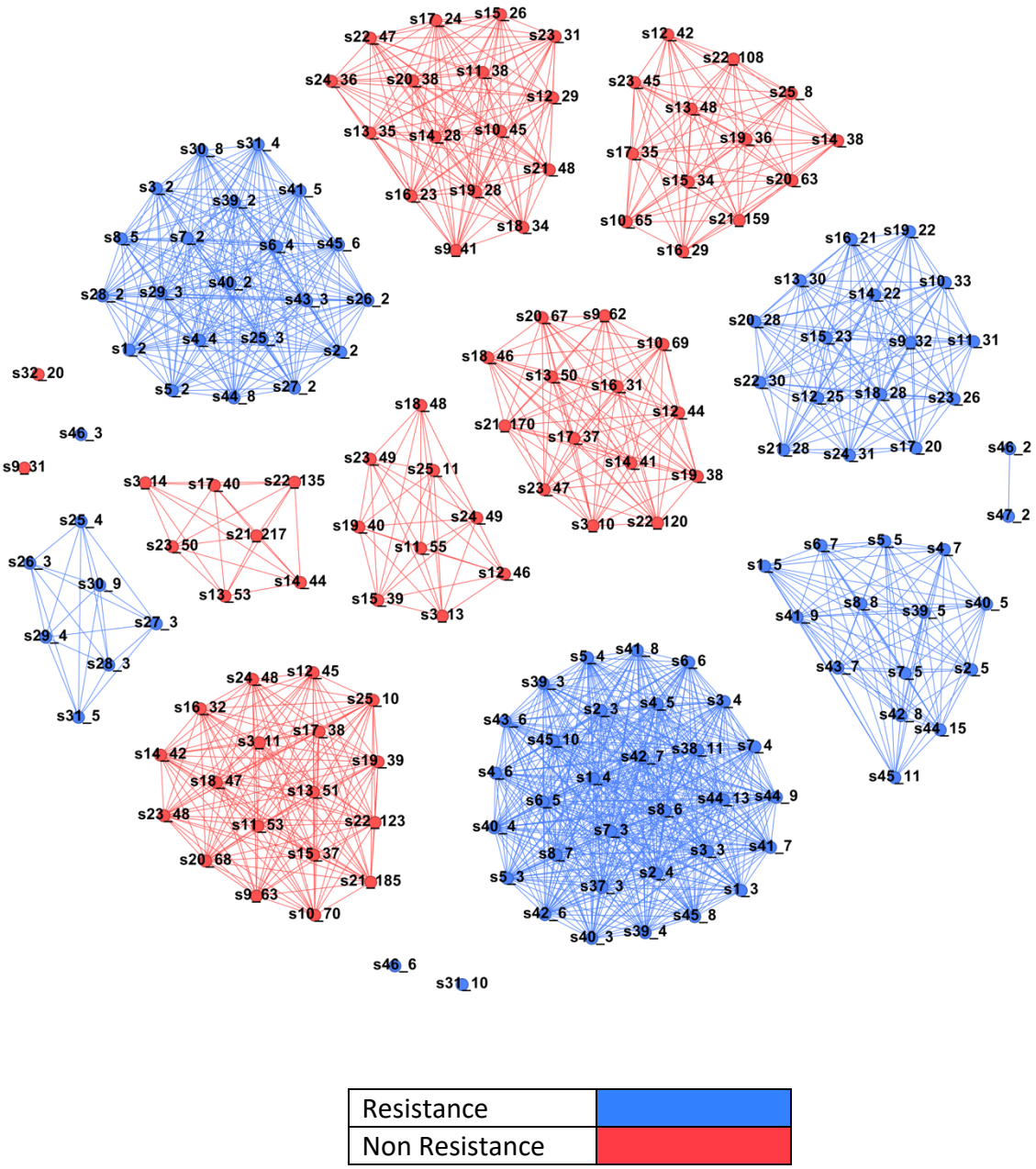
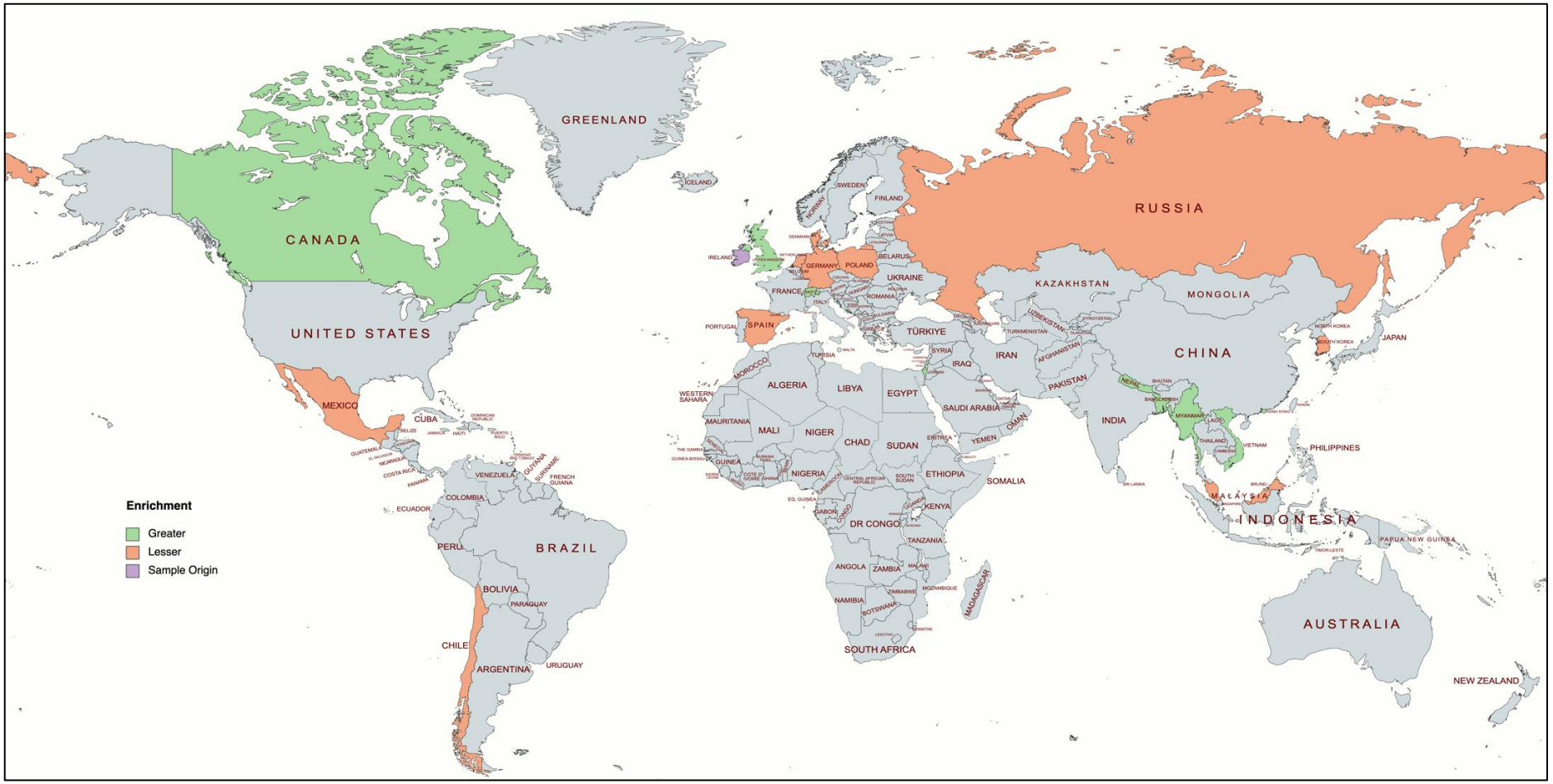


Figure 13: Global plasmid mapping



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Chapter 6

Discussion

6. Thesis Discussion

Antibiotic resistance will remain a prevalent global threat to both human health and the economy, with deaths related to antibiotic resistance expected to overtake cancer by 2050 [103]. The functional importance of plasmid mediated AMR mobility is widely understood, however, despite this, the relative understanding of their role within specific microcosms is generally undetermined. The objective of this work was to further develop this understanding in the microbiome of WWTP effluent and create a basis for future studies into this known hotspot of AMR persistence and dissemination.

The preliminary research discussed sheds light on the resistance patterns of faecal coliforms found in the effluent from two WWTPs in Ireland. It has been shown previously that wastewaters with faecal contamination can be considered a reservoir of both ARB and ARGs within the environment [104,105]. This study was able to establish the higher prevalence of amoxicillin and ampicillin resistance which coincided with the high levels of β-lactam resistance that had been noted previously in *Enterobacteriaceae* in an urban WWTP [106]. In comparison the lower resistance rates of tetracycline, cefotaxime and ciprofloxacin where also noted in this study. The higher resistance rate of *E. coli* to ampicillin and tetracycline as well as lower rates of resistance to ciprofloxacin were detected in WWTPs in Portugal [107]. Raw sewage studies previously have shown a 100% sensitivity of *E. coli* to ciprofloxacin and resistance levels of 50-75% for amoxicillin and tetracycline. The proportions of ciprofloxacin resistant faecal coliforms were 31.42% in WWTP A effluent and 26.47% in WWTP B with this level of resistance being higher than has previously been determined in other WWTPs [106,108,109]. When viewing the studied WWTPs from

a wider context, differences in antibiotic resistance levels were found between WWTP A and WWTP B. Particularly in colistin, trimethoprim and kanamycin. As these two WWTPs are using the same treatment process, this difference may be associated with their location or other external factors.

The presence of colistin and carbapenem resistant isolates in WWTP effluents raises the possibility of ARG dissemination and its subsequent risk to human health. These antibiotics are considered 'last resort' treatments to MDR bacterial infections and the presence of resistance pertaining to both is of great concern. At the time of this study, research in colistin resistant Enterobacteriaceae had mainly focused on food human and animal samples [110–113] with a relative gap in WWTP related knowledge for *mcr* genes [114–118]. This study bolstered evidence of colistin resistance presence within WWTP with 114 faecal coliform isolates resistant to colistin (28 from WWTP A and 86 from WWTP B). Despite the lack of *mcr* genes and the proportion of colistin resistant coliforms in our study being lower than those in the studies of Igwaran et al. [115] , the plasmids extracted from 43 isolates were successfully transferred to recipient *E. coli* DH5α in transformation studies thus providing vital context to the potential of colistin resistance dissemination from faecal coliforms in WWTPs. The percentage of carbapenem resistance phenotypes in our work were considered high in comparison to previous studies in wastewater [119,120]. However, there were no known carbapenem resistance genes detected in the carbapenem resistant coliforms. This suggests that other resistance mechanisms are responsible for the resistance phenotypes and thus require further study to characterise these novel mechanisms.

Multi-drug resistant faecal coliforms were retrieved at high rates from all effluent samples. In the study of Lefkowitz and Durán [121], 60% of *E. coli* in WWTP effluent were resistant to two or more antibiotics and 25% to four or more antibiotics. The study of Garcia, S et. al. [122] in WWTP effluent showed no more than 12% of *E. coli* were resistant to two antibiotics and less than 10% to three or more antibiotics. *Escherichia coli* (34.3%) were found to be resistant to two or more antibiotics and 8.8% to four or more antibiotics in treated wastewater in Portugal (Ferreira Da Silva et al., 2007). The MDR faecal coliforms in our study were found in the same range of the study of Lefkowitz and Durán, but at a higher percentage than in others. The ESBL or AmpC producing faecal coliforms were recovered from all WWTP effluent samples. The rate of ESBL producing *Enterobacteriaceae* in our work were within the range of previous studies. It was considerably high in comparison to some studies of WWTP effluent (0.5–9.8%) [124–128]. However it was lower than those studies in wastewater (45–100%) without treatment [129,130]. The high load of bacteria and rich nutrient environment in WWTPs facilitates the transfer of ARGs among bacteria [131,132]. These may explain the relatively high rates of ESBL producers in WWTP effluent. When discussing this study in the context of specific ARGs, the *bla*$_{\text{TEM}}$ were the most prevalent beta-lactamase group found, which coincides with a study on hospital wastewater in Brazil [130]. However, this is in contrast with other findings with *bla*$_{\text{CTX-M}}$ being the most frequent ESBL genes from various samples including in hospital effluent, surface water and WWTPs [127,133,134]. These results were confirmed by qPCR data in another related project which showed the detection of *bla*$_{\text{TEM}}$ genes, where *bla*$_{\text{SHV}}$ and *bla*$_{\text{CTX-M}}$ group 1 were not detected [135]. Most of ESBL genes were found in *E. coli*, others were carried by Klebsiella spp. in our work. These results are in an agreement with previous findings which indicated

that *E. coli* were the most common ESBL-producers among Enterobacteriaceae [133]. Transformation of plasmids carrying ESBL genes were successful for 88% of ESBL donor isolates in this work. Different replicon variants were found in the ESBL plasmids. The most prevalent replicon was IncF group which were also reported in other studies of plasmid replicon typing in Enterobacteriaceae [136–138]. These replicons have a narrow host range and can be transfer easily among *E. coli* species [139]. The cross-resistance of ESBL producers to other antibiotic causes of great concern as ESBL genes are frequently located on conjugative plasmids carrying other ARGs (Gniadko wski, 2001). In this work 8 of 52 transferable plasmids carrying ESBL genes expressed a MDR phenotype.

Within this study effluent samples from two WWTPs demonstrated the presence of ARB and MDR and, of particular importance, a source of a relatively high proportion of ESBL-producing, carbapenem and colistin resistant Enterobacteriaceae. Although the bacteria were phenotypically resistant to colistin or carbapenems no known mobile resistance mechanisms were detected, despite the ability to transfer the resistance phenotype via transformation. Thus, faecal coliforms from WWTP effluent are sources of novel ARGs conferring resistance to antibiotics of last line of defence. The ability of these bacteria to survive in water has been demonstrated for many years. This study's significance lies in recognising WWTPs as potential control points to curb or halt the dissemination of resistant bacteria and genes into the environment from upstream sources like human or animal waste. Moreover, it paves the way for implementing additional treatment technologies in WWTPs to prevent or diminish the entry of ARB and ARGs into global water systems. Its finding also highlighted the urgency that was required in developing an understanding of possible plasmid based

AMR dissemination pathways as well as AMR transfer in under studied environmental biomes.

As presented previously, there is a clear need for further elucidation of possible plasmid transfer within environmental biomes. When isolating a specific niche to develop a greater understanding, studies have suggested that soil is an important source of bacterial transfer to the phyllosphere [14,15,141–146] and despite its importance no studies have analysed the commonality of the total resistome between soil and the phyllosphere and we have yet to identify any studies that have analysed the total resistome of grass. The study presented clearly demonstrated that the phyllosphere of grass harboured a wider array of both ARGs and MGs while also containing a greater relative abundance of said genes than could be found in soil. The wealth of studies performed on soil in regards to its source as wide range and relative abundance ARGs provided a suitable comparator when developing insight into the relatively unknown resistome of grass. While this study did find that ARGs conferring resistance to multiple antimicrobial classes were shared between soil and grass, grass contained many unique ARGs and also had a significantly higher evenness and richness in its resistome when compared to soil. When determining the possible transmission of ARGs and MGEs between soil and grass it could be seen that despite containing similar genes, soil and grass held significantly different MGEs, thus suggesting that they did not transfer between each other or had transferred to different MGEs once situated on grass. Additionally, it was indicated that common ARGs and MGs in soil had either transferred to different bacteria in grass or were mobilised from sources other than soil due to the disparate correlations between ARG and MGEs with grass and soil bound bacteria. This in turn highlights the range of resistance mobilome

diversity that can be present in proximal niches while providing additional understanding of the crucial necessity to investigate different ecosystems. The discrepancies observed in the relative abundance of ARGs between soil and grass suggest that either the bacteria harbouring these ARGs or the MGEs carrying them are more prevalent on grass than in soil leading to the identification of grass as a largely under studied reservoir of ARGs and possible MGE induced AMR dissemination. Despite requiring further verification, due to the persistence of detected resistomes temporally and consistencies with other studies it could be considered that these findings may be representative of grass resistomes globally. The relative importance of this suggestion cannot be understated due the use of such agricultural lands in the feeding processes of both ruminants and herbivores, thus giving a possible dissemination pathway to the food-chain for a large pool of ARGs.

Our study observed that the common phyla we identified in grass and soil did not exhibit any significant impact when exposed to slurry. A number of studies demonstrating that slurry-derived ARGs and ARB can exist in treated soils for weeks and years post application and also allow for subsequent ARG transfer via HGT [147–155]. According to other studies, slurry-amended soils have been associated with increased detection of ARGs and ARB on lettuce and root vegetables but this result has not been associated with all crops or ARGs, which was further bolstered by our study despite the abundance of ARGs detected. A previous study [156] performed within our research group on soil and lettuce showed contrasting results to those observed in this study on grass and soil. Therefore, this raises the question of whether the lack of impact on the identified phyla is specific to the rhizosphere and phyllosphere of grass. Compositional microbiome analysis in this study found that evenness and PCoA

results were different between soil and grass, reflecting results from Vorholt et al., who found that the communities on leaves not in contact with the soil did not converge with the soil communities this suggests that environmental factors other than slurry may have impacted these significant differences. When comparing our study with the few comparative microbiome studies performed on soil and grass it could be seen that the genera overlap detected in our work (98.7%) was consistent with other findings and despite the overlap, Shannon index and PCoA of the Yan et al. study demonstrated significant differences between the phyllosphere and soil which was mirrored in our study. Despite the overlaps the relative abundancies present varied considerably suggests that while genera are shared across soil and grass, the phyllosphere provides a selective environment for the relative abundances of genera different to the soil to flourish and that filtering of the composition occurs on the grass.

Resistome analysis has previously focused on soil as a potential hotbed of resistance within the environment. This study provided novel insight into the additional potential of grass as a significant reservoir of ARGs and MGEs, even more so than soil. The use of grass in agriculture thus links this pool to animal and human food chains which further enforces the need for surveillance of a diverse set of microbiomes to develop necessary steps to mitigate the global spread of AMR.

Surveillance of global AMR patterns has primarily focused on culturable pathogens, their resistance phenotypes and the identification of associated ARGs. It has been previously show that plasmids play a significant role in the mediation of AMR and MDR gene transfer bacteria, commensals or pathogenic bacteria of humans, animals and the environment [157]. Despite this, most genomic studies have focused on specific

AMR genes or the whole genome sequences determined without resolving the possible plasmid content of isolated samples. This is due to the technical difficulty of plasmid assembly from short-read data due to their inherent repetition and fragmentation. Long-read sequencing technology has allowed for an improved resolution of such plasmids an therefore was used in this study. Our study, through the context of a One Health approach, identified a definitive spread of plasmids globally with distinct relationships to our dataset. However, despite this, the lowest coverage of the One Health triangle determined was that of the environment, which relates to the relatively low number of completed plasmid sequences from environmental samples . This identified a clear gap in knowledge surrounding the AMR present in water, soil or plant samples.

A clear demonstration of whole plasmid global mobility across 63 different countries was demonstrated in this study through the mapping of plasmids and associated metadata. This in turn suggested that the widespread dissemination of AMR genes and host pathogens across was also bolstered by the transfer of entire plasmids. The variability of sequenced plasmids was also of note as no specific pattern from antimicrobial resistance genes, metal resistance genes, virulence genes and replicon types could coalesce all plasmids into a notifiable group. The framework created within this study also suggested a novel path for the investigation of plasmid AMR presence and dissemination in a more global One Health based context. This study also determined the possible reservoir of novel AMR genes that are present within plasmid populations due to the lack of detectable ARGs that conferred resistance to associated phenotypic results. Supportive findings also reinforced the concept of plasmids being pools of AMR with genes conferring a wide range resistance being

found on the same plasmid and multiple resistance plasmids being found in the same sample.

There are many studies of the ESBL profiles and known ARGs conferring resistance in E. *coli* globally across One Health but relatively little information about the plasmids conferring ESBL resistance or other AMR in food animals and their link to those identified in humans, other animals or the environment [158]. Colicin E-type plasmids lacking AMR, virulence genes, or MRGs were identified, indicating that the transfer of colicin E plasmids may occur along with AMR plasmids. This suggests that the use of antimicrobials might also be promoting the selection of colicin plasmids. It is well-established that colicin is a plasmid-borne bacteriocin that selectively kills other *E. coli* cells that lack the same plasmid [159]. Metal resistance and virulence genes associated with extraintestinal pathogenic *E. coli* (ExPEC) and avian pathogenic *E. coli* (APEC). The virulence factors of APEC can include *hlyE*, *cvaC*, *iss*, *fimC*, *tsh*, *lucC* and *sitA* [160]. However, despite this there is no definitive list of virulence genes common to APEC and while it is the agent of coliobacilliosis it is also a commensal of the poultry gut. The plasmids identified contained only *iss*, *sitA* and *cvaC* (*n*=2), from the APEC virulence genes. The *iss* and *cvaC* are significant virulence factors found in neonatal meningitis *E. coli* (NMEC), but not in uropathogenic *E. coli*. However, the plasmids still contained the core ExPEC genes that are present in NMEC, including *iroBCDEN, icsA/sopA, sitABCD*, and *hlyF* (n=4). One plasmid contained *iucABCD* and *iutA*, but none had *ompT* nor *bor*. Although the virulence factors do not conclusively indicate that the plasmid confers virulence in a particular ExPEC, the presence of these genes suggests that the plasmids have the potential to confer virulence in hosts such as poultry and humans.

When viewing plasmid matching results from a temporal perspective, it could be seen that there was a notable bias towards the past decade due to the relevant fall in sequencing costs which subsequently allowed for an increase in frequency of the use genomic sequence analysis. Overall matching also showed a bias towards *Enterobacteriaceae*, specifically the genera of *Escherichia, Salmonella* and *Shigella.* Throughout all groups of plasmids, there was a common trend in which resistance genes and virulence factors were periodically integrated and then lost over time. This implies that there are various evolutionary pressures acting on these plasmids. Nonetheless, the plasmid network remains highly successful and adaptable, demonstrating a flexible and dynamic plasmid backbone. Furthermore, since the plasmid network is non-transitively retained, this suggests that plasmids with similar characteristics are more likely to be transferred between bacteria, contributing to the widespread dissemination of these traits throughout bacterial populations. Despite the global persistence of most plasmid groups, neither resistance nor virulent genotypes seem to be largely geographically enriched. Three of four plasmid groups in this study were most associated with clinical isolates, while one was associated with agriculture, every plasmid except one could also be associated with food products. Genes conferring resistance to β-lactams and tetracyclines were found across all samples and these commonly observed in agricultural isolates, showed a clear consistency with our current understanding [161]. The detection of *Siphoviridae* phage and its associated Ig domain-like genes suggested its important role in the development of wider virulence as these proteins have been found to function as invasins and adhesins in both enteropathogenic E. coli (EPEC) and enterohaemorrhagic E. coli (EHEC) promoting the development of diarrhoeal disease [162].

This study provided a novel perspective into the global nature of resistance and virulence when viewed through a temporal, food-chain bound lens. Compelling evidence of the globetrotting nature of AMR plasmids and the subsequent necessary increase in surveillance that must be completed was determined while also providing a novel framework in which such analysis can be performed with a global One Health Outlook. The presence of plasmids that may have contained novel AMR mechanisms as well as the possibility of co-selection through metal and QAC use was also identified and thus elucidated the need for further investigation into plasmids role in the dissemination of ARG and AMR worldwide and their risks to human, animal environmental health. This study further identified the distinct need to investigate plasmids impact on the dissemination and persistence of AMR within a One Health context globally.

The previous studies discussed in this thesis have developed the understanding that environmental and animal microbiomes worldwide have a significant impact on the possible dissemination and transfer of AMR. A significant observation identified through these studies was the key role plasmids play in this dissemination within their specific niches as well as the prevalence and extended reach these plasmids can have when looking at AMR spread in a global context. The culmination of these efforts pointed towards the unique threat that plasmid bound AMR HGT poses for humanity in both health an economic terms and thus further enquiries into their relevance to this process were established.

As has been previously mentioned, WWTPs are considered notable reservoirs of AMR. For this reason, the relationship of HGT through plasmids in this environmental niche was determined to be an ideal basis for the development of understanding into possible AMR dissemination and subsequent global interactions. With the advent of long-read sequencing, there has been a notable advantage in plasmid sequencing that was previously unavailable. While short-read WGS had been utilised in the past, its specific flaws in regards to full plasmid resolution led to a distinct lack in thorough plasmidome studies. In this study, hybrid sequencing was utilised as a novel pathway for the resolution of fully closed plasmids and their subsequent genotypic characteristics. The high quality sequences produce through this method allowed for an accurate analysis of mobile ARG presence and related characteristics while also providing a foundation for further comparative environmental plasmidome analysis.

Circularisation of 173 plasmids was completed in this study with two distinct phylogenetic grouping appearing. This phylogenetic distinction was mirrored in the presence of both ARGs and MGEs with one group containing no identifiable resistance mechanisms while the other contained plasmids harbouring a diverse range of ARGs, MGEs and virulence genes. This confirmed previous reporting that WWTP was a significant potential source of AMR [163]. The prominence of plasmids lacking ARGs, MGEs and VFs is of particular note within this study. The classification of 46% of isolated plasmids within Irish WWTP effluent that appear to persist despite having no apparent selective advantage nor confer a selective marker has not been previously noted. The identification of toxin-antitoxin systems within some plasmids of this group could point to an explanation of their presence, however a notable lack of obvious genes that could explain their persistence was found. The determination of

mobility in this group and their relative small size did however suggest that these plasmids could be candidates for possible co-transfer with larger conjugative plasmids found in this study. The presence of numerous hypothetical genes could also suggest that these plasmids contain currently uncharacterised mechanisms that could account for their ongoing survival. The ancestry of these plasmids also showed that they form distinct groups unrelated to those that contain known drivers of persistence such as ARGs. This suggests that the plasmids have not likely previously held ARGs and instead have evolved separately and consequently must have their own factors for persistence and survival. This observation leads to the possibility that these non-resistant plasmids are more common than expected and could have been inadvertently ignored in previous studies due to the studies focus on ARG/MGE determination and short-read exclusive sequencings inability to fully resolve circularised plasmids thus leading to their characterisation as fragmented assemblies [59].

The identified statistical enrichment within this sample population for megaplasmids was of note due to the abundance of ARGs, MGEs and VFs they contained. When looking in a comparative context, their enrichment when compared to the PLSDB plasmid database suggested that an explicit rationale or justification is necessary for their continued existence despite their hypothesised high metabolic fitness cost. Previously it has been shown, that Irish WWTP effluent contains some of the highest antibiotic concentrations in Europe [164] and this could provide a setting where the collective selective pressure against multiple antibiotic classes allows for the development and perseverance of MDR megaplasmids. Coincidentally, the plasmids also contain a large number of MR and VFs which could possibly bring about survival through effective mutualism with their host, when present in pathogenic bacteria.

Some however, contained TA-systems that would suggest a more parasitic persistence coevolutionary synergy[165]. The plasmids large size, multiple interspersed incompatibility groups, and polarised compositional distances between otherwise unrelated plasmids could also suggest that these megaplasmids are the result of the fusion of smaller plasmids coexisting in the WWTP environment.

Further comparison with the PLSDB plasmid dataset identified significant enrichment for both animal and human waste related plasmid matches while those relating to environmental samples were decreased. The findings imply that the existence of these plasmids in the effluent environment of the WWTP might be attributable to the infiltration of animal waste into the water source. This corresponds with the specific animal-associated resistance profiles mentioned earlier, indicating a possible increased necessity for monitoring to mitigate any possible public health hazards. When looking in a global context enrichment and purification of PLSDB plasmid matches did not show any specific geographic trends which suggests to the global nature of resistant plasmid transfer or possible convergent evolution through shared microbial niche pressures.

This study consolidated the efforts of the studies mentioned previously to develop a key insight into the prevalence and perseverance of mobile plasmid bound resistance. Through the utilisation of long-read NGS, and relevant computational methodologies a framework for the further analysis of mobile AMR was created. The identified global nature of the analysed WWTP effluent plasmids also bolstered the ever-growing evidence to suggest periodical and consistent surveillance of environmental reservoirs of AMR in order to effectively mitigate potential public health issues.

When discussing the previously presented studies and their impact on the field of AMR research, it can clearly be seen that they have provided novel insight into key areas of possible plasmid persistence and dissemination. The broad range of microbiomes studied consolidated a clear theme of resistance transfer through plasmid HGT mechanisms while also providing the necessary microbiome characterisation to give context within each study. The utilisation of state of the art methodologies and techniques also provided a novel framework from which future plasmidomic work can be performed while developing a prescient view towards plasmids role as a global purveyor of antimicrobial resistance.

Chapter 7

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7. Bibliography

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