

Antibiotic resistance in *Escherichia coli* isolated from United Kingdom dairy cow faeces

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Abbreviations

2YT	Double yeast extract tryptone broth
ABC	ATP binding cassette family
ABR	Antibiotic resistance
AHDB	Agricultural and Horticulture Development Board
AMC	Amoxicillin-clavulanate
AMP	Ampicillin
AMR	Antimicrobial resistance
AMU	Antimicrobial usage
APHA	Animal and plant health agency
ARG-ANNOT	Antibiotic resistance gene-annotation tool
ASL	Antibiotic susceptibility level
ASP	Antibiotic susceptibility phenotype
ASR	Antibiotic susceptibility result
AST	Antibiotic susceptibility testing
C	Chloramphenicol
CARD	Comprehensive antibiotic resistance database
CFU	Colony forming unit
CIA	Critically important antimicrobials
CIP	Ciprofloxacin
CLSI	Clinical and laboratory standards institute
CTX	Cefotaxime
DCA	Dairy calf
DEFRA	Department for environment, food, and rural affairs
DEFRA	The Department for Environment Food and Rural Affairs
DMSO	Dimethyl sulfoxide
dNTP	Deoxyribonucleotide
EcOH	The database of known *E. coli* O-groups and H-types
EDTA	Ethylenediaminetetraacetic acid
EFSA	European food safety authority
EMA	European medicines agency
ERIC	Enterobacterial repetitive intergenic consensus
ESBL	Extended spectrum β-lactamase
EUCAST	European committee on antimicrobial susceptibility testing
HP-CIA	Highest priority-critically important antimicrobials
K	Kanamycin
KST	Kolmogorov-Smirnov test
KWT	Kruskal-Wallis ANOVA with Dunn's multiple comparisons test
LB	Luria-Bertani broth
LCA	Lowest common ancestor
MAG	Metagenome assembled genome
MARG	Metagenome assembled genome containing antibiotic resistance gene
MASD	Median antibiotic susceptibility distribution

MDR	Multidrug resistant
MFS	Major facilitator superfamily
mg	Milligram
mg/kg	Milligram of active ingredient of an antimicrobial per kg of average animal during treatment
MGE	Mobile genetic element
MH	Mueller-Hinton Agar
MIC	Minimum inhibitory concentration
MLST	Multilocus sequence typing
MMR	Medicated milk replacement
MRSA	Methicillin resistant *Staphylococcus aureus*
NA	Nutrient agar
NB	Nutrient broth
NCBI ARG	NCBI bacterial antimicrobial resistance reference gene database
OD	Optical density at 600nm
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PPD	Post-parturition dry cow
PPL	Post-parturition lactating cow
PRP	Pre-parturition cow
RND	Resistance nodulation cell division superfamily
RO	Reverse osmosis
RUMA	Responsible use of medicines in agriculture alliance
S10	Streptomycin
S300	Sulfonamide
SB Farm	The Centre for Dairy Science Innovation on the Sutton Bonington Dairy farm
SIE	Susceptible with increased exposure
SVMS	University of Nottingham School of Veterinary Medicine and Science
TBX	Tryptone bile X-glucuronide agar
TE	Tetracycline
TEB	Tris EDTA buffer
UTI	Urinary tract infection
VARSS	Veterinary antimicrobial resistance and sales surveillance report
VCD	VARSS clinical surveillance of antibiotic resistance dataset
VMD	Veterinary medicines directorate
WGS	Whole genome sequencing
WHO	World health organisation
ZOI	Zone of inhibition

Ethical approval

This research project was reviewed and approved by the University of Nottingham School of Veterinary Medicine and Science (SVMS) Ethical Review Committee, approval number 2200 180202.

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Abstract

Antimicrobials are essential tools in modern human and veterinary medicine, but antimicrobial resistance (AMR) is reducing their effectiveness. The global AMR objective of increased stewardship to achieve sustainable antimicrobial use (AMU) has been a priority for many years, with increased attention after 2015. This approach has significantly reduced AMU overall in some key areas. However, the impact of reduced AMU on the risk of AMR needs to be better understood through surveillance of AMU and AMR. This thesis directly contributes to this global objective, making it a crucial part of the ongoing fight against AMR. Specific challenges in the dairy industry have resulted in significant AMU and AMR data gaps. Is the available data sufficient to understand the current risk of resistance, gauge the success of current approaches and inform future decision-making? This work presents a baseline for the antibiotic susceptibility of bovine faecal *Escherichia coli (E. coli)* from dairy cows on a single farm in the United Kingdom (UK); it also investigates the impact of chemotherapeutic treatment given to cows and the physiological development of cows. Over three years, 38 convenience faecal samples were collected from different bovines across several developmental stages from a single farm. *E. coli* was selected as the model organism for faecal bacteria as *E. coli* are well-characterised commensal bacteria and opportunistic pathogens in humans and bovines. A combined approach of direct culture on selective media and antibiotic susceptibility testing of faecal *E. coli* (the current gold standard) with next-generation short-read sequencing, shotgun metagenomics, and *in silico* phenotype prediction was used. The total outputs of this combined approach were the isolation of over 800 bovine faecal *E. coli*

with antibiograms to six classes of antibiotics, the identification of three transmissible MDR phenotypes, 160 bovine faecal *E. coli* whole genomes, and 20 bovine faecal metagenomes. Resistance to several important antibiotics and the associated antibiotic resistance genes have been identified. The genetic diversity of these *E. coli* and the diversity of the faecal metagenomes from which they were isolated were also investigated. This thesis demonstrates that chemotherapy increases the proportion of antibiotic resistant *E. coli* in faeces and that this proportion decreases as time after treatment increases. The antibiotic susceptibility phenotypes of the *E. coli* from all of the adult animals were similar. However, a higher level of antibiotic resistance was identified in the *E. coli* sourced from dairy calves. The differences in the faecal metagenomes of these developmental groups provide a possible explanation for these differences in *E. coli* antibiotic susceptibility. Differences in animal physiology and diet affect the faecal metagenome, affecting the microbiome composition. The findings of this thesis contribute evidence for the data gaps around AMR in the UK dairy industry and inform discussions around antimicrobial stewardship, dairy cow faecal microbiomes, and the need for biosecurity measures focusing on calf health as a priority. In conclusion, this thesis represents a significant investigation of antibiotic resistance in *E. coli* isolated from UK dairy cow faeces.

Declaration

I declare that the work in this dissertation was carried out in accordance with the regulations of the University of Nottingham.

The work is original and has not been submitted for any other degree at the University of Nottingham or elsewhere.

Name: Caleb Jonathan Marsh

Signature:



Date: 20/12/2024

Chapter 1 Introduction

1.1 Antimicrobial resistance, a global challenge of critical importance

Antimicrobial resistance (AMR) is the evolution of microorganisms to survive in the presence of antibiotics, antiprotozoal, antiviral, and antifungal compounds. This ability of adaptation to protect against selective pressures (such as antibiotics) is ancient, but human activities have been identified as key drivers in increasing the prevalence of AMR (Perron *et al.*, 2015; Booton *et al.*, 2021). Unlike traditional pandemics caused by a single organism, such as SARS-CoV-2, the challenge of AMR involves multiple pathogens and diseases. These pathogens can harbour different antibiotic resistance genes (ARG) and are spread across various sectors (World Health Organization *et al.*, 2023). It is undeniable that AMR currently represents a global threat to human health (Murray *et al.*, 2022). In 2014, the cost of antibiotic resistance (ABR) in hospitals in the United States of America exceeded $2 billion (Thorpe, Joski and Johnston, 2018). Models that assumed no significant response to AMR between 2016 and 2050 predicted up to 10 million deaths annually by 2050 and economic losses of $100 trillion cumulatively (KPMG, 2014; Taylor *et al.*, 2014; O'Neill, 2016). The models underpinning these predictions have been criticised for lacking context when discussing veterinary use and for assuming the loss of all current medicines used to treat major bacterial pathogens and HIV (de Kraker, Stewardson and Harbarth, 2016; NOAH, 2016). Other attempts at modelling the future of AMR in *E. coli* and *Salmonella* predict that 50% of the isolates from Africa and South America will be resistant to penicillins or tetracyclines in the next 2-12 years (Zhao *et al.*, 2024). During the same period, 50% of the isolates from Asia are predicted to be resistant to

penicillins or sulfonamides (Zhao *et al.*, 2024). The worst-case scenario does not need to be realised for AMR to have significant impacts; bacterial AMR alone was estimated to have resulted in 1.27 million human deaths and was associated with a further 4.95 million deaths in 2019 (Murray *et al.*, 2022). This has been predicted to increase to 1.91 million human deaths due to bacterial AMR and 8.22 million deaths associated with AMR per year by 2050 (GBD 2021 Antimicrobial Resistance Collaborators, 2024). The importance of AMR has been stated by the United Nations and its agencies, such as the World Health Organization (WHO), the Food and Agriculture Organization, and the World Organization for Animal Health. These organisations published a global action plan in 2015 and outlined a One Health research agenda for AMR in 2023 (WHO, 2015; 2023). A recommendation of this global action plan was the development of national-specific documents, with 161 currently listed in the WHO library of national action plans (WHO, 2024). Context from veterinary medicine is an area that many of these plans highlight, with 118 countries reporting antimicrobial use (AMU) in animals in 2017 (World Organisation for Animal Health, 2021). This veterinary context is vital because AMR is a global problem affecting multiple sectors beyond human medicine, including veterinary medicine (Zhao *et al.*, 2024). Dairy cattle specifically have been identified globally as reservoirs of antibiotic resistant bacteria that represent a risk to animal, environmental, and human health (Sawant *et al.*, 2007; Mao *et al.*, 2015; Madoshi *et al.*, 2016; Thomas *et al.*, 2017; Doster *et al.*, 2018; X. Li *et al.*, 2018; Barlow *et al.*, 2022; Shoaib *et al.*, 2023). Animals are an important reservoir of infectious agents, approximately 60% of emerging human infectious diseases are of zoonotic origin, with 72% of these traced back to wildlife (Jones *et al.*, 2008). The environment is a further reservoir of AMR,

with high concentrations of antimicrobials identified in the environment around antibiotic manufacturing facilities, as well as waste water treatment plants, rivers, seawater, and agricultural land (Tahrani *et al.*, 2016; Bielen *et al.*, 2017; Chukwu *et al.*, 2023; Khmaissa *et al.*, 2024). Environmental reservoirs of AMR are also maintained through AMU, with 30% - 90% of the antibiotics prescribed in humans and animals excreted into the environment in urine and faeces (Halling-Sørensen *et al.*, 1998; Alcock, Sweetman and Jones, 1999; Winckler and Grafe, 2001; Heberer, 2002; Jjemba, 2002b, 2002a; Bound and Voulvoulis, 2004; Phillips *et al.*, 2004; Kumar *et al.*, 2005). The amount of antibiotic active ingredient and metabolic residuals excreted vary by the antibiotic, the dosage, the species of the patient, and the age of the patient (Zhao, Dong and Wang, 2010). The manure of animals in industrial farming has been identified as a significant source of veterinary antibiotics in the environment (Burkhardt *et al.*, 2005; Kay, Blackwell and Boxall, 2005; Kim and Carlson, 2005; Kim *et al.*, 2005). Tetracyclines are an example of highly stable antimicrobials that have been shown to persist in soil for longer than a year, with only slight degradation of the active ingredient after 180 days (Zuccato *et al.*, 2000; Hamscher *et al.*, 2002). Oxytetracycline, tetracycline, and chlortetracycline residues were identified at higher frequencies in soil samples that had been treated with animal manure two days before sampling (93%, 88%, and 93%), many times greater compared to untreated soil (38x, 13x, and 12x) (Zhang, Luo and Zhou, 2008; Zhang, Wang and Zheng, 2008; Zhang, Zhang and Gu, 2008). This multi-sector importance of AMR (human, animal, and environment) makes it a One Health challenge, requiring solutions that consider the risks in each sector and the connections to other sectors of global health (WHO, 2015).

1.2 Determining the antibiotic susceptibility of micro-organisms

Antibiotic susceptibility is contextual; for clinicians and veterinarians, this term informs their prescribing and treatment decision making. If a given infection will not be killed by certain antibiotics this equates to resistance, but this varies by host, pathogen, and site of infection. It is not possible to simulate physiological conditions in a laboratory environment, meaning that it is not possible to determine if a given isolate is susceptible to a given antibiotic directly. Antibiotic susceptibility testing (AST) involves highly standardised assays with an extensive database of clinical isolates to provide breakpoint concentrations, correlating antibiotic concentration to clinical outcomes of antibiotic chemotherapy for important pathogens (EUCAST, 2019).

The Kirby-Bauer disk diffusion method uses paper disks containing a known concentration of an antibiotic along with tightly controlled variables to provide zones of inhibition around these disks. The reliability of the zone of inhibition is increased by controlling variables such as the media composition, inoculum, and temperature. The inhibition presents as a zone based upon the rate of diffusion of the antibiotic and the minimum inhibitory concentration (MIC) of the isolate. A susceptible isolate will have a lower MIC and therefore a larger zone of inhibition (ZOI). EUCAST collects data from several sources and relates susceptibility observed in clinical settings to zones of inhibition surrounding paper disks containing the antibiotic of interest. By controlling temporal and spatial variables, the results can be more reliably compared across labs around the world. There have been discussions around interpretation of disk diffusion results, with the traditional options being susceptible, intermediate, and resistant. Intermediate was used as a tolerance

for error in the method as well as a suggestion that clinical outcomes may be more variable. EUCAST has changed terminology shifting to susceptible, susceptible with increased exposure (SIE), and resistant. This new definition of intermediate as SIE provides clinicians with more information on how to proceed in treating an infection. If the site of the infection allows for increased exposure then therapy can continue, however if the site of infection is pharmacokinetically privileged then an alternate antibiotic will be selected. This approach is highly standardised to enable comparison of tests completed by different operators, at different times and in different regions.

1.2.1 The importance of the interpretation of antibiotic susceptibility testing

Incorrectly interpreting SIE as non-susceptible could result in inefficient antibiotic prescription negatively impacting stewardship efforts. The risk in grouping SIE with resistant is that if an organism presents as SIE to all antibiotics except one (making it a CIA) then clinicians would select the antibiotic that the organism is susceptible to as opposed to increasing the dosing regimen of the non-CIA. This outcome has been observed in the prescription practices of meropenem for *Pseudomonas aeruginosa* (*P. aeruginosa*) at Lausanne University Hospital, Switzerland (Munting *et al.*, 2022). When SIE was introduced, *P. aeruginosa* MIC cut-offs for susceptibility to piperacillin-tazobactam, ceftazidime, and cefepime reduced while cut-offs for resistance did not change. This change recharacterized the majority of previously susceptible *P. aeruginosa* strains to SIE. This was found to increase the proportion of meropenem prescription (3.2% to 30.2%) and decrease the proportion of piperacillin-tazobactam prescription (52% to 35.3%) for *P. aeruginosa* (Munting *et al.*, 2022). Over time this prescription

practice would likely increase the proportion of strains that are resistant to the last remaining fully effective antibiotic.

The antibiotic susceptibility of the *E. coli* isolates collected in this work will not be used to guide prescription decisions. Therefore, SIE in this thesis can be interpreted differently to how it would be interpreted in a clinic. In this thesis, SIE to a given antibiotic is interpreted as unpredictable antibacterial efficacy with an increased likelihood of resistance emerging. The antibiotic susceptibility of the *E. coli* isolates collected in this work will not be used to guide prescription decisions, therefore SIE is interpreted as a given antibiotic would have unpredictable antibacterial efficacy and that the likelihood of resistance emerging from a population of SIE isolates is greater than from a population of susceptible isolates. Antibiotic susceptibility level (ASL) descriptors are used to provide context to an antibiotic susceptibility result (ASR) (European Food Safety Authority, 2022; Veterinary Medicines Directorate, 2023b). Without this context, it is not possible to determine the level of risk associated with a given proportion of resistant isolates.

1.3 The United Kingdom dairy herd: its antibiotic usage and level of antibiotic resistance

The Agriculture and Horticulture Development Board (AHDB) analysed data from the Department for the Environment, Food and Rural Affairs (DEFRA) on the size of the UK milking herd and annual milk production. From 1995 to 2010, the average size of the UK milking herd declined from 2.619 million head to 1.853 million head, a 29.25% reduction (Figure 1.1) (AHDB, 2023). Since 2010, the size of the UK milking herd increased to 1.906 million in 2015 and reduced to 1.844 million head cows in 2022. Over the same period of 1995 to

2022, the average annual milk yield per cow increased from 7406 litres to 8090 litres (Figure 1.1) (AHDB, 2023). Practitioners will likely continue attempting to optimise their approaches for maximum output with minimum cost. In 2013, more than 90% of dairy cattle had access to external pasture, with only 6% of milking cattle housed continuously (Table 1.1) (DEFRA, 2013).

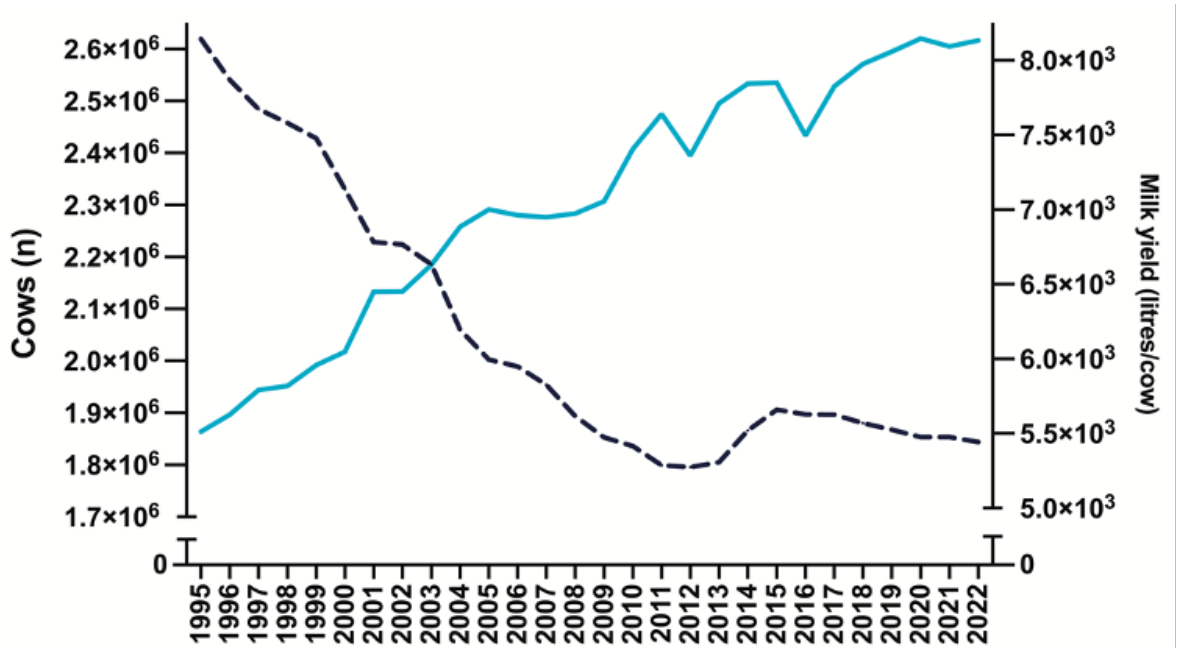


Figure 1.1: Average number of UK milking dairy cows and average annual milk yield 1995-2022. The number of cows is shown by the dark blue dotted line and the left y-axis, the average annual milk yield per cow is shown by the light blue solid line and the right y-axis (AHDB, 2023 Date accessed: 30/03/2024).

Table 1.1: Housing arrangements of UK cattle in 2013.

	Milking cows		Dry cows	
Housing	% of holdings	95% CI	% of holdings	95% CI
Regular access to external pasture*	94	± 2	93	± 2
Access to a loafing area only	2	± 1	2	± 1
Continuously housed	6	± 2	5	± 2
Intended to be continuously housed in the future	7	± 2	5	± 2

*could include cows housed continuously for periods (e.g., winter). CI = confidence intervals. Data from (DEFRA, 2013).

In 2019, 87% of cattle farms used a mixture of grazing and housed systems, but larger farms (>150 head) were more likely to use year-round housing than smaller farms (<150 head) (DEFRA, 2019). The dairy industry is moving away from traditional all-summer grazing, incorporating more year-round indoor

feeding and continuous housing (March *et al.*, 2014). This shift is likely due to a several reasons: intensification to meet increased demand, feed consistency, increased herd size for a given farm, reduced pasture availability, robotic milking, and climactic factors (Arnott, Ferris and O'connell, 2017). Practitioners and consumers perceive that farms that provide animals access to pasture offer a higher standard of animal welfare (Ellis *et al.*, 2009). The welfare implications of continuous housing compared to pasture grazing have been reviewed, with access to pasture linked to reducing dairy cow mortality (Arnott, Ferris and O'connell, 2017). Access to pasture improves animal welfare by reducing the prevalence of lameness, mastitis, and uterine infections (Arnott, Ferris and O'connell, 2017). However, regular access to pasture also increases the risk of endoparasitic infections and disease transmission from neighbouring farms (Arnott, Ferris and O'connell, 2017).

There is a possibility that improved animal welfare would result in reduced AMU, with evidence found in Sweden (Grundin *et al.*, 2020). Low animal welfare was found on two Italian dairy farms that used no antibiotics, suggesting that limited AMU could result in unpredictable consequences across the dairy farming sector (Mazza *et al.*, 2021). An investigation into the impact of dairy farm housing on AMU or AMR could not be identified. A higher level of veterinary AMU has been linked to increased agricultural AMR nationally across several European nations (Chantziaras *et al.*, 2014). The annual Veterinary Antimicrobial Resistance and Sales Surveillance (VARSS) reports UK agricultural antimicrobial sales and AMU for sectors in which it is available. The volume of antimicrobials sold and their intended use are the predominant data for AMU in the VARSS report. There are currently forty-two antibiotics, across ten classes, approved for use in bovines in the UK (Veterinary Medicines Directorate, 2023b) (Table 1.2).

Table 1.2: Antimicrobials and routes of administration approved for use in bovines in the UK.

Antibiotic class/active ingredient	Administration route
Tetracyclines	
Chlortetracycline	Cutaneous spray, oral/water, premix
Oxytetracycline	Tablet, injectable, premix, oral/water, cutaneous spray
Tetracycline	Tablet, oral
Trimethoprim/sulfonamides	
Sulfadiazine	Tablet, oral/water, injectable, premix, intramammary suspension
Sulfadimidine	Injectable
Sulfadoxine	Injectable
Trimethoprim	Tablet, oral/water, premix, intramammary suspension
***β*-lactams**	
Penicillins[+]	
Amoxicillin	Injectable, tablet, oral/water, premix, intramammary suspension, top dressing
Ampicillin	Injectable, tablet, intramammary suspension
Benzylpenicillin	Injectable, oral/water, intramammary suspension
Cloxacillin	Intramammary suspension, eye ointment
Nafcillin	Intramammary suspension
1st generation cephalosporins	
Cefalexin	Tablet, injectable, intramammary suspension
Cefalonium	Intramammary suspension
Cefapirin	Intramammary suspension, intrauterine suspension
3rd generation cephalosporins[+]	
Cefoperazone	Intramammary suspension
Cefovecin	Intramammary suspension
Ceftiofur	Injectable
4th generation cephalosporins[+]	
Cefquinome	Injectable, intramammary suspension/ointment
Aminoglycosides[+]	
Apramycin	Premix, oral/water
Dihydrostreptomycin	Injectable, intramammary suspension
Framycetin	Injectable, intramammary suspension, ear drops
Kanamycin	Intramammary suspension
Neomycin	Injectable, oral/water, intramammary suspension, ear drops
Paronamycin	Oral/water
Spectinomycin	Injectable, premix, oral/water
Streptomycin	Injectable, oral/water, intramammary suspension
Fluoroquinolones[+]	
Danofloxacin	Injectable
Difloxacin	Injectable, tablet, oral/water
Enrofloxacin	Injectable, tablet, oral/water
Marbofloxacin	Tablet, injectable, ear drops
Macrolides	
Gamithromycin	Injectable
Spiramycin	Injectable, tablet
Tildipirosin	Injectable
Tilmicosin	Injectable, premix, oral/water
Tulathromycin	Injectable
Tylosin	Oral/water, premix, injectable
Amphenicols	
Florfenicol	Injectable, oral/water, premix, ear gel
Lincomycins	
Lincomycin	Oral/water, premix, injectable, intramammary solution
Pirlimycin	Intramammary solution
Polymyxins[+]	
Colistin[+]	Oral/water
Other Antibiotics	
Novobiocin	Intramammary suspension

Information taken from VARSS (Veterinary Medicines Directorate, 2021b).

A significant issue with the VARSS report is the lack of cattle data, representing 25% of UK livestock (Veterinary Medicines Directorate, 2021a). Many antimicrobials sold in the UK are used in both cattle and sheep, and 80% of UK farms registered as dairy farms also have sheep. Therefore, the sales of antimicrobials to most UK dairy farms cannot be grouped into livestock types (Veterinary Medicines Directorate, 2021b). However, the VARSS report does include data on products stated for intramammary use. It has been estimated that 70% of intramammary products are used in dairy cattle, but intramammary use represented 6.5% of antimicrobial active ingredients sold for use on 358 UK dairy farms in 2016 (Hyde *et al.*, 2017; Kingshay, 2021). While most of these 358 UK dairy farms had AMU lower than the UK livestock average, 25% of these farms represented over 50% of AMU by mass of active ingredient (Hyde *et al.*, 2017). The Responsible Use of Medicines in Agriculture Alliance (RUMA) established several targets for AMU in UK dairy to be achieved by 2020 (RUMA, 2020b) (Table 1.3).

Table 1.3: RUMA 2020 targets for UK dairy AMU.

Target	Status
Reduce to 21.5 mg/kg* overall use	Data unavailable
10% reduction in intramammary lactating cow tube sales	Achieved 2019 (Veterinary Medicines Directorate, 2020)
20% reduction in intramammary dry cow tube sales	Achieved 2019 (Veterinary Medicines Directorate, 2020)
Increase sealant tube sales from 0.5 to 0.7 courses/cow	No longer a target (RUMA, 2023)
Halve sales of highest priority intramammary tubes	Achieved 2018 & 2019 (Veterinary Medicines Directorate, 2020)

*milligram of active ingredient/ kg of typical animal treated (mg/kg) (RUMA, 2020b).

VARSS reports have insufficient data to confirm if the first RUMA target has been achieved. In a privately produced report that assessed 940 herds, including 550 herds continually evaluated over three years, the AMU in 79% of these herds was below the 21 mg/kg RUMA target set for 2020 (Kingshay, 2021). This suggests that while the dairy industry has reduced AMU and that farmers are invested in measures to control AMR, the majority of use is occurring on a select group of farms (Kingshay, 2021). Sales of antimicrobials for veterinary use in 2022 totalled 193 tonnes, a 43% decrease since 2014 (Veterinary Medicines Directorate, 2023a) (Figure 1.2). Sales are an indirect measure of AMU as some compounds have a shelf life of several years, and therefore, the actual reduction in AMU may be more gradual than sales data suggests as stockpiles are depleted.

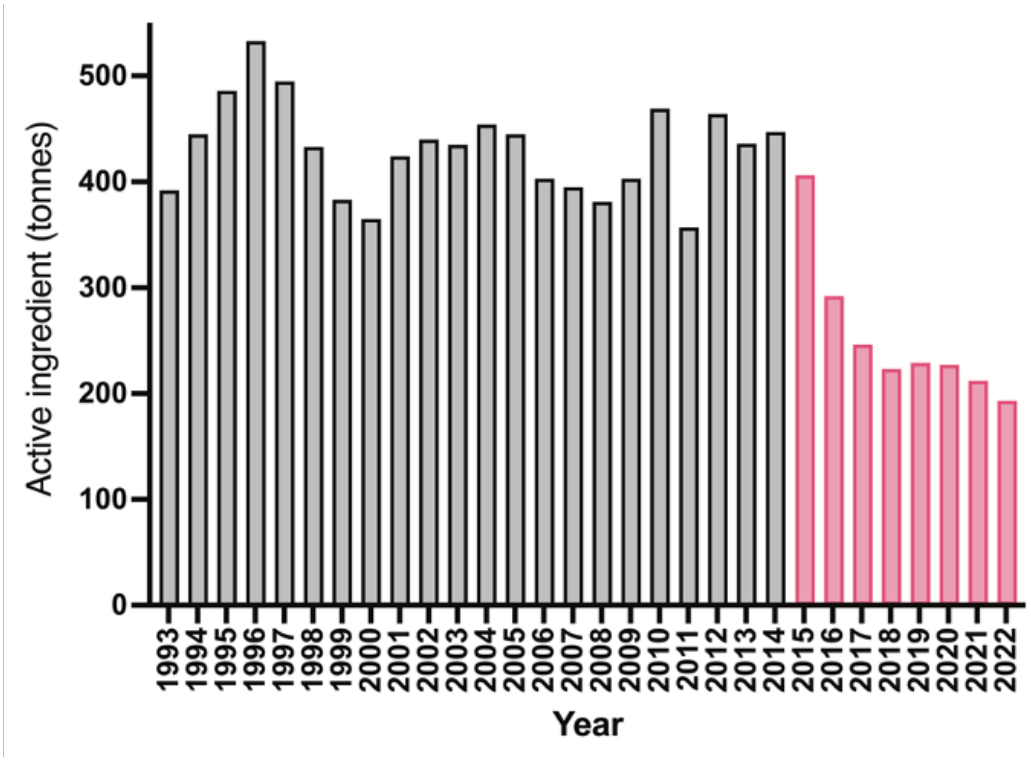


Figure 1.2: Total tonnes of antibiotic active ingredient sold for UK veterinary use 1993-2022. Data was collated from multiple VARSS reports (UK VARSS 2007-2023).

The most purchased antibiotic classes for food producing animals in 2022 were tetracyclines (8.3 mg/kg) and penicillins (7.3 mg/kg); these have been the most purchased annually since 2014 (Veterinary Medicines Directorate, 2023a) (Table 1.4). Trimethoprim/sulfonamides were the third most purchased from 2014-2021, but more aminoglycosides and macrolides were purchased in 2022.

Table 1.4: Active ingredient (mg/kg) of antibiotics sold for use in food producing animals in the UK 2014-2022.

	2014	2015	2016	2017	2018	2019	2020	2021	2022
Tetracyclines	26.1	23.6	15	13.1	11.7	10.2	10.2	9.6	8.3
Penicillins	11.4	9.8	7.9	7.3	6.7	7.5	8	7.6	7.3
Trimethoprim/ sulfonamides	10	9.7	7	3.3	3.2	3.5	3.5	3.1	2.2
Aminoglycosides	3.6	3.5	2.2	2.5	2.5	3.1	3	3	3.1
Macrolides	7.2	5.5	4	3.2	2.3	2.3	3	2.7	2.3
Pleuromutilins	1.8	2.2	1.4	1.4	1.2	2.1	1	0.9	1
Lincosamides	1	0.9	0.5	0.3	0.4	0.6	0.7	0.5	0.7
Amphenicols	0.4	0.4	0.6	0.6	0.6	0.6	0.6	0.6	0.5
1st/2nd generation cephalosporins	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Imidazole derivatives	0	0	0	0	0	0.005	0.005	0.005	0.005
Aminocoumarins	0.03	0.03	0.02	0.02	0.02	0.02	0	0	0
Fluoroquinolones	0.35	0.35	0.22	0.16	0.15	0.13	0.1	0.1	0.1
3rd/4th generation cephalosporins	0.19	0.17	0.14	0.11	0.06	0.03	0.04	0.02	0.02
Colistin	0.12	0.13	0.02	0.0006	0.0007	0.002	0.00007	0	0
Total	62.97	57.15	39.38	32.36	29.21	30.57	30.34	28.42	25.82

Data from VARSS ((Veterinary Medicines Directorate, 2023a).

The size and milk yield of the UK dairy herd provides context for the reductions in AMU (Figure 1.1, Figure 1.2, and Table 1.4). The size of the milking herd has increased in recent years, but antimicrobial stewardship has produced consistent reductions in AMU. Crucially, this reduced AMU has not reduced the efficiency of the milking herd in terms of milk yield. Within the EU, overall sales of antimicrobials for veterinary use in 2011 for 25 countries totalled 8420 tonnes, while in 2016 for 30 countries, sales totalled 7787.1 tonnes (European

Medicines Agency, 2013, 2018). This reduction in sales across a larger region suggests a reduction in use across Europe, but the same issues of antibiotic stockpiles and a lack of reporting affect this approach.

The WHO maintains a document that acts as a risk assessment for all antimicrobials used in humans (World Health Organization, 2019). This document defines criteria and prioritisation factors based on clinical context to categorise antimicrobials for sustainable use (World Health Organization, 2019). Every antimicrobial used in human medicine is categorised as important and warrants stewardship. Antimicrobials are categorised as Critically important antimicrobials (CIA) with two criteria: if they are the only or one of the limited available options to treat a serious bacterial infection in humans, and if they are used to treat infections caused by possibly zoonotic bacteria or infections with resistance genes from non-human sources. Three prioritisation factors categorise the priority of CIA; they are categorised as Highest priority critically important antimicrobials (HP-CIA) if they are used to treat a large number of people, are frequently used in medicine or high-risk groups, and are used to treat infections with extensive evidence of resistance emerging from non-human sources. Antimicrobials that only satisfy one criterion and fewer than three prioritisation factors, they categorised as highly important antimicrobials. In Europe, HP-CIA should only be used in veterinary medicine as a last resort after susceptibility testing and when no other antimicrobial will provide adequate treatment (European Medicines Agency, 2019; RUMA, 2020a). Since 2014, the sales of HP-CIA represent a small proportion of UK veterinary antibiotic sales and by 2022, fluoroquinolones reduced by 72%, 3rd/4th generation cephalosporins reduced by 90%, and

colistin sales reduced by 100% to zero (Veterinary Medicines Directorate, 2023a) (Table 1.4). Over the same period of 2014-2022, the sales of injectable HP-CIA products licenced for cattle have reduced by 81% from 1.10 mg/kg to 0.20 mg/kg (Veterinary Medicines Directorate, 2023a).

1.3.1 VARSS antibiotic resistance monitoring in *E. coli* from UK cattle samples

The VARSS clinical surveillance of antibiotic resistance dataset (VCD) reports sample veterinary pathogens from food producing animals in the UK to monitor ABR in several sectors. The VCD includes bovine *E. coli* collected from clinical mastitis samples and gastrointestinal samples from cattle carcasses. The VARSS report assumes that the *E. coli* collected from mastitis cases are of faecal origin (Veterinary Medicines Directorate, 2023b). The VCD for cattle comprises isolates from cattle of different ages as part of routine diagnostic samples; the Veterinary Medicines Directorate and Animal and Plant Health Agency have established a scheme to collect information from private veterinary labs to include these data in national monitoring. The 2023 VCD included 984 gastrointestinal *E. coli* from all food-producing animal species; 814 isolates were sampled from neonatal cattle, pre-weaning calves, and adult cattle. Multidrug resistant (MDR) is a term for microorganisms that are resistant to an antimicrobial in three or more different antimicrobial classes (Magiorakos *et al.*, 2012). In the VCD, 24% of the isolates were classified as MDR, with 40% of the isolates from cattle classified as MDR. The susceptibility results of the VCD are presented with SIE and resistance results combined as resistance.

The annual VCD data were compiled and summarised from 2018-2022 (Table 1.5), the 2022 results grouped by cow developmental stage (Table 1.6), and data on *E. coli* collected from clinical mastitis cases during 2018-2022 (Table 1.7). Antibiotic susceptibility is presented in the VARSS reports as resistance levels, with descriptors associated with the proportion of resistant isolates. The level descriptors are assigned to the percentage ranges; <0.1% (rare), 0.1% to 1% (very low), >1% to 10% (low), >10% to 20% (moderate), >20% to 50% (high), >50% to 70% (very high), and >70% (extremely high) (European Food Safety Authority, 2022; Veterinary Medicines Directorate, 2023b). The proportion of the isolates resistant to β-lactams decreased from 2018-2022 but remained very high for ampicillin (58.2%) and high for both amoxicillin-clavulanate (27.5%) and cefpodoxime (22.9%). In 2022, the β-lactam resistance level was higher in the isolates from neonatal and pre-weaning cattle, and a high level of ampicillin resistance (41%) was also found in isolates from clinical mastitis cases. Aminoglycoside resistance decreased overall, with very high levels of isolates resistant to both neomycin (64.8%) and streptomycin (52.9%) in 2018, and high levels of resistance to neomycin (38.8%) and streptomycin (34.7%) in 2022. In isolates from mastitis, streptomycin resistance was moderate in isolates collected yearly except when there was a low level (7.1%) in 2021. In 2022, Aminoglycoside resistance was higher in the neonatal cattle and highest in the pre-weaning cattle (extremely high resistance level to streptomycin (72.7%), but spectinomycin resistance was high in the isolates from neonates (33.3%), pre-weaning cattle (35.7%), and adult cattle (38.1%). Trimethoprim/sulfonamide resistance levels decreased annually from very high (57.3%) in 2018 to high

(35.6%) in 2022, with high resistance levels in the isolates from all developmental groups. Trimethoprim/sulfonamide resistance levels in the *E. coli* from mastitis samples fluctuate between low (2018, 2019, 2021) and moderate (2020 and 2022). Phenicol resistance was high in the isolates collected every year and in all developmental sample groups (the lowest proportion was 23.6% of the isolates collected in 2021 were chloramphenicol resistant, and the highest proportion was 45.5% of the isolates from pre-weaning cattle in 2022). Phenicols were not included in the panel of antibiotics mastitis samples were tested with. The level of quinolone resistance was monitored using enrofloxacin only. In 2018, a high proportion of the isolates were resistant (27.2%), which decreased to a low level (7.6%) by 2020 and has remained low. In 2022, the level of enrofloxacin resistance was moderate in the isolates from neonatal cattle (19.1%), low in pre-weaning cattle (3.2%), and rare in adult cattle (0%). In the isolates from mastitis samples, the levels of enrofloxacin resistance were low in all years except 2019, when levels were rare (0%). The levels of resistance to tetracycline were extremely high in the isolates collected in 2018 (77%), and this reduced to very high (53.6%) in 2022, where more resistance was found in the isolates from pre-weaning cattle (67.4%) and adult cattle (56.5), than in the isolates from neonates (48.1%). The levels of resistance to tetracycline in the *E. coli* from mastitis samples increased from moderate (13.6%) in 2018 to high (20.5%) in 2022.

Table 1.5: Antibiotic susceptibility and resistance levels of *E. coli* collected for the VCD across the UK 2018-2022.

Antibiotic	2018		2019		2020		2021		2022	
	Resistance proportion*	Resistance level	Resistance proportion*	Resistance level	Resistance proportion*	Resistance level	Resistance proportion*	Resistance level	Resistance proportion*	Resistance level
Amikacin	0/189 (0)	Rare	0/130 (0)	Rare	0/141 (0)	Rare	0/127 (0)	Rare	0/105 (0)	Rare
Ampicillin	572/710 (80.6)	Extremely high	320/427 (74.9)	Extremely high	314/499 (62.9)	Very high	282/466 (60.5)	Very high	237/407 (58.2)	Very high
Amoxicillin-clavulanate	341/707 (48.2)	High	175/426 (41.1)	High	147/499 (29.5)	High	124/466 (26.6)	High	112/407 (27.5)	High
Apramycin	49/614 (8)	Low	15/395 (3.8)	Low	9/358 (2.5)	Low	39/346 (11.3)	Moderate	8/306 (2.6)	Low
Cefotaxime	22/190 (11.6)	Moderate	15/135 (11.1)	Moderate	13/142 (9.2)	Low	6/128 (4.7)	Low	5/106 (4.7)	Low
Cefpodoxime	208/437 (47.6)	High	NA	NA	54/260 (20.8)	High	64/204 (31.4)	High	46/201(22.9)	High
Ceftazidime	9/190 (4.7)	Low	5/135 (3.7)	Low	6/142 (4.2)	Low	6/128 (4.7)	Low	1/106 (0.9)	Very low
Neomycin	444/685 (64.8)	Very high	212/405 (52.3)	Very high	165/477 (34.6)	High	174/439 (39.6)	High	152/392 (38.8)	High
Spectinomycin	124/321 (38.6)	High	97/286 (33.9)	High	79/292 (27.1)	High	61/262 (23.3)	High	81/231 (35.1)	High
Streptomycin	100/189 (52.9)	Very high	72/139 (51.8)	Very high	114/259 (44)	High	65/127 (51.2)	Very high	66/190 (34.7)	High
Trimethoprim/ sulfonamide	405/707 (57.3)	Very high	211/427 (49.4)	High	202/499 (40.5)	High	173/466 (37.1)	High	145/407 (35.6)	High
Chloramphenicol	82/189 (43.4)	High	52/130 (40)	High	60/139 (43.2)	High	30/127 (23.6)	High	41/105 (39)	High
Florfenicol	286/646 (44.3)	High	137/329 (41.6)	High	113/305 (37)	High	85/272 (31.3)	High	94/232 (40.5)	High
Enrofloxacin	192/707 (27.2)	High	83/435 (19.1)	Moderate	38/499 (7.6)	Low	46/466 (9.9)	Low	36/407 (8.8)	Low
Tetracycline	546/709 (77)	Extremely high	302/425 (71.1)	Extremely high	263/499 (52.7)	Very high	272/466 (58.4)	Very high	218/407 (53.6)	Very high

*The number of resistant isolates/the number of isolates tested (the resistant proportion of isolates). Levels follow VARSS and EFSA standards defined as rare (<0.1%), very low (0.1-1%), low (1-10%), moderate (10-20%), high (20-50%), very high (50-70%), and extremely high (>70%) (European Food Safety Authority, 2022; Veterinary Medicines Directorate, 2023b). Data used with permission(Veterinary Medicines Directorate, 2021a, 2022a, 2023a).

Table 1.6: Antibiotic susceptibility and resistance levels of *E. coli* collected for the 2023 VCD across sample groups.

Antibiotic	All cattle		Neonatal cattle		Pre-weaning cattle		Adult cattle	
	Resistant proportion*	Resistance level	Resistant proportion*	Resistance level	Resistant proportion*	Resistance level	Resistant proportion*	Resistance level
Amikacin	0/105 (0)	Rare	0/83 (0)	Rare	0/22 (0)	Rare	NA	NA
Ampicillin	237/407 (58.2)	Very high	152/266 (57.1)	Very high	63/95 (66.3)	Very high	22/46 (47.8)	High
Amoxicillin-clavulanate	112/407 (27.5)	High	75/266 (28.2)	High	31/95 (32.6)	High	6/46 (13)	Moderate
Apramycin	8/306 (2.6)	Low	7/180 (3.9)	Low	0/84 (0)	Rare	1/42 (2.4)	Low
Cefotaxime	5/106 (4.7)	Low	5/84 (6)	Low	0/22 (0)	Rare	NA	NA
Cefpodoxime	46/201(22.9)	High	44/167 (26.3)	High	1/11 (9.1)	Low	1/23 (4.3)	Low
Ceftazidime	1/106 (0.9)	Very low	1/84 (1.2)	Low	0/22 (0)	Rare	NA	NA
Neomycin	152/392 (38.8)	High	121/266 (45.5)	High	25/84 (29.8)	High	6/42 (14.3)	Moderate
Spectinomycin	81/231 (35.1)	High	35/105 (33.3)	High	30/84 (35.7)	High	16/42 (38.1)	High
Streptomycin	66/190 (34.7)	High	50/168 (29.8)	High	16/22 (72.7)	Extremely high	NA	NA
Trimethoprim/sulfonamide	145/407 (35.6)	High	98/266 (36.8)	High	36/95 (37.9)	High	11/46 (23.9)	High
Chloramphenicol	41/105 (39)	High	31/83 (37.3)	High	10/22 (45.5)	High	NA	NA
Florfenicol	94/232 (40.5)	High	72/165 (43.6)	High	13/44 (29.5)	High	9/23 (39.1)	High
Enrofloxacin	36/407 (8.8)	Low	33/266 (12.4)	Moderate	3/95 (3.2)	Low	0/46 (0)	Rare
Tetracycline	218/407 (53.6)	Very high	128/266 (48.1)	High	64/95 (67.4)	Very high	26/46 (56.5)	Very high

*The number of resistant isolates/the number of isolates tested (the resistant proportion of isolates). Levels follow VARSS and EFSA standards defined as rare (<0.1%), very low (0.1-1%), low (1-10%), moderate (10-20%), high (20-50%), very high (50-70%), and extremely high (>70%) (European Food Safety Authority, 2022; Veterinary Medicines Directorate, 2023b). Data used with permission(Veterinary Medicines Directorate, 2021a, 2022a, 2023a).

Table 1.7: Antibiotic susceptibility and resistance levels of *E. coli* collected from mastitis in UK cattle 2018-2022 for the VCD.

Antibiotic	2018 (n = 110)		2019 (n = 94)		2020 (n = 55)		2021 (n = 42)		2022 (n = 39)	
	Resistance proportion	Resistance level	Resistance proportion	Resistance level	Resistance proportion	Resistance level	Resistance proportion	Resistance level	Resistance proportion	Resistance level
Ampicillin	24 (21.8)	High	37 (39.4)	High	15 (45.5)	High	17 (7.1)	Low	16 (41)	High
Amoxicillin-clavulanate	6 (5.5)	Low	5 (5.3)	Low	4 (7.3)	Low	1 (2.4)	Low	3 (7.7)	Low
Cefpodoxime	1 (0.9)	Very low	1 (1.1)	Low	1 (1.8)	Low	0 (0)	Rare	0 (0)	Rare
Neomycin	3 (2.7)	Low	5 (5.3)	Low	3 (5.5)	Low	1 (2.4)	Low	1 (2.6)	Low
Streptomycin	11 (10)	Moderate	11 (11.7)	Moderate	7 (12.7)	Moderate	3 (7.1)	Low	7 (17.9)	Moderate
Trimethoprim/sulfonamide	7 (6.4)	Low	7 (7.4)	Low	6 (10.9)	Moderate	3 (7.1)	Low	5 (12.8)	Moderate
Enrofloxacin	3 (2.7)	Low	0 (0)	Rare	1 (1.8)	Low	1 (2.4)	Low	1 (2.6)	Low
Tetracycline	15 (13.6)	Moderate	12 (12.8)	Moderate	8 (14.5)	Moderate	4 (14.3)	Moderate	8 (20.5)	High

* The number of resistant isolates/the number of isolates tested (the resistant proportion of isolates). Levels follow VARSS and EFSA standards defined as rare (<0.1%), very low (0.1-1%), low (1-10%), moderate (10-20%), high (20-50%), very high (50-70%), and extremely high (>70%) (European Food Safety Authority, 2022; Veterinary Medicines Directorate, 2023b). Data used with permission(Veterinary Medicines Directorate, 2021a, 2022a, 2023a).

1.3.2 The Centre for Dairy Science Innovation on the Sutton Bonington Dairy

The Centre for Dairy Science Innovation on the Sutton Bonington Dairy farm (SB farm) produces over two million litres of milk annually and is part of the 445-hectare University of Nottingham farm. The SB farm is a working farm and research facility focusing on dairy cow nutrition, optimisations in the built environment of dairy farms, and investigations of disease pathology and development of therapeutics. The herd of ~200 Holstein-Friesian are housed inside on concrete floors permanently, with different developmental stages housed separately (Ibrahim *et al.*, 2016; Baker *et al.*, 2022). The predominant AMU on this farm previously consisted of aminocoumarins, aminoglycoside and β-lactams in combination, β-lactams, and tetracyclines (Baker *et al.*, 2022). In another study on the same farm, which sampled slurry tanks from May 2017 to November 2017, the last recorded use of 4th generation cephalosporins was in 2015, sulfonamides and 3rd generation cephalosporins was in 2016, and 1st generation cephalosporins was April 2017 (Baker *et al.*, 2022). Of the 811 *E. coli* isolates collected from the slurry tanks on this farm, 37% had an MDR phenotype (Baker *et al.*, 2022).

1.3.3 Physiological development of the dairy cow digestive tract

The dairy cow (*Bos taurus*) gastrointestinal microbiota is a complex ecosystem that aids in the fermentation of feed providing improved nutrition to the host consisting of; archaea, bacteria, fungi, protozoa, and viruses (Noel *et al.*, 2019). The physiology of the digestive tract is fundamental in providing the ecological niches required to support this complex ecosystem, and this complex ecosystem is fundamental in the development of this physiology (Van Soest, 1994; Dill-Mcfarland, Breaker and Suen, 2017). The cow digestive tract consists of a major oesophageal expansion forming the forestomachs (rumen,

reticulum, and omasum) followed by the stomach (abomasum) and the small and large intestines (Singh, 2016). The adult cow stomach capacity varies from 100-200 litres depending on body size (Budras and Budras, 2003). The adult cow stomach is composed of three non-glandular components (rumen, reticulum, and omasum) and one compartment with glandular mucosa (abomasum) (Budras and Budras, 2003). These compartments vary in size, with the post-mortem volume of each estimated as rumen, 80%; reticulum, 5%; omasum, 4%; and abomasum, 13% (Singh, 2016). Due to the stratified squamous epithelial mucosa composition of the forestomachs they do not produce digestive enzymes, but instead rely on microorganisms (bacteria, protozoa, and fungi) for fermentation of organic compounds in ruminant feed through anaerobic degradation (Sjaastad, Sand and Hove, 2016). At birth, the ruminant stomach is adapted for the digestion of milk, the abomasum is at 60% adult volume and attained nearly complete structural maturity (Sjaastad, Sand and Hove, 2016). Dairy calves are monogastric and lack a fully formed reticulorumen, their milk feed bypasses the forestomach's via the reticular groove and travels from the oesophagus into the abomasum (Heinrichs and Lesmeister, 2005). After the first feed, the abomasum stretches from the liver/diaphragm to the pelvic entrance, flank to flank, abdomen floor into the upper half of the abdomen (Singh, 2016). When feeding, the reticular groove contracts by reflex, forcing the milk to bypass the forestomachs and enter straight into the abomasum via the reticulo-omasal orifice (Reece *et al.*, 2015; Singh, 2016). The rumen does not begin to mature until the calf begins grazing/eating solid feed, at this point the walls thicken and the microbiota establish. The process is further activated by products of fermentation such as butyric acid, and the reticulorumen expands with the formation of the

recognizable papillae for increased surface area and friction to aid digestion of fibrous materials (Sjaastad, Sand and Hove, 2016). The reticular groove enables growing calves to establish their rumen microbiomes while consuming milk, ensuring they receive the proteins present in the milk and avoid it souring in the rumen (Reece *et al.*, 2015) while the rumen quickly develops and stomach composition reaches maturity by 20 weeks post birth (Figure 1.3) (Godfrey, 1961; Murray, Tulloh and Winter, 1977).

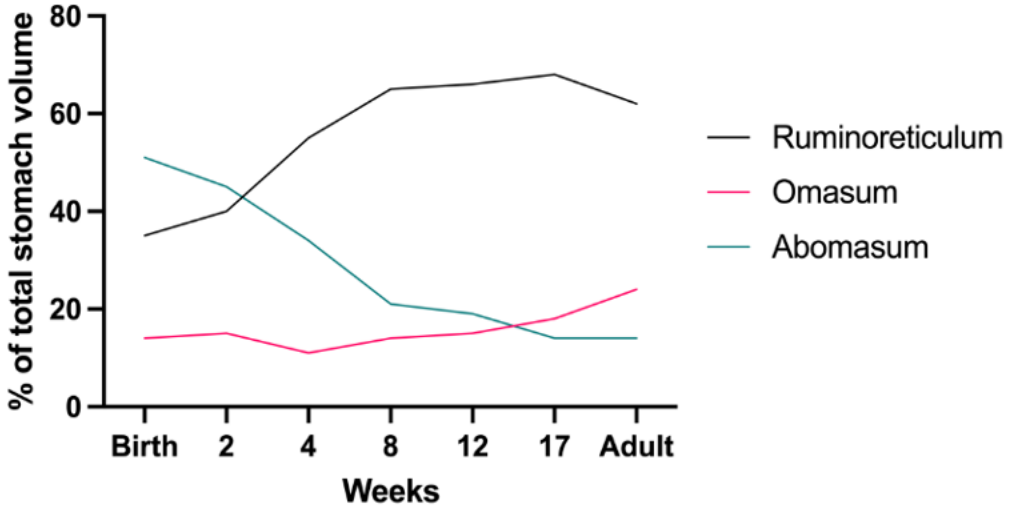


Figure 1.3: Percentage composition of each stomach component from birth to maturation. Data in this figure was adapted from (Godfrey, 1961; Murray, Tulloh and Winter, 1977).

This results in minimal mixing of ruminant bacteria into the abomasum and further into the digestive tract for excretion in the faeces (Dill-McFarland *et al.*, 2019; Mu *et al.*, 2019; Noel *et al.*, 2019). A diet that increases the amount of available nitrogen in the hindgut does not appear to cause a significant change in the hindgut microbiota, suggesting that nitrogen sourced from the rumen and minimal dietary protein is sufficient to sustain hindgut populations (Frey *et al.*, 2010). The variation in cellulolytic microbial populations has been shown to differ more between animals than differences observed across populations with different diets (Weimer *et al.*, 1999). Some reports have found that the faeces and rumen microbiota differ significantly, with archaea present in both, protozoa virtually absent and bacteria in greater abundance in the faeces

(Tapio *et al.*, 2016). However, others have identified similar taxonomical compositions and levels of bacterial diversity in the bovine stomach and large intestine microbiota (Wang *et al.*, 2022). With the abundance of *Ruminococcaceae*, *Rikenellaceae*, and *Bacteroidaceae* reported to be higher in the bovine large intestine microbiota than in the stomach (Wang *et al.*, 2022). Crucially, the variability of microbiota in the mucosa and digesta in specific sites of the rumen, and rectum have shown significant variation, suggesting that generalisations about microbiota could confound interpretations (Mao *et al.*, 2015).

1.3.3.1 The effect of dairy calf nutrition on the microbiome

The diet of dairy calves differs significantly from post weaned animals, due to the differences described in the gastrointestinal physiology previously. While in the womb dairy calves do not receive immunoglobulins leaving them vulnerable to infection (Erickson and Kalscheur, 2019). Colostrum, milk produced by the dam in the days immediately after the birth, is high in immunoglobulins and generally seen as an essential practice in the dairy industry. Colostrum consumption is also correlated with increased milk production when calves mature (Erickson and Kalscheur, 2019). Weaning off of milk has been shown to result in significant differences in total microbial communities and specific taxa, but these dissimilarities did not correlate with growth or milk production (Dill-McFarland *et al.*, 2019). Direct culture and culture-independent methods have established that the gastrointestinal tract of young ruminants after birth are rapidly colonised by aerobic and facultative anaerobic bacteria, followed by replacement with anaerobic bacteria as they mature (Bryant *et al.*, 1958; Fonty *et al.*, 1987; Minato *et al.*, 1992; Jami *et al.*, 2013).

1.4 Stewardship to preserve our antimicrobial arsenal and mitigate the risk of antimicrobial resistance

Antimicrobial stewardship has been a global objective for many years, eliminating unnecessary use and reducing the risk of emerging resistance (WHO, 2001, 2024; European Medicines Agency, 2005; European Food Safety Authority, 2022; World Health Organization, 2022). However, sub-inhibitory concentrations of antimicrobials select for AMR *in vitro*, with similar adaptations to those seen in clinical resistant isolates (Gullberg *et al.*, 2011). Some have observed that reductions in AMU in humans have been linked to reductions in AMR in the community and hospitals (Guillemot *et al.*, 2005; Livermore *et al.*, 2013). In comparison, others have been unable to find a direct correlation between reductions in AMU in humans and a reduction in AMR in the community and hospitals (Enne *et al.*, 2001; Sundqvist *et al.*, 2010; Guthrie *et al.*, 2019; Peñalva *et al.*, 2020). In 2015, the National Health Service (NHS) implemented a Quality Premium that rewards trusts for achieving objectives; 10% of the available reward focused on antimicrobial prescription reduction targets, specifically in bacteraemia and urinary tract infection (UTI) (NHS, 2015, 2017a, 2017b). The English Surveillance Programme For Antimicrobial Usage and Resistance (ESPAUR) monitors the rates of priority pathogen infections, AMR, and AMU in NHS England (United Kingdom Health Security Agency, 2023). The 2020 ESPAUR showed that between April 2015 and March 2019, AMU was reduced in primary care, specifically amoxicillin-clavulanate and some quinolones (Public Health England, 2020). Other studies also observed a reduction in primary care prescriptions (Bou-Antoun *et al.*, 2018; Anyanwu *et al.*, 2020). However, levofloxacin prescribing increased from 2014 to 2018 (Public Health England, 2020; Aliabadi *et al.*,

2021). Levofloxacin use for UTIs has been shown to increase the rates of ciprofloxacin-resistant *E. coli* in UTIs (Pouwels *et al.*, 2019). The reduction in amoxicillin-clavulanate prescriptions in primary healthcare was mirrored by increased amoxicillin-clavulanate prescriptions in hospitals since 2015. This relationship between hospital and clinic prescriptions highlights how any reduction in one area may have unexpected consequences in other health system sectors (Public Health England, 2020). The impact of reducing AMU has been estimated to take up to a decade for reductions in AMR to be realised (Enne *et al.*, 2001). While AMR is a global multisectoral challenge, this thesis focuses on understanding ABR in *E. coli* collected from the faeces of cattle in the United Kingdom (UK). The information collected in this literature review discusses broad themes but focuses on ABR *E. coli* and UK dairy.

1.5 *Escherichia coli*: environmental sentinel and microbiology work horse

E. coli is a Gram-negative, rod shaped, facultative anaerobic *Enterobacteriaceae* that has been a key species in microbiology research since its discovery in 1885 (Escherich, 1885; Madigan *et al.*, 2018). Isolated from a human infant stool sample, it is now understood to be a component of the gut microbiota of most mammals. *E. coli* are an important human pathogen, the most frequently isolated Gram-negative organism isolated from bacteraemia and UTI in the UK (Escherich, 1885; United Kingdom Health Security Agency, 2023). Due to its status as an important pathogen, *E. coli* is included in programmes such as the Global Antimicrobial Resistance and Use Surveillance System (GLASS) and more specifically monitoring ABR in the dairy farm environment (Moreno *et al.*, 2000; Ibrahim *et al.*, 2016; Astorga *et al.*, 2019; Suzuki *et al.*, 2022; World Health Organization, 2022). The UK continues to follow the harmonized monitoring framework, which includes

monitoring animal carcasses and meat products for carbapenemase positive *E. coli* (European Union, 2020).

1.5.1 Bovine adapted *E. coli* are opportunistic pathogens

E. coli that are adapted to the bovine niche are characterised as faecal commensals and opportunistic pathogens (X. Li *et al.*, 2018). *E. coli* are important pathogens in the dairy industry and represent one of the main agents of acute clinical mastitis in dairy cattle (Shpigel, Elazar and Rosenshine, 2008; Gao *et al.*, 2017). In a herd of Holstein cattle from Israel, *E. coli* mastitis resulted in an average loss of 200 litres per cow over a lactation period, with persistent infections resulting in 1500 litres per cow over the same period (Blum, Heller and Leitner, 2014). *E. coli* are a leading cause of diarrhoea in calves until they are three days old, with pathogenic *E. coli* avoiding the immunity provided by colostrum (Constable *et al.*, 2016).

1.6 Approaches to investigate pathogen epidemiology and clonality

Prokaryotic organisms undergo asexual reproduction, creating a copy of a single genome passed vertically to progenitors. Random mutations within this genome result in differentiation and eventually speciation, but several generations are required for these processes to occur. Prokaryotic genomes can shift rapidly by sharing genomic information horizontally through mobile genetic elements (MGE). Clonality describes the balance between vertical (through cell division) and horizontal inheritance (facilitated by MGE) within a bacterial population. A population with no horizontal inheritance would be expected to have slower rates of recombination and therefore individuals would be more clonal. Molecular typing methods have been used to identify species within clusters of highly recombinant bacteria, to assess the clonality of the isolates in outbreaks, and to investigate the global population of several

pathogens (Selander, Beltran, Smith, Barker, *et al.*, 1990; Feil *et al.*, 2004; Hanage, Fraser and Spratt, 2005; Lefébure and Stanhope, 2007; Didelot and Maiden, 2010; Pérez-Losada *et al.*, 2013).

1.6.1 Pulse field gel electrophoresis and its use investigating *E. coli*

Pulse field gel electrophoresis (PFGE) uses restriction enzymes and multiple electric fields, which are alternately pulsed perpendicularly across an agarose gel to provide molecular fingerprints (Schwartz *et al.*, 1982; Schwartz and Cantor, 1984). Reptation describes the movement of polymers in the absence of a field, in the example of DNA in an agarose gel, the polymer is moving because of an electric field and therefore is subject to biased reptation without orientation (de Gennes, 1979; Doi and Edwards, 1986; Slater, 2009). A large molecule of DNA can align to the direction of the electrical field and travel through the agarose gel at a rate that is equivalent to a significantly smaller fragment (Schwartz and Cantor, 1984). The benefit of PFGE is that larger molecules of DNA are constantly forced to realign by perpendicular electrical fields. Therefore, large molecules can be run through an agarose gel at a rate that is proportional to their size.

PFGE has previously been used to investigate the relatedness of *E. coli*, including O157 serotype isolates from food borne outbreaks in humans and different dairy herds across 24 total farms (Barrett *et al.*, 1994; Lee *et al.*, 1996; Shere, Bartlett and Kaspar, 1998). It has also been shown to outperform other subtyping methods such as bacteriophage typing, antimicrobial susceptibility testing, plasmid typing, and ribotyping (Barrett *et al.*, 1994; Farber, 1996; Krause, Thomson-Carter and Hugh Pennington, 1996; Olive and Bean, 1999; Hopkins and Hilton, 2000).

1.6.2 Arbitrarily primed polymerase chain reaction to investigate *E. coli* clonality

Arbitrarily primed polymerase chain reaction (AP-PCR) use polymerase chain reaction (PCR) primers that are designed to capture highly variable loci in isolates (Welsh and Mcclelland, 1990). The underlying theory is that these PCR primers can be expected to anneal to several sequences, with mismatches. Some of these annealing sites will be sufficiently close to each other and on opposing strands, with the sequence segments between these positions amplified. The extent of amplification of each segment depends on the priming efficiency at each annealing site and the extension efficiency. The segments that prime most efficiently in early cycles will predominate, and those that amplify most efficiently will predominate in later cycles (Welsh and Mcclelland, 1990). The main advantage of this approach is that minimal information about the genome of the target organism is required, as the primers are not designed to target a specific region or gene.

1.6.3 The enterobacterial repetitive intergenic consensus repeats polymerase chain reaction to investigate *E. coli* clonality

The enterobacterial repetitive intergenic consensus (ERIC) PCR is a molecular genotyping technique developed in 1991 (Versalovic, Koeuth and Lupski, 1991). ERIC-PCR was developed to meet the need for rapid, reliable, and cost-effective methods for bacterial genotyping (Versalovic, Koeuth and Lupski, 1991). The technique is based on the presence of highly conserved, intergenic, and repetitive 127 nucleotide ERIC sequences (Hulton, Higgins and Sharp, 1991; Versalovic, Koeuth and Lupski, 1991). These sequences are randomly distributed throughout the genomes of *Enterobacteriaceae*, with variable lengths of DNA between ERIC sequences. Following PCR

amplification, these variable lengths of DNA generate unique electrophoretic banding patterns for different isolates (Versalovic, Koeuth and Lupski, 1991). These unique electrophoretic banding patterns can be used to track pathogen transmission and understand a bacterial population's genetic diversity (Zoolkifli and Mutalib, 2013; Ranjbar, Pezeshknejad, *et al.*, 2017; Ranjbar, Tabatabaee, *et al.*, 2017). ERIC-PCR has been used for genotyping bacterial isolates in various contexts, including clinical, agricultural, and environmental settings (Versalovic, Koeuth and Lupski, 1991; Meacham *et al.*, 2003; Ibrahim *et al.*, 2016, 2023; Chen *et al.*, 2021). ERIC-PCR has been used to genotype *E. coli* isolates associated with human and animal health. Studies have demonstrated its effectiveness in differentiating uropathogenic *E. coli* strains responsible for urinary tract infections (Afkhami Ardakani and Ranjbar, 2016). The application of ERIC-PCR for genotyping *E. coli* from dairy cows has provided insights into the genetic diversity and populations of these bacteria. For example, ERIC-PCR has been used for genotyping *E. coli* from dairy cows, even differentiating Shiga toxin-producing and non-pathogenic *E. coli* isolated from dairy products (Leung *et al.*, 2004; Costa, Nunes and França, 2011; Oltramari *et al.*, 2014). This data is particularly relevant in understanding zoonoses, or the transmission of pathogenic strains from animals to humans, including through the consumption of contaminated food products (Pusparini *et al.*, 2018; Yar *et al.*, 2022). ERIC-PCR's use extends beyond the characterization of pathogenic strains; it has also been used to study commensal *E. coli* populations in healthy cattle (Madoshi *et al.*, 2016). ERIC-PCR has been used in investigations of AMR among *E. coli* strains from dairy cattle, providing critical information for managing antibiotic resistance in agricultural settings (Ibrahim *et al.*, 2016; K. Chen *et al.*, 2020; Thuan *et al.*,

2022). In addition to its applications in dairy cow studies, ERIC-PCR has been employed in various research contexts involving *E. coli*, including isolates from water samples, providing insights into environmental reservoirs of pathogenic *E. coli* (Duan *et al.*, 2009; Ranjbar, Pezeshknejad, *et al.*, 2017). ERIC-PCR has also been applied in clinical settings to monitor the spread of MDR *E. coli* strains, identify the potential sources of outbreaks, and inform public health responses (Moosavian and Emam, 2019; Chen *et al.*, 2021; Ibrahim *et al.*, 2023). Comparative studies with other molecular typing methods, such as PFGE, support the robustness of ERIC-PCR as a genotyping tool while being more cost-effective and easier to perform (Leung *et al.*, 2004; Fajs *et al.*, 2013). Therefore, ERIC-PCR represents an attractive option for laboratories with limited resources or where access to advanced molecular techniques may be restricted. However, others have questioned the method's reliability, showing that the primers used in ERIC-PCR can amplify DNA that does not contain EIRC sequences (Wilson and Sharp, 2006). The original developers state that ERIC-PCR has some versatility in typing organisms outside of the *Enterobacteriaceae*, including *Treponema*, *Deinococcus*, *Thermus*, and even a member of the Archaea (Versalovic, Koeuth and Lupski, 1991). This suggests that the primers are binding to sites other than the intended ERIC sequences. The method's repeatability has also been questioned, with distinct banding patterns identified from replicate PCRs of the same isolate (Meacham *et al.*, 2003).

1.6.4 Multilocus sequence typing to investigate bacterial clonality

Multilocus sequence typing (MLST) is a molecular typing technique employed in studying bacterial population genetics since its inception in the late 1990s. At the time of MLST development, PFGE and arbitrarily primed PCR methods

had been successfully used to investigate methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus*, *E. coli* O157:H7, and meningococcal meningitis outbreaks (De Lencastre, De Lencastre and Tomasz, 1996; Gouveia *et al.*, 1998; Jelfs *et al.*, 1998; Roberts *et al.*, 1998; Besser, Griffin and Slutsker, 1999; Morrison *et al.*, 1999). However, these schemes did not always agree; for example, global population studies of the *Salmonella enterica* Typhi serovar using PFGE indicated great diversity, while Multilocus enzyme electrophoresis (MLEE) indicated two closely related types (Selander, Beltran, Smith, Helmuth, *et al.*, 1990). The major drawback shared by PFGE, PCR, and MLEE approaches is inter-lab method variability, making it difficult to compare results from different regions and nations (Maiden *et al.*, 1998). MLST was developed by adapting MLEE to identify nucleotides of housekeeping genes, as opposed to the electrophoretic probe of the enzymes they encode. Focusing on nucleotide sequence rather than the enzymatic profile of isolates increases the resolution of the detected variation between isolates and allows results to be more easily compared between groups, as separate gel images are not directly comparable (Maiden *et al.*, 1998). Initially, MLST was developed to characterise *Neisseria meningitidis* strains (Maiden *et al.*, 1998). MLST identifies alleles of typically seven housekeeping genes that are 300 - 500 nucleotides in length. The alleles of each gene in an isolate constitute the allelic profile, with each allelic profile assigned a unique sequence type (ST) (Maiden *et al.*, 1998; Jolley and Maiden, 2014; Jolley, Bray and Maiden, 2018). Isolates from the same ST are expected to be more closely related and have similar phenotypes. ST complexes are composed of two parts: a “consensus group” (a group of bacteria that are identical at all loci) and single locus variants (SLVs) (bacteria that are identical at all but one locus)

(Smith, Feil and Smith, 2000). Each SLV likely leaves a consensus group by a single mutation or recombination event; the number of SLVs in a population provides direct evidence of the relative contributions of these processes to clonal diversification. Therefore, this enables comparisons between species and subpopulations of a species (Smith, Feil and Smith, 2000). MLST schemes have since been developed for various bacterial species, including *E. coli*, and MLST has become a cornerstone in microbial epidemiology and phylogenetics (Jolley and Maiden, 2014; Jolley, Bray and Maiden, 2018). The establishment of databases such as PubMLST and software such as BIGSdb have facilitated the development of MLST; these resources enable the efficient storage and analysis of sequencing data to expand MLST databases (Jolley and Maiden, 2010; Jolley, Bray and Maiden, 2017, 2018).

1.6.4.1 Achtman seven-loci multilocus sequence typing scheme

The Achtman seven-loci MLST scheme has become one of the most widely used for typing *E. coli*. This scheme was designed to capture the genetic diversity of *E. coli* and to identify high-risk clones associated with extraintestinal infections (Jolley and Maiden, 2010; Clermont, Gordon and Denamur, 2015). The Achtman scheme utilises seven highly conserved housekeeping genes, allowing for a robust comparison of genetic variation (Wirth *et al.*, 2006; Jolley and Maiden, 2010; Clermont, Gordon and Denamur, 2015). The scheme has been pivotal in tracking the spread of pathogenic *E. coli* strains, particularly those associated with foodborne outbreaks and antibiotic resistance (Roer *et al.*, 2017; Manges *et al.*, 2019). The 15 loci Michigan and eight loci Pasteur schemes are additional conventional MLST schemes for *E. coli* sub-typing (Reid *et al.*, 2000; Jaureguy *et al.*, 2008). There is some evidence that these alternate seven loci schemes outperform the

Achtman scheme (Clermont, Gordon and Denamur, 2015). However, the Achtman scheme incorporates twice as many genomes as the Michigan scheme and seven times as many as the Pasteur scheme (Clermont, Gordon and Denamur, 2015). The evolution of MLST has been driven by the need for higher resolution in strain typing, particularly in epidemiological studies and outbreak investigations. This development has produced the core genome MLST, ribosomal MLST, and whole genome MLST schemes. Generally, these developments have leveraged the improved accuracy and accessibility of whole genome sequencing (WGS), increasing the number of loci screened and, therefore, increasing the discriminatory power of the typing method.

1.6.4.2 Core genome multilocus sequence typing

The cgMLST approach emerged as a response to the limitations of traditional MLST, which often relied on a small number of loci that may not be representative of the genetic diversity within a species (Jolley *et al.*, 2012). In cgMLST, alleles are identified in 100 - 1000 core genes, which are conserved across the genomes of related strains (Moura *et al.*, 2016). The first cgMLST scheme was developed for *Listeria monocytogenes* (Moura *et al.*, 2016). Subsequent cgMLST schemes have differentiated between closely related strains of pathogens, such as *Klebsiella pneumoniae* (*K. pneumoniae*) and *P. aeruginosa*, providing insights into their epidemiology and population structure (Moura *et al.*, 2016; Zhou *et al.*, 2017; de Sales *et al.*, 2020). The *E. coli* cgMLST scheme has been used to differentiate highly similar strains and track the source of contaminants in a food production facility in Japan (Nakamura *et al.*, 2021).

1.6.4.3 Ribosomal multilocus sequence typing

Another significant development was rMLST, focusing on the sequences of 53 loci encoding ribosomal protein subunits (*rps* genes). The rMLST scheme was originally curated from 1900 bacterial genomes across the domain of bacteria, providing a robust framework for phylogenetic analysis (Jolley *et al.*, 2012). The *rps* genes used in this scheme were identified as targets for universal characterisation because they are shared across diverse bacterial taxa, are distributed throughout the chromosome, and encode functionally conserved proteins (Jolley *et al.*, 2012). However, MLST, cgMLST, and rMLST schemes do have limitations. The reliance on a restricted number of genes restricts the investigation of the total genetic diversity of a species or environmental niche, particularly in cases where horizontal gene transfer has occurred (Patiño *et al.*, 2018). Additionally, the loci included in MLST, cgMLST, and rMLST may not sufficiently differentiate isolates that only differ by a few nucleotides. Considering that these single nucleotide differences could change the antibiotic susceptibility of an isolate (e.g., *gyrA* mediated fluoroquinolone resistance), this level of detail is required for studies attempting to understand the spread of antibiotic resistance.

1.6.4.4 Whole genome multilocus sequence typing

Whole genome MLST (wgMLST) schemes expand on the previous MLST schemes, analysing a broader range of genetic information across the entire genome. This method provides a higher resolution for differentiating closely related strains and has been increasingly adopted in studies of *E. coli* (Tré-Hardy *et al.*, 2023). The wgMLST approach is valuable in understanding the genetic basis of ABR, as it encompasses a significantly greater number of loci than other MLST schemes (Tré-Hardy *et al.*, 2023). The higher resolution of

MLST, cgMLST, and wgMLST between similar strains has identified clusters that would not have been identified by lower-resolution techniques such as PFGE (Jolley *et al.*, 2012). Some investigations has employed hybrid approaches, utilising less discriminatory approaches such as cgMLST to cluster isolates into ST and subsequent clustering of these ST with wgMLST to refine intercluster variability (Jagadeesan *et al.*, 2019).

1.7 Enterobase enables investigations of the genomic epidemiology of pathogenic bacteria

Enterobase, a comprehensive database for enterobacteria, has emerged as a key resource for investigating these pathogens (Zhou *et al.*, 2020). Enterobase currently contains over one million bacterial genomes from *E. coli* as well as *Salmonella, Mycobacterium, Streptococcus, Clostridoides, Vibrio, Helicobacter, Yersinia,* and *Moraxella* (Zhou *et al.*, 2020).

This platform provides access to a significant collection of *E. coli* genomes and offers tools for MLST analysis, including the Achtman seven-loci MLST, cgMLST, and wgMLST schemes (Zhou *et al.*, 2020). Facilitating the identification of STs, and enabling comparisons between a dataset and a global database (Zhou *et al.*, 2020; Hayer *et al.*, 2022). Enterobase has been used to track the genetic relatedness of *E. coli* isolates from various sources, including animals, food, wastewater, and clinical samples (Ramadan *et al.*, 2021; Hayer *et al.*, 2022). Research utilising Enterobase and MLST has shown that particular *E. coli* STs, such as ST131 and ST410, are prevalent in dairy cattle from Egypt and New Zealand and potentially pose a risk of transmission to humans via the food chain (Collis *et al.*, 2022; Magaña-Lizárraga *et al.*, 2022). Identifying these high-risk clones through MLST has informed plans for monitoring and controlling the spread of ABR *E. coli* in agricultural settings

(Collis *et al.*, 2022; Magaña-Lizárraga *et al.*, 2022). Enterobase also facilitates the integration of MLST data with other analyses, such as serotyping, plasmid characterisation, and virulence gene profiling. These complimentary analyses can provide a more complete understanding of the factors that contribute to the pathogenicity of *E. coli* (Ramadan *et al.*, 2021; Magaña-Lizárraga *et al.*, 2022). Filtering the Enterobase *E. coli* database for genomes of the isolates collected from bovine sources between 2018 and 2024, resulted in 6743 genomes from 25 countries (Table 1.8).

Table 1.8: Isolation continent and country of *E.coli* genomes submitted to Enterobase.

Continent	Country	Number of genomes
North America	USA	5671
	Canada	33
	Total	5704
Europe	UK	431
	Switzerland	275
	France	23
	Portugal	11
	Spain	4
	Czechia	2
	Norway	2
	Hungary	2
	Luxembourg	1
	Total	751
South America	Chile	12
	Ecuador	11
	Brazil	9
	Uruguay	8
	Argentina	4
	Total	44
Asia	Pakistan	14
	China	12
	Thailand	12
	No country stipulated	1
	Total	39
Oceania	Australia	14
	Total	14
Africa	South Africa	3
	Ethiopia	3
	Djibouti	3
	Egypt	1
	Rwanda	1
	Total	11

Genomes isolated from bovine sources submitted between 2018 and 2024.

Across these 6743 genomes, 905 STs and 40 ST complexes were identified according to the *E. coli* whole genome MLST scheme. The most common STs in descending order were ST11 (7%), ST10 (4.81%), ST58 (4.72%), ST679 (3.37%), and ST8427 (3.16%) (Table 1.9). The most common ST complexes in descending order were no complex (36.99%), ST10 complex (8.62%), ST155 complex (8.17%), ST11 complex (7.64%), and ST446 complex (5.52%) (Table 1.9).

Table 1.9: Sequence types and sequence type complexes identified in *E. coli* genomes submitted to Enterobase.

ST	*E. coli* genomes		ST complex	*E. coli* genomes	
	Number	Percent		Number	Percent
11	472	7.00	No complex	2494	36.99
10	324	4.80	10	581	8.62
58	318	4.72	155	551	8.17
679	227	3.37	11	515	7.64
8427	213	3.16	446	372	5.52
21	169	2.51	469	361	5.35
17	146	2.17	29	342	5.07
278	139	2.06	20	251	3.72
154	105	1.56	23	229	3.40
101	98	1.45	278	210	3.11
164	97	1.44	101	102	1.51
155	93	1.38	69	97	1.44
109	92	1.36	206	74	1.10
16	91	1.35	399	72	1.07
69	81	1.20	86	70	1.04
441	72	1.07	32	70	1.04
297	71	1.05			
88	70	1.04			

Only sequence type (ST) and ST complexes identified in ≥1% of genomes from bovine sources that were submitted between 2018 and 2024 are presented.

Enterobase includes a plotting functionality to create GrapeTree plots, which creates a minimum spanning tree of isolates. The ST with the greatest number of SLV is placed at the centre of the tree, each SLV of this central node forms a branch. The process of identifying SLV is repeated for the terminal node and a tree that clusters similar ST together originating from the ST of least overall variance is produced (Figure 1.4) (Zhou *et al.*, 2018). This GrapeTree plot displays the global spread of the major STs identified in *E. coli* genomes isolated in samples from bovine sources, with the caveat that the vast majority of the isolates in the database were sampled from North America, mostly the USA (Table 1.8).

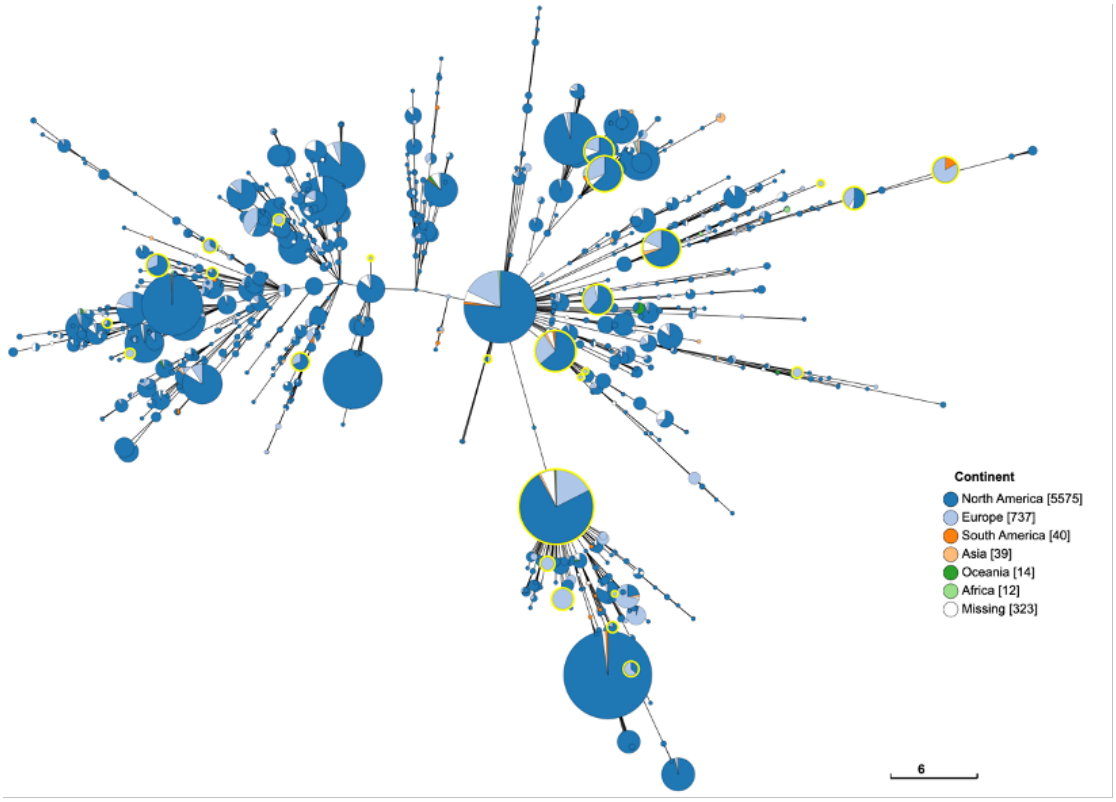


Figure 1.4: MSTree V2 GrapeTree plot of 6743 *E. coli* genomes. All isolates are from bovine sources collected between 2018 and 2024 grouped by the Achtman seven gene MLST scheme and coloured by continent of sample. This figure also includes 170 genomes of the isolates collected in this thesis, nodes highlighted by yellow circle include these genomes.

1.7.1 *In silico* serotyping *E. coli* to investigate diversity

Serotyping was developed to differentiate haemolytic *streptococci* using antibodies generated in rabbits (Lancefield, 1933). A curated scheme for *coliform* bacteria that differentiated strains on 25 O, 55 K, and 20 H-antigens followed (Kauffmann, 1947). Serological typing (serotyping) of the polymorphic O (somatic), K (capsule) and H (flagellar) antigens has been used to differentiate *E. coli* and identify pathogenic lineages. There are 182 O-antigens and 53 H-antigens in traditional serotyping schemes for *E. coli* (Ørskov and Ørskov, 1984; Scheutz *et al.*, 2004). The O-antigen is a polysaccharide component of outer bacterial membrane lipopolysaccharides, usually consisting of 10 - 25 repeating units of oligosaccharides. The O units are polymerised and translocated by the O-antigen processing proteins, polymerase (encoded by *wzy*) and flippase (encoded by *wzx*) (Whitfield, 1995; Valvano, 2003). The K-antigens inhibit the identification of O-antigens and are identified by typing a given strain before and after heat inactivation (Kauffmann, 1947). The H-antigen is a polymeric filament composed of flagellin repeats, identified as potent immunogens (Ada *et al.*, 1967; Joys, 1988). Flagella are amongst the earliest discoveries in prokaryotes, described by Antonie van Leeuwenhoek in a letter sent to the Royal Society in 1677 (Dobell, 1919). Flagellar filament structural protein (FliC) determines the H-antigen of a strain and expression is determined by several genes, *fliC*, *flkA*, *fllA*, *flmA*, and *flnA* (Ratiner, 1998; Wang *et al.*, 2003; Tominaga, 2004; Feng *et al.*, 2008; Ratiner *et al.*, 2010). Serogroups are composed of strains with similar O-antigens, serotypes are composed of strains with similar O and H-antigens, and pathotypes are composed of serotypes with shared virulence factors and implicated in the same disease (Nataro and Kaper, 1998).

Enteropathogenic, enterotoxigenic, enteroinvasive, enterohaemorrhagic, enteroaggregative, Shiga toxin-producing, diffusely adherent, and adherent invasive are the most well defined *E. coli* pathotypes (Nataro and Kaper, 1998; Fratamico *et al.*, 2016). Serotyping has known flaws, with agglutination results varying for the same strain between different laboratories (Ørskov *et al.*, 1975). The assumption that serotype can serve as a marker to differentiate *E. coli* was due to the vast number of possible combinations of known *E. coli* O and H-antigens (>10,000) (Ingle *et al.*, 2016). However, strong selective pressures from bacteriophage and host immune cells on the O-antigen lead to a high recombination frequency at this locus (Ingle *et al.*, 2016). Therefore, distantly related *E. coli* strains can have the same serotype, while closely related isolates may have different serotypes (Ingle *et al.*, 2016). Molecular methods and online platforms were developed to serotype strains through PCR amplification to identify the genes for O and H-antigens from DNA and assembled genomes respectively, but these methods are still too slow for public health laboratories (Fratamico *et al.*, 2009; Joensen *et al.*, 2015). The database of known *E. coli* O-groups and H-types (EcOH) predicts the serotype of *E. coli* from raw sequencing reads with no assembly required, identifying matching nucleotide sequences in a curated database of O and H-antigen genes (Ingle, 2016; Ingle *et al.*, 2016).

1.8 Antibiotic mechanisms of action, resistance mechanisms, and associated resistance genes

Different antibiotic classes produce their inhibitory effects through several mechanisms of action and bacteria have evolved a diverse set of resistance mechanisms in response. This section contains the mechanisms of action of

classes of antibiotics in use in the veterinary industry (Veterinary Medicines Directorate, 2021b) and the mechanisms of resistance found in *E. coli*.

1.8.1 The presence of an antibiotic resistance gene is not equivalent to phenotypic antibiotic resistance in an isolate or a population

The presence of an antibiotic resistance gene (ARG) does not necessarily equate to phenotypic ABR in an isolate or population. In order to understand the risk of AMR in a population, several factors must be assessed, including mutation rates, the level of resistance conferred, fitness costs of resistance mechanisms, and selective pressures.

1.8.1.1 Mutation rates and resistance emergence

Mutation rates play a key role in the emergence of resistance. The mutation supply rate depends on population size, mutation rates, and horizontal gene transfer (HGT) frequencies (Worby, Lipsitch and Hanage, 2014). For bacteria, typical mutation rates are $\leq 10^{-10}$ per nucleotide per generation, resulting in a genomic mutation rate of ~0.003 per genome per generation for *E. coli* (Abdulkarim and Hughes, 1996; Drake *et al.*, 1998; Drake, 1999; Foster, 2006; Lynch, 2010). Rearrangement mutations, including insertions, deletions, and duplications, occur at much higher rates of 10^{-5} to 10^{-3} per cell per generation (Reams *et al.*, 2010, 2012). HGT frequencies are highly variable, ranging from 50% in 10-minute conjugation experiments to undetectable levels ($<10^{-9}$) depending on transfer mechanisms, bacterial species, and transfer conditions (Dunny, Yuhasz and Ehrenfeld, 1982; Cabezón *et al.*, 2015). However, the rate of resistance mutation alone does not correlate with clinical resistance emergence, likely because isolates often harbour ARGs before treatment begins (Hughes and Andersson, 2017b). Therefore, relying solely on mutation rates is insufficient for predicting AMR risk. A more comprehensive approach

includes screening metagenomes for ARGs, assessing their likelihood of horizontal transfer, and evaluating their stability post-transfer (Andersson, 2015).

1.8.1.2 Variability in the resistance levels conferred

The degree of resistance conferred by mutations (e.g., increases in MIC) influences a resistant isolate's success. Resistance levels depend on the mechanism involved and the environmental or in vitro conditions under which they are assessed. Some mechanisms allow growth to persist at increasing antibiotic concentrations, while others inhibit growth until the MIC threshold is reached (Nilsson *et al.*, 2003; Gullberg *et al.*, 2011; Thulin, Sundqvist and Andersson, 2015). Environmental conditions and bacterial interactions further modulate phenotypic resistance, influencing the effects of ARG expression (Thulin, Sundqvist and Andersson, 2015). For example, physiological responses or interactions with metabolites can impact phenotypic outcomes (Kubicek-Sutherland *et al.*, 2015; Hughes and Andersson, 2017a).

1.8.1.3 Fitness costs of resistance mechanisms

The fitness cost of ARG expression is critical to understanding resistance emergence and persistence. Fitness refers to the ability of an isolate to compete and replicate within a population. Although exceptions exist, most resistance mechanisms investigated in vitro reduce relative fitness (Andersson and Levin, 1999; Andersson and Hughes, 2010; Hughes and Andersson, 2015). Fitness costs influence the rate of resistance emergence and reversibility when selective pressures decrease or are removed. This dynamic has been demonstrated theoretically, epidemiologically, and *in vitro* (Andersson and Levin, 1999; Andersson and Hughes, 2010; zur Wiesch *et al.*, 2011; Wiser, Ribeck and Lenski, 2013). For example, in clinical

Mycobacterium tuberculosis and *Staphylococcus aureus*, ARGs with lower fitness costs were more prevalent epidemiologically (Bottger *et al.*, 1998; Sander *et al.*, 2002; O'Neill *et al.*, 2006; Brandis *et al.*, 2015). Selection at sub-inhibitory antibiotic concentrations can promote ARGs that confer small resistance increases but minimal fitness costs. These low-cost mutations accelerate resistance emergence, reduce reversibility rates, and increase the persistence of susceptible isolates capable of acquiring ARGs through HGT or further mutation (Andersson and Hughes, 2014).

1.8.1.4 Epistasis and compensatory mechanisms

Epistasis describes how genetic interactions influence phenotypes, such as altering an ARG's fitness cost or resistance level. For instance, secondary mutations can compensate for fitness costs, enabling resistant mutants to persist and spread (Hughes and Andersson, 2017b). Mutations in genes affecting cell wall structure, membrane permeability, or efflux activity can modulate the MIC values of antibiotics (Gwynn *et al.*, 2010; Silver, 2011). In *E. coli*, fluoroquinolone resistance via *gyrA* target modifications requires functioning efflux systems for clinically significant resistance (Oethinger *et al.*, 2000). Epistatic interactions can also reduce fitness costs associated with ARG expression for multiple antibiotic classes, including aminoglycosides, rifamycins, fusidic acid, and fluoroquinolones (Mariam *et al.*, 2011; Brandis *et al.*, 2012; Schrag, Perrot and Levin, 2012; Brandis and Hughes, 2013; Lázár *et al.*, 2014).

1.8.2 Multidrug resistance and associated increase risk to public health

Bacteria with a MDR phenotype have been identified as a serious public health problem (WHO, 2015). MDR bacteria either carry multiple resistance genes or express multidrug efflux pumps (Nikaido, 2009). Isolates resistant to at least

one antimicrobial in all but two or fewer classes are termed extensively drug resistant, and isolates resistant to all antimicrobials in all classes are termed pan drug resistant (Magiorakos *et al.*, 2012). The rates of MDR *E. coli* in dairy samples range from 29% in mastitis cases in Canada, to 44.4% from udders in China (Majumder *et al.*, 2021; Shoaib *et al.*, 2023). Previous studies on the SB Farm found that 37% of *E. coli* in slurry tank samples were MDR (Baker *et al.*, 2022).

1.8.3 Structure and function of multidrug efflux pump families

There are six families of multidrug efflux pumps, in *Enterobacteriaceae;* Resistance nodulation cell division superfamily (RND), Major facilitator superfamily (MFS), and ATP binding cassette family (ABC) are the predominant efflux families (Du *et al.*, 2018; Davin-Regli, Pages and Ferrand, 2021). The Colicin tolerance (TolC) trimeric outer membrane protein is comprised of a periplasmic alpha barrel connected to a beta barrel anchored in and spanning the outer membrane (Nagel de Zwaig and Luria, 1967; Koronakis *et al.*, 1997). TolC acts as a subunit of several tripartite multidrug efflux complexes across RND, ABC, and MFS efflux pumps (Koronakis *et al.*, 2000). Many pumps are termed 'multidrug efflux, but the physiological purpose of these pumps extends beyond antimicrobial compounds with evidence linking them to metabolism, biofilm formation, and persistence. The structure of an efflux pump determines which molecules are substrates, and if an antibiotic is substrate of a pump this confers reduced susceptibility or resistance. Several efflux pumps have been identified in *E. coli* with a range of substrates conferring several resistance phenotypes, including MDR (Table 1.10).

Table 1.10: Antibiotic efflux resistance genes identified in *E. coli*.

Gene	Resistance phenotype	Reference
	RND superfamily efflux pumps	
acrAB-tolC	β-lactams, phenicols, macrolides, quinolones, aminocoumarins, tetracyclines, and linezolid	(Swick *et al.*, 2011)
acrCD	Aminoglycosides	(Ferrand *et al.*, 2020)
acrEF	β-lactams and fluoroquinolones	(Teelucksingh, Thompson and Cox, 2020)
mdtABC	Aminocoumarins	
mdtEF	Penicillins, fluoroquinolones, and macrolides	
oqxAB-tolC	Quaternary ammonium compounds, phenicols, quinolones, and nitrofurantoin	(Hansen *et al.*, 2007)
	MFS efflux pumps	
emrAB-tolC	Aminocoumarins and quinolones	(Nishino, Latifi and Groisman, 2006)
emrD	Phenicols	(Sulavik *et al.*, 2001)
mdfA	Phenicols, macrolides, aminoglycosides, tetracyclines, and quinolones	(Edgar and Bibi, 1997)
mdtG	Quaternary ammonium compounds	(Fàbrega *et al.*, 2010)
mdtH	Quinolones	(Li and Nikaido, 2009)
mdtM		(Holdsworth and Law, 2013)
mdtN		(Sulavik *et al.*, 2001)
mdtO		
mdtP		
kpnH	Macrolides, β-lactams, aminoglycosides, quinolones, colistin	(Srinivasan *et al.*, 2014)
	ABC efflux pumps	
macAB-tolC	Macrolides	(Kobayashi, Nishino and Yamaguchi, 2001)
msbA	Nitromidazoles	(Singh *et al.*, 2016)
yojI	Microcin	(Delgado *et al.*, 2005)
ydcV	Increased expression found in strains with high prevalence of resistance to penicillin, quinolone, and aminoglycosides.	(Zeng *et al.*, 2020)
ydcU		
ydcS		
ydcT		
cysP		
	MATE efflux	
mdtK	Quinolones	(Nishino, Latifi and Groisman, 2006)
	Multidrug transporters	
emrE	Quaternary ammonium compounds	(Rotem and Schuldiner, 2004)

1.8.3.1 Resistance nodulation division superfamily efflux pumps

Resistance nodulation division (RND) transmembrane domains contain a conserved internal structure, with a highly variable periplasmic luminal domain (Du *et al.*, 2018). RND structure can be generalised into three domains; a transmembrane domain, a pore domain, and a docking domain. The transmembrane domain is the site of proton movement, coupled to drug/substrate movement in the pore domain (Murakami *et al.*, 2002, 2006; Eicher *et al.*, 2014). The pore domain is comprised of four subdomains, which are enriched with several charged, polar, and aromatic residues conferring each pocket with different substrate preferences (Murakami *et al.*, 2002; Eicher *et al.*, 2014). The docking domain consists of two subdomains and these interact with periplasmic proteins in tripartite assemblies (Murakami *et al.*, 2002). The Acriflavine resistance protein family (Acr), specifically the inner membrane AcrA and periplasmic AcrB are major contributors to efflux mediated resistance in *E. coli* (Nakamura, 1966; Ma *et al.*, 1993). AcrAB-tolC is a major RND superfamily efflux pump in *E. coli*, undergoing a conformational change due to drug binding (Du *et al.*, 2018). AcrB is a trimeric protein, with each trimer occupying one of three states when the pump is active; open, tight, and loose (Seeger *et al.*, 2006). These trimers work co-operatively to move substances unidirectionally, out of the cell from the periplasm in a peristaltic fashion (Seeger *et al.*, 2006).

1.8.3.2 Adenosine triose phosphate binding cassette family efflux pumps

Adenosine triose phosphate binding cassette (ABC) transporters mediate adenosine triose phosphate (ATP) dependent solute import/export (Du *et al.*, 2018). ABC transporters are transmembrane proteins that contain binding pockets to interact with substrates and nucleotide binding domains to

hydrolyse ATP (Du *et al.*, 2018). ABC transporters undergo conformational changes between inward-open, occluded, and outward-open states, driven by nucleotide binding domain dimerization and ATP binding/hydrolysis (Khare *et al.*, 2009). In Gram-negative bacteria, ABC transporters can either pump substrates across the inner membrane or across both membranes and the periplasm by forming a tripartite system with a periplasmic membrane protein (e.g., MacA) and an outer membrane protein (e.g., TolC) (Du *et al.*, 2018).

1.8.3.3 Major facilitator superfamily efflux pumps

The major facilitator superfamily (MFS) is the largest and most diverse family of transporters including uniporters, symporters, and antiporters (Yan, 2013). MFS transporters alternate access, with two domains undergoing a conformational switch between outward-open and inward-open states in a similar fashion to ABC transporters (Yan, 2013). Unlike ABC, MFS are not ATP dependent, conformational changes are driven by ligand binding with the rate of transport controlled by electrochemical proton gradients (Yan, 2013). MdfA encodes a membrane protein involved in drug transport conferring resistance to a broad range of antimicrobial compounds including several classes of antibiotics (Edgar and Bibi, 1997). Some MFS antiporters can form tripartite assemblies with partner proteins to pump across Gram-negative double membranes, VceB in *Vibrio cholerae* is an example (Federici *et al.*, 2005).

1.8.3.4 *E. coli* efflux pump regulatory genes

The presence of several regulatory mechanisms suggests an increased likelihood of over-expression if a selective pressure was present in the environment (Appendix 1).

1.8.4 Mobile genetic elements in antibiotic resistance

Horizontal gene transfer significantly increases the complexity of the resistome as microbes can move resistance genes within their genome as well as acquire and share them with other microbes of different species (Figure 1.5) (Partridge *et al.*, 2018).

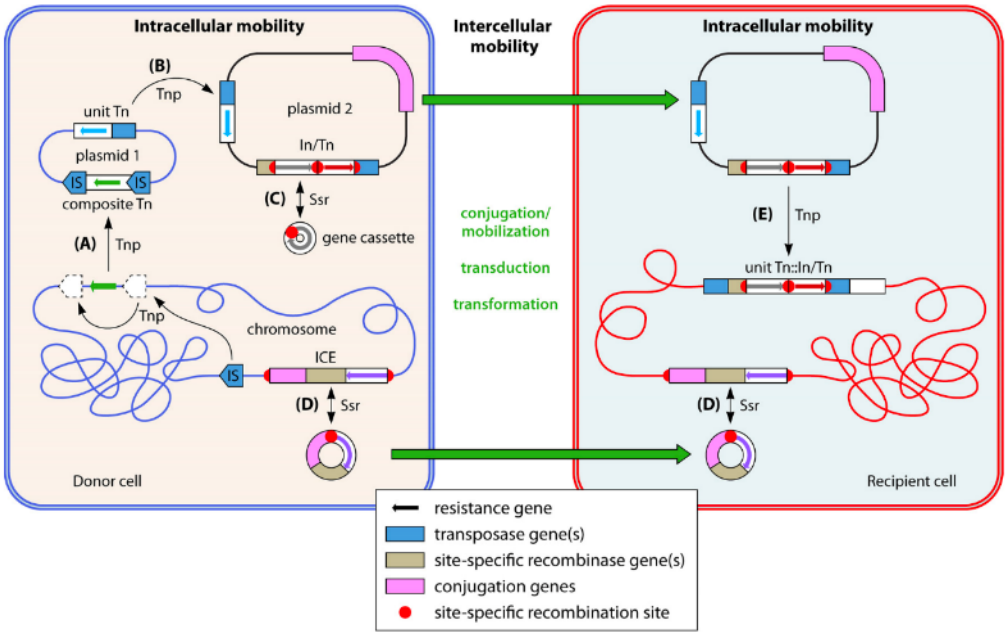


Figure 1.5: Examples of mobile genetic elements and processes involved in ARG transfer. The blue cell is the donor containing its chromosome and two plasmids, the red cell is the recipient containing its chromosome and a conjugated plasmid from donor. Gene function shown in the key. ARG associated with different mobile genetic elements are shown by small arrows of various colours. Intracellular process are represented by thin black arrows, those mediated by transposase protein and by a site-specific recombinase protein are labelled. thick green arrows represent intercellular transfer (Partridge *et al.*, 2018).

Genomic vectors of AMR are mobile genetic elements, segments of DNA that encode enzymes and proteins, which in turn are involved in the movement of DNA in genomes (Frost *et al.*, 2005). They include insertion sequences, plasmids, transposons, integrons and integrative conjugative elements (Frost *et al.*, 2005). These vectors can move DNA intracellularly and intercellularly. Intercellular movement can occur through multiple routes, including transformation, conjugation, or by transduction (Frost *et al.*, 2005). Insertion sequences are generally small mobile elements, typically carrying one or two transposase genes (Siguier *et al.*, 2015). Plasmids are a collection of functional genetic modules that are organized into a stable, self-replicating replicon which is smaller than the cellular chromosome (Frost *et al.*, 2005). Essential requirements for a plasmid are backbone genes that encode replicative functions and variable accessory genes that encode processes distinct from the chromosome (Thomas, 2000; Dahlberg and Chao, 2003). Plasmids are grouped into incompatibility groups, determined by the genes they require for replication, plasmids of the same type (requiring the same replication systems) compete and ultimately one will replicate more successfully and the other is eliminated. This concept was identified in bacteriophage and plasmids (Watanabe *et al.*, 1964; Datta and Hedges, 1973). Transposons are sections of DNA that can move from one location in a genome to another (Pray, 2008). Integrons are genetic elements that encode an integrase enzyme, which assembles tandem arrays of genes or fragments while providing a promoter for expression (Frost *et al.*, 2005).

1.8.5 Tetracycline antibiotics mechanism of action and resistance mechanisms

Tetracyclines inhibit bacterial growth by binding the 16S ribosomal RNA (16S rRNA) target within the 30S sub-unit, reducing translation by interfering with aminoacyl-transfer RNA during elongation (Maxwell, 1967; Brodersen *et al.*, 2000; Pioletti *et al.*, 2001). In Gram-negative cells, tetracycline passively diffuses through outer membrane porins (OmpF and OmpC) while chelated with Magnesium (McMurry *et al.*, 1981; Argast and Beck, 1985; Mortimer and Piddock, 1993; Nikaido and Thanassi, 1993). Tetracyclines traverse the bacterial cytoplasmic membranes via facilitated diffusion using proton motive force and phosphate bond hydrolysis (McMurry and Levy, 1978; Argast and Beck, 1984; Yamaguchi *et al.*, 1991). To date, resistance to tetracyclines has been described through three mechanisms: efflux, ribosomal protection, and enzymatic modification of tetracyclines (Table 1.11).

1.8.5.1 Tetracycline efflux resistance mechanisms

Most genes encoding tetracycline efflux proteins belong to the major facilitator superfamily, containing over 300 protein products, of which nine have been shown to facilitate tetracycline efflux in *E. coli* (Paulsen, Brown and Skurray, 1996; Roberts and Schwarz, 2015). In *E. coli* the transport of small molecules across the outer membranes is facilitated by a membrane fusion protein that connects to the outer membrane protein channel (Chopra and Roberts, 2001). In addition to major facilitator superfamily efflux, the chromosomal *mar* operon is involved in differential expression of over 60 genes including a tetracycline efflux system associated with MDR in *E. coli* (Anthony M George and Levy, 1983; Levy, 1992; Barbosa and Levy, 2000). In this operon, MarA is a transcriptional activator that induces increased expression of the multidrug

efflux pump AcrAB and decreased expression of the porin OmpF (Anthony M George and Levy, 1983; Cohen, Mcmurry and Levy, 1988; Okusu, Ma and Nikaido, 1996).

1.8.5.2 Ribosomal protection mediated tetracycline resistance mechanisms

Several genes have been identified that protect the ribosome from tetracycline binding. The best characterised of these are *tet(M)* and *tet(O)* (Chopra and Roberts, 2001; Connell *et al.*, 2003). Elongation factors that catalyse the binding of an aminoacyl-transfer RNA to the ribosome share sequence similarity with *tet(M)* and *tet(O)* (Sanchez-Pescador *et al.*, 1988), *tet*(O) and *tet*(M) confer resistance by dislodging tetracycline from the ribosome in a guanosine triphosphate dependent manner (Burdett, 1996; Trieber *et al.*, 1998).

1.8.5.3 Enzymatic modification of tetracycline resistance mechanisms

The *tet(X)* family encode flavin-dependent monooxygenases confer enzymatic resistance by covalently inactivating all tetracyclines groups by adding a hydroxyl group to the tetracycline core structure (Speer, Bedzyk and Salyers, 1991; Yang *et al.*, 2004; Moore, Hughes and Wright, 2005). Self-transmissible plasmids and transposons containing *tet(X)* have been described and found in environmental, farm, and clinical settings (Wang and Shao, 2012; Leski *et al.*, 2013; Deng *et al.*, 2014; Forsberg *et al.*, 2015).

Table 1.11: Tetracycline resistance mechanism and associated resistance genes.

Gene	Reference
Efflux	
tet(A)	(Tuckman *et al.*, 2007; Wang *et al.*, 2014)
tet(B)	(Lancashire *et al.*, 2005; Tuckman *et al.*, 2007)
tet(C)	(Schnabel and Jones, 1999; Tuckman *et al.*, 2007)
tet(D)	(Tuckman *et al.*, 2007; Roberts, 2021)
tet(E)	(Tuckman *et al.*, 2007)
tet(G)	(Schnabel and Jones, 1999; Tuckman *et al.*, 2007)
tet(J)	(Roberts, 2021)
tet(L)	(Tuckman *et al.*, 2007)
tet(Y)	(Tuckman *et al.*, 2007)
tet(Z)*	(Tauch *et al.*, 2000)
tet(30), *tet*(31), and *tet*(33)*	(Roberts, 2021)
tet(57)*	(Huang, Zhang and Wang, 2015)
tet(35), *tet*(39), *tet*(41), *tet*(K), *tet*(L), *tet*(38), *tet*(45), *tet*(58), *tet*(63), *tetA*(P), *tet*(40), *otr*(B), *otr*(C), *tcr3, tet*(42), *tet*(43), *tet*AB(46), *tet*AB(60), *tet*(62), and *tet*(64)*	(Roberts, 2021)
emrK *emrY*	(Tanabe *et al.*, 1987)
Ribosomal protection	
tet(M)	(Tuckman *et al.*, 2007; Dönhöfer *et al.*, 2012)
tet(W)	(Chopra and Roberts, 2001; Aires, Doucet-Populaire and Butel, 2007)
tet(O), *tet*(S), *tet*(32), *tet*(Q), *tet*(T), *tet*(36), *tet*(61), *otr*(A), *tetB*(P), and *tet*(44)*	(Roberts, 2021)
Enzymatic modification	
tet(X)	(Yang *et al.*, 2004)
tet(37), *tet(34),* *tet*(47), *tet*(48), *tet*(49), *tet*(50), *tet*(51), *tet*(52), *tet*(53), *tet*(54), *tet*(55), and *tet*(56)*	(Roberts, 2021)

*resistance genes characterised in bacteria other than *E. coli*. Adapted from Roberts and Schwarz, 2015.

1.8.6 β-lactam antibiotics mechanism of action and resistance mechanisms

β-lactams target the cell membrane by mimicking the natural D-Ala-D-Ala substrate of the penicillin-binding protein (PBP) family, interacting with these proteins in the periplasmic space in Gram-negative bacteria (Tipper and Strominger, 1965; Wise and Park, 1965; Worthington and Melander, 2013). The interaction of β-lactams with PBPs inhibits peptidoglycan cross linking during bacterial cell wall formation resulting in eventual cell lysis (Tipper and Strominger, 1965; Wise and Park, 1965; Bush and Bradford, 2016). There are several classes of β-lactam antibiotics; penicillins, cephalosporins, carbapenems, and monobactams, but only a selection are approved for use in UK bovines (Table 1.2). Penicillins were derived from *Penicillium* fungi by Alexander Fleming in 1929 (Fleming, 1929). Penicillins can be split into four subclasses; natural, penicillinase resistant, aminopenicillins, and extended spectrum (Miller, 2002). The emergence of resistance to natural penicillins drove the development of novel aminopenicillin synthetic derivatives, with the subsequent resistance against these compounds driving the development of alternative β-lactams (cephalosporins, carbapenems, and monobactams) (Kahan *et al.*, 1979; Sykes *et al.*, 1982; Bo, 2000; Kong, Schneper and Mathee, 2010; Bush and Bradford, 2016). Different species of micro-organisms carry different PBPs and β-lactamases resulting in some β-lactams having a narrow spectrum of activity, while others have a broad spectrum of activity which can be extended with the addition of β-lactamase inhibitors (Bush and Bradford, 2016). The general β-lactam resistance mechanisms are enzymatic degradation, alteration of PBPs through substitution and mutation, and restricted access to PBPs through regulation of entry and efflux.

1.8.6.1 Enzymatic modification of β-lactam antibiotics

Production of β-lactamases is the most prevalent β-lactam resistance mechanism in bacteria (Pitout, Sanders and Sanders, 1997; Worthington and Melander, 2013). By 2009, over 890 unique protein sequences confirmed to be β-lactamases were known; these are currently organised by two classification schemes (Bush and Jacoby, 2010). The Ambler system is a molecular classification system, dividing enzymes based upon their amino acid sequence into classes, which hydrolyse β-lactams with serine (A, C, and D) and a class of metalloenzymes, which require divalent zinc ions for hydrolysis (B) (Ambler, 1980; Hall and Barlow, 2005). The Bush-Jacoby system is a functional classification system that takes into account substrate and inhibitor profiles to correlate enzymes and clinical phenotypes. The Bush-Jacoby system divides β-lactamases into: group 1 (Cephalosporinases), group 2 (broad spectrum inhibitor-resistant serine carbapenemases and extended spectrum β-lactamases), and group 3 (metallo-β-lactamases) (Bush, Jacoby and Medeiros, 1995; Bush and Jacoby, 2010). Both classification schemes are represented in Table 1.12.

Table 1.12: Classification of bacterial β-lactamases (Illustration of Ambler and Bush-Jacoby classifications).

Ambler	Bush-Jacoby	Characteristics	Example Enzymes	Substrate
A	2a	Penicillinases inhibited by CA	PC1	Penicillins
	2b	Broad spectrum enzymes inhibited by CA	TEM-1, TEM-2, TEM-13, SHV-1, SHV-11	Penicillins, cephalothin
	2be	extended spectrum broad spectrum enzymes inhibited by CA	TEM-3, TEM-10, TEM-26, SHV-2, SHV-3, CTX-M, PER, VEB	Penicillins, oxyimino-cephalosporins (CTX, CTD, CFT, CFP), monobactams
	2br	Broad spectrum enzymes with reduced binding to CA (inhibitor resistant TEMS)	TEM-30, TEM-31, SHV-10, SHV-72	Penicillins, resistant to CA, tazobactam and sulbactam
	2ber	Extended spectrum enzymes with relative resistance to CA	TEM-50, TEM-158	Penicillins, oxyimino-cephalosporins, monobactams, resistant to CA, tazobactam and sulbactam
	2c	CB-hydrolysing enzymes inhibited by CA	PSE-1, CARB-3	Penicillins, CB
	2ce	Extended spectrum carbenicillinase	RTG-4 (CARB-10)	CB, CFP
	2e	Cephalosporinases inhibited by CA	CepA	Cephalosporins
	2f	Carbapenem hydrolysing non-metallo-β-lactamases	KPC, SME, GES, IMI-1, TEM150	All β-lactams, including carbapenems (Shakil and Khan, 2013)
B	3	Metallo-β-lactamase	IMP, VIM, IND	All β-lactams, including carbapenems, with the exception of monobactams
C	1	Cephalosporinases not inhibited by CA	ACT-1, FOX-1, MIR-1, CMY, AmpC, AmpH	Narrow and extended-spectrum cephalosporins, including cephamycins
D	2d	Cloxacillin hydrolysing enzymes with variable inhibition by CA	OXA-1, OXA-2, OXA-10,	Cloxacillin, oxacillin
	2de		OXA-11, OXA-15	Cloxacillin, oxacillin, oxyimino-cephalosporins, monobactams
	2df		OXA-23, OXA-51, OXA-58	Cloxacillin, oxacillin, carbapenems, penicillins
	4	Penicillinase not inhibited by CA	Penicillinases from *Burkholderia cepacia*	Penicillins

CA (clavulanic acid), CTX (cefotaxime), CTD (ceftazidime), CFT (ceftriaxone), CFP (cefepime), CB (carbenicillin). Adapted from (Tang, Apisarnthanarak and Hsu, 2014).

1.8.6.2 Restricted access to penicillin-binding proteins

Access to PBPs is restricted due to the additional membrane present around Gram-negative bacteria, a loss of porins or increased efflux prevent antibiotics from reaching the target site (Worthington and Melander, 2013). Alteration to a PBP is rare in Gram-negative bacteria but plays a key role in Gram-positive cocci, e.g., *Staphylococcus aureus* and *Streptococcus pneumoniae*, PBP2a is the key resistance determinant in methicillin resistant *Staphylococcus aureus* (MRSA) (Pitout, Sanders and Sanders, 1997; Worthington and Melander, 2013).

1.8.6.3 β-lactam antibiotics resistance through alteration of the penicillin-binding proteins

Various alterations to the PBPs in bacterial membranes are possible; a low affinity alternative PBP can be overexpressed or acquired, endogenous PBPs can be altered by homologous recombination or point mutations, and a combination of these alterations is also possible (Worthington and Melander, 2013). PBPs are divided by their structures, into high molecular mass and low molecular mass, with most bacterial species carrying sets of 3-8 distinctive PBPs (Georgopapadakou and Liu, 1980). The reduced susceptibility of PBP2a in MRSA is due to a combination of acylation and decreased binding of β-lactams to the active site (Fuda *et al.*, 2004).

1.8.7 Trimethoprim and sulfonamides mechanism of action and resistance mechanisms

Although trimethoprim and sulfonamides are two separate classes of antimicrobials they are synthetic antibacterial agents belonging to the diaminopyrimidine group of compounds and they are often used together

clinically, as a formulation of trimethoprim and sulfamethoxazole (Seydel *et al.*, 1972; Shin *et al.*, 2014).

Sulfonamides are analogues of p-aminobenzoic acid; exhibiting their antimicrobial properties by competitively inhibiting the bacterial enzyme dihydropteroate synthase (DHPS), disrupting the reaction sequence from p-aminobenzoic acid leading to dihydrofolic acid (Woods, 1940; Henry, 1944; Skold, 2001). Trimethoprim acts by competitively inhibiting the action of dihydrofolate reductase (DHFR), therefore disrupting the synthesis of DNA thymine (Skold, 2001). Resistance occurs through non-allelic and drug-resistant variants of the target enzymes DHPS and DHFR respectively (Skold and Widh, 1974; Wise and Abou Donia, 1975; Skold, 1976) (Table 1.13).

Table 1.13: Sulfonamide and Trimethoprim resistance genes found in *E. coli.*

Gene	Reference
Sulfonamide	
*sul*1	(Aarestrup *et al.*, 2003)
*sul*2	
*sul*3	(Perreten and Boerlin, 2003)
Trimethoprim	
dfrA1	(Skold and Widh, 1974; Blahna *et al.*, 2006)
dfrA3	(Blahna *et al.*, 2006)
dfrA5	
dfrA6	
dfrA7	
dfrA8	
dfrA9	
dfrA10	
dfrA12	
dfrA13	
dfrA14	
dfrA15	
dfrA16	
dfrA17	
dfrA18	
dfrA19	
dfrA20	

Adapted from (Shin et al., 2014).

1.8.8 Aminoglycoside antibiotics mechanism of action and resistance mechanisms

A typical aminoglycoside is a six membered ring with amino group substituents, linked to an amino containing or non-amino containing sugar (Doi, Wachino and Arakawa, 2016). Aminoglycosides enter the cell in three stages: initially an energy-independent step (ionic binding), followed by two energy dependent phases (EDPI and EDPII) (Taber *et al.*, 1987; Vakulenko and Mobashery, 2003; Veyssier and Bryskier, 2014). Aminoglycosides enter bacteria by binding to anionic sites on cell surface membranes, in Gram-negative bacteria these sites include: lipopolysaccharides, heads of phospholipids, and outer membrane proteins (Ramirez and Tolmasky, 2010). This binding displaces divalent cations that cross-bridge adjacent lipopolysaccharides, reducing membrane permeability and allowing aminoglycosides to enter the periplasm (Vanhoof, Sonck and Hannecart-Pokorni, 1995). EDPI follows ionic binding, aminoglycosides cross the cytoplasmic membrane because of the membrane potential and induce errors in protein synthesis; EDPI has been shown to be affected by growth rate with the suggested hypothesis being that this is due to the number of active ribosomes in the cell (Bryan and Van den Elzen, 1976; Muir, van Heeswyck and Wallace, 1984). The errors induced in EDPI trigger EDPII, which has been shown to be affected by inhibition of electron transport and oxidative phosphorylation (Bryan and Van Den Elzen, 1977). After entry, aminoglycosides bind at the aminoacyl-transfer RNA site of the 16S rRNA, resulting in inhibited synthesis of polypeptides and cell death (Doi, Wachino and Arakawa, 2016). Aminoglycosides are not metabolised, but instead excreted as active compounds (Wenk *et al.*, 1979; Veyssier and Bryskier,

2014). Aminoglycoside resistance is characterised into several mechanisms; enzymatic modification and inactivation, aminoglycoside efflux, and infrequently ribosomal modification (Shaw et al., 1993; Ramirez and Tolmasky, 2010; Magiorakos et al., 2012) (Table 1.14).

1.8.8.1 Enzymatic modification of aminoglycoside antibiotics

There are three distinct groups of aminoglycoside modifying enzymes: Aminoglycoside *N*-acetyltransferases (AAC), Aminoglycoside *O*-nucleotidyltransferases (ANT), Aminoglycoside *O*-phosphotransferases (APH) (Ramirez and Tolmasky, 2010) (Table 1.14). AAC's belong to the GCN5-related *N*-acetyltransferase (GNAT) protein superfamily (Vetting *et al.*, 2005). They catalyse acetylation of NH_2 groups of aminoglycosides using acetyl coenzyme A as the donor substrate (Ramirez and Tolmasky, 2010). ANT's catalyse the transfer of an Adenosine monophosphate group from ATP to a hydroxyl group of an aminoglycoside molecule (Ramirez and Tolmasky, 2010). APH's catalyse transfer of a phosphate group to the aminoglycoside molecule (Wright and Thompson, 1999).

1.8.8.2 Aminoglycoside efflux resistance mechanisms

Several multidrug pumps and some aminoglycoside specific pumps are known to impact aminoglycoside efficacy (Table 1.14). In *E. coli*, AcrD has been identified as an aminoglycoside specific efflux pump (Rosenberg, Ma and Nikaido, 2000). AcrD belongs to the RND family, known to pump lipophilic molecules, amphiphilic molecules or toxic divalent cations (Saier *et al.*, 1994; Nikaido, 1996; Rosenberg, Ma and Nikaido, 2000). The two component potassium transport system KdpD/KdpE has been shown to increase intracellular survival, virulence, and confer resistance to kanamycin (Hirakawa, Nishino, Hirata, *et al.*, 2003; Freeman, Dorus and Waterfield, 2013).

1.8.8.3 Aminoglycoside antibiotic resistance associated with ribosomal modification

Aminoglycoside producing organisms have been found to produce 16S ribosomal RNA methyl-transferases (16S-RMTases) to protect these organisms from their antimicrobial metabolites (Doi, Wachino and Arakawa, 2016). These enzymes are involved in posttranscriptional methylation at either G^{1405} or A^{1408} of 16S rRNA (Beauclerk and Cundliffe, 1987). Several have been identified in multiple bacterial species including *E. coli* (Doi, Wachino and Arakawa, 2016) (Table 1.14).

Table 1.14: Aminoglycoside resistance genes and associated mechanisms in *E. coli*.

	System/Enzyme	Gene	Genetic location	Reference
			Enzymatic modification	
N-acetyltransferases	AAC(1)			(Lovering, White and Reeves, 1987; Sunada *et al.*, 1999)
	AAC(3)-Ia C	*aac(3)-Ia, aacC1*	Plasmid, transposon, integron	(Wohlleben *et al.*, 1989; Javier Terán *et al.*, 1991)
	AAC(3)-IIb	*aac(3)-IIb, aac(3)-Vb*		(Rather *et al.*, 1992; Dahmen *et al.*, 2010)
	AAC(3)-IIc	*aac(3)-IIc, aacC2*	Plasmid	(Dubois *et al.*, 2008)
	AAC(3)-IVa	*aac(3)-IVa*	Plasmid	(Bräu, Pilz and Piepersberg, 1984; Heuer *et al.*, 2002)
	AAC(3)-VIa	*aac(3)-VIa*	Plasmid	(Rather *et al.*, 1993; Call *et al.*, 2010)
	AAC(6')-Ia	*aac(6')-Ia, aacA1*	Plasmid, transposon, integron	(Tenover *et al.*, 1988; Parent and Roy, 1992)
	AAC(6')-Im	*aac(6')-Im*	Plasmid	(Chow *et al.*, 2001)
O-nucleotidyltransferases	ANT(3")-Ia	*aadA2*	Plasmid, integron	(Y. T. Chen *et al.*, 2007)
		aadA3	Plasmid, transposon, integron	(Parent and Roy, 1992)
		aadA4	Plasmid, chromosome	(Parkhill *et al.*, 2003; Périchon *et al.*, 2008)
		aadA5	Plasmid, transposon	(Sandvang, 1999)
		aadA7	Integron	(Ahmed *et al.*, 2004)
		aadA10	Plasmid, integron	(Partridge, Collis and Hall, 2002; Fiett *et al.*, 2006)
		aadA11	Integron	(Llanes *et al.*, 2006)
		aadA12	Integron	(Ajiboye *et al.*, 2009)
		aasA13	Plasmid, integron	(Revilla *et al.*, 2008)
		aadA16	Plasmid, integron	(Wei *et al.*, 2009)
		aadA22	Plasmid, integron	(Herrero *et al.*, 2008)
O-phosphotransferases	APH(4)-Ia	*aph(4)Ia, hph, strA*	Plasmid	(Kaster *et al.*, 1983)
	APH(6)-Ic	*aph(6)-Ic*	Transposon	(Mazodier *et al.*, 1985; Steiniger-White, Rayment and Reznikoff, 2004)
	APH(6)-Id	*aph(6)-Id, strB, orfI*	Plasmid, integrative conjugative element, genomic islands	(Scholz *et al.*, 1989; Daly *et al.*, 2005; Gordon *et al.*, 2008; Meyer, 2009)
	APH(3')-Ia	*aph(3')-Ia, aphA-1*	Transposon	(Oka, Sugisaki and Takanami, 1981)
	APH(3')-Ib	*aph(3')-Ib, aphA-like*	Plasmid	(Pansegrau *et al.*, 1987)
	APH(3')-IIa C	*aph(3')-Iia, aphA-2*	Transposon	(Beck *et al.*, 1982)
	APH(2")-IIa C	*aph(2")-Iia, aph(2')-Ib*	Chromosome	(Kao *et al.*, 2000)
			Efflux	
	AcrD	*acrD*	Plasmid, Chromosome	(Rosenberg, Ma and Nikaido, 2000)
	kdpD	*kpdD*	Chromosome	(Hirakawa, Nishino, Hirata, *et al.*, 2003; Freeman, Dorus and Waterfield, 2013)
	kdpE	*kdpE*	Chromosome	
			Ribosome modification	
16S-RMTases	ArmA	*armA*	Plasmid	(Du *et al.*, 2009; Yong *et al.*, 2009)
	RmtB	*rmtB*	Plasmid	(Chen *et al.*, 2007; Deng *et al.*, 2011; Rahman *et al.*, 2014)
	RmtC	*rmtC*	Plasmid	(Mushtaq *et al.*, 2011; Rahman *et al.*, 2014)
	RmtF	*rmtF*	Plasmid	(Rahman *et al.*, 2014)
	RmtE	*rmtE*	Class 1 Integron	(Davis *et al.*, 2010; Lee *et al.*, 2014)
	NpmA	*npmA*	Plasmid	(Wachino *et al.*, 2007)

16S ribosomal methyl-transferases (16s-RMTases).

1.8.9 Phenicol antibiotics mechanism of action and resistance mechanisms

Phenicols inhibit protein synthesis by preventing peptide chain elongation, via reversible binding to the peptidyltransferase centre of the 50S ribosomal subunit (Schlünzen *et al.*, 2001). Reported mechanisms of *E. coli* phenicol resistance include enzymatic inactivation and efflux pumps (Murray and Shaw, 1997; Shaw, 2002) (Table 1.15).

1.8.9.1 Enzymatic inactivation of phenicol antibiotics

The main mechanism of phenicol resistance in bacteria is the production of phenicol inactivating chloramphenicol acetyltransferase (CAT). CAT enzymes are trimeric proteins with three active sites at the site of monomer interface, with two structural conformations: type A and type B (Leslie, Moody and Shaw, 1988). The two types of CAT differ in primary and secondary structure, but their molecular strategy of *O*-acetylation is likely to be conserved. Both types contain a conserved histidine residue in their active site, which is critical for the deprotonation of the nucleophilic hydroxyl group of chloramphenicol (Murray, Lewendon and Shaw, 1991). Chloramphenicol was banned for use in food producing animals in Europe in 1994, due to low efficacy compared to other antibiotics and adverse side effects such as aplastic anaemia (Schwarz *et al.*, 2004; Hanekamp and Bast, 2015). Florfenicol does not have the same link to adverse side effects and due to the fluorine instead of the hydroxyl group, florfenicol is resistant to the action of CATs and it is used in agriculture in Europe (Schwarz *et al.*, 2004).

1.8.9.2 Efflux of phenicol antibiotics

Phenicols are substrates of the AcrAB-TolC multidrug efflux system, with overexpression resulted in resistance (McMurry, George and Levy, 1994; Okusu, Ma and Nikaido, 1996; Lee *et al.*, 2000). MdfA has also been identified as a multidrug pump in *E. coli* capable of transporting phenicols (Nilsen *et al.*, 1996; Edgar and Bibi, 1997). The presence of MdfA and AcrAB-TolC produces an additive and multiplicative effect on the phenicol resistance of *E. coli* (Lee *et al.*, 2000) The multiple antibiotic resistance (*mar)* locus was first identified on a transposon in *E. coli* (A. M. George and Levy, 1983). The AraC transcriptional regulator MarA, contained within the *mar* locus, was identified through genetic screening and demonstrated to cause the MDR phenotype associated with the *mar* locus (Cohen, Hachler and Levy, 1993; Gambino, Gracheck and Miller, 1993). MarA increases phenicol resistance in *E. coli* by promoting the expression of MdfA and AcrAB-tolC while also activating *micF,* a small inhibitory RNA, decreasing cell permeability through inhibition of the outer membrane porin F (OmpF) translation (Cohen, Mcmurry and Levy, 1988).

Table 1.15: Phenicol resistance genes and resistance mechanism in *E. coli*.

System/Enzyme	Gene	Genetic location	Reference
Enzymatic modification of drug			
Type A chloramphenicol acetyltransferases			
A1	*cat*		(Shaw, 2002)
	catI	Transposon and chromosome	(Alton and Vapnek, 1979)
	catII	Plasmid	(Murray *et al.*, 1990)
Type B chloramphenicol acetyltransferases			
B1	*catB2*	Plasmid, transposon	(Parent and Roy, 1992)
Efflux			
E-1	*cmlA5*	Transposon	(Poirel *et al.*, 2000)
E-2	*cml*	Plasmid	(Dorman, Foster and Shaw, 1986)
E-3	*flo*	Plasmid	(Bischoff *et al.*, 2002)
		Plasmid	(Cloeckaert *et al.*, 2000)
	floR	Plasmid and chromosome	(White *et al.*, 2000; Blickwede and Schwarz, 2004)
	acrAB-tolC		
	mdfA		

Adapted from (Schwarz et al., 2004).

1.8.10 Polymyxin mechanism of action and resistance mechanisms

The antibacterial activity of colistin (Polymyxin E) is driven by an electrostatic interaction between positively charged colistin and the phosphate groups of the lipid A membrane. This increases the permeability of the outer membrane by destabilizing the lipopolysaccharide (Dixon and Chopra, 1986). Reported mechanisms of *E. coli* polymyxin resistance include enzymatic inactivation of the cellular target (Table 1.16).

1.8.10.1 Enzymatic modification of colistin target

Plasmid-mediated mobilized colistin resistance (Mcr) enzymes are members of the phosphoethanolamine (PEA) transferase family of proteins (Table 1.16). The Mcr enzymes confer colistin resistance by decorating the lipid A head groups of lipopolysaccharide with PEA (H. Li *et al.*, 2018). This reduces the net negative charge and prevents colistin from binding to the lipid A moiety. In *E. coli* from pigs in China the *mcr*-3 was found less frequently than *mcr*-1 and *mcr*-2 (Hernandez et al., 2017, Roer et al., 2017, Yin *et al.*, 2017). This suggests that the majority of MDR including colistin resistance, in *E. coli* could be attributed to *mcr*-1 and *mcr*-2. As opposed to a Mcr independent resistance mechanism for MDR including colistin in *K. pneumoniae* and *P. aeruginosa* (Nicolet et al., 2016). The *mcr-4* gene was discovered in *Salmonella* spp. and *E. coli* in pigs in Italy, Spain, and Belgium (Carattoli *et al.*, 2017). The efficacy of antimicrobial peptides and polymyxins in Gram-negative bacteria are reduced by *pmrE, pmrF,* and *pmrC*. These genes are are all involved in the synthesis and transfer of 4-amino-4-deoxy-L-arabinose (Ara4N) to Lipid A, *pmrC* also mediates Lipid A modification through addition of phosphoethanolamines (Gunn *et al.*, 1998; Lee *et al.*, 2004). The two component system PhoP-PhoQ reduces susceptibility to antimicrobial

peptides and polymyxins by altering the physiology of the membrane to reduce binding (Groisman, Duprey and Choi, 2021).

Table 1.16: Polymyxin resistance genes identified globally 2016-2021.

Gene	Reference
mcr-1	(Liu *et al.*, 2016)
mcr-2	(Xavier *et al.*, 2016)
mcr-3	(Yin *et al.*, 2017)
mcr-4	(Carattoli *et al.*, 2017)
mcr-5	(Borowiak *et al.*, 2017)
mcr-6	(AbuOun *et al.*, 2017)
mcr-7	(Yang *et al.*, 2018)
mcr-8	(Wang *et al.*, 2019)
mcr-9	(Carroll *et al.*, 2019)
mcr-10	(Wang *et al.*, 2020)
phoQ *phoP*	(Groisman, Duprey and Choi, 2021)
ugd (pmrE) *pmrF*	(Gunn *et al.*, 1998)
eptA (pmrC)	(Lee *et al.*, 2004)

1.8.11 Quinolone antibiotics mechanism of action and resistance mechanisms

Quinolones are synthetic antimicrobials, by-products of chloroquine synthesis (Lesher *et al.*, 1962). They act upon bacterial type II topoisomerase's; gyrase and topoisomerase IV (Sugino *et al.*, 1977; Kato *et al.*, 1990; Miura-Masuda and Ikeda, 1990). These enzymes supercoil bacterial DNA chromosome as part of their normal function (Levine, Hiasa and Marians, 1998; Anderson and Osheroff, 2001; Champoux, 2001; Deweese and Osheroff, 2009; Pommier *et al.*, 2010). Quinolones inhibit this essential cellular machinery (Gellert *et al.*, 1977; Kreuzer and Cozzarelli, 1979). Fluoroquinolone resistance in *Enterobacteriaceae* occurs through target modification, inhibition of target binding, and efflux mechanisms.

1.8.11.1 Topoisomerase target modification

Point mutations in the DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*) inhibit the binding of quinolones, specifically in the quinolone resistance determining region (Yoshida *et al.*, 1991). Commonly a non-polar

amino acid such as leucine or phenylalanine is substituted for the polar amino acid serine at position 83 of GyrA (Jaktaji and Mohiti, 2010). Modifications in both *gyrA* and *parC have* been demonstrated to confer an increased level of resistance than a mutation in one subunit (Jalal and Wretlind, 1998). Crucially, for high level fluoroquinolone resistance in *E. coli*, functional efflux pumps are necessary in addition to topoisomerase modifications (Oethinger *et al.*, 2000).

1.8.11.2 Efflux mediated quinolone resistance

Active efflux mediated quinolone resistance was identified in studies investigating cell entry (Cohen *et al.*, 1988). With *acrAB-tolC* identified as the predominant efflux pump in fluoroquinolone resistance (Ma *et al.*, 1993; Fralick, 1996). An RND pump (OqxAB) classically associated with *K pneumoniae* has been identified on plasmids in *E. coli* (Hansen *et al.*, 2007).

1.8.11.3 Inhibition of quinolone antibiotic target binding

Quinolone resistance proteins (Qnr) are encoded by the *qnr* genes, these penta-peptide repeats reduce susceptibility to quinolones by preventing their binding to DNA topoisomerases (Martínez-Martínez, Pascual and Jacoby, 1998; Nordmann and Poirel, 2005).

Table 1.17: Quinolone resistance genes in Gram-negative bacteria.

Gene	Location	Reference
gyrA/gyrB	Chromosome	(Gellert *et al.*, 1977; Kreuzer and Cozzarelli, 1979; Yoshida *et al.*, 1991)
parC/parE		(Yoshida *et al.*, 1991)
Binding inhibition		
qnrA1 to *qnrA6* *qnrB1* to *qnrB6* *qnrS1* to *qnrS2*	Plasmid	(Martínez-Martínez, Pascual and Jacoby, 1998; Cattoir *et al.*, 2007)
Efflux		
emrA *emrR*	Chromosome	(Lomovskaya, Lewis and Matin, 1995)
acrAB	Chromosome	(Ma *et al.*, 1993)
oqxAB	Chromosome and plasmid	(Hansen *et al.*, 2007)

1.8.12 Lincosamide antibiotics mechanism of action and resistance mechanisms

Lincosamides inhibit the action of peptidyl transferase through dissociation of peptidyl tRNA from the 23S rRNA of the 50S bacterial ribosome, therefore reducing protein synthesis (Douthwaite, 1992; Poulsen, Kofoed and Vester, 2000; Tenson, Lovmar and Ehrenberg, 2003). Resistance has been observed via modification of the ribosomal target and enzymatic inactivation of the drug. In *E. coli*, efflux pumps confer reduced susceptibility, but not resistance.

1.8.12.1 Ribosome modification mediated lincosamide resistance

The predominant resistance mechanism affecting lincosamides is ribosomal modification of 23S rRNA, the erythromycin ribosome methylation genes (*erm*) act on adenine 2508 specifically in *E. coli* (Skinner, Cundliffe and Schmidt, 1983). This modification also confers resistance to macrolides, lincosamides, and streptogramin B antibiotics (Weisblum and Demohn, 1969).

1.8.12.2 Enzymatic inactivation of lincosamide antibiotics

The lincosamide nucleotidyltransferases (Lnu) drive enzymatic inactivation mediated resistance, specifically inhibiting lincomycin but not erythromycin (Dutta and Devriese, 1982). These enzymes nucleotidylate the lincomycin sugar moiety at carbon three or four (Argoudelis and Coats, 1969).

1.8.12.3 Efflux of lincosamide antibiotics

E. coli strains that lack the outer membrane component of several efflux pumps were observed to be more susceptible to lincosamides, suggesting that resistance nodulation cell division superfamily pumps are involved in intrinsic resistance (Leclercq and Courvalin, 1991; Schwarz *et al.*, 2016).

Table 1.18: Lincosamide resistance genes in *E. coli*.

Gene	Reference
Enzymatic inactivation	
lnuA	(Petinaki *et al.*, 2008)
lnuB	
lnuD	
lnuC	(Achard *et al.*, 2005)
Target modification	
ermA	(Skinner, Cundliffe and Schmidt, 1983; Leclercq, 2002; Ojo *et al.*, 2004)
ermB	
ermC	

1.9 Understanding biology through genomic sequencing and the short-read revolution

DNA sequencing took many years of research and development after the structure of DNA was solved using crystallographic data produced by Rosalind Franklin and Maurice Wilkins (Watson and Crick, 1953; Zallen, 2003). Initial methods could only determine the nucleotide composition of a sample and not the order (Holley *et al.*, 1961). An additional process utilising selective ribonuclease treatments was developed to produce fully and partially degraded fragments, facilitating the first complete nucleotide sequence, that of alanine tRNA from *Saccharomyces cerevisiae* (Holley *et al.*, 1951; Holley, Madison and Zamir, 1964). Radiolabelling after 2D fractionation was another method used to increase the number of known tRNA sequences (Sanger, Brownlee and Barrell, 1965). The first protein coding sequence was completed in 1972, with the associated complete genome of MS2 bacteriophage following in 1976 (Min Jou *et al.*, 1972; Fiers *et al.*, 1976). Using chemical analogues of nucleotides that lacked the 3' hydroxyl group necessary for bonding to the following 5' phosphate of the next nucleotide revolutionised sequencing (Maxam and Gilbert, 1977) These deoxyribonucleotides (dNTPs) incorporated into a DNA extension reaction would randomly bind into an extending chain

and terminate that chain; over a reaction, every possible length of DNA was created. Four parallel reactions containing each individual dNTP run on polyacrylamide gels can be used to infer the sequence of the template strand. This process is commonly called Sanger sequencing or first-generation sequencing (Sanger, Nicklen and Coulson, 1977). Sanger sequencing was optimised for a single reaction, exchanging radiolabelling with fluorescence labelling and capillary electrophoresis. Utilising luminescent reactions to measure pyrophosphate synthesis defines second-generation DNA sequencing. This involves a two-step enzyme process converting pyrophosphate into ATP, which is then a substrate for luciferase and produces light proportionally to the pyrophosphate concentration (Nyrén and Lundin, 1985). The DNA template is fixed, and each nucleotide is washed over separately; when the complimentary nucleotide reacts with the template, the enzymatic reaction occurs, and a measurable quantity of light is emitted (Hyman, 1988). Incremental optimisations continued, and the technology eventually became known as Roche 454 sequencing and enabled mass parallelisation of sequencing reactions (Margulies *et al.*, 2005). Illumina short-read sequencing technology is based on "bridge amplification". Oligonucleotides are bound to a flow cell, and DNA molecules bracketed with complimentary adapter brackets are passed through the flow cell. Solid-phase PCR produces clusters of clonal populations from each of the original flow-cell binding DNA strands (Fedurco *et al.*, 2006; Bentley *et al.*, 2008). The sequencing process is completed synchronously, using fluorescent dNTPs that cannot immediately bind subsequent nucleotides as the fluorophore occupies the 3' hydroxyl position (Turcatti *et al.*, 2008). DNA polymerase and modified dNTPs are washed over a primed flow-cell containing clusters of

sample DNA, the fluorophores are excited and measured, the fluorophore is removed enzymatically, and the cycle is repeated for the following position. Paired-end reads, or reading the flow-cell bound DNA molecules in both directions, increased the accuracy of short-read sequencing and improved mapping to reference sequences.

1.10 Long-read sequencing provides greater detail and compliments previous technologies

Long-read sequencing technology represents the most anticipated technology in third-generation DNA sequencing, underpinned by the technology of nanopores, which have been used to detect and quantify a broad array of chemical and biological molecules (Haque *et al.*, 2013). A single-stranded DNA or RNA molecule can be electrophoretically driven through large α-hemolysin ion channels in a lipid bilayer. As the molecule passes through the channel, ion flow is blocked, and a decrease in the current proportion to the length of the nucleic acid can be measured (Kasianowicz *et al.*, 1996). Solid-state, non-biological nanopore technology can be engineered to fit double-stranded DNA molecules, simplifying the sample preparation and overall robustness of the sequencing platform (Li *et al.*, 2001; Dekker, 2007). The technology has been used in remote regions without access to molecular laboratory facilities, enabling the safe and rapid detection of pathogens such as the Ebola virus (Hayden, 2015). The main benefit of long-read sequencing is the clarification of repetitive genomic regions (Zhang, Jain and Aluru, 2020). This benefit has been demonstrated for de novo genome assembly novel variant detection, RNA sequencing analysis, metagenomics, and epigenetics (Chaisson *et al.*, 2015; Gordon *et al.*, 2015; Loman, Quick and Simpson, 2015; Chin *et al.*, 2016; Rand *et al.*, 2017; Simpson *et al.*, 2017; Jain *et al.*, 2018;

Sedlazeck *et al.*, 2018; De Maio *et al.*, 2019; Dilthey *et al.*, 2019). Initially, the high error rate of long-read sequencing was criticised, but these concerns have been mitigated with error-correcting algorithms (Goodwin *et al.*, 2015; Laver *et al.*, 2015; Cretu Stancu *et al.*, 2017; Jain *et al.*, 2017, 2018; Tyson *et al.*, 2017; Zhang, Jain and Aluru, 2020).

1.11 Capturing the antibiotic resistome

The antibiotic resistome includes all ARG and how they exist within a microbiome (pathogens and non-pathogens) (Wright, 2010). Direct culture approaches are insufficient as it is estimated that less than 1% of bacteria are known because the remainder are not culturable *in vitro* (Bodor *et al.*, 2020). Shotgun metagenomics on total DNA extractions from samples enables investigation of all available genomic information, and has been used to investigate the cattle resistome in faecal samples, environmental soil, wastewater, and slurry tanks (Noyes *et al.*, 2016; Rovira *et al.*, 2019; Baker *et al.*, 2022). Distinct microbiomes and resistomes have been consistently identified in dairy calves, with lower diversity in older cows (Noyes *et al.*, 2016). The faecal resistome is informed by the calf microbiome, which is informed by diet composition (Liu *et al.*, 2019). As the underlying technology and bioinformatic pipelines continue to develop, future metagenomics studies will likely enrich samples for genes of interest using sequence probes or fluorescence-tagged plasmids and fluorescence-activated cell sorting (Pinilla-Redondo *et al.*, 2018; Guitor *et al.*, 2019). This reduces the overall volume of genomic data produced while increasing the data relevant for understanding the antibiotic resistome. After obtaining high-quality DNA and sequencing it, the raw sequencing reads must be assembled into contiguous sections of DNA (contigs). These contigs can then be screened using ARG databases to

identify genes with sufficient evidence of phenotypic ABR. When coding sequences (CDS) within contigs are mapped to specific taxa, it is possible to link identified ARGs to their taxonomical source. This linkage facilitates targeted studies, where taxa identified as more likely sources of ARG can be selectively enriched in subsequent experiments, thereby collecting focused sequencing data that is more relevant to understanding ABR in the original sample.

1.11.1 Antibiotic resistance gene annotation tool to identify antibiotic resistance genes

ARG-ANNOT (Antibiotic resistance gene annotation) is a bioinformatic tool built to detect bacterial ARG (Gupta *et al.*, 2014). This tool completes a nucleotide basic local alignment search tool (BLAST) and amino acid sequence homology modeling to identify matches in a curated database of 1689 ARG with corresponding protein sequences collected from the National center for biotechnology information (NCBI) GenBank database. ARG-ANNOT can identify point mutations, acquired ARG, and putative ARG with homology to known ARG (Gupta *et al.*, 2014).

1.11.2 The Comprehensive antibiotic resistance database to identify antibiotic resistance genes

The Comprehensive antibiotic resistance database (CARD) combines reference nucleotide and amino acid sequences, detection models to identify ARG to annotate and interpret resistome data (Jia *et al.*, 2017; Alcock *et al.*, 2020, 2023). This database contains 3567 ABR ontologies supported by 2136 publications (Jia *et al.*, 2017).

1.11.3 The MEGARes antibiotic resistance gene database

The Microbial ecology group created and curate MEGARes, an AMR database and annotation structure designed for AMR analysis in metagenomes. There are 1345 genes covering 220 resistance mechanisms to 57 classes of antimicrobials in this database (Doster *et al.*, 2020).

1.11.4 The ResFinder tool to identify antibiotic resistance genes

The Center for genomic epidemiology was established to develop accessible bioinformatics tools, one of which was ResFinder (Zankari *et al.*, 2012a; Florensa *et al.*, 2022). The ResFinder database collated acquired ARG from several publications and reviews, with reference sequences sourced from the NCBI nucleotide database. The resulting ResFinder database includes over 1400 ARG and the ResFinder tool can Detects existing ARG with ≥50% sequence similarity and at least 40% sequence coverage of the reference gene (Zankari *et al.*, 2012b, 2012a).

1.11.5 The Bacterial antimicrobial resistance reference gene database

The NCBI hosts the Bacterial antimicrobial resistance reference gene database (NCBI ARG). This database is a collection of ARG reference sequences collected from 1751 publications manually curated by RefSeq staff to maintain consistency in gene names, and sequences 100 bp either side of coding regions where available (NCBI, 2016).

1.12 Antibiotic resistance genes in the National antimicrobial resistance monitoring system for Enteric Bacteria

The National antimicrobial resistance monitoring system for Enteric Bacteria (NARMS) is a public health surveillance system that tracks AMR in enteric and foodborne bacteria (Holmes and Chiller, 2004). This database is curated by several labs across the US in collaboration with the Centers for Disease Control and Prevention (CDC), the US Food and Drug Administration, the Center for Veterinary Medicine, and the US department of Agriculture, Agricultural Research Services. Sequences can be submitted from around the world and included in the database for comparison. The goals of NARMS are to enhance sampling for foodborne pathogens in a One Health framework, use technology to understand the evolution and spread of resistance among foodborne pathogens, improve data sharing, communication, and collaboration, and conduct research to assess the sources and impact of resistance and the effectiveness of prevention practices (CDC, US Food and Drug Administration and US Department of Agriculture, 2020). NARMS uses the NCBI ARG to identify ARG in these sequences for several food-borne pathogens, including *E. coli* (NCBI, 2016). Between 2019 and 2023 there were 5643 sequences of *E. coli* isolates sourced from cows and 18321 sequences of *E. coli* isolates sourced from humans submitted to NARMS. In the isolates

from cows, 5442 ARG were identified and 17803 ARG were identified in *E. coli* from humans. The ARG that were identified in this selection of NARMS sequences are summarised in Table 1.19. The identified aminoglycoside ARG were *aph(3")-Ib* (30.31% of the isolates from cows and 21.35% of the isolates from humans), *aph(6)-Id* (30.25% of the isolates from cows and 21.37% of the isolates from humans) and *aadA1* (7.69% of the isolates from cows and 8.92% of the isolates from humans). The identified β-lactam ARG in this subset were all β lactamases *TEM-1* (9.73% of the isolates from cows and 20.16% of the isolates from humans), *CTX-M1* (2.63% of the isolates from cows and 0.45% of the isolates from humans), *CMY-2* (1.69% of the isolates from cows and 1.1% of the isolates from humans), *ampC* (1.58% of the isolates from cows and 0.92% of the isolates from humans), *CTX-M14* (1.47% of the isolates from cows and 2.24% of the isolates from humans), *CTX-M15* (1.44% of the isolates from cows and 9.07% of the isolates from humans), and *OXA-1* (1.15% of the isolates from cows and 3.76% of the isolates from humans). The identified phenicol ARG in *E. coli* in this subset were *floR* (8.25% of the isolates from cows and 4.83% of the isolates from humans), *catA1* (3.28% of the isolates from cows and 3.13% of the isolates from humans), and *catB3* (0.46% of the isolates from cows and 3.41% of the isolates from humans). The identified quinolone ARG in the *E. coli* in this subset were *gyrA S83L* (6.37% of the isolates from cows and 17.92% of the isolates from humans), *parC S80I* (4.75% of the isolates from cows and 10.45% of the isolates from humans), *gyrA D87N* (3.69% of the isolates from cows and 9.61% of the isolates from humans), *qnrS1* (0.96% of the isolates from cows and 5.57% of the isolates from humans), and *qnrB19* (0.35% of the isolates from cows and 5.21% of the isolates from humans). The identified sulfonamide/trimethoprim ARG in the *E.*

coli in this subset were *sul2* (18.23% of the isolates from cows and 22.655 of the isolates from humans), *sul1* (7.65% of the isolates from cows and 14.66% of the isolates from humans), *dfrA1* (4.08% of the isolates from cows and 5.59% of the isolates from humans), *dfrA17* (1.97% of the isolates from cow and 8.42% of the isolates from humans), *sul3* (1.49% of the isolates from cows and 2.71% of the isolates from humans), *dfrA12* (1.17% of the isolates from cows and 2.95% of the isolates from humans), and *dfrA14* (1.05% of the isolates from cows and 3.76% of the isolates from humans). The identified tetracycline ARG in the *E. coli* in this subset were *tetA* (13.68% of the isolates from cows and 19.02% of the isolates from humans), *tetB* (11.84% of the isolates from cows and 7.46% of the isolates from humans), *tetC* (1.83% of the isolates from cows and 0.04% of the isolates from humans), and *tetM* (1.22% of the isolates from cows and 0.8% of the isolates from humans).

Table 1.19: Antibiotic resistance genes identified in *E. coli* sequences sourced from cows and humans submitted to NARMS in 2019-2022.

		Source			
		Cow/dairy		Human	
		Number	Percent of isolates	Number	Percent of isolates
Class					
All	Isolates	5634	-	18321	-
	Genes	5442	-	17803	-
Ami	Genes	3500	-	14723	-
	aph(3")-Ib	1061	18.83	3911	21.35
	aph(6)-Id	1059	18.80	3915	21.37
	aadA1	433	7.69	1635	8.92
β-lac	Genes	1194	-	8907	-
	blaTEM-1	548	9.73	3693	20.16
	blaCTX-M-1	148	2.63	82	0.45
	blaCMY-2	95	1.69	201	1.10
	ampC	89	1.58	168	0.92
	blaCTX-M-14	83	1.47	411	2.24
	blaCTX-M-15	81	1.44	1661	9.07
	blaOXA-1	65	1.15	688	3.76
Phe	Genes	734	-	2793	-
	floR	465	8.25	884	4.83
	catA1	185	3.28	573	3.13
	catB3	26	0.46	625	3.41
Qui	Genes	1133	-	12976	-
	gyrA_S83L	359	6.37	3284	17.92
	parC_S80I	211	3.75	1914	10.45
	gyrAD87N	208	3.69	1760	9.61
	qnrS1	54	0.96	1021	5.57
	qnrB19	20	0.35	955	5.21
Sulfonamide/ trimethoprim	Genes	2158	-	12178	-
	sul2	1027	18.23	4149	22.65
	sul1	431	7.65	2685	14.66
	dfrA1	230	4.08	1024	5.59
	dfrA17	111	1.97	1542	8.42
	sul3	84	1.49	497	2.71
	dfrA12	66	1.17	541	2.95
	dfrA14	59	1.05	689	3.76
Tet	Genes	1632	-	5108	-
	tet(A)	771	13.68	3485	19.02
	tet(B)	667	11.84	1365	7.45
	tet(C)	103	1.83	8	0.04
	tet(M)	69	1.22	146	0.80

Antibiotic classes: Ami (aminoglycosides), *β*-lac (*β*-lactams), Phe (phenicols), Qui (quinolones), and Tet (tetracyclines).

1.13 National database of antibiotic resistant organisms

The National database of antibiotic resistant organisms (NDARO) (National Center for Biotechnology Information, 2023) contains 357027 *E. coli* and shigella isolates and in the period of 2019 to 2023, 289900 isolates were added. These isolates were filtered further to include 8164 *E. coli* isolates labelled as sourced from bovines, bovine faeces, or calf faeces. The terms that were used to filter the NDARO database to produce this set of isolates are in Table 1.20. The NDARO uses the NCBI ARG to identify ARG (NCBI, 2016). In this set of *E. coli* genomes from 21 countries, 99 distinct ARG were identified covering genes linked to multidrug efflux, cell permeability, and ARG specific to nine separate classes of antibiotics. Table 1.21 contains the ARG that were identified in the genomes of ≥1% of all isolates, with regional prevalence also shown. All of the ARG identified are presented in Appendix 2 (regional prevalence) and Appendix 3 (isolate sample source prevalence).

Table 1.20: Filter terms used to collect *E. coli* isolates from the NDARO.

Filter	Label		Number
Taxa	*E. coli* and Shigella		357027
Upload date	01.01.2019-31.12.2023		289900
Isolate sample source*	All included		8164
	Bovine (4434)	animal-cattle-dairy cow	215
		Animal-Cattle-Dairy Cow (cecal)	384
		cattle	623
		cow bovine	221
		bovine	2991
	Faeces (2634)	Cattle feces	1624
		Cattle faeces	354
		feces bovine	395
		cattle fecal samples	261
	Calf faeces (1096)	dairy calf feces	839
		Calf fecal composite	257
Country of submission	North America (7122)	Canada	458
		Mexico	4
		United States of America	6660
	Central/South America (30)	Chile	20
		Ecuador	10
	Australasia (259)	Australia	36
		New Zealand	223
	Asia (4)	China	4
	Europe (645)	United Kingdom	166
		Denmark	2
		Finland	1
		France	188
		Germany	118
		Ireland	12
		Italy	10
		Netherlands	40
		Norway	14
		Spain	48
		Sweden	46
	Africa (62)	Kenya	61
		Uganda	1
	None (42)		42

*Bovine, Faeces, and Calf faeces are author groupings of the database filter labels, labels within these groupings are included as they appear in the database.

The identified aminoglycoside resistance genes were *aph(3")-Ib* (20.58% of all the isolates), *aph(6)-Id* (20.19%), *aadA1* (7.81%), and *aph(3')-Ia* (7.62%). Half of the isolates from Asia and Europe contained aminoglycoside resistance genes. The predominant β-lactam resistance gene identified was *blaEC* (98.54%), also known as *ampC*. North America was the only region with isolates where *blaEC* was not identified (98.33% of the North American isolates). The other identified β-lactam resistance genes were *blaTEM-1* (6.9%), *blaCMY-2* (6.23%), and *blaCTX-M-1* (3.71%). While only identified in 1.54% of all the isolates, *blaTEM* was identified in 33.3% of the isolates from Central and South America and 4.65% of the isolates from Europe. The identified genes involved in antibiotic efflux or cell permeability were *mdtM* (96.30%), *acrF* (75.48%), and *emrD* (6.66%). The most prevalent phosphonic acids resistance gene was a point mutation at position 448 in *glpT* (91.82%) changing a glutamic acid to lysine. The other identified phosphonic acids resistance genes were a point mutation at position 352 in *cyaA* (4.41%) changing a serine to threonine, a point mutation at position 350 in *uhpT* (1.78%) changing a glutamic acid to glutamine, and *fosA7.5* (1.03%). The identified macrolide resistance gene was *mph(A)* (4.40%), *mph(A)* was identified in 50% of the isolates from Asia and 6.98% of the isolates from Europe. The most prevalent phenicols resistance gene was *catA1* (2.32%), *catA1* was identified in 22.48% of the isolates from Europe. The most prevalent resistance gene for polymyxins was a point mutation at position 358 of *pmrB* (78.75%) changing a tyrosine to asparagine. This mutation was identified at lower prevalences in isolates from Central and South America (60% of the isolates from CSA), Europe (54.11% of the isolates from Europe) and Africa (69.35% of the isolates from Africa). The identified quinolones resistance

genes were *floR* (11.33%); two separate point mutations in *gyrA*, a change from serine to leucine at position 83 (4.03%), and a change from aspartic acid to asparagine at position 87 (2.69%); and a serine to isoleucine point mutation in *parC* at position 80 (1.64%). Several quinolone resistance genes were more prevalent in the isolates from Europe the *gyrA* S83L (22.02% of the isolates from Europe), *gyrA* D87N (17.36% of the isolates from Europe), and *parC* S80I (5.74% of the isolates from Europe). The identified sulfonamide/trimethoprim resistance gene was *sul2* (14.17%). The other common but less prevalent sulfonamide/trimethoprim resistance genes were *sul1* (6.31%), *dfrA1* (4.78%), *dfrA12* (2.54%), and *dfrA17* (1.89%). The sulfonamide/trimethoprim resistance genes were found in higher proportions of the isolates from Africa and Europe, *sul2* (30.56% of the isolates from Africa and 18.60% of the isolates from Europe), *sul1* (12.90% and 8.99%), *dfrA1* (6.45% and 17.98%), and *dfrA17* (6.45% and 8.68%). The tetracyclines resistance gene *tet(A)* was identified in 11.29% of all the isolates, in 25% of the isolates from Asia, and in 24.19% of the isolates from Africa. Other tetracycline resistance genes, *tet(B)* (7.73%) and *tet(C)* (1.02%), were also identified.

Table 1.21: ARG identified in ≥1% of *E. coli* collected from dairy cows submitted to the NDARO 2019-2023.

			Region							
			Global	NA	CSA	AUS	Asia	Europe	Africa	None
Class	Gene	Number	8164	7122	30	259	4	645	62	42
			Percent							
Aminoglycosides	*aph(3'')-Ib*	1680	20.58	18.58	6.67	10.42	50.0	47.29	33.87	0
	aph(6)-Id	1648	20.19	18.2	10	10.04	50.0	47.29	25.81	0
	aadA1	638	7.81	5.17	23.33	0	0	40.16	6.45	0
	aph(3')-Ia	622	7.62	5.90	6.67	1.16	0	30.54	0	0
	aadA2	260	3.18	2.99	10	0	0	6.67	1.61	0
	aadA5	168	2.06	1.49	0	0	0	8.99	6.45	0
β-lactams	*blaEC*	8045	98.54	98.33	100	100	100	100	100	100
	blaTEM-1	563	6.90	4.61	6.67	0	50	34.11	17.74	00
	blaCMY-2	516	6.32	7.16	6.67	0	0	0.62	0	0
	blaCTX-M-1	303	3.71	2.2	0	0.0	0	22.64	0	0
	ampC_C-42T	157	1.92	1.45	0	13.13	0	3.10	0	0
	blaTEM	126	1.54	1.21	33.33	0	0	4.65	0	0
	blaCTX-M-14	109	1.34	0.41	0	0	0	12.40	0	0
	blaCTX-M-15	100	1.22	0.86	3.33	0	0	5.89	0	0
Efflux/ permeability	*mdtM*	7862	96.3	97.63	80	100	100	80.31	100	100
	acrF	6162	75.48	75.95	100	59.07	75	77.83	100	7.14
	emrD	544	6.66	4.91	13.33	27.8	0	17.98	3.23	0
Phosphonic acids	*glpT_E448K*	7496	91.82	92.78	86.67	100	100	77.67	90.32	100
	cyaA_S352T	360	4.41	4	10	0	0	10.85	3.23	0
	uhpT_E350Q	145	1.78	1.9	0	0	0	1.24	3.23	0
	fosA7.5	84	1.03	1.17	0	0	0	0.16	0	0
Macrolides	*mph(A)*	359	4.4	4.35	3.33	0	50	6.98	1.61	0
Phenicols	*catA1*	189	2.32	0.6	0	0	0	22.48	1.61	0
Polymyxin	*pmrB_Y358N*	6429	78.75	80.24	60	100	75	54.11	69.35	100
Quinolones	*floR*	925	11.33	11.49	23.33	0	50	15.19	0	0
	gyrA_S83L	329	4.03	2.56	13.33	0	25	22.02	0	0
	gyrA_D87N	220	2.69	1.45	16.67	0	0	17.36	0	0
	parC_S80I	134	1.64	1.35	3.33	0	0	5.74	0	0
Sulfonamide/ trimethoprim	*sul2*	1157	14.17	14.14	0	4.25	0	18.6	30.65	0
	sul1	515	6.31	6.3	0	0	0	8.99	12.9	0
	dfrA1	390	4.78	3.78	3.33	0	0	17.98	6.45	0
	dfrA12	207	2.54	2.67	6.67	0	0	2.17	1.61	0
	dfrA17	154	1.89	1.31	3.33	0	0	8.68	6.45	0
Tetracyclines	*tet(A)*	922	11.29	11.81	6.67	2.7	25	8.68	24.19	0
	tet(B)	631	7.73	8.14	0	2.32	0	6.67	3.23	0
	tet(C)	83	1.02	1.07	0	0	0	1.09	0	0

NA = North America, CSA = Central and South America, AUS = Australasia. Genes identified in <1% of all isolates can be found in Appendix 2 and Appendix 3.

1.14 Aims

The aim of this thesis was to establish an understanding of antibiotic resistance in *E. coli* from dairy cow faeces collected from one farm in the East Midlands of the UK. This understanding is critical for evidence-based decision making; by practitioners, veterinarians, and policy-makers. This investigation initially focused on culturing *E. coli* from dairy cow faecal samples and determining the antibiotic susceptibility phenotype of these isolates through the Kirby Bauer disk diffusion method. Differences in the antibiotic susceptibility of the isolates from the same sample, and between samples across several animals at different bovine developmental stages on the farm were assessed. Transmissible MDR was examined as a potential higher risk phenotype using conjugation methods. Additionally, short-read WGS was employed to identify antibiotic resistance genes and to understand the genetic context of AMR in *E. coli*. Finally, short-read shotgun metagenomic sequencing was used to investigate the bovine faecal metagenome and resistome.

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Chapter 2 Materials and methods

2.1 Media selection and preparation

All the media used were purchased as a powder and dissolved in reverse osmosis (RO) water before sterilisation by autoclaving for 15 minutes, at 121ºC, and at 15 psi. The molten media was then allowed to cool to ca. 50°C before adding any supplements and antibiotics. Tryptone bile X-glucuronide agar (TBX) (OXOID, UK) was used for the selective isolation of presumptive non-toxigenic *E. coli* from faecal samples. *E. coli* colonies have a blue/green colony morphology on TBX, because they contain glucuronidase that degrades a chromophore-glucuronide complex contained in the media. Nutrient Agar (NA) (OXOID) was used for maintaining working stocks of the isolates and bacterial enumeration. Nutrient broth (NB) (OXOID) was used for preparing liquid overnight cultures of bacteria for bio-assays. Mueller Hinton Agar (MH) (OXOID) was used for Kirby-Bauer Disk diffusion assays as stipulated by EUCAST guidelines. Luria-Bertani (LB) broth (OXOID) and agar (OXOID) were used to culture *E. coli* for DNA extraction and molecular assays following a published protocol (Ibrahim *et al.*, 2016). Double Yeast Extract Tryptone broth (2YT) (SIGMA, UK) was used as an enriched medium to culture a high biomass of *E. coli*, for WGS following instructions from MicrobesNG (Birmingham, UK).

2.2 Preparation of solutions

The solutions necessary for this work were prepared according to the following methodologies. Hydrochloric acid (SIGMA) and sodium hydroxide (SIGMA) were used when solutions required pH balancing.

Phosphate buffered saline (PBS) (OXOID) tablets were dissolved in RO water and sterilised by autoclaving for 15 minutes, at 121°C, and at 15 psi. Tris EDTA buffer (TEB) contained 10 mM Tris-Cl (SIGMA) and 1 mM Ethylenediaminetetraacetic acid buffer (EDTA) (SIGMA) in RO water, balanced to a final pH of 7.0. Tris acetate EDTA buffer (TAE) contained 40 mM Tris, 20 mM ethanoic acid (Thermo Fisher Scientific, UK), and 1 mM EDTA in RO water, balanced to a final pH of 8.0 (Behle and Pawlowski, 2018).

2.3 Preparation, storage, and use of antibiotic stocks

The antibiotic solutions used in this study were prepared as concentrated stocks, with the appropriate solvents depending upon solubility. Antibiotic salts and Dimethyl sulfoxide (DMSO) were obtained from SIGMA. The dissolved antibiotic stocks were filter sterilised with a 0.25 µM nylon filter (Sartorius, UK), before diluting to the desired concentration in the relevant pre-sterilised media. Molten agars were cooled to ca. 50°C before the addition of the antibiotic. Concentrations of the antibiotic stocks and their dissolving solvents used in this study are listed in Table 2.1.

Table 2.1: Antibiotic stocks, dissolving solvent, and concentration.

Antibiotic	Solvent	Stock concentration mg/ml	Working concentration ug/ml
Ampicillin	Water	100	128
Streptomycin	Water and DMSO*	100	100
Kanamycin	Water	100	64[1]
Cefotaxime	Water	100	2[1]
Sulfonamide	Water and DMSO*	100	512[1]
Tetracycline	70% Ethanol	100	64

*DMSO added dropwise until antibiotic had dissolved, working solutions were below 1% DMSO.
[1]Concentration taken from CLSI guidelines (CLSI, 2022).

2.4 Collection, storage, and processing of faecal samples

Bovine faecal samples were collected on the SB farm in two distinct sets. The α samples were taken at three distinct time points from 18 different post-parturition lactating cows (PPL) in the herd (Table 2.2). The β samples were taken at one time point in May 2021, from 20 different animals representing four bovine developmental stages; five dairy calves (DCA), five pre-parturition cows (PRP), five PPL, and five post-parturition dry cows (PPD) (Table 2.3). On the SB farm, these developmental groups are housed separately from each other. When samples were taken from the 18 α PPL, three months of clinical treatment history for each PPL were also gathered. Due to COVID-19 access constraints, treatment records for the bovines included in the β samples were not collected at the time of sampling. The majority of the β samples records were retrieved from the farm management software Uniform-AGRI (Uniform-AGRI, UK) at a later date. Unfortunately, the treatment history of several sample IDs could not be identified in the database, leaving the treatment history unknown. Faecal samples were collected using examination gloves if a rectal exam was clinically required. If an examination was not necessary, convenience samples were taken by waiting for an animal to defecate. Each sample was aseptically placed into a sterile container and labelled with the animal's tag number. The samples were then transported to the University of Nottingham School of Veterinary Medicine and Science (SVMS) within four hours of collection. Upon arrival at SVMS, samples were stored at 4°C and processed within 24 hours. The contribution of Dr Ginny Sherwin for collecting the α samples, and Dr Ryan Cook for collecting the β samples is acknowledged with gratitude.

Table 2.2: α samples metadata and treatment history.

Sample ID	Cow ID	Isolates	Sample Date	Treatment date	Days before sampling	Treatment	
1	28	43	21/6/19	15/6/19-17/6/19	4-6	Amoxicillin	β-lactam
2	+	31	21/6/19	None	90	None	
3	+	30	21/6/19	None	90	None	
4	436	30	21/6/19	18/6/19-20/6/19	1-3	Amoxicillin	β-lactam
				17/6/19-20/6/19	1-4	Tetra-Delta*	Aminocoumarin, aminoglycoside, β-lactam, and anti-inflammatory
				12/6/19 & 20/6/19	1 & 9	Carprofen	Anti-inflammatory
5	55	29	21/6/19	5/6/19-8/6/19	13-15	Amoxicillin	β-lactam
				2/6/19	19	Tulathromycin	Macrolide
6	+	21	21/6/19	None	90	None	
7	725	27	25/9/19	2/09/19	23	Meloxicam	Anti-inflammatory
				28/8/19-2/9/19	23-28	Tetra-Delta*	Aminocoumarin, aminoglycoside, β-lactam, and anti-inflammatory
				2/9/19	23	Tetracycline	Tetracycline
8	807	28	25/9/19	None	90	None	
9	447	25	25/9/19	None	90	None	
10	524	26	25/9/19	None	90	None	
11	602	25	3/10/19	None	90	None	
12	166	24	3/10/19	None	90	None	
13	501	25	3/10/19	27/9/19	6	Meloxicam	Anti-inflammatory
				25/9/19-27/9/19	6-8	Amoxicillin	β-lactam
				22/9/19	11	Carprofen	Anti-inflammatory
14	504	25	3/10/19	11/9/19	22	Rotavec Corona	Vaccine
				119/19	22	Bismuth subnitrate	Teat sealant
15	209	39	3/10/19	None	90	None	
16	446	25	3/10/19	None	90	None	
17	148	30	3/10/19	None	90	None	
18	84	25	3/10/19	None	90	None	

+Sample IDs unknown, but it is known that no treatment had been administered 90 days before sampling. *Tetra-Delta contains streptomycin, neomycin, albamycin, penicillin G, and prednisolone.

Table 2.3: β samples metadata and treatment history.

Date	Group	Sample ID	Cow ID	Age (Days)	Treatment date	Days before sampling	Treatment	
11/05/2021	Dairy calf (DCA)	A	3809	33	No known treatments	-	-	
		B	3818	23	28/04/21	13	Metacam	Anti-inflammatory
		C	3821	20	No known treatments	-	-	
		D	3819	20	No known treatments	-	-	
		E	3817	23	22/04/21-28/04/21	13-19	Metacam	Anti-inflammatory
	Pre-parturition (PRP)	F	3360	431	No known treatments	-	-	
		G	3293	486	No known treatments	-	-	
		H	3399	406	No known treatments	-	-	
		I	3282	498	14/04/21	27	Metacam	Anti-inflammatory
		J	3397	406	No known treatments	-	-	
	Post-parturition dry (PPD)	K	678	966	No known treatments	-	-	
		L	7272*	-	-	-	-	
		M	918	953	No known treatments	-	-	
		N	92*	-	-	-	-	
		O	3184	631	No known treatments	-	-	
	Post-parturition lactating (PPL)	P	2998	830	22/04/21	19	Lactation treatment	
		Q	3725*	-	-	-	-	
		R	9690*	-	-	-	-	
		S	629*	-	-	-	-	
		T	546*	-	-	-	-	

*Recorded cow ID have no match in farm database. Bovine developmental stage is correct as samples were collected from distinct groups on the dairy farm.

2.5 Culture conditions of *E. coli* in solid and liquid media

All incubations were carried out under aerobic conditions at 37°C for 18-20 hours. Solid agar plates were incubated in a standing incubator. Liquid overnight cultures, consisting of 3 ml of media in a 30 ml container, were incubated in a Forma Orbital shaking incubator (Thermo Fisher Scientific) at 180 rpm.

2.6 Enumeration of *E. coli* isolates from faeces

The sampled faeces were placed into sterile sample tubes (Thermo Fisher Scientific) and suspended in sterile PBS at a 1:1 weight-to-volume ratio, then vortexed thoroughly for 15 seconds (Thermo Fisher Scientific). A fivefold decimal serial dilution was prepared with PBS (1 $g.ml^{-1}$ to $1x10^{-6}$ $g.ml^{-1}$). Aliquots of 100 µl of each dilution was spread onto TBX and TBX supplemented with antibiotics (Table 2.1). Colonies with a green/blue morphology were counted, picked, assigned an isolate ID, and passaged onto TBX with no antibiotic supplementation. These colonies were treated as presumptive isolates of non-toxigenic *E. coli*, each was inoculated into a 20% v/v glycerol and NB cryovial tube (Eppendorf, UK). The inoculated cryovials were vortexed for three seconds before they were placed in a -80°C freezer for long term storage. When isolates were required regularly for culturing, cryovial tubes were temporarily moved to a -20°C freezer.

2.7 Antibiotic susceptibility testing through Kirby-Bauer disk diffusion

The antibiotic susceptibility of the isolates was assessed by the Kirby-Bauer disk diffusion method, following EUCAST protocols with minor adjustments (EUCAST, 2022). The isolates were resuscitated from cryovial tubes on TBX, and liquid NA overnight cultures were inoculated with an individual green/blue morphology colony. Following overnight incubation, the optical density at 600 nm (OD) was measured by a Libra S22 spectrophotometer (Biochrom, UK) and adjusted with NB to 0.10 ± 0.02. A 100 µl aliquot of OD adjusted culture was spread onto a 90mm round plate or a 200 µl aliquot was spread onto a 120 mm square plate of MH. Aliquots were spread evenly across the plate with a sterile plastic spreader. Sterile tweezers were used to place paper disks containing antibiotics onto inoculated MH. The tweezers were sterilised ahead of each assay by autoclaving for 15 minutes, at 121°C, and 15 psi. To maintain sterility, the tweezers were washed with 70% alcohol and sterilized in a flame between the placement of each disk and between isolates. A maximum of 20 minutes elapsed between the completion of each phase of the protocol; OD adjustment of overnight culture, MH plate inoculation, placement of antibiotic disks, and incubation. The antibiotic disks included in the antibiotic susceptibility testing panel covered a broad range of antibiotics used in bovine veterinary medicine, and include antibiotics reserved for human use (Table 2.4). The diameter of the ZOI of growth around each disk was measured, with the 6mm diameter of the disk being the minimum size. The EUCAST interpretive criteria (EUCAST, 2022) were followed to interpret each isolates ZOI for each antibiotic as resistant, SIE, or susceptible.

Table 2.4: Antibiotic panel for isolate antibiotic susceptibility testing.

Antibiotic	Disk content (µg)	Breakpoints (mm)[2]		
		S ≥	SIE	R ≤
***β*-lactams**				
Ampicillin (AMP)* [1]	10	17	14-16	13
Amoxicillin-clavulanic acid (AMC)[1]	20/10	18	14-17	13
Cefotaxime (CTX) [1]	30	26	23-25	22
Aminoglycoside				
Streptomycin (S10)*	10	15	12-14	11
Kanamycin (K)[+]	10	18	14-17	13
Sulfonamide/complex				
Sulfonamide (S300)	300	17	13-16	12
Quinolones				
Ciprofloxacin (CIP)[1]	5	26	22-25	21
Phenicol				
Chloramphenicol (C) [1]	30	18	13-17	12
Tetracycline				
Oxytetracycline (TE)* [1]	30	15	12-14	11

Breakpoints are taken from EUCAST version 12.0 published 2022.
*Approved for use in bovines within the European Union.
[1]Included in European Union resistance monitoring legislation.
[+]Kanamycin was not included in α sample disk diffusions, but was included in β sample disk diffusions.

The ASLs were given descriptors following VARSS methodology and EU standards (European Food Safety Authority, 2022; Veterinary Medicines Directorate, 2023b) (Table 2.5).

Table 2.5: Antibiotic susceptibility level descriptors used in VARSS reports and European Union AMR monitoring.

Descriptor	Equivalent percentage resistance range
Rare	<0.1%
Very low	0.1% to 1%
Low	>1% to 10%
Moderate	>10% to 20%
High	>20% to 50%
Very high	>50% to 70%
Extremely high	>70%

Levels follow VARSS and EFSA standards (European Food Safety Authority, 2022; Veterinary Medicines Directorate, 2023b).

2.8 Enterobacterial repetitive intergenic consensus polymerase chain reaction to investigate *E. coli* clonality

E. coli isolates were resuscitated from cryovial tubes and incubated in 3 ml of LB broth, 50 µl aliquots of the overnight cultures were spread onto LB agar and incubated. A single colony was picked and dispersed into a 1.5 ml microcentrifuge tube (Eppendorf) containing 1000 µl of TEB before heating in a BTD dry block heater (Grant, UK) at 100°C for 30 minutes to lyse the cells. Lysates were centrifuged at 13,000 xg for 15 minutes in a Heraeus Pico 17 Centrifuge (Thermo Fisher Scientific) to pellet the cell debris. The supernatant containing DNA was collected, and 5 µl aliquots were stored at -20°C. A PCR master mix was prepared for the appropriate number of volumes of DNA according to the ERIC-PCR protocol (Table 2.6). The primer sequences were listed in the original method paper and used in more recent work (Versalovic, Koeuth and Lupski, 1991; Ibrahim *et al.*, 2016) (Table 2.6).

Table 2.6: ERIC-PCR protocol master mix composition.

Reagent	Volume	Sequence
Red taq hot start (2x) (Sigma, UK)	9.5 µl	NA
Nuclease free water (Sigma, UK)	7.5 µl	NA
DNA supernatant	1 µl	NA
ERIC-Forward (10 µM) (Eurofins, DE)	1 µl	ATGTAAGCTCCTGGGGATTCAC
ERIC-Reverse (10 µM) (Eurofins, DE)	1 µl	AAGTAAGTGACTGGGGTGAGCG

ERIC-PCRs followed optimised cycling conditions in a Techne TC-512 thermal cycler (Keison, UK) (Table 2.7).

Table 2.7: ERIC-PCR cycling conditions.

Cycles	Temperature (°C)	Time (minutes)	Comment
1	100	4	Heated lid
1	94	3	
35	94	0.5	
	52	1	
	65	4	
1	65	8	

Agarose (Thermo Fisher Scientific) was suspended in 1x TAE buffer for a final 2% weight per volume. Dissolved agar was boiled in a microwave, and set in a gel tray with comb insert (Biorad, UK). An aliquot of 1 µl of 6x Loading dye (New England Biolabs, USA) was added to 5 µl of PCR product before each product was loaded into the appropriate well in the agarose gel. A 1 kb plus DNA ladder (New England Biolabs) was included to allow comparison between gel images. The loaded gel was electrophoresed at 120 V for two hours using Consort EV233 powerpack (Consort, Belgium). After electrophoresis was completed, the gel was stained with 1% ethidium bromide (Thermo Fisher Scientific) in RO water for 10 minutes, followed by destaining in RO water for 10 minutes. The stained gel was imaged using a Gel-Doc XR system (Bio-Rad), and the image was analysed using FPQuest gel analysis software V4.5 (Bio-Rad).

2.9 Determination of transmissible multidrug resistant phenotypes through conjugation

Kanamycin resistance was induced in DH5-α competent *E. coli* (New England Biolabs) by plating DH5-α onto MH2 containing 64 μg.ml^{-1} of kanamycin. Colonies on these plates were picked and kanamycin resistance was confirmed through the Kirby-Bauer disk diffusion method. This DH5-α kanamycin resistant strain (recipient) was used as a recipient for any potential MGE from kanamycin susceptible MDR α isolates (donors). Kanamycin was not included in the AST panel for the α isolates. To screen the α isolates for kanamycin susceptibility, all of the MDR α isolates were plated onto MH2 plates containing 64 μg.ml^{-1} of kanamycin. Suspected susceptible isolates, indicated by no visible colonies were confirmed susceptible through the Kirby-Bauer disk diffusion method. The recipient strain and donor isolates were resuscitated from cryovial tubes on TBX, and green/blue colony morphology confirmed *E. coli* identity. A single colony was picked and inoculated into NB for overnight culture. Aliquots of 500 μl of the recipient strain overnight culture and 500 μl of the donor isolate overnight culture were mixed together in a 1.5 ml microcentrifuge tube to prepare a mixed culture. An aliquot of 100 μl of mixed culture was pipetted onto sterile filter paper on NA and incubated. Growth on the filter paper was streaked onto TBX containing 100 μg.ml^{-1} of streptomycin and 64 μg.ml^{-1} of kanamycin to identify successful conjugants (Table 2.8). This work was carried out during COVID-19 related lab restrictions and due to time constraints surrounding lab access and consumables, streptomycin was selected as the only additional selective pressure as a large portion of MDR isolates presented with streptomycin resistance.

Table 2.8: Selective media used to confirm successful conjugation of donor phenotype into recipient strain.

Antibiotic in media	Concentration ($\mu g.ml^{-1}$)	Donor growth	Recipient growth	Successful conjugant growth	Unsuccessful conjugant growth
None	n/a	Yes	Yes	NA	NA
S10	100	Yes	No	NA	NA
K	64	No	Yes	NA	NA
S10 + K	100 + 64	No	No	Yes	No

Streptomycin (S10) and kanamycin (K).

Donor isolates are resistant to streptomycin and susceptible to kanamycin, DH5 α recipient is susceptible to streptomycin and resistant to kanamycin, and only the successful conjugants are resistant to both and identifiable by growth on multidrug selective media (Figure 2.1).

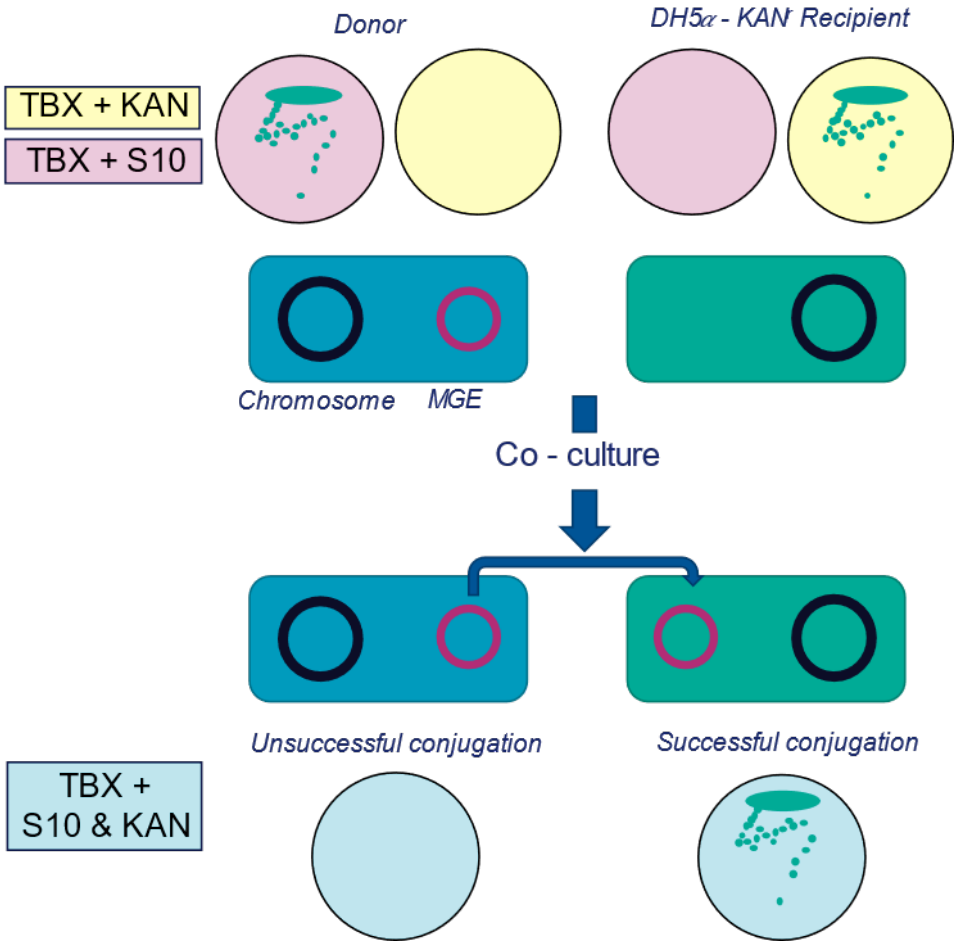


Figure 2.1: Diagrammatic representation of positive selection of successful conjugants.

2.10 Short-read whole genome sequencing to identify antibiotic resistance genes

Transmissible MDR α isolates were selected as candidates for short-read WGS. High quality DNA extractions were completed according to manufacturer's instructions using the DNeasy Ultraclean Microbial Kit (QIAGEN, UK). DNA was quantified on Qubit 4 Fluorometer according to manufacturer's instructions (Thermo Fisher Scientific). Extracted DNA was frozen and shipped to MicrobesNG (Birmingham, UK) for 2 x 250 paired end Illumina sequencing (Illumina, USA), in a 96 well polystyrene plate (Thermo Fisher Scientific) sealed in a thermal cycler. Isolates from the β samples across the bovine developmental stages were selected for WGS, isolates were selected to investigate dissimilarity in isolates from similar aged animals and capture the majority of disk diffusion diversity (Table 2.9).

Table 2.9: β samples selected for NGS of all picked isolates.

Sample	Age (days)	Selection criteria
DCA 1	30	Oldest DCA
DCA 3	20	Same age as DCA 4, youngest in samples
DCA 4	20	Same age as DCA 3, youngest in samples
DCA 5	24	Received Metacam, only known treatment in DCA samples
PRP 3	406	Same age as Heifer 4, closest to mean age in samples (409)
PRP 5	406	Same age as Heifer 3, closest to mean age in samples (409)
PPD 1	966	Oldest cow in samples
PPD 3	953	Second oldest cow in samples
PPL 1	Unknown	Only PPL with treatment history
PPL 5	Unknown	Most different DD phenotype from PPL 1

Isolates were prepared for shipping in DNA/RNA shield (Zymo Research, USA) following instructions from MicrobesNG. Isolates were plated from frozen stocks onto TBX before overnight incubation. A single colony was picked and dispersed in 10 ml of 2xYT broth for overnight incubation. Overnight cultures were grown to minimum 1 OD (OD 600) measured on Varioskan LUX multimode (Thermo Fisher Scientific). Overgrowth was diluted to 1 OD with PBS and the diluent volume was removed for 10 ml of 1 OD. Labelled tubes containing 10 ml of 1 OD solutions were centrifuged at 5000 xg for 10 minutes in Avanti J-E centrifuge (Beckman Coulter, USA). The cell free supernatant was discarded and the cell pellet resuspended in 1 ml of PBS. Aliquots of 1 ml of 10 OD were placed into a 1.5 ml microcentrifuge tube and centrifuged at 4000 xg for 10 minutes. The cell free supernatant was discarded and aliquots of 500 µl of DNA/RNA shield were added for resuspension of pellet before pipetting into labelled and barcoded isolate tubes. Isolates were shipped according to UN3373 to MicrobesNG for 2 x 250 paired end Illumina sequencing (Illumina, USA).

2.11 Whole genome sequencing analysis to identify antibiotic resistance genes and investigate *E. coli* diversity

MicrobesNG provide assembled annotated genomes (MicrobesNG, UK). Their standard pipeline identifies the closest reference genome with Kraken (v1.1.1), mapping reads to this genome with BWA-MEM (v0.7.17) (Li, 2013; Wood and Salzberg, 2014). *De novo* assemblies of reads are made in SPAdes (v3.15.3) with reads mapped to these again with BWA-MEM (Bankevich *et al.*, 2012; Li, 2013). Prokka (v1.14.5) performs an automated annotation of these assemblies (Seemann, 2014). ABRicate (v1.0.0) was used to screen contigs

against a selection of gene databases (Seemann, no date, Accessed: December 2022). To identify acquired ARG the databases used included CARD (v3.1.4), MEGARes (v2.0), NCBI ARG (v3.10.18), ResFinder (v4.0), and ARG-ANNOT (v4.0) (Zankari *et al.*, 2012a; Gupta *et al.*, 2014; Jia *et al.*, 2017; Feldgarden *et al.*, 2019; Doster *et al.*, 2020). EcOH was used for *in silico* *E. coli* serotyping (v2.0) (Ingle *et al.*, 2016). PlasmidFinder (v2.1.1) was used to identify plasmid incompatibility groups present in the sequences (Carattoli *et al.*, 2014). *E. coli* genomes were submitted to the *Escherichia/Shigella* database hosted on the Enterobase online resource for analysing and visualising enteric bacteria genomic variation (Zhou *et al.*, 2020). Genomes were compared to the Achtman seven gene MLST scheme as well as the whole genome MLST scheme (wgMLST) to identify ST and investigate clonality. Genomic variation was visualised using the GrapeTree plot functionality of Enterobase using the MSTreeV2 Algorithm (Zhou *et al.*, 2018).

2.12 Metagenomic sequencing of total DNA extracts from faecal samples

Total DNA extractions were taken from each sample in the β sample set following the manufacturer's instructions for the PowerFecal DNA extraction kit (QIAGEN, UK), and DNA was quantified with broad range dsDNA Qubit 4 Fluorometer according to manufacturer's instructions (Thermo Fisher Scientific). Extracted DNA was frozen and shipped to the University of Leeds Institute of Molecular Medicine (Leeds, UK) for NextSeq 2K 300 cycle lane, 150 bp paired-end metagenomic sequencing (Illumina, USA) in a 96-well plate sealed in a thermal cycler.

2.13 Metagenomics analysis to produce metagenome assembled genomes, investigate metagenome diversity, and identify antibiotic resistance genes

Metagenome data was compiled into a database and analysed in R using SQMtools (v0.6.2) and the SqueezeMeta (v1.4.0) pipeline (Tamames and Puente-Sánchez, 2019). The pipeline is composed of several open-access tools (Table 2.10). The community ecology R package vegan (v2.6-4) was used to investigate the diversity of metagenomes (Dixon, 2003).

Table 2.10: Tools included in the Squeezemeta bioinformatics pipeline.

Task	Tool	Reference
Short contig removal (<1000bp)	Prinseq	(Schmieder and Edwards, 2011)
Contig statistics		
Redundant contig removal	cd-hit	
Contig merging	Minimus2	(Treangen *et al.*, 2012)
Predicting RNA sequences	Barrnap (prokka)	(Seemann, 2014)
16S rRNA taxonomic classification	RDP Classifier	(Wang *et al.*, 2007)
Assembly	MEGAHIT	(Li *et al.*, 2015)
Predicting tRNA/tmRNA sequences	Aragorn	(Laslett and Canback, 2004)
Predicting ORF's	Prodigal	(Hyatt *et al.*, 2010)
GenBank similarity search	DIAMOND	(Buchfink, Xie and Huson, 2014)
eggnog similarity search		
KEGG similarity search		
Pfam database HMM homology search	HMMER3	(Eddy, 2009)
Taxonomic assignment of genes	MyTaxa	(Luo, Rodriguez-R and Konstantinidis, 2014)
Read mapping against contigs	BWA	(Li and Durbin, 2009)
Binning genomes	MaxBin2	(Wu, Simmons and Singer, 2016)
	MetaBAT2	(Kang *et al.*, 2019)
Combination of binning	DAS tool	(Sieber *et al.*, 2018)
Bin statistics	CheckM	(Parks *et al.*, 2015)
KEGG pathway prediction	MinPath	(Ye and Doak, 2009)
MetaCyc pathway prediction		

2.14 Software used to prepare figures

All graphs were created in GraphPad Prism 9 (version 9.4.1); when a different software was used, it is stated in the figure legend.

2.15 Software used to complete statistical analysis

Statistical analyses were carried out in GraphPad Prism 9 (version 9.4.1) or in R using the vegan package (Oksanen *et al.*, 2022). The Kolmogorov-Smirnov test (KST) was used as a non-parametric test to compare the difference between the distributions of unmatched groups. The Mann Whitney test was used as a non-parametric test to compare the differences between the distributions of unmatched groups. The Kruskal-Wallis ANOVA with Dunn's multiple comparisons test (KWT) was used as a non-parametric test to determine if the ranks of the means or medians of three or more distributions were significantly different.

Chapter 3 Antibiotic susceptibility of *Escherichia coli* and interpreting risk in cows from one UK dairy herd

To determine the risk posed by ABR, it has to be contextualised to its source. Every bacteria is considered an individual with a single nucleotide change potentially resulting in a different phenotype. Each cow in a dairy herd could be viewed as an individual environmental niche, providing a different selective environment where isolates compete. The housing, treatment, and diet of dairy cows are tightly controlled to maintain high levels of biosecurity, health, and milk yield. While the environment that each cow inhabits in a single farm is nearly universal, the selection pressures in the microbiome of each animal may be different. For instance, there will be variations in chemotherapeutic treatment between individual animals based on their clinical need. Antibiotic treatment of dairy cows, particularly cephalosporins and penicillins, has been reported to increase the risk of ABR faecal *E. coli* or bacteria (Donaldson *et al.*, 2006; Singer, Patterson and Wallace, 2008; Chambers *et al.*, 2015). However, the magnitude and persistence of this shift varies across different studies (Donaldson *et al.*, 2006; Sawant *et al.*, 2007; Singer, Patterson and Wallace, 2008; Feng, Chambers and Knowlton, 2020). Some have reported that antibiotic treatment given to bovines resulted in no difference in the abundance of ARG in faecal *E. coli* (Feng *et al.*, 2020; Feng, Chambers and Knowlton, 2020). Additionally, housing conditions and standard practices are not preserved across different farms and regions of the UK and the globe. Differences in farming practices have been identified as contributors to the risk of ABR, with higher proportions of ABR bacteria reported in conventional farming than organic systems in the United States (Halbert *et al.*, 2006). This

chapter presents the overall antibiotic susceptibility of 403 *E. coli* isolates collected from the faeces of 18 PPL to understand the risk of AMR present in faecal *E. coli* in this herd. The ASRs are a combination of the ASLs and the median antibiotic susceptibility distributions (MASD). The treatment records of the PPL for the three months preceding sampling provided insight into the selective pressures that may have been present in each PPL and how these pressures could have influenced the observed antibiotic susceptibility of the faecal *E. coli*. This chapter focused on understanding ABR in PPL faecal *E. coli*, but the number of the isolates in a sample with resistance phenotypes may represent a small fraction of the overall population. While this fraction may not be representative of the whole, if present, it must be captured through enrichment. To enrich for antibiotic resistant isolates, the faecal samples were plated on media supplemented with different antibiotics to increase the probability of capturing these isolates. The shift in the overall ASPs of this enriched dataset is considered to investigate if this enrichment could improve surveillance of ABR. It is important to understand how this enrichment affects the overall abundance of bacteria, significant reductions in the overall abundance would increase the relative proportion of resistant isolates and artificially increase the perception of risk in an environment. This dataset was compared with the VCD as this is the predominant dataset collected from UK bovines, summarised in Section 1.3.1. The VCD data is presented as susceptibility proportions with minimal context for the source of the isolates. This introduces unknown biases into the data as it is likely that some of these isolates have come from animals suffering ill health and potentially receiving antibiotic treatment.

3.1 Aims

1. To establish the antibiotic susceptibility of PPL faecal *E. coli*.
2. To investigate how much antibiotic susceptibility vary across PPL faecal *E. coli* from samples:
    a. In a single herd.
    b. Plated on TBX with or without antibiotics.
    c. Collected from the PPL that did or did not receive chemotherapy before sampling
    d. Where varying durations of time have elapsed since chemotherapeutic treatment.
3. To assess the strengths and limitations of ASL metrics compared to MASDs.

3.2 Methods

3.2.1 Sample collection and purification of presumptive *E. coli* isolates

Detailed sampling and isolate purification methodology is available in sections 2.4, 2.5, and 2.6. TBX was used successfully to isolate 403 non-toxigenic *E. coli* (α isolates) from 18 PPL faeces samples (α samples) between June and October 2019. Convenience samples were taken from veterinarians' gloves upon completion of routine work and delivered to SVMS. Samples were stored at 4°C, and aliquots were plated within 24 hours of collection. Colonies on TBX with a green/blue morphology were picked and re-streaked onto fresh TBX. Glycerol stocks were inoculated when isolates had a confirmed pure green/blue morphology and stored at -80°C.

3.2.2 Enumeration of *E. coli* on TBX supplemented with different antibiotics

Details of the enumeration method are contained in section 2.6. The abundance of *E. coli* was measured through total viable counts of green/blue

morphology colonies on TBX and the abundance of bacteria was measured through total viable counts on NA. Aliquots of faecal samples were weighed and the appropriate volume of PBS was added to prepare a 1 $g.ml^{-1}$ solution. Serial dilutions were prepared and plated onto solid media. The number of colonies were counted after incubation and the *E. coli* CFU g^{-1} of faeces was calculated.

3.2.3 Kirby-Bauer disk diffusion method of antibiotic susceptibility testing

Detailed methodology for determining antibiotic susceptibility is in section 2.7. Aliquots of overnight cultures were adjusted to OD 0.1 before plating on MH. Paper antibiotic disks were placed onto inoculated plates before incubation at 37°C for 20 hours. ZOIs were measured with a ruler and compared to EUCAST breakpoints to determine antibiotic susceptibility.

3.2.4 Determining antibiotic susceptibility levels of the α *E. coli* for each antibiotic

The ASL descriptors are used by the VARSS report and EFSA AMR monitoring (Table 2.5) (European Food Safety Authority, 2022; Veterinary Medicines Directorate, 2023b). The VARSS report group resistant and SIE into resistant, this thesis reports each individually following EUCAST guidelines on interpretation of AST (EUCAST, 2022).

3.2.5 Median antibiotic susceptibility distributions to display the antibiotic susceptibility of a population of *E. coli*

The MASD present the median ZOI and the ASR for each antibiotic tested, for all of the isolates in a population (e.g. all of the samples, a PPL treatment group, or a single sample). The MASD also presents the interquartile range, the minimum, and the maximum ZOI to display the range of antibiotic susceptibility in a population.

3.2.6 Identifying statistically different median antibiotic susceptibility distributions in the isolates from different PPL chemotherapy treatment groups

The non-parametric KST was used to compare the MASDs of the isolates taken from PPL that received chemotherapy before sampling, to the isolates taken from PPL that did not receive chemotherapy before sampling. This test identifies differences in the distribution of data about the median, rather than between the medians. The non-parametric KWT was used to identify significant differences in medians of antibiotic ZOI for the isolates grouped by the time between PPL treatment and sampling.

3.3 Results

3.3.1 Abundance of *Escherichia coli* in α faecal samples

The treatment records that could be linked to the sampled PPL and found in the on-farm database were collected (Table 2.2). High levels of *E. coli* ranging between $1.77x10^5$ CFU g^{-1} and $2.4\ x10^9$ CFU g^{-1} were collected from the faeces of PPL (Figure 3.1). The faecal sample from PPL 1 contained $2.4x10^9$ CFU g^{-1} *E. coli*, with 45 isolates stored at -80°C. The PPL 2 faecal sample contained $1.77x10^5$ CFU g^{-1} *E. coli,* and 31 isolates were stored. The faecal sample from PPL 3 contained $1.47x10^7$ CFU g^{-1} *E. coli*, with 30 isolates stored. The PPL 4 faecal sample contained $4.33x10^6$ CFU g^{-1} *E. coli*, and 30 isolates were stored. The faecal sample from PPL 5 contained $5.55x10^6$ CFU g^{-1} *E. coli*, with 29 isolates stored. The PPL 6 faecal sample contained $1.27x10^7$ *E. coli*, and 21 isolates were stored. The faecal sample from PPL 7 contained $9.8x10^7$ CFU g^{-1} *E. coli*, with 27 isolates stored. The PPL 8 faecal sample contained $1.49x10^7$ CFU g^{-1} *E. coli,* and 28 isolates were stored. The faecal sample from PPL 9 contained $3.5x10^8$ CFU g^{-1} *E. coli*, with 25 isolates stored.

The PPL 10 faecal sample contained 5x10^{9} CFU g^{-1} *E. coli,* and 26 isolates were stored. The faecal sample from PPL 11 contained 4x10^{8} CFU g^{-1} *E. coli*, with 25 isolates stored. The PPL 12 faecal sample contained 3.42x10^{7} CFU g^{-1} *E. coli,* and 24 isolates were stored. The faecal sample from PPL 13 contained 5.4x10^{7} CFU g^{-1} *E. coli*, with 25 isolates stored. The PPL 14 faecal sample contained 4x10^{7} CFU g^{-1} *E. coli,* and 25 were stored. The faecal sample from PPL 15 contained 5.7x10^{8} CFU g^{-1} *E. coli*, with 39 isolates stored. The PPL 16 faecal sample contained 5.44x10^{8} CFU g^{-1} *E. coli*, and 25 isolates were stored. The faecal sample from PPL 17 contained 3.86x10^{8} CFU g^{-1} *E. coli*, with 30 isolates stored. The PPL 18 faecal sample contained 1.9x10^{7} CFU g^{-1} *E. coli*, and 25 isolates were stored.

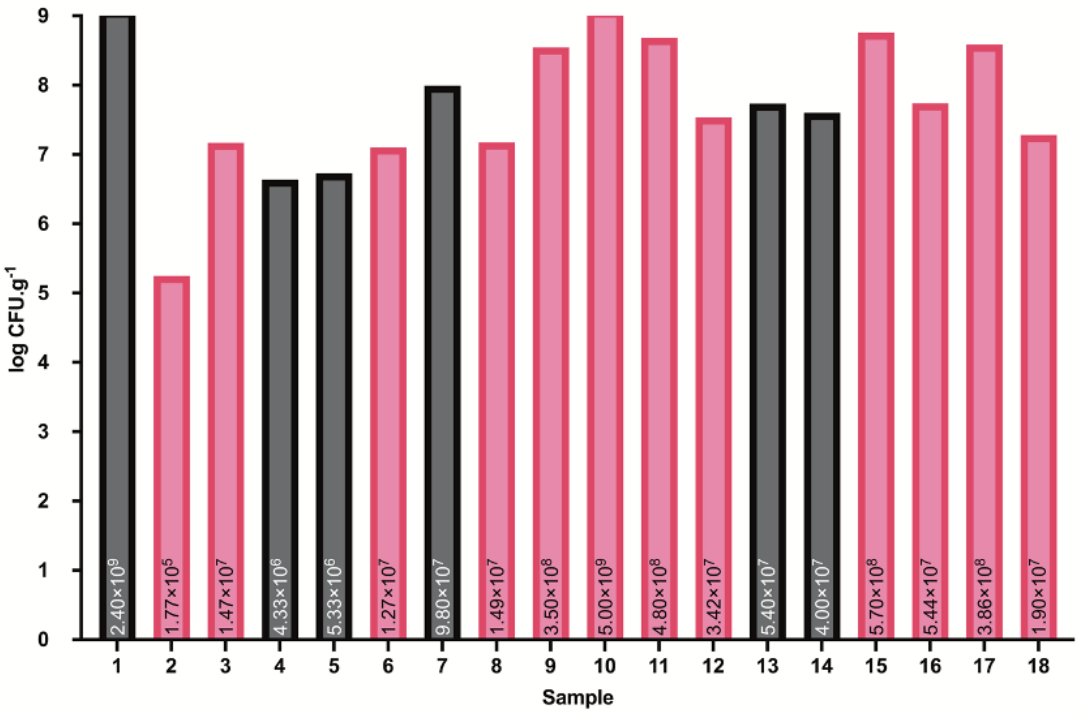


Figure 3.1: *E. coli* abundance from PPL faeces. Bacteria were enumerated on TBX and green/blue morphology colonies were presumed to be *E. coli*. The *E. coli* colony forming units per gram of faeces (CFU g^{-1}) are presented on a logarithmic scale. The pink bars are the counts of *E. coli* from PPL that had no recorded chemotherapeutic treatments in the three months before sampling. The black bars are the counts of *E. coli* from PPL that had recorded chemotherapeutic treatments in the three months before sampling.

Records of chemotherapeutic treatment given to PPL were not found to significantly alter the abundance of *E. coli*, with no significant difference observed between the median CFU g^{-1} of samples from the treated and the

untreated PPL (Figure 3.2). The samples from the treated PPL contained a median of 4.7 x10^7 CFU g^{-1} and a mean of 4.34 x10^8 CFU g^{-1}, while the samples from the untreated PPL contained a median of 4.43 x10^7 CFU g^{-1} and a mean of 5.78 x10^8 CFU g^{-1} (Figure 3.2).

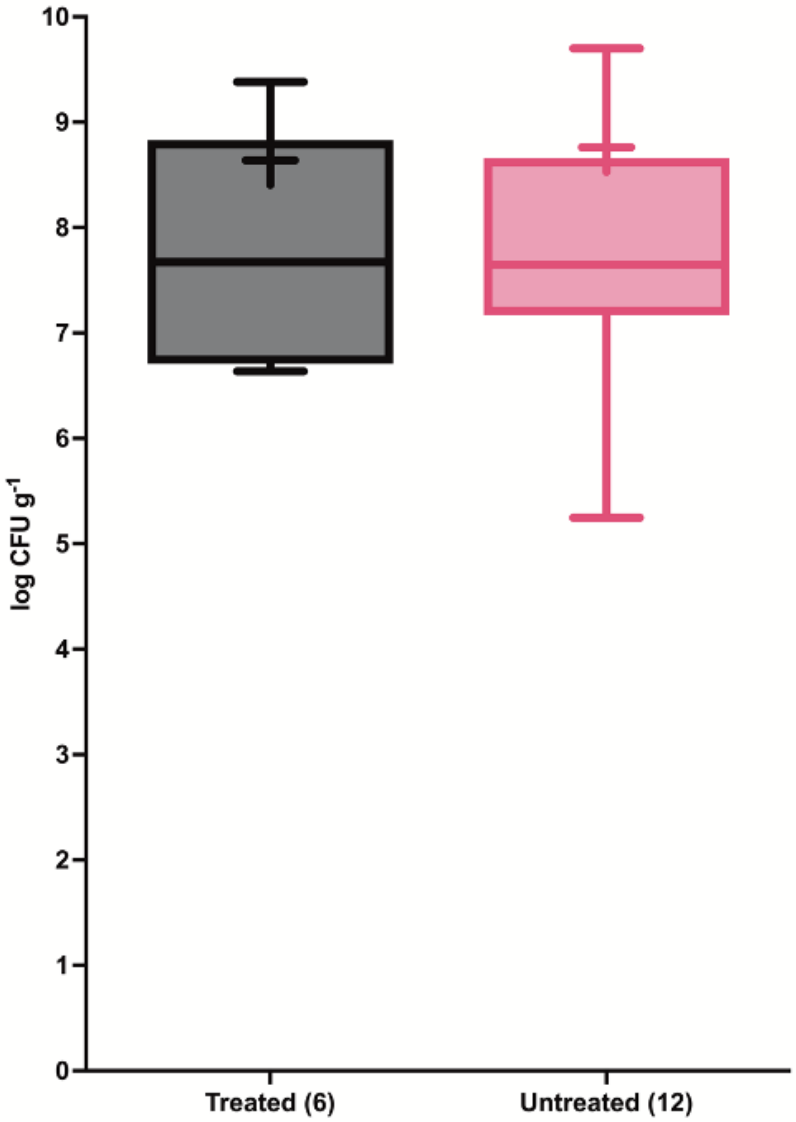


Figure 3.2: Impact of chemotherapeutic treatment on *E. coli* abundance. The *E. coli* CFU g^{-1} of faeces is plotted for each PPL treatment group. The black box represents samples from the treated PPL, and the pink box represents samples from the untreated PPL. The group median *E. coli* CFU g^{-1} is represented by the mid-line, the cross represents the mean *E. coli* CFU g^{-1}, the midline to the bottom of the box represents the 25% interquartile range, the midline to the top of the box represents the 75% interquartile range, and the whiskers represent min-max values. A Mann Whitney test confirms the median counts were not significantly different (ρ = 0.7503).

3.3.2 Antibiotic susceptibility levels of the α sample faecal *Escherichia coli*

ASRs were determined by the Kirby-Bauer disk diffusion method with EUCAST clinical breakpoints used for interpretation. ASLs in accordance with VARSS and EFSA descriptors are presented to provide veterinary context to these ASR's (Table 2.5). The ASRs and ASLs for all 403 isolates are presented in Table 3.1, and the ASLs for each antibiotic are described in this section. To ampicillin, a high level of the isolates were susceptible (39.2%), SIE (33.7%), and resistant (27%). An extremely high level (87.8%) of the isolates were susceptible to amoxicillin-clavulanate, with low levels of resistance (7.7%) and SIE (4.5%). To cefotaxime, a very high level of the isolates were susceptible (69.5%), a high level were SIE (25.3%), and a low level were resistant (5%). Moderate levels of the isolates were susceptible (18.4%) and resistant (16.9%) to streptomycin, with a very high level of SIE (64.8%). To sulfonamide, a very high level of the isolates were susceptible (63.3%), a high level were SIE (22.3%), and a moderate level were resistant (14.4%). An extremely high level of the isolates were susceptible (97.5%), a low level were SIE (1.5%), and a low level were resistant (1%) to chloramphenicol. To ciprofloxacin, an extremely high level of the isolates were susceptible (86.4%), a moderate level were SIE (11.9%), and a low level were resistant (1.7%). To tetracycline, an extremely high level of the isolates were susceptible (94%), a low level were SIE (5.9%), and a low level were resistant (5.5%).

Table 3.1: Overall antibiotic susceptibility and susceptibility levels of the 403 *α E. coli* isolates from the 18 PPL faecal samples.

	AMP	AMC	CTX	S10	S300	C	CIP	TE
Susceptible	158	354	281	74	255	393	348	379
SIE	136	18	102	261	90	6	48	2
Resistant	109	31	20	68	58	4	7	22
% Susceptible	39.2	87.8	69.5	18.4	63.3	97.5	86.4	94
Susceptible level	High	Extremely high	Very high	Moderate	Very high	Extremely high	Extremely high	Extremely high
% SIE	33.7	4.5	25.3	64.8	22.3	1.5	11.9	0.5
SIE level	High	Low	High	Very high	High	Low	Moderate	Very low
% Resistant	27.0	7.7	5.0	16.9	14.4	1	1.7	5.5
Resistance level	High	Low	Low	Moderate	Moderate	Low	Low	Low

SIE = susceptible increased exposure, AMP = ampicillin, AMC = amoxicillin-clavulanate, CTX = cefotaxime, S10 = streptomycin, K = kanamycin, S300 = sulfonamide, C = chloramphenicol, CIP = ciprofloxacin, TE = tetracycline. The level descriptors follow VARSS and EFSA standards defined as rare (<0.1%), very low (0.1-1%), low (1-10%), moderate (10-20%), high (20-50%), very high (50-70%), and extremely high (>70%) (European Food Safety Authority, 2022; Veterinary Medicines Directorate, 2023b).

3.3.3 Herd median antibiotic susceptibility distributions

The MASD provides a median ZOI, the ASR, and the spread of the interquartile range of ZOI for each antibiotic tested. The α sample isolates' median ASRs were susceptible to six antibiotics from five classes (amoxicillin-clavulanate, cefotaxime, sulfonamide, chloramphenicol, ciprofloxacin, and tetracycline), and SIE to two antibiotics from two classes (ampicillin and streptomycin) (Figure 3.3). This MASD profile is unchanged when including α samples across all TBX compositions (Appendix 9). The median ZOI, ASR, and interquartile range of ZOI for each antibiotic are described in this section. The median ZOI for ampicillin was 16 mm (SIE) and an interquartile range of 11-17 mm. The median ZOI for amoxicillin-clavulanate was 20 mm (susceptible) and an interquartile range of 19-21 mm. The median ZOI for cefotaxime was 27 mm (susceptible) and an interquartile range of 25-29 mm. The median ZOI for streptomycin was 13 mm (SIE) and an interquartile range of 12-14 mm. The median ZOI for sulfonamide was 19 mm (susceptible) and an interquartile range of 15-22 mm. The median ZOI for chloramphenicol was 24 mm (susceptible) and an interquartile range of 22-25 mm. The median ZOI for ciprofloxacin was 31 mm (susceptible) and an interquartile range of 27-33 mm. The median ZOI for tetracycline was 21 mm (susceptible) and an interquartile range of 20-22 mm.

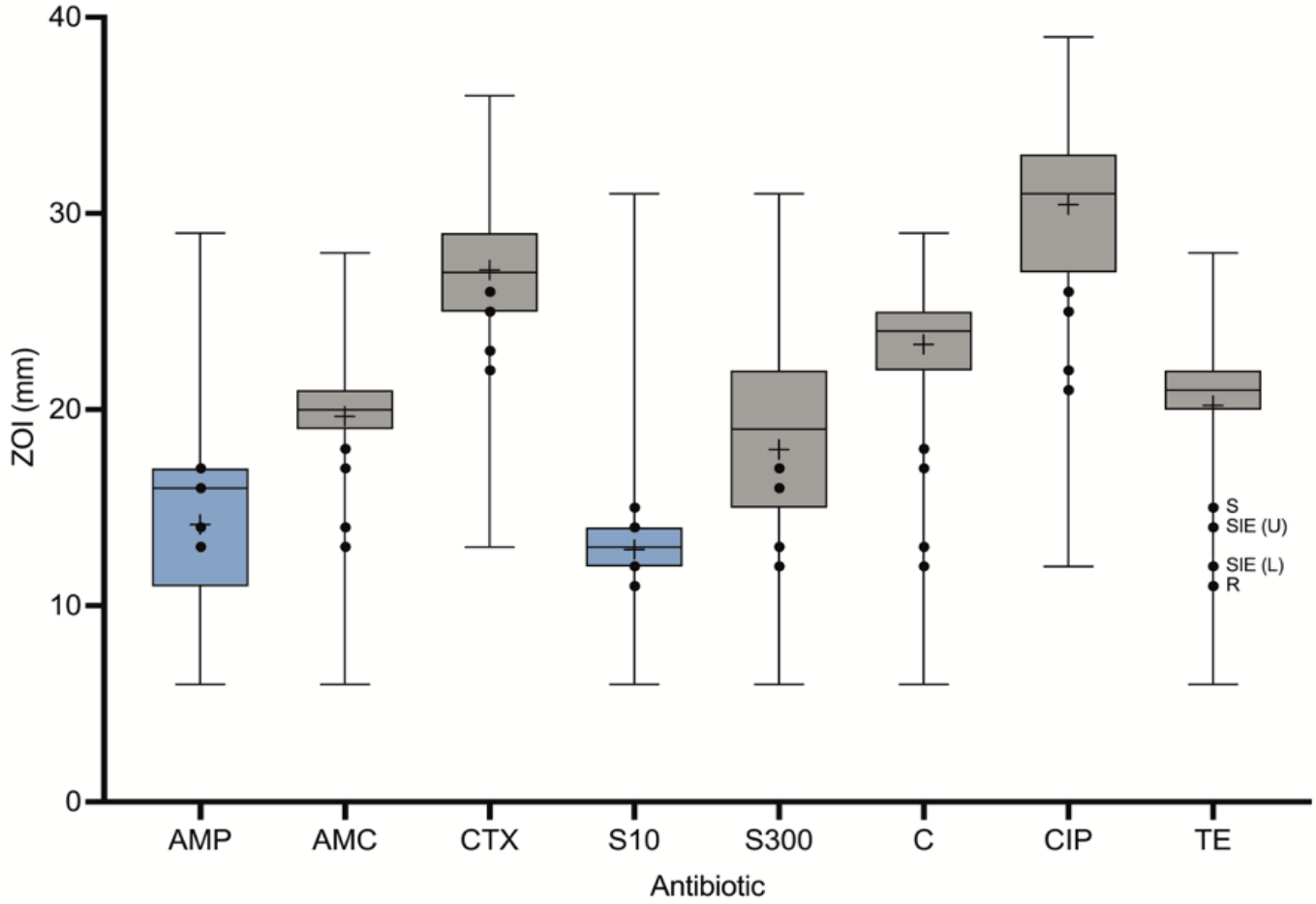


Figure 3.3: Median antibiotic susceptibility distibutions of the 403 *α E. coli* isolates. The midline represents the median zone of inhibition (ZOI) across all isolates, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown as black dots: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility: grey for susceptible and blue for SIE. Abbreviations: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), TE (tetracycline).

3.3.4 Post-parturition lactating dairy cow antibiotic susceptibility levels

To investigate the variability of *E. coli* ASLs between individuals in the same herd, *E. coli* can be grouped by PPL source. The levels of resistance and SIE for all of the isolates grouped by sample PPL are in Table 3.2, with all levels moderate or greater described in this section. The ASRs and ASLs grouped by sample PPL are presented in Appendix 4 - Appendix 8.

The 43 *E. coli* isolates from PPL 1 had extremely high levels of resistance to ampicillin (100%), streptomycin (97.7%), sulfonamide (97.7%), and tetracycline (97.7%), with low levels (2.3%) of SIE to amoxicillin-clavulanate, chloramphenicol, and ciprofloxacin. The 31 PPL 2 *E. coli* isolates had low levels of resistance to ampicillin (9.7%), cefotaxime (3.2%), streptomycin (9.7%), sulfonamide (6.5%), and ciprofloxacin (3.2%) with extremely high levels of SIE to ampicillin (80.6%) and streptomycin (87.1%), and moderate levels of SIE to sulfonamide (19.4%) and ciprofloxacin (19.4%). The 30 *E. coli* isolates from PPL 3 had a moderate level of resistance to ampicillin (10%), an extremely high level of SIE to streptomycin (93.3%), and high levels of SIE to ampicillin (26.7%) and cefotaxime (23.3%). The 30 PPL 4 *E. coli* isolates had an extremely high level of resistance to ampicillin (100%), high levels of resistance to amoxicillin-clavulanate (33.3%) and tetracycline (23.3%), and high levels of SIE to amoxicillin-clavulanate (26.7%) and streptomycin (30%). The 29 *E. coli* isolates from PPL 5 had a very high level of resistance to ampicillin (58.6%), moderate levels of resistance to streptomycin (17.2%) and sulfonamide (13.8%), a very high level of SIE to streptomycin (62.1%), high levels of SIE to ampicillin (34.5%) and ciprofloxacin (41.4%), and a moderate level of SIE to cefotaxime (10.3%). The 21 PPL 6 *E. coli* isolates had a

moderate level of resistance to ampicillin (14.3%) and an extremely high level of SIE to streptomycin (71.4%). The 27 *E. coli* isolates from PPL 7 had high levels of resistance to ampicillin (48.1%) and sulfonamide (25.9%), very high levels of SIE to ampicillin (33.3%) and cefotaxime (33.3%), a high level of SIE to amoxicillin-clavulanate (22.2%), and moderate levels of SIE to streptomycin (85.2%), sulfonamide (11.1%), and ciprofloxacin (18.5%). The 28 PPL 8 *E. coli* isolates had an extremely high level of resistance to ampicillin (92.9%); high levels of resistance to amoxicillin-clavulanate (32.1%), streptomycin (50%), and sulfonamide (21.4%); moderate levels of resistance to cefotaxime (10.7%) and tetracycline (14.3%); and high levels of SIE to cefotaxime (32.1%), streptomycin (50%), and sulfonamide (39.2%). The 25 *E. coli* isolates from PPL 9 had very high levels of resistance to ampicillin (68%) and amoxicillin-clavulanate (56%), a high level of resistance to streptomycin (36%), a moderate level of resistance to sulfonamide (20%), very high levels of SIE to cefotaxime (56%) and streptomycin (56%), a high level of SIE to sulfonamide (48%), and a moderate level of SIE to ampicillin (16%). The 26 PPL 10 *E. coli* isolates had an extremely high level of SIE to streptomycin (84.6%) and a moderate level of SIE to ampicillin (11.5%). The 25 *E. coli* isolates from PPL 11 had a moderate level of resistance to ampicillin (12%), a very high level of SIE to streptomycin (68%), high levels of SIE to sulfonamide (28%) and ampicillin (44%), and moderate levels of SIE to cefotaxime (16%) and ciprofloxacin (16%). The 24 PPL 12 *E. coli* isolates had high levels of resistance to streptomycin (33.3%) and sulfonamide (29.2%); moderate levels of resistance to chloramphenicol (16.7%) and SIE to streptomycin (12.5%); and high levels of SIE to ampicillin (33.3%), cefotaxime (20.8%), and

ciprofloxacin (25%). The 25 *E. coli* isolates from PPL 13 had a very high level of SIE to streptomycin (68%), a high level of SIE to ampicillin (48%), and a moderate level of SIE to ciprofloxacin (16%). The 25 PPL 14 *E. coli* isolates had an extremely high level of SIE to streptomycin (88%), a high level of SIE to ampicillin (32%), and moderate levels of SIE to cefotaxime (20%), and sulfonamide (20%). The 39 *E. coli* isolates from PPL 15 had a moderate level of resistance to streptomycin (10.3%), very high levels of SIE to ampicillin (64.1%) and streptomycin (69.2%), and high levels of SIE to cefotaxime (43.6%) and sulfonamide (50%). The 25 PPL 16 *E. coli* isolates had a moderate level of resistance to sulfonamide (12%), a very high level of SIE to streptomycin (68%), and high levels of SIE to ampicillin (24%), cefotaxime (31.8%), sulfonamide (24%), and ciprofloxacin (20%). The 30 *E. coli* isolates from PPL 17 had high levels of resistance to streptomycin (26.7%) and sulfonamide (30%); moderate levels of resistance to ampicillin (20%), chloramphenicol (16.7%), and tetracycline (16.7%); extremely high levels of SIE to ampicillin (73.3%) and streptomycin (73.3%); high levels of SIE to cefotaxime (33.3%) and sulfonamide (43.3%); and a moderate level of SIE to ciprofloxacin (16.7%). The 25 PPL 18 *E. coli* isolates had high levels of resistance to streptomycin (28%) and sulfonamide (24%), a very high level of SIE to streptomycin (68%), and high levels of SIE to ampicillin (48%), cefotaxime (44%), and sulfonamide (28%) (Table 3.2).

Table 3.2: Overall levels of antibiotic resistance and susceptibility with increased exposure of the isolates from each PPL.

PPL	Level	AMP	AMC	CTX	S10	S300	C	CIP	TE
1	SIE	Rare	Low	Rare	Rare	Rare	Low	Low	Rare
	R	E. high	Low	Low	E. high	E. high	Rare	Rare	E. high
2	SIE	E. high	Rare	Low	E. high	Mod	Rare	Mod	Rare
	R	Low	Rare	Low	Low	Low	Rare	Low	Low
3	SIE	High	Rare	High	E. high	Low	Rare	Low	Rare
	R	Mod	Rare	Rare	Rare	Rare	Rare	Low	Low
4	SIE	Rare	High	Rare	High	Rare	Rare	Low	Low
	R	E. high	High	Rare	Low	Low	Rare	Rare	High
5	SIE	High	Rare	Mod	V. high	Rare	Rare	High	Rare
	R	V. high	Rare	Low	Mod	Mod	Rare	Low	Rare
6	SIE	Low	Low	Rare	E. high	Low	Rare	Low	Rare
	R	Mod	Rare	Rare	Low	Low	Rare	Low	Low
7	SIE	V. high	High	V. high	Mod	Mod	Low	Mod	Rare
	R	High	Rare	Low	Low	High	Rare	Low	Low
8	SIE	Low	Low	High	High	High	Low	Low	Rare
	R	E. high	High	Mod	High	High	Rare	Rare	Mod
9	SIE	Mod	Low	V. high	V. high	High	Low	Low	Low
	R	V. high	V. high	Rare	High	Mod	Rare	Rare	Rare
10	SIE	Mod	Rare	Low	E. high	Low	Rare	Rare	Rare
	R	Low	Rare	Rare	Low	Rare	Rare	Rare	Rare
11	SIE	High	Low	Mod	V. high	High	Low	Mod	Rare
	R	Mod	Rare	Low	Low	Low	Rare	Rare	Rare
12	SIE	High	Rare	High	Mod	Low	Rare	High	Rare
	R	Rare	Rare	Low	High	High	Mod	Low	Rare
13	SIE	High	Rare	Low	V. high	Low	Rare	Mod	Rare
	R	Rare	Rare	Low	Rare	Rare	Rare	Low	Rare
14	SIE	High	Rare	Mod	E. high	Mod	Rare	Rare	Rare
	R	Rare	Rare	Low	Low	Low	Rare	Rare	Rare
15	SIE	V. high	Low	High	V. high	High	Rare	Low	Rare
	R	Low	Rare	Low	Mod	Low	Rare	Rare	Low
16	SIE	High	Low	High	V. high	High	Rare	High	Rare
	R	Low	Rare	Low	Rare	Mod	Rare	Rare	Rare
17	SIE	E. high	Rare	High	E. high	High	Rare	Mod	Rare
	R	Mod	Rare	Low	High	High	Mod	Low	Mod
18	SIE	High	Rare	High	V. high	High	Rare	Low	Rare
	R	Low	Rare	Low	High	High	Rare	Rare	Rare

Grey shaded cells are cows known to have received treatment within one month before sampling. AMP = ampicillin, AMC = amoxicillin-clavulanate, CTX = cefotaxime, S10 = streptomycin, S300 = sulfonamide, C = chloramphenicol, CIP = ciprofloxacin, TE = tetracycline. Levels follow VARSS and EFSA standards (European Food Safety Authority, 2022; Veterinary Medicines Directorate, 2022b).

Rare	Very low (V. low)	Low	Moderate (Mod)	High	Very high (V. high)	Extremely high (E. high)
<0.1%	0.1%-1%	>1%-10%	>10%-20%	>20%-50%	>50%-70%	>70%

3.3.5 Median antibiotic susceptibility distributions of *Escherichia coli* from post-parturition lactating dairy cow faeces

PPL 1 received amoxicillin (β-lactam) six days before sampling (Table 2.2). The median ASRs of the PPL 1 *E. coli* isolates were susceptibility to four antibiotics from three classes (amoxicillin-clavulanate, cefotaxime, chloramphenicol, and ciprofloxacin) and resistance to four antibiotics from four classes (ampicillin, streptomycin, sulfonamide, and tetracycline) (Figure 3.4). The median ZOI for ampicillin was 6 mm (resistant) with no interquartile range. The median ZOI for amoxicillin-clavulanate was 20 mm (susceptible) and an interquartile range of 19-20 mm. The median ZOI for cefotaxime was 30 mm (susceptible) and an interquartile range of 29-31 mm. The median ZOI for streptomycin was 6 mm (resistant) with no interquartile range. The median ZOI for sulfonamide was 6 mm (resistant) with no interquartile range. The median ZOI for chloramphenicol was 24 mm (susceptible) and an interquartile range of 24-25 mm. The median ZOI for ciprofloxacin was 30 mm (susceptible) and an interquartile range of 30-32 mm. The median ZOI for tetracycline was 9 mm (resistant) and an interquartile range of 8-9 mm (Figure 3.4).

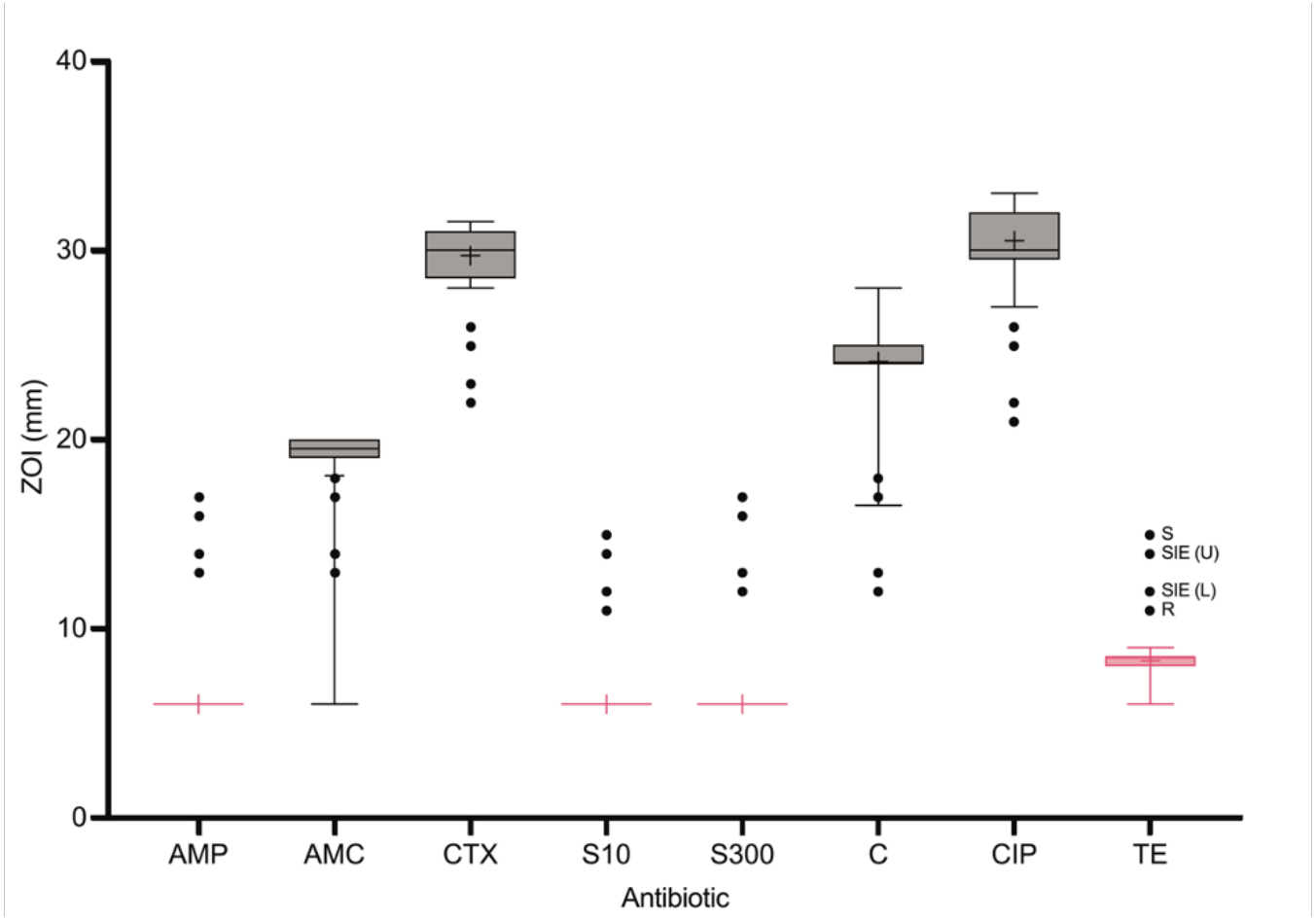


Figure 3.4: PPL 1 *E. coli* isolates median antibiotic susceptibility distributions. The midline represents the median zone of inhibition (ZOI) across all isolates, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown as black dots: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility: pink for resistant, grey for susceptible, and blue for SIE. Abbreviations: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), TE (tetracycline).

The *E. coli* isolates from PPL 2 had median ASRs of susceptibility to six antibiotics from five classes (amoxicillin-clavulanate, cefotaxime, sulfonamide, chloramphenicol, ciprofloxacin, and tetracycline) and SIE to two antibiotics from two classes (ampicillin and streptomycin) (Figure 3.5). The median ZOI for ampicillin was 15 mm (SIE) and an interquartile range of 14-16 mm. The median ZOI for amoxicillin-clavulanate was 20 mm (susceptible) and an interquartile range of 20-21 mm. The median ZOI for cefotaxime was 28 mm (susceptible) and an interquartile range of 27-30 mm. The median ZOI for streptomycin was 13 mm (SIE) and an interquartile range of 12-14 mm. The median ZOI for sulfonamide was 20 mm (susceptible) and an interquartile range of 19-21 mm. The median ZOI for chloramphenicol was 23 mm (susceptible) and an interquartile range of 23-24 mm. The median ZOI for ciprofloxacin was 27 mm (susceptible) and an interquartile range of 27-30 mm. The median ZOI for tetracycline was 21 mm (susceptible) and an interquartile range of 20-22 mm (Figure 3.5).

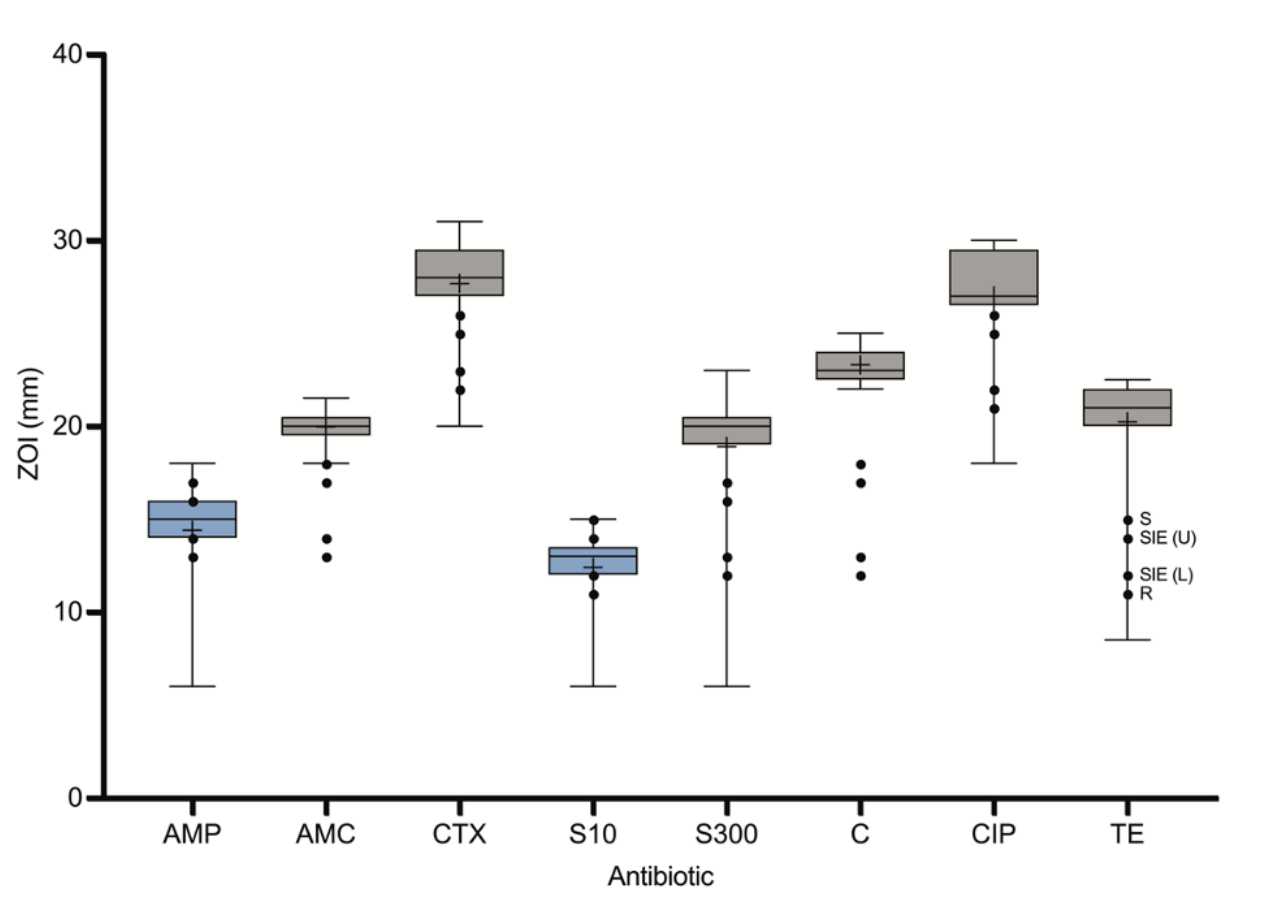


Figure 3.5: PPL 2 *E. coli* isolates median antibiotic susceptibility distibutions. The midline represents the median zone of inhibition (ZOI) across all isolates, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown as black dots: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility: grey for susceptible and blue for SIE. Abbreviations: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), TE (tetracycline).

The median ASRs of the PPL 3 *E. coli* isolates were susceptibility to seven antibiotics from five classes (ampicillin, amoxicillin-clavulanate, cefotaxime, sulfonamide, chloramphenicol, ciprofloxacin, and tetracycline) and SIE to streptomycin (Figure 3.6). The median ZOI for ampicillin was 18 mm (susceptible) and an interquartile range of 17-18 mm. The median ZOI for amoxicillin-clavulanate was 21 mm (susceptible) and an interquartile range of 21-22 mm. The median ZOI for cefotaxime was 28 mm (susceptible) and an interquartile range of 25-30 mm. The median ZOI for streptomycin was 13 mm (SIE) and an interquartile range of 13-15 mm. The median ZOI for sulfonamide was 20 mm (susceptible) and an interquartile range of 19-22 mm. The median ZOI for chloramphenicol was 25 mm (susceptible) and an interquartile range of 24-26 mm. The median ZOI for ciprofloxacin was 30 mm (susceptible) and an interquartile range of 29-32 mm. The median ZOI for tetracycline was 23 mm (susceptible) and an interquartile range of 21-23 mm (Figure 3.6).

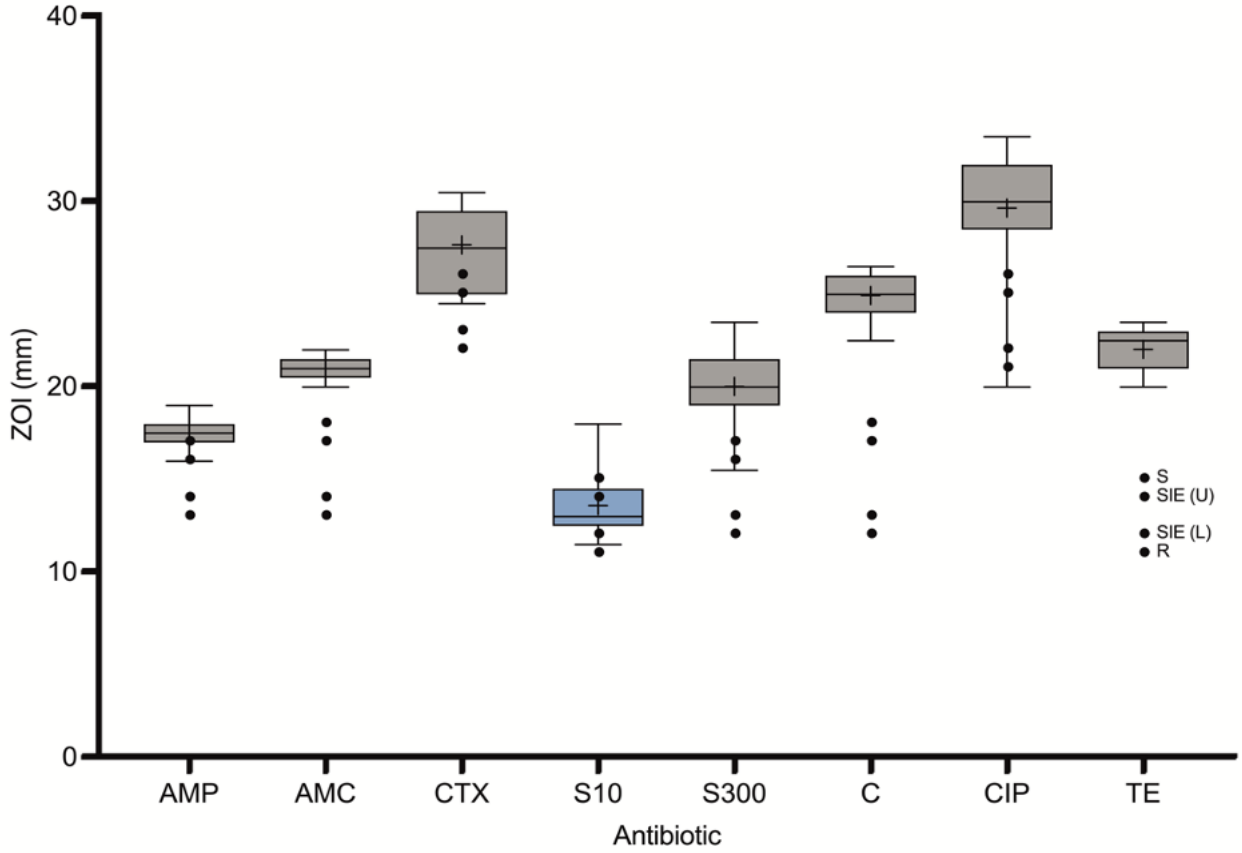


Figure 3.6: PPL 3 *E. coli* isolates median antibiotic susceptibility distibutions. The midline represents the median zone of inhibition (ZOI) across all isolates, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown as black dots: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility: grey for susceptible and blue for SIE. Abbreviations: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), TE (tetracycline).

PPL 4 received amoxicillin one to three days before sampling, Tetra-Delta (aminocoumarin, aminoglycoside, β-lactam, and anti-inflammatory) one to four days before sampling, and carprofen (anti-inflammatory) one day and nine days before sampling (Table 2.2). The *E. coli* isolates from PPL 4 had median ASRs of susceptibility to six antibiotics from six classes (cefotaxime, streptomycin, sulfonamide, chloramphenicol, ciprofloxacin, and tetracycline), SIE to amoxicillin-clavulanate, and resistance to ampicillin (Figure 3.7). The median ZOI for ampicillin was 6 mm (resistant) and an interquartile range of 6-7 mm. The median ZOI for amoxicillin-clavulanate was 15 mm (SIE) and an interquartile range of 13-17 mm. The median ZOI for cefotaxime was 30 mm (susceptible) and an interquartile range of 29-32 mm. The median ZOI for streptomycin was 15 mm (susceptible) and an interquartile range of 14-16 mm. The median ZOI for sulfonamide was 26 mm (susceptible) and an interquartile range of 24-27 mm. The median ZOI for chloramphenicol was 25 mm (susceptible) and an interquartile range of 24-26 mm. The median ZOI for ciprofloxacin was 32 mm (susceptible) and an interquartile range of 28-33 mm. The median ZOI for tetracycline was 21 mm (susceptible) and an interquartile range of 20-21 mm (Figure 3.7).

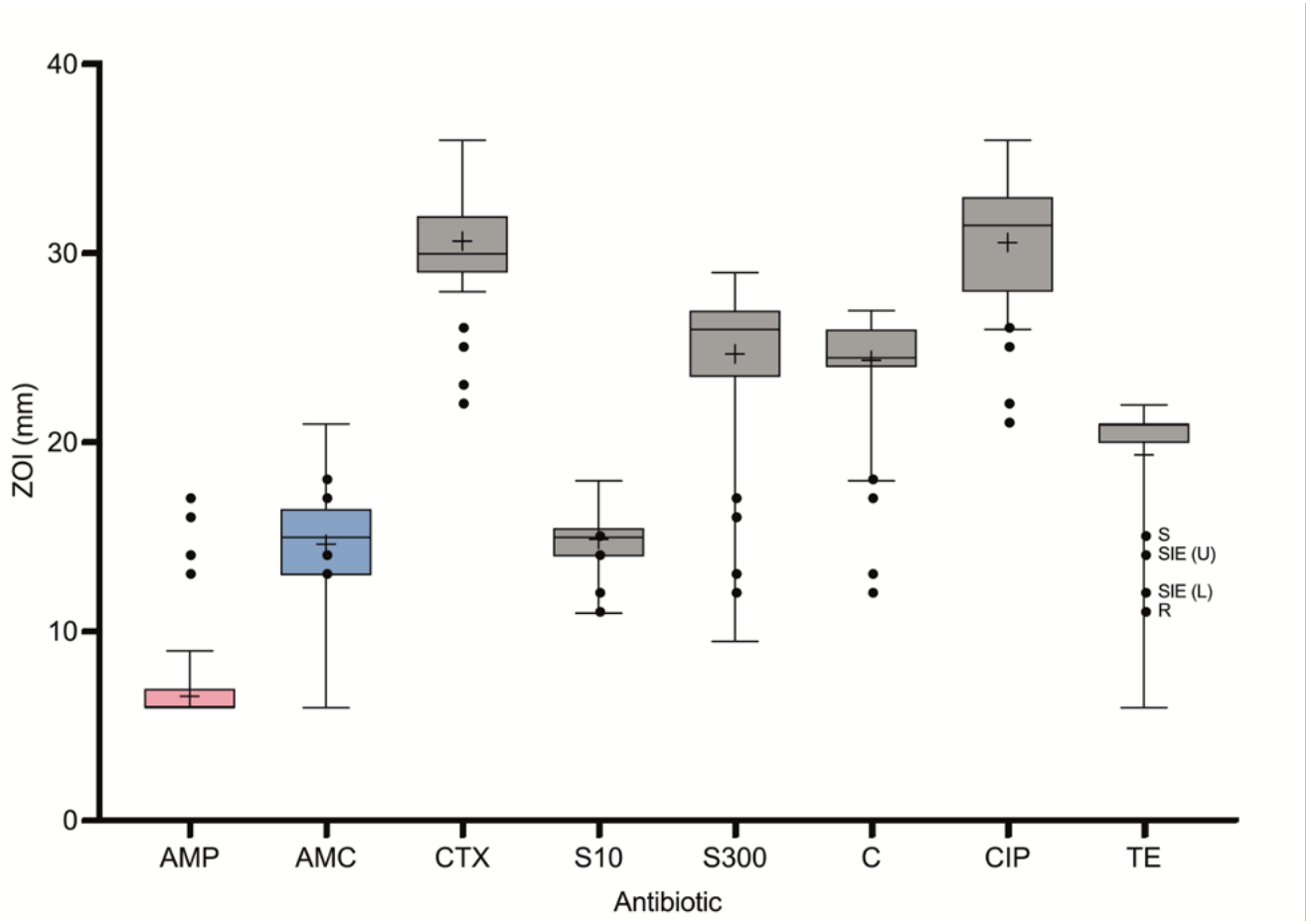


Figure 3.7: PPL 4 *E. coli* isolates median antibiotic susceptibility distibutions. The midline represents the median zone of inhibition (ZOI) across all isolates, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown as black dots: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility: grey for susceptible and blue for SIE. Abbreviations: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), TE (tetracycline).

PPL 5 received amoxicillin 13 to 15 days before sampling and tulathromycin (macrolide) 19 days before sampling (Table 2.2). The median ASRs of the PPL 5 *E. coli* isolates were susceptibility to six antibiotics from five classes (amoxicillin-clavulanate, cefotaxime, sulfonamide, chloramphenicol, ciprofloxacin, and tetracycline), SIE to streptomycin, and resistance to ampicillin (Figure 3.8). The median ZOI for ampicillin was 6 mm (resistant) with no interquartile range. The median ZOI for amoxicillin-clavulanate was 20 mm (susceptible) and an interquartile range of 20-21 mm. The median ZOI for cefotaxime was 29 mm (susceptible) and an interquartile range of 25-30 mm. The median ZOI for streptomycin was 14 mm (SIE) and an interquartile range of 12-15 mm. The median ZOI for sulfonamide was 25 mm (susceptible) and an interquartile range of 23-27 mm. The median ZOI for chloramphenicol was 23 mm (susceptible) and an interquartile range of 21-24 mm. The median ZOI for ciprofloxacin was 25 mm (susceptible) and an interquartile range of 24-28 mm. The median ZOI for tetracycline was 21 mm (susceptible) and an interquartile range of 20-21 mm (Figure 3.8).

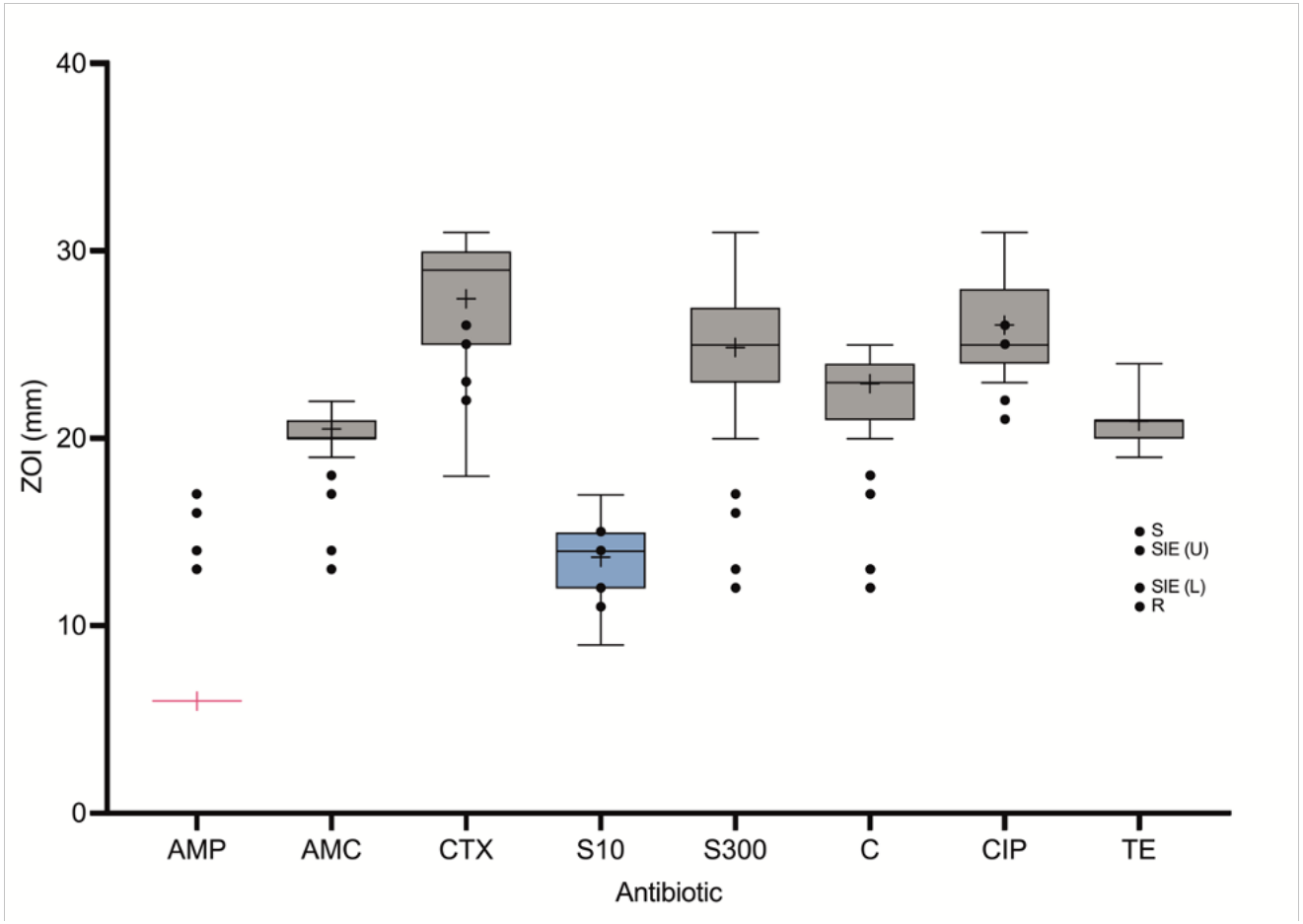


Figure 3.8: PPL 5 *E. coli* isolates median antibiotic susceptibility distibutions. The midline represents the median zone of inhibition (ZOI) across all isolates, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown as black dots: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility: pink for resistant, grey for susceptible, and blue for SIE. Abbreviations: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), TE (tetracycline).

The *E. coli* isolates from PPL 6 had median ASRs of susceptibility to seven antibiotics from five classes (ampicillin, amoxicillin-clavulanate, cefotaxime, sulfonamide, chloramphenicol, ciprofloxacin, and tetracycline) and SIE to streptomycin (Figure 3.9). The median ZOI for ampicillin was 19 mm (susceptible) and an interquartile range of 18-20 mm. The median ZOI for amoxicillin-clavulanate was 22 mm (susceptible) and an interquartile range of 21-22 mm. The median ZOI for cefotaxime was 30 mm (susceptible) and an interquartile range of 29-31 mm. The median ZOI for streptomycin was 14 mm (SIE) and an interquartile range of 13-15 mm. The median ZOI for sulfonamide was 22 mm (susceptible) and an interquartile range of 19-23 mm. The median ZOI for chloramphenicol was 26 mm (susceptible) and an interquartile range of 24-27 mm. The median ZOI for ciprofloxacin was 32 mm (susceptible) and an interquartile range of 29-35 mm. The median ZOI for tetracycline was 22 mm (susceptible) and an interquartile range of 21-23 mm (Figure 3.9).

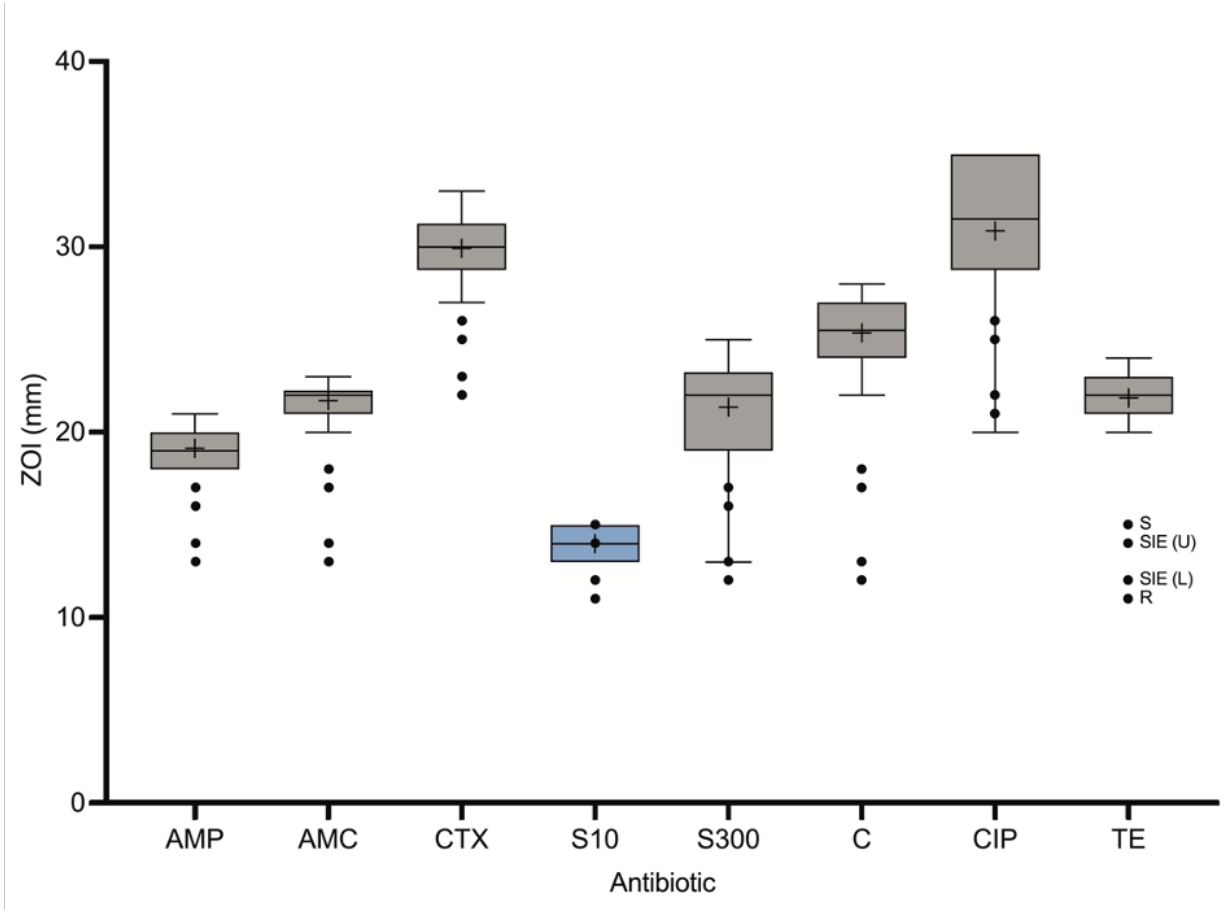


Figure 3.9: PPL 6 *E. coli* isolates median antibiotic susceptibility distibutions. The midline represents the median zone of inhibition (ZOI) across all isolates, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown as black dots: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility: grey for susceptible and blue for SIE. Abbreviations: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), TE (tetracycline).

PPL 7 received meloxicam (anti-inflammatory) 23 days before sampling, Tetra-Delta 23 to 28 days before sampling, and tetracycline 23 days before sampling (Table 2.2). The median ASRs of the PPL 7 *E. coli* isolates were susceptibility to six antibiotics from five classes (amoxicillin-clavulanate, cefotaxime, sulfonamide, chloramphenicol, ciprofloxacin, and tetracycline) and SIE to two antibiotics from two classes (streptomycin and ampicillin) (Figure 3.10). The median ZOI for ampicillin was 15 mm (SIE) and an interquartile range of 6-16 mm. The median ZOI for amoxicillin-clavulanate was 19 mm (susceptible) and an 18-20 mm interquartile range. The median ZOI for cefotaxime was 26 mm (susceptible) and an interquartile range of 25-29 mm. The median ZOI for streptomycin was 13 mm (SIE) and an interquartile range of 12-13 mm. The median ZOI for sulfonamide was 17 mm (susceptible) and an interquartile range of 12-20 mm. The median ZOI for chloramphenicol was 22 mm (susceptible) and an interquartile range of 21-25 mm. The median ZOI for ciprofloxacin was 30 mm (susceptible) and an interquartile range of 26-33 mm. The median ZOI for tetracycline was 20 mm (susceptible) and an interquartile range of 19-21 mm (Figure 3.10).

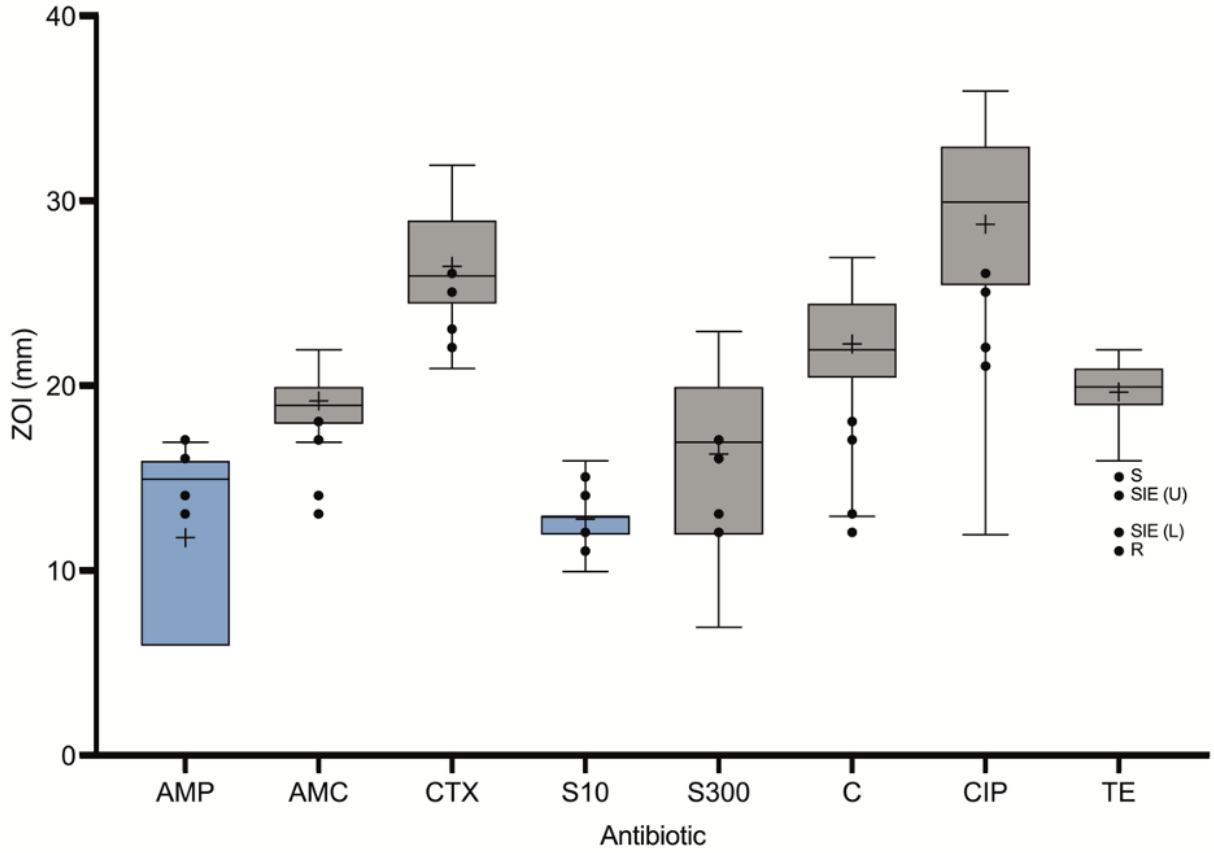


Figure 3.10: PPL 7 *E. coli* isolates median antibiotic susceptibility distibutions. The midline represents the median zone of inhibition (ZOI) across all isolates, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown as black dots: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility: grey for susceptible and blue for SIE. Abbreviations: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), TE (tetracycline).

The *E. coli* isolates from PPL 8 had median ASRs of susceptibility to five antibiotics from four classes (amoxicillin-clavulanate, cefotaxime, chloramphenicol, ciprofloxacin, and tetracycline), SIE to two antibiotics from two classes (streptomycin and sulfonamide), and resistance to ampicillin (Figure 3.11). The median ZOI for ampicillin was 6 mm (SIE) with no interquartile range covering. The median ZOI for amoxicillin-clavulanate was 19 mm (susceptible) and an interquartile range of 12-20 mm. The median ZOI for cefotaxime was 26 mm (susceptible) and an interquartile range of 25-29 mm. The median ZOI for streptomycin was 12 mm (SIE) and an interquartile range of 11-12 mm. The median ZOI for sulfonamide was 16 mm (susceptible) and an interquartile range of 14-19 mm. The median ZOI for chloramphenicol was 22 mm (susceptible) and an interquartile range of 20-25 mm. The median ZOI for ciprofloxacin was 32 mm (susceptible) and an interquartile range of 28-34 mm. The median ZOI for tetracycline was 20 mm (susceptible) and an interquartile range of 19-20 mm (Figure 3.11).

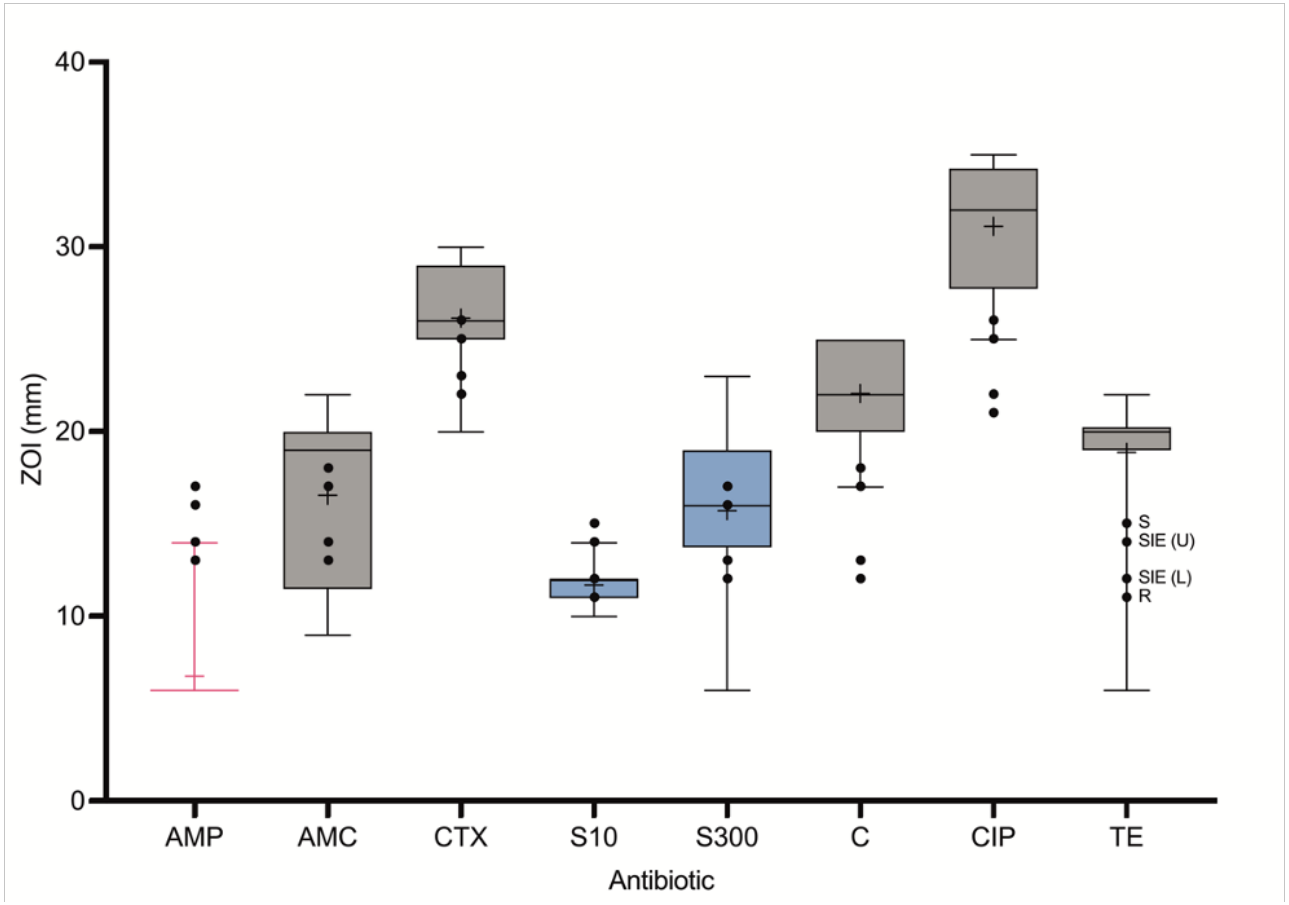


Figure 3.11: PPL 8 *E. coli* isolates median antibiotic susceptibility distibutions. The midline represents the median zone of inhibition (ZOI) across all isolates, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown as black dots: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility: pink for resistant, grey for susceptible, and blue for SIE. Abbreviations: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), TE (tetracycline).

The median ASRs of the PPL 9 *E. coli* isolates were susceptibility to four antibiotics from four classes (cefotaxime, chloramphenicol, ciprofloxacin, and tetracycline), SIE to two antibiotics from two classes (streptomycin and sulfonamide), and resistance to two antibiotics from one class (ampicillin and amoxicillin-clavulanate) (Figure 3.12). The median ZOI for ampicillin was 6 mm (resistant) and an interquartile range of 6-15 mm. The median ZOI for amoxicillin-clavulanate was 11 mm (resistant) and an interquartile range of 9-20 mm. The median ZOI for cefotaxime was 25 mm (susceptible) and an interquartile range of 25-27 mm. The median ZOI for streptomycin was 12 mm (SIE) and an interquartile range of 11-13 mm. The median ZOI for sulfonamide was 15 mm (SIE) and an interquartile range of 14-17 mm. The median ZOI for chloramphenicol was 22 mm (susceptible) and an interquartile range of 21-24 mm. The median ZOI for ciprofloxacin was 32 mm (susceptible) and an interquartile range of 30-33 mm. The median ZOI for tetracycline was 20 mm (susceptible) and an interquartile range of 19-21 mm (Figure 3.12).

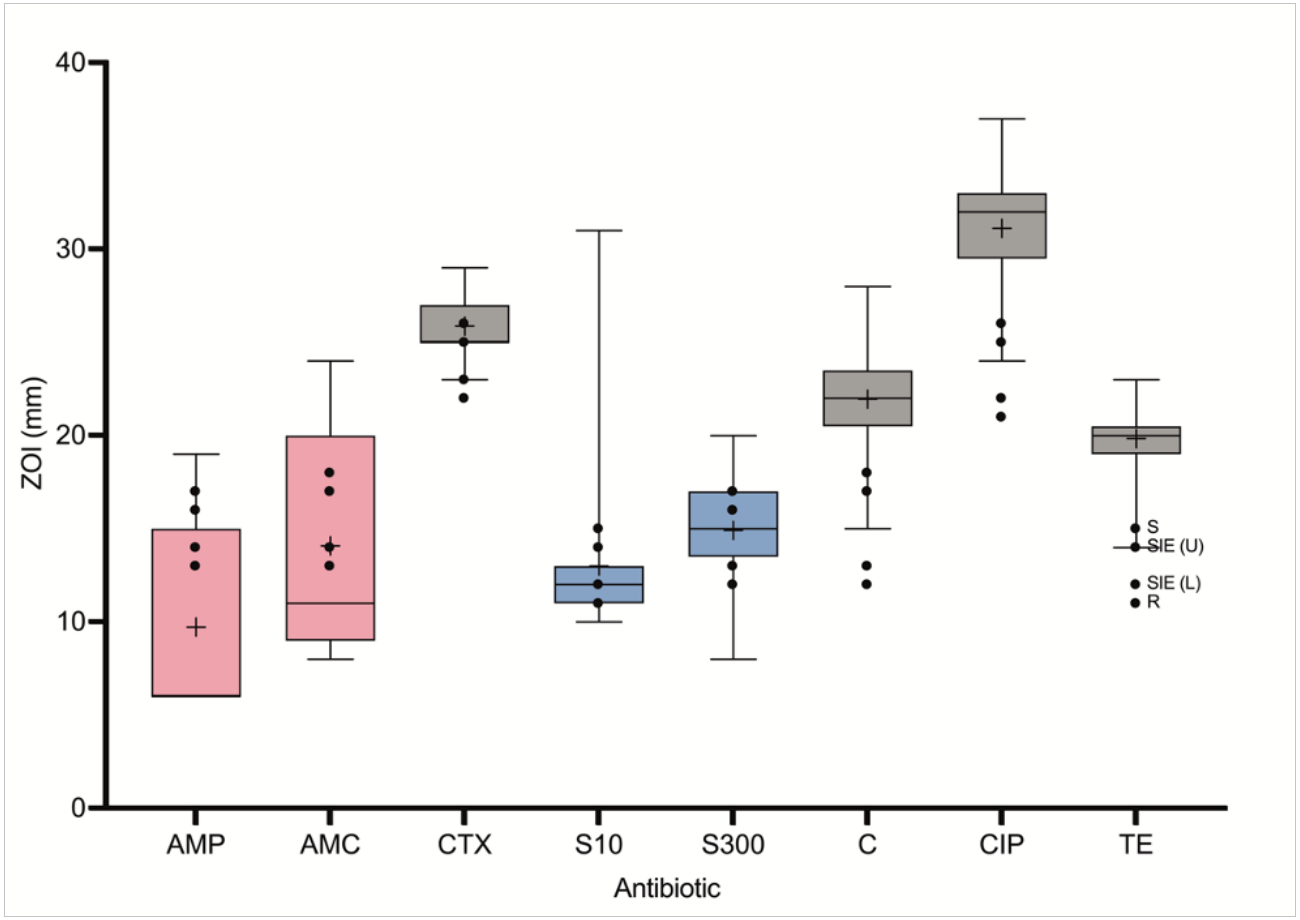


Figure 3.12: PPL 9 *E. coli* isolates median antibiotic susceptibility distibutions. The midline represents the median zone of inhibition (ZOI) across all isolates, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown as black dots: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility: pink for resistant, grey for susceptible, and blue for SIE. Abbreviations: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), TE (tetracycline).

The *E. coli* isolates from PPL 10 had median ASRs of susceptibility to seven antibiotics from five classes (ampicillin, amoxicillin-clavulanate, cefotaxime, sulfonamide, chloramphenicol, ciprofloxacin, and tetracycline) and SIE to streptomycin (Figure 3.13). The median ZOI for ampicillin was 17 mm (susceptible) with no interquartile range. The median ZOI for amoxicillin-clavulanate was 21 mm (susceptible) and an interquartile range of 20-21 mm. The median ZOI for cefotaxime was 28 mm (susceptible) and an interquartile range of 27-29 mm. The median ZOI for streptomycin was 14 mm (SIE) and an interquartile range of 13-14 mm. The median ZOI for sulfonamide was 22 mm (SIE) and an interquartile range of 20-23 mm. The median ZOI for chloramphenicol was 23 mm (susceptible) and an interquartile range of 22-24 mm. The median ZOI for ciprofloxacin was 32 mm (susceptible) and an interquartile range of 32-34 mm. The median ZOI for tetracycline was 20 mm (susceptible) and an interquartile range of 20-21 mm (Figure 3.13).

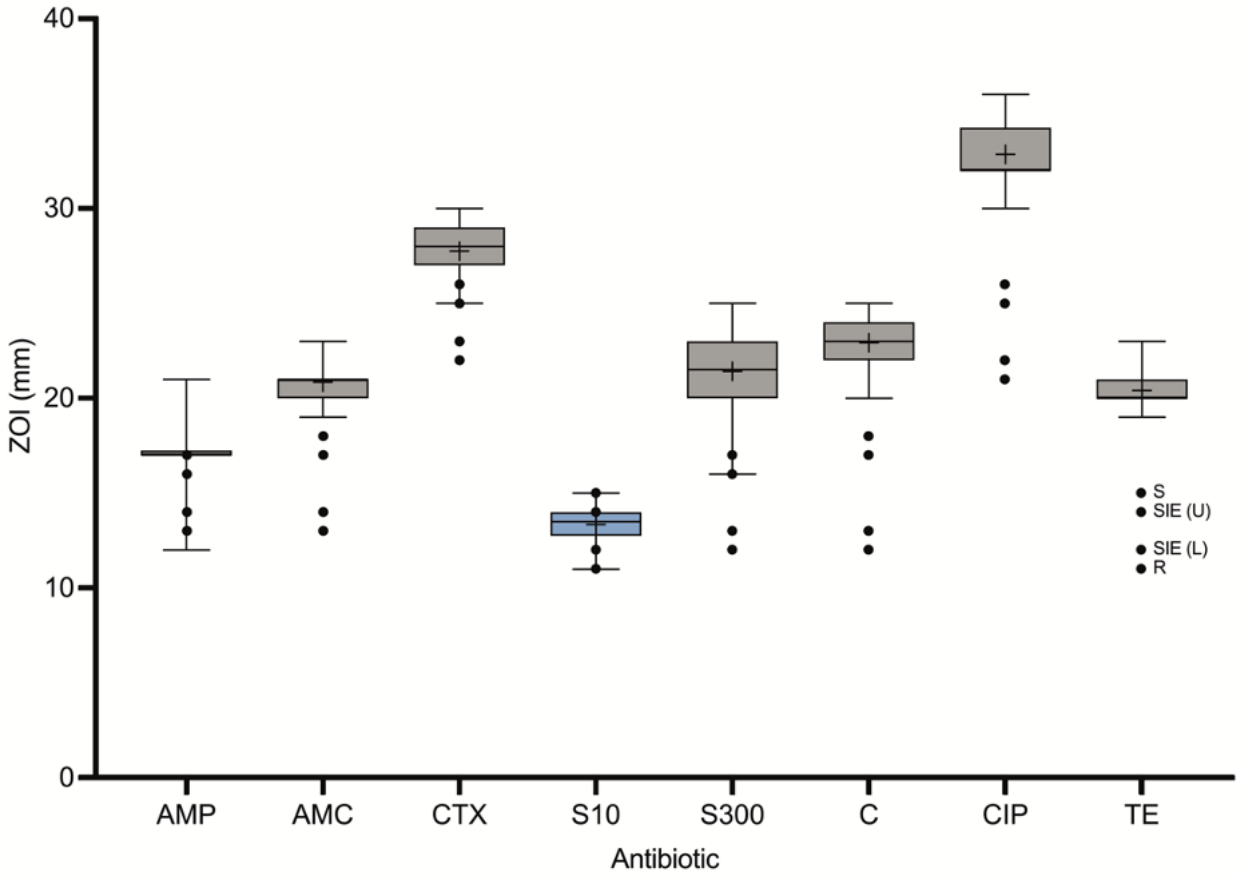


Figure 3.13: PPL 10 *E. coli* isolates median antibiotic susceptibility distibutions. The midline represents the median zone of inhibition (ZOI) across all isolates, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown as black dots: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility: grey for susceptible and blue for SIE. Abbreviations: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), TE (tetracycline).

The median ASRs of the PPL 11 *E. coli* isolates were susceptibility to six antibiotics from five classes (amoxicillin-clavulanate, cefotaxime, sulfonamide, chloramphenicol, ciprofloxacin, and tetracycline) and SIE to two antibiotics from two classes (ampicillin and streptomycin) (Figure 3.14). The median ZOI for ampicillin was 16 mm (SIE) and an interquartile range of 15-18 mm. The median ZOI for amoxicillin-clavulanate was 20 mm (susceptible) and an interquartile range of 20-22 mm. The median ZOI for cefotaxime was 29 mm (susceptible) and an interquartile range of 26-30 mm. The median ZOI for streptomycin was 13 mm (SIE) and an interquartile range of 12-15 mm. The median ZOI for sulfonamide was 20 mm (susceptible) and an interquartile range of 16-24 mm. The median ZOI for chloramphenicol was 22 mm (susceptible) and an interquartile range of 20-24 mm. The median ZOI for ciprofloxacin was 31 mm (susceptible) and an interquartile range of 27-34 mm. The median ZOI for tetracycline was 20 mm (susceptible) and an interquartile range of 20-22 mm (Figure 3.14).

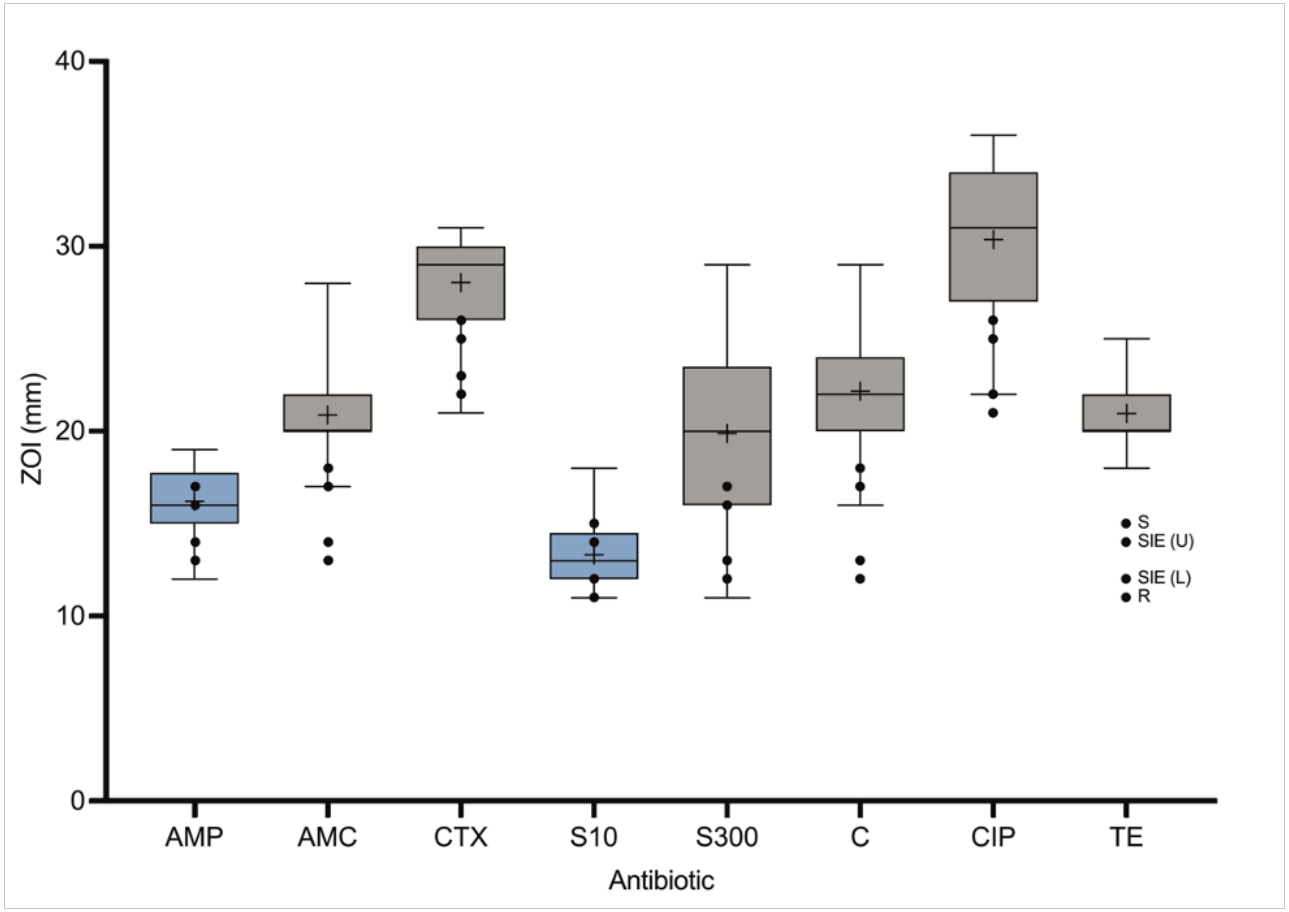


Figure 3.14: PPL 11 *E. coli* isolates median antibiotic susceptibility distibutions. The midline represents the median zone of inhibition (ZOI) across all isolates, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown as black dots: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility: grey for susceptible and blue for SIE. Abbreviations: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), TE (tetracycline).

The *E. coli* isolates from PPL 12 had median ASRs of susceptibility to all eight antibiotics from six classes (Figure 3.15). The median ZOI for ampicillin was 17 mm (susceptible) and an interquartile range of 16-19 mm. The median ZOI for amoxicillin-clavulanate was 22 mm (susceptible) and an interquartile range of 20-23 mm. The median ZOI for cefotaxime was 27 mm (susceptible) and an interquartile range of 25-30 mm. The median ZOI for streptomycin was 15 mm (susceptible) and an interquartile range of 10-15 mm. The median ZOI for sulfonamide was 22 mm (susceptible) and an interquartile range of 7-25 mm. The median ZOI for chloramphenicol was 25 mm (susceptible) and an interquartile range of 21-26 mm. The median ZOI for ciprofloxacin was 29 mm (susceptible) and an interquartile range of 25-32 mm. The median ZOI for tetracycline was 22 mm (susceptible) and an interquartile range of 21-23 mm (Figure 3.15).

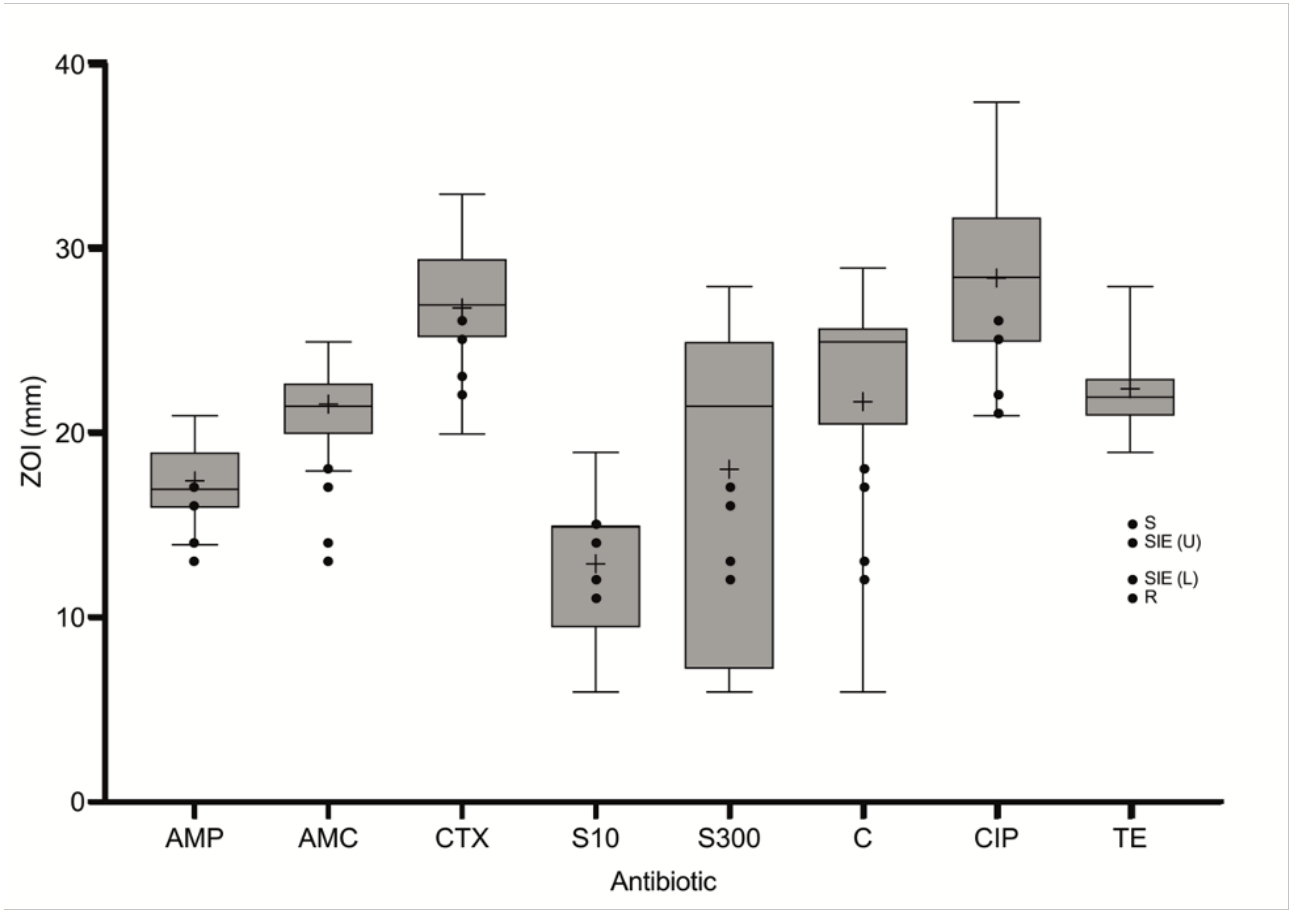


Figure 3.15: PPL 12 *E. coli* isolates median antibiotic susceptibility distibutions. The midline represents the median zone of inhibition (ZOI) across all isolates, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown as black dots: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The median antibiotic susceptibility result for all antibiotics is susceptible. Abbreviations: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), TE (tetracycline).

PPL 13 received meloxicam six days before sampling, amoxicillin six to eight days before sampling, and carprofen six days before sampling (Table 2.2). The median ASRs of the PPL 13 *E. coli* isolates were susceptibility to seven antibiotics from six classes (ampicillin, amoxicillin-clavulanate, cefotaxime, sulfonamide, chloramphenicol, ciprofloxacin, and tetracycline) and SIE to streptomycin (Figure 3.16). The median ZOI for ampicillin was 17 mm (susceptible) and an interquartile range of 16-18 mm. The median ZOI for amoxicillin-clavulanate was 20 mm (susceptible) and an interquartile range of 20-21 mm. The median ZOI for cefotaxime was 29 mm (susceptible) and an interquartile range of 27-34 mm. The median ZOI for streptomycin was 14 mm (SIE) and an interquartile range of 13-15 mm. The median ZOI for sulfonamide was 21 mm (susceptible) and an interquartile range of 18-23 mm. The median ZOI for chloramphenicol was 23 mm (susceptible) and an interquartile range of 23-25 mm. The median ZOI for ciprofloxacin was 32 mm (susceptible) and an interquartile range of 27-34 mm. The median ZOI for tetracycline was 21 mm (susceptible) and an interquartile range of 20-22 mm (Figure 3.16).

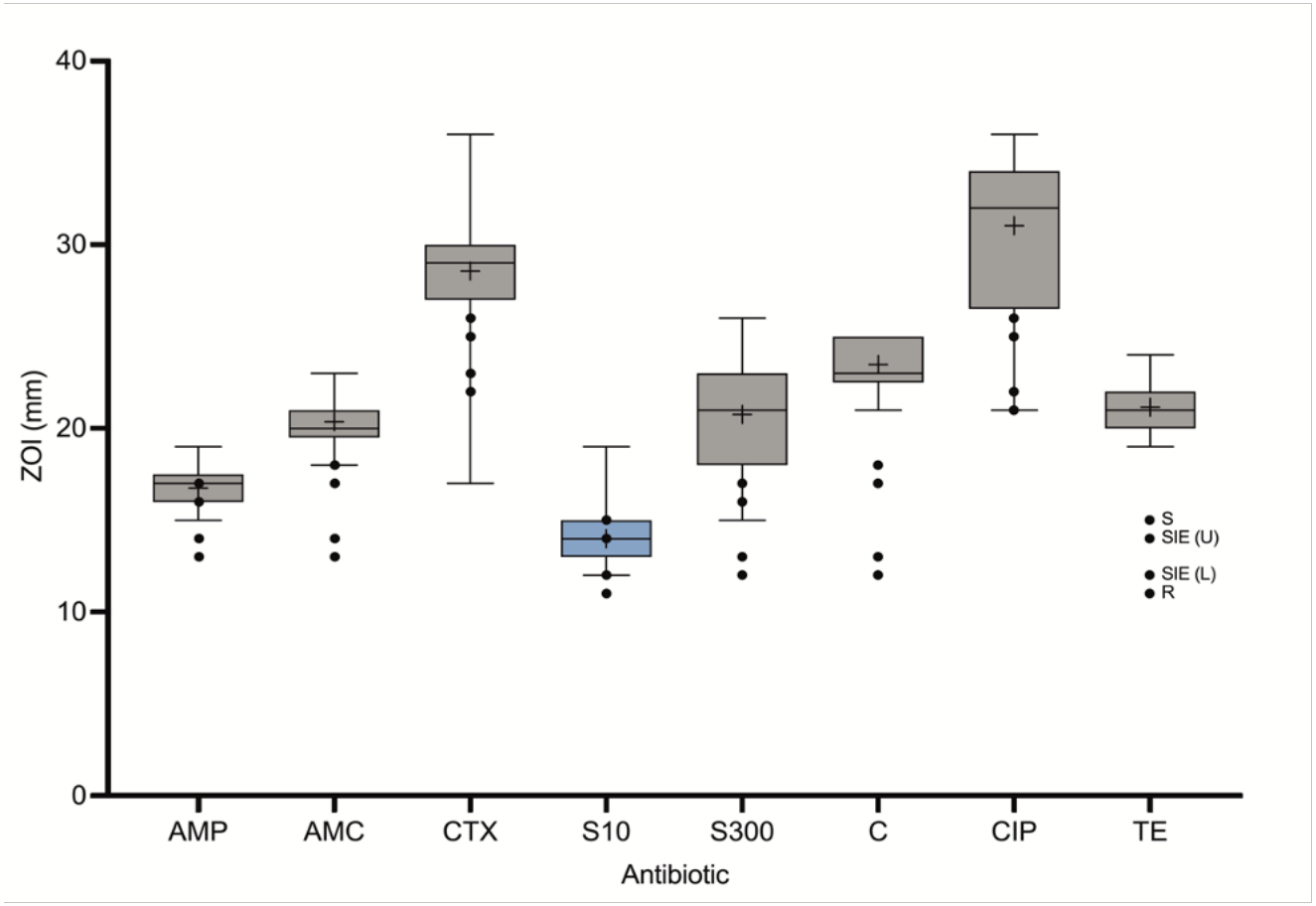


Figure 3.16: PPL 13 *E. coli* isolates median antibiotic susceptibility distibutions. The midline represents the median zone of inhibition (ZOI) across all isolates, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown as black dots: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility: grey for susceptible and blue for SIE. Abbreviations: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), TE (tetracycline).

PPL 14 received rotavec corona (vaccine) and bismuth subnitrate (teat sealant) 22 days before sampling (Table 2.2). The *E. coli* isolates from PPL 14 had median ASRs of susceptibility to seven antibiotics from six classes (ampicillin, amoxicillin-clavulanate, cefotaxime, sulfonamide, chloramphenicol, ciprofloxacin, and tetracycline) and SIE to streptomycin (Figure 3.17). The median ZOI for ampicillin was 17 mm (susceptible) and an interquartile range of 16-19 mm. The median ZOI for amoxicillin-clavulanate was 22 mm (susceptible) and an interquartile range of 21-24 mm. The median ZOI for cefotaxime was 27 mm (susceptible) and an interquartile range of 26-28 mm. The median ZOI for streptomycin was 12 mm (SIE) and an interquartile range of 12-13 mm. The median ZOI for sulfonamide was 19 mm (susceptible) and an interquartile range of 16-22 mm. The median ZOI for chloramphenicol was 24 mm (susceptible) and an interquartile range of 23-25 mm. The median ZOI for ciprofloxacin was 34 mm (susceptible) and an interquartile range of 33-36 mm. The median ZOI for tetracycline was 21 mm (susceptible) and an interquartile range of 20-22 mm (Figure 3.17).

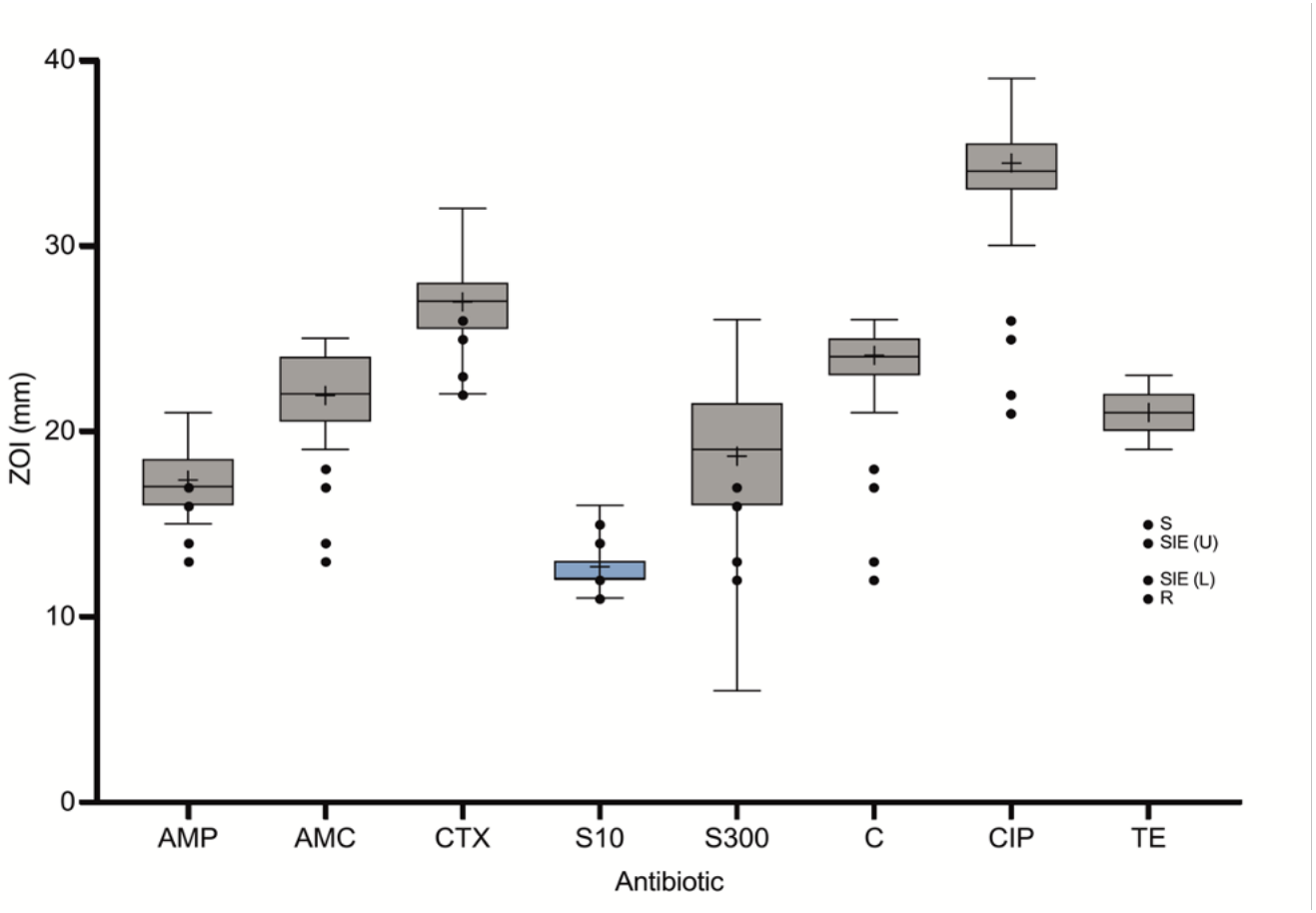


Figure 3.17: PPL 14 *E. coli* isolates median antibiotic susceptibility distibutions. The midline represents the median zone of inhibition (ZOI) across all isolates, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown as black dots: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility: grey for susceptible and blue for SIE. Abbreviations: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), TE (tetracycline).

The median ASRs of the PPL 15 *E. coli* isolates were susceptibility to five antibiotics from four classes (amoxicillin-clavulanate, cefotaxime, chloramphenicol, ciprofloxacin, and tetracycline), SIE to three antibiotics from three classes (ampicillin, streptomycin and sulfonamide) (Figure 3.18). The median ZOI for ampicillin was 16 mm (SIE) and an interquartile range of 15-18 mm. The median ZOI for amoxicillin-clavulanate was 20 mm (susceptible) and an interquartile range of 20-21 mm. The median ZOI for cefotaxime was 26 mm (susceptible) and an interquartile range of 25-27 mm. The median ZOI for streptomycin was 13 mm (SIE) and an interquartile range of 12-14 mm. The median ZOI for sulfonamide was 16 mm (SIE) and an interquartile range of 15-20 mm. The median ZOI for chloramphenicol was 24 mm (susceptible) and an interquartile range of 23-25 mm. The median ZOI for ciprofloxacin was 34 mm (susceptible) and an interquartile range of 31-35 mm. The median ZOI for tetracycline was 21 mm (susceptible) and an interquartile range of 20-22 mm (Figure 3.18).

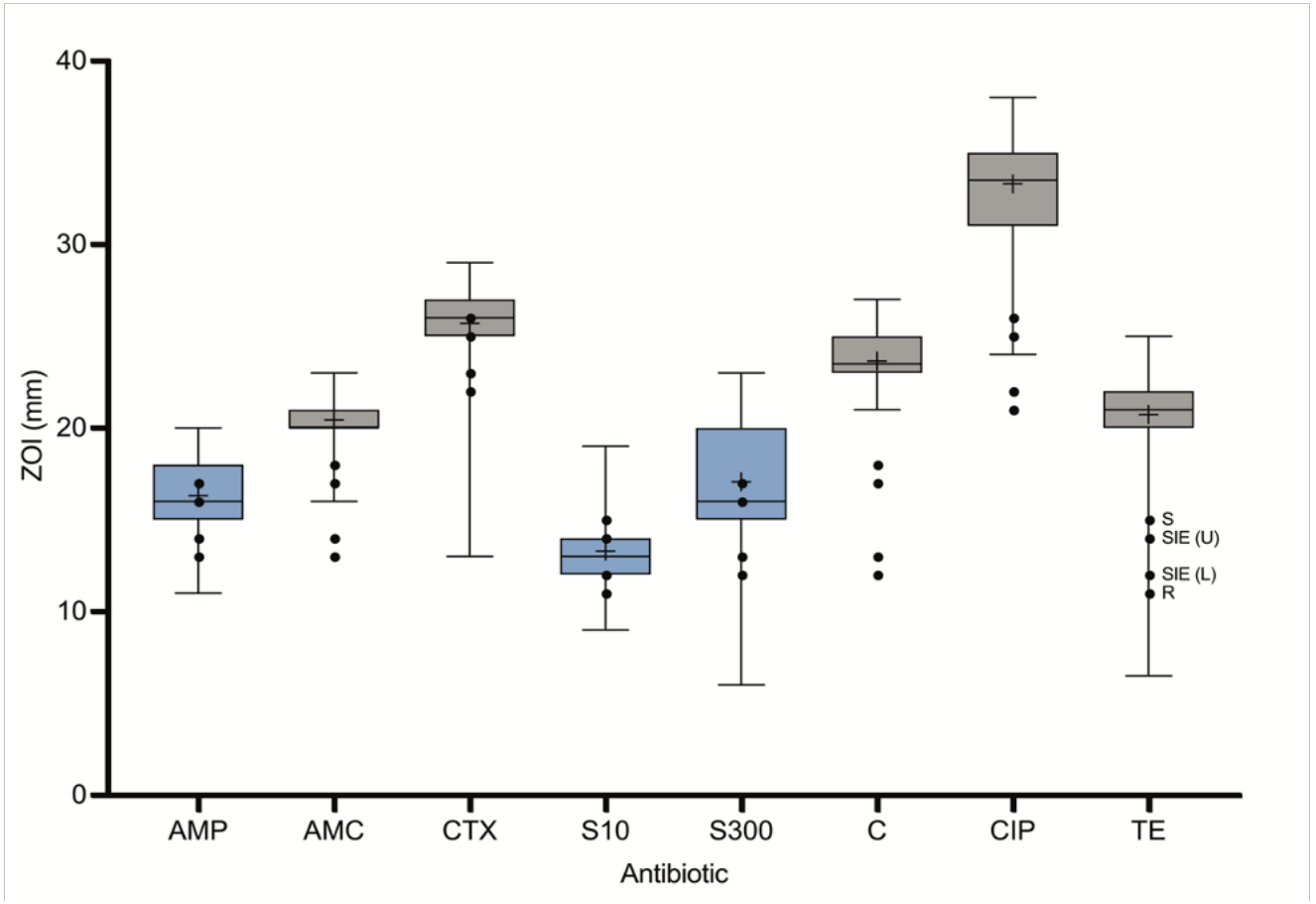


Figure 3.18: PPL 15 *E. coli* isolates median antibiotic susceptibility distibutions. The midline represents the median zone of inhibition (ZOI) across all isolates, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown as black dots: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility: grey for susceptible and blue for SIE. Abbreviations: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), TE (tetracycline).

The *E. coli* isolates from PPL 16 had median ASRs of susceptibility to seven antibiotics from six classes (ampicillin, amoxicillin-clavulanate, cefotaxime, sulfonamide, chloramphenicol, ciprofloxacin, and tetracycline) and SIE to streptomycin (Figure 3.19). The median ZOI for ampicillin was 17 mm (susceptible) and an interquartile range of 16-19 mm. The median ZOI for amoxicillin-clavulanate was 21 mm (susceptible) and an interquartile range of 20-23 mm. The median ZOI for cefotaxime was 26 mm (susceptible) and an interquartile range of 25-29 mm. The median ZOI for streptomycin was 14 mm (SIE) and an interquartile range of 13-15 mm. The median ZOI for sulfonamide was 18 mm (susceptible) and an interquartile range of 16-20 mm. The median ZOI for chloramphenicol was 25 mm (susceptible) and an interquartile range of 24-25 mm. The median ZOI for ciprofloxacin was 30 mm (susceptible) and an interquartile range of 26-33 mm. The median ZOI for tetracycline was 21 mm (susceptible) and an interquartile range of 20-23 mm (Figure 3.19).

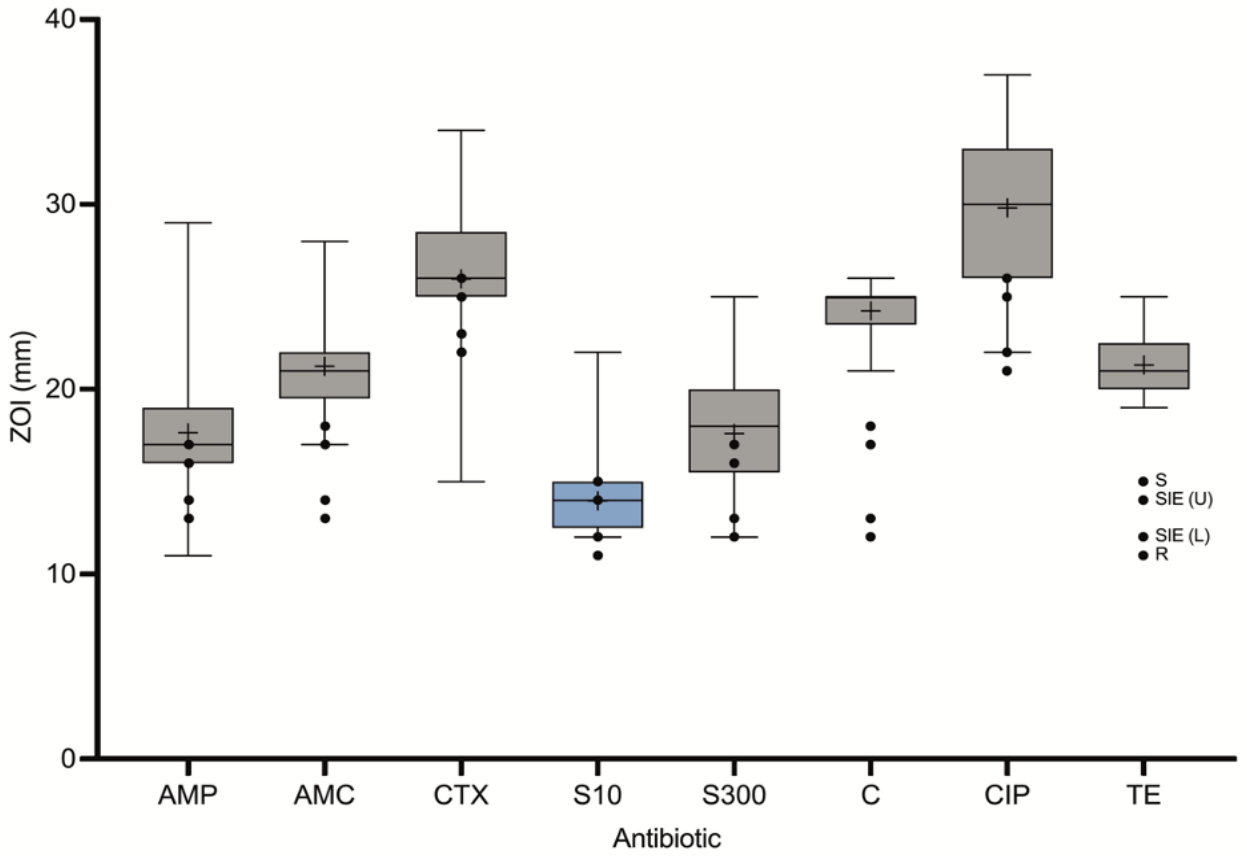


Figure 3.19: PPL 16 *E. coli* isolates median antibiotic susceptibility distibutions. The midline represents the median zone of inhibition (ZOI) across all isolates, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown as black dots: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility: grey for susceptible and blue for SIE. Abbreviations: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), TE (tetracycline).

The median ASRs of the PPL 17 *E. coli* isolates were susceptibility to four antibiotics from four classes (amoxicillin-clavulanate, chloramphenicol, ciprofloxacin, and tetracycline) and SIE to three antibiotics from two classes (ampicillin, cefotaxime, streptomycin, and sulfonamide) (Figure 3.20). The median ZOI for ampicillin was 16 mm (SIE) and an interquartile range of 16-19 mm. The median ZOI for amoxicillin-clavulanate was 20 mm (susceptible) and an interquartile range of 19-20 mm. The median ZOI for cefotaxime was 25 mm (SIE) and an interquartile range of 25-27 mm. The median ZOI for streptomycin was 12 mm (SIE) and an interquartile range of 12-14 mm. The median ZOI for sulfonamide was 16 mm (SIE) and an interquartile range of 14-18 mm. The median ZOI for chloramphenicol was 24 mm (susceptible) and an interquartile range of 23-25 mm. The median ZOI for ciprofloxacin was 27 mm (susceptible) and an interquartile range of 26-29 mm. The median ZOI for tetracycline was 20 mm (susceptible) and an interquartile range of 20-22 mm (Figure 3.20).

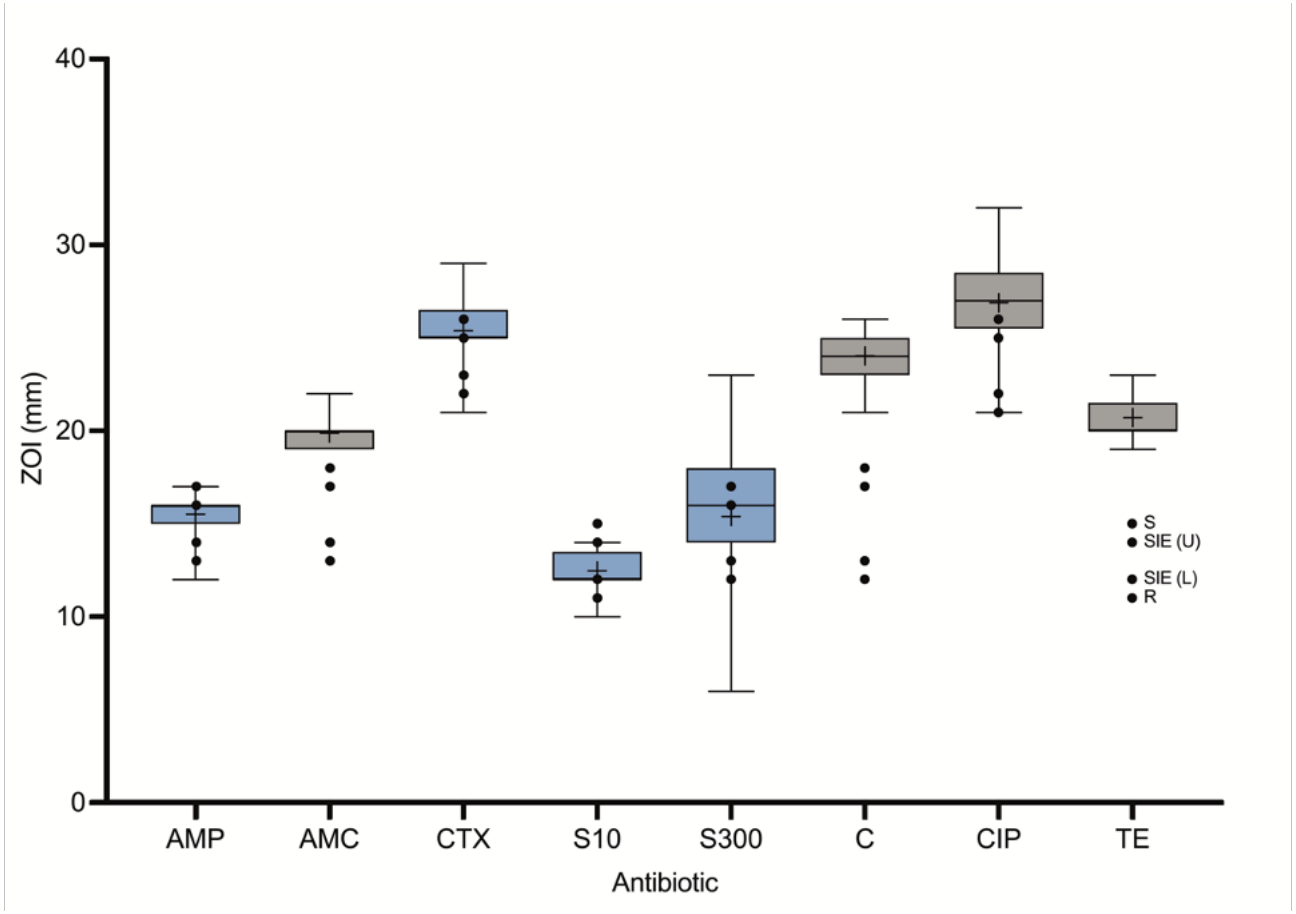


Figure 3.20: PPL 17 *E. coli* isolates median antibiotic susceptibility distibutions. The midline represents the median zone of inhibition (ZOI) across all isolates, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown as black dots: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility: grey for susceptible and blue for SIE. Abbreviations: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), TE (tetracycline).

The *E. coli* isolates from PPL 18 had median ASRs of susceptibility to four antibiotics from four classes (amoxicillin-clavulanate, chloramphenicol, ciprofloxacin, and tetracycline), SIE to three antibiotics from three classes (ampicillin, streptomycin, and sulfonamide), and resistance to cefotaxime (Figure 3.21). The median ZOI for ampicillin was 16 mm (SIE) and an interquartile range of 15-18 mm. The median ZOI for amoxicillin-clavulanate was 30 mm (susceptible) and an interquartile range of 28-32 mm. The median ZOI for cefotaxime was 20 mm (resistant) and an interquartile range of 19-20 mm. The median ZOI for streptomycin was 12 mm (SIE) and an interquartile range of 11-13 mm. The median ZOI for sulfonamide was 16 mm (SIE) and an interquartile range of 11-29 mm. The median ZOI for chloramphenicol was 24 mm (susceptible) and an interquartile range of 23-25 mm. The median ZOI for ciprofloxacin was 26 mm (susceptible) and an interquartile range of 24-28 mm. The median ZOI for tetracycline was 20 mm (susceptible) and an interquartile range of 20-22 mm (Figure 3.21).

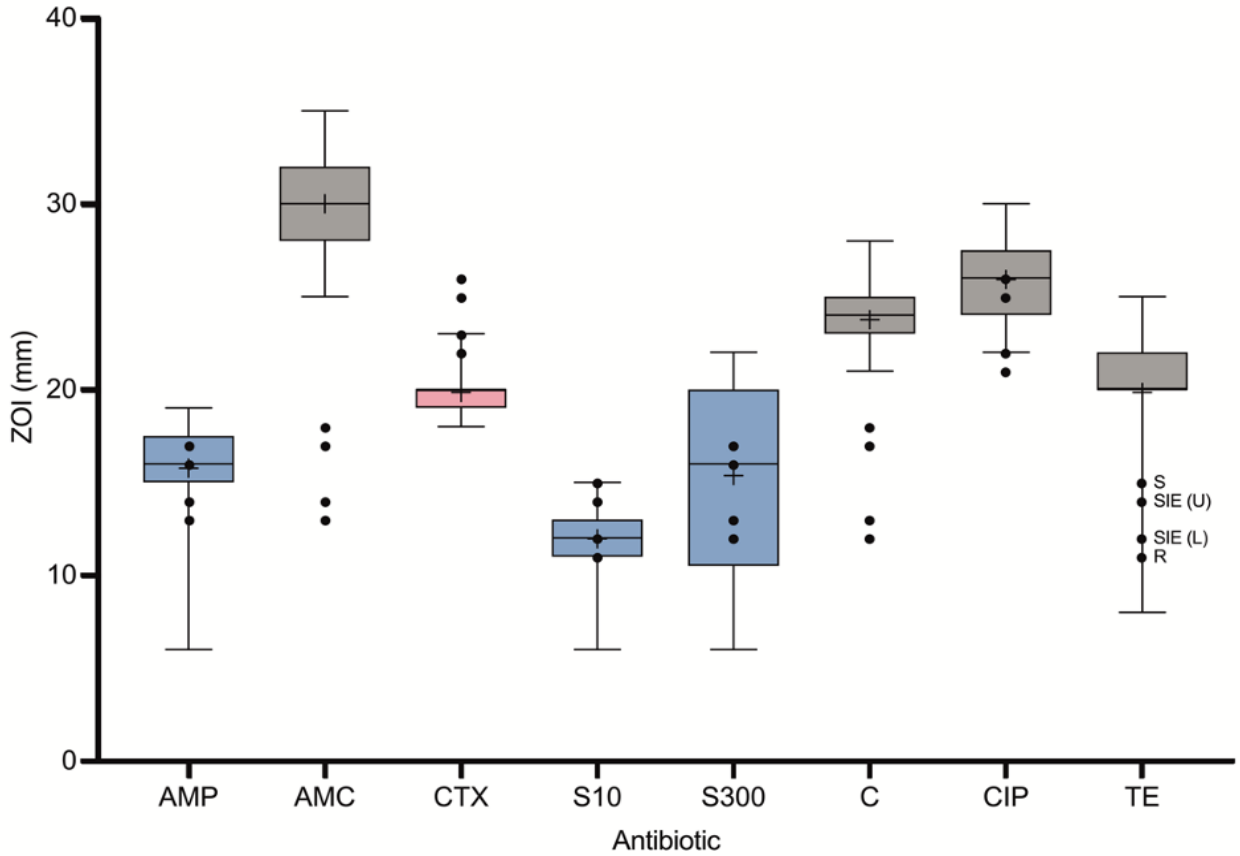


Figure 3.21: PPL 18 *E. coli* isolates median antibiotic susceptibility distibutions. The midline represents the median zone of inhibition (ZOI) across all isolates, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown as black dots: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility: pink for resistant, grey for susceptible, and blue for SIE. Abbreviations: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), TE (tetracycline).

3.3.6 Impact of media composition on *E. coli* isolation and susceptibility

The intent of antibiotic supplementation was to investigate the impact of additional selective pressures in the isolation media. The inclusion of antibiotics increased the prevalence of non-susceptible isolates but also significantly reduced the number of the isolates that were able to successfully grow *in vitro*. The faecal samples were plated on TBX and on TBX supplemented with several antibiotics (Table 3.3). Plating the faeces from PPL 1 on TBX enumerated 2.4 $x10^9$ CFU $g^{-1}$of *E. coli*, with 3.5 $x10^9$ CFU g^{-1} (145.8%) enumerated when penicillin was included in the media and 2 $x10^9$ CFU g^{-1} (83.3%) enumerated when spectinomycin was included in the media. Plating the faeces from PPL 2 on TBX enumerated 1.77 $x10^5$ CFU $g^{-1}$of *E. coli*, including penicillin in the media enumerated 2.03 $x10^5$ CFU g^{-1} (114.7%) and including spectinomycin enumerated 1 $x10^4$ (5.6%). Plating the faeces from PPL 3 on TBX enumerated 1.47 $x10^7$ CFU g^{-1} of *E. coli*, with 6.63 $x10^5$ CFU g^{-1} (4.5%) enumerated when penicillin was included and no growth when spectinomycin was included in the media. Plating the faeces from PPL 4 on TBX enumerated 4.33 $x10^6$ CFU g^{-1} of *E. coli*, including penicillin in the media enumerated 2.33 $x10^6$ CFU g^{-1} (52.6%) and including spectinomycin resulted in no growth. Plating the faeces from PPL 5 on TBX enumerated 5.33 $x10^6$ CFU $g^{-1}$of *E. coli*, with 1.17 $x10^7$ CFU g^{-1} (219.5%) enumerated when penicillin was included in the media and no growth when spectinomycin was included in the media. Plating the faeces from PPL 6 on TBX enumerated 1.27 $x10^7$ CFU $g^{-1}$of *E. coli*, including penicillin in the media enumerated 1 $x10^6$ CFU g^{-1} (7.9%) and no growth with streptomycin was included in the media. Plating the faeces from PPL 7 on TBX enumerated 9.8 $x10^7$ CFU $g^{-1}$of *E. coli*, with 2 $x10^5$

CFU g^{-1} (0.2%) enumerated when tetracycline (30 $\mu g.ml^{-1}$) was included in the media. Plating the faeces from PPL 8 on TBX enumerated 1.49 $x10^7$ CFU g^{-1} of *E. coli*, with 2 $x10^5$ CFU g^{-1} (2%) enumerated when tetracycline (30 $\mu g.ml^{-1}$) was included in the media. Plating the faeces from PPL 9 on TBX enumerated 3.5 $x10^8$ CFU g^{-1} of *E. coli* and including tetracycline (30 $\mu g.ml^{-1}$) in the media resulted in no growth. Plating the faeces from PPL 10 on TBX enumerated 5 $x10^9$ CFU g^{-1} of *E. coli* and including tetracycline (30 $\mu g.ml^{-1}$) in the media resulted in no growth. Plating the faeces from PPL 11 on TBX enumerated 4.8 $x10^8$ CFU $g^{-1}$of *E. coli*, including tetracycline (6.25 $\mu g.ml^{-1}$) or cefotaxime (30 $\mu g.ml^{-1}$) in the media resulted in no growth. Plating the faeces from PPL 12 on TBX enumerated 3.42 $x10^7$ CFU $g^{-1}$of *E. coli* and including tetracycline (6.25 $\mu g.ml^{-1}$) or cefotaxime (30 $\mu g.ml^{-1}$) in the media resulted in no growth. Plating the faeces from PPL 13 on TBX enumerated 5.4 $x10^7$ CFU g^{-1} of *E. coli* and including tetracycline (6.25 $\mu g.ml^{-1}$) or cefotaxime (30 $\mu g.ml^{-1}$) in the media resulted in no growth. Plating the faeces from PPL 14 on TBX enumerated 4 $x10^7$ CFU g^{-1} of *E. coli*, including tetracycline (6.25 $\mu g.ml^{-1}$) enumerated 2 $x10^5$ CFU g^{-1} (0.71%) and including cefotaxime (30 $\mu g.ml^{-1}$) resulted in no growth. Plating the faeces from PPL 15 on TBX enumerated 5.7 $x10^8$ CFU g^{-1} of *E. coli*, including tetracycline (6.25 $\mu g.ml^{-1}$) in the media enumerated 2 $x10^5$ CFU g^{-1} (0.08%) and including cefotaxime (30 $\mu g.ml^{-1}$) in the media resulted in no growth. Plating the faeces from PPL 16 on TBX enumerated 5.44 $x10^7$ CFU g^{-1} of *E. coli*, including tetracycline (6.25 $\mu g.ml^{-1}$) in the media enumerated 2 $x10^5$ CFU g^{-1} (0.26%) and including cefotaxime (30 $\mu g.ml^{-1}$) in the media resulted in no growth. Plating the faeces from PPL 17 on TBX enumerated 3.86 $x10^8$ CFU g^{-1} of *E. coli*, including tetracycline (6.25 $\mu g.ml^{-1}$)

in the media enumerated 4 x10^5 CFU g^{-1} (0.06%) and including cefotaxime (30 µg.ml^{-1}) in the media resulted in no growth. Plating the faeces from PPL 18 on TBX enumerated 1.9 x10^7 CFU g^{-1} of *E. coli* and including tetracycline (6.25 µg.ml^{-1}) or cefotaxime (30 µg.ml^{-1}) in the media resulted in no growth.

Table 3.3: Percentage growth of *E. coli* on TBX supplemented with different antibiotics compared to TBX.

Sample	TBX CFU g^{-1}	Percentage growth				
		PCN (30µg)	SPT (30µg)	TET (30µg)	TET (6.25µg)	CTX (30µg)
1	2.40E+09	145.8	83.3	NA	NA	NA
2	1.77E+05	114.7	5.6	NA	NA	NA
3	1.47E+07	4.5	0	NA	NA	NA
4	4.43E+06	52.6	0	NA	NA	NA
5	5.33E+06	219.5	0	NA	NA	NA
6	1.27E+07	7.9	0	NA	NA	NA
7	9.80E+07	NA	NA	0.20	NA	NA
8	1.00E+07	NA	NA	2	NA	NA
9	3.50E+08	NA	NA	0	NA	NA
10	5.00E+09	NA	NA	0	NA	NA
11	3.52E+08	NA	NA	NA	0	0
12	8.00E+06	NA	NA	NA	0	0
13	4.00E+06	NA	NA	NA	0	0
14	2.80E+07	NA	NA	NA	0.71	0
15	2.64E+08	NA	NA	NA	0.08	0
16	7.60E+07	NA	NA	NA	0.26	0
17	6.94E+08	NA	NA	NA	0.06	0
18	4.00E+07	NA	NA	NA	0	0

Grey samples are from PPL that received chemotherapeutic treatment within one month before sampling.
NA = samples were not plated on this media composition.

Samples from the untreated PPL formed 66% of the α samples, from which 64.8% of the isolates were collected, whereas treated PPL samples accounted for 33% of the α samples, and 35.2% of the isolates collected (Table 3.4). Of the α isolates, 76% were picked from TBX without antibiotic supplementation, followed by 16% from TBX with penicillin, 3% from TBX with spectinomycin, and 2% from TBX with tetracycline (Table 3.4). A greater portion of the isolates from the treated PPL samples, than from the untreated PPL samples, were picked on TBX with penicillin (23.5% v 11.2%) and TBX with spectinomycin (8.4% v 0.6%) and a greater portion of the isolates from the untreated PPL samples, than from the treated PPL samples, were picked on TBX with tetracycline (2.1% v 1.1%) (Table 3.4).

Table 3.4: Proportions of α isolates across both PPL treatment groups and all isolation media.

	All	%	Treated PPL	%	Untreated PPL	%
Samples	18	100	6	33	12	66
Isolates	508	100	179	35.2	329	64.8
TBX	403	79	120	67	283	86
TBC + PCN	79	16	42	23.5	37	11.2
TBX + SPT	17	3	15	8.4	2	0.6
TBX + TE	9	2	2	1.1	7	2.1

3.3.6.1 Antibiotic susceptibility levels of the α *E. coli* picked from TBX

The ASRs and ASLs of the 403 *E. coli* isolates from TBX are shown in Table 3.5, and the ASLs of these isolates are described in this section. A high level were resistant to ampicillin (27%); moderate levels were resistant to streptomycin (16.9%) and sulfonamide (14.4%); low levels were resistant to amoxicillin-clavulanate (7.7%), cefotaxime (5.2%), chloramphenicol (1.2%), ciprofloxacin (2%), and tetracycline (5.7%). A very high level were SIE to ampicillin (33.7%); a high level were SIE to cefotaxime (23.8%); a moderate level were SIE to ciprofloxacin (11.7%); low levels were SIE to amoxicillin-clavulanate (4.5%) and chloramphenicol (1.2%); and a very low level were SIE to tetracycline (0.2%). Extremely high levels were susceptible to amoxicillin-clavulanate (87.8%), cefotaxime (71%), chloramphenicol (97.5%), ciprofloxacin (86.4%), and tetracycline (94%); a very high level were susceptible to sulfonamide (64%); a high level were susceptible to ampicillin (39.2%); and a moderate level were susceptible to streptomycin (19.1%).

Table 3.5: Overall antibiotic susceptibility and susceptibility levels of the 403 *α E. coli* isolates picked from TBX.

	AMP	AMC	CTX	S10	S300	C	CIP	TE
Susceptible	158	354	286	77	258	393	348	379
SIE	136	18	96	258	87	5	47	1
Resistant	109	31	21	68	58	5	8	23
% Susceptible	39.2	87.8	71	19.1	64	97.5	86.4	94
Susceptible level	High	Extremely high	Extremely high	Moderate	Very high	Extremely high	Extremely high	Extremely high
% SIE	33.7	4.5	23.8	64	21.6	1.2	11.7	0.2
SIE Level	High	Low	High	Very high	High	Low	Moderate	Very low
% Resistant	27	7.7	5.2	16.9	14.4	1.2	2	5.7
Resistance level	High	Low	Low	Moderate	Moderate	Low	Low	Low

SIE = susceptible increased exposure, AMP = ampicillin, AMC = amoxicillin-clavulanate, CTX = cefotaxime, S10 = streptomycin, K = kanamycin, S300 = sulfonamide, C = chloramphenicol, CIP = ciprofloxacin, TE = tetracycline. The level descriptors follow VARSS and EFSA standards defined as rare (<0.1%), very low (0.1-1%), low (1-10%), moderate (10-20%), high (20-50%), very high (50-70%), and extremely high (>70%) (European Food Safety Authority, 2022; Veterinary Medicines Directorate, 2023b)

3.3.6.2 Antibiotic susceptibility levels of the α *E. coli* picked from TBX supplemented with penicillin

The ASRs and ASLs of the 79 *E. coli* isolates from TBX supplemented with penicillin are shown in Table 3.6, with the ASLs of these isolates described in this section. High levels were resistant to ampicillin (44.3%), streptomycin (25.3%), sulfonamide (24.1%), and tetracycline (29.1%); low levels were resistant to amoxicillin-clavulanate (5.1%), ciprofloxacin (1.3%), and cefotaxime (1.3%); and a rare level were resistant to chloramphenicol (0%). A very high level was SIE to streptomycin (57%); high levels were SIE to ampicillin (38%) and ciprofloxacin (21.5%); low levels were SIE to amoxicillin-clavulanate (1.3%), cefotaxime (5.1%), and sulfonamide (2.5%); and rare levels were SIE to chloramphenicol (0%) and tetracycline (0%). Extremely high levels were susceptible to amoxicillin-clavulanate (93.7%), cefotaxime (93.7%), sulfonamide (73.4%), chloramphenicol (100%), ciprofloxacin (77.2%), and tetracycline (70.9%); and moderate levels were susceptible to ampicillin (17.7%) and streptomycin (17.7%).

Table 3.6: Overall antibiotic susceptibility and susceptibility levels of the 79 *α E. coli* isolates picked from TBX supplemented with penicillin.

	AMP	AMC	CTX	S10	S300	C	CIP	TE
Susceptible	14	74	74	14	58	79	61	56
SIE	30	1	4	45	2	0	17	0
Resistant	35	4	1	20	19	0	1	23
% Susceptible	17.7	93.7	93.7	17.7	73.4	100	77.2	70.9
Susceptible level	Moderate	Extremely high	Extremely high	Moderate	Extremely high	Extremely high	Extremely high	Extremely high
% SIE	38	1.3	5.1	57	2.5	0	21.5	0
SIE Level	High	Low	Low	Very high	Low	Rare	High	Rare
% Resistant	44.3	5.1	1.3	25.3	24.1	0	1.3	29.1
Resistance level	High	Low	Low	High	High	Rare	Low	High

SIE = susceptible increased exposure, AMP = ampicillin, AMC = amoxicillin-clavulanate, CTX = cefotaxime, S10 = streptomycin, K = kanamycin, S300 = sulfonamide, C = chloramphenicol, CIP = ciprofloxacin, TE = tetracycline. The level descriptors follow VARSS and EFSA standards defined as rare (<0.1%), very low (0.1-1%), low (1-10%), moderate (10-20%), high (20-50%), very high (50-70%), and extremely high (>70%) (European Food Safety Authority, 2022; Veterinary Medicines Directorate, 2023b).

3.3.6.3 Antibiotic susceptibility levels of the α *E. coli* picked from TBX supplemented with spectinomycin

The ASRs and ASLs of the 17 *E. coli* isolates picked from TBX supplemented with spectinomycin are described in Table 3.7, with the ASLs of these isolates described in this section.

Extremely high levels were resistant to ampicillin (100%), streptomycin (94.1%), sulfonamide (94.1%), and tetracycline (82.4%); a low level was resistant to amoxicillin-clavulanate (5.9%); and rare levels were resistant to cefotaxime (0%), chloramphenicol (0%), and ciprofloxacin (0%). A moderate level was SIE to amoxicillin-clavulanate (11.8%); a low level was SIE to tetracycline (5.9%); and rare levels were SIE to ampicillin (0%), cefotaxime (0%), streptomycin (0%), sulfonamide (0%), chloramphenicol (0%), and ciprofloxacin (0%). Extremely high levels were susceptible to amoxicillin-clavulanate (82.4%), cefotaxime (100%), chloramphenicol (100%), and ciprofloxacin (100%); a moderate level was susceptible to tetracycline (11.8%); low levels were susceptible to streptomycin (5.9%) and sulfonamide (5.9%); and a rare level was susceptible to ampicillin (0%).

Table 3.7: Overall antibiotic susceptibility and susceptibility levels of the 17 *α E. coli* isolates picked from TBX supplemented with spectinomycin.

	AMP	AMC	CTX	S10	S300	C	CIP	TE
Susceptible	0	14	17	1	1	17	17	2
SIE	0	2	0	0	0	0	0	1
Resistant	17	1	0	16	16	0	0	14
% Susceptible	0	82.4	100	5.9	5.9	100	100	11.8
Susceptible level	Rare	Extremely high	Extremely high	Low	Low	Extremely high	Extremely high	Moderate
% SIE	0	11.8	0	0	0	0	0	5.9
SIE Level	Rare	Moderate	Rare	Rare	Rare	Rare	Rare	Low
% Resistant	100	5.9	0	94.1	94.1	0	0	82.4
Resistance level	Extremely high	Low	Rare	Extremely high	Extremely high	Rare	Rare	Extremely high

SIE = susceptible increased exposure, AMP = ampicillin, AMC = amoxicillin-clavulanate, CTX = cefotaxime, S10 = streptomycin, K = kanamycin, S300 = sulfonamide, C = chloramphenicol, CIP = ciprofloxacin, TE = tetracycline. The level descriptors follow VARSS and EFSA standards defined as rare (<0.1%), very low (0.1-1%), low (1-10%), moderate (10-20%), high (20-50%), very high (50-70%), and extremely high (>70%) (European Food Safety Authority, 2022; Veterinary Medicines Directorate, 2023b).

3.3.6.4 Antibiotic susceptibility levels of the α *E. coli* picked from TBX supplemented with tetracycline

The ASRs and ASLs of the nine *E. coli* isolates picked from TBX supplemented with tetracycline are described in Table 3.8, and the ASLs of these isolates are described in this section. Extremely high levels were resistant to ampicillin (100%), streptomycin (88.9%), sulfonamide (77.8%), and tetracycline (100%); a very high level was resistant to chloramphenicol (55.6%); a moderate level was resistant to cefotaxime (11.1%); and rare levels were resistant to amoxicillin-clavulanate (0%) and ciprofloxacin (0%). A moderate level was SIE to streptomycin (11.1%) and rare levels were SIE to ampicillin (0%), amoxicillin-clavulanate (0%), cefotaxime (0%), sulfonamide (0%), chloramphenicol (0%), ciprofloxacin (0%), and tetracycline (0%). Extremely high levels were susceptible to amoxicillin-clavulanate (100%), cefotaxime (88.9%), and ciprofloxacin (100%); high levels were susceptible to sulfonamide (22.2%) and chloramphenicol (44.4%); and rare levels were susceptible to ampicillin (0%), streptomycin (0%), and tetracycline (0%).

Table 3.8: Overall antibiotic susceptibility and susceptibility levels of the nine *α E. coli* isolates picked from TBX supplemented with tetracycline.

	AMP	AMC	CTX	S10	S300	C	CIP	TE
Susceptible	0	9	8	0	2	4	9	0
SIE	0	0	0	1	0	0	0	0
Resistant	9	0	1	8	7	5	0	9
% Susceptible	0	100	88.9	0	22.2	44.4	100	0
Susceptible level	Rare	Extremely high	Extremely high	Rare	High	High	Extremely high	Rare
% SIE	0	0	0	11.1	0	0	0	0
SIE Level	Rare	Rare	Rare	Moderate	Rare	Rare	Rare	Rare
% Resistant	100	0	11.1	88.9	77.8	55.6	0	100
Resistance level	Extremely high	Rare	Moderate	Extremely high	Extremely high	Very high	Rare	Extremely high

SIE = susceptible increased exposure, AMP = ampicillin, AMC = amoxicillin-clavulanate, CTX = cefotaxime, S10 = streptomycin, K = kanamycin, S300 = sulfonamide, C = chloramphenicol, CIP = ciprofloxacin, TE = tetracycline. The level descriptors follow VARSS and EFSA standards defined as rare (<0.1%), very low (0.1-1%), low (1-10%), moderate (10-20%), high (20-50%), very high (50-70%), and extremely high (>70%) (European Food Safety Authority, 2022; Veterinary Medicines Directorate, 2023b).

3.3.7 Chemotherapeutic treatment increases faecal *E. coli* antibiotic resistance

The ASRs and ASLs of the 120 *E. coli* from the PPL that did receive chemotherapy before sampling, and the 283 *E. coli* from the PPL that did not are presented in Table 3.9. The ASLs of these *E. coli* subsets are described in this section.

A high level of the isolates from the untreated PPL (44.2%) and the treated PPL (29.2%) were susceptible to ampicillin. A high level of the isolates from the untreated PPL (37.1%) and the treated PPL (24.2%) were SIE to ampicillin. A moderate level of the isolates from the untreated PPL (18.7%) and a high level of the isolates from the treated PPL (46.7%) were resistant to ampicillin. An extremely high level of the isolates from the untreated PPL (90.1%) and the treated PPL (91.7%) were susceptible to amoxicillin-clavulanate. A low level of the isolates from the untreated PPL (1.8%) and the treated PPL (5%) were SIE to amoxicillin-clavulanate. A low level of the isolates from the untreated PPL (8.1%) and the treated PPL (3.3%) were resistant to amoxicillin-clavulanate. A very high level of the isolates from the untreated PPL (66.8%) and an extremely high level of the isolates from the treated PPL (80%) were susceptible to cefotaxime. A high level of the isolates from the untreated PPL (27.9%) and a moderate level of the isolates from the treated PPL (15.8%) were SIE to cefotaxime. A low level of the isolates from the untreated PPL (5.3%) and the treated PPL (4.2%) were resistant to cefotaxime. A moderate level of the isolates from the untreated PPL (18.7%) and the treated PPL (13.3%) were susceptible to streptomycin. A very high level of the isolates from the untreated PPL (64.3%) and the treated PPL (58.3%) were SIE to

streptomycin. A moderate level of the isolates from the untreated PPL (17%) and a high level of the isolates from the treated PPL (28.3%) were resistant to streptomycin. A very high level of the isolates from the untreated PPL (60.4%) and the treated PPL (59.2%) were susceptible to sulfonamide. A high level of the isolates from the untreated PPL (27.9%) and a low level of the isolates from the treated PPL (8.3%) were SIE to sulfonamide. A moderate level of the isolates from the untreated PPL (11.7%) and a high level of the isolates from the treated PPL (32.5%) were resistant to sulfonamide. An extremely high level of the isolates from the untreated PPL (97.2%) and the treated PPL (97.5%) were susceptible to chloramphenicol. A low level of the isolates from the untreated PPL (1.4%) and the treated PPL (2.5%) were SIE to chloramphenicol. A low level of the isolates from the untreated PPL (1.4%) and a rare level of the isolates from the treated PPL (0%) were resistant to chloramphenicol. An extremely high level of the isolates from the untreated PPL (88%) and the treated PPL (83.3%) were susceptible to ciprofloxacin. A moderate level of the isolates from the untreated PPL (10.2%) and the treated PPL (15%) were SIE to ciprofloxacin. A low level of the isolates from the untreated PPL (1.8%) and the treated PPL (1.7%) were resistant to ciprofloxacin. An extremely high level of the isolates from the untreated PPL (97.5%) and the treated PPL (75%) were susceptible to tetracycline. A very low level of the isolates from the untreated PPL (0.4%) and a rare level of the isolates from the treated PPL (0%) were SIE to tetracycline. A low level of the isolates from the untreated PPL (2.1%) and a high level of the isolates from the treated PPL (25%) were resistant to tetracycline (Table 3.9).

Table 3.9: Antibiotic susceptibility levels of the *α E. coli* isolates in different PPL treatment groups.

		AMP	AMC	CTX	S10	S300	C	CIP	TE
Isolates from untreated PPL (n = 283)	Susceptible	125	255	189	53	171	275	249	276
	SIE	105	5	79	182	79	4	29	1
	Resistant	53	23	15	48	33	4	5	6
	% Susceptible	44.2	90.1	66.8	18.7	60.4	97.2	88	97.5
	Susceptible level	High	Extremely high	Very high	Moderate	Very high	Extremely high	Extremely high	Extremely high
	% SIE	37.1	1.8	27.9	64.3	27.9	1.4	10.2	0.4
	SIE Level	High	Low	High	Very high	High	Low	Moderate	Very low
	% Resistant	18.7	8.1	5.3	17	11.7	1.4	1.8	2.1
	Resistance level	Moderate	Low	Low	Moderate	Moderate	Low	Low	Low
Isolates from treated PPL (n = 120)	Susceptible	35	110	96	16	71	117	100	90
	SIE	29	6	19	70	10	3	18	0
	Resistant	56	4	5	34	39	0	2	30
	% Susceptible	29.2	91.7	80	13.3	59.2	97.5	83.3	75
	Susceptible level	High	Extremely high	Extremely high	Moderate	Very high	Extremely high	Extremely high	Extremely high
	% SIE	24.2	5	15.8	58.3	8.3	2.5	15	0
	SIE Level	High	Low	Moderate	Very high	Low	Low	Moderate	Rare
	% Resistant	46.7	3.3	4.2	28.3	32.5	0	1.7	25
	Resistance level	High	Low	Low	High	High	Rare	Low	High

SIE = susceptible increased exposure, AMP = ampicillin, AMC = amoxicillin-clavulanate, CTX = cefotaxime, S10 = streptomycin, K = kanamycin, S300 = sulfonamide, C = chloramphenicol, CIP = ciprofloxacin, TE = tetracycline. The level descriptors follow VARSS and EFSA standards defined as rare (<0.1%), very low (0.1-1%), low (1-10%), moderate (10-20%), high (20-50%), very high (50-70%), and extremely high (>70%) (European Food Safety Authority, 2022; Veterinary Medicines Directorate, 2023b).

The median ASRs of the isolates from both treatment groups were SIE to two antibiotics from two classes (ampicillin and streptomycin) and susceptibility to six antibiotics from five classes (amoxicillin-clavulanate, cefotaxime, sulfonamide, chloramphenicol, ciprofloxacin, and tetracycline) (Figure 3.22). The MASDs of these *E. coli* subsets were different and will be described, and statistically investigated in the next section.

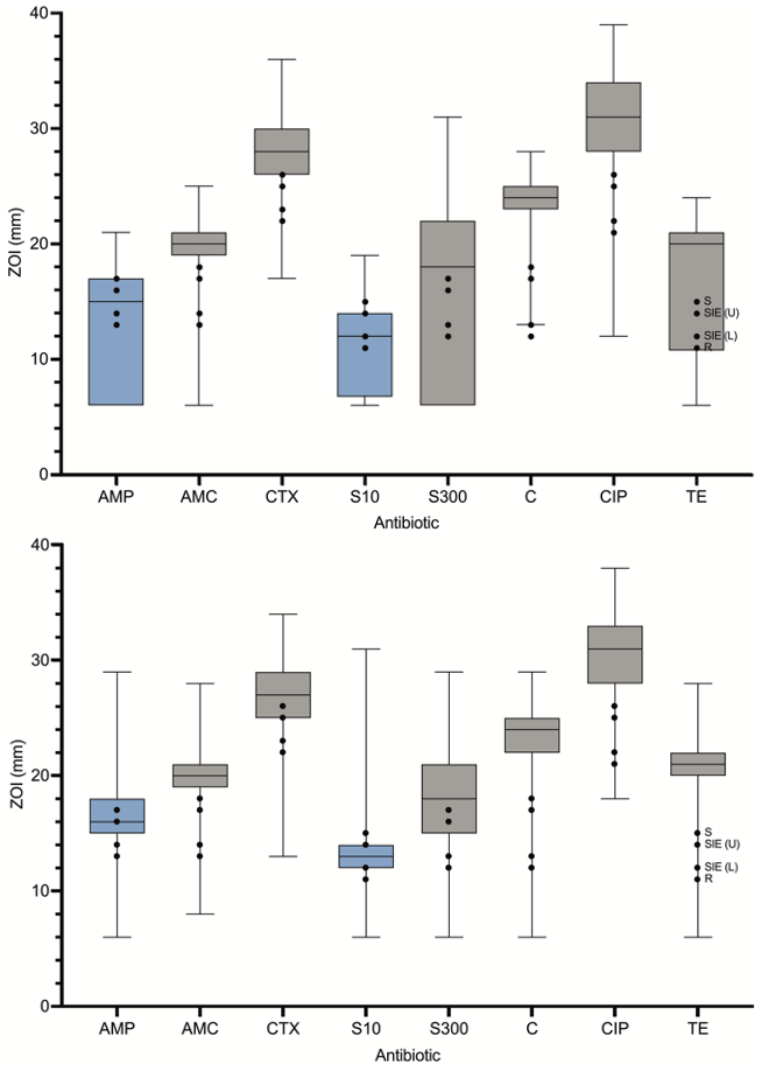


Figure 3.22: Median antibiotic susceptibility distributions of *E. coli* from PPL in different treatment groups. The midline represents the median zone of inhibition (ZOI) across all isolates, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown as black dots: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility: grey for susceptible and blue for SIE. Abbreviations: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), TE (tetracycline). The top graph is the isolates from treated PPL (n = 120) and the bottom graph is the isolates from untreated PPL (n = 283).

3.3.7.1 Significant differences in the median antibiotic susceptibility distributions of the isolates from different PPL treatment groups

The distributions of the ZOIs of each antibiotic for the isolates in both PPL treatment groups were compared using the KST. The median ZOI for ampicillin in the isolates from treated PPL was 15 mm (SIE) with an interquartile range of 6-17 mm, and in the isolates from untreated PPL was 16 mm (SIE) with an interquartile range of 15-18 mm. The KST confirmed that these distributions were significantly different (ρ <0.0001) (Figure 3.23).

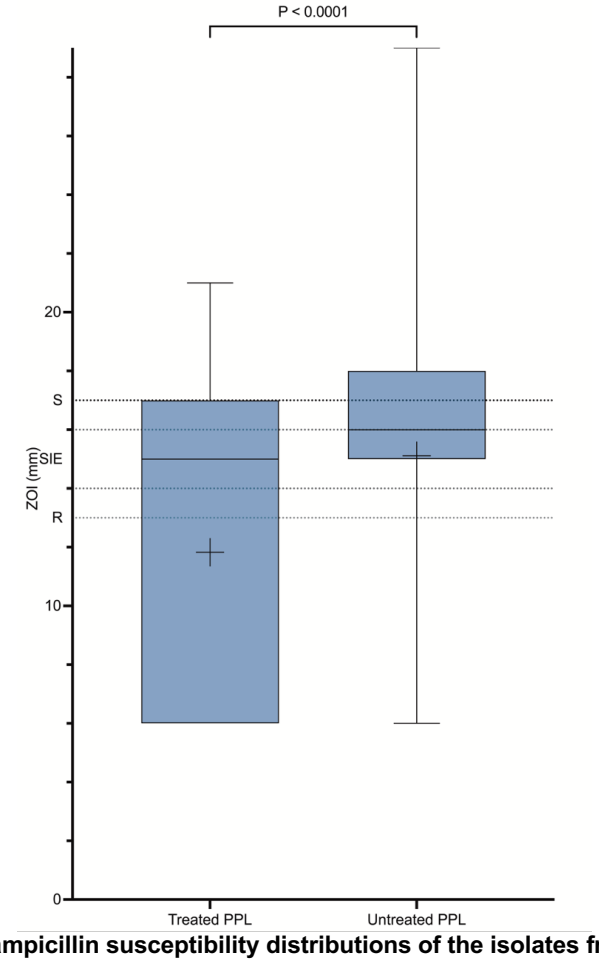


Figure 3.23: Median ampicillin susceptibility distributions of the isolates from both of the PPL treatment groups. The midline represents the median zone of inhibition (ZOI) across all isolates, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown by dotted ticks on y-axis: S (susceptible), SIE (susceptible with increased exposure), and R (resistant). The box colour indicates the median antibiotic susceptibility result: blue for SIE. The left box is the isolates from treated PPL (n = 120) and the right box is the isolates from untreated PPL (n = 283). The Kolmogorov-Smirnov test identified significantly different distributions of these isolates (ρ <0.0001).

The median ZOIs for amoxicillin-clavulanate in the isolates from the treated and untreated PPL was 20 mm (susceptible) with an interquartile range of 19-21 mm (Figure 3.24). The distributions of ZOIs for amoxicillin-clavulanate were not significantly different between the isolates from the different PPL treatment groups (Figure 3.24).

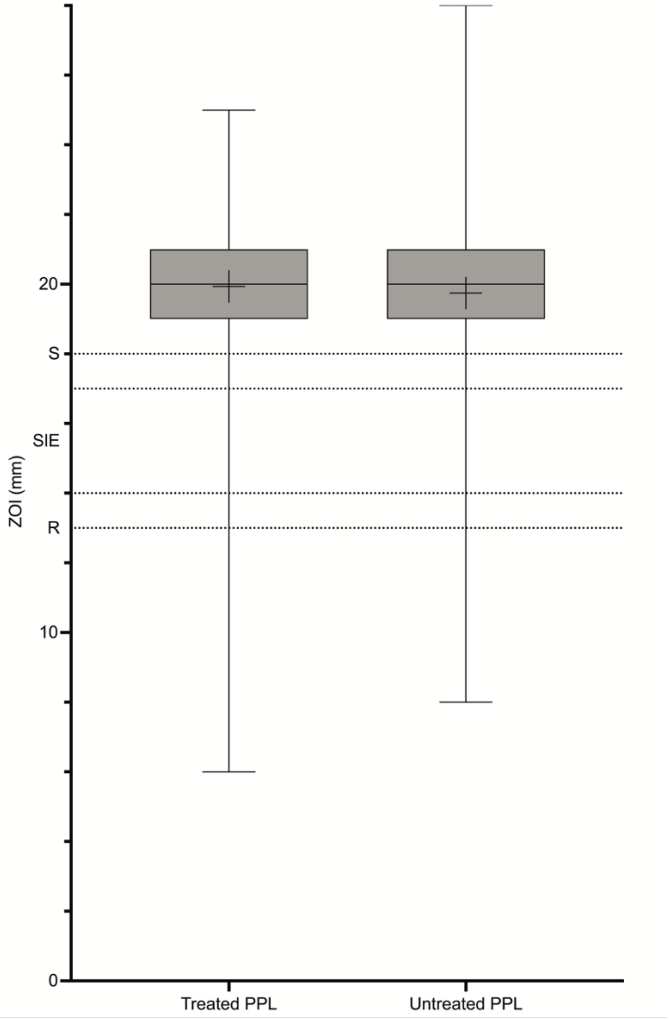


Figure 3.24: Median amoxicillin-clavulanate susceptibility distributions of the isolates from both of the PPL treatment groups. The midline represents the median zone of inhibition (ZOI) across all isolates, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown by dotted ticks on y-axis: S (susceptible), SIE (susceptible with increased exposure), and R (resistant). The box colour indicates the median antibiotic susceptibility result: grey for susceptibility. The left box is the isolates from treated PPL (n = 120) and the right box is the isolates from untreated PPL (n = 283). The Kolmogorov-Smirnov test found no significant difference in the distributions of these isolates.

The median ZOI for cefotaxime in the isolates from the treated PPL was 28 mm (susceptible) with an interquartile range of 26-30 mm, and in the isolates from the untreated PPL was 27 mm (susceptible) with an interquartile range of 25-29 mm (Figure 3.25). The distributions of ZOI for cefotaxime were significantly different for the isolates from the different PPL treatment groups (ρ = 0.0003) (Figure 3.25).

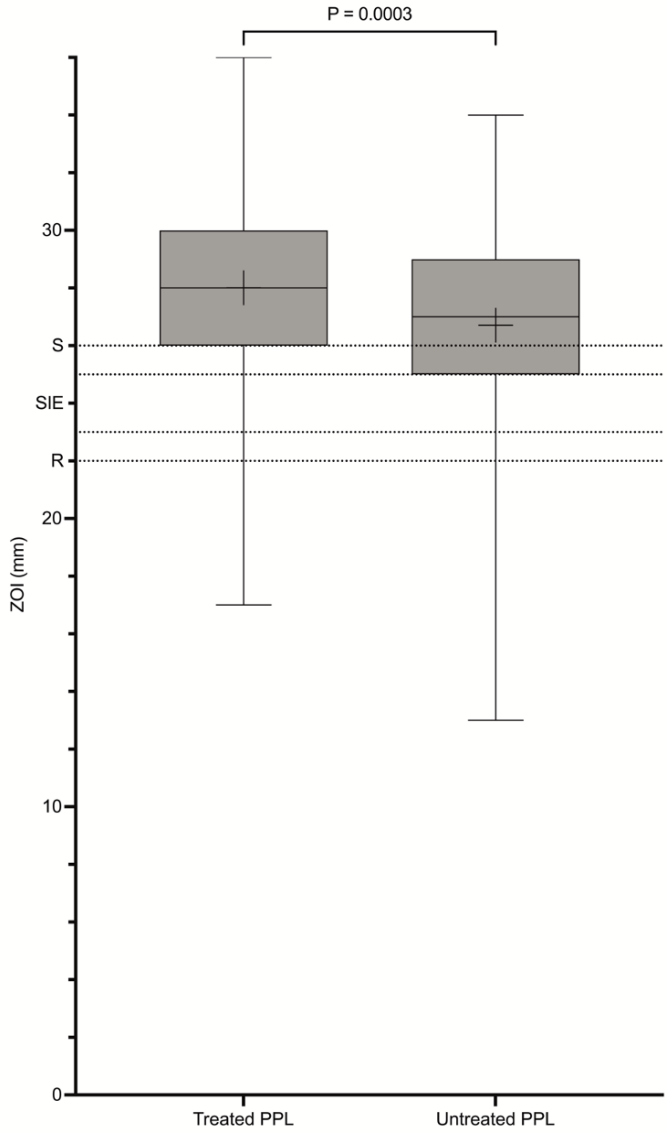


Figure 3.25: Median cefotaxime susceptibility distributions of the isolates from both of the PPL treatment groups. The midline represents the median zone of inhibition (ZOI) across all isolates, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown by dotted ticks on y-axis: S (susceptible), SIE (susceptible with increased exposure), and R (resistant). The box colour indicates the median antibiotic susceptibility result: grey for susceptible. The left box is the isolates from treated PPL (n = 120) and the right box is the isolates from untreated PPL (n = 283). The Kolmogorov-Smirnov test identified significantly different distributions in these isolates (ρ = 0.0003).

The median ZOI for streptomycin in the isolates from the treated PPL was 12 mm (SIE) with an interquartile range of 7-14 mm, and in the isolates from the untreated PPL was 13 mm (SIE) with an interquartile range of 12-14mm (Figure 3.26). The distribution of ZOIs for streptomycin was significantly different for the isolates from the different PPL treatment groups (ρ = 0.0002) (Figure 3.26).

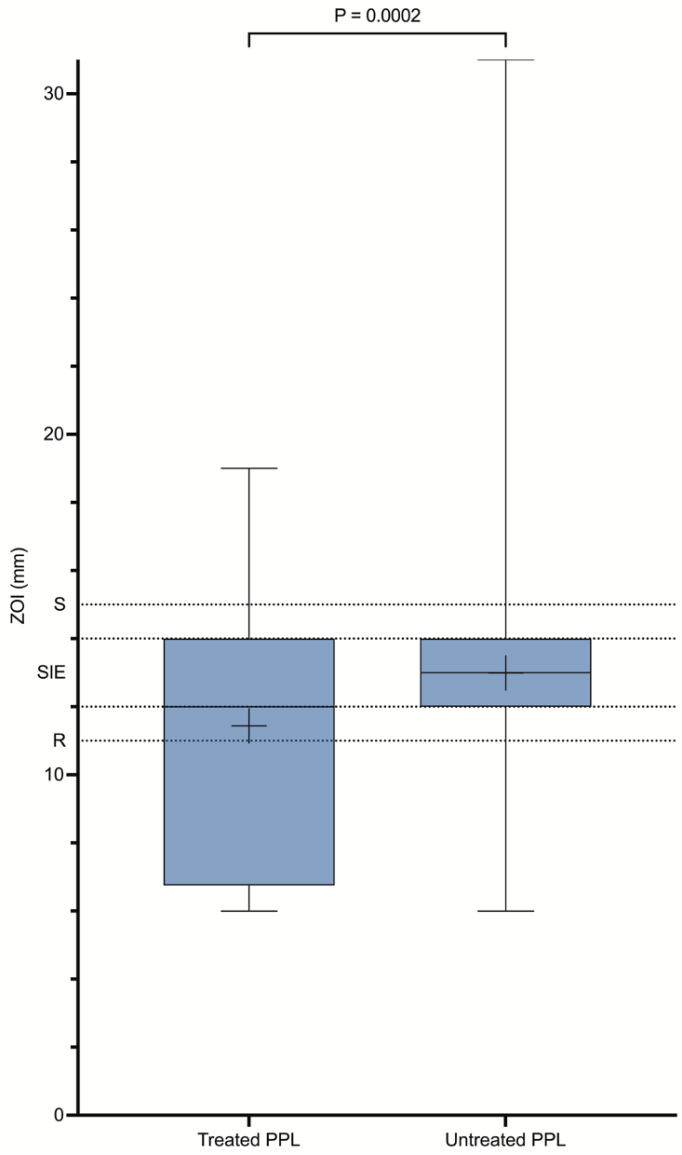


Figure 3.26: Median streptomycin susceptibility distributions of the isolates from both of the PPL treatment groups. The midline represents the median zone of inhibition (ZOI) across all isolates, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown by dotted ticks on y-axis: S (susceptible), SIE (susceptible with increased exposure), and R (resistant). The box colour indicates the median antibiotic susceptibility result: blue for SIE. The left box is the isolates from treated PPL (n = 120) and the right box is the isolates from untreated PPL (n = 283). The Kolmogorov-Smirnov test identified significantly different distributions in these isolates (ρ = 0.0002).

The distributions of ZOIs for sulfonamide were significantly different between the isolates from the different PPL treatment groups (ρ = 0.0002) (Figure 3.27). The median ZOI for sulfonamide in the isolates from the treated PPL was 18 mm (susceptible) with an interquartile range of 6-22 mm, and in the isolates from the untreated PPL was 18 mm (susceptible) with an interquartile range of 15-21 mm (Figure 3.27).

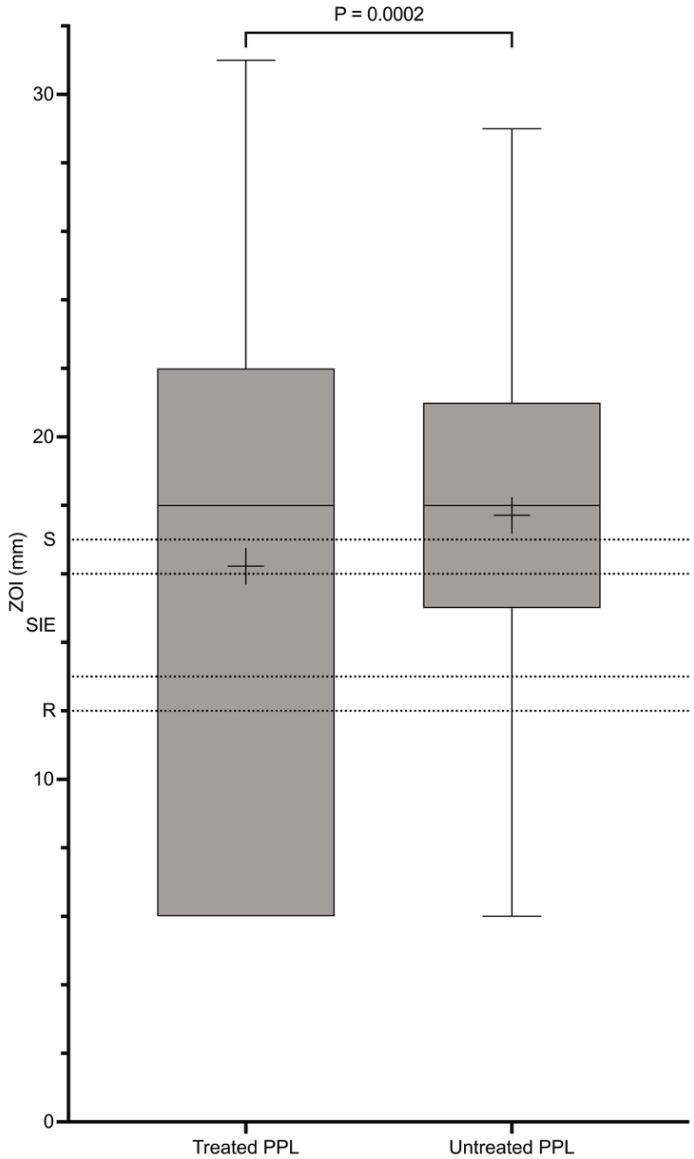


Figure 3.27: Median sulfonamide susceptibility distributions of the isolates from both of the PPL treatment groups. The midline represents the median zone of inhibition (ZOI) across all isolates, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown by dotted ticks on y-axis: S (susceptible), SIE (susceptible with increased exposure), and R (resistant). The box colour indicates the median antibiotic susceptibility result: grey for susceptibility. The left box is the isolates from treated PPL (n = 120) and the right box is the isolates from untreated PPL (n = 283). The Kolmogorov-Smirnov test identified significantly different distributions in these isolates (ρ = 0.0002).

The distributions of ZOI for chloramphenicol were not significantly different between the isolates from the different treatment groups (Figure 3.28). The median ZOI for chloramphenicol in the isolates from the treated PPL was 24 mm (susceptible) with an interquartile range of 23-25 mm, and in the isolates from the untreated PPL was 24 mm (susceptible) with an interquartile range of 22-25 mm (Figure 3.28).

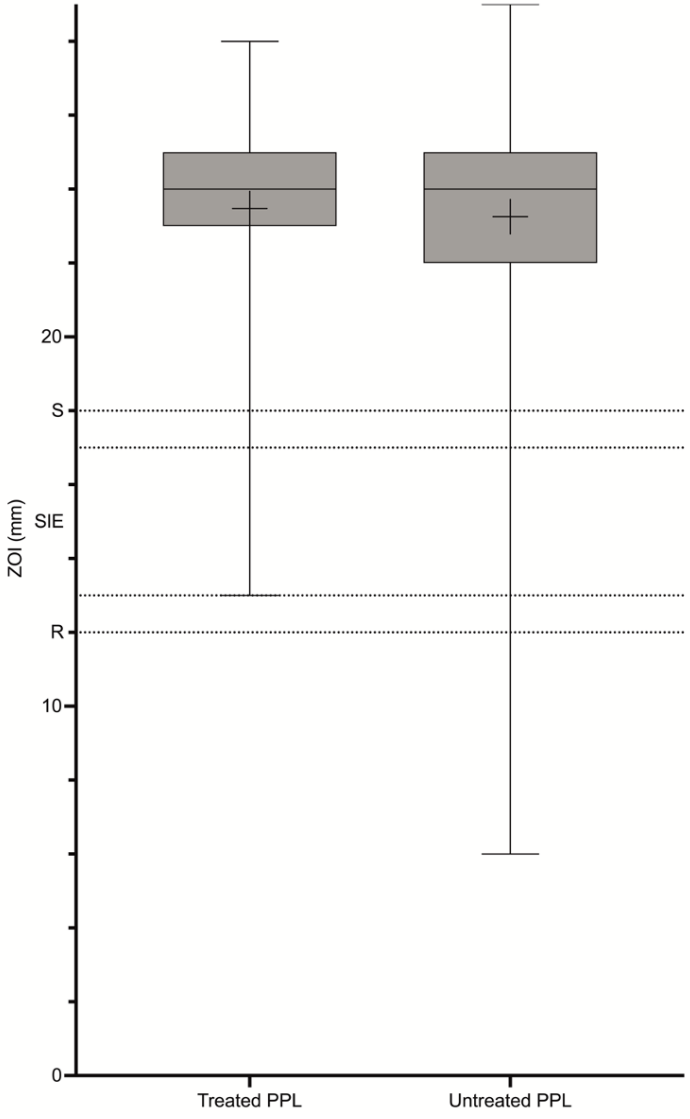


Figure 3.28: Median chloramphenicol susceptibility distributions of the isolates from both of the PPL treatment groups. The midline represents the median zone of inhibition (ZOI) across all isolates, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown by dotted ticks on y-axis: S (susceptible), SIE (susceptible with increased exposure), and R (resistant). The box colour indicates the median antibiotic susceptibility result: grey for susceptible. The left box is the isolates from treated PPL (n = 120) and the right box is the isolates from untreated PPL (n = 283). The Kolmogorov-Smirnov test did not identify significantly different distributions in these isolates.

The distributions of ZOI for ciprofloxacin were not significantly different between the isolates from the different PPL treatment groups (Figure 3.29). The median ZOI for ciprofloxacin in the isolates from the treated PPL was 31 mm (susceptible) with an interquartile range of 28-34 mm, and in the isolates from the untreated PPL was 31 mm (susceptible) with an interquartile range of 28-33 mm (Figure 3.29).

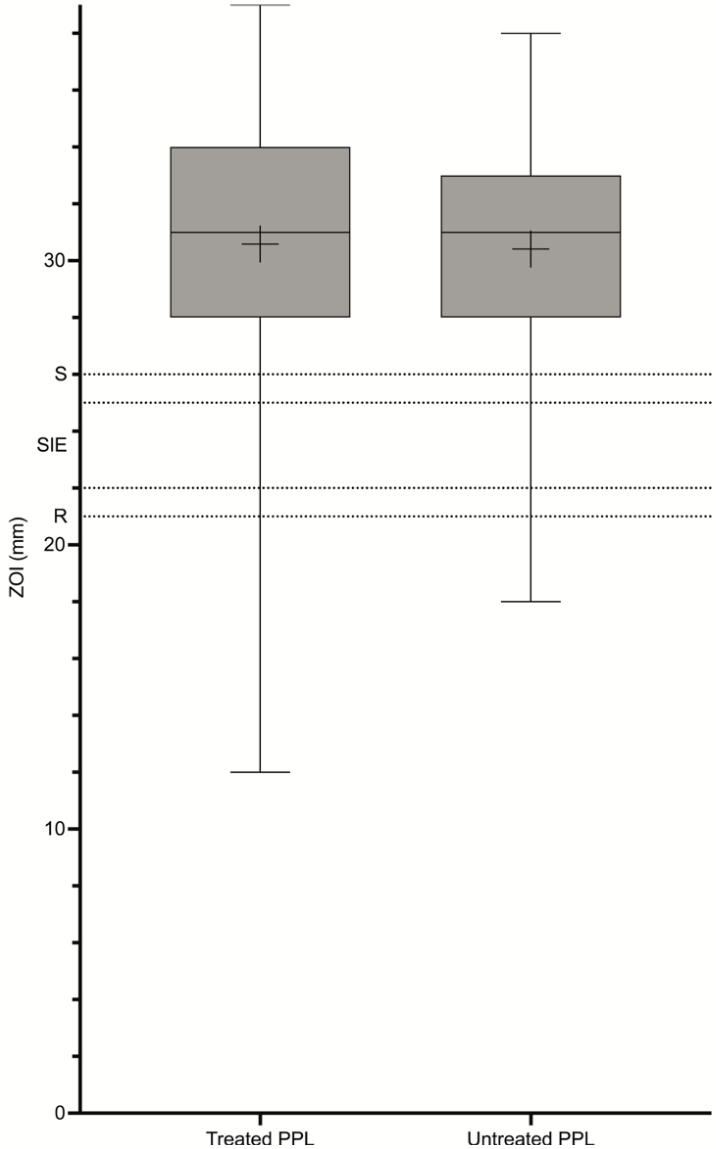


Figure 3.29: Median ciprofloxacin susceptibility distributions of the isolates from both of the PPL treatment groups. The midline represents the median zone of inhibition (ZOI) across all isolates, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown by dotted ticks on y-axis: S (susceptible), SIE (susceptible with increased exposure), and R (resistant). The box colour indicates the median antibiotic susceptibility result: grey for susceptible. The left box is the isolates from treated PPL (n = 120) and the right box is the isolates from untreated PPL (n = 283). The Kolmogorov-Smirnov test did not identify significantly different distributions in these isolates.

The distributions of ZOI for tetracycline were significantly different between the isolates from the different PPL treatment groups (ρ <0.0001) (Figure 3.30). The median ZOI for tetracycline in the isolates from the treated PPL was 20 mm (susceptible) with an interquartile range of 11-21 mm, and in the isolates from the untreated PPL was 21 mm (susceptible) with an interquartile range of 20-22mm (Figure 3.30).

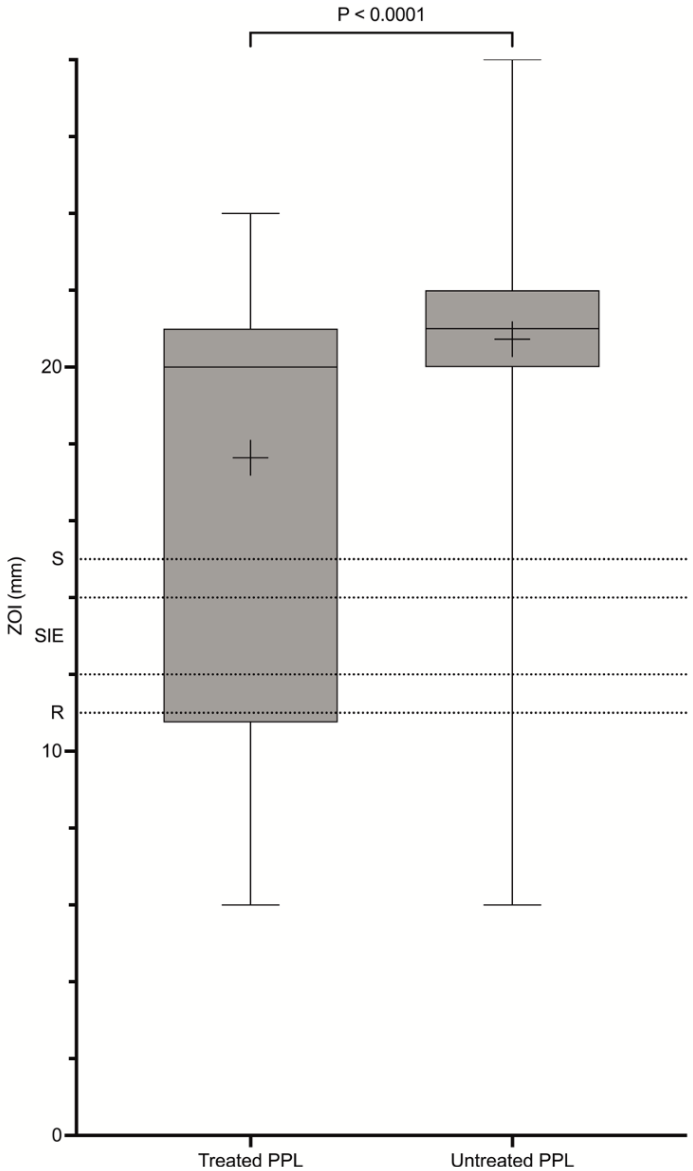


Figure 3.30: Median tetracycline susceptibility distributions of the isolates from both of the PPL treatment groups. The midline represents the median zone of inhibition (ZOI) across all isolates, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown by dotted ticks on y-axis: S (susceptible), SIE (susceptible with increased exposure), and R (resistant). The box colour indicates the median antibiotic susceptibility result: grey for susceptible. The left box is the isolates from treated PPL (n = 120) and the right box is the isolates from untreated PPL (n = 283). The Kolmogorov-Smirnov test identified significantly different distributions in these isolates (ρ <0.0001).

3.3.7.2 Levels of antibiotic resistant *E. coli* decreases as time after chemotherapy treatment increases

This study did not sample the same PPL twice; therefore, it is not possible to directly investigate the longitudinal link between AMU and AMR. However, the samples from treated PPL were collected at various times after treatment. A total of 120 isolates were collected from the six treated PPL, of which 55 isolates were from PPL that received treatment <10 days before sampling, and 65 isolates were from PPL that received treatment 10-30 days before sampling. The levels of antibiotic susceptible isolates increased, while the levels of SIE, and resistance decreased as time between treatment and sampling increased. The ASRs and ASLs of the isolates from untreated PPL are included in the subsequent tables and figures for comparison, but they have been described previously in this chapter (3.3.7.1). The ASRs and ASLs for the isolates from PPL treated within 10 days before sampling and from PPL treated 10-30 days before sampling can be found in Table 3.10. High levels of the isolates from the PPL treated within 10 days before sampling (23.6%) and from the PPL treated 10-30 days before sampling (33.8%) were susceptible to ampicillin. High levels of the isolates from the PPL treated within 10 days before sampling (21.8%) and from the PPL treated 10-30 days before sampling (26.2%) were SIE to ampicillin. Very high levels of the isolates from the PPL treated within 10 days before sampling (54.5%) and from the PPL treated 10-30 days before sampling (40%) were resistant to ampicillin. Extremely high levels of the isolates from the PPL treated within 10 days before sampling (92.7%) and from the PPL treated 10-30 days before sampling (90.8%) were susceptible to amoxicillin-clavulanate. A rare level of

the isolates from the PPL treated within 10 days before sampling (0%) and a low level of the isolates from the PPL treated 10-30 days before sampling (9.2%) were SIE to amoxicillin-clavulanate. A low level of the isolates from the PPL treated within 10 days before sampling (7.3%) and a rare level of the isolates from the PPL treated 10-30 days before sampling (0%) were resistant to amoxicillin-clavulanate. An extremely high level of the isolates from the PPL treated within 10 days before sampling (94.5%) and a very high level of the isolates from the PPL treated 10-30 days before sampling (67.7%) were susceptible to cefotaxime. A low level of the isolates from the PPL treated within 10 days before sampling (3.6%) and a high level of the isolates from the PPL treated 10-30 days before sampling (26.2%) were SIE to cefotaxime. A low level of the isolates from the PPL treated within 10 days before sampling (1.8%) and from the PPL treated 10-30 days before sampling (6.2%) were resistant to cefotaxime. Moderate levels of the isolates from the PPL treated within 10 days before sampling (14.5%) and from the PPL treated 10-30 days before sampling (12.3%) were susceptible to streptomycin. A high level of the isolates from the PPL treated within 10 days before sampling (30.9%) and an extremely high level of the isolates from the PPL treated 10-30 days before sampling (81.5%) were SIE to streptomycin. A very high level of the isolates from the PPL treated within 10 days before sampling (54.5%) and a low level of the isolates from the PPL treated 10-30 days before sampling (6.2%) were resistant to streptomycin. A high level of the isolates from the PPL treated within 10 days before sampling (41.8%) and an extremely high level of the isolates from the PPL treated 10-30 days before sampling (73.8%) were susceptible to sulfonamide. A low level of the isolates from the PPL treated

within 10 days before sampling (3.6%) and a moderate level of the isolates from the PPL treated 10-30 days before sampling (12.3%) were SIE to sulfonamide.

A very high level of the isolates from the PPL treated within 10 days before sampling (54.5%) and a moderate level of the isolates from the PPL treated 10-30 days before sampling (13.8%) were resistant to sulfonamide. Extremely high levels of the isolates from the PPL treated within 10 days before sampling (96.4%) and from the PPL treated 10-30 days before sampling (98.5%) were susceptible to chloramphenicol. Low levels of the isolates from the PPL treated within 10 days before sampling (3.6%) and from the PPL treated 10-30 days before sampling (1.5%) were SIE to chloramphenicol. Rare levels of the isolates from the PPL treated within 10 days before sampling (0%) and from the PPL treated 10-30 days before sampling (0%) were resistant to chloramphenicol. Extremely high levels of the isolates from the PPL treated within 10 days before sampling (90.9%) and from the PPL treated 10-30 days before sampling (76.9%) were susceptible to ciprofloxacin. A low level of the isolates from the PPL treated within 10 days before sampling (7.3%) and a high level of the isolates from the PPL treated 10-30 days before sampling (21.5%) were SIE to ciprofloxacin. Low levels of the isolates from the PPL treated within 10 days before sampling (1.8%) and from the PPL treated 10-30 days before sampling (1.5%) were resistant to ciprofloxacin. A high level of the isolates from the PPL treated within 10 days before sampling (45.5%) and an extremely high level of the isolates from the PPL treated 10-30 days before sampling (100%) were susceptible to tetracycline. Rare levels of the isolates from the PPL treated within 10 days before sampling (0%) and from the PPL

treated 10-30 days before sampling (0%) were SIE to tetracycline. A very high level of the isolates from the PPL treated within 10 days before sampling (54.5%) and a rare level of the isolates from the PPL treated 10-30 days before sampling (0%) were resistant to tetracycline.

Table 3.10: Antibiotic susceptibility levels of the isolates from faeces of untreated PPL and PPL faeces sampled at different times after treatment.

		AMP	AMC	CTX	S10	S300	C	CIP	TE
Faeces sampled <10 days after PPL treatment (n = 55)	S	13	51	52	8	23	53	50	25
	SIE	12	0	2	17	2	2	4	0
	R	30	4	1	30	30	0	1	30
	% S	23.6	92.7	94.5	14.5	41.8	96.4	90.9	45.5
	S level	High	E. high	E. high	Mod	High	E. high	E. high	High
	% SIE	21.8	0	3.6	30.9	3.6	3.6	7.3	0
	SIE level	High	Rare	Low	High	Low	Low	Low	Rare
	% R	54.5	7.3	1.8	54.5	54.5	0	1.8	54.5
	R level	V. high	Low	Low	V. high	V. high	Rare	Low	V. high
Faeces sampled 10-30 days after PPL treatment (n = 65)	S	22	59	44	8	48	64	50	65
	SIE	17	6	17	53	8	1	14	0
	R	26	0	4	4	9	0	1	0
	% S	33.8	90.8	67.7	12.3	73.8	98.5	76.9	100
	S level	High	E. high	V. high	Mod	E. high	E. high	E. high	E. high
	% SIE	26.2	9.2	26.2	81.5	12.3	1.5	21.5	0
	SIE level	High	Low	High	E. high	Mod	Low	High	Rare
	% R	40	0	6.2	6.2	13.8	0	1.5	0
	R level	High	Rare	Low	Low	Mod	Rare	Low	Rare
Faces sampled from untreated PPL (n = 283)	S	125	255	189	53	171	275	249	276
	SIE	105	5	79	182	79	4	29	1
	R	53	23	15	48	33	4	5	6
	% S	44.2	90.1	66.8	18.7	60.4	97.2	88	97.5
	S level	High	E. high	V. high	Mod	V. high	E. high	E. high	E. high
	% SIE	37.1	1.8	27.9	64.3	27.9	1.4	10.2	0.4
	SIE level	High	Low	High	V. high	High	Low	Mod	V. low
	% R	18.7	8.1	5.3	17	11.7	1.4	1.8	2.1
	R level	Mod	Low	Low	Mod	Mod	Low	Low	Low

S = susceptible, SIE = susceptible increased exposure, R = resistant, AMP = ampicillin, AMC = amoxicillin-clavulanate, CTX = cefotaxime, S10 = streptomycin, K = kanamycin, S300 = sulfonamide, C = chloramphenicol, CIP = ciprofloxacin, TE = tetracycline. The level descriptors follow VARSS and EFSA standards defined as rare (<0.1%), v. low = very low (0.1-1%), low (1-10%), mod = moderate (10-20%), high (20-50%), v. high = very high (50-70%), and e. high = extremely high (>70%) (European Food Safety Authority, 2022; Veterinary Medicines Directorate, 2023b).

The median ASRs of the isolates from the PPL treated 10 days before sampling were MDR, with resistance to four antibiotics from four classes (ampicillin, streptomycin, sulfonamide, and tetracycline) and susceptibility to four antibiotics from three classes (amoxicillin-clavulanate, cefotaxime, chloramphenicol and ciprofloxacin) (Figure 3.31). The median ASRs of the isolates from the PPL treated 10-30 days before sampling w the same as the profile of the isolates from the untreated PPL, SIE to two antibiotics from two classes (ampicillin and streptomycin), and susceptibility to six antibiotics from five classes (amoxicillin-clavulanate, cefotaxime, sulfonamide, chloramphenicol, ciprofloxacin, and tetracycline) (Figure 3.31).

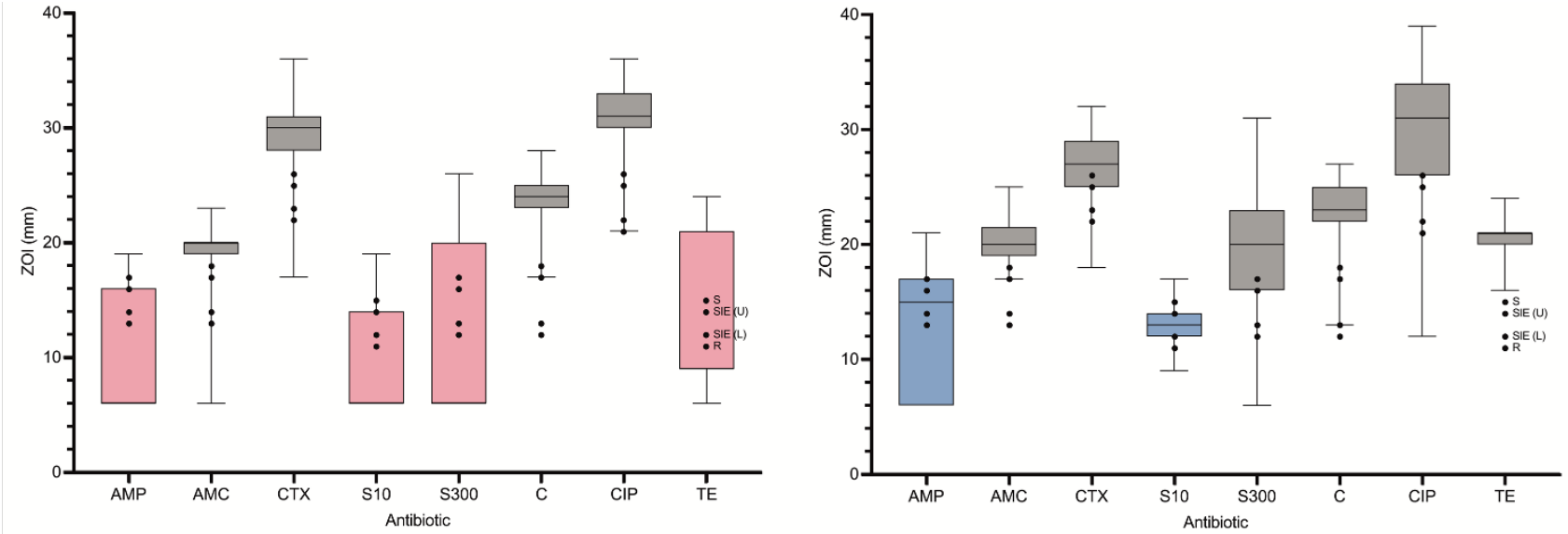


Figure 3.31: Median antibiotic susceptibility distributions of the isolates as time between PPL treatment and sampling increased. The midline represents the median zone of inhibition (ZOI) across all isolates, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown as black dots: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility: pink for resistant, grey for susceptible, and blue for SIE. Abbreviations: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), TE (tetracycline). The left graph is the isolates from PPL treated within 10 days before sampling (n = 55) and the right graph is the isolates from PPL treated between 10 and 30 days before treatment (n = 65).

The KWT was used to identify significant differences in medians of antibiotic ZOI for the isolates grouped by the time between PPL treatment and sampling. The median ZOI for ampicillin in the 55 isolates from the PPL treated within 10 days before sampling was 6 mm (resistance) with an interquartile range of 6-16 mm, in the 65 isolates from the PPL treated 10-30 days before sampling was 15 mm (SIE) with an interquartile range of 6-17 mm, and in the 283 isolates from the untreated PPL was 16 mm (SIE) with an interquartile range of 15-18 mm (Figure 3.32). The median ZOI for ampicillin in the isolates from the PPL treated 10 days before sampling was significantly different to the isolates from the untreated PPL (ρ <0.0001), and the median ZOI for ampicillin in the isolates from the PPL treated 10-30 days before sampling was also significantly different to the isolates from the untreated PPL (ρ = 0.0118) (Figure 3.32).

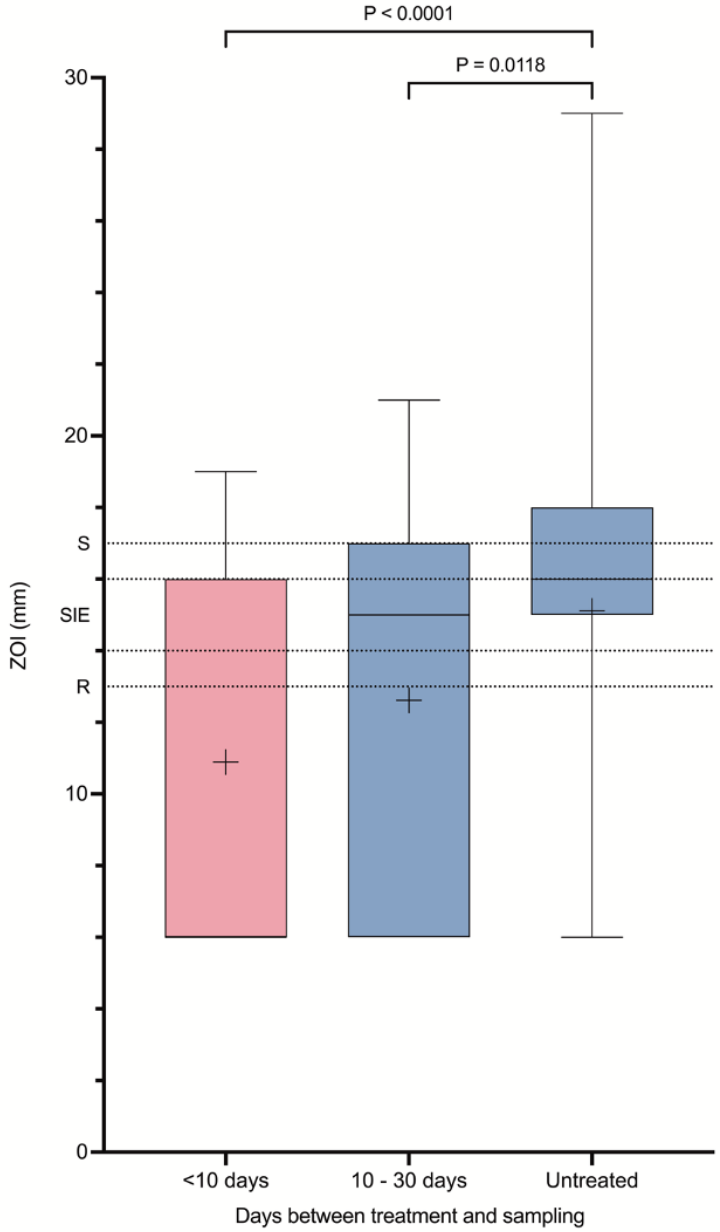


Figure 3.32: Median ampicillin susceptibility distributions with increasing time between PPL treatment and sampling. The midline represents the median zone of inhibition (ZOI) across all isolates, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown by dotted ticks on y-axis: S (susceptible), SIE (susceptible with increased exposure), and R (resistant). The box colour indicates the median antibiotic susceptibility result: grey for susceptibility. The left box is the isolates from the PPL treated <10 days before sampling (n = 55), the middle box is the isolates from the PPL treated 10-30 days before sampling (n = 65), and the right box is the isolates from untreated PPL (n = 283). The Kruskal-Wallis ANOVA test with Dunn's multiple comparisons test found significant differences in the median ZOI for ampicillin in the isolates from the PPL treated <10 days before sampling compared to the isolates from the untreated PPL (ρ <0.0001), and significant differences in the median ZOI for ampicillin in the isolates from the PPL treated 10-30 days before sampling compared to the isolates from the untreated PPL (ρ = 0.0118).

The median ZOI for amoxicillin-clavulanate in the 55 isolates from the PPL treated within 10 days before sampling was 20 mm (susceptible) with an interquartile range of 19-20 mm, in the 65 isolates from the PPL treated 10-30 days before sampling was 20 mm (susceptible) with an interquartile range of 19-21.5 mm, and in the 283 isolates from the untreated PPL was 20 mm (susceptible) with an interquartile range of 19-21 mm (Figure 3.33). The difference in the median ZOIs for amoxicillin-clavulanate in the isolates from the PPL treated 10 days before sampling compared the isolates from PPL treated 10-30 days before sampling was found to be significant (ρ = 0.0232). The median ZOIs for amoxicillin-clavulanate in the isolates from the PPL treated 10 days before sampling compared to the isolates in the untreated PPL were significantly different (ρ = 0.0232) (Figure 3.33).

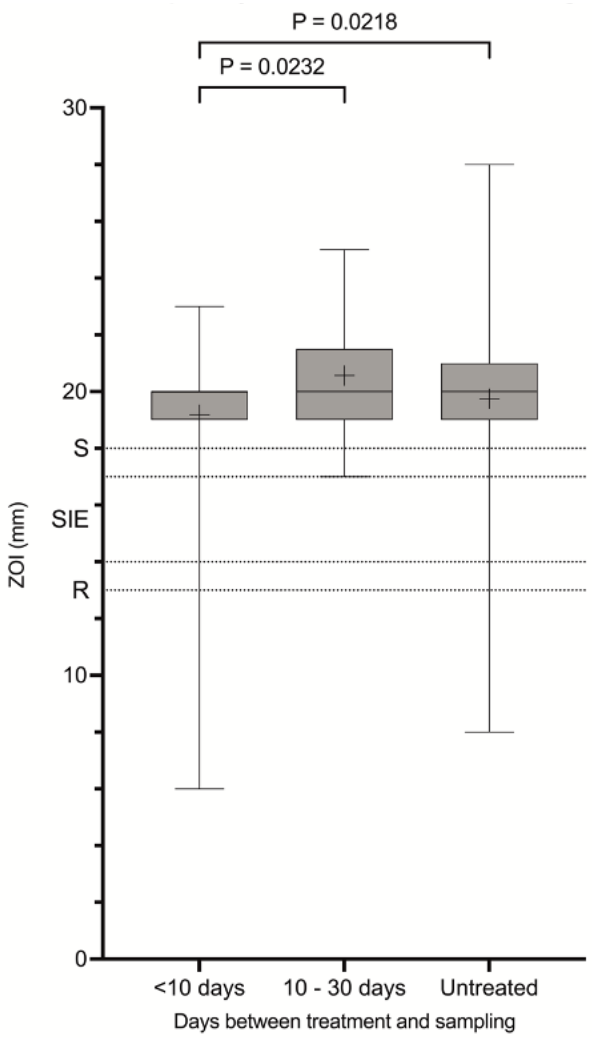


Figure 3.33: Median amoxicillin-clavulanate susceptibility distributions with increasing time between PPL treatment and sampling. The midline represents the median zone of inhibition (ZOI) across all isolates, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown by dotted ticks on y-axis: S (susceptible), SIE (susceptible with increased exposure), and R (resistant). The box colour indicates the median antibiotic susceptibility result: grey for susceptibility. The left box is the isolates from the PPL treated <10 days before sampling (n = 55), the middle box is the isolates from the PPL treated 10-30 days before sampling (n = 65), and the right box is the isolates from untreated PPL (n = 283).The Kruskal-Wallis ANOVA test with Dunn's multiple comparisons test found significant differences in the median ZOIs for amoxicillin-clavulanate in the isolates from the PPL treated <10 days before sampling compared to the isolates from the untreated PPL (ρ = 0.0218), and compared to the isolates from the PPL treated 10-30 days before sampling (ρ = 0.0118).

The median ZOI for cefotaxime in the 55 isolates from the PPL treated within 10 days before sampling was 30 mm (susceptible) with an interquartile range of 28-31 mm, in the 65 isolates from the PPL treated 10-30 days before sampling was 27 mm (susceptible) with an interquartile range of 25-29 mm, and in the 283 isolates from the untreated PPL was 27 mm (susceptible) with an interquartile range of 25-29 mm (susceptible) (Figure 3.34). A significant difference was found in the median ZOIs for cefotaxime in the isolates from the PPL treated 10 days before sampling compared to the isolates from PPL treated 10-30 days before sampling (ρ <0.0001). The median ZOI for cefotaxime in the isolates from the PPL treated 10 days before sampling was also significantly different compared to the isolates from the untreated PPL (ρ <0.0001) (Figure 3.34).

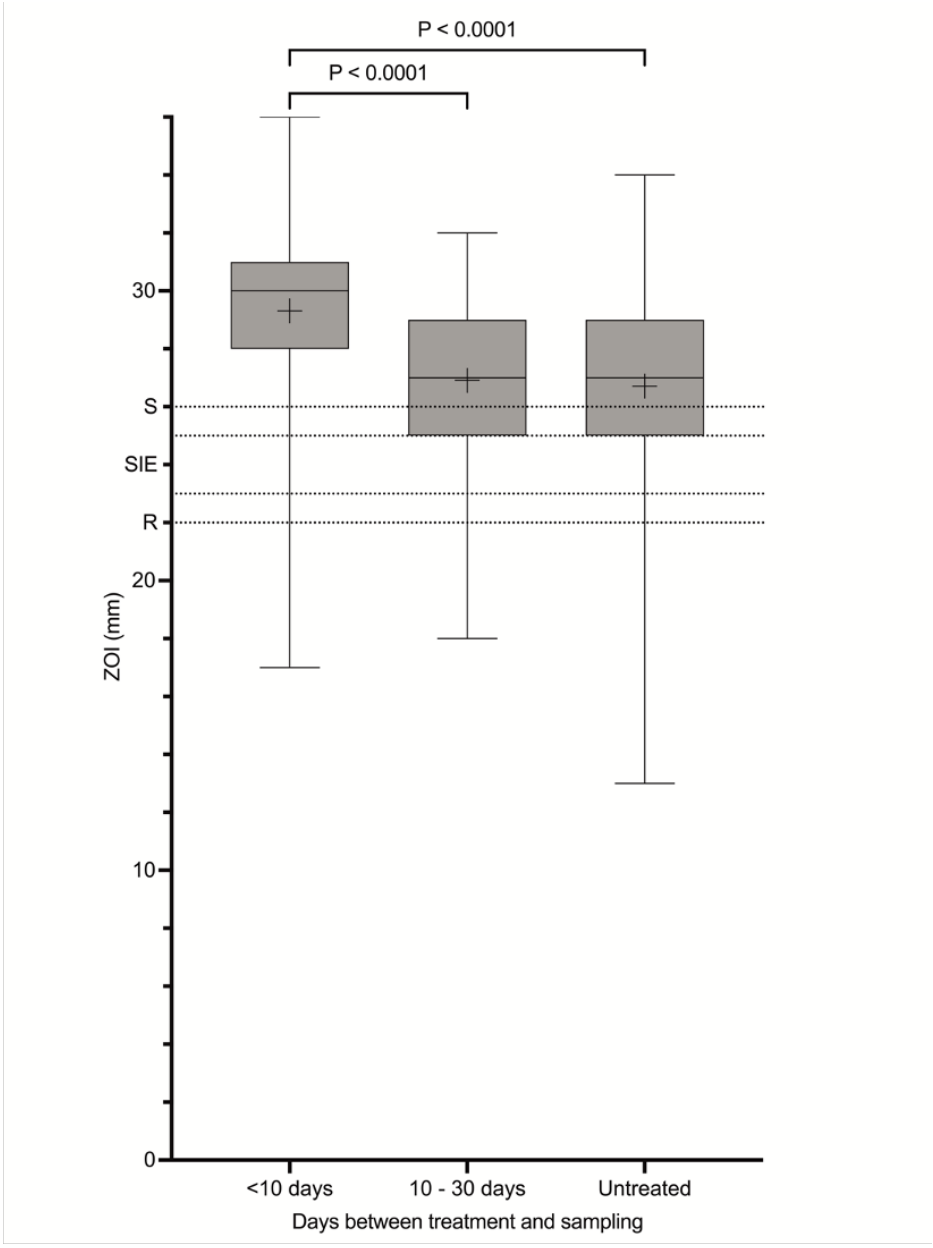


Figure 3.34: Median cefotaxime susceptibility distributions with increasing time between PPL treatment and sampling. The midline represents the median zone of inhibition (ZOI) across all isolates, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown by dotted ticks on y-axis: S (susceptible), SIE (susceptible with increased exposure), and R (resistant). The box colour indicates the median antibiotic susceptibility result: grey for susceptibility. The left box is the isolates from the PPL treated <10 days before sampling (n = 55), the middle box is the isolates from the PPL treated 10-30 days before sampling (n =65), and the right box is the isolates from untreated PPL (n = 283). The Kruskal-Wallis ANOVA test with Dunn's multiple comparisons test found significant differences in the median ZOI for cefotaxime in the isolates from the PPL treated <10 days before sampling compared to the isolates from the untreated PPL (ρ <0.0001). A significant difference was found in the median ZOIs for cefotaxime in the isolates from the PPL treated <10 days before sampling compared the isolates from the PPL treated 10-30 days before sampling (ρ <0.0001).

The median ZOI for streptomycin in the 55 isolates from the PPL treated within 10 days before sampling was 6 mm (resistant) with an interquartile range of 6-14 mm, in the 65 isolates from the PPL treated 10-30 days before sampling was 13 mm (SIE) with an interquartile range of 12-14 mm, and in the 283 isolates from the untreated PPL was 13 mm (SIE) with an interquartile range of 12-14 mm (Figure 3.35). The difference in the median ZOIs for streptomycin in the isolates from the PPL treated 10 days before sampling compared to the isolates from PPL treated 10-30 days before sampling was found to be significant (ρ = 0.001), and a significant difference was found in the median ZOIs for streptomycin in the isolates from the PPL treated 10 days before sampling compared to the isolates from the untreated PPL (ρ <0.0001) (Figure 3.35).

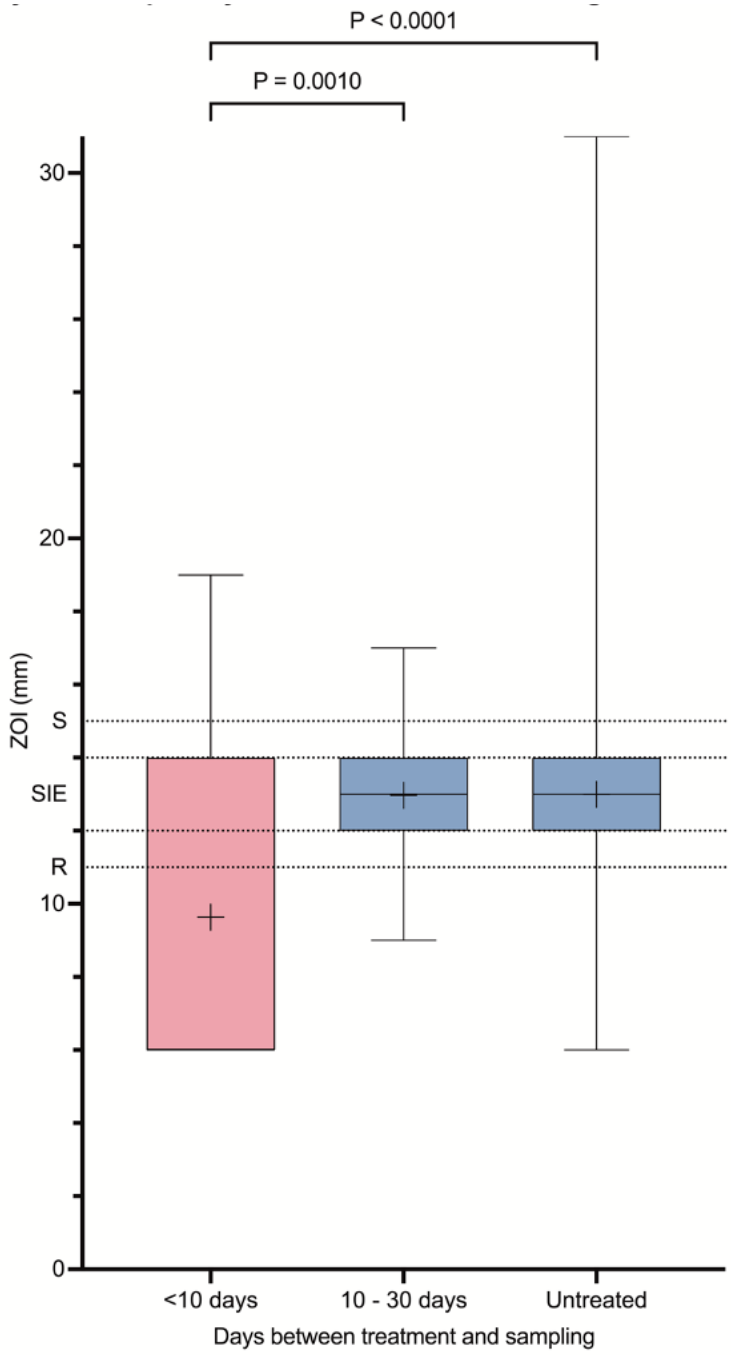


Figure 3.35: Median streptomycin susceptibility distributions with increasing time between PPL treatment and sampling. The midline represents the median zone of inhibition (ZOI) across all isolates, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown by dotted ticks on y-axis: S (susceptible), SIE (susceptible with increased exposure), and R (resistant). The box colour indicates the median antibiotic susceptibility result: pink for resistance and blue for SIE. The left box is the isolates from the PPL treated <10 days before sampling (n = 55), the middle box is the isolates from the PPL treated 10-30 days before sampling (n =65), and the right box is the isolates from untreated PPL (n = 283). The Kruskal-Wallis ANOVA test with Dunn's multiple comparisons test found significant differences in the median ZOIs for streptomycin in the isolates from the PPL treated <10 days before sampling compared to the isolates from the untreated PPL (ρ <0.0001) A significant difference in the median ZOIs for streptomycin in the isolates from the PPL treated <10 days before sampling compared to the isolates from the PPL treated 10-30 days before sampling (ρ = 0.001).

The median ZOI for sulfonamide in the 55 isolates from the PPL treated within 10 days before sampling was 6 mm (resistant) with an interquartile range of 6-20 mm, in the 65 isolates from the PPL treated 10-30 days before sampling was 20 mm (susceptible) with an interquartile range of 16-23 mm, and in the 283 isolates from the untreated PPL was 18 mm (susceptible) with an interquartile range of 15-21 mm (Figure 3.36). The difference in the median ZOIs for sulfonamide in the isolates from the PPL treated 10 days before sampling compared to the isolates from PPL treated 10-30 days before sampling was significant (ρ <0.0001). The median ZOIs for sulfonamide in the isolates from the PPL treated 10 days before sampling was also significantly different compared the isolates from the untreated PPL (ρ = 0.0002) (Figure 3.36).

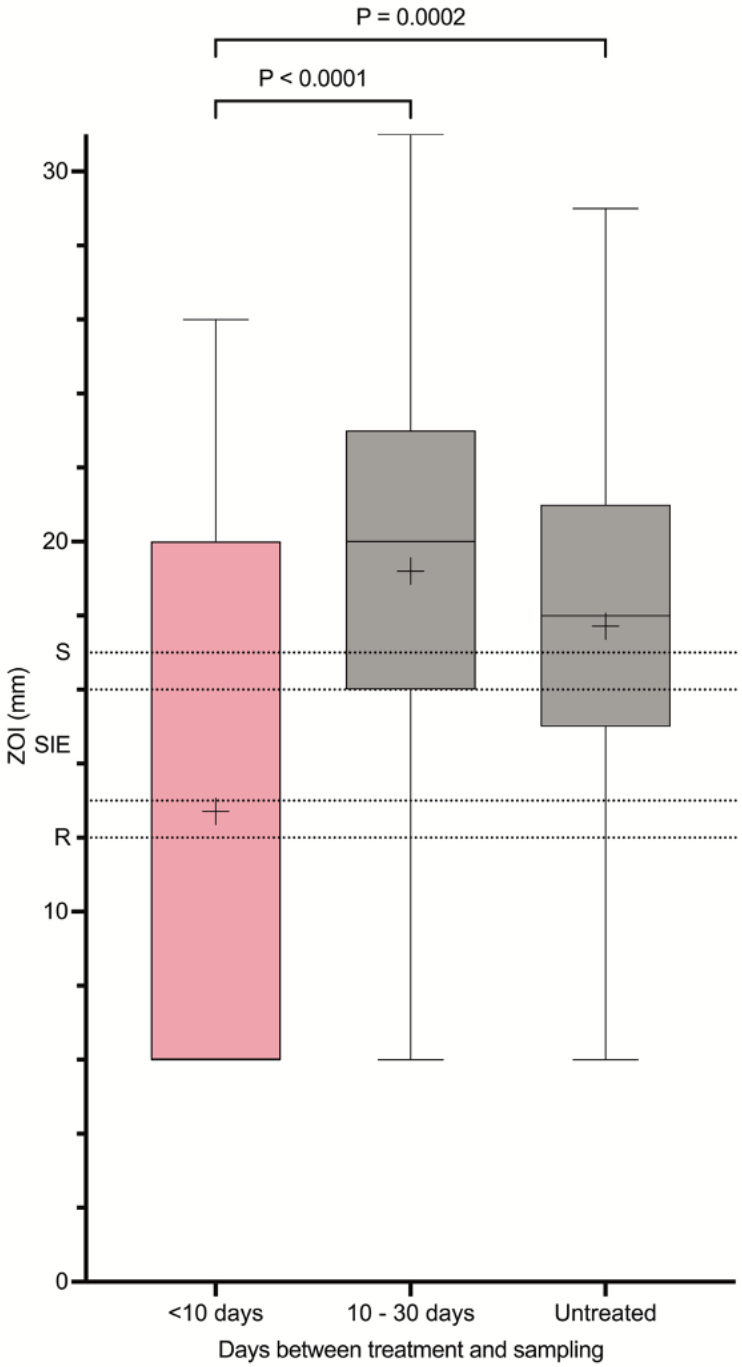


Figure 3.36: Median sulfonamide susceptibility distributions with increasing time between PPL treatment and sampling. The midline represents the median zone of inhibition (ZOI) across all isolates, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown by dotted ticks on y-axis: S (susceptible), SIE (susceptible with increased exposure), and R (resistant). The box colour indicates the median antibiotic susceptibility result: pink for resistance and grey for susceptibility. The left box is the isolates from the PPL treated <10 days before sampling (n = 55), the middle box is the isolates from the PPL treated 10-30 days before sampling (n =65), and the right box is the isolates from untreated PPL (n = 283). The Kruskal-Wallis ANOVA test with Dunn's multiple comparisons test found significant differences in the median ZOIs for sulfonamide in the isolates from the PPL treated <10 days before sampling compared to the isolates from the untreated PPL (ρ = 0.0002). A significant difference was found in the median ZOIs for sulfonamide in the isolates from the PPL treated <10 days before sampling compared to the isolates from the PPL treated 10-30 days before sampling (ρ <0.0001).

The median ZOI for chloramphenicol in the 55 isolates from the PPL treated within 10 days before sampling was 24 mm (susceptible) with an interquartile range of 23-25 mm, in the 65 isolates from the PPL treated 10-30 days before sampling was 23 mm (susceptible) with an interquartile range of 22-25 mm, and in the 283 isolates from the untreated PPL was 24 mm (susceptible) with an interquartile range of 22-25 mm (Figure 3.37). No significant differences were found in the median ZOIs for chloramphenicol in the isolates across the PPL treatment groups (Figure 3.37).

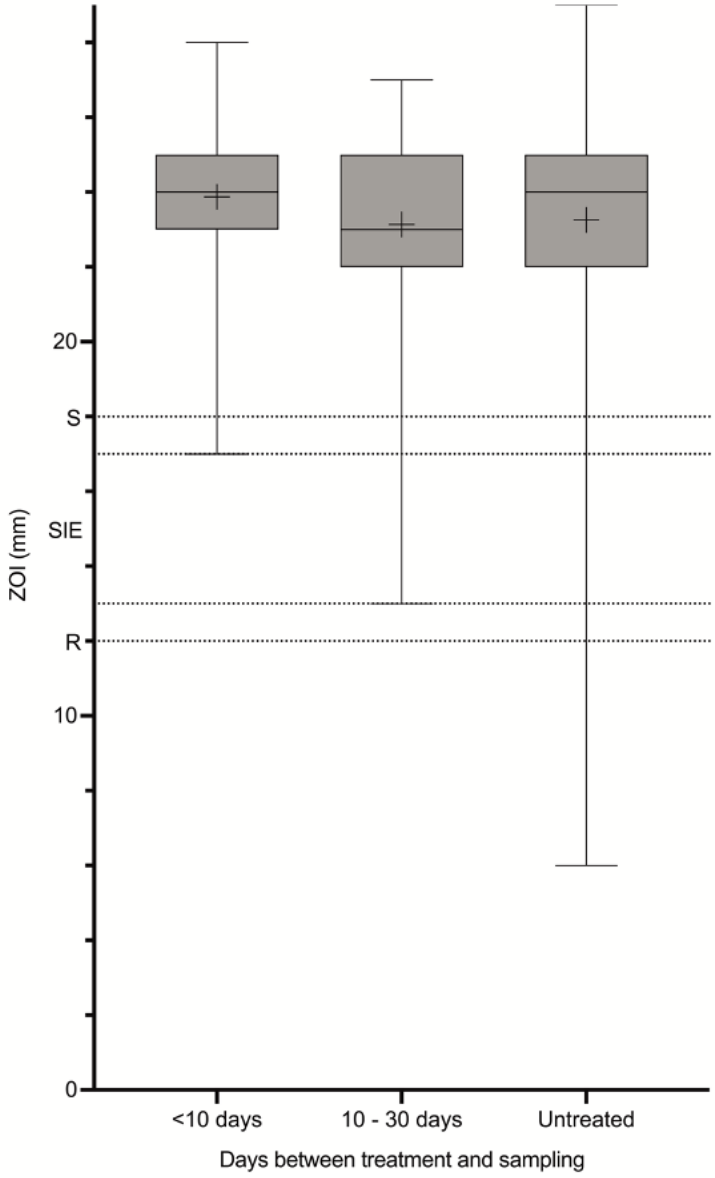


Figure 3.37: Median chloramphenicol susceptibility distributions with increasing time between PPL treatment and sampling. The midline represents the median zone of inhibition (ZOI) across all isolates, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown by dotted ticks on y-axis: S (susceptible), SIE (susceptible with increased exposure), and R (resistant). The box colour indicates the median antibiotic susceptibility result: grey for susceptibility. The left box is the isolates from the PPL treated <10 days before sampling (n = 55), the middle box is the isolates from the PPL treated 10-30 days before sampling (n =65), and the right box is the isolates from untreated PPL (n = 283). The Kruskal-Wallis ANOVA test with Dunn's multiple comparisons test found no significant differences in the median ZOIs for chloramphenicol in the isolates across the PPL treatment groups.

The median ZOI for ciprofloxacin in the 55 isolates from the PPL treated within 10 days before sampling was 31 mm (susceptible) with an interquartile range of 30-33 mm, in the 65 isolates from the PPL treated 10-30 days before sampling was 31 mm (susceptible) with an interquartile range of 26-34 mm, and in the 283 isolates from the untreated PPL was 31 mm (susceptible) with an interquartile range of 28-33 mm (Figure 3.38). No significant differences were found in the median ZOI for ciprofloxacin in the isolates across the PPL treatment groups (Figure 3.38).

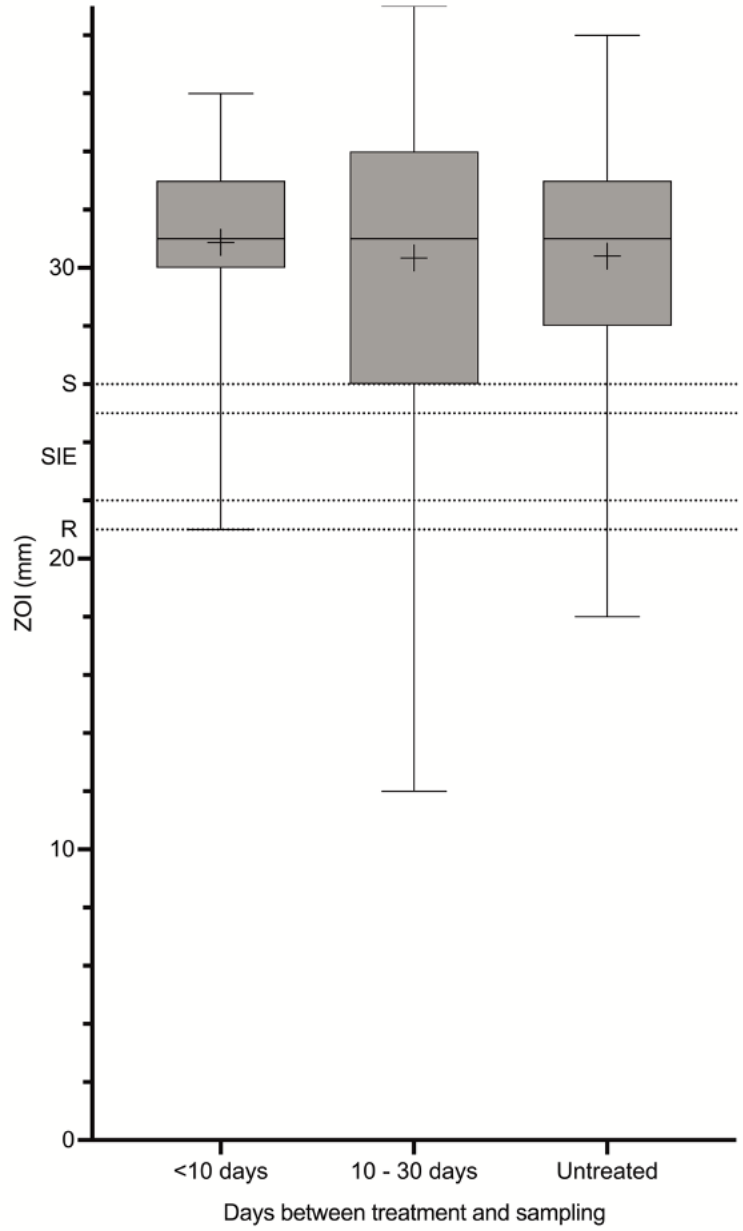


Figure 3.38: Median ciprofloxacin susceptibility distributions with increasing time between PPL treatment and sampling. The midline represents the median zone of inhibition (ZOI) across all isolates, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown by dotted ticks on y-axis: S (susceptible), SIE (susceptible with increased exposure), and R (resistant). The box colour indicates the median antibiotic susceptibility result: grey for susceptibility. The left box is the isolates from the PPL treated <10 days before sampling (n = 55), the middle box is the isolates from the PPL treated 10-30 days before sampling (n =65), and the right box is the isolates from untreated PPL (n = 283). The Kruskal-Wallis ANOVA test with Dunn's multiple comparisons test found no significant differences in the median ZOIs for ciprofloxacin in the isolates across the PPL treatment groups.

The median ZOI for tetracycline in the 55 isolates from the PPL treated within 10 days before sampling was 9 mm (resistant) with an interquartile range of 9-21 mm, in the 65 isolates from the PPL treated 10-30 days before sampling was 21 mm (susceptible) with an interquartile range of 20-21 mm, and in the 283 isolates from the untreated PPL was 21 mm (susceptible) with an interquartile range of 20-22 mm (Figure 3.39). The difference in the median ZOIs for tetracycline in the isolates from the PPL treated 10 days before sampling compared to those from PPL treated 10-30 days before sampling was significant (ρ = 0.0007). The median ZOIs for tetracycline in the isolates from the PPL treated 10 days before sampling was significantly different compared to the isolates from the untreated PPL (ρ <0.0001) (Figure 3.39).

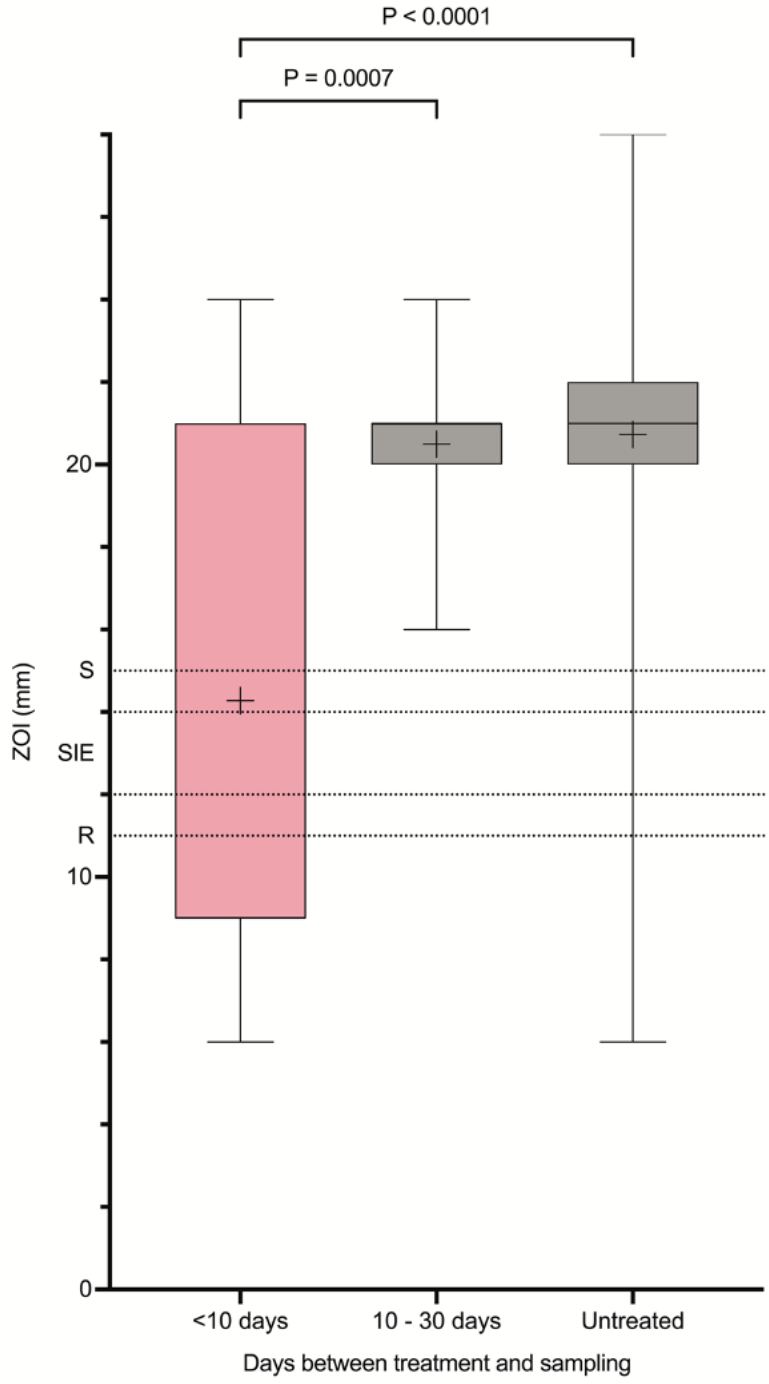


Figure 3.39: Median tetracycline susceptibility distributions with increasing time between PPL treatment and sampling. The midline represents the median zone of inhibition (ZOI) across all isolates, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown by dotted ticks on y-axis: S (susceptible), SIE (susceptible with increased exposure), and R (resistant). The box colour indicates the median antibiotic susceptibility result: pink for resistance and grey for susceptibility. The left box is the isolates from the PPL treated <10 days before sampling (n = 55), the middle box is the isolates from the PPL treated 10-30 days before sampling (n = 65), and the right box is the isolates from untreated PPL (n = 283).The Kruskal-Wallis ANOVA test with Dunn's multiple comparisons test found a significant differences in the median ZOIs for tetracycline in the isolates from the PPL treated <10 days before sampling compared to the isolates from the untreated PPL (ρ <0.0001). A significant difference was found in the median ZOIs for tetracycline in the isolates from the PPL treated 10-30 days before sampling compared to the isolates from the PPL treated 10-30 days before sampling (ρ = 0.0007).

3.4 Discussion

These results represent a focused approach aimed at understanding the antibiotic susceptibility of faecal *E. coli* on a single farm. These results show the overall antibiotic susceptibility of 403 *E. coli* isolates collected from 18 PPL on one farm, comparable in size to the annual VARSS dataset for England and Wales (Veterinary Medicines Directorate, 2023b). While a representative sample of the 1.844 million cows in the UK milking herd is not feasible, the VARSS dataset is the most representative currently available, being the largest available collected across the UK (AHDB, 2023). *E. coli* were collected directly from faeces samples to link the ABR in these isolates to the PPL from which they were taken. Investigating ABR in bovine faeces is important as this is a known source of pathogenic bacteria that impacts the health and productivity of UK dairy herds. ASRs in the VARSS report are presented and discussed as ASLs, specifically levels of SIE and resistance (i.e., what proportion of the isolates were not susceptible to a given antibiotic). This thesis proposes an additional analysis for interpreting antibiotic susceptibility in a population of the isolates, the MASD, plotting the median ZOI for all isolates or a subset of the isolates against each antibiotic. The MASDs could provide more detail and nuance around antibiotic susceptibility and shifts within the population over time than a percentage alone. MASDs would require no additional sampling or laboratory work yet could improve our ability to measure the outcome of antibiotic stewardship policy. Is it possible to improve ABR monitoring using metrics such as the MASDs and the established ASLs, and could this improved understanding inform better data-driven policy design?

3.4.1 Successful isolation of *E. coli* from PPL faeces samples on TBX

The first challenge in this work was sample collection and rapid processing. Samples had to be collected by a qualified vet as part of their routine work and transported to SVMS. These samples were processed rapidly to collect the most representative and diverse set of *E. coli.* Toxigenic *E. coli* were avoided for the safety of further work and to remove the need for a microbiology safety cabinet. TBX media was used as it selects for *E. coli* and removes toxigenic strains; this simplifies the process of identifying *E. coli* and reduces the safety risk of culturing *E. coli* from dairy cows, which are considered reservoirs of toxigenic strains (Hancock *et al.*, 1994; Mechie, Chapman and Siddons, 1997). However, when samples are stored on TBX, white colonies grow, making it challenging to identify the blue/green morphology of non-toxigenic *E. coli.* This limitation resulted in a bottleneck that limited the number of *E. coli* that could be isolated from a single sample within the study parameters. While not a significant result of this work, the successful isolation of *E. coli* from all 18 samples on TBX was critical in all work that followed.

3.4.2 Estimating bacterial abundance in faecal samples through viable counts

Unfortunately, collecting reliable bacterial counts was challenging due to the variation in sample compositions impacting the accuracy of pipetting in the serial dilution. The consistency and the amount of fibrous and gritty aggregates was variable across the faecal samples. For several samples, the bacterial count was recorded as greater than the limit of quantification because every aliquot of the lowest dilution was above the reliably countable range. To avoid this in future work, the ratio of solid sample and liquid added could be

adjusted. This dilution step would reduce pipetting errors and increase the accuracy of all subsequent dilutions. Additionally, samples could be filtered through filter paper or centrifuged at speeds sufficient to remove large aggregates without removing bacteria. To confirm that any filtration/centrifugation does not remove a significant proportion of bacteria, replicates of aliquots which have not undergone the additional process can be plated and counted in parallel.

3.4.3 Antibiotic supplementation of TBX to enrich samples for antibiotic resistant *E. coli*

Antibiotics were included in isolation media to ensure that if resistant isolates were exceedingly rare in the samples, they could be enriched. This method has also been used to determine antibiotic resistant proportions in samples, rather than picking and purifying isolates before completing AST, which would have been significantly faster and would have screened a greater proportion of the isolates in a given sample (Hoyle *et al.*, 2004). However, several isolates that grew on selective plates were later confirmed to be susceptible to that class of antibiotic, therefore, this procedure does not guarantee that only resistant isolates can grow. Cefotaxime (2 $\mu g.ml^{-1}$) was one of the antibiotics added to TBX, and no colonies grew on this supplemented media. However, 20 isolates picked from TBX media with no antibiotic selection were confirmed as cefotaxime resistant through disk diffusion. This apparent difference in antibiotic susceptibility may be due to inconsistencies in antibiotic concentration in the agar or differences in the diffusion of antibiotics in TBX rather than MH2, the validated media (EUCAST, 2022). These results suggest that growth on TBX supplemented with an antibiotic is insufficient to determine

the susceptibility of an isolate to that antibiotic. Enrichment with antibiotics successfully isolates more resistant and SIE isolates than TBX alone. However, antibiotics also reduced the number of *E. coli* isolated from a sample. The inclusion of penicillin increased the abundance of *E. coli*; this may be due to the inherent resistance of *E. coli* to penicillin, reducing the abundance of other bacteria, which may grow slowly on TBX. Including all other antibiotics in TBX reduced the abundance of *E. coli*. This may have decreased the diversity of *E. coli* isolates collected by selecting for clonal isolates, but investigating this further was outside the scope of this work. The antibiotic susceptibility of the isolates collected from antibiotic supplemented media was determined, but these isolates were not included in this analysis. The exclusion of the isolates from TBX supplemented with antibiotics was to avoid unintentionally and immeasurably biasing the data; the ASL and MASD of all isolates are available (Appendix 9 and Appendix 10).

3.4.4 Overall faecal *E. coli* antibiotic susceptibility levels

As stated previously, ASLs are the preferred method for reporting AMR in the UK and the EU; this approach takes varied and complex samples and distils the antibiotic resistome into categorical values for comparison. These AST results provide context for antibiotic prescriptions on this dairy farm and raise essential questions about interpreting the risk of emerging ABR. Most of the *E. coli* isolates from these PPL samples are treatable with the antibiotics tested. Ampicillin was the only antibiotic with more than 20% of the isolates shown to be resistant, which is a concern. These were moderate levels of resistance to streptomycin and sulfonamide and low levels of resistance to amoxicillin-clavulanate, cefotaxime, chloramphenicol, ciprofloxacin, and

tetracycline. These findings indicate a minimal risk of ABR in faecal *E. coli* when these samples were collected. The risk of resistance in these PPL faecal *E. coli* isolates is higher against penicillins without β-lactamase inhibitors, aminoglycosides, and sulfonamides. Very high and high levels of non-susceptibility to ampicillin, streptomycin and sulfonamide are cause for concern, especially considering the most commonly used antibiotics given to PPL known to have received chemotherapy were β-lactams and aminoglycosides. How AST results are interpreted is crucial for assessing the risk of ABR in a sample. Grouping the SIE results with the resistant results (non-susceptibility), as presented in the VARRS report, more than 20% of the isolates are resistant to ampicillin, cefotaxime, streptomycin, and sulfonamide. The higher level of non-susceptibility may be due to the higher use of these antibiotics on this farm. However, without the AMU of the SB farm, it is not possible to know if the AMU seen in these PPLs is representative of the farm. The most used antibiotics on the SB farm in 2017 were aminocoumarins, aminoglycoside and β-lactams in combination, β-lactams, and tetracyclines (Baker *et al.*, 2022). If this AMU continued into the period when the α samples were collected in 2019, then the levels of β-lactam and aminoglycoside resistance are not surprising. Additionally, if these antibiotics have been routinely used and remain effective, this suggests that resistance in faecal *E. coli* to these antibiotics is not currently affecting clinical outcomes on the SB farm. This underscores the importance of ongoing surveillance to identify trends in the risk of antibiotic resistance in bovine faecal *E. coli*.

3.4.4.1 Comparing the antibiotic susceptibility levels of the α *E. coli* to the VARSS *E. coli* clinical surveillance dataset

The levels of non-susceptibility (resistance and SIE) in the α *E. coli* isolates can be compared to the VCD (Veterinary Medicines Directorate, 2021a, 2022a, 2023a). The *E. coli* isolates from the α samples had higher non-susceptible levels to cefotaxime (30% v 5%), streptomycin (80% v 40%), and fluoroquinolones (13% v 8%); equivalent non-susceptible levels to ampicillin (60%) and sulfonamide (36% v 40%); and lower non-susceptible levels to amoxicillin-clavulanate (12% v 30%), chloramphenicol (2.5% v 40%), and tetracycline (6% v 54%). The VCD also included *E. coli* collected from clinical mastitis cases; the *E. coli* isolates from the α samples had higher non-susceptible levels to amoxicillin-clavulanate (12% v 6%), streptomycin (80% v 5%), sulfonamide (36% v 10%) and fluoroquinolones (13% v 2%), with an equivalent non-susceptible level to ampicillin (60%), and a lower non-susceptible level to tetracycline (6% v 13%).

The similar ASL observed against some of the antibiotics in this thesis and the national VCD provide some weight to the validity of these results. These similarities suggest that more focused regional sampling could produce data of equivalent quality, at a fraction of the cost of nationwide schemes. However, the ASL for some of the antibiotics tested were very dissimilar. In these cases, the α isolates benefit from linked metadata that could provide explanations for increased ASL. The optimum dataset would be the national VCD with linked metadate back to the sample source.

A new approach is needed for further antimicrobial stewardship; switching the antibiotic of choice would not lower the overall level of resistance, but would only shift it from one antibiotic to another. This shift would likely result in no net change to the risk posed by AMR in a herd of dairy cows.

3.4.5 Benefits of the median antibiotic susceptibility distribution

MASDs are presented to investigate the antibiotic susceptibility of these isolates further. The MASD combines the ZOI of all the isolates from a population to provide the median antibiotic susceptibility and the range of ZOI for each antibiotic. The MASD can provide more information about the diversity of antibiotic susceptibility of the *E. coli* collected from these samples than the ASL alone. This approach presents the median ZOI. Therefore, if the breakpoints change, the susceptibility result can be updated. Including the ZOI of a data set would enable the comparison of data sets collected at different times with the most up-to-date clinical breakpoints.

3.4.6 The variability of antibiotic susceptibility differs depending on the antibiotic

Variability in the antibiotic susceptibility of *E. coli* across the PPL differed across the antibiotics tested. Across all of the isolates, there were low levels of non-susceptibility to chloramphenicol, ciprofloxacin, and tetracycline. Across most isolates, there were low levels of non-susceptibility to amoxicillin-clavulanate and cefotaxime. However, the isolates from some PPL had concerning levels of non-susceptibility. There were high levels of non-susceptibility to ampicillin, streptomycin, and sulfonamide across most isolates, while the isolates from a few PPL had high levels of susceptibility. The MASDs of the isolates from PPL 1 and PPL 9 were distinct from the

isolates from other PPLs. Only PPL 1 had a median MDR susceptibility profile, with resistance to antibiotics from four different classes, and only PPL 12 was susceptible to all antibiotics tested. While PPL 1 was not the only PPL to receive chemotherapy, it did receive amoxicillin four to six days before sampling; this might suggest that the full impact of antibiotic treatment on the faecal microbiome is not instantaneous. These ASRs suggest a stable faecal *E. coli* antibiotic resistome, likely due to the consistent housing and feeding conditions of these PPL.

3.4.7 Impact of bovine chemotherapeutic treatment on the antibiotic susceptibility of faecal *E. coli*

In the samples from known treated PPL, amoxicillin was the most commonly used antibiotic, and the second most common was Tetra-Delta (aminocoumarin, aminoglycoside, β-lactam, and anti-inflammatory). If this is assumed to be the same across the herd, then the high levels of ampicillin resistance, moderate levels of streptomycin resistance, and very high levels of streptomycin SIE are clinically concerning but not surprising. Veterinarians might choose alternate antimicrobials, knowing the level of non-susceptibility present on the farm, but the only option may be to progress with the standard course while assuming resistance is likely. There were observable differences between the isolates from the treated PPL and the untreated PPL MASD. At the same time, the susceptibility profile remained the same; a greater proportion of the isolates had lower ZOIs, displayed by the larger range of the lower quartile. Significantly different MASDs and lower median ZOIs were found for ampicillin, streptomycin, sulfonamide, and tetracycline. This significance was determined by the KGS test comparing the cumulative

distributions; It is important to state that this test does not determine if two medians are significantly different; it determines if the data around two medians are distributed differently. These differences suggest that the common use of β-lactams, tetracyclines, and aminoglycosides might contribute to increased levels of ABR in the dairy industry. The impact of observed *E. coli* resistance on the clinical outcomes in the sampled PPL is unknown, but the resistant organisms and the genes they carry are shed into the environment, and faecal *E. coli* are implicated in conditions such as mastitis, with long-term impacts and significant financial implications (Shpigel, Elazar and Rosenshine, 2008; Blum, Heller and Leitner, 2014; Rózańska *et al.*, 2019). Antibiotic treatment of dairy cows, particularly β-lactams, is known to increase the risk of ABR faecal *E. coli* or bacteria (Donaldson *et al.*, 2006; Singer, Patterson and Wallace, 2008; Chambers *et al.*, 2015). However, the magnitude and persistence of this shift varies, or was not observed across different studies (Donaldson *et al.*, 2006; Sawant *et al.*, 2007; Singer, Patterson and Wallace, 2008; Feng *et al.*, 2020; Feng, Chambers and Knowlton, 2020).

3.4.8 Impact of time after bovine chemotherapeutic treatment on the antibiotic susceptibility results of faecal *E. coli*

Several reports have found that proportions of ABR isolates rise after antibiotic treatment and fall within 17 days (Jiang *et al.*, 2006; Singer, Patterson and Wallace, 2008). The bacterial diversity of bovine faecal microbiomes have been reported to decrease after antibiotic treatment, and then gradually recover at variable rates for individual microbiomes (Collis *et al.*, 2024).

This data in this chapter supports the hypothesis that there is an increased risk of ABR faecal *E. coli* after chemotherapeutic treatment, and that this risk decreases as the selective pressure is reduced. Most of the difference between isolates from treated and untreated PPL can be attributed to the isolates from PPL that had received treatment within 10 days before sampling. The difference can be seen in the MDR median ASRs, specifically the significantly different MASDs against all antibiotics except chloramphenicol and ciprofloxacin. A result of critical importance is the differences observed between isolates from PPL that received treatment less than 10 days before sampling and from PPL that received treatment 10-30 days before sampling. The ASLs in the isolates from PPL that received chemotherapy 10-30 days before sampling had mostly returned to those observed in the isolates from untreated PPL. This could guide policies and farm practices on appropriate handling of animal waste for 10 days after treatment. For instance, it may become cost-effective to take more care when cleaning this animal as faecal contamination with antibiotic resistant *E. coli* would be harder to treat. Other work has assessed the risk posed when spreading faeces as fertiliser, and a time-dependent reduction in ARG prevalence was observed in the slurry tanks on the SB dairy farm (Baker *et al.*, 2022). Baker *et al.,* 2022 suggest a 60 day storing period providing a 99% reduction in cephalosporin resistance; the shift in antibiotic susceptibility as time after PPL treatment increases adds evidence to allowing a ‘cool off’ period post chemotherapeutic treatment to minimise the risk of spreading resistant bacteria.

3.5 Limitations and future work

This study used convenience samples to reduce the impact on the well-being of the PPL and to reduce the time required of the veterinarian to collect the samples. Therefore, it was not possible to select a PPL of particular interest or to sample the faeces of the same PPL more than once. The random availability of samples resulted in fewer samples taken from PPL that received chemotherapy before sampling than those from PPL that did not receive chemotherapy before sampling. If the sampling methodology could be designed to collect representative samples of faeces from different PPL treatment groups or even sample a group of PPL longitudinally, this could provide more robust data to investigate the effect of chemotherapeutic treatment on faecal *E. coli* antibiotic susceptibility.

Due to methodology, the number of isolates that could be picked from a single sample was limited. Samples could only be plated up to 24 hours following sample collection to reduce the potential impact of bacterial growth and competition after sampling, which would not reflect the environment in PPL. This work did not assess the impact of storing samples in the fridge for more than 24 hours after sampling. However, no significant difference was found in the composition of the microbial communities of human faecal samples stored at room temperature or 4°C (24 hours) and -20°C or -80°C (1 week) (Tedjo *et al.*, 2015). While the 403 *E. coli* represent a large dataset, the mean abundance of *E. coli* in these samples was 1.77×10^5 CFU g^{-1} of faeces and 2.4×10^9 CFU g^{-1} of faeces. Therefore, it is likely that the true diversity of faecal *E. coli* in these PPLs has not been captured. The actual number of individual *E. coli* in the α isolates may be lower than 403 as this chapter has not

investigated clonality. These 403 *E. coli* could be cultured from glycerol stocks, allowing for further investigation.

If resistant isolates had represented such a small proportion of the population that they could not be identified on TBX alone, antibiotics were added to media to inhibit the growth of susceptible isolates and enrich the samples for resistance phenotypes. This approach has been utilised in this thesis as well as resistance surveillance in several regions, and while it was successful in identifying resistant strains, it reduced the number of isolates that were collected overall (Ibrahim *et al.*, 2016, 2023; Baker *et al.*, 2022; European Food Safety Authority, 2022; Veterinary Medicines Directorate, 2023b). The EU harmonised monitoring protocol to isolate ESBL or AmpC-producing *E. coli* calls for pre-enrichment of the sample with low levels of a carbapenem (e.g., 0.125 $\mu g.ml^{-1}$ meropenem) before supplementing MacConkey agar with a carbapenem (1 $\mu g.ml^{-1}$) (Hasman *et al.*, 2015). The VARSS data reported for harmonised monitoring in 2022 supplemented MacConkey agar with 1 $\mu g.ml^{-1}$ cefotaxime to identify ESBL or AmpC in caecal samples from healthy broilers and turkeys at slaughter (Veterinary Medicines Directorate, 2023b). While the UK has left the EU and has no legal requirement to continue this work, if the UK intends to sell produce to the EU, the UK may be required to match the standards required for EU food production. The method used in this thesis would be improved by plating control strains with known resistance phenotypes to validate that the concentration of antibiotics in the plate is sufficient to inhibit the growth of susceptible isolates but sub-inhibitory for resistant isolates. Unfortunately, the ASLs found in the α isolates are not directly comparable with the VCD due to differences in the antibiotics in the

respective testing panels. The VCD collected antibiotic susceptibility for trimethoprim with sulfonamide in combination rather than sulfonamide alone and enrofloxacin rather than ciprofloxacin. However, the ARG necessary for resistance to the different antibiotics tested are shared; if an isolate is resistant to the antibiotics tested in the VCD, it will likely be resistant to the antibiotic of the same class the α isolates were tested with. The median ZOIs can be statistically compared, but the statistical power is low for each sample due to the low sample size of *E. coli* collected from each sample, and the statistical power is low comparing medians across all samples due to the high variability within and between samples. Ultimately, longitudinal sampling of the same PPL before, during, and after treatment is required to determine the impact of chemotherapy on AMR.

3.6 Conclusion

The AST of these *E. coli* isolates represents a significant increase in the data available on the levels of ABR in UK dairy cows, providing more evidence to understand the risks associated with current practices. The high levels of resistance and SIE to ampicillin and streptomycin in these isolates are concerning. However, penicillins and aminoglycosides are commonly used antibiotics from the known treatment records. Therefore, the observed resistance is expected. This information may encourage vets on this farm to consider alternate antibiotics when necessary. There is an observable relationship between chemotherapeutic treatment and the level of ABR in faecal *E. coli* and the time for resistant phenotypes to be selected and removed from a population. The ASL is the standard method for AMR surveillance in Europe when interpreting AST results for large datasets. The ASL distils

potentially large datasets into categorical values for simple communication and enables comparison of data from studies with different sample sizes, geographical regions, and ecological niches. The additional analysis of MASD can provide more nuanced information, interpreting population antibiotic susceptibility through the median and the range of ZOI. The MASD presents the average susceptibility result but could also highlight the presence of different populations. For example, a small proportion of resistant isolates, which may have a competitive advantage when antibiotics or other selective agents are present. Assessing only the ASL could miss the early warning of broad MASDs or significant levels of SIE. Broad MASDs suggest that while the median ASR may be susceptible, and susceptible levels may be low to moderate, a population of isolates are present that could become prevalent as soon as a selective pressure is introduced. This analysis could inform AMU decisions and improve the likelihood of successful outcomes of antibiotic stewardship. Importantly, MASDs can be plotted for all historical datasets based on the same disk diffusion results used to determine ASLs.

A low level of MDR phenotypes were identified in these isolates, which is a positive result. However, the level of transmissible MDR phenotypes has not been determined in this chapter. Transmissible MDR would be more concerning as these phenotypes can persist in the environment with minimal selective pressure. The next chapter will assess the risk of transmissible MDR phenotypes through *in vitro* conjugation experiments and short-read WGS.

Chapter 4 Investigating *E. coli* clonality and transmissible MDR

While 508 *E. coli* colonies were picked across all media types from the α sample set, it is important to understand how distinct these isolates were from one another. This informs discussions on the diversity of opportunistic pathogens such as *E. coli* in dairy cows on this dairy farm. In the context of ABR, reduced diversity of *E. coli* paired with high levels of ABR would suggest there are selective pressures in this ecological niche that are selecting for ABR isolates. MGE can increase the diversity of a population and reduces the fitness cost of carrying ARG when selective pressures are minimal. This chapter will use conjugation experiments with MDR *E. coli* to identify candidates for short-read WGS. The *E. coli* genomes produced in this process will be screened to identify ARG and plasmid incompatibility replicons, as well as investigate the clonality of these isolates with MLST and *in silico* serotype prediction.

4.1 Introduction

4.2 Molecular genotyping of *E.coli* with the *Enterobacteriaceae* repetitive intergenic consensus polymerase chain reaction

ERIC-PCR is a molecular genotyping technique that has been used previously on the SB Farm and in other studies of *Enterobacteriaceae* (Versalovic, Koeuth and Lupski, 1991; Oltramari *et al.*, 2014; Ibrahim *et al.*, 2016; Chukwu *et al.*, 2019). Concerns on the discriminatory power and reproducibility of this technique have been reported previously (Meacham *et al.*, 2003; Wilson and Sharp, 2006).

4.3 Multidrug resistance has been identified in *E. coli* from the SB farm and in national and global datasets

MDR describes microorganisms that are resistant to an antimicrobial in three or more different antimicrobial classes (Magiorakos *et al.*, 2012). MDR bacteria have been identified as a serious public health problem (WHO, 2015). The reported rates of MDR *E. coli* in dairy samples range from 29% in mastitis cases in Canada, to 44.4% sampled from udders in China (Majumder *et al.*, 2021; Shoaib *et al.*, 2023). The VCD reported high levels of MDR *E. coli* from cattle samples in 2020 (43%), 2021 (41%), and 2022 (40%) (Veterinary Medicines Directorate, 2023b). On the SB farm; a high level (37%) of *E. coli* collected from the slurry tank in 2017 were MDR (Baker *et al.*, 2022), while very high levels (57.9%) of MDR *E. coli* were collected from bovine faecal and slurry tank samples, with a moderate level (19.8%) of plasmid mediated ESBL-positive *E. coli* between 2012 and 2014 (Ibrahim *et al.*, 2016). MDR phenotypes, such as these ESBL-positive *E. coli* that can be shared through MGE are more concerning. There is evidence of transmission of ARG between bacteria of the same and different species, as well as transmission of ARG between animals and humans (Rafaï *et al.*, 2015; Madoshi *et al.*, 2016; Salerno *et al.*, 2022; Thuan *et al.*, 2022).

4.4 Aims

This chapter aimed to investigate the MDR α *E. coli* collected in Chapter 3. These isolates were used to;

1. Asses the molecular genotyping approach of ERIC-PCR to determine the clonality of the α *E. coli*
2. Determine the level of MDR *E. coli* , and how was the level of MDR affected by;
    a. Antibiotics supplemented into TBX
    b. PPL treatment group
3. Conduct conjugation experiments to identify *E. coli* with transmissible MDR phenotypes
4. Screen short-read WGS with ABRicate for ARG, ST, serotype, and plasmid incompatibility groups

4.5 Methods

4.5.1 DNA extraction for ERIC-PCR

The method of DNA extraction outlined in section 2.8 was selected for its high throughput, low cost nature. Aliquots of overnight cultures were boiled at 100°C for 5 minutes to lyse the cells. Lysates were centrifuged, cell debris pellets were discarded, and the DNA supernatants were collected.

4.5.2 ERIC-PCR protocol and analysis

The ERIC-PCR protocol was adapted from previous work (Woods *et al.*, 1993; Ibrahim *et al.*, 2016). Details on the reaction components and conditions are listed in section 2.8, Table 2.6, and Table 2.7.

4.5.3 Generation of kanamycin resistance in *E. coli* DH5 α

DH5 α was spread onto a MH2 plate supplemented with kanamycin (64 $\mu g.ml^{-1}$) and after overnight incubation, colonies were picked. The kanamycin resistance and streptomycin susceptibility of these isolates was confirmed by Kirby-Bauer disk diffusion. The first confirmed kanamycin resistant and streptomycin susceptible isolate was selected and stored in a glycerol cryovial (DH5 α-KAN_R) (section 2.9).

4.5.4 Screening α isolates for kanamycin susceptibility

Kanamycin was not included in the α sample antibiotic susceptibility testing panel. Each isolate was streaked onto MH2 supplemented with kanamycin (64 $\mu g.ml^{-1}$). The isolates that grew on this media were assumed resistant to kanamycin and not included in subsequent work. The susceptibility to kanamycin of the isolates which did not grow on this media was confirmed by the Kirby-Bauer disk diffusion method (section 2.9). This approach of

screening all of the isolates before completing AST was necessary because of COVID related laboratory access constraints at the time of work.

4.5.5 Bacterial conjugation to identify transmissible MDR phenotypes

The detailed methodology for the conjugation protocol is details in section 2.9 and a diagrammatic representation is available (Figure 2.1). An overnight culture of the DH5-α KAN_R recipient strain and each potential donor isolate was prepared. Equal volumes of each donor and the recipient strain were added to 100 µl aliquots. A piece of sterile filter paper was placed onto NA for each mixed culture, before the 100 µl aliquot were pipetted onto their respective filter papers. After overnight incubation, growth was picked from these plates and streaked onto TBX containing streptomycin and kanamycin. A single colony of each transconjugant was picked for each mixed aliquot, and named 'donor isolate ID'X. The antibiotic susceptibility of the transconjugant isolates was determined by the Kirby-Bauer disk diffusion method (section 2.7).

4.5.6 DNA extraction for short-read whole genome sequencing

To collect high quality DNA extractions for WGS, the manufacturer's instructions for the DNeasy Ultraclean Microbial Kit (QIAGEN) were followed (section 2.10).

4.5.7 Using ABRicate to identify ARG, sequence type, serotype, and plasmid incompatibility groups in *E. coli* whole genomes

A detailed breakdown of the bioinformatic approach in this chapter is available (section 2.11). Assembled annotated genomes were provided by MicrobesNG and ABRicate (v1.0.0) was used to screen contigs against a selection of ARG databases (Seemann, no date, Accessed: December 2022). To identify

acquired ARG the databases used included CARD (v3.1.4), MEGARes (v2.0), NCBI ARG (v3.10.18), ResFinder (v4.0), and ARG-ANNOT (v4.0) (Zankari *et al.*, 2012a; Gupta *et al.*, 2014; Jia *et al.*, 2017; Feldgarden *et al.*, 2019; Doster *et al.*, 2020). The EcOH database was screened for *in silico* serotyping of *E. coli* (v2.0) (Ingle *et al.*, 2016). The PlasmidFinder (v2.1.1) database was screened to identify plasmid incompatibility groups present in the contigs (Carattoli *et al.*, 2014). STs were assigned using the Achtman seven gene MLST scheme available in Enterobase (Zhou *et al.*, 2020). The clonality of the genomes was also investigated with the whole genome MLST scheme (wgMLST). Genomic variation was visualised using the GrapeTree plot functionality of Enterobase using the MSTreeV2 Algorithm (Zhou *et al.*, 2018).

4.6 Results

4.6.1 Assessment of isolate clonality by ERIC-PCR

A pilot study was completed with four α isolates to investigate the feasibility of screening all isolates by ERIC-PCR. The isolates included were selected because their distinct ASPs suggested non-clonal isolates. A sample of DNA was extracted from each isolate and triplicate PCR reactions were completed. ERIC-PCR provided a unique banding pattern for each isolate. However, the same banding pattern was not produced for each PCR reaction of the same DNA extraction (Figure 4.1).

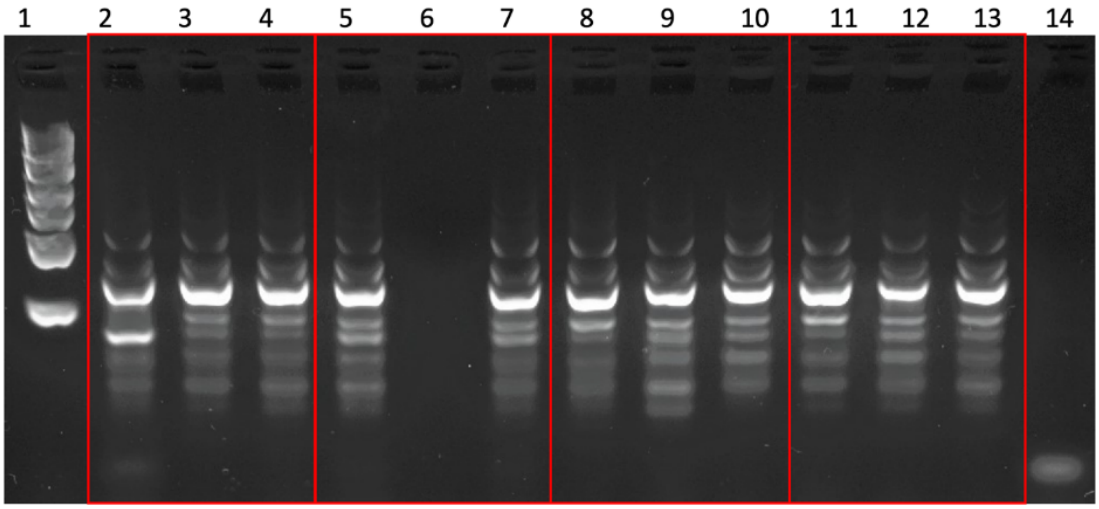


Figure 4.1: Gel image of ERIC-PCR products. Lane 1 contains a 1kB ladder. Lanes 2-4 are PCR products of isolate 2. Lanes 5 & 7 are PCR products of isolate 26. PCR products from isolate 26 were also loaded into lane 6, but this well had collapsed and contents did not pass through the gel. Lanes 8-10 are PCR products of isolate 38. Lanes 11-13 are PCR products of isolate 52. Lanes 14 is a no DNA control. These isolates were picked due to their phenotypic differences in disk diffusions, with the intention of producing distinct banding patterns. All PCR products were technical triplicates from an individual DNA extract.

4.6.2 Successful isolation of multidrug resistant faecal *E. coli*

The number of the isolates that were resistant to a given number of antibiotic classes and the number which were MDR across all samples and isolation medias are presented in Table 4.1. Across the 508 isolates collected from all media types, 64 isolates, or a moderate level (12%) were MDR. The MDR level was low (6.7%) for the isolates picked from TBX without antibiotic supplementation, and moderate (17.7%) for the isolates picked from TBX supplemented with penicillin. The levels of MDR was extremely high for the isolates picked from TBX supplemented with spectinomycin (94.1%) and TBX supplemented with tetracycline (77.7%).

Table 4.1: Number of α isolates that were resistant to a given number of antibiotic classes isolated on different media compositions.

		Resistance to antibiotic classes (n)								
		0	1	2	3	4	5	6		
	Isolation media	Non-MDR (n)			MDR (n)				MDR (%)	MDR level
E. coli isolates	All (n = 508)	269	125	50	9	50	5	0	12	Moderate
	TBX (n = 403)	230	110	36	8	19	0	0	6.7	Low
	TBX + PCN (n = 79)	39	14	12	0	14	0	0	17.7	Moderate
	TBX + SPT (n = 17)	0	1	0	1	15	0	0	94.1	Extremely high
	TBX + TET (n = 9)	0	0	2	0	2	5	0	77.7	Extremely high

Penicillin (PCN), spectinomycin (SPT), and tetracycline (TET). Multidrug resistance (MDR) is defined as resistance to three or more classes of antibiotics. The level descriptors follow VARSS and EFSA standards defined as rare (<0.1%), very low (0.1-1%), low (1-10%), moderate (10-20%), high (20-50%), very high (50-70%), and extremely high (>70%) (European Food Safety Authority, 2022; Veterinary Medicines Directorate, 2023b).

The number of the isolates that were resistant to a given number of antibiotic classes and the number which were MDR across all isolation medias grouped by PPL treatment group are presented in Table 4.2. A low level (5.8%) of the isolates from the untreated PPL and a high level (40.8%) of the isolates from the treated PPL were MDR. An extremely high level (63.5%) of the isolates

from the untreated PPL and a high level (33.5%) of the isolates from the treated PPL were resistant to none of the antibiotic classes.

Table 4.2: Number of α isolates that were resistant to a given number of antibiotic classes from samples taken from untreated and treated PPL.

		Resistance to antibiotic classes (n)						
		0	1	2	3	4	5	6
Untreated PPL (n = 329)	Isolates (n)	209	69	32	7	7	5	0
	Percentage	63.5	21.0	9.7	2.1	2.1	1.5	0
	Level	E. high	High	Low	Low	Low	Low	Rare
	Non-MDR	94.2% (Extremely high)			NA			
	MDR	NA			5.8% (Low)			
Treated PPL (n = 179)	Isolates (n)	60	34	12	1	72	0	0
	Percentage	33.5	19.0	6.7	0.6	40.2	0	0
	Level	High	Moderate	Low	V. low	High	Rare	Rare
	Non-MDR	59.2% (Very high)			NA			
	MDR	NA			40.8% (High)			

Multidrug resistance (MDR) is defined as resistance to three or more classes of antibiotics. The level descriptors follow VARSS and EFSA standards defined as rare (<0.1%), V. low = very low (0.1-1%), low (1-10%), moderate (10-20%), high (20-50%), very high (50-70%), and E. high = extremely high (>70%) (European Food Safety Authority, 2022; Veterinary Medicines Directorate, 2023b).

4.6.3 Identification of transmissible MDR phenotypes by conjugation

This section investigated how many of the MDR isolates had ARG that were transmissible through bacterial conjugation. Of the 64 MDR α *E. coli* isolates, 59 were streptomycin resistant, and 48 of these isolates were kanamycin susceptible. These 48 isolates were selected as potential MDR donors and were co-cultured with the recipient strain (DH5 α-KAN_R). From the co-culture, 10 isolates with resistance to streptomycin and kanamycin were termed successful transconjugants named 'donor isolate ID'X (Table 4.3). The DH5 α-KAN_R strains, the 10 MDR α *E.coli* donors, and the 10 transconjugant isolates are collectively the MDR conjugation panel.

Table 4.3: Number of the α *E. coli* isolates that met each selection criteria.

Criteria	All	MDR	Streptomycin resistant	Kanamycin susceptible	Conjugation
Isolates (n)	508	64	59	48	10

Antibiotic susceptibility was determined by the Kirby-Bauer disk diffusion method.

4.6.4 Conjugation did not transfer the entire antibiotic susceptibility phenotype

There were six ASPs in the 10 MDR donor *E. coli* and six different ASPs in the 10 transconjugant *E. coli* isolates. None of the MDR donor *E. coli* were resistant to all of the antibiotics tested and the *E. coli* DH5 α-KAN$_R$ recipient strain was only resistant to kanamycin. None of the ASPs of the 10 donor *E. coli* isolates were shown to be fully transmissible (Figure 4.2).

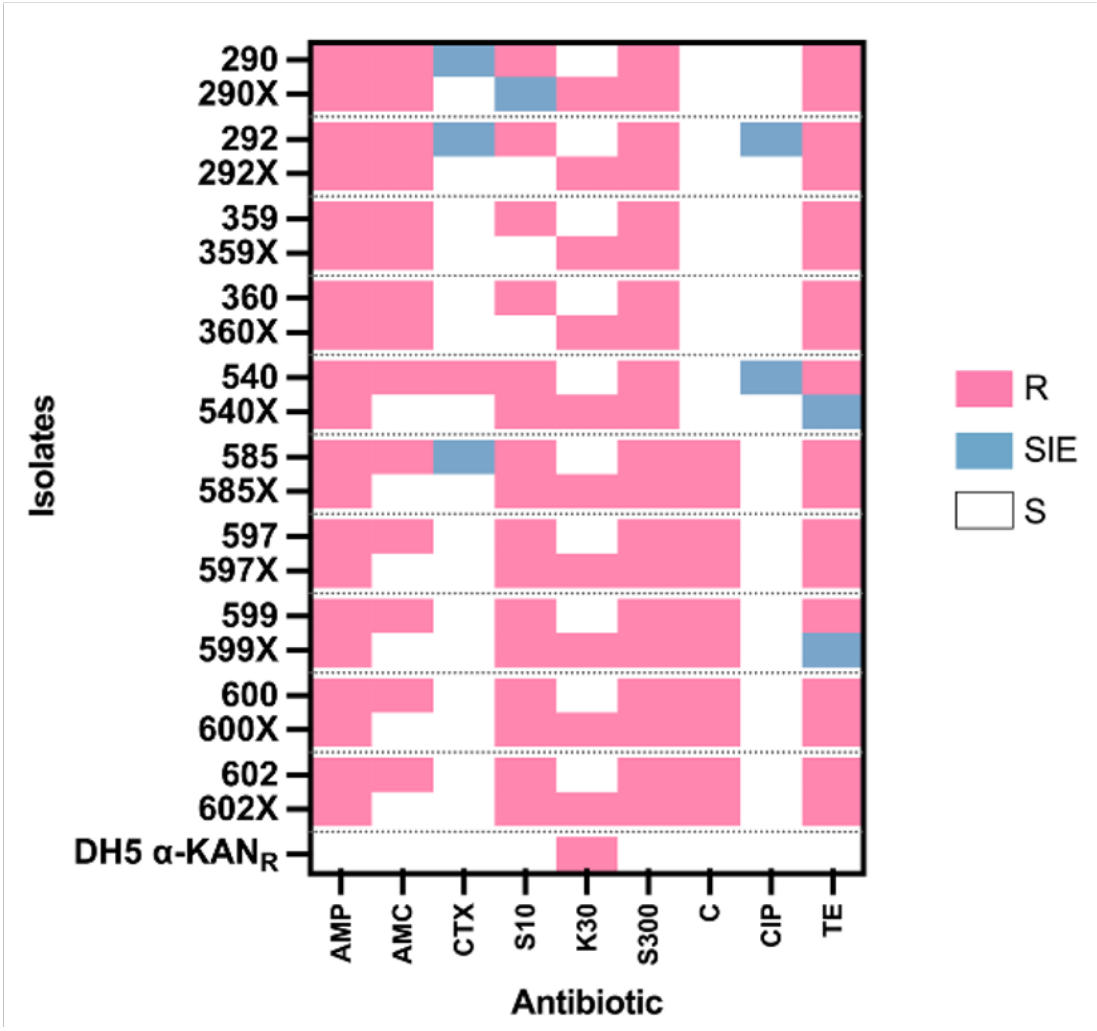


Figure 4.2: Antibiotic susceptibility phenotypes of the MDR conjugation panel. The isolates in the conjugation panel are listed on the y-axis, and the black dotted horizontal lines separate the results for each MDR donor isolate and transconjugant pair from the others. Each antibiotic tested by Kirby-Bauer disk diffusion is listed on the x-axis: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), K30 (kanamycin), S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), and TE (tetracycline). The box colour displays the antibiotic susceptibility result: pink for R (resistant), blue for SIE (susceptible, increased exposure), and white for S (susceptible).

E. coli 290 was resistant to five antibiotics from four classes (ampicillin, amoxicillin-clavulanate, streptomycin, sulfonamide, and tetracycline), SIE to cefotaxime, and susceptible to three antibiotics from three classes (kanamycin, chloramphenicol, and ciprofloxacin). *E. coli* 290X was resistant to five antibiotics from four classes (ampicillin, amoxicillin-clavulanate, kanamycin, sulfonamide, and tetracycline), SIE to streptomycin, and susceptible to three antibiotics from three classes (cefotaxime, chloramphenicol, and ciprofloxacin). Therefore, the resistances to ampicillin, amoxicillin-clavulanate, sulfonamide, and tetracycline in *E. coli* 290 were confirmed as transmissible. However, the resistance to streptomycin in *E. coli* 290 and SIE to streptomycin in *E. coli* 290X suggests this phenotype may be transmissible, while the SIE to cefotaxime was not transmissible from *E. coli* 290. *E. coli* 292 was resistant to five antibiotics from four classes (ampicillin, amoxicillin-clavulanate, streptomycin, sulfonamide, and tetracycline), SIE to two antibiotics from two classes (cefotaxime and ciprofloxacin), and susceptible to two antibiotics from two classes (kanamycin and chloramphenicol). *E. coli* 292X was resistant to five antibiotics from four classes (ampicillin, amoxicillin-clavulanate, kanamycin, sulfonamide, and tetracycline) and susceptible to four antibiotics from four classes (cefotaxime, streptomycin, chloramphenicol, and ciprofloxacin). Therefore, the resistances to ampicillin, amoxicillin-clavulanate, sulfonamide, and tetracycline in *E. coli* 292 were confirmed as transmissible. However, the resistance to streptomycin and SIE to cefotaxime and ciprofloxacin were not transmissible from *E. coli* 292. *E. coli* 359 and 360 were resistant to five antibiotics from four classes (ampicillin, amoxicillin-clavulanate, streptomycin, sulfonamide, and

tetracycline) and susceptible to four antibiotics from four classes (cefotaxime, kanamycin, chloramphenicol, and ciprofloxacin). *E. coli* 359X and 360X were resistant to five antibiotics from four classes (ampicillin, amoxicillin-clavulanate, streptomycin, sulfonamide, and tetracycline) and susceptible to four antibiotics from four classes (cefotaxime, kanamycin, chloramphenicol, and ciprofloxacin). Therefore, the resistances to ampicillin, amoxicillin-clavulanate, sulfonamide, and tetracycline in *E. coli* 359 and 360 were confirmed as transmissible. However, the resistance to streptomycin was not transmissible from *E. coli* 359 and 360. *E. coli* 540 was resistant to six antibiotics from four classes (ampicillin, amoxicillin-clavulanate, cefotaxime, streptomycin, sulfonamide, and tetracycline), SIE to ciprofloxacin, and susceptible to two antibiotics from two classes (kanamycin and chloramphenicol). *E. coli* 540X was resistant to four antibiotics from three classes (ampicillin, streptomycin, kanamycin, and sulfonamide), SIE to tetracycline, and susceptible to four antibiotics from three classes (amoxicillin-clavulanate, cefotaxime, chloramphenicol, and ciprofloxacin). Therefore, the resistances to ampicillin, streptomycin, and sulfonamide resistance in *E. coli* 540 were confirmed as transmissible. However, the resistance to tetracycline in *E. coli* 540 and SIE to tetracycline in *E. coli* 540X suggests this phenotype may be transmissible, while the SIE to ciprofloxacin was not transmissible from *E. coli* 540. *E. coli* 585 was resistant to six antibiotics from five classes (ampicillin, amoxicillin-clavulanate, streptomycin, sulfonamide, chloramphenicol, and tetracycline), SIE to cefotaxime, and susceptible to two antibiotics from two classes (kanamycin and ciprofloxacin). *E. coli* 585X was resistant to six antibiotics from five classes (ampicillin, amoxicillin-clavulanate,

streptomycin, sulfonamide, chloramphenicol, and tetracycline) and susceptible to three antibiotics from three classes (cefotaxime, kanamycin, and ciprofloxacin). *E. coli* 597, 599, 600, and 602 were resistant to six antibiotics from five classes (ampicillin, amoxicillin-clavulanate, streptomycin, sulfonamide, chloramphenicol, and tetracycline) and susceptible to three antibiotics from three classes (cefotaxime, kanamycin, and ciprofloxacin). *E. coli* 597X, 600X, and 602X were resistant to six antibiotics from five classes (ampicillin, streptomycin, kanamycin, sulfonamide, chloramphenicol, and tetracycline) and susceptible to three antibiotics from two classes (amoxicillin-clavulanate, cefotaxime, and ciprofloxacin); the same ASP as *E. coli* 585X. Therefore, the resistances to ampicillin, streptomycin, sulfonamide, chloramphenicol, and tetracycline in *E. coli* 585, 597, 600, and 602 were confirmed as transmissible. However, the resistance to amoxicillin-clavulanate was not transmissible from *E. coli* 585, 597, 600, and 602. *E. coli* 599X was resistant to five antibiotics from four classes (ampicillin, streptomycin, kanamycin, sulfonamide, and chloramphenicol), SIE to tetracycline, and susceptible to three antibiotics from two classes (amoxicillin-clavulanate, cefotaxime, and ciprofloxacin). Therefore, the resistances to ampicillin, streptomycin, sulfonamide, and chloramphenicol in *E. coli* 599 were confirmed as transmissible. However, the resistance to tetracycline in *E. coli* 599 and SIE to tetracycline in *E. coli* 599X suggests this phenotype may be transmissible, while the resistance to amoxicillin-clavulanate was not transmissible from *E. coli* 599 (Figure 4.2).

4.6.5 Short-read whole genome sequencing of MDR *E. coli* to investigate clonality and identify transmissible ARG and associated MGE vectors

DNA samples of the MDR donor isolates, the DH5 α-KAN_R recipient strain, and the successful transconjugants were extracted and whole genomes were assembled from the subsequent short-read sequencing. ARGs, serotypes, and plasmid incompatibility groups were identified from these genomes using ABRicate to screen against several ARG gene databases. These genomes were submitted to Enterobase to assign ST following the seven gene Achtman MLST scheme.

4.6.5.1 Confirming adequate data quality of short-read whole genome sequencing

Before using short-read sequencing to investigate the whole genomes of donor, recipient and transconjugant isolates, the quality of the sequencing and assemblies must be investigated (Figure 4.3). The median number of reads per genome was $1.12x10^6$ base pairs. The mean of all genomes mean contig coverage was 113 reads per contig. The median N50 across the contigs of all genomes was $1.57x10^5$ base pairs (Figure 4.3).

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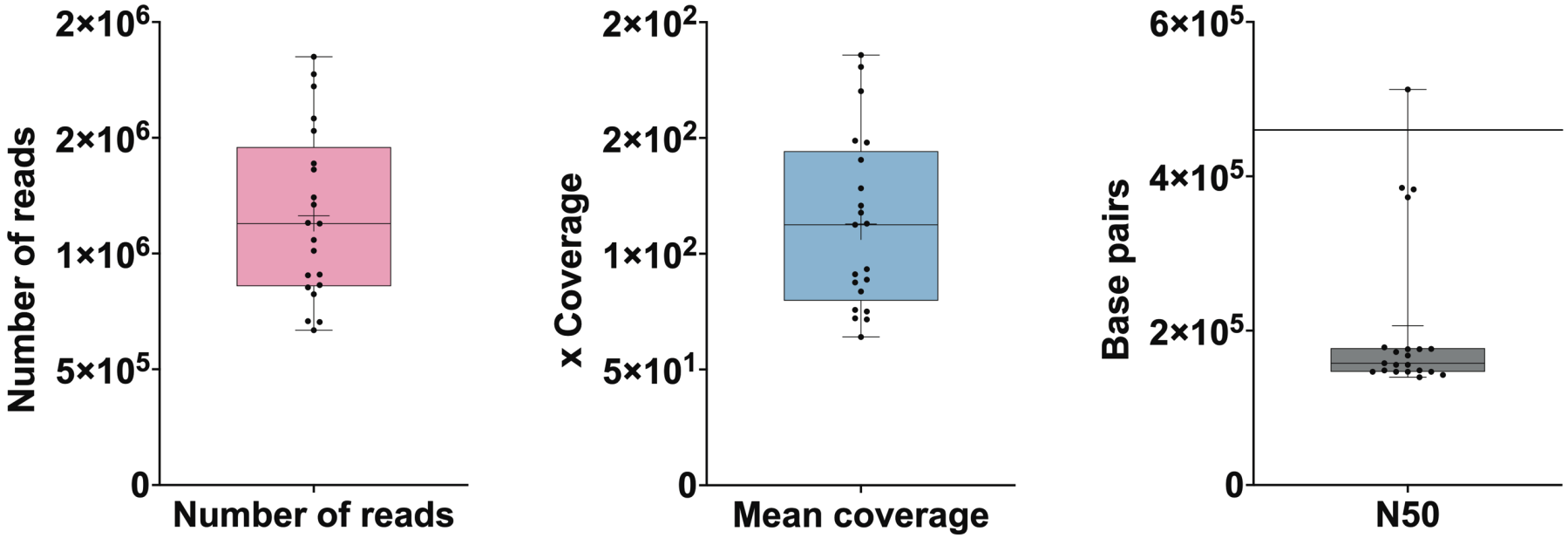


Figure 4.3: Sequencing and assembly quality metrics of the whole genomes from the MDR conjugation panel. The midline represents the median value across all sequences, with the box indicating the interquartile range (25th to 75th percentile) of all values. Whiskers extend to the minimum and maximum values and the mean value is denoted by a cross. Pink is the total number of sequencing reads for all genomes, blue: Mean coverage of reads per contig for all genomes, and black: N50, contig length to include 50% of contigs in each genome, the solid line represents the average length of an *E. coli* genome.

4.6.5.2 Identification of antibiotic resistance genes by screening genomes in ARG databases with ABRicate

Many ARGs were identified in the genomes of this panel of MDR isolates across several ARG databases (Appendix 11-Appendix 20). The focus of this section was the ARG responsible for the transmissible ASP (Table 4.4 and Figure 4.4).

Several aminoglycoside O-phosphotransferases were transmissible from all MDR donor isolates. Specifically, *aph6* genes were identified in the MEGARes database, *aph6* and *aph3* genes were identified by all other databases, and the ResFinder database identified two variants of *aph(3")-Ib.* The genomes of *E. coli* 290, 292, 359, and 360 had sequence matches for *aph(3")-Ib_2,* while the *E. coli* 540, 585, 597, 599, 600, and 602 genomes had sequence matches for *aph(3")-Ib_5*. Only the genome of *E. coli* 599X did not have a sequence match for *aph(3")-Ib_5* that its MDR donor parent carried, but it did have a sequence match for *aph(3")-Ib_2,* which was not found in the genomes of *E. coli* 599 or DH5 α-KAN$_R$. Non-transmissible β-lactamase *ampC* was identified, as BLAEC, by MEGARes in the genomes of the MDR donor *E. coli*. Sequence matches were identified for other β-lactamases that were transmissible (*TEM-1* and *TEM-105*) and were not transmissible (*blaEC-18*, *blaEC-15*, *blaTEM-1B_1*). The *E. coli* 290, 290X, 292, 292X, 359, 359X, 360, 360X, and 599X genomes had 84% nucleotide identity sequence matches for the MEGARes database *TEM* reference sequence. The genomes of *E. coli* 540, 540X, 585, 585X, 597, 597X, 599, 600, 600X, 602, and 602x had 98% nucleotide identity sequence matches for the MEGARes database *TEM* reference sequence. Sequence matches from the NCBI ARG database for *blaEC-18* were identified

in the *E. coli* 290, 292, 359, and 360 genomes. A sequence match from the NCBI ARG database for *blaEC15* was identified in the *E. coli* 540 genome. Non-transmissible *emrE* was identified by CARD in all of the MDR donor *E. coli* genomes. Transmissible sequence matches in all ARG databases for the phenicol efflux gene *floR* were identified in the genomes of *E. coli* 585, 585X, 597, 597X, 599, 599X, 600, 600X, 602, and 602X. Transmissible sequence matches for the sulfonamide target replacement gene *sul II* were identified in all of the MDR donor *E. coli* and transconjugant *E. coli.* Sequence matches in the ResFinder ARG database identified *sul2_3* in the genome of *E. coli* 540 and *sul2_2* in the genomes of the other MDR donor *E. coli* and transconjugant *E. coli.* All of the ARG databases identified transmissible sequence matches for mutations in the *dfra5* gene that confer resistance to trimethoprim in the genomes of *E. coli* 540 and *E. coli* 540X. The MEGARes ARG database identified the gene as DFRA, and all of the other ARG databases specified *dfra5*. All of the MDR donor *E. coli* and transconjugant *E. coli* 97.8% nucleotide identity sequence matches for the transmissible tetracycline efflux gene *tet(A)_6* according to the ResFinder ARG database. The CARD ARG database identified transmissible sequence matches for *tet(A)* in all of the MDR donor *E. coli* and transconjugant *E. coli.* Transmissible sequence matches for the tetracycline efflux gene *tetR* were also identified in all of the MDR donor *E. coli* and transconjugant *E. coli* with the ARG-ANNOT database (Table 4.4).

Table 4.4: Transmissibility of antibiotic resistance genes identified in MDR donor isolates.

	Transmissible genes	Isolates	Non-transmissible genes	Isolates
MEGARes	*APH6*	All	*MVRC*	All
	dfra	540	*BLAEC*	All
	floR	585, 597, 599, 600, 602	*PBP-4B*	540
	sul II	All		
	TEM	All		
	tetA	All		
NCBI ARG	*aph(3")-lb*	All	*blaEC-15*	540
	aph(6)-ld	All	*blaEC-18*	290, 292, 359, 360
	blaTEM-1	All		
	dfrA17	540		
	floR	585, 597, 599, 600, 602		
	sul II	All		
	tet(A)	All		
ResFinder	*aph(3")-lb_2*	290, 292, 359, 360	*aph(3")-lb_5*	599
	aph(3")-lb_5	540, 597,600,602		
	aph(6)-ld	All		
	blaTEM-1	All		
	dfrA5_1	540		
	floR	585, 597, 599, 600, 602		
	sul2_2	all excluding 540		
	sul2_3	540		
	tet(A)_6	All		
ARG-ANNOT	*strA*	All		
	strB	All		
	TEM-105	All		
	floR	585, 597, 599, 600, 602		
	sul II	All		
	tetA	All		
	tetR	All		
	dfrA5	540		
CARD	*aph(3")lb*	All	*emrE*	All
	aph(6)-ld	All		
	TEM-1	All		
	dfra-5	540		
	floR	585, 597, 599, 600, 602		
	sul II	All		
	tet(A)	All		

Transmissible genes were not identified in the whole genome sequence of DH5 α-KAN_R, but were identified in the whole genome sequences of MDR donor isolates and their matching transconjugant. Non-transmissible genes were identified in the whole genome sequence of the MDR donor isolates only. ARG that were identified in the whole genome sequence of DH5 α-KAN_R could not be confirmed as transmissible. All isolates = 290, 292, 359, 360, 540, 585, 597, 599, 600, and 602.

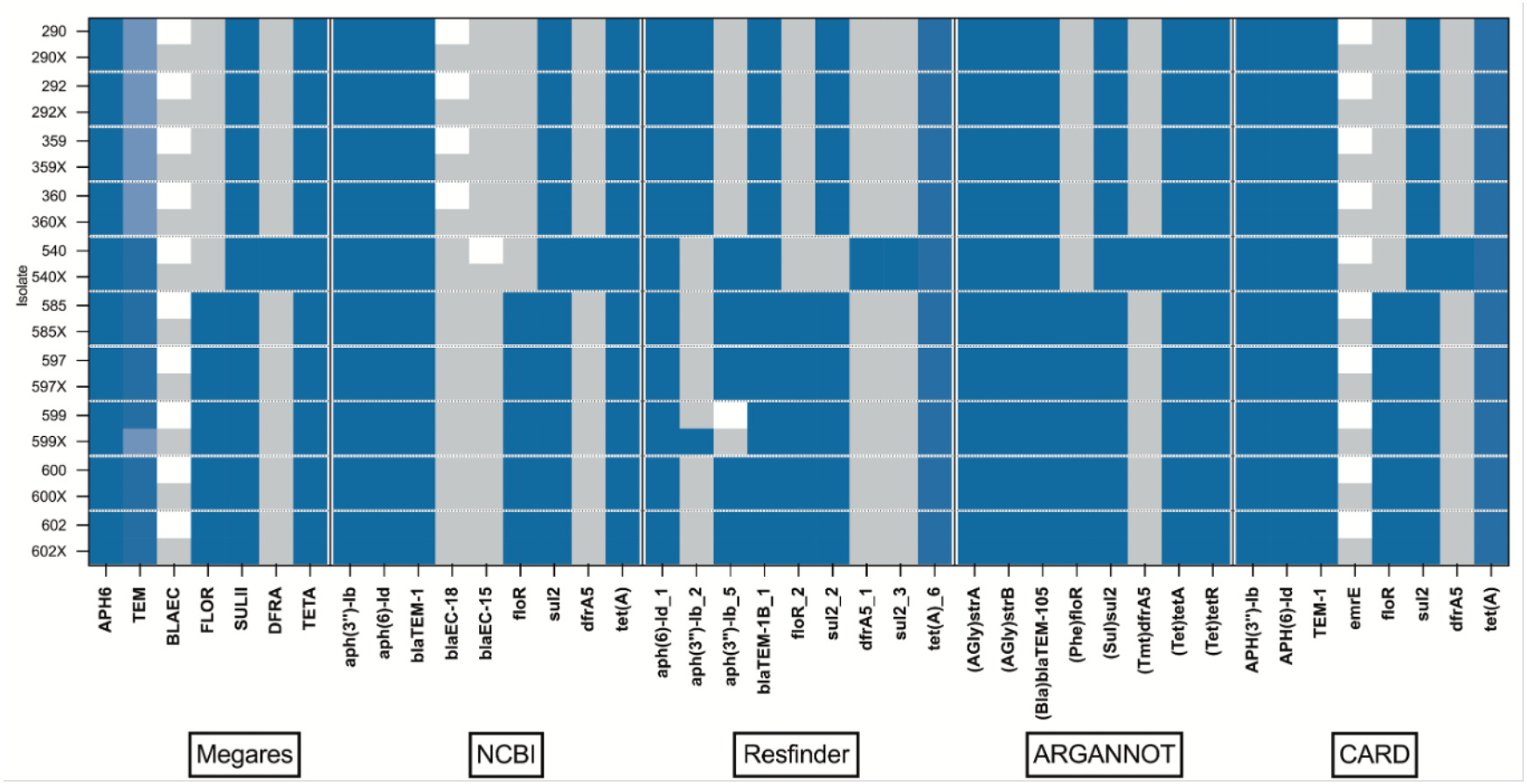


Figure 4.4: Transmissible ARG in the MDR conjugation panel identified in different ARG databases with ABRicate. The isolates in the MDR conjugation panel are listed on the y-axis, and the black dotted horizontal lines separate the results for each MDR donor isolate and transconjugant pair from the others. ARG that were identified in the genomes of these isolates are listed on the x-axis, and the black vertical bars separate ARG by the database they were identified in. The ARG databases are listed below the x-axis in line with the genes that were identified in each. The percentage identity of the isolate sequence compared to the database sequence is shown by dark blue (100%) and light blue (minimum value 84%). The presence of a gene that was confirmed as non-transmissible is shown by white and the lack of a gene is shown by grey. The ARG names are taken from the corresponding ARG database.

4.6.5.3 Identifying clusters of β *E. coli* isolates through *in silico* serotyping and mulitlocus sequence typing

Whole genome sequences of MDR donor *E. coli*, transconjugant *E. coli,* and the *E. coli* DH5 α-KAN$_R$ recipient were submitted to the Enterobase database. This enabled MLST assignment with the Achtman seven gene MLST scheme. ABRicate was also used to screen whole genome sequences for matches in the EcOH database for *in silico* serotype predictions (Table 4.5 and Figure 4.5). The MDR donor *E. coli* in the conjugation were split into three groups identified by MLST and EcOH. *E. coli* 290, 292, 359, and 360 from PPL 8 were all serotype O163:H7 and ST1304. *E. coli* 540 from PPL 18 was serotype O7:H4, ST93 and ST complex 168. *E. coli* 585, 597, 599, 600, and 602 from PPL 17 were all serotype O15:H16 and ST325. The DH5 α-KAN$_R$ recipient strain and transconjugants were all serotype O16:H48 and ST1060 (Table 4.5).

Table 4.5: Sequence type and sequence type complex determined by the Achtman seven gene MLST scheme and serotype predicted by EcOH.

			MLST		EcOH
Source	Isolation media	Isolate	ST	ST complex	Serotype
PPL 8	TBX	290	1304	NA	O163:H7
		292			
	TBX + TET	359			
		360			
PPL 18	TBX	540	93	168	O7:H4
PPL 17	TBX + TET	585	325	NA	O15:H16
		597			
		599			
		600			
		602			
Transconjugant	Not applicable	290X	1060	NA	O16:H48
		292X			
		359X			
		360X			
		540X			
		585X			
		597X			
		599X			
		600X			
		602X			
New England Biolabs		DH5 α-KAN$_R$			

MLST assignment completed in Enterobase and EcOH prediction completed using ABRicate. ST complex NA = none identified in Enterobase. TET = tetracycline.

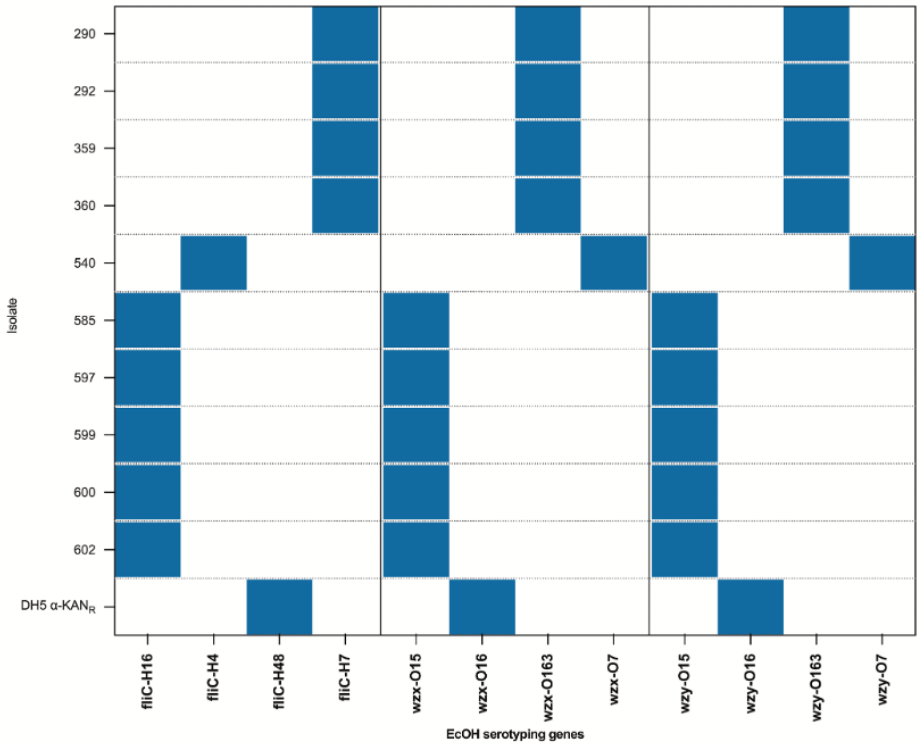


Figure 4.5: Genes in EcOH serotyping database identified in the genomes of the MDR donor isolates and DH5 α-KAN$_R$ strain. The genomes from the MDR donor and recipient isolate are listed on the y-axis separated by dotted black lines. The identified O and H antigens contained in EcOH are listed along the x-axis, and the vertical black lines separate the database sequences into broad categories of O and H antigens. These antigens are flagellar filament structural protein (*fliC*), flippase (*wzx*), and polymerase (*wzy*). Blue boxes indicate a sequence match between an isolate genome and the database reference sequence, while white boxes indicate no sequence match.

The DH5 α-KAN$_R$ recipient and transconjugant *E. coli* were all classified as ST1060. However, there was one cluster containing *E. coli* DH5 α-KAN$_R$, 290X, 292X, 359X, 360X, and 599X; and another closely related cluster containing 540X, 597X, 600X, and 602X. The ST1304 *E. coli* (290, 292, 359, and 360) clustered together and the ST325 *E. coli* (585, 597, 599, 600, and 602) clustered together. The ST1304 cluster and ST325 clusters differed from the ST1060 cluster to a similar degree, but at different loci. The ST93 *E. coli* (540) was more similar to the ST1060 cluster than all of the other MDR donor *E. coli* and therefore it was positioned closer to the ST1060 cluster.

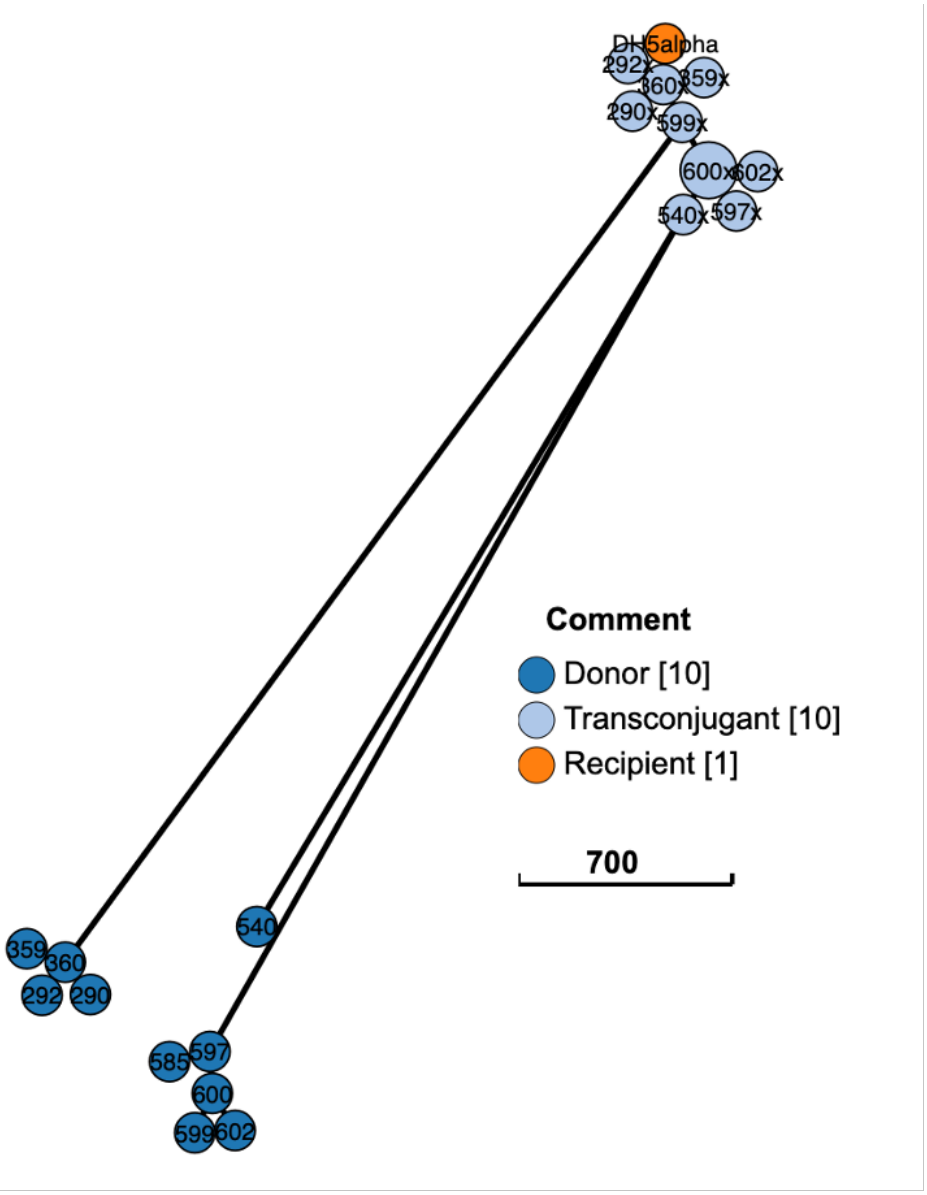


Figure 4.6: MSTree V2 GrapeTree plot of the MDR conjugation panel. The dark blue circles are the 10 MDR donor *E. coli*, the light blue circles are the 10 transconjugant *E. coli*, and the orange circle is the DH5 α-KAN$_R$ recipient strain. Each circles is labelled with the isolate ID, isolate 585X and isolate 600X had zero loci differences and are both represented by the larger light blue circle labelled 600X. ST were assigned by the wgMLST scheme in Enterobase. The scale represents the distance associated with 700 single loci differences.

4.6.5.4 Screening the PlasmidFinder database for genes associated with plasmid incompatibility groups in the β *E. coli* genomes

The PlasmidFinder database identified similar plasmids sequences in the MDR donor *E. coli* assigned the same ST and serotypes, and their transconjugants (Figure 4.7). A sequence match for *IncC1* plasmids was identified in the genomes of *E. coli* 290, 290X 292, 292X 359, 359X, 360, and 360X. A sequence match for *IncFIB* plasmids was found in the genomes of *E.*

coli 290, 292, 359, and 360. Sequence matches for *IncFIB* and *IncFII* plasmids were identified in the genomes of *E. coli* 540 and 540X. A sequence match for *IncB/O/K/Z* plasmids was identified in the genomes of *E. coli* 585, 585X, 597, 597X, 599, 599X, 600, 600X, 602, and 602X. A sequence match for *IncFIB* plasmids was present in the genomes of *E. coli* 585, 597, 599, 600, and 602. A sequence match for *IncCII* plasmids was identified in the genome of *E. coli* 599X, but it was not identified in the genomes of *E. coli* 599 or DH5 α-KAN$_R$. No sequence matches for the PlasmidFinder database were identified in the DH5 α-KAN$_R$ genome (Figure 4.7).

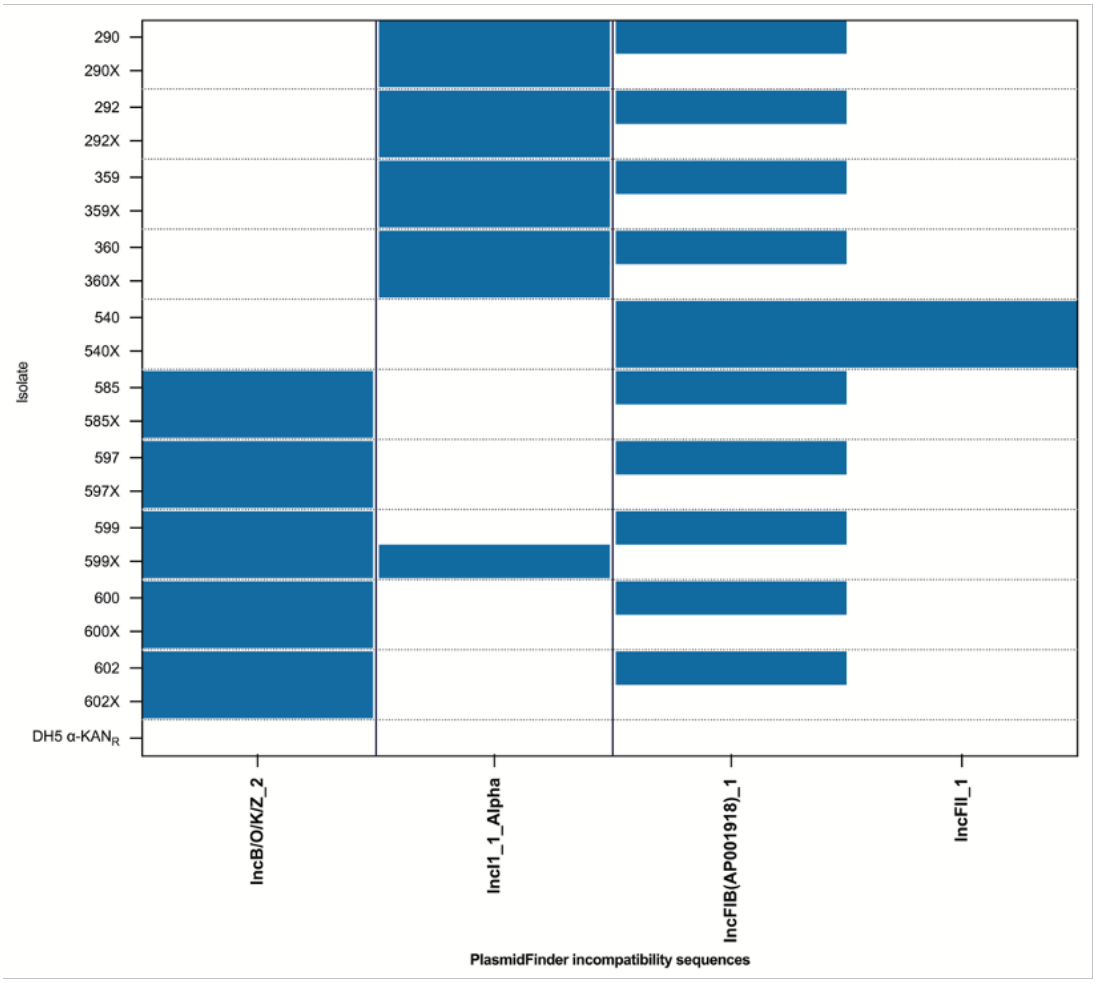


Figure 4.7: Plasmid incompatibility sequences in the PlasmidFinder database identified in the MDR conjugation panel. The genomes from the MDR donor isolates, the transconjugant isolates, and the DH5 α-KAN$_R$ recipient strain are listed on the y-axis. The dotted horizontal black lines separate α *E. coli* MDR donor and transconjugant pairs from the other pairs. The sequences corresponding to specific plasmid incompatibility groups contained in the PlasmidFinder database are listed along the y-axis. The vertical black lines separate the database sequences into categories of incompatibility groups. Blue boxes indicate a sequence match between a genome and the database reference sequence, while white boxes indicate no sequence match.

4.7 Discussion

This chapter aimed to investigate the MDR α *E. coli* collected in Chapter 3. These isolates were used to;

1. Asses the molecular genotyping approach of ERIC-PCR to determine the clonality of the α *E. coli*
2. Determine the level of MDR *E. coli* , and how was the level of MDR affected by;
    a. Antibiotics supplemented into TBX
    b. PPL treatment group
3. Conduct conjugation experiments to identify *E. coli* with transmissible MDR phenotypes
4. Screen short-read WGS with ABRicate for ARG, ST, serotype, and plasmid incompatibility groups

4.7.1 Limitations of ERIC-PCR to investigate bacterial diversity

ERIC-PCR was identified as a potential method for investigating the diversity of *E. coli* isolates. However, the pilot study in this thesis demonstrated that ERIC-PCR could not provide consistent fingerprints for the same DNA sample. Therefore, ERIC-PCR is not a reliable method for differentiating between isolates. Issues with this method have been highlighted and explained extensively by others (Wilson and Sharp, 2006). Primarily, the ERIC regions are not randomly distributed across bacterial genomes and are often associated with highly expressed genes. This association suggests that ERIC sequences may have functional roles with evidence they are evolutionarily conserved, limiting this discriminatory power for typing closely related isolates. Additionally, the ERIC-PCR primers can introduce bias as they preferentially

bind some ERIC sequences while not detecting others. Finally, PCR techniques are liable to high variability between users and between laboratories, limiting comparisons between datasets (Wilson and Sharp, 2006). To illustrate these issues, different PCR products of the same isolate were placed on the opposite end of a dendrogram (Meacham *et al.*, 2003). If this work was expanded, more robust methods such as PFGE could be utilised as they have been shown to produce reliable molecular fingerprints. Crucially, six or more restriction enzymes would need to be used to adequately differentiate *E. coli* to determine genetic relatedness (Davis *et al.*, 2003). A PFGE investigation of clonality could not be conducted in this thesis as the necessary equipment was not available for use in the SVMS.

4.7.2 Comparison of multidrug resistant proportions in the α isolates to other datasets

If it is assumed that AST results across studies are comparable, the trend for MDR on the SB Farm is declining, in 2012 – 2014 very high levels (57.9%) were identified from the slurry tanks and faecal samples, while a high level (37%) were MDR in the slurry tank in 2017 (Ibrahim *et al.*, 2016; Baker *et al.*, 2022). In the α isolates, a moderate level (12%) of the isolates had a MDR susceptibility phenotype, and of these a moderate level were shown to have a transmissible phenotype. A high level (40.8%) of the isolates from the PPL that received chemotherapeutic treatment were identified as MDR, suggesting that AMU is linked to an increased risk of MDR. This level aligns with the 40% of *E. coli* from cattle in the VCD, this could suggest that a significant portion of the isolates in the VCD are from animals that had recently received chemotherapeutic treatment (Veterinary Medicines Directorate, 2023b). The

overall low level of MDR *E. coli* is a positive result, and suggests that the *E. coli* on the SB Farm are less likely to have a MDR phenotype than the national population. The results of two additional studies on the SB Farm, in combination with the results in this chapter could suggest that there is a mechanism reducing the fitness cost associated with MDR in the animals or slurry tank on the SB Farm, which is causing the increased proportion. The longevity of different antimicrobial compounds could be an explanation for this increased proportion, tetracyclines specifically persist longer than other commonly used antibiotics such as β-lactams in slurry (Ibrahim *et al.*, 2016; Baker *et al.*, 2022).

4.7.3 Identifying *E. coli* with a transmissible multidrug resistance phenotype

It was not feasible to complete WGS for every α isolate. Transmissible MDR has been identified as a source of infection associated with increased risk for animals and humans (WHO, 2015). Critically, this phenotype can be identified through *in vitro* conjugation assays and AST as completed in this chapter. This approach identified three distinct transmissible phenotypes, all of which horizontally transfer phenotypic resistance or SIE to β-lactams, aminoglycosides, and tetracycline. This is concerning as these antibiotic classes represent 77% of active ingredient (by weight) sold for use in food producing animals in 2021 (Veterinary Medicines Directorate, 2022b). None of these isolates were able to transfer cefotaxime non-susceptibility and none of them were resistant to ciprofloxacin, this is a positive sign as these antibiotics are both HP-CIA and not approved for use in bovines, therefore resistance would likely be as a result of co-selection caused by the use of other antimicrobials. This work focused on transmission between *E. coli*, because

successful conjugation is more likely between similar hosts (Levin, Stewart and Rice, 1979; Kosterlitz *et al.*, 2022). The pilot study was conducted before the COVID pandemic and after the return to research other experiments were prioritised. To investigate this panel of 21 isolates further and identify ARG, short-read WGS was completed.

4.7.4 Screening short-read whole genomes against antibiotic resistance databases to identify ARG

Before discussing the results of the sequencing, the quality metrics presented confirm the sequencing data is of good quality. The high number of reads and high coverage indicate that each contig and the subsequent base calls are likely to be accurate. The quality of the genomes assemblies were mixed, with a range of N50 values suggesting that some of the genomes were assembled with shorter sequencing reads. However, all of the genomes are sufficient for MLST in Enterobase and using ABRicate for the identification of ARG and plasmid incompatibility replicons, and for *in silico* serotyping. Screening these *E. coli* genomes for ARG has linked resistance genes to susceptibility phenotypes. ARG were identified that facilitate multidrug efflux and others that confer resistance to aminoglycosides, β-lactams, fluoroquinolones, macrolides, phenicols, polymyxins, sulfonamides, trimethoprim, and tetracyclines. Experimental design also enabled the identification of mobile genes. The genes that were present in the donor and transconjugant isolates, but not in the recipient strain are proven to be transmissible from the donor isolates. This is not possible with short-read sequencing of the donors or the transconjugants alone, because bacterial chromosomes and plasmids contain several highly repetitive sequences that confound assembly. The isolates in this section were all MDR and represent 2% of all of the *E. coli* isolated from the α samples, this provides insight into the kind of resistance genes that are found in the dairy farm environment. These isolates contained mobile resistance genes capable of rendering all approved antibiotics ineffective; this presents a real risk to the UK dairy industry if not adequately monitored.

4.7.4.1 β-lactam resistance genes that determined ampicillin, amoxicillin-clavulanate, and cefotaxime susceptibility

The genetic determinants for all of the β-lactam susceptibility in the conjugation panel have not been conclusively identified. The *TEM* β-lactamase is the likely source of transmissible ampicillin resistance, as it was identified in all of the donor and transconjugant genomes. *TEM* are class A broad spectrum enzymes, capable of hydrolysing penicillins and cephalosporins, and have been identified on transposons and conjugable plasmids (Datta and Kontomichalou, 1965; Bush, 1989; Bush and Jacoby, 2010). The results for amoxicillin-clavulanate and cefotaxime susceptibility in these isolates paired with the ARG identified in these genomes suggest that additional regulatory genes are required to change the breakpoint of *E. coli* to these antibiotics. The specific *TEM* genes in these isolates appear to be inhibited by clavulanic acid and are not active against third generation cephalosporins, as they do not explain the observed amoxicillin-clavulanate or cefotaxime ASRs. The amoxicillin-clavulanate resistance observed in the donor isolates is not fully explained by the *TEM* genes. The phenotype was transmissible in the ST1304 isolates from PPL 8, but not transmissible in the ST93 isolate from PPL 18, or the ST325 isolates from PPL 17. Screening genomes against the NCBI ARG identified sequence matches for *bla-ec15* in the ST1304 isolates, and *bla-ec18* in the ST93 isolate. Both of these genes are class C β-lactamases and have been linked to extended penicillinase and cephalosporin activity, and therefore amoxicillin-clavulanate non-susceptibility would have been the expected result. Isolate 540 was the only donor isolate with a cefotaxime resistance, while isolates 290, 292, and 585 were

cefotaxime SIE. None of these phenotypes have been shown to be transmissible, suggesting that none of the β-lactamases identified in the transconjugant isolates are the source of cefotaxime non-susceptibility.

4.7.4.2 Aminoglycoside resistance genes that determined kanamycin and streptomycin susceptibility

The transmissible streptomycin resistance of the MDR donor isolates was likely due to the aminoglycoside phosphotransferases *aph(6)-Id* and *aph(3")-Ib*. These genes were identified in all the conjugation panel genomes except for the DH5 α-KAN_R recipient genome, proving they are transmissible *in vitro* between *E. coli.* These genes are known to be streptomycin specific, exist on broad host range conjugable plasmids, and have been found in bacteria from humans, animals, plants, and the environment (Sundin and Bender, 1996). They have also been previously described in *E. coli* from dairy cows; a moderate level (19%) of 593 *E. coli* from 101 dairy farms in Canada were resistant to streptomycin, and this was linked to the *aph(6')-Id* and *aph(3")-Ib* genes (Massé *et al.*, 2021). The kanamycin resistance of the DH5 α-KAN_R recipient and the transconjugant isolates is likely due to another aminoglycoside phosphotransferase, *aph(3')-IIa*. This gene was not identified in any of the MDR donor isolates but it was in the DH5 α-KAN_R recipient.

4.7.4.3 Sulfonamide/ trimethoprim resistance genes that determined sulfonamide susceptibility

The likely sulfonamide resistance determinant was the dihydropteroate synthase *sul2* gene. This gene was identified in all the MDR donor and transconjugant genomes. The 540 and 540X genomes also had sequence matches for dihydrofolate reductase *dfrA* variants, *dfrA17* or *dfrA5* depending on which ARG database was screened.

4.7.4.4 Phenicol resistance genes that determined chloramphenicol susceptibility

The likely determinant of the transmissible chloramphenicol resistance in the MDR conjugation panel was the *floR* MFS efflux gene. This gene has been implicated in transmissible phenicol and quinolone resistance in bacteria from freshwater and agriculture previously (Cloeckaert *et al.*, 2000; Doublet *et al.*, 2005; Gordon *et al.*, 2008).

4.7.4.5 Tetracycline resistance genes determining tetracycline susceptibility

All of the MDR donors and transconjugants were not susceptible to tetracycline, and the tetracycline efflux gene *tetA* was identified in all of the genomes of these isolates. Interestingly, isolates 540X and 599X were SIE to tetracycline while their MDR donors were resistant. This suggests that there may be non-transmissible regulatory mechanisms involved in tetracycline resistance, which have not been identified in this approach.

4.7.4.6 Quinolone resistance genes that determined fluoroquinolone susceptibility

Isolates 292 and 540 had a non-transmissible ciprofloxacin SIE phenotype, and they were the only donor isolates that were not susceptible to ciprofloxacin. The approach taken in this chapter has not identified a genetic determinant for these ciprofloxacin phenotypes. The only quinolone specific resistance gene identified in any of the ARG databases was the efflux genes *emrA*, and *emrE*. Thes genes were identified in all genomes by the MEGAres database, but the CARD database identified sequence matches for these genes in the MDR donor isolate genomes only. The structural and regulatory genes required for several families of multidrug efflux pumps were identified in all of the genomes, further work investigating the expression levels across these isolates could provide insight to the contribution of efflux.

4.7.5 Investigating *E. coli* clonality and mobile genetic elements with short-read sequencing

The approach taken collecting and selecting these isolates has resulted in similar isolates being assessed from each sample. EcOH serotyping and MLST data determined that the *wt E. coli* cluster into three groups originating from different cows. These groups were categorised into three different STs; specifically ST1304, ST93, and ST325. ST 93 and ST1304 *E. coli* have been identified in poultry packing facilities in Switzerland, these isolates were associated with $bla_{CTX\text{-}M\text{-}1}$ positive plasmids providing transmissible third generation cephalosporin resistance (Vogt *et al.*, 2014). An *E. coli* ST325 isolate with resistance to β-lactams, aminoglycosides, and tetracyclines has previously been identified in a dairy cow in Australia (Barlow *et al.*, 2022). The

ST325 isolates were from samples collected in September 2019, while the ST93 and ST1304 isolates were from samples collected in October 2019. Therefore, transmissible MDR phenotypes have been identified on the farm, but they have not been shown to transfer between different animals over time. Plasmidfinder provides information on the incompatibility groups present in the sequencing data, to investigate if the resistance phenotype observed is plasmid based then plasmid preps would need to be prepared and sequenced. Two *IncF* sequences were identified in isolate 540 that cannot be from the same plasmid, suggesting it has two plasmids. *IncF*-type plasmids have been associated with MDR bacteria including *K. pneumoniae*, *P. aeruginosa*, *Acinetobacter baumanii, and E. coli* isolated from humans in a clinic (Chen *et al.*, 2024). Isolate 599X had an *Inc1* incompatibility group that neither the donor isolate or recipient do, this could be a sequencing/identification error. More work would be needed to investigate the origin of this sequence in isolate 599X, as the obvious explanation of contamination is not supported by ST, serotyping, or susceptibility profiles.

4.8 Limitations and future work

4.8.1 Investigating the clonality of the α *E. coli*

Unfortunately, the clonality of the *E. coli* isolates collected in the α samples has not been investigated thoroughly.

4.8.1.1 ERIC-PCR does not provide reproducible molecular fingerprints

ERIC-PCR was identified as an accessible molecular approach to fingerprint these isolates (Versalovic, Koeuth and Lupski, 1991). However, these results demonstrate that this method does not provide a reproducible output. Others have identified that *E. coli* ERIC sequences are too few and distributed too far apart in the genome of K12 and 10 other *E. coli* to provide the small fragments required to differentiate strains (Wilson and Sharp, 2006). This is believed to explain the poor repeatability of this method, with the same strain being categorised on opposite ends of a dendrogram determined by ERIC-PCR banding patterns (Meacham *et al.*, 2003). Additionally, ERIC-PCR was shown to amplify genomic fragments from *Treponema, Deinococcus, Thermus,* and even Archaea that do not contain the ERIC sequences the primers are designed for (Versalovic, Koeuth and Lupski, 1991). Alternate molecular methods such as (PFGE) could be employed. However, this method also appears to suffer from a lack of resolution with six or more enzymes required to investigate genetic relationships (Davis *et al.*, 2003). Additionally, PFGE banding patterns could be changed to the same degree through minor single nucleotide changes and major changes such as long insertions or deletions (Tenover *et al.*, 1995).

4.8.1.2 Focused investigation on isolates with a transmissible multidrug resistant phenotype

Due to COVID related access constraints, the conjugation work had to be limited in its scope. Ideally, tailored selection protocols would have been designed to test all the MDR isolates for transmissibility. Generating resistance to different antibiotics in *E. coli* DH5 α, and co-culturing potential MDR donors. This focused approach identified 10 isolates, representing 2% of the α *E. coli*. Therefore, these genomes are unlikely to be representative of these samples, or of the *E. coli* on the SB farm. This could be investigated further in the future because all the isolates from this work are stored in glycerol stocks in the -80°C.

4.8.2 Determining the genomic source of antibiotic resistance using ABRicate

4.8.2.1 Differences between antibiotic resistance gene databases

The choice of ARG database is crucial, for example, the ResFinder database appeared to have provided the greatest detail for the aminoglycoside resistance genes, stipulating that one variant of *aph(6)-Id* was identified, while two variants of *aph(3")-Ib* were identified in the donor isolates. However, *aph(3")-Ib_5* was identified in the genome of isolate 599, and *aph(3")-Ib_2* was identified in the genome of isolate 599X. No variant of this gene was identified in the DH5 α-KAN_R recipient, therefore, the only source for an *aph(3")-Ib* gene should have been the variant identified in isolate 599. Assuming that a spontaneous mutation did not occur during the conjugation, this suggests that there are minor differences in the reference sequences of these variants. If this is true, small errors in the sequencing data could lead to the identification of the incorrect gene variant. It is not clear from these results if this potential

erroneous variant calling is due to the quality of the short-read sequencing data, the approach that ABRicate uses to identify sequence matches, or in the ARG databases themselves. To definitively identify a specific variant of an ARG, a set of PCR primers could be designed for variants of interest in the ARG database. There are curated databases of PCR primer pairs already available that identify several ARG in the Bacterial antimicrobial resistance reference gene database and the NCBI ARG database (Gorecki *et al.*, 2019). Alternatively, the quality of the whole genomes could be improved through other technologies such as long read sequencing. Hybrid assemblies of long read and short-read sequencing can identify mobile elements, but long read sequencing is more costly (Juraschek *et al.*, 2021). Additionally, it has been reported that long-read genome assemblers can struggle with plasmids smaller than 10kb in size (Johnson, Soehnlen and Blankenship, 2023).

The isolates from this project are stored in glycerol and the short-read sequencing data is of sufficient quality to combine with long read sequencing. These hybrid assemblies would add more genetic context to the ARG already identified and potentially identify more genes associated with MGE.

4.9 Conclusion

Molecular approaches can be used to provide insight, but ERIC-PCR does not work as described by the original authors. The limitations of ERIC-PCR have been identified by the pilot study in this thesis and the work of others (Johnson *et al.*, 2001; Meacham *et al.*, 2003).

A moderate level of the isolates had a MDR susceptibility phenotype, and of these a low level were shown to have a transmissible phenotype. Therefore, the increase in risk is low. The next experimental step with these isolates

would be to repeat the conjugation experiments with different genera of bacteria from the dairy farm environment, *e.g., Streptococcus*, *Staphylococcus*, *Campylobacter*, and *Pseudomonas*. Transmissible phenotypes that can be shared between different species are unlikely but not impossible and would pose a greater risk. Long-read sequencing could also be completed to create hybrid assemblies, which would capture more of the genetic context surrounding ARG, improving understanding of the MGE causing the MDR transmissible phenotypes. The STs of these MDR transmissible isolates have been identified in *E. coli* from poultry isolates associated with ESBL plasmids, and with mastitis isolates that were resistant to β-lactams, aminoglycosides, and tetracyclines.

In the next chapter, additional isolates will be collected from samples taken across bovine developmental stages on the dairy farm. Antibiotic susceptibility and MDR profiles will be investigated, and a large selection will be submitted for short-read WGS and screened with ABRicate.

Chapter 5 *E. coli* antibiotic susceptibility sampled across bovine developmental stages

This chapter considered the variation of antibiotic susceptibility of the isolates within hosts, between hosts, and between bovine developmental stages across the dairy farm herd. DCA, PPD, PRP, and PPL are all kept in separate housing, with specific diets, and potential treatments. The most significant differences in the conditions and husbandry of these groups are found in the diet, due to the underdeveloped forestomachs of the DCA.

5.1 Dairy calves represent a critical stage for herd management, and mitigation for the risk of antibiotic resistance

Dairy calves suffer increased mortality due to common diseases such as diarrhoea, with *E. coli* identified as a major causative pathogen. Diarrhoea occurs in 48.2% of heifers and has been estimated to cause 1-2% of calf mortality in the UK (DEFRA, 2015; Johnson *et al.*, 2017). This increased susceptibility is likely because the bovine placenta separates the foetal and maternal blood supplies, preventing the exchange of maternal antibodies in utero. As a result of this, young calves do not have a functional immune system and are more likely to suffer pathogenic infections and likely to receive antibiotics at the earliest sign of disease. Potentially as a result of this, dairy calves have been identified as sources of a higher proportion of ABR bacteria; with animal physiological differences, and differences in the farming practices surrounding dairy calves also identified as potential causes (Villarroel, 2007; Cao, 2015; X. Li *et al.*, 2018; Salerno *et al.*, 2022; Shoaib *et al.*, 2023). In the VCD, the *E. coli* from neonatal and pre-weaning cattle had higher proportions

of resistance to ampicillin, amoxicillin-clavulanate, cefpodoxime, neomycin, trimethoprim/ sulfonamide, florfenicol, and enrofloxacin than the *E. coli* from adult cattle (Table 1.6). Conventionally, young calves are thought to rely on colostrum as the primary source of maternal antibodies (Chase, Hurley and Reber, 2008; Godden, 2008). Colostrum is certainly crucial in the early stage of calf development and several factors can influence the efficacy of immunoglobulin transfer. These include the time between birth and milking, the colostrum quality, the breed and age of the dam, and even the season of calving (Dixon, Weigle and Vasquez, 1961; Muller and Ellinger, 1981; Nardone *et al.*, 1997; McGuirk and Collins, 2004; Swan *et al.*, 2007; Chigerwe *et al.*, 2008). However, the vaginal microbiome could be a more direct contributor to the early colonisation of newborn dairy cattle. The microbiota of colostrum and calf faecal microbiota two days after birth have been reported to be significantly different, while the calf microbiota was more similar to the Dams vaginal microbiota (Klein-Jöbstl *et al.*, 2019). The umbilical cord microbiota has also been identified as a key contributor of early colonisation of the calf hindgut, with additional associations identified between the calf meconium microbiota and the microbiota of the placenta, umbilical cord, colostrum, and amniotic fluid (Zhu *et al.*, 2021). The conventional approach of preventing diarrhoea was adding antibiotics to milk products or medicated milk replacement (MMR), which has been banned in the UK since 2012. Younger calves (0 – 180 days) have been reported to be at higher risk of harbouring ABR and MDR *E. coli* than older calves (>180 days), and the likelihood of isolating ABR and MDR *E. coli* was highest immediately after antibiotic treatment, and only decreasing two weeks after treatment (Villarroel, 2007). A

study assessed the presence of ABR and MDR *E. coli* in composite manure samples across 80 Dairy farms in Pennsylvania, USA (Cao, 2015). ABR *E. coli* were identified in 85% of pre-weaned calf manure samples, 70% of post-weaned calf manure samples, 44% of lactating cow manure samples, and 28% of dry cow manure samples. MDR *E. coli* were identified in 70% of pre-weaned calf manure samples, 39% of post-weaned calf manure samples, 20% of lactating cow manure samples, and 6% of dry cow manure samples (Cao, 2015). MMR feeding has been demonstrated to increase the likelihood of ABR in faecal *E. coli* over time. Additional antibiotic treatment given to the calves that had received MMR was not associated with increase in the likelihood of isolating ABR *E. coli*, but it was in the calves that had not received MMR (Berge, Moore and Sischo, 2006).

5.1.1 Sequence types of bovine *E. coli* genomes

The STs and ST complexes identified in this chapter will be compared to two subsets of bovine *E. coli* collected from around the globe. The dataset collected from Enterobase for comparison in this thesis contained 6743 *E. coli* genomes from bovine sources submitted between 2018 and 2024. The STs and ST complexes that were identified in more than 1% of those genomes are shown in Table 1.9, with a GrapeTree plot displaying the minimum spanning tree of all of the genomes grouped by whole genome ST in Figure 1.4. A large scale genome analysis of 523 *E. coli* collected from bovine sources in Belgium, France, the USA, and Japan between 2010 and 2018 identified 209 ST (Arimizu *et al.*, 2019) (Table 5.1). Genomes with an unknown ST were the most common, representing 78 genomes (14.94%), 22 of the genomes (4.21%) were ST58, 19 of the genomes (3.64%) were ST10, 15 of the

genomes (2.87%) were ST101, and 11 of the genomes (2.3%) were ST906. The remaining 204 STs were identified in 10 genomes or fewer each.

Table 5.1: STs identified in 523 bovine commensal *E. coli* in a large scale genome analysis.

Genomes (n)	STs identified
78	No ST identified
22	58
19	10
15	101
11	906
9	205, 327, and 765
7	1079, 297, and 278
6	164, 718, and 677
5	679, 56, 154, 32, 223, 109, 206, and 20
4	1423, 155, 300, 332, 6126, 446, 29, and 795
3	156, 187, 201, 1172, 2008, 342, 1304, 6488, 295, 329, 1308, 17, 517, 5487, and 441
2	95, 38, 398, 1294, 847, 5937, 939, 4038, 13, 7895, 21, 2385, 2387, 448, 162, 392, 7867, 10?, 11, 443, 7889, 6118, 642, 937, 1510, 1125, 7907, 679*, 679?, 345?, 388, 301, 132, 442, and 345*
1	73, 88, 1204, 219, 26, 345, 5236, 763, 376, 5973, 2388, 8355, 336, 3489, 5552, 38?, 1723?, 2480?, 5738*, 118, 691, 7894, 7893, 6353, 7883, 7873, 7616, 583, 515, 4942, 491?, 7868, 137, 7888, 5487*, 196, 602, 2520, 1326, 419, 135, 7871, 7905, 119, 7897, 657, 43, 647*, 1196, 7899, 2079, 3519, 1324, 348, 85, 7869, 7900, 7593, 655, 7616*, 111, 192, 1890, 5978, 1724, 2766, 1163, 4212, 1122?, 278*?, 7902, 3494, 730, 549, 295?, 711, 6646, 2705, 1592, 4088, 53, 2565, 6625, 1293, 7543, 7912, 661, 7864, 5971, 1727, 40, 1431, 4467, 2035, 7876, 4977, 7879, 88*, 325, 351, 1642, 2473, 1049, 4035, 3891, 8813, 4642, 5022, 6595, 1426, 1616, 1252, 3858, 3090, 2524, 4398, 753, 2602, 1001, 675, 5289, 8694, 2521, 1610, 7289, 1423?, 4767, 1400, and 154*

* = the best scoring allele had ≥ 1 mismatch, ? = uncertainty in the result because the best scoring allele had some low-depth bases. Data from supplementary information (Arimizu *et al.*, 2019).

5.2 Aims

1. This chapter will investigate the differences in *E. coli* collected from the faeces of different bovine developmental groups. Specifically in:
    a. Bacterial abundance
    b. Antibiotic susceptibility
2. Short-read WGS will be completed for select isolates to further investigate the differences in *E. coli* from these development groups. Specifically in:
    a. ARG absence
    b. ST
    c. Plasmid incompatibility groups
3. To contextualise these results, they are all are compared with national, regional, and global datasets

5.3 Methods

5.3.1 Sample collection and purification of the β *E. coli* isolates

Detailed sampling and isolate purification methodology is available in sections 2.4, 2.5, and 2.6. TBX was used successfully to isolate 300 non-toxigenic *E. coli* (β isolates) The β samples were taken at one time point in May 2021, from 20 different animals representing four bovine developmental stages; five DCA, five PRP, five PPL, and five PPD (Table 2.3). Due to COVID-19 access constraints, treatment records for these animals were not collected at the time of sampling. The treatment records for the β samples were retrieved from the farm management software Uniform-AGRI (Uniform-AGRI, UK) at a later date. Unfortunately, the treatment history of several sample IDs could not be identified in the database, leaving the treatment history unknown.

Convenience samples were taken from veterinarians' gloves upon completion of routine work and delivered to SVMS. Samples were stored at 4°C, and aliquots were plated within 24 hours of collection. Colonies on TBX with a green/blue morphology were picked and re-streaked onto fresh TBX. Glycerol stocks were inoculated when isolates had a confirmed pure green/blue morphology and stored at -80°C.

5.3.2 Enumeration of the *β E. coli* on TBX supplemented with different antibiotics

Details of the enumeration method are contained in section 2.6. The abundance of *E. coli* was measured through total viable counts of green/blue morphology colonies on TBX and the abundance of bacteria was measured through total viable counts on NA. Aliquots of faecal samples were weighed and the appropriate volume of PBS was added to prepare a 1 $g.ml^{-1}$ solution. Serial dilutions were prepared and plated onto solid media. The number of colonies were counted after incubation and the *E. coli* CFU g^{-1} of faeces was calculated.

5.3.3 Kirby-Bauer disk diffusion method of antibiotic susceptibility testing

Detailed methodology for determining antibiotic susceptibility is in section 2.7. Aliquots of overnight cultures were adjusted to OD 0.1 before plating on MH. Paper antibiotic disks were placed onto inoculated plates before incubation at 37°C for 20 hours. ZOIs were measured with a ruler and compared to EUCAST breakpoints to determine antibiotic susceptibility.

5.3.4 Determining antibiotic susceptibility levels of the *β E. coli* for each antibiotic

The ASL descriptors are used by the VARSS report and EFSA AMR monitoring (Table 2.5) (European Food Safety Authority, 2022; Veterinary Medicines Directorate, 2023b). The VARSS report group resistant and SIE into resistant, this thesis reports each individually following EUCAST guidelines on interpretation of AST (EUCAST, 2022).

5.3.5 Median antibiotic susceptibility distributions to display the antibiotic susceptibility of a population of the *β E. coli*

The MASD present the median ZOI and the ASR for each antibiotic tested, for all of the isolates in a population (e.g. all of the samples, a sample group, or a single sample). The MASD also presents the interquartile range, the minimum, and the maximum ZOI to display the range of antibiotic susceptibility in a population.

5.3.6 Identifying statistically different median antibiotic susceptibility distributions in isolates from samples taken from the bovine developmental groups on the SB Farm

The non-parametric KWT was used to compare the ranks of groups means and group medians of three or more distributions were significantly different.

5.3.7 Culturing isolates to ship for high quality DNA extraction and short-read whole genome sequencing

Isolates from the β samples across the bovine developmental stages were selected for short-read WGS, isolates were selected to investigate dissimilarity in isolates from similar aged animals and capture the majority of disk diffusion diversity (Table 2.9). Isolates were prepared for shipping in DNA/RNA shield

(Zymo Research, USA) following instructions from MicrobesNG, with detailed methods in section 2.10 and the MirobesNG bioinformatic pipeline at the time of analysis in section 2.11.

5.3.8 Using ABRicate to identify antibiotic resistance genes, serotype, and plasmid incompatibility groups in *E. coli* whole genomes

ABRicate (v1.0.0) was used to screen contigs against a selection of gene databases (Seemann, no date, Accessed: December 2022). To identify acquired ARG the databases used included CARD (v3.1.4), MEGARes (v2.0), NCBI ARG (v3.10.18), ResFinder (v4.0), and ARG-ANNOT (v4.0) (Zankari *et al.*, 2012a; Gupta *et al.*, 2014; Jia *et al.*, 2017; Feldgarden *et al.*, 2019; Doster *et al.*, 2020). EcOH was used for *in silico E. coli* serotyping (v2.0) (Ingle *et al.*, 2016). PlasmidFinder (v2.1.1) was used to identify plasmid incompatibility groups present in the sequences (Carattoli *et al.*, 2014).

5.3.9 Identifying clusters of *E. coli* isolates with *in silico* mulitlocus sequence typing in Enterobase

E. coli genomes were submitted to the *Escherichia/Shigella* database hosted on the Enterobase online resource for analysing and visualising enteric bacteria genomic variation (Zhou *et al.*, 2020). Genomes were compared to the Achtman seven gene MLST scheme as well as the whole genome MLST scheme (wgMLST) to identify ST and investigate clonality. Genomic variation was visualised using the GrapeTree plot functionality of Enterobase using the MSTreeV2 Algorithm (Zhou *et al.*, 2018).

5.4 Results

TBX media was used to select non-toxigenic *E. coli*. The abundance of *E. coli* in faeces from different bovine groups and on different media compositions was investigated. The samples were all collected at the same time point, from four bovine developmental groups to investigate how the ASPs vary within and between the age groups. Isolates collected from media supplemented with antibiotics were included in this sample set and the impact of this will also be assessed.

5.4.1 Abundance of *E. coli* in the β samples

E. coli were successfully isolated on TBX from 20 faecal samples, and the mean abundance of *E. coli* on TBX across all samples was 2.08 $x10^{11}$ CFU g^{-1} (Figure 5.1). The mean abundance of *E. coli* in the DCA samples was 2.97 $x10^{11}$ CFU g^{-1}, in the DCA A sample was 6.64 $x10^{10}$ CFU g^{-1}, in the DCA B sample was 4.43 $x10^{11}$ CFU g^{-1}, in the DCA C sample was 3.65 $x10^{11}$ CFU g^{-1}, in the DCA D sample was 1.3 $x10^{11}$ CFU g^{-1}, and in the DCA E sample was greater than 4.8 $x10^{11}$ CFU g^{-1}. The mean abundance of *E. coli* in the PRP samples was 5.51 $x10^{8}$ CFU g^{-1}, in the PRP F sample was 1.82 $x10^{8}$ CFU g^{-1}, in the PRP G sample was 3.92 $x10^{9}$ CFU g^{-1}, in the PRP H sample was 4.24 $x10^{9}$ CFU g^{-1}, in the PRP I sample was 2.48 $x10^{11}$ CFU g^{-1}, and in the PRP J sample was 9.2 $x10^{8}$ CFU g^{-1}. The mean *E. coli* abundance in the PPD samples was 1.94 $x10^{11}$ CFU g^{-1}, in the PPD K sample was greater than 4.8 $x10^{11}$ CFU g^{-1}, in the PPD L sample was 7.2 $x10^{8}$ CFU g^{-1}, in the PPD M sample was greater than 4.8 $x10^{11}$ CFU g^{-1}, and in the PPD N sample was 7.2 $x10^{9}$ CFU g^{-1}. The mean *E. coli* abundance in the PPL samples was 2.88 $x10^{11}$ CFU g^{-1}, in the PPL O sample was 1.6 $x10^{9}$ CFU g^{-1}, in the PPL P

sample was greater than 4.8 x10^{11} CFU g^{-1}, in the PPL Q sample was 1.24 x10^{8} CFU g^{-1}, in the PPL R sample was greater than 4.8 x10^{11} CFU g^{-1}, in the PPL S sample was 8 x10^{7} CFU g^{-1}, and in the PPL T sample was greater than 4.8 x10^{11} CFU g^{-1} (Figure 5.1).

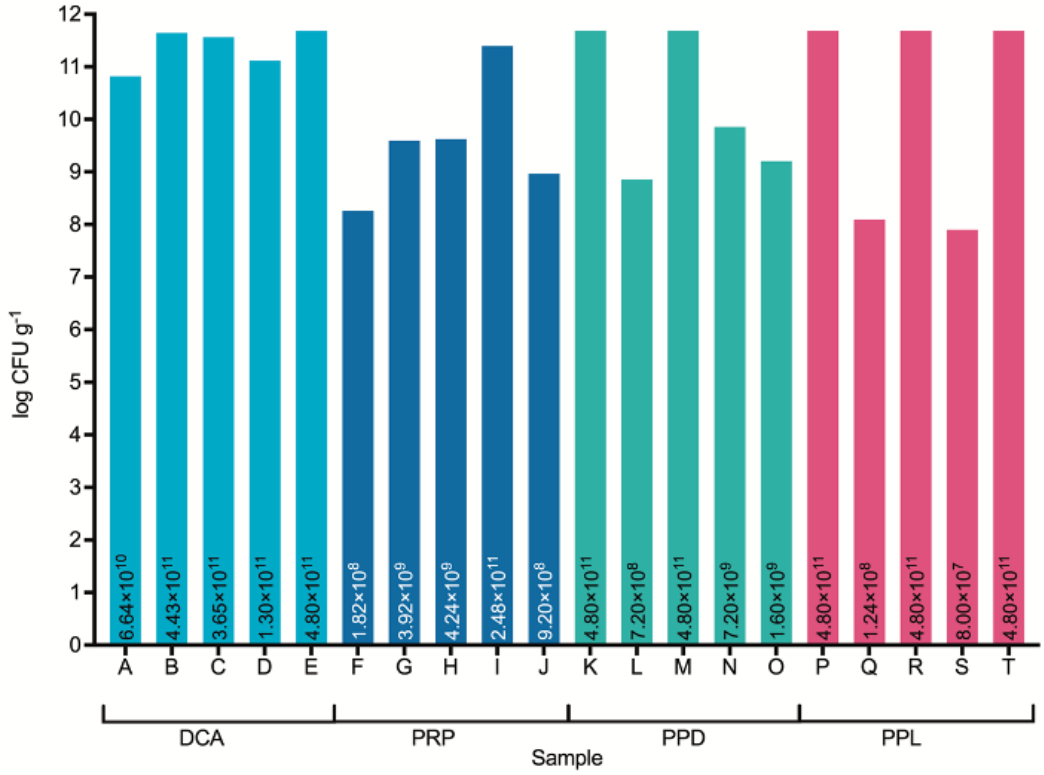


Figure 5.1: Mean abundance of *E. coli* from each of the *β* samples. The number of colony formng units per gram of faecal sample (CFU g^{-1}) is shown on a logarithmic scale on the y-axis. Each sample is represented on the x-axis, the light blue bars (A-E) are dairy calf (DCA) samples, the dark blue bars (F-J) are pre-parturition (PRP) samples, the green bars (K-O) are post-parturition dry cow (PPD) samples, and the pink bars (P-T) are post-parturition lactating cows (PPL) samples. The CFU g^{-1} of each sample is displayed at the bottom of each bar. Sample E, K, M, P, R, and T had counts above the limit of quantification for the greatest serial dilution. Therefore, the actual CFU g^{-1} was greater than the 4.8x10^{11} displayed in this figure.

From the 20 samples across four bovine developmental groups, 300 non-toxigenic *E. coli* were isolated on several different TBX compositions (Table 5.2). A limited number of the isolates (n = 12) from the PPL samples could be picked from TBX supplemented with ampicillin or tetracycline, while slightly fewer isolates (n = 23) from the PPD samples could be picked from TB supplemented with tetracycline. Equivalent numbers of the isolates (n = 25) from all of the other samples could be isolated on all TBX compositions (Table 5.2).

Table 5.2: Number of *E. coli* isolates picked from each isolation media for each developmental group.

Sample group	TBX	TBX + AMP	TBX + TE	Total
DCA	25	25	25	75
PRP	25	25	25	75
PPD	27	25	23	75
PPL	63	4	8	75
Total	140	79	81	300

When plating the faecal samples on TBX, a KWT determined there were no significant differences in the mean ranks of *E. coli* CFU g^{-1} from the DCA, PRP, PPD, and PPL samples (Figure 5.3). On TBX with ampicillin and TBX with tetracycline, the mean *E. coli* CFU g^{-1} from the DCA samples was significantly higher than from the PPL samples (ρ = 0.0003 and ρ = 0.0004) (Figure 5.4 and Figure 5.6). Overall bacterial abundance was investigated with counts from NA, where the mean of all of the samples was 1.75 $x10^{11}$ CFU g^{-1} (Figure 5.5). The mean bacterial abundance was significantly higher in the DCA than the PPL samples on NA (ρ = 0.0146), NA with ampicillin (ρ = 0.0008), and NA with tetracycline (ρ = 0.0006); it was also significantly higher in the DCA samples than the PPD samples on NA with ampicillin (ρ = 0.0353) (Figure 5.3, Figure 5.5, and Figure 5.7). The abundance of *E. coli* and bacteria for each sample group across the media types were also compared (Appendix 22). In the DCA samples, there were no significant differences in abundance across all media (Appendix 22). In the PRP samples, there was a significantly greater abundance on TBX and NA than on TBX with ampicillin (ρ = 0.0172 and ρ = 0.0019) (Appendix 22). In the PPD samples, there was a significantly higher *E. coli* abundance on TBX than on TBX with ampicillin (ρ = 0.0122) and there was a significantly higher bacterial abundance on NA than on TBX with ampicillin and NA with ampicillin (ρ = 0.0048 and ρ = 0.0374) (Appendix 22). In the PPL samples, there was a significantly higher *E. coli* abundance on TBX than on TBX with ampicillin and TBX with tetracycline (ρ = 0.0017 and ρ = 0.0215). The PPL samples also had a significantly higher bacterial abundance on NA than on TBX with ampicillin (ρ = 0.0046) (Appendix 22).

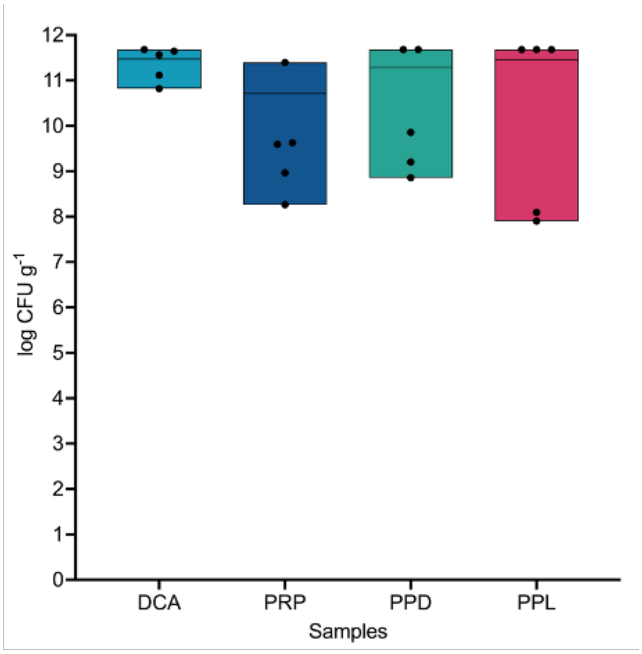


Figure 5.2: Grouped mean *E. coli* abundance per gram of faeces on tryptone bile X-glucuronide agar. The light blue box shows the range of counts from the dairy calf (DCA) samples, the dark blue box shows the range of counts from the pre-parturition cow (PRP) samples, the green box shows the range of counts from the post-parturition dry cow (PPD) samples, and the pink box shows the range of counts from the post-parturition lactating cow (PPL) samples. The number of colony forming units per gram of faecal samples (CFU g^{-1}) is represented with a logarithmic scale on the y-axis. The midline of the boxes shows the sample group mean colony forming units per gram of faeces (CFU g^{-1}), and the dots show the CFU g^{-1} of each sample. These counts include only the colonies with a green/blue morphology. The Kruskal-Wallis ANOVA with Dunn's multiple comparisons test was used to determine if significant differences existed between the ranks of the grouped means.

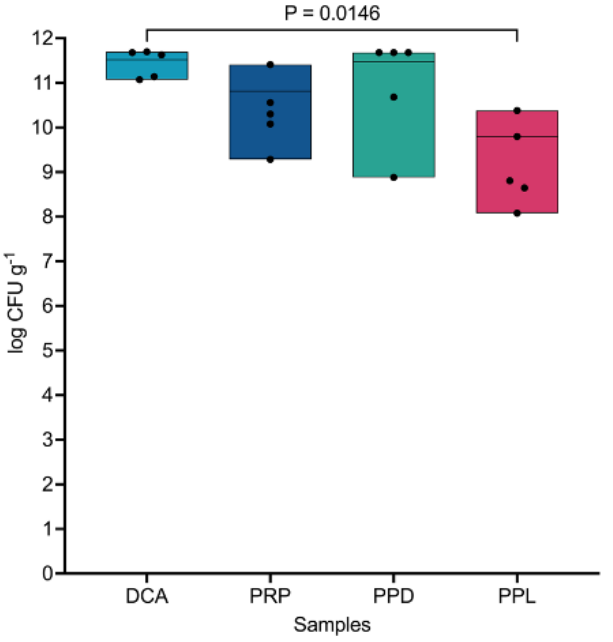


Figure 5.3: Grouped mean bacterial abundance per gram of faeces on nutrient agar. The light blue box shows the range of counts from the dairy calf (DCA) samples, the dark blue box shows the range of counts from the pre-parturition cow (PRP) samples, the green box shows the range of counts from the post-parturition dry cow (PPD) samples, and the pink box shows the range of counts from the post-parturition lactating cow (PPL) samples. The number of colony forming units per gram of faecal samples (CFU g^{-1}) is represented with a logarithmic scale on the y-axis. The midline of the boxes shows the sample group mean colony forming units per gram of faeces (CFU g^{-1}), and the dots show the CFU g^{-1} of each sample. These counts include all colony morphologies. The Kruskal-Wallis ANOVA with Dunn's multiple comparisons test was used to determine if significant differences existed between the ranks of the grouped means.

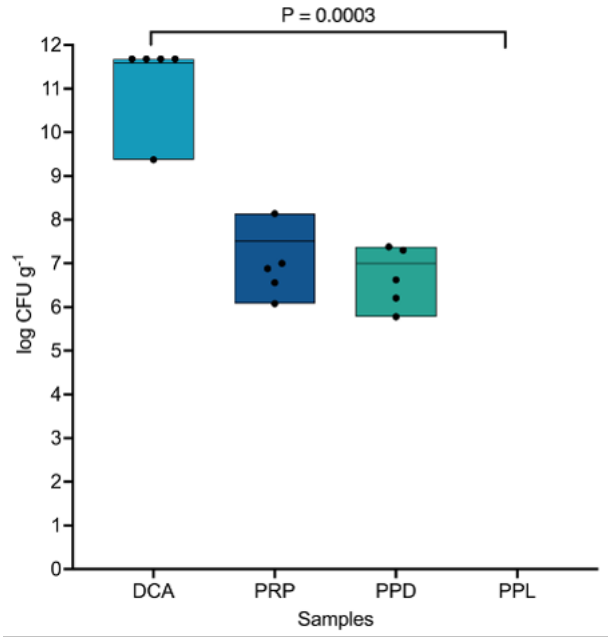


Figure 5.4: Grouped mean *E. coli* abundance per gram of faeces on tryptone bile X-glucuronide agar supplemented with ampicillin. The light blue box shows the range of counts from the dairy calf (DCA) samples, the dark blue box shows the range of counts from the pre-parturition cow (PRP) samples, the green box shows the range of counts from the post-parturition dry cow (PPD) samples, and the pink box shows the range of counts from the post-parturition lactating cow (PPL) samples. The number of colony forming units per gram of faecal samples (CFU g^{-1}) is represented with a logarithmic scale on the y-axis. The midline of the boxes shows the sample group mean colony forming units per gram of faeces (CFU g^{-1}), and the dots show the CFU g^{-1} of each sample. These counts include only the colonies with a green/blue morphology. The Kruskal-Wallis ANOVA with Dunn's multiple comparisons test was used to determine if significant differences existed between the ranks of the grouped means.

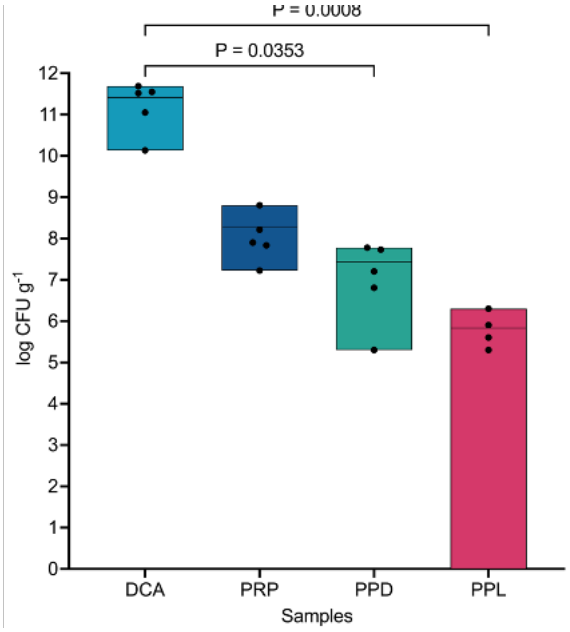


Figure 5.5: Grouped mean bacterial abundance per gram of faeces on nutrient agar supplemented with ampicillin. The light blue box shows the range of counts from the dairy calf (DCA) samples, the dark blue box shows the range of counts from the pre-parturition cow (PRP) samples, the green box shows the range of counts from the post-parturition dry cow (PPD) samples, and the pink box shows the range of counts from the post-parturition lactating cow (PPL) samples. The number of colony forming units per gram of faecal samples (CFU g^{-1}) is represented with a logarithmic scale on the y-axis. The midline of the boxes shows the sample group mean colony forming units per gram of faeces (CFU g^{-1}), and the dots show the CFU g^{-1} of each sample. These counts include all colony morphologies. The Kruskal-Wallis ANOVA with Dunn's multiple comparisons test was used to determine if significant differences existed between the ranks of the grouped means.

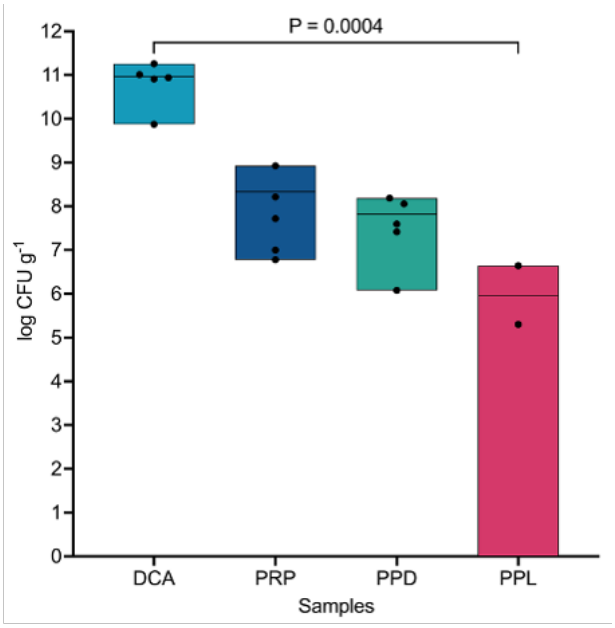


Figure 5.6: Grouped mean *E. coli* abundance per gram of faeces on tryptone bile X-glucuronide agar supplemented with tetracycline. The light blue box shows the range of counts from the dairy calf (DCA) samples, the dark blue box shows the range of counts from the pre-parturition cow (PRP) samples, the green box shows the range of counts from the post-parturition dry cow (PPD) samples, and the pink box shows the range of counts from the post-parturition lactating cow (PPL) samples. The number of colony forming units per gram of faecal samples (CFU g^{-1}) is represented with a logarithmic scale on the y-axis. The midline of the boxes shows the sample group mean colony forming units per gram of faeces (CFU g^{-1}), and the dots show the CFU g^{-1} of each sample. These counts include only the colonies with a green/blue morphology. The Kruskal-Wallis ANOVA with Dunn's multiple comparisons test was used to determine if significant differences existed between the ranks of the grouped means.

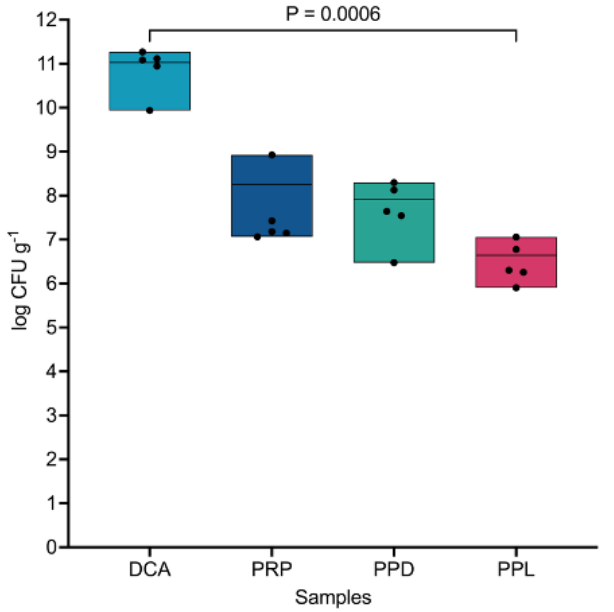


Figure 5.7: Grouped mean bacterial abundance per gram of faeces on nutrient agar supplemented with tetracycline. The light blue box shows the range of counts from the dairy calf (DCA) samples, the dark blue box shows the range of counts from the pre-parturition cow (PRP) samples, the green box shows the range of counts from the post-parturition dry cow (PPD) samples, and the pink box shows the range of counts from the post-parturition lactating cow (PPL) samples. The number of colony forming units per gram of faecal samples (CFU g^{-1}) is represented with a logarithmic scale on the y-axis. The midline of the boxes shows the sample group mean colony forming units per gram of faeces (CFU g^{-1}), and the dots show the CFU g^{-1} of each sample. These counts include all colony morphologies. The Kruskal-Wallis ANOVA with Dunn's multiple comparisons test was used to determine if significant differences existed between the ranks of the grouped means.

5.4.2 Antibiotic susceptibility and susceptibility levels of the *E. coli* from the β samples

ASLs in accordance with VARSS and EFSA descriptors are presented to provide veterinary context to these ASR's (Table 2.5). The ASRs and ASLs for all 300 isolates are presented in Table 5.3, and the ASLs for each antibiotic are described in this section.

Across all 300 isolates, a very high level of susceptibility (59.7%), a very low level of SIE (0.7%), and a high level of resistance (39.7%) was found to ampicillin. An extremely high level of susceptibility (80.3%), a low level of SIE (9.3%), and a moderate level of resistance (10.3%) was found to amoxicillin-clavulanate. A high level of susceptibility (35%), a very high level of SIE (57.3%), and a low level of resistance (7.7%) was found to cefotaxime. A high level of susceptibility (35%), a very high level of SIE (59%), and a moderate level of resistance (18.7%) was found to streptomycin. A high level of susceptibility (37.3%), a very high level of SIE (61%), and a low level of resistance (1.7%) was found to kanamycin. A very high level of susceptibility (67%), a moderate level of SIE (12%), and a high level of resistance (21%) was found to sulfonamide. An extremely high level of susceptibility (82%), a very low level of SIE (0.7%), and a moderate level of resistance (17.3%) was found to chloramphenicol. An extremely high level of susceptibility (81.7%), a low level of SIE (7%), and a moderate level of resistance (11.3%) was found in these isolates to ciprofloxacin. A very high level of susceptibility (50.3%), a low level of SIE (6%), and a high level of resistance (43.7%) was found to tetracycline.

Table 5.3: Overall antibiotic susceptibility and susceptibility levels of the 300 *β E.coli* isolates.

	AMP	AMC	CTX	S10	K	S300	C	CIP	TE
Susceptible	179	241	105	67	112	201	246	245	151
SIE	2	28	172	177	183	36	2	21	18
Resistant	119	31	23	56	5	63	52	34	131
% Susceptible	59.7	80.3	35.0	22.3	37.3	67.0	82.0	81.7	50.3
Susceptible level	Very high	Extremely high	High	High	High	Very high	Extremely high	Extremely high	Very high
% SIE	0.7	9.3	57.3	59	61	12	0.7	7	6
SIE Level	Very low	Low	Very high	Very high	Very high	Moderate	Very low	Low	Low
% Resistant	39.7	10.3	7.7	18.7	1.7	21	17.3	11.3	43.7
Resistance level	High	Moderate	Low	Moderate	Low	High	Moderate	Moderate	High

SIE = susceptible increased exposure, AMP = ampicillin, AMC = amoxicillin-clavulanate, CTX = cefotaxime, S10 = streptomycin, K = kanamycin, S300 = sulfonamide, C = chloramphenicol, CIP = ciprofloxacin, TE = tetracycline. The level descriptors follow VARSS and EFSA standards defined as rare (<0.1%), very low (0.1-1%), low (1-10%), moderate (10-20%), high (20-50%), very high (50-70%), and extremely high (>70%) (European Food Safety Authority, 2022; Veterinary Medicines Directorate, 2023b).

5.4.3 Determining the level of multidrug resistant *E. coli* in the β samples

An extremely high level of the isolates were classified as non-MDR (78.3%), with resistances to two or fewer classes. A high level of the isolates were susceptible to all (35.7%) or one (30.3%) of the antibiotic classes tested. A moderate level of the isolates were resistant to at least two of the antibiotic classes tested (12.7%). There was a high level of MDR isolates (21.3%), with 64 of the isolates were resistant to between three and six antibiotic classes. Separating the MDR isolates by the number of classes they were resistant to, a low level were resistant to three (6.3%) or four (3%) antibiotic classes, a very low level were resistant to five antibiotic classes (0.7%), and a moderate level were resistant to six antibiotic classes (11.3%) (Table 5.4).

Table 5.4: Number of resistant β isolates and susceptibility levels against the antibiotic classes tested.

Classes (n)	0	1	2	3	4	5	6
Isolates (n)	107	91	38	19	9	2	34
Percentage	35.7	30.3	12.7	6.3	3.0	0.7	11.3
Level	High	High	Moderate	Low	Low	Very low	Moderate
Non-MDR	78.7 (Extremely high)			NA			
MDR	NA			21.3 (High)			

The number of isolates from each sample group with resistance to a given number of antibiotic classes and the susceptibility level descriptors are presented in Table 5.5 and described in this section. A very high level (58.7%) of the isolates from the DCA samples were MDR, with resistance to 6 antibiotic classes the most common phenotype (44%, a high level). A low level (4%) of the isolates from the PRP samples were MDR, with resistance to one class of antibiotic the most common phenotype (42.7%, a high level). A low level (5.3%) of the isolates from the PPL samples were MDR, with resistance to no classes of antibiotic the most common phenotype (68%, a very high level). A

moderate level (17.3%) of the isolates from the PPD samples were MDR, with resistance to one class and six classes of antibiotics the most common phenotypes (44%, high levels).

Table 5.5: Number of resistant β isolates and susceptibility levels against the antibiotic classes tested from each sample group.

Sample group	Classes (n)	0	1	2	3	4	5	6
Dairy calves	Isolates (n)	10	6	15	10	1	0	33
	Percentage	13.3	8	20	13.3	1.3	0	44.0
	Level	Moderate	Low	High	Moderate	Low	Rare	High
	Non-MDR	41.3 (High)			NA			
	MDR	NA			58.7 (Very high)			
Pre-parturition cows	Isolates (n)	21	32	19	0	1	2	0
	Percentage	28	42.7	25.3	0	1.3	2.7	0
	Level	High	High	High	Rare	Low	Low	Rare
	Non-MDR	96 (Extremely high)			NA			
	MDR	NA			4 (Low)			
Post-parturition lactating cows	Isolates (n)	51	20	0	1	3	0	0
	Percentage	68	26.7	0	1.3	4	0	0
	Level	Very high	High	Rare	Low	Low	Rare	Rare
	Non-MDR	94.7 (Extremely high)			NA			
	MDR	NA			5.3 (Low)			
Post-parturition dry cows	Isolates (n)	25	33	4	8	4	0	1
	Percentage	33.3	44	5.3	10.7	5.3	0	1.3
	Level	High	High	Low	Moderate	Low	Rare	Low
	Non-MDR	82.7 (Extremely high)			NA			
	MDR	NA			17.3 (Moderate)			

Percentage of the isolates from a given samples group (the number of the isolates collected from each sample group was the same, n = 75).

5.4.4 Enriching samples for antibiotic resistant *E. coli*

When samples were plated on TBX without antibiotic supplementation, 140 *E. coli* isolates were picked. The ASRs and ASLs of these isolates are shown in Table 5.6, and the ASLs of this subset of the isolates for each antibiotic are described in this section. To ampicillin, an extremely high level were susceptible (89.3%), a very low level were SIE (0.7%), and a low level were resistant (10%). To amoxicillin-clavulanate, an extremely high level were susceptible (91.4%), a low level were SIE (5%), and a low level were resistant (4%). To cefotaxime, a high level were susceptible (32.9%), a very high level were SIE (64.3%), and a low level were resistant (3%). To streptomycin, a high level were susceptible (27.1%), a very high level were SIE (61.4%), and a moderate level were resistant (11%). To kanamycin, a high level were susceptible (42.9%), a very high level were SIE (54.2%), and a low level were resistant (3%). To sulfonamide, an extremely high level were susceptible (85%), a moderate level were SIE (10.7%), and a low level were resistant (4%). To chloramphenicol, a very high level were susceptible (65.7%), a high level were SIE (30.7%), and a low level were resistant (4%). To ciprofloxacin, an extremely high level were susceptible (97.1%), a very low level were SIE (0.7%), and a low level were resistant (2%). To tetracycline, an extremely high level were susceptible (92.9%), a rare level were SIE (0%), and a low level were resistant (7%).

Table 5.6: Overall antibiotic susceptibility and susceptibility levels of the 140 *β E. coli* isolates picked from TBX without antibiotic supplementation.

	AMP	AMC	CTX	S10	K	S300	C	CIP	TE
Susceptible	125	128	46	38	60	119	92	136	130
SIE	1	7	90	86	76	15	43	1	0
Resistant	14	5	4	16	4	6	5	3	10
% Susceptible	89.3	91.4	32.9	27.1	42.9	85.0	65.7	97.1	92.9
Susceptible level	Extremely high	Extremely high	High	High	High	Extremely high	Very high	Extremely high	Extremely high
% SIE	0.7	5	64.3	61.4	54.3	10.7	30.7	0.7	0
SIE Level	Very low	Low	Very high	Very high	Very high	Moderate	High	Very low	Rare
% Resistant	10	4	3	11	3	4	4	2	7
Resistance level	Low	Low	Low	Moderate	Low	Low	Low	Low	Low

SIE = susceptible increased exposure, AMP = ampicillin, AMC = amoxicillin-clavulanate, CTX = cefotaxime, S10 = streptomycin, K = kanamycin, S300 = sulfonamide, C = chloramphenicol, CIP = ciprofloxacin, TE = tetracycline. The level descriptors follow VARSS and EFSA standards defined as rare (<0.1%), very low (0.1-1%), low (1-10%), moderate (10-20%), high (20-50%), very high (50-70%), and extremely high (>70%) (European Food Safety Authority, 2022; Veterinary Medicines Directorate, 2023b).

When samples were plated on TBX supplemented with ampicillin, 79 *E. coli* isolates were picked. The ASRs and ASLs of these isolates are shown in Table 5.7, and the ASLs for each antibiotic were as follows. To ampicillin, a rare level were susceptible (0%), a rare level were SIE (0%), and an extremely high level were resistant (100%). To amoxicillin-clavulanate, a very high level were susceptible (57%), a high level were SIE (25%), and a moderate level were resistant (18%). To cefotaxime, a high level were susceptible (44%), a very high level were SIE (51%), and a low level were resistant (5%). To streptomycin, a high level were susceptible (24%), a very high level were SIE (53%), and a high level were resistant (23%). To kanamycin, a high level were susceptible (41%), a very high level were SIE (58%), and a low level were resistant (1%). To sulfonamide, a very high level were susceptible (52%), a low level were SIE (6%), and a high level were resistant (42%). To chloramphenicol, a high level were susceptible (33%), a high level were SIE (32%), and a high level were resistant (35%). To ciprofloxacin, an extremely high level were susceptible (87%), a low level were SIE (3%), and a low level were resistant (10%). To tetracycline, a high level were susceptible (27%), a high level were SIE (23%), and a very high level were resistant (51%).

Table 5.7: Overall antibiotic susceptibility and susceptibility levels of the 79 *β E. coli* isolates picked from TBX supplemented with ampicillin.

	AMP	AMC	CTX	S10	K	S300	C	CIP	TE
Susceptible	0	45	35	19	32	41	26	69	21
SIE	0	20	40	42	46	5	25	2	18
Resistant	79	14	4	18	1	33	28	8	40
% Susceptible	0	57	44	24	41	52	33	87	27
Susceptible level	Rare	Very high	High	High	High	Very high	High	Extremely high	High
% SIE	0	25	51	53	58	6	32	3	23
SIE Level	Rare	High	Very high	Very high	Very high	Low	High	Low	High
% Resistant	100	18	5	23	1	42	35	10	51
Resistance level	Extremely high	Moderate	Low	High	Very low	High	High	Low	Very high

SIE = susceptible increased exposure, AMP = ampicillin, AMC = amoxicillin-clavulanate, CTX = cefotaxime, S10 = streptomycin, K = kanamycin, S300 = sulfonamide, C = chloramphenicol, CIP = ciprofloxacin, TE = tetracycline. The level descriptors follow VARSS and EFSA standards defined as rare (<0.1%), very low (0.1-1%), low (1-10%), moderate (10-20%), high (20-50%), very high (50-70%), and extremely high (>70%) (European Food Safety Authority, 2022; Veterinary Medicines Directorate, 2023b).

When samples were plated on TBX supplemented with tetracycline, 81 *E. coli* isolates were picked. The ASRs and ASLs of these isolates are shown in Table 5.8, and the ASLs for each antibiotic were as follows. To ampicillin, a very high level was susceptible (67%), a low level was SIE (1%), and a high level was resistant (32%). To amoxicillin-clavulanate, an extremely high level was susceptible (84%), a low level was SIE (1%), and a moderate level was resistant (15%). To cefotaxime, a high level was susceptible (30%), a very high level was SIE (52%), and a moderate level was resistant (19%). To streptomycin, a moderate level was susceptible (12%), a very high level was SIE (60%), and a high level was resistant (27%). To kanamycin, a high level was susceptible (25%), an extremely high level was SIE (75%), and a rare level was resistant (0%). To sulfonamide, a very high level was susceptible (51%), a high level was SIE (20%), and a high level was resistant (30%). The susceptibility levels of these isolates to chloramphenicol were a high level of susceptible (38%), SIE (35%), and resistant (27%). The susceptibility levels of these isolates to ciprofloxacin were an extremely high level of susceptible (77%), a low level of SIE (5%), and a moderate level of resistant (19%). The susceptibility levels of these isolates to tetracycline were a rare level of susceptible (0%), a rare level of SIE (0%), and an extremely high level of resistant (100%) (Table 5.8).

Table 5.8: Overall antibiotic susceptibility and susceptibility levels of the 81 *β E. coli* isolates picked from TBX supplemented with tetracycline.

	AMP	AMC	CTX	S10	K	S300	C	CIP	TE
Susceptible	54	68	24	10	20	41	31	62	0
SIE	1	1	42	49	61	16	28	4	0
Resistant	26	12	15	22	0	24	22	15	81
% Susceptible	67	84	30	12	25	51	38	77	0
Susceptible level	Very high	Extremely high	High	Moderate	High	Very high	High	Extremely high	Rare
% SIE	1	1	52	60	75	20	35	5	0
SIE Level	Low	Low	Very high	Very high	Extremely high	High	High	Low	Rare
% Resistant	32	15	19	27	0	30	27	19	100
Resistance level	High	Moderate	Moderate	High	Rare	High	High	Moderate	Extremely high

SIE = susceptible increased exposure, AMP = ampicillin, AMC = amoxicillin-clavulanate, CTX = cefotaxime, S10 = streptomycin, K = kanamycin, S300 = sulfonamide, C = chloramphenicol, CIP = ciprofloxacin, TE = tetracycline. The level descriptors follow VARSS and EFSA standards defined as rare (<0.1%), very low (0.1-1%), low (1-10%), moderate (10-20%), high (20-50%), very high (50-70%), and extremely high (>70%) (European Food Safety Authority, 2022; Veterinary Medicines Directorate, 2023b).

5.4.5 Antibiotic susceptibility levels of the *β E. coli* isolates from different developmental stages

The ASRs and ASLs of the isolates from the DCA samples are shown in Table 5.9, from the PRP samples are shown in Table 5.10, from the PPL samples are shown in Table 5.11, and from the PPD samples are shown in Table 5.12. The ASLs are described in this section for each antibiotic against the isolates from each sample group.

To ampicillin, the levels of resistance was extremely high from the DCA samples (82.7%), high from the PRP samples (37.3%) and the PPD samples (33.3%), and low from the PPL samples (5.3%). To ampicillin, the levels of SIE was low from the PPL samples (1.3%) and the PPD samples (1.3%), and rare from the DCA samples (0%) and the PRP samples (0%). The levels that were susceptible to ampicillin was low from the DCA samples (17.3%), very high from the PRP samples (62.7%) and the PPD samples (65.3%), and extremely high from the PPL samples (93.3%). To amoxicillin-clavulanate, the levels of resistance was high from the DCA samples (40%), very low from the PRP samples (1.3%), and rare from the PPL samples (0%) and the PPD samples (0%). To amoxicillin-clavulanate, the levels of SIE was moderate from the DCA samples (18.7%) and the PRP samples (17.3%), low from the PPD samples (1.3%), and rare from the PPL samples (0%). The levels that were susceptible to amoxicillin-clavulanate was extremely high from the PRP samples (81.3%), the PPL samples (100%), and the the PPD samples (98.7%), with a high level from the DCA samples (41.3%). To cefotaxime, the levels of resistance was moderate from the PRP samples (14.7%), with a low level from the DCA samples (4%), the PPL samples (5.3%), and the PPD samples (6.7%). The

levels that were SIE to cefotaxime was extremely high from the PPL samples (81.3%) and the PPD samples (77.3%), very high from the PRP samples (64%), and low from the DCA samples (6.7%). The levels that were susceptible to cefotaxime was extremely high from the DCA samples (89.3%), high from the PRP samples (21.3%), and moderate from the PPL samples (13.3%) and the PPD isolates (16%). To streptomycin, the level of resistance was very high from the DCA samples (57.3%), moderate from the PPL samples (10.7%), and low from the PRP samples (5.3%) and the PPD samples (1.3%). The levels that were SIE to streptomycin was extremely high from the PRP samples (78.7%) and the PPD samples (86.7%), very high from the PPL samples (57.3%), and moderate from the DCA samples (13.3%). The levels that were susceptible to streptomycin was high from the DCA samples (29.3%) and the PPL samples (32%), and moderate from the PRP samples (16%) and the PPD samples (12%). The levels that were resistant to kanamycin was low from the PRP samples (2.7%) and the PPL samples (4%), and rare from the DCA samples (0%) and the PPD samples (0%). The levels that were SIE to kanamycin was very high from the DCA samples (54.7%), the PRP samples (66.7%), the PPL samples (56%), and the PPD samples (66.7%). The levels that were susceptible to kanamycin was high from the DCA samples (45.3%), the PRP samples (30.7%), the PPL samples (40%), and the PPD samples (33.3%). The levels that were resistant to sulfonamide was very high from the DCA samples (56%), moderate from the PPD samples (18.7%), and low from the PRP samples (4%) and the PPL samples (5.3%). The levels that were SIE to sulfonamide was high from the PRP samples (25.3%), moderate from the PPL samples (12%), and low from the DCA samples (2.7%) and the PPD samples (8%). The levels that were susceptible to sulfonamide was extremely

high from the PRP samples (70.7%), the PPL samples (82.7%), and the PPD samples (73.3%), with a high level from the DCA samples (41.3%). The levels that were resistant to chloramphenicol was high from the DCA samples (44%), moderate from the PPD samples (17.3%), and low from the PRP samples (2.7%) and the PPL samples (5.3%). The levels that were SIE to chloramphenicol was low from the PRP samples (1.3%) and the PPL samples (1.3%), and rare from the DCA samples (0%) and the PPD samples (0%). The levels that were susceptible to chloramphenicol was extremely high from the PRP samples (96%), the PPL samples (93.3%), and the PPD samples (82.7%), with a high level from the DCA samples (41.3%). The levels that were resistant to ciprofloxacin was high from the DCA samples (44%), low from the PPD samples (1.3%), and rare from the PRP samples (0%) and the PPL samples (0%). The levels that were SIE to cefotaxime was low from the DCA samples (4%), the PRP samples (9.3%), the PPL samples (5.3%), and the PPD samples (9.3%). The levels that were susceptible to ciprofloxacin was extremely high from the PRP samples (90.7%), the PPL samples (94.7%), and the PPD samples (89.3%), with a very high level from the DCA samples (56%). The levels that were resistant to tetracycline was extremely high from the DCA samples (73.3%), very high from the PRP samples (50.7%) and the PPD samples (37.3%), and moderate from the PPL samples (13.3%). The levels that were SIE to tetracycline was moderate from the PPD samples (14.7%), and low from the DCA samples (1.3%), the PRP samples (5.3%), and the PPL samples (2.7%). The levels that were susceptible to tetracycline was extremely high from the PPL samples (84%), and high from the DCA samples (25.3%), the PRP samples (44%), and the PPD samples (48%).

Table 5.9: Overall antibiotic susceptibility results and susceptibility levels of the 75 *β E. coli* isolates from five DCA faecal samples.

	AMP	AMC	CTX	S10	K	S300	C	CIP	TE
Susceptible	13	31	67	22	34	31	42	39	19
SIE	0	14	5	10	41	2	0	3	1
Resistant	62	30	3	43	0	42	33	33	55
% Susceptible	17.3	41.3	89.3	29.3	45.3	41.3	56	52	25.3
Susceptible level	Moderate	High	Extremely high	High	High	High	Very high	Very high	High
% SIE	0	18.7	6.7	13.3	54.7	2.7	0	4	1.3
SIE Level	Rare	Moderate	Low	Moderate	Very high	Low	Rare	Low	Low
% Resistant	82.7	40	4	57.3	0	56	44	44	73.3
Resistance level	Extremely high	High	Low	Very high	Rare	Very high	High	High	Extremely high

SIE = susceptible increased exposure, AMP = ampicillin, AMC = amoxicillin-clavulanate, CTX = cefotaxime, S10 = streptomycin, K = kanamycin, S300 = sulfonamide, C = chloramphenicol, CIP = ciprofloxacin, TE = tetracycline. The level descriptors follow VARSS and EFSA standards defined as rare (<0.1%), very low (0.1-1%), low (1-10%), moderate (10-20%), high (20-50%), very high (50-70%), and extremely high (>70%) (European Food Safety Authority, 2022; Veterinary Medicines Directorate, 2023b).

Table 5.10: Overall antibiotic susceptibility results and susceptibility levels of the 75 *β E. coli* isolates from five PRP faecal samples.

	AMP	AMC	CTX	S10	K	S300	C	CIP	TE
Susceptible	47	61	16	12	23	53	72	68	33
SIE	0	13	48	59	50	19	1	7	4
Resistant	28	1	11	4	2	3	2	0	38
% Susceptible	62.7	81.3	21.3	16	30.7	70.7	96	90.7	44
Susceptible level	Very high	Extremely high	High	Moderate	High	Extremely high	Extremely high	Extremely high	High
% SIE	0	17.3	64.0	78.7	66.7	25.3	1.3	9.3	5.3
SIE Level	Rare	Moderate	Very high	Extremely high	Very high	High	Low	Low	Low
% Resistant	37.3	1.3	14.7	5.3	2.7	4	2.7	0	50.7
Resistance level	High	Low	Moderate	Low	Low	Low	Low	Rare	Very high

SIE = susceptible increased exposure, AMP = ampicillin, AMC = amoxicillin-clavulanate, CTX = cefotaxime, S10 = streptomycin, K = kanamycin, S300 = sulfonamide, C = chloramphenicol, CIP = ciprofloxacin, TE = tetracycline. The level descriptors follow VARSS and EFSA standards defined as rare (<0.1%), very low (0.1-1%), low (1-10%), moderate (10-20%), high (20-50%), very high (50-70%), and extremely high (>70%) (European Food Safety Authority, 2022; Veterinary Medicines Directorate, 2023b).

Table 5.11: Overall antibiotic susceptibility results and susceptibility levels of the 75 *β E. coli* isolates from five PPL faecal samples.

	AMP	AMC	CTX	S10	K	S300	C	CIP	TE
Susceptible	70	75	10	24	30	62	70	71	63
SIE	1	0	61	43	42	9	1	4	2
Resistant	4	0	4	8	3	4	4	0	10
% Susceptible	93.3	100	13.3	32	40	82.7	93.3	94.7	84
Susceptible level	Extremely high	Extremely high	Moderate	High	High	Extremely high	Extremely high	Extremely high	Extremely high
% SIE	1.3	0	81.3	57.3	56	12	1.3	5.3	2.7
SIE Level	Low	Rare	Extremely high	Very high	Very high	Moderate	Low	Low	Low
% Resistant	5.3	0	5.3	10.7	4	5.3	5.3	0	13.3
Resistance level	Low	Rare	Low	Moderate	Low	Low	Low	Rare	Moderate

SIE = susceptible increased exposure, AMP = ampicillin, AMC = amoxicillin-clavulanate, CTX = cefotaxime, S10 = streptomycin, K = kanamycin, S300 = sulfonamide, C = chloramphenicol, CIP = ciprofloxacin, TE = tetracycline. The level descriptors follow VARSS and EFSA standards defined as rare (<0.1%), very low (0.1-1%), low (1-10%), moderate (10-20%), high (20-50%), very high (50-70%), and extremely high (>70%) (European Food Safety Authority, 2022; Veterinary Medicines Directorate, 2023b).

Table 5.12: Overall antibiotic susceptibility results and susceptibility levels of the 75 *β E. coli* isolates from five PPD faecal samples.

	AMP	AMC	CTX	S10	K	S300	C	CIP	TE
Susceptible	49	74	12	9	25	55	62	67	36
SIE	1	1	58	65	50	6	0	7	11
Resistant	25	0	5	1	0	14	13	1	28
% Susceptible	65.3	98.7	16	12	33.3	73.3	82.7	89.3	48.0
Susceptible level	Very high	Extremely high	Moderate	Moderate	High	Extremely high	Extremely high	Extremely high	High
% SIE	1.3	1.3	77.3	86.7	66.7	8	0	9.3	14.7
SIE Level	Low	Low	Extremely high	Extremely high	Very high	Low	Rare	Low	Moderate
% Resistant	33.3	0	6.7	1.3	0	18.7	17.3	1.3	37.3
Resistance level	High	Rare	Low	Low	Rare	Moderate	Moderate	Low	High

SIE = susceptible increased exposure, AMP = ampicillin, AMC = amoxicillin-clavulanate, CTX = cefotaxime, S10 = streptomycin, K = kanamycin, S300 = sulfonamide, C = chloramphenicol, CIP = ciprofloxacin, TE = tetracycline. The level descriptors follow VARSS and EFSA standards defined as rare (<0.1%), very low (0.1-1%), low (1-10%), moderate (10-20%), high (20-50%), very high (50-70%), and extremely high (>70%) (European Food Safety Authority, 2022; Veterinary Medicines Directorate, 2023b).

The median ASRs of the isolates from the DCA samples were susceptible to cefotaxime, SIE to amoxicillin-clavulanate, and resistant to ampicillin, streptomycin, sulfonamide, and tetracycline (Figure 5.8). The DCA median ASRs were MDR, with resistance to four antibiotics from four distinct classes. The median ASRs of the isolates from the PRP samples were resistance to tetracycline, SIE to cefotaxime, streptomycin, and kanamycin, and susceptibility to ampicillin, amoxicillin-clavulanate, sulfonamide, chloramphenicol, and ciprofloxacin (Figure 5.9). The median ASRs of the isolates from the PPD samples were SIE to cefotaxime, streptomycin, kanamycin, tetracycline, susceptibility to ampicillin, amoxicillin-clavulanate, sulfonamide, chloramphenicol, and ciprofloxacin (Figure 5.10). The median ASRs of the isolates from the PPL samples were SIE to cefotaxime, streptomycin, kanamycin, susceptibility to ampicillin, amoxicillin-clavulanate, sulfonamide, chloramphenicol, ciprofloxacin, and tetracycline (Figure 5.11).

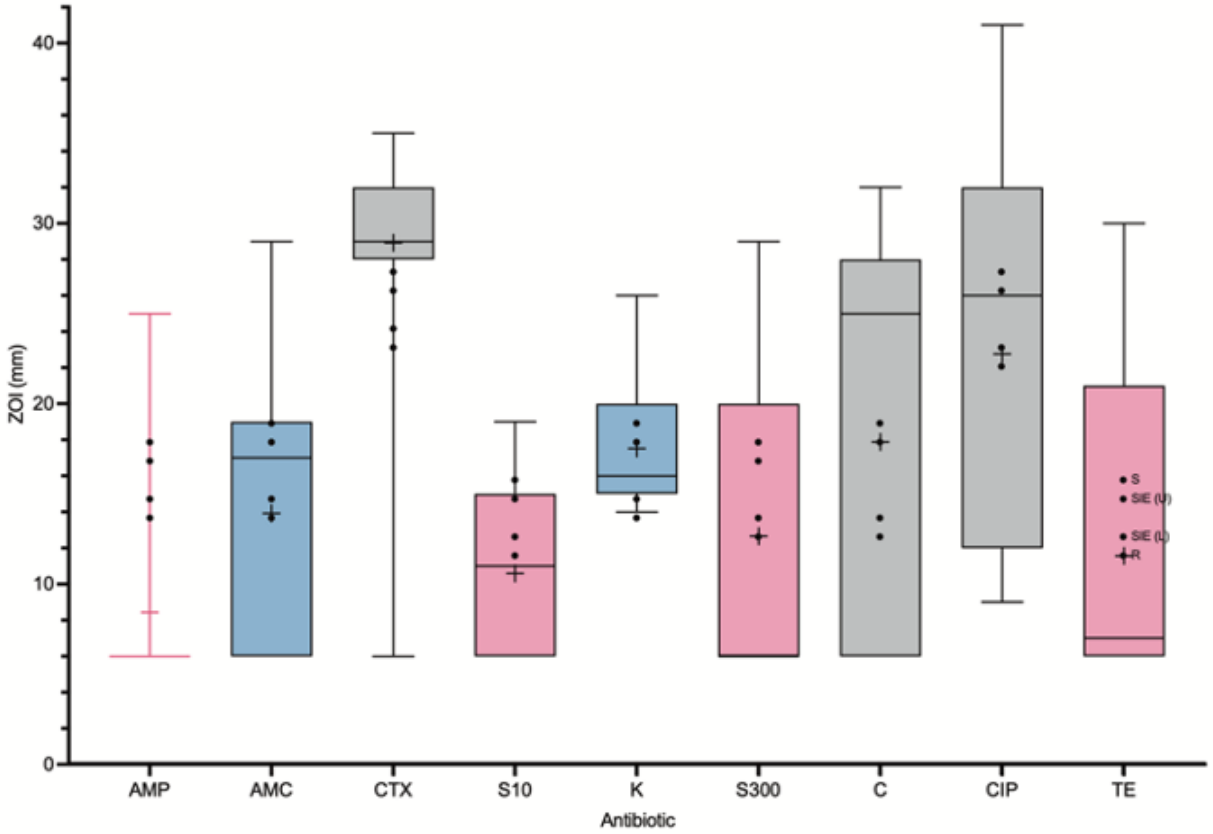


Figure 5.8: Median antibiotic susceptibility distributions of the isolates from the DCA samples. The midline represents the median zone of inhibition (ZOI) across all isolates from the dairy calf (DCA) samples, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown as black dots: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility: pink for resistant, grey for susceptible, and blue for SIE. Abbreviations: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), K (kanamycin), S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), TE (tetracycline).

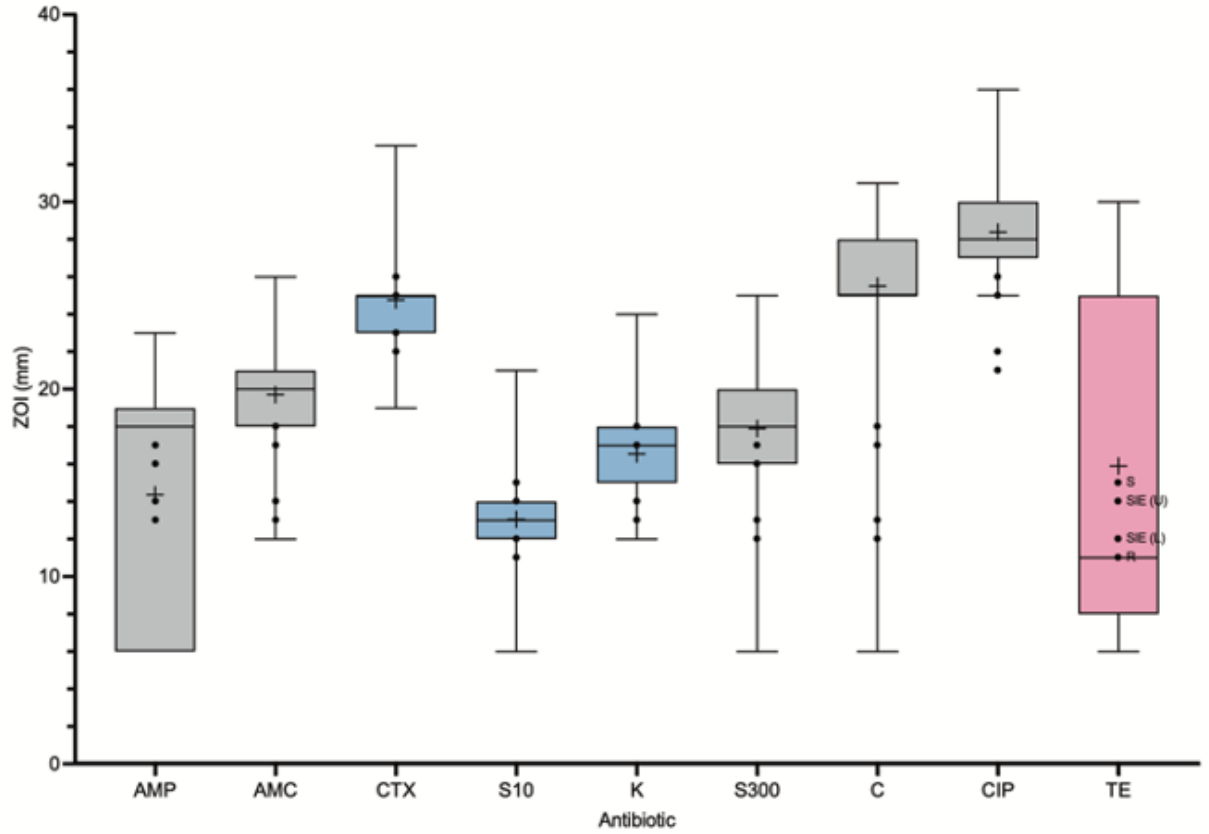


Figure 5.9: Median antibiotic susceptibility distributions of the isolates from the PRP samples. The midline represents the median zone of inhibition (ZOI) across all isolates from the pre-parturition cow (PRP) samples, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown as black dots: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility: pink for resistant, grey for susceptible, and blue for SIE. Abbreviations: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), K (kanamycin), S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), TE (tetracycline).

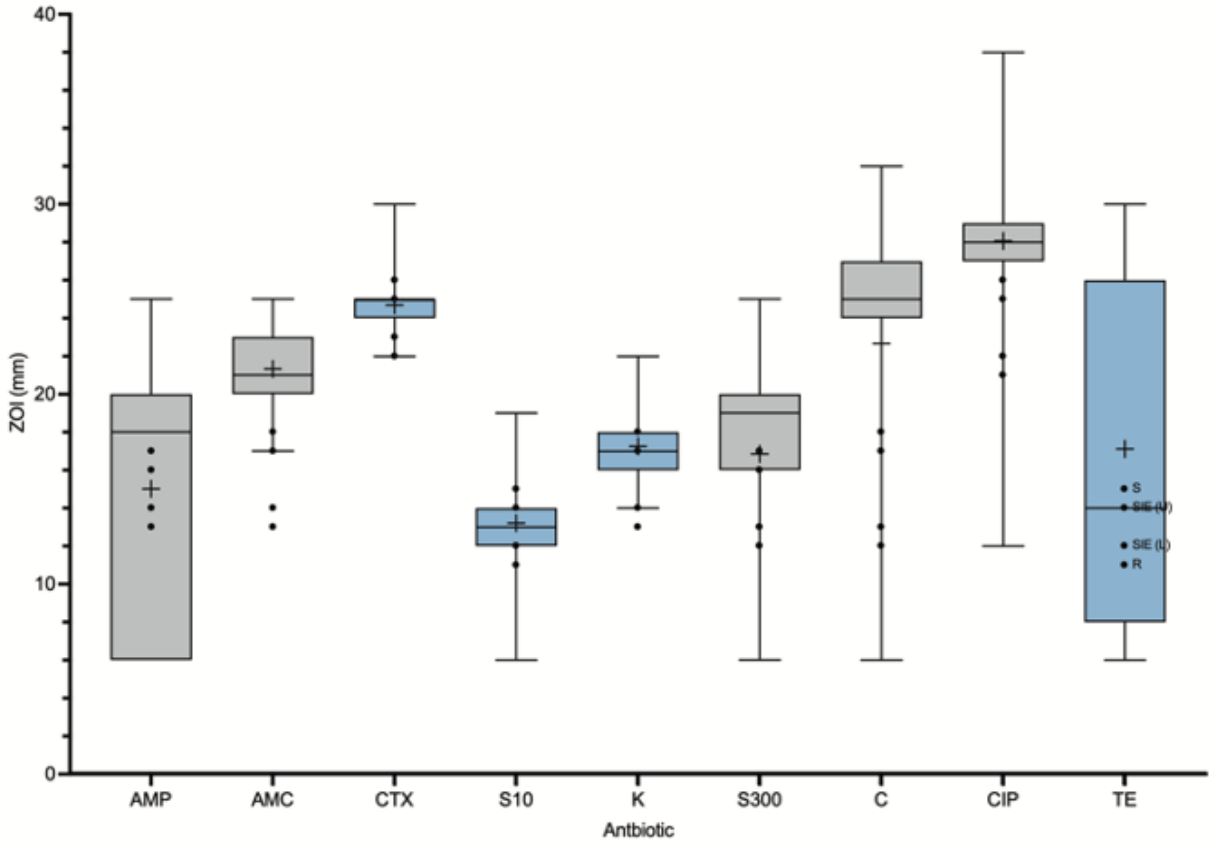


Figure 5.10: Median antibiotic susceptibility distributions of the isolates from the PPD samples. The midline represents the median zone of inhibition (ZOI) across all isolates from the post-parturition dry cow(PPD) samples, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown as black dots: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility: pink for resistant, grey for susceptible, and blue for SIE. Abbreviations: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), K (kanamycin), S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), TE (tetracycline).

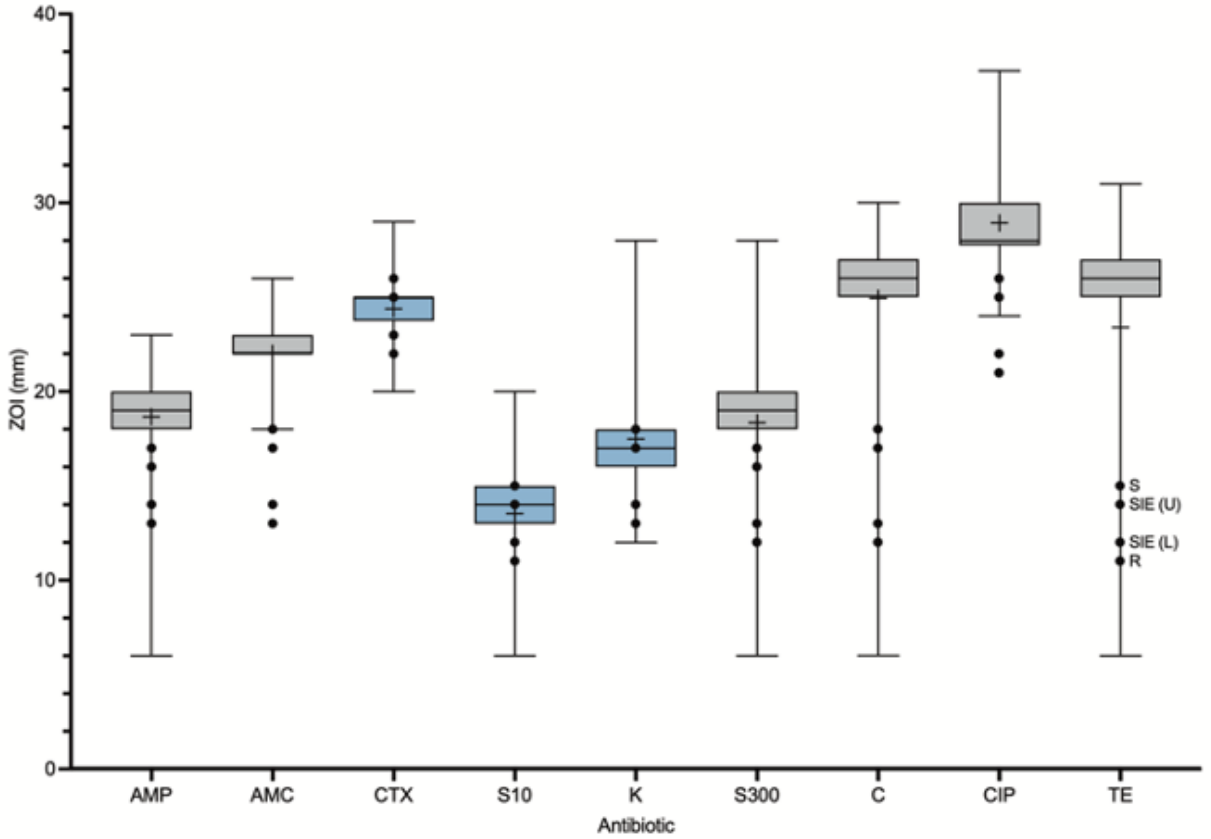


Figure 5.11: Median antibiotic susceptibility distributions of the isolates from the PPL samples. The midline represents the median zone of inhibition (ZOI) across all isolates from the post-parturition lactating cow (PPL) samples, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown as black dots: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility: pink for resistant, grey for susceptible, and blue for SIE. Abbreviations: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), K (kanamycin), S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), TE (tetracycline).

The median ZOI for ampicillin was significantly smaller in the isolates from the DCA samples compared to the isolates from the other groups (ρ <0.0001). The median ZOIs for ampicillin were not significantly different in the isolates from the PRP and the PPD samples. However, they were both significantly smaller than the median ZOI for ampicillin in the isolates from the PPL samples (ρ = 0.0005 & ρ = 0.0095) (Figure 5.12).

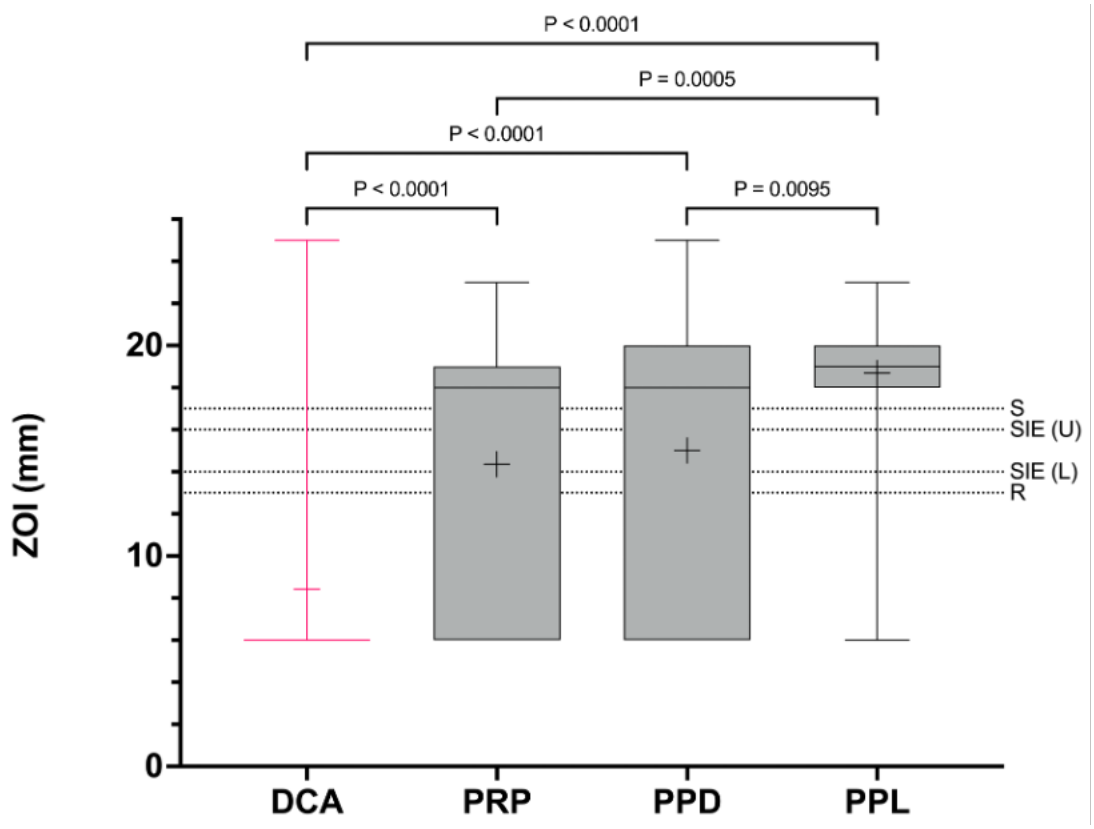


Figure 5.12: Comparison of the median ampicillin susceptibility distributions of the β isolates sampled from different bovine developmental stages. The midline represents the median zone of inhibition (ZOI) across all isolates, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown by the labelled horizontal dotted black lines: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility results, pink for resistant and grey for susceptible. The developmental stages are dairy calves (DCA), pre-parturition dairy cows (PRP), post-parturition dry cows (PPD), and post-parturition lactating cows (PPL). The Kruskal-Wallis ANOVA with Dunn's multiple comparisons test was used to compare the median ZOIs of all groups.

The median ZOI for amoxicillin-clavulanate was significantly smaller in the isolates from the DCA samples compared to the isolates from the other groups (ρ <0.0001). The median ZOI for amoxicillin-clavulanate was significantly smaller in the isolates from the PRP samples compared to the isolates from the PPD or the PPL samples (ρ = 0.0009 & ρ <0.0001), which were not significantly different from each other (Figure 5.13).

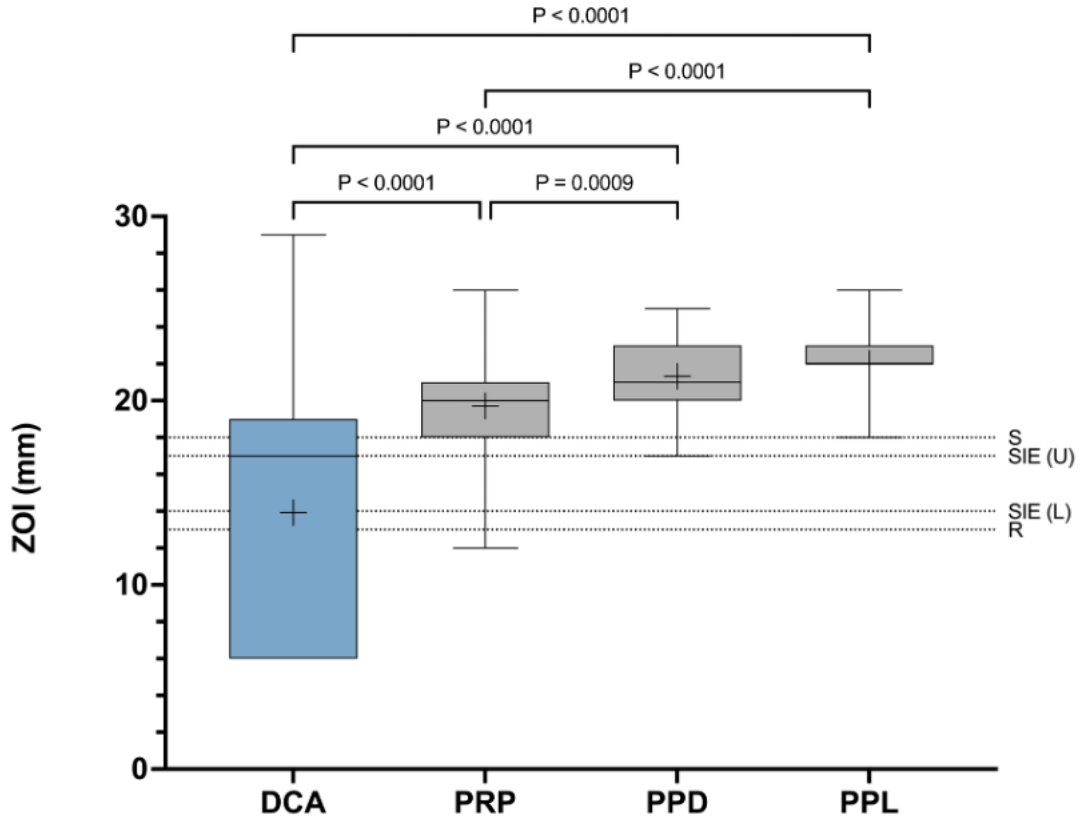


Figure 5.13: Comparison of the median amoxicillin-clavulanate susceptibility distributions of the *β* isolates sampled from different bovine developmental stages. The midline represents the median zone of inhibition (ZOI) across all isolates, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown by the labelled horizontal dotted black lines: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility results, grey for susceptible and blue for SIE. The developmental stages are dairy calves (DCA), pre-parturition dairy cows (PRP), post-parturition dry cows (PPD), and post-parturition lactating cows (PPL). The Kruskal-Wallis ANOVA with Dunn's multiple comparisons test was used to compare the median ZOIs of all groups.

The median ZOI for cefotaxime was significantly larger in the isolates from the DCA samples compared to the isolates from the other groups (ρ <0.0001), with no other significant differences observed (Figure 5.14).

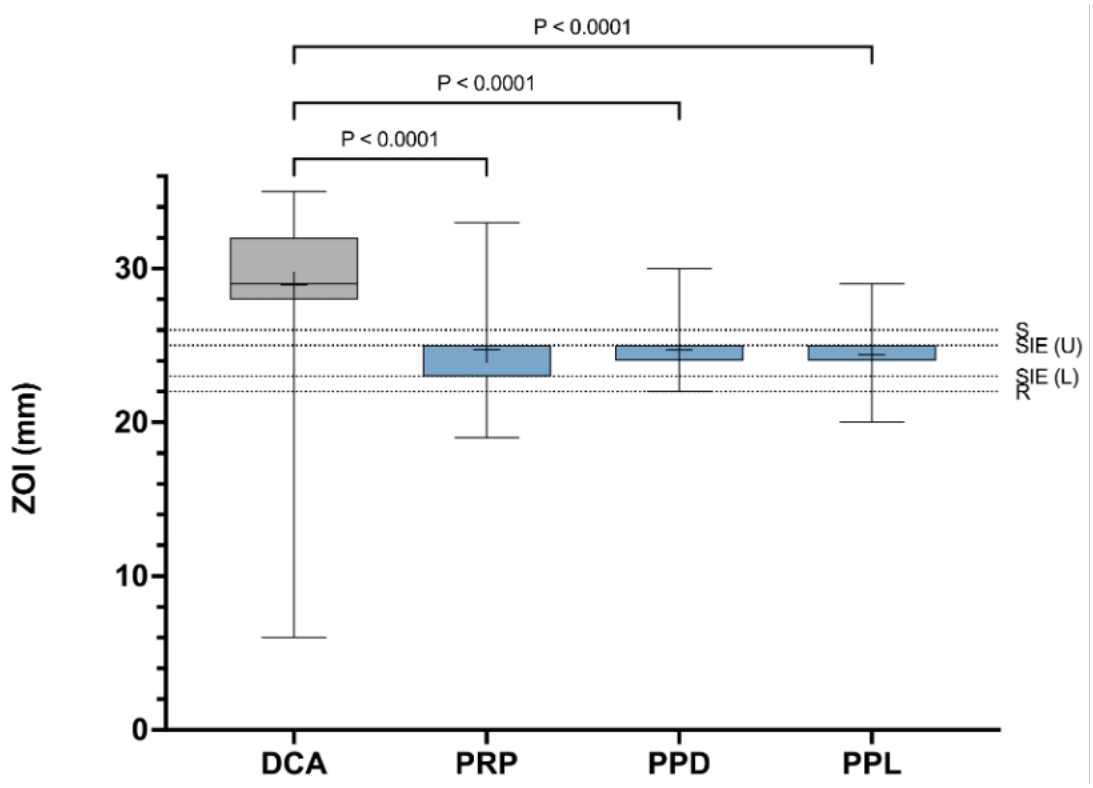


Figure 5.14: Comparison of the median cefotaxime susceptibility distributions of the β isolates sampled from different bovine developmental stages. The midline represents the median zone of inhibition (ZOI) across all isolates, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown by the labelled horizontal dotted black lines: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility results, grey for susceptible and blue for SIE. The developmental stages are dairy calves (DCA), pre-parturition dairy cows (PRP), post-parturition dry cows (PPD), and post-parturition lactating cows (PPL). The Kruskal-Wallis ANOVA with Dunn's multiple comparisons test was used to compare the median ZOIs for cefotaxime across all groups.

The median ZOI for streptomycin was significantly smaller in the isolates from the DCA samples compared to the isolates from the PPD and the PPL samples (ρ = 0.0245 & ρ <0.0001), with no other significant differences observed (Figure 5.15).

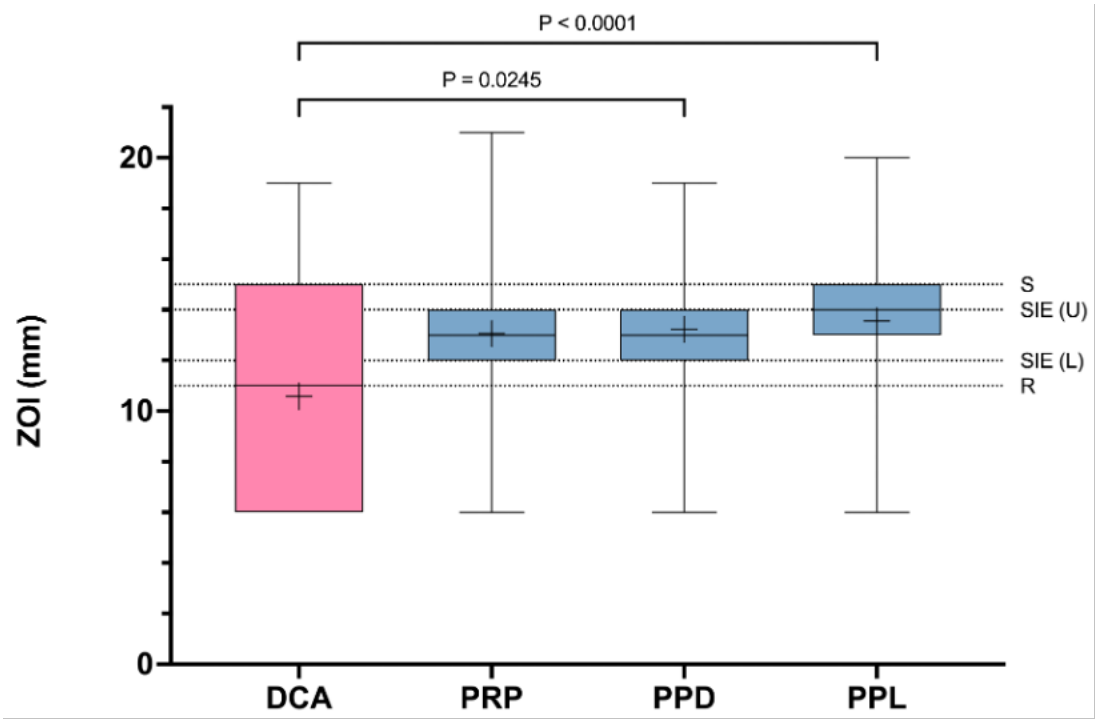


Figure 5.15: Comparison of the median streptomycin susceptibility distributions of the β isolates sampled from different bovine developmental stages. The midline represents the median zone of inhibition (ZOI) across all isolates, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown by the labelled horizontal dotted black lines: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility results, pink for resistant and blue for SIE. The developmental stages are dairy calves (DCA), pre-parturition dairy cows (PRP), post-parturition dry cows (PPD), and post-parturition lactating cows (PPL). The Kruskal-Wallis ANOVA with Dunn's multiple comparisons test was used to compare the median ZOIs for streptomycin across all groups.

The median ZOI for kanamycin was significantly smaller in the isolates from the PRP samples compared to the isolates from the PPL samples ($\rho = 0.0234$), with no other significant differences observed (Figure 5.16).

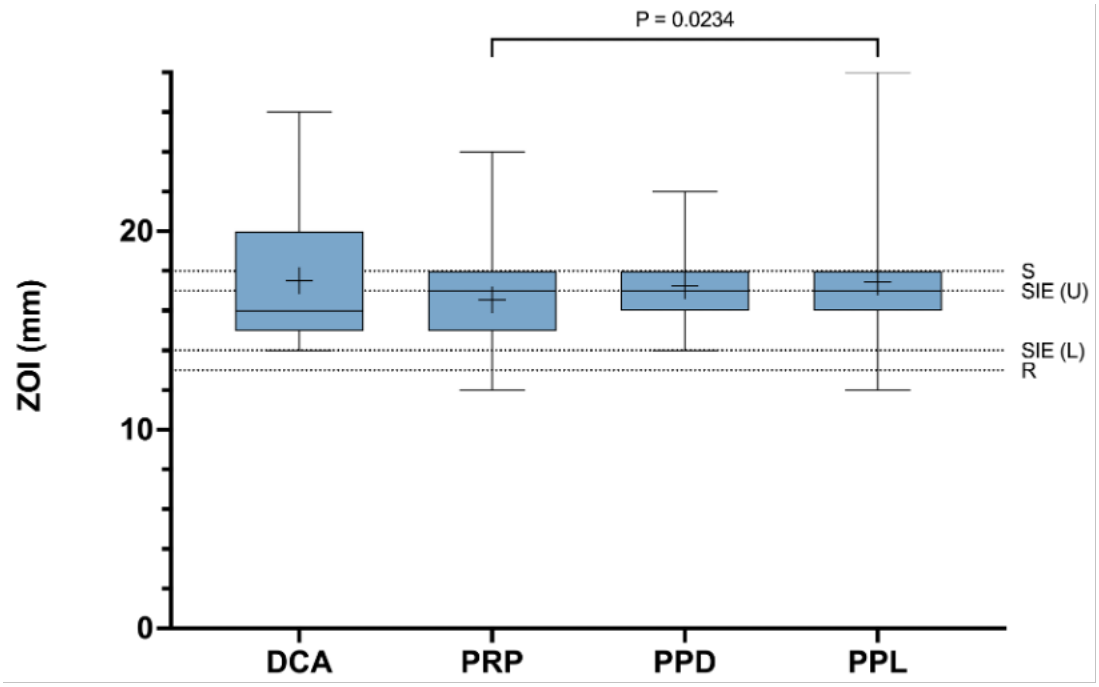


Figure 5.16: Comparison of the median kanamycin susceptibility distributions of the β isolates sampled from different bovine developmental stages. The midline represents the median zone of inhibition (ZOI) across all isolates, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown by the labelled horizontal dotted black lines: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The blue box colour indicates the median antibiotic susceptibility results of SIE. The developmental stages are dairy calves (DCA), pre-parturition dairy cows (PRP), post-parturition dry cows (PPD), and post-parturition lactating cows (PPL). The Kruskal-Wallis ANOVA with Dunn's multiple comparisons test was used to compare the median ZOIs for kanamycin across all groups.

The median ZOI for sulfonamide was significantly smaller in the isolates from the DCA samples compared to the isolates from the PRP, the PPD, and the PPL samples (ρ = 0.0066, ρ = 0.0059, and ρ <0.0001), with no other significant differences observed (Figure 5.17).

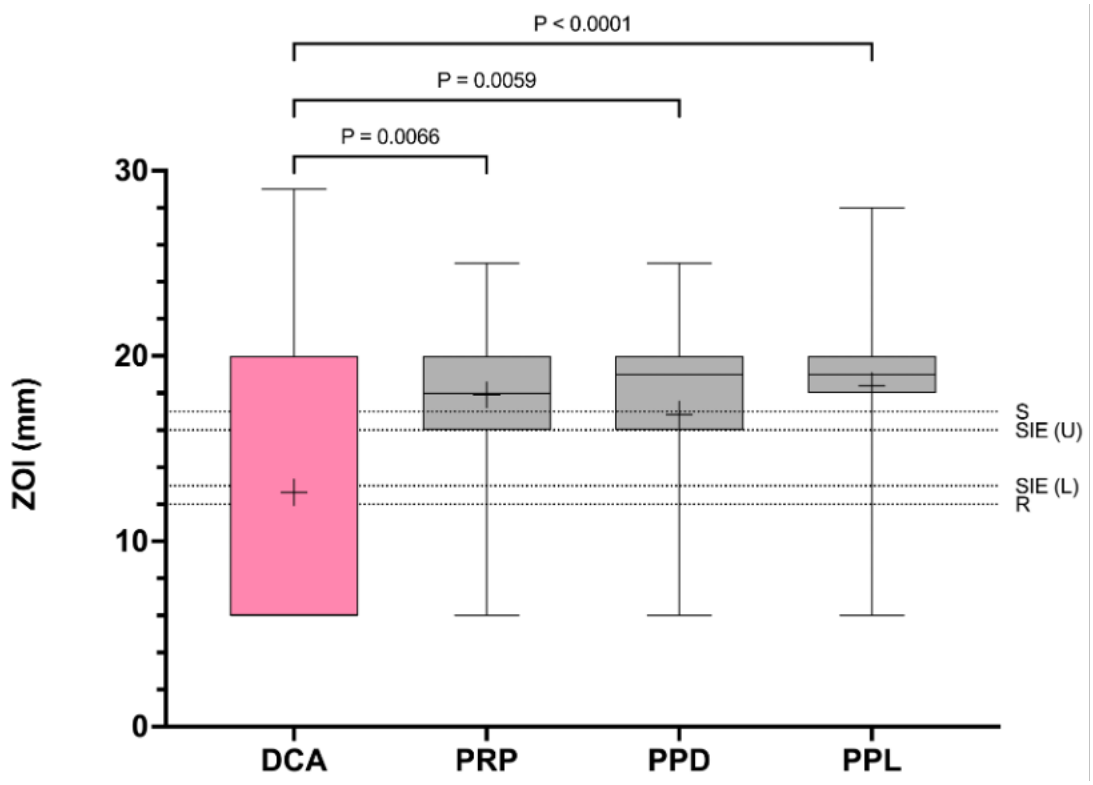


Figure 5.17: Comparison of the median sulfonamide susceptibility distributions of the β isolates sampled from different bovine developmental stages. The midline represents the median zone of inhibition (ZOI) across all isolates, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown by the labelled horizontal dotted black lines: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility results, pink for resistant and grey for susceptible. The developmental stages are dairy calves (DCA), pre-parturition dairy cows (PRP), post-parturition dry cows (PPD), and post-parturition lactating cows (PPL). The Kruskal-Wallis ANOVA with Dunn's multiple comparisons test was used to compare the median ZOIs for sulfonamide across all groups.

The median ZOI for chloramphenicol was significantly smaller in the isolates from the DCA samples compared to the isolates from the PRP and the PPL samples (ρ = 0.0368 and ρ = 0.0183), with no other significant differences observed (Figure 5.18).

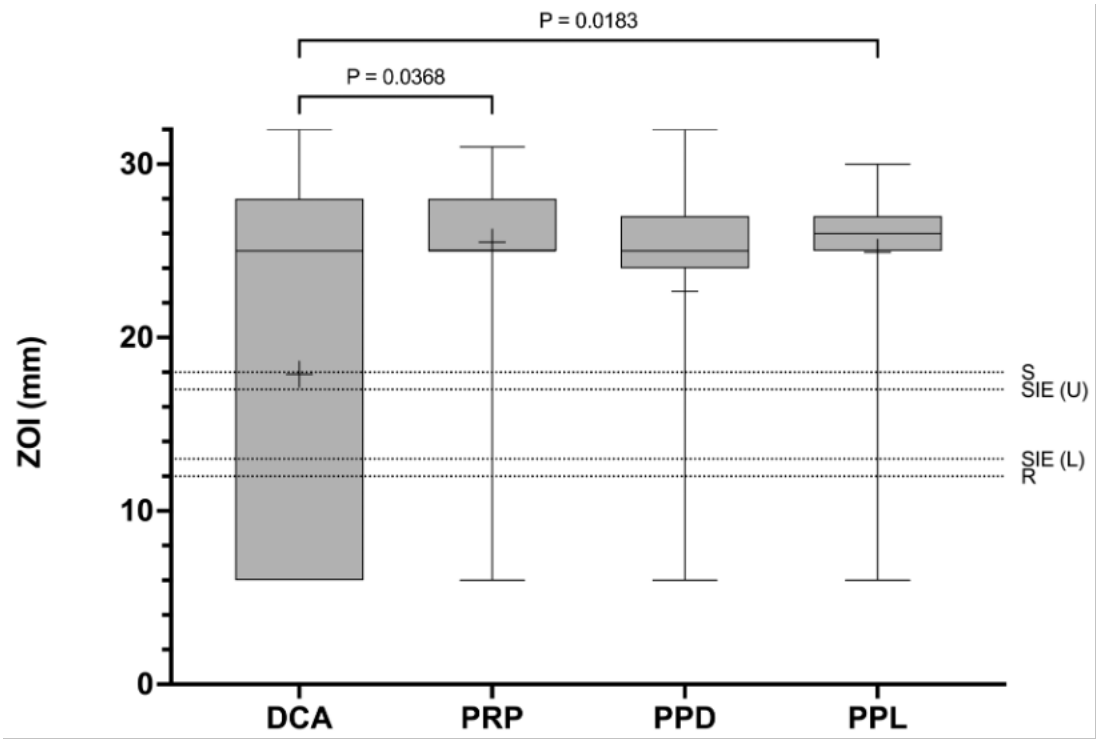


Figure 5.18: Comparison of the median chloramphenicol susceptibility distributions of the β isolates sampled from different bovine developmental stages. The midline represents the median zone of inhibition (ZOI) across all isolates, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown by the labelled horizontal dotted black lines: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The grey box colour indicates the median antibiotic susceptibility results of susceptible. The developmental stages are dairy calves (DCA), pre-parturition dairy cows (PRP), post-parturition dry cows (PPD), and post-parturition lactating cows (PPL). The Kruskal-Wallis ANOVA with Dunn's multiple comparisons test was used to compare the median ZOIs for chloramphenicol across all groups.

The median ZOI for ciprofloxacin was significantly smaller in the isolates from the DCA samples compared to the isolates from the PPL samples (ρ = 0.0181), with no other significant differences observed (Figure 5.19).

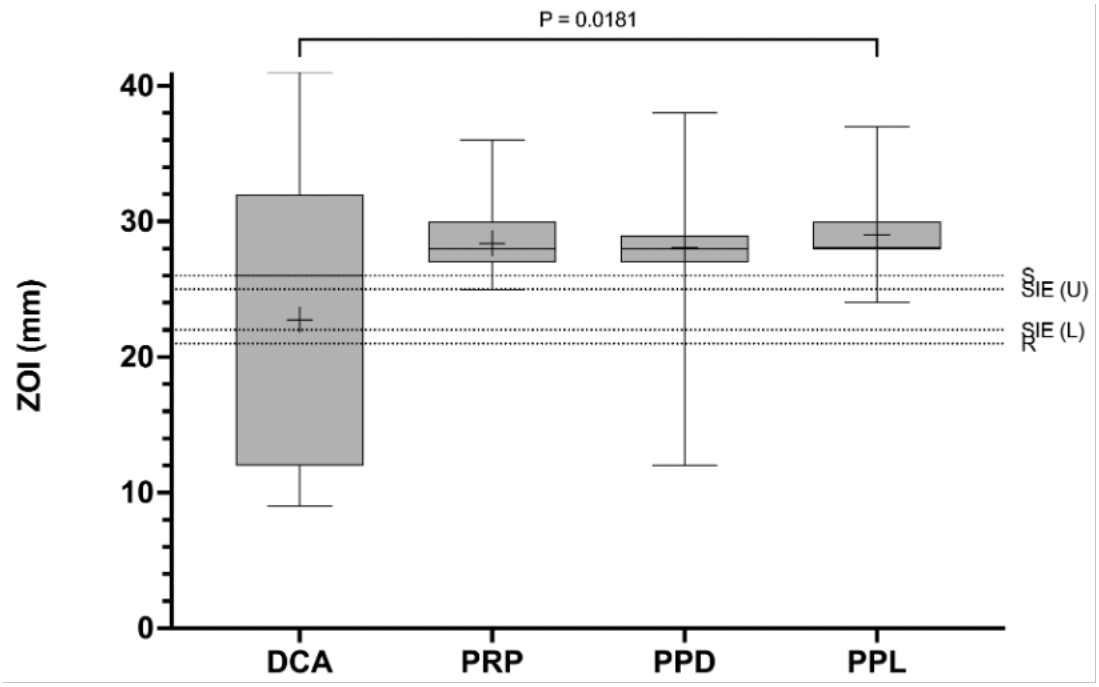


Figure 5.19: Comparison of the median ciprofloxacin susceptibility distributions of the β isolates sampled from different bovine developmental stages. The midline represents the median zone of inhibition (ZOI) across all isolates, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown by the labelled horizontal dotted black lines: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The grey box colour indicates the median antibiotic susceptibility results of susceptible. The developmental stages are dairy calves (DCA), pre-parturition dairy cows (PRP), post-parturition dry cows (PPD), and post-parturition lactating cows (PPL). The Kruskal-Wallis ANOVA with Dunn's multiple comparisons test was used to compare the median ZOIs for ciprofloxacin across all groups.

The median ZOI for tetracycline was significantly smaller in the isolates from the DCA samples compared to the isolates from the PRP, the PPD, and the PPL samples (ρ = 0.052, ρ <0.0001, and ρ <0.0001). The median ZOI for tetracycline was significantly smaller in the isolates from the PRP samples compared to the isolates from the PPL samples (ρ <0.0001), and significantly smaller in the isolates from the PPD samples compared to the isolates from the PPL samples (ρ = 0.0002) (Figure 5.20).

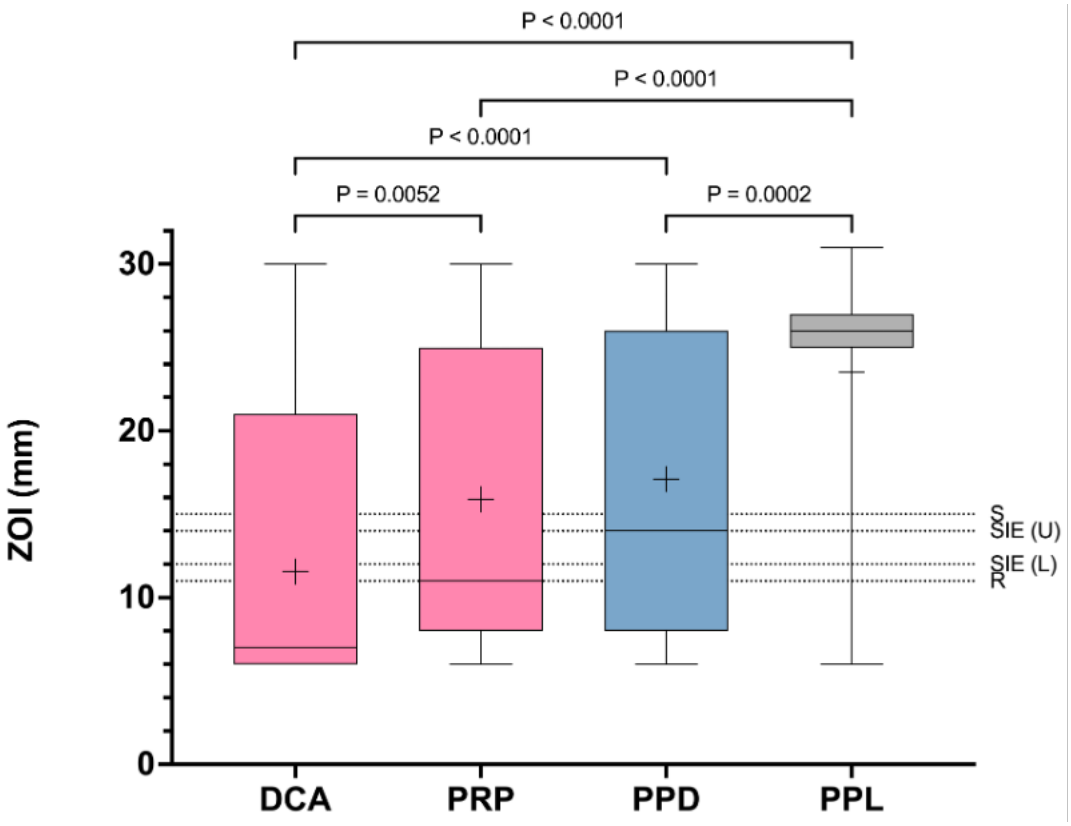


Figure 5.20: Comparison of the median tetracycline susceptibility distributions of the β isolates sampled from different bovine developmental stages. The midline represents the median zone of inhibition (ZOI) across all isolates, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown by the labelled horizontal dotted black lines: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility results: pink for resistant, grey for susceptible, and blue for SIE. The developmental stages are dairy calves (DCA), pre-parturition dairy cows (PRP), post-parturition dry cows (PPD), and post-parturition lactating cows (PPL). The Kruskal-Wallis ANOVA with Dunn's multiple comparisons test was used to compare the median ZOIs of tetracycline across all groups.

The median ASRs of the DCA A isolates were resistance to ampicillin, SIE to amoxicillin-clavulanate, and susceptibility to seven antibiotics from six classes (cefotaxime, streptomycin, kanamycin, sulfonamide, chloramphenicol, ciprofloxacin, and tetracycline) (Figure 5.21).

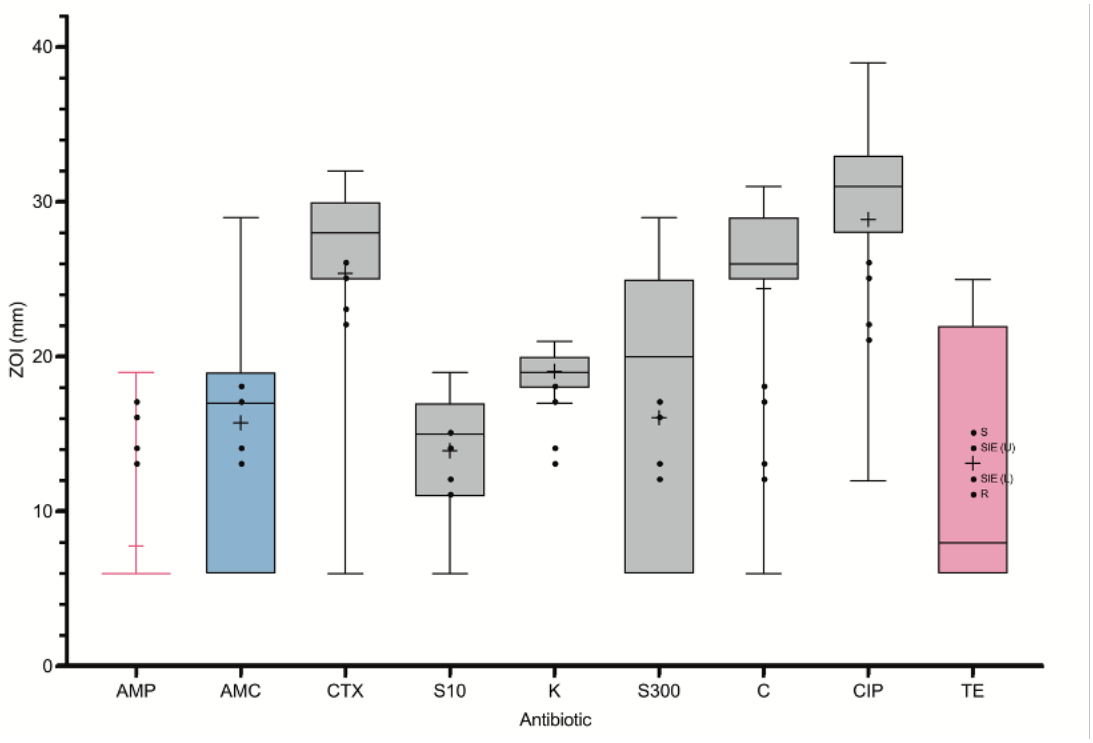


Figure 5.21: Median antibiotic susceptibility distributions of the isolates from the DCA A sample. The midline represents the median zone of inhibition (ZOI) across all isolates from the dairy calf (DCA) A sample, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown as black dots: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility: pink for resistant, grey for susceptible, and blue for SIE. Abbreviations: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), K (kanamycin), S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), TE (tetracycline).

The median ASRs of the DCA B isolates were MDR, with resistance to six antibiotics from five classes (ampicillin, amoxicillin-clavulanate, streptomycin, sulfonamide, chloramphenicol, tetracycline), and susceptibility to three antibiotics from three classes (cefotaxime, kanamycin, and ciprofloxacin) (Figure 5.22).

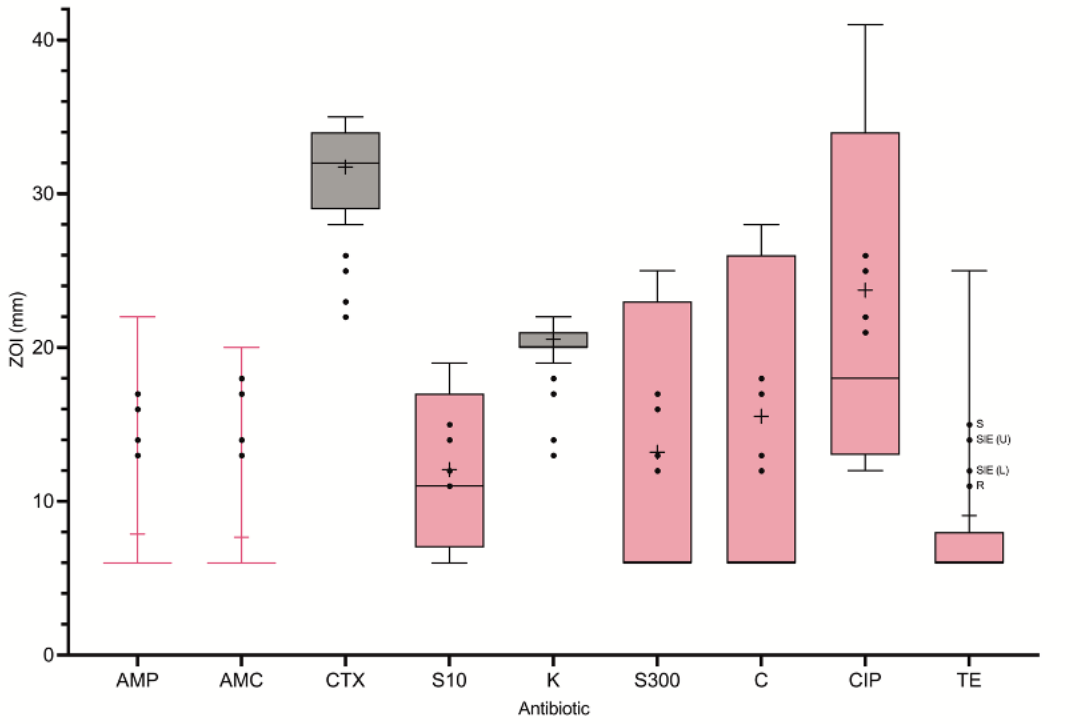


Figure 5.22: Median antibiotic susceptibility distributions of the isolates from the DCA B sample. The midline represents the median zone of inhibition (ZOI) across all isolates from the dairy calf (DCA) B sample, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown as black dots: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility: pink for resistant, grey for susceptible, and blue for SIE. Abbreviations: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), K (kanamycin), S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), TE (tetracycline).

The median antibiotic susceptibility of the DCA C isolates results were MDR, with resistance to eight antibiotics from six classes (ampicillin, amoxicillin-clavulanate, streptomycin, sulfonamide, chloramphenicol, ciprofloxacin, and tetracycline), SIE to kanamycin, and susceptibility to cefotaxime (Figure 5.23).

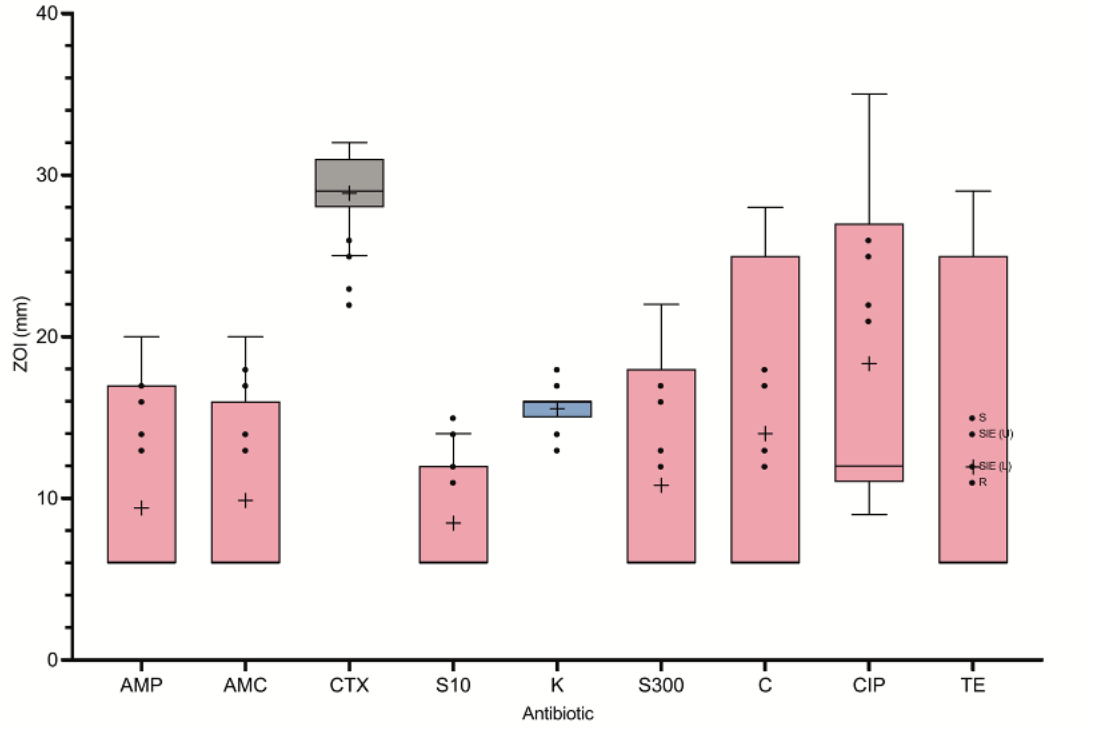


Figure 5.23: Median antibiotic susceptibility distributions of the isolates from the DCA C sample. The midline represents the median zone of inhibition (ZOI) across all isolates from the dairy calf (DCA) C sample, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown as black dots: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility: pink for resistant, grey for susceptible, and blue for SIE. Abbreviations: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), K (kanamycin), S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), TE (tetracycline).

The median ASRs of the DCA D isolates were MDR, with resistance to five antibiotics from five classes (ampicillin, streptomycin, sulfonamide, chloramphenicol, and tetracycline), SIE to two antibiotics from two classes (kanamycin and ciprofloxacin) and susceptibility to two antibiotics from one class (amoxicillin-clavulanate, and cefotaxime) (Figure 5.24).

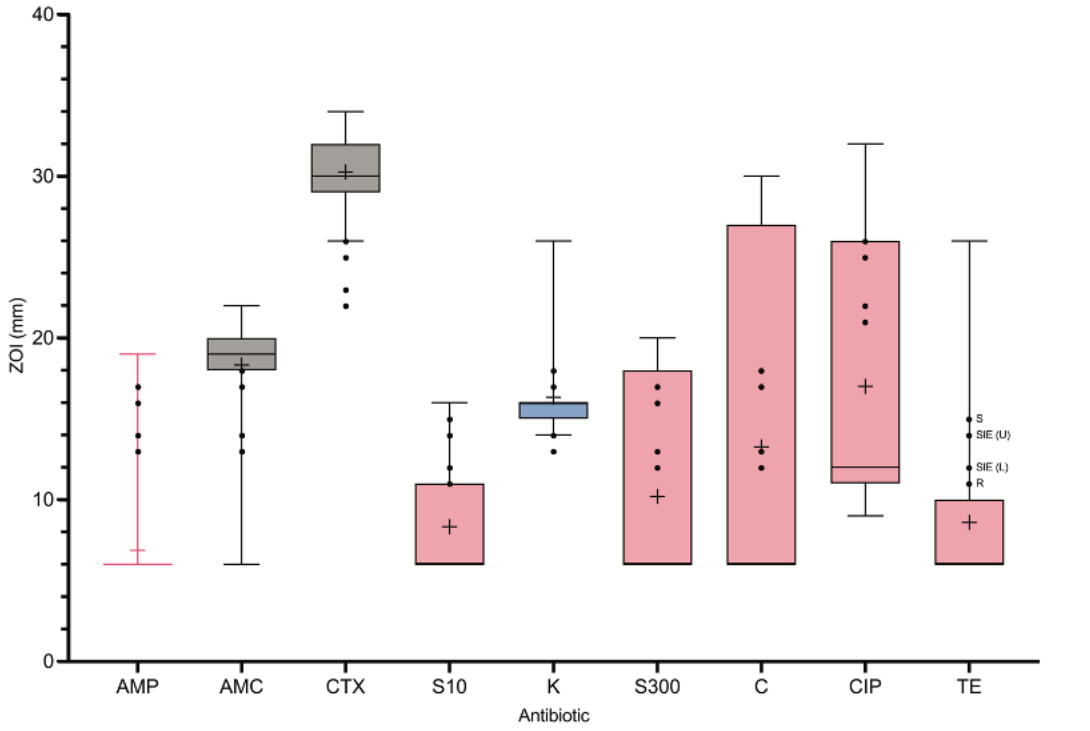


Figure 5.24: Median antibiotic susceptibility distributions of the isolates from the DCA D sample. The midline represents the median zone of inhibition (ZOI) across all isolates from the dairy calf (DCA) D sample, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown as black dots: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility: pink for resistant, grey for susceptible, and blue for SIE. Abbreviations: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), K (kanamycin), S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), TE (tetracycline).

The median ASRs of the DCA E isolates were MDR, with resistance to three antibiotics from three classes (amoxicillin-clavulanate, streptomycin, and tetracycline), SIE to kanamycin, and susceptibility to five antibiotics from four classes (amoxicillin-clavulanate, cefotaxime, sulfonamide, chloramphenicol, and ciprofloxacin) (Figure 5.25).

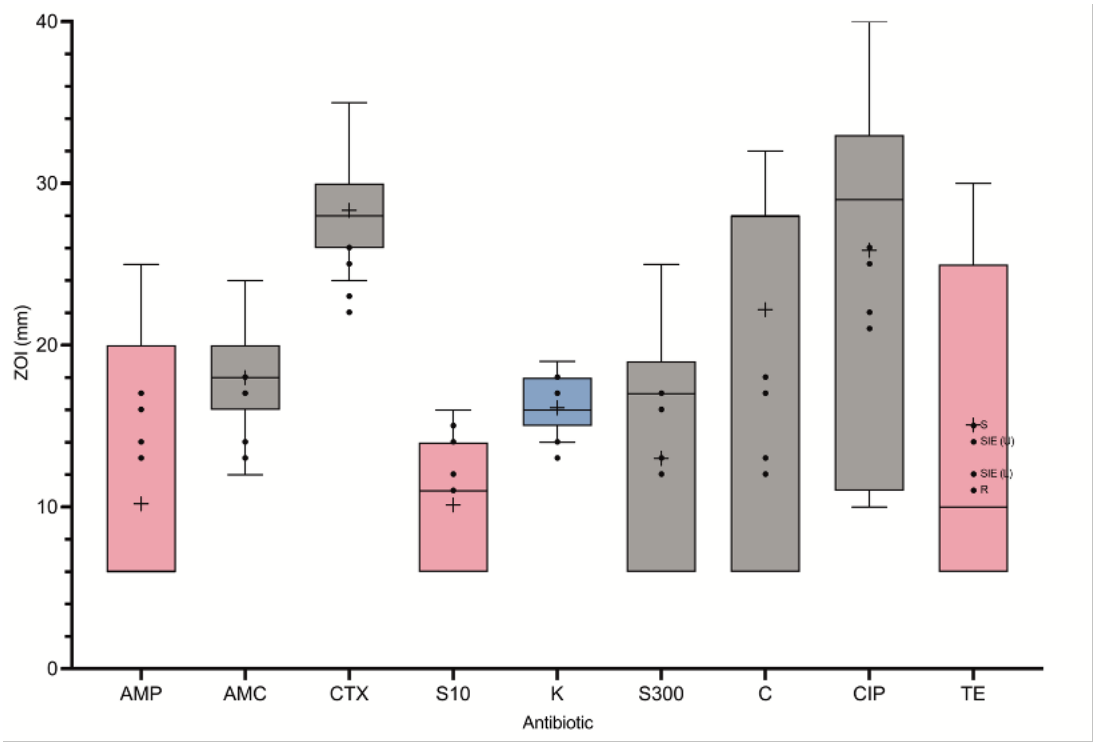


Figure 5.25: Median antibiotic susceptibility distributions of the isolates from the DCA E sample. The midline represents the median zone of inhibition (ZOI) across all isolates from the dairy calf (DCA) E sample, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown as black dots: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility: pink for resistant, grey for susceptible, and blue for SIE. Abbreviations: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), K (kanamycin), S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), TE (tetracycline).

The median ASRs of the PRP F isolates were resistance to tetracycline, SIE to three antibiotics from two classes (cefotaxime, streptomycin, and kanamycin), and susceptibility to five antibiotics from four classes (ampicillin, amoxicillin-clavulanate, cefotaxime, sulfonamide, chloramphenicol, and ciprofloxacin) (Figure 5.26).

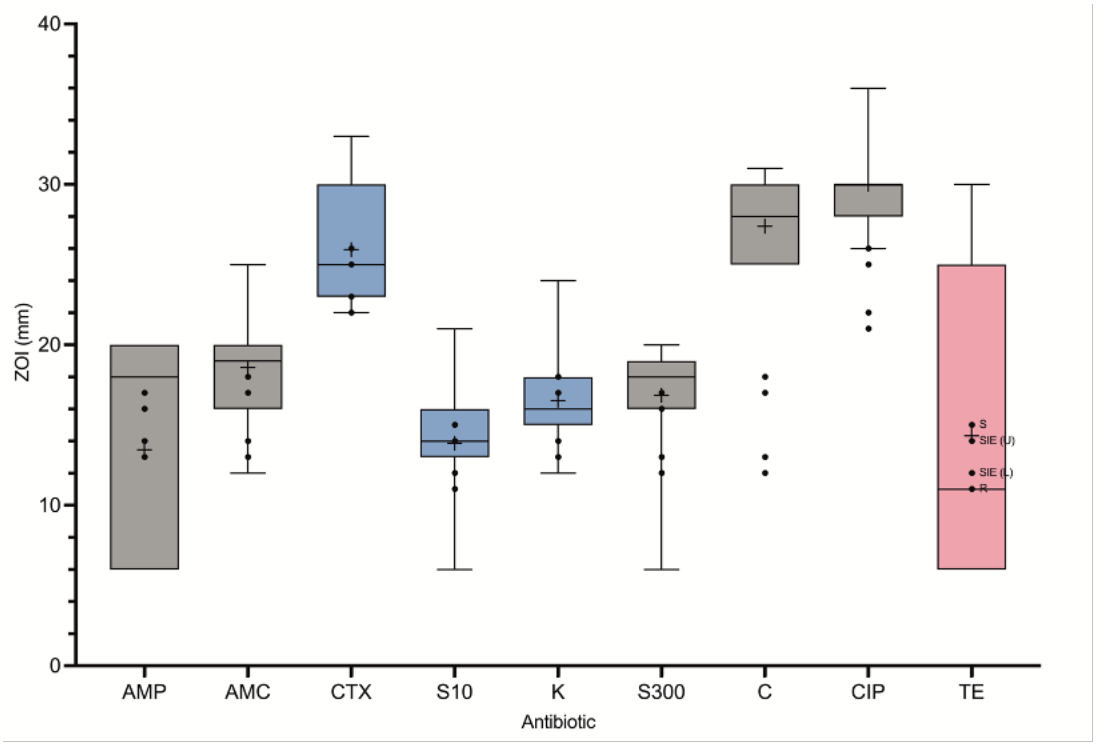


Figure 5.26: Median antibiotic susceptibility distributions of the isolates from the PRP F sample. The midline represents the median zone of inhibition (ZOI) across all isolates from the pre-parturition cow (PRP) F sample, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown as black dots: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility: pink for resistant, grey for susceptible, and blue for SIE. Abbreviations: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), K (kanamycin), S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), TE (tetracycline).

The median ASRs of the PRP G isolates were resistance to tetracycline, SIE four antibiotics from three classes (cefotaxime, streptomycin, kanamycin, and sulfonamide), and susceptibility to four antibiotics from three classes (ampicillin, amoxicillin-clavulanate, chloramphenicol, and ciprofloxacin) (Figure 5.27).

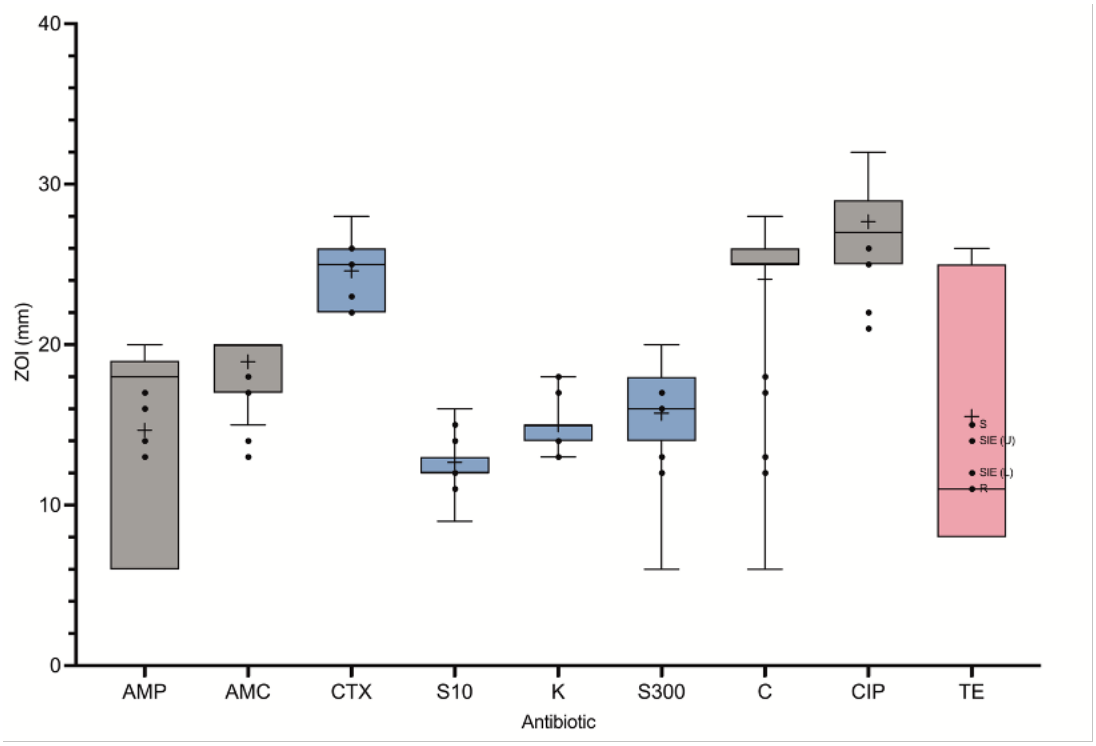


Figure 5.27: Median antibiotic susceptibility distributions of the isolates from the PRP G sample. The midline represents the median zone of inhibition (ZOI) across all isolates from the pre-parturition cow (PRP) G sample, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown as black dots: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility: pink for resistant, grey for susceptible, and blue for SIE. Abbreviations: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), K (kanamycin), S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), TE (tetracycline).

The median ASRs of the PRP H isolates were SIE to four antibiotics from three classes (cefotaxime, streptomycin, kanamycin, and tetracycline) and susceptibility to five antibiotics from four classes (ampicillin, amoxicillin-clavulanate, sulfonamide, chloramphenicol, ciprofloxacin, and tetracycline) (Figure 5.28).

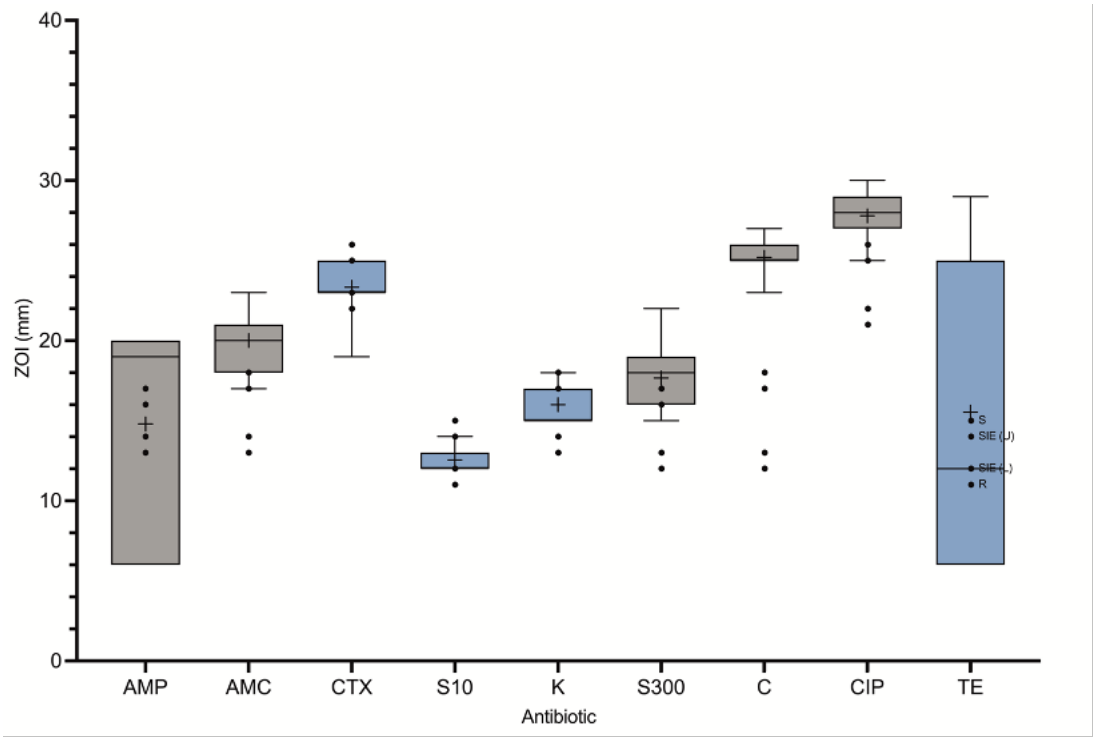


Figure 5.28: Median antibiotic susceptibility distributions of the isolates from the PRP H sample. The midline represents the median zone of inhibition (ZOI) across all isolates from the pre-parturition cow (PRP) H sample, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown as black dots: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility: pink for resistant, grey for susceptible, and blue for SIE. Abbreviations: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), K (kanamycin), S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), TE (tetracycline).

The median ASRs of the PRP I isolates were SIE to four antibiotics from four classes (cefotaxime, streptomycin, kanamycin, and tetracycline) and susceptibility to five antibiotics from four classes (ampicillin, amoxicillin-clavulanate, sulfonamide, chloramphenicol, and ciprofloxacin) (Figure 5.29).

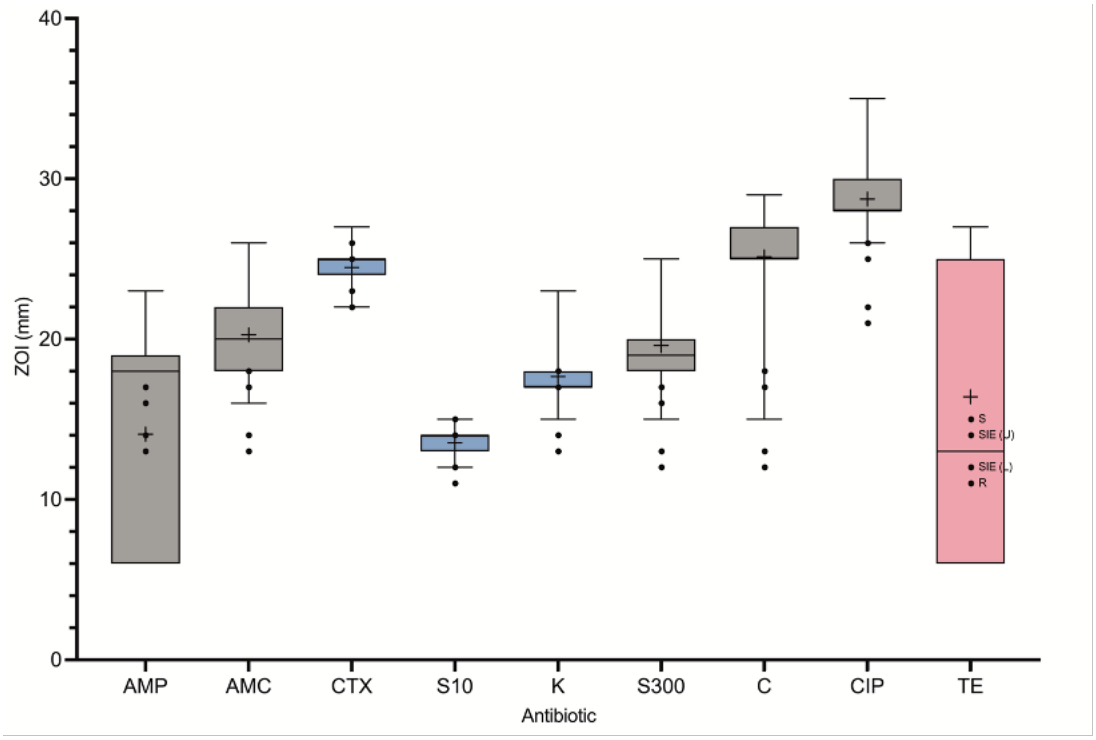


Figure 5.29: Median antibiotic susceptibility distributions of the isolates from the PRP I sample. The midline represents the median zone of inhibition (ZOI) across all isolates from the pre-parturition cow (PRP) I sample, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown as black dots: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility: pink for resistant, grey for susceptible, and blue for SIE. Abbreviations: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), K (kanamycin), S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), TE (tetracycline).

The median ASRs of the PRP J isolates were SIE to two antibiotics from two classes (cefotaxime and streptomycin) and susceptible to seven antibiotics from six classes (ampicillin, amoxicillin-clavulanate, kanamycin, sulfonamide, chloramphenicol, ciprofloxacin, and tetracycline) (Figure 5.30).

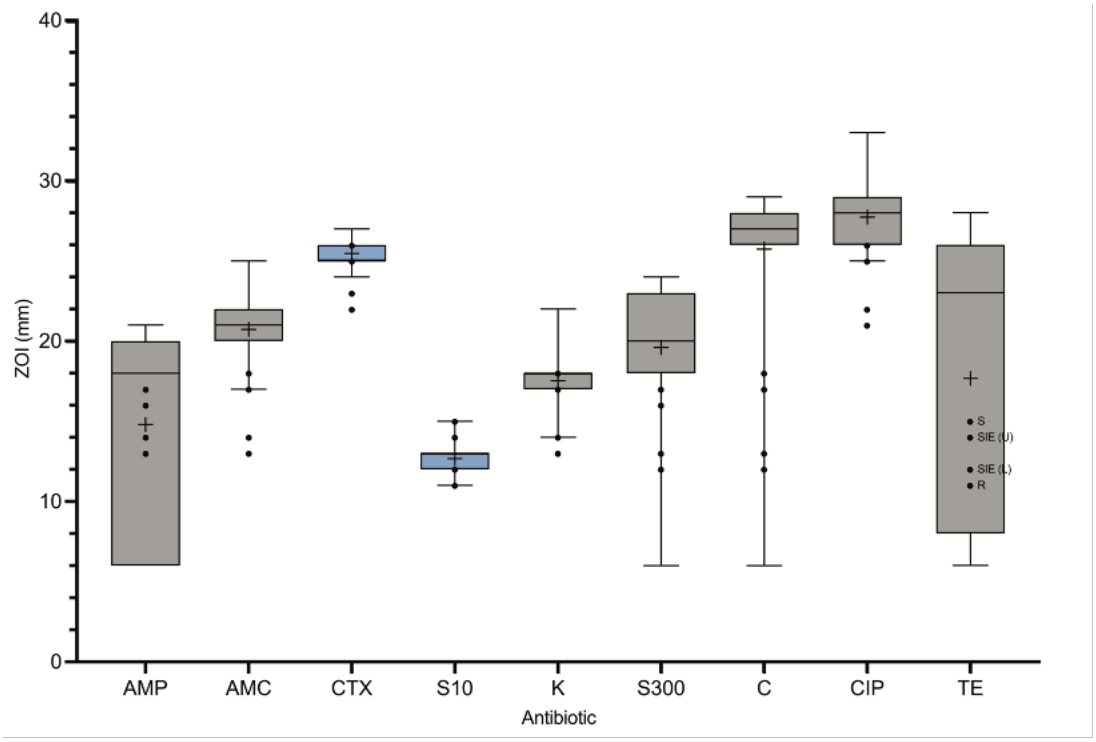


Figure 5.30: Median antibiotic susceptibility distributions of the isolates from the PRP J sample. The midline represents the median zone of inhibition (ZOI) across all isolates from the pre-parturition cow (PRP) J sample, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown as black dots: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility: pink for resistant, grey for susceptible, and blue for SIE. Abbreviations: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), K (kanamycin), S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), TE (tetracycline).

The median ASRs for the isolates in each PPL sample were SIE to three antibiotics from two classes (cefotaxime, streptomycin, and sulfonamide) and susceptibility to six antibiotics from five classes (ampicillin, amoxicillin-clavulanate, sulfonamide, chloramphenicol, ciprofloxacin, and tetracycline) (Figure 5.31-Figure 5.35).

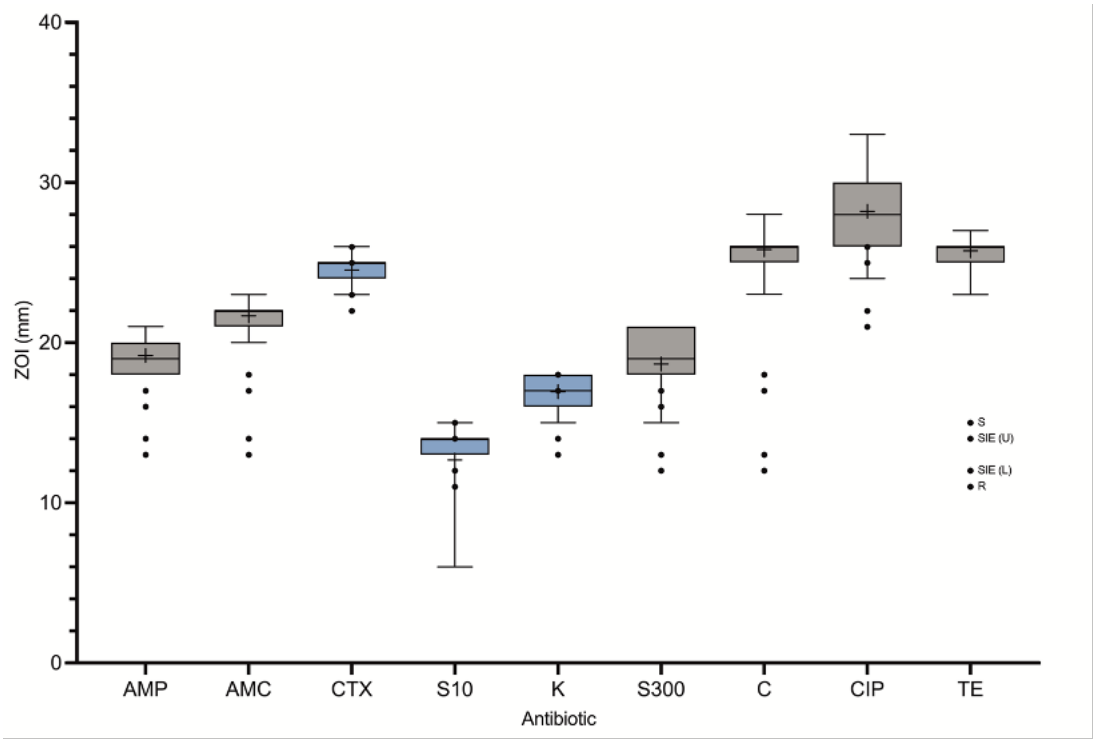


Figure 5.31: Median antibiotic susceptibility distributions of the isolates from the PPL P sample. The midline represents the median zone of inhibition (ZOI) across all isolates from the post-parturition lactating cow (PPL) P sample, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown as black dots: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility: pink for resistant, grey for susceptible, and blue for SIE. Abbreviations: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), K (kanamycin), S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), TE (tetracycline).

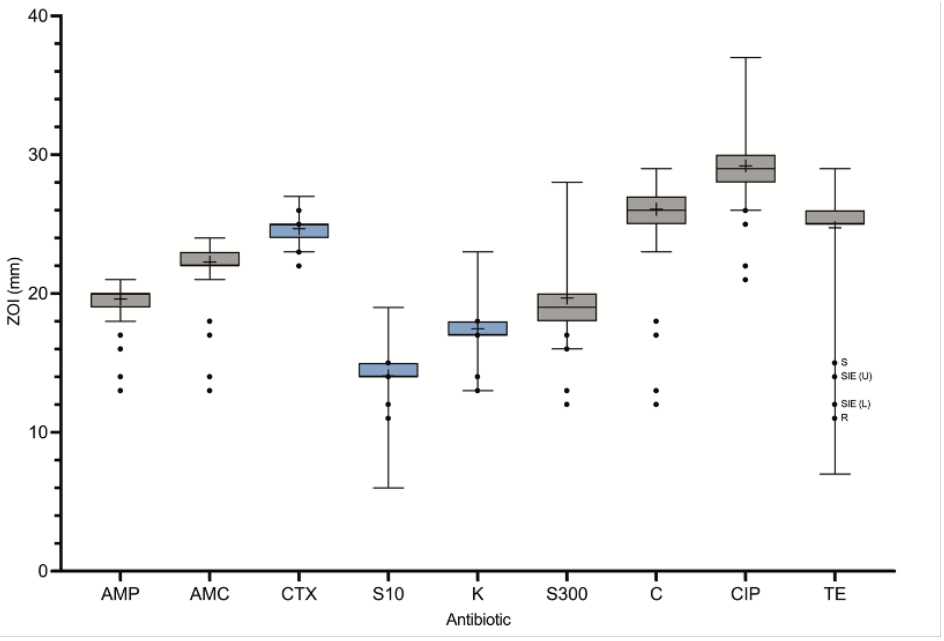


Figure 5.32: Median antibiotic susceptibility distributions of the isolates from the PPL Q sample. The midline represents the median zone of inhibition (ZOI) across all isolates from the post-parturition lactating cow (PPL) Q sample, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown as black dots: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility: pink for resistant, grey for susceptible, and blue for SIE. Abbreviations: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), K (kanamycin), S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), TE (tetracycline).

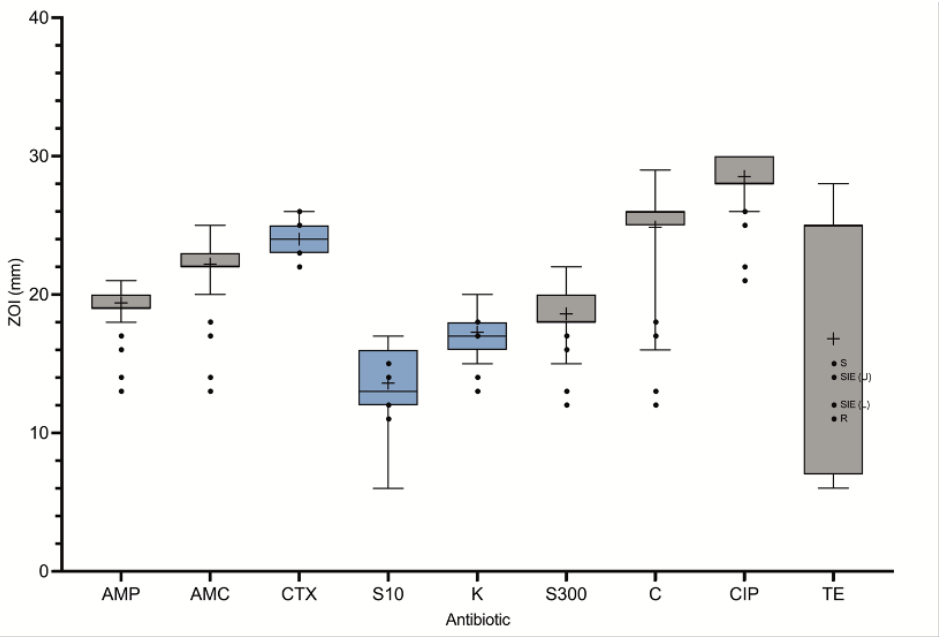


Figure 5.33: Median antibiotic susceptibility distributions of the isolates from the PPL R sample. The midline represents the median zone of inhibition (ZOI) across all isolates from the post-parturition lactating cow (PPL) R sample, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown as black dots: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility: pink for resistant, grey for susceptible, and blue for SIE. Abbreviations: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), K (kanamycin), S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), TE (tetracycline).

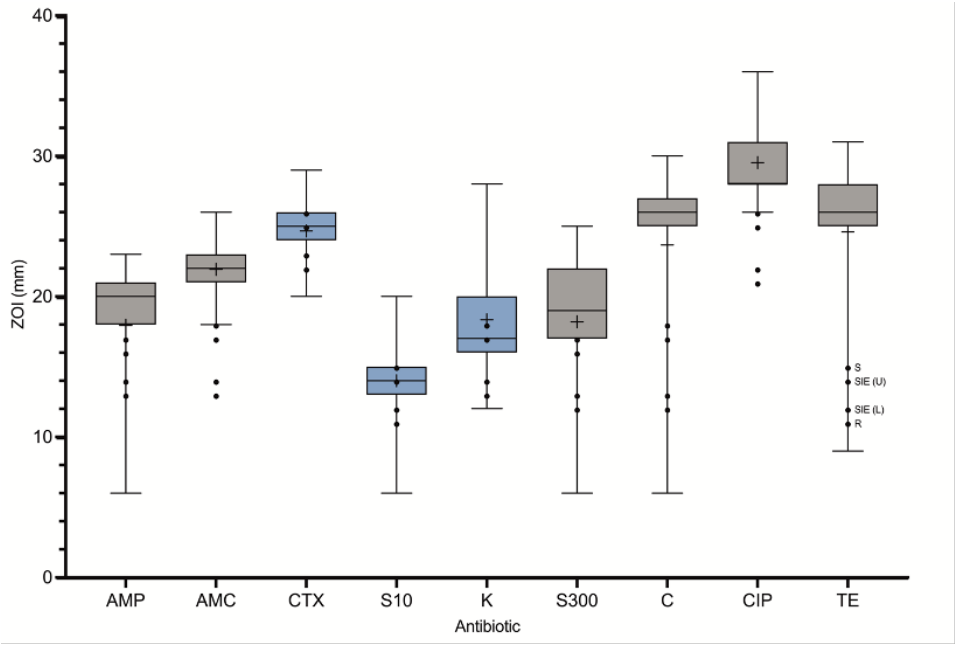


Figure 5.34: Median antibiotic susceptibility distributions of the isolates from the PPL S sample. The midline represents the median zone of inhibition (ZOI) across all isolates from the post-parturition lactating cow (PPL) S sample, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown as black dots: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility: pink for resistant, grey for susceptible, and blue for SIE. Abbreviations: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), K (kanamycin), S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), TE (tetracycline).

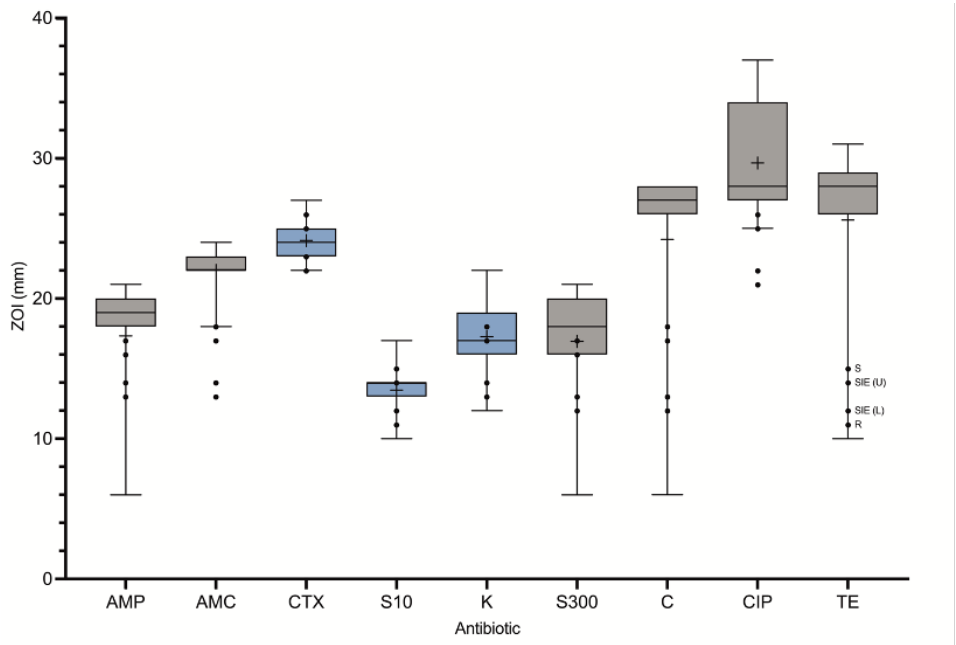


Figure 5.35: Median antibiotic susceptibility distributions of the isolates from the PPL T sample. The midline represents the median zone of inhibition (ZOI) across all isolates from the post-parturition lactating cow (PPL) T sample, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown as black dots: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility: pink for resistant, grey for susceptible, and blue for SIE. Abbreviations: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), K (kanamycin), S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), TE (tetracycline).

The median ASRs of the PPD K isolates were SIE to four antibiotics from three classes (cefotaxime, streptomycin, kanamycin, and tetracycline) and susceptibility to five antibiotics from four classes (ampicillin, amoxicillin-clavulanate, sulfonamide, chloramphenicol, and ciprofloxacin) (Figure 5.36).

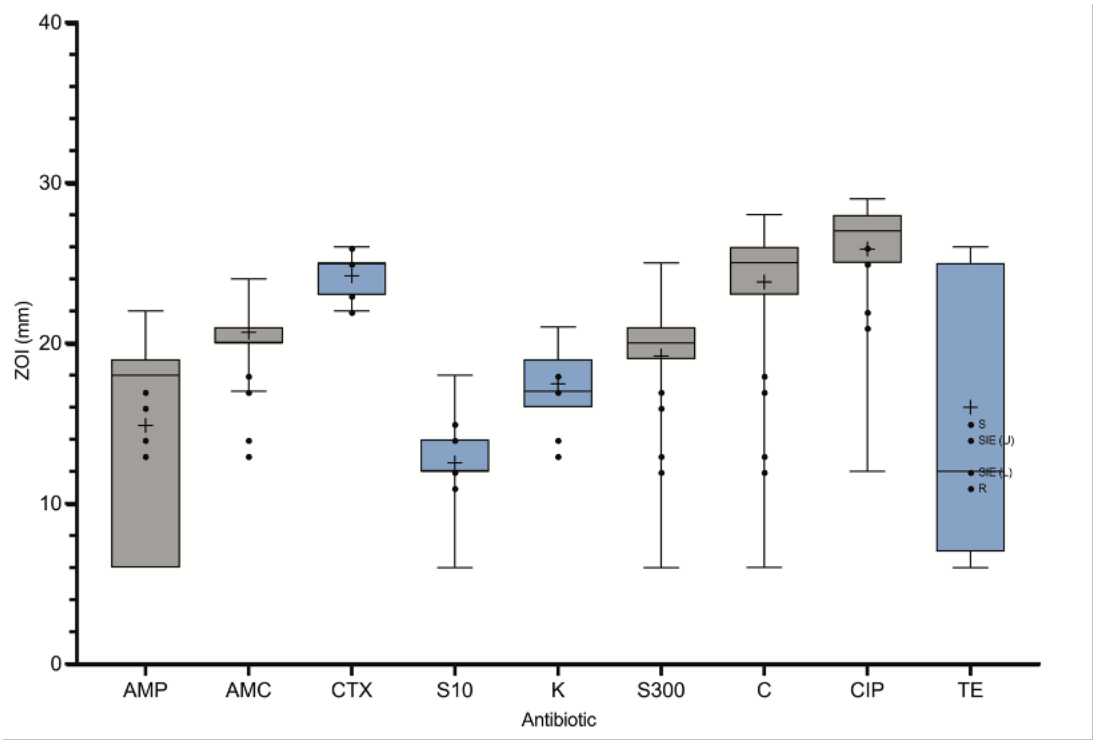


Figure 5.36: Median antibiotic susceptibility distributions of the isolates from the PPD K sample. The midline represents the median zone of inhibition (ZOI) across all isolates from the post-parturition dry cow (PPD) K sample, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown as black dots: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility: pink for resistant, grey for susceptible, and blue for SIE. Abbreviations: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), K (kanamycin), S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), TE (tetracycline).

The median ASRs of the PPD L isolates were SIE to streptomycin and susceptibility to eight antibiotics from six classes (ampicillin, amoxicillin-clavulanate, cefotaxime, kanamycin, sulfonamide, chloramphenicol, ciprofloxacin, and tetracycline) (Figure 5.37).

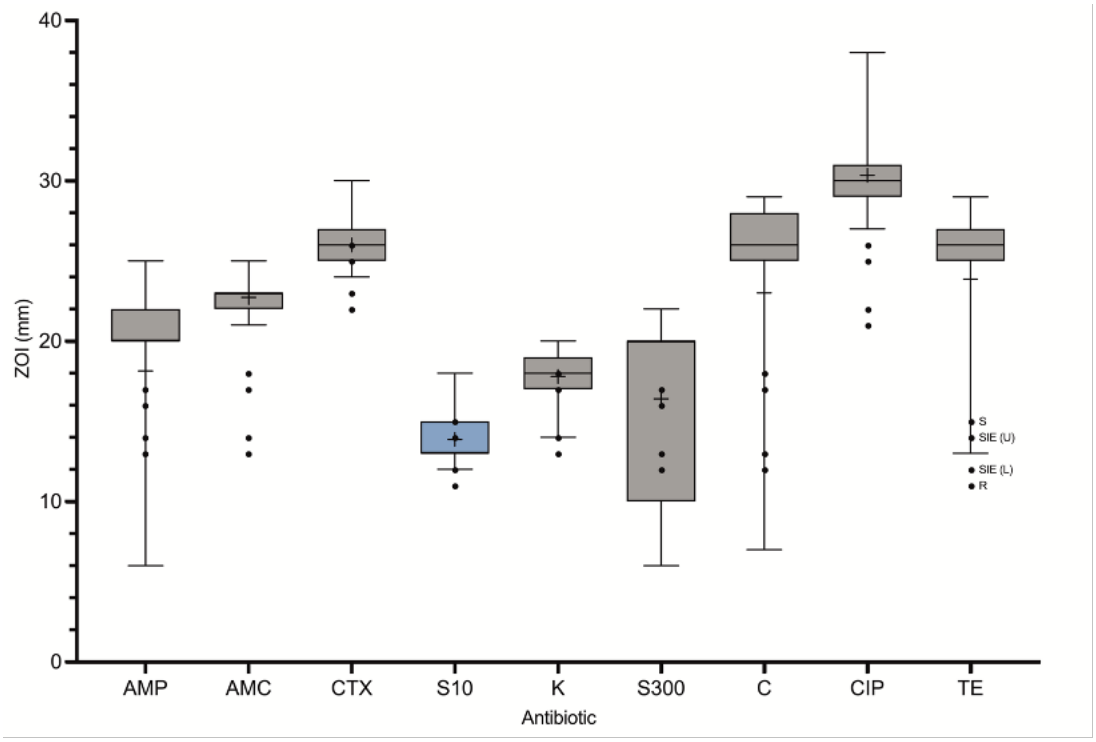


Figure 5.37: Median antibiotic susceptibility distributions of the isolates from the PPD L sample. The midline represents the median zone of inhibition (ZOI) across all isolates from the post-parturition dry cow (PPD) L sample, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown as black dots: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility: pink for resistant, grey for susceptible, and blue for SIE. Abbreviations: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), K (kanamycin), S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), TE (tetracycline).

The median ASRs of the PPD M isolates were resistance to tetracycline, SIE to four antibiotics from two classes (ampicillin, cefotaxime, streptomycin, and kanamycin), and susceptibility to four antibiotics from four classes (amoxicillin-clavulanate, sulfonamide, chloramphenicol, and ciprofloxacin) (Figure 5.38).

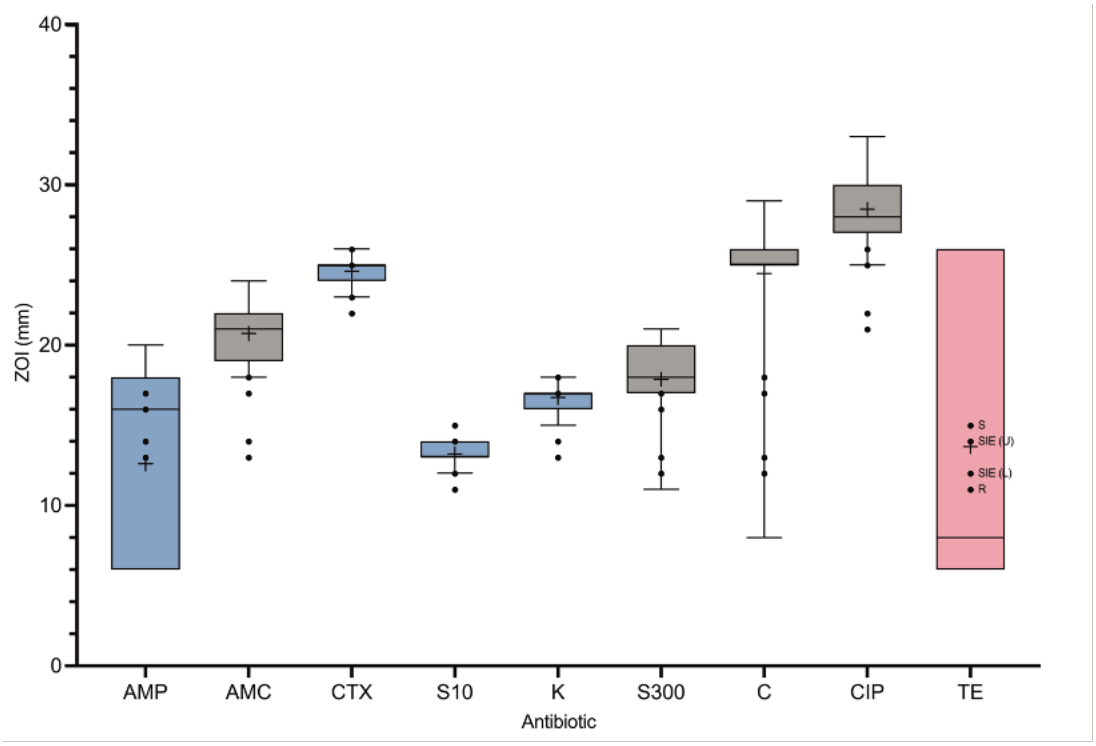


Figure 5.38: Median antibiotic susceptibility distributions of the isolates from the PPD M sample. The midline represents the median zone of inhibition (ZOI) across all isolates from the post-parturition dry cow (PPD) M sample, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown as black dots: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility: pink for resistant, grey for susceptible, and blue for SIE. Abbreviations: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), K (kanamycin), S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), TE (tetracycline).

The median ASRs of the PPD N isolates were resistance to tetracycline, SIE to four antibiotics from three classes (cefotaxime, streptomycin, kanamycin, and sulfonamide), and susceptibility to four antibiotics from three classes (ampicillin, amoxicillin-clavulanate, chloramphenicol, and ciprofloxacin) (Figure 5.39).

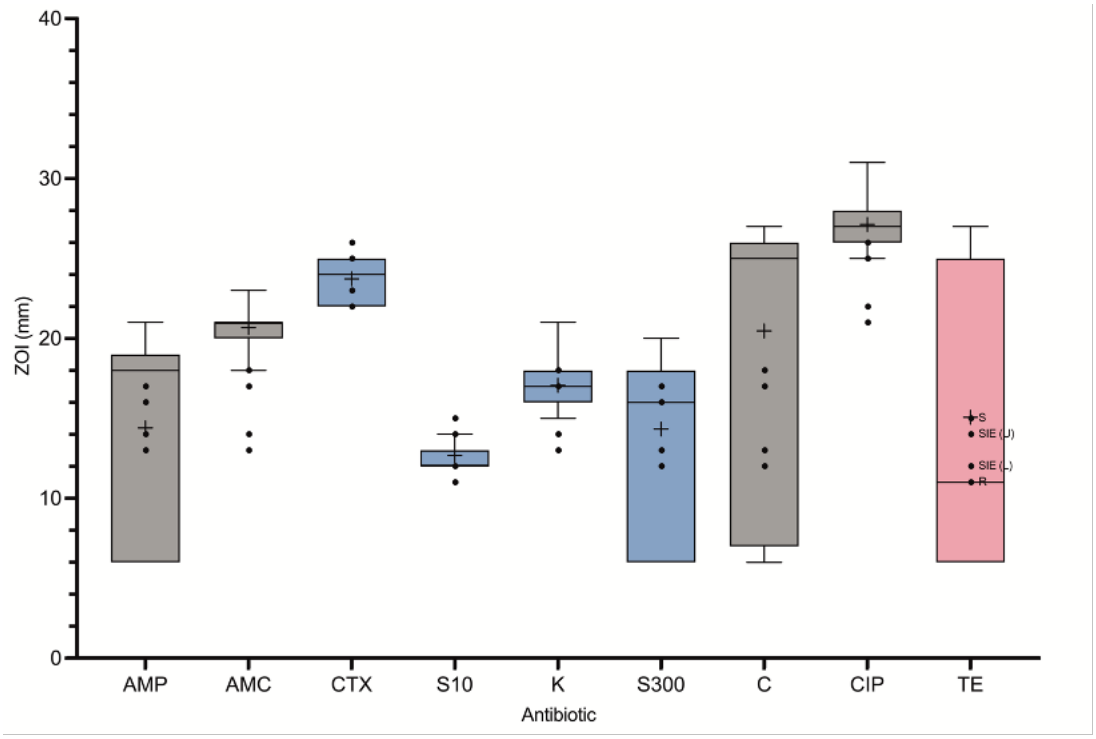


Figure 5.39: Median antibiotic susceptibility distributions of the isolates from the PPD N sample. The midline represents the median zone of inhibition (ZOI) across all isolates from the post-parturition dry cow (PPD) N sample, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown as black dots: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility: pink for resistant, grey for susceptible, and blue for SIE. Abbreviations: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), K (kanamycin), S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), TE (tetracycline).

The median ASRs of the PPD O isolate were SIE to four antibiotics from three classes (cefotaxime, streptomycin, kanamycin, and tetracycline) and susceptibility to five antibiotics from four classes (ampicillin, amoxicillin-clavulanate, sulfonamide, chloramphenicol, ciprofloxacin, and tetracycline) (Figure 5.40).

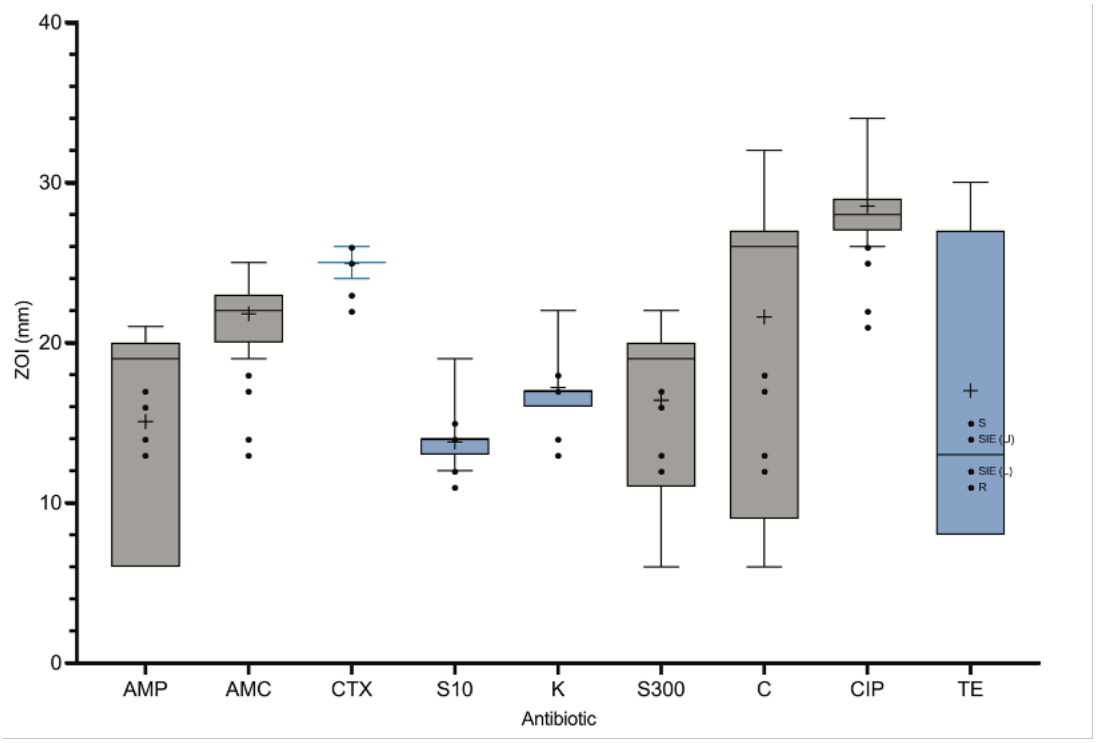


Figure 5.40: Median antibiotic susceptibility distributions of the isolates from the PPD O sample. The midline represents the median zone of inhibition (ZOI) across all isolates from the post-parturition dry cow (PPD) O sample, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown as black dots: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility: pink for resistant, grey for susceptible, and blue for SIE. Abbreviations: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), K (kanamycin), S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), TE (tetracycline).

5.4.6 Short-read whole genome sequencing

Out of the 300 *E. coli* isolated from the β samples, 150 isolates were selected across the bovine developmental groups for short-read WGS. More DCA isolates were selected for sequencing as they had higher levels of non-susceptibility and the DCA samples were distinct from the other bovine developmental group samples in physical appearance (Appendix 21). The quality of the sequencing and assembly were assessed, before ARG, serotyping, and plasmid replicons were identified across several databases using ABRicate. The clonality of the isolates was also investigated by submitting genomes to Enterobase to determine MLST.

Candidate isolates for sequencing were selected from the β samples to investigate what ARG were present in faecal *E. coli* collected across the DCA, the PRP, the PPD, and the PPL samples. The selection process is detailed in the methods section, ideally the genomes of all 300 isolates would have been sequenced but this was not feasible (Table 2.9). The isolates that were selected for sequencing had a variety of antibiotic susceptibility phenotypes (Figure 5.41).

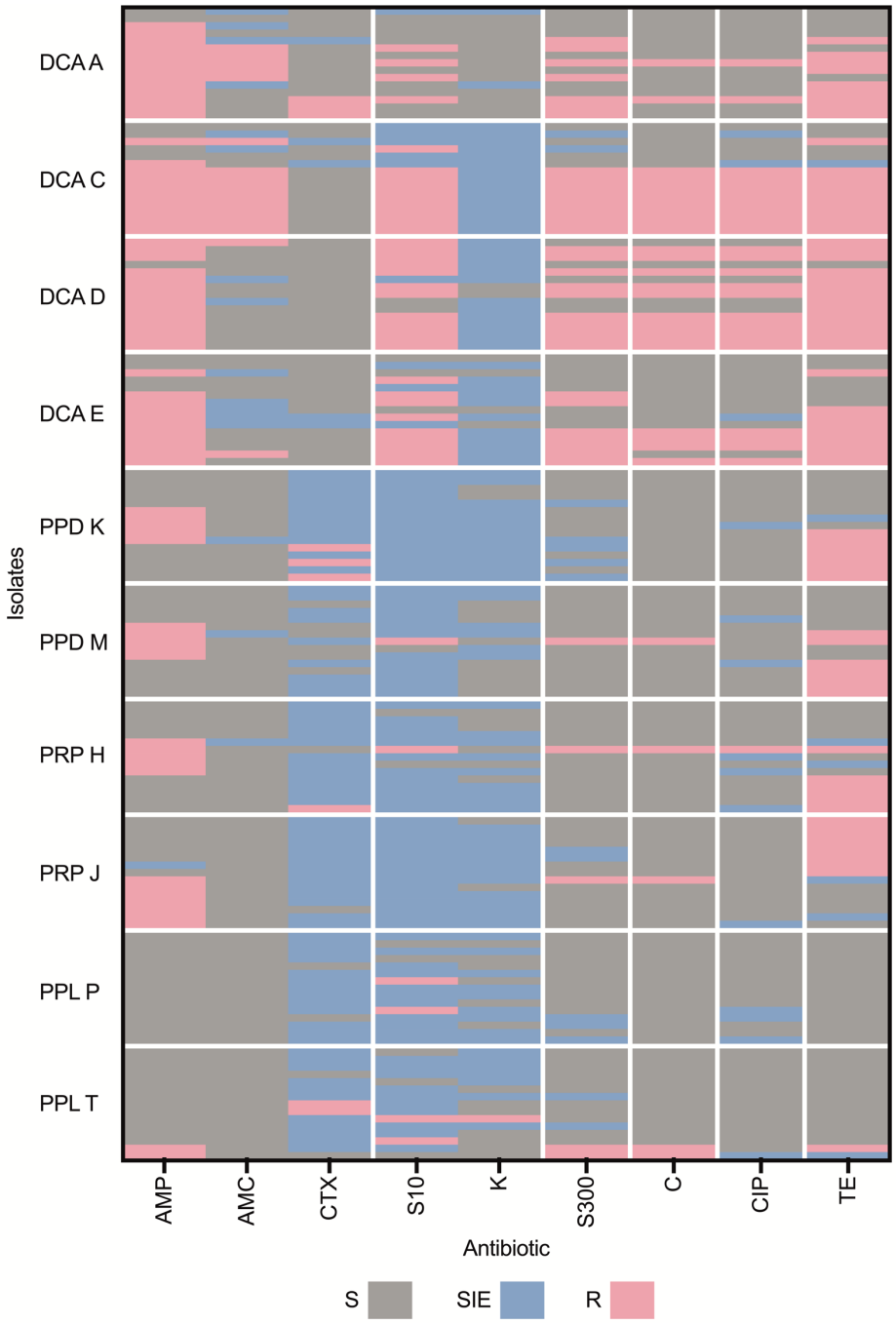


Figure 5.41:Antibiotic susceptibility phenotypes of β sample isolates with whole genome sequences. Columns are different antibiotics with each class separated with a vertical gap, rows are individual isolates with each host separated with a horizontal gap. The box colour indicates the median antibiotic susceptibility: grey for susceptible and blue for SIE. Abbreviations: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), K (kanamycin), S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), TE (tetracycline).

5.4.6.1 Quality of the Illumina short-read sequencing data and *E. coli* genome assembly

The sequencing quality metrics for the genomes of the isolates from each sample source are presented in Figure 5.42.

The metrics presented include the number of sequencing reads per genome, the mean coverage of reads per contig in the genome, and the N50 contig length (shortest length contig that includes half of all contigs).

The median number of sequencing reads was $1.07x10^6$ in the DCA, $1.27x10^6$ in the PPD, $1.10x10^6$ in the PRP, and $1.24x10^6$ in the PPL genomes. The medians of mean read coverage per contigs was x105 in the DCA, x123 in the PPD, x101 in the PRP, and x118 in the PPL genomes. The N50 median contig lengths were $2.4x10^5$ Mbp in the DCA, $2.7x10^5$ Mbp in the PPD, $2.7x10^5$ Mbp in the PRP, and $2.9x10^5$ Mbp in the PPL genomes. The C1A1, C3C5, C5E2, M1P12, M5T5, M5T7, M5T11, and M5T13 genomes had N50 values greater than the average *E. coli* genome length.

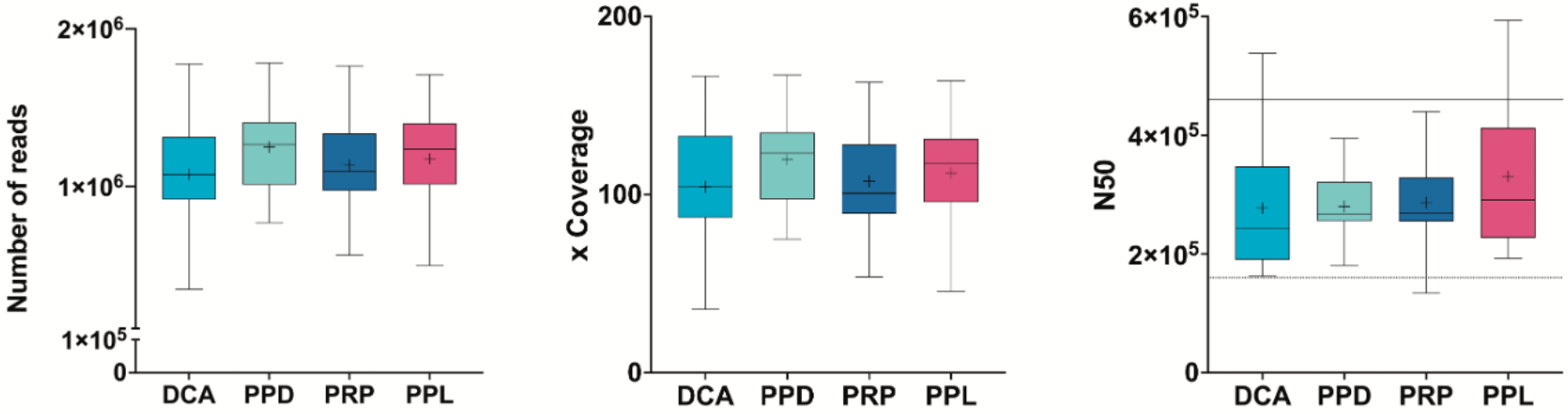


Figure 5.42: Summary of key quality metrics of sequencing and assembly quality. The midline is the median; the box displays the 25th to 75th percentile. Whisker bars extend to the maximum and minimum results. The cross shows the mean of all genomes. Light blue = DCA (Dairy calf), light green = PPD (post-parturition dry cow), dark blue = PRP (pre-parturition cow), pink = PPL (post-parturition lactating cow). (Top left) Total number of sequencing reads for all genomes by sample group. (Top right) The coverage of reads per contig for all genomes by sample group. (Bottom left) N50, shortest contig length to include 50% of contigs. Dotted line represents the length of 16S rRNA sequences used for taxonomic identification and solid line represents the average length of an *E. coli* genome.

5.4.7 Screening the β sample *E. coli* genomes for antibiotic resistance genes in the ARG-ANNOT database

ABRicate was used to compare genome contigs against the ARG-ANNOT ARG database. The percentage of genomes that contained ARG sequence matches from the ARG-ANNOT database are presented in Table 5.13 and described in this section. Sequence matches for the aminoglycoside phosphotransferase genes *strA and strB* were identified in 21.3% of the genomes, including the genomes from 47% of the DCA isolates, D1K7, D3M9, M5T14, and M5T15. Only the ARG-ANNOT database identified sequence matches for *strA* in these genomes. The genomes of C1A6, C1A10, C5E6, and C5E7 had 88% nucleotide identity matches for the *strB* gene reference sequence. Sequence matches for aminoglycoside nucleotidyltransferase genes were identified in various genomes. Sequence matches for *aadA5* were identified in 16.7% of the genomes, including the genomes from 40% of the DCA isolates and D1K7. Sequence matches for *aadA22* were present in the genomes of M1P7 and M1P11. A sequence match for *aadA1-pm* was identified in the genome of H5J8. Finally, a sequence match for *aadA2* was found in the genome of C5E14. The *aadA1-pm* was a unique variant of the *aadA1* gene identified by the ARG-ANNOT database. Every genome had sequence matches for the *E. coli* penicillin-binding protein genes, *PBP_ E. coli*, this was a gene variant only identified by the ARG-ANNOT database. Every genome had sequence matches for the β-lactamase genes *ampC2_ E. coli,* and *ampH*. The *ampC2_ E. coli* gene was a unique variant of this gene identified by the ARG-ANNOT database. The β-lactamase *ampC1* was identified in 95% of the genomes. Only the D1K4, H3H2, M5T5, M5T7, M5T10,

M5T11, and M5T11 genomes did not have sequence matches for *ampC1*. The β-lactamase *blaTEM-105* was identified in 25.3% of the genomes, including from 42% of the DCA isolates, 30% of the PPD isolates, and 13% of the PRP isolates. The β-lactamase *blaTEM-135* was identified in 14.7% of the genomes, including from 25% of the DCA isolates, D1K6, D3M14, H3H6, H3H7, H3H9, H3H10, and H5J7. Only the ARG-ANNOT database identified sequence matches for *blaTEM-105* and *blaTEM-135* in these genomes. The β-lactamase *blaTEM-150* was identified in 2.7% of the genomes, specifically D3M9, H5J8, M5T14, and M5T15. Only the C5E14 genome had a sequence match for the β-lactamase *CARB-3*. The H5J3 genome had a 93.94% identity match for the lincosamide nucleotidyltransferase gene *lnuD.* The macrolide phosphotransferase *mphA* gene was identified in 16.7% of the genomes, including the genomes from 40% of the DCA isolates, and D1K7. The C5E14 genome had the only sequence match for the macrolide esterase gene *ereB*. The phenicol inactivating acetyltransferase gene *catA1* was identified in 17.3% of the genomes, including the genomes from 40% of the DCA isolates, D1K7, and H5J8. The phenicol efflux gene *floR* was identified in 2% of the genomes, specifically D3M10, M5T14, and M5T15. The sulfonamide target modification gene *sul2* was identified in 21.3% of the genomes, including the genomes from 47% of the DCA isolates, D1K7, D3M9, M5T14, and M5T15. The sulfonamide target modification gene *sul1* was identified in 18% of the genomes, including the genomes from 38% of the DCA isolates, D1K7, and H5J8. The trimethoprim target modification gene *dfra17* was identified in 16.7% of the genomes, including the genomes from 40% of the DCA isolates, and D1K7. The tetracycline efflux gene *tetB* was identified in 36% of the genomes,

including the genomes from 50% of the DCA isolates, 46% of the PPD isolates, and 33% of the PRP isolates. The tetracycline efflux gene *tetR* was identified in 15.3% of the genomes, including the genomes from 20% of the DCA isolates, D1K6, D3M9, D3M14, H5J7, H5J8, M5T14, and M5T15. The tetracycline efflux gene *tetA* was identified in 14.7% of genomes, including the genomes from 18% of the DCA isolates, D1K6, D3M14, H3H6, H3H7, H3H9, and H3H10. The D3M9, M5T14, and M5T15 genomes had *tetA* sequence matches with 97.8% nucleotide identity.

Table 5.13: ARG-ANNOT antibiotic resistance genes, mechanism, and abundance in the *β E. coli* genomes.

Class	Resistance mechanism	Gene	Presence (%)
Aminoglycoside	Inactivation	(AGly)strA	21.3
		(AGly)strB	21.3
		(AGly)aadA5	16.7
		(AGly)aadA22	1.3
		(AGly)aadA1-pm*	0.7
		(AGly)aadA2	0.7
β-lactam	Target replacement	(Bla)PBP_Ecoli*	100
	Inactivation	(Bla)AmpC2_Ecoli*	100
		(Bla)ampH_Ecoli	100
		(Bla)AmpC1_Ecoli	95.3
		(Bla)blaTEM-105	25.3
		(Bla)blaTEM-135	14.7
		(Bla)blaTEM-150	2.7
		(Bla)CARB-3	0.7
Lincosamide	Inactivation	(MLS)lnu(D)	0.7
Macrolide	Inactivation	(MLS)mph(A)	16.7
		(MLS)ere(B)	0.7
Phenicol	Inactivation	(Phe)catA1	17.3
	Efflux	(Phe)floR	2
Sulfonamide/ trimethoprim	Target replacement	(Sul)sul2	21.3
		(Sul)sul1	18
		(Tmt)dfrA17	16.7
Tetracycline	Efflux	(Tet)tetB	36
		(Tet)tetR	15.3
		(Tet)tetA	14.7

Grey shading highlights a gene only identified by this database, and * highlights a unique variant identified by this database.

5.4.8 Screening the β sample *E. coli* genomes for antibiotic resistance genes in the CARD database

ABRicate was used to compare genome contigs against the CARD ARG database. The percentage of genomes that contained ARG sequence matches from the CARD database are presented in Table 5.14 and described in this section.

Aminoglycoside efflux related genes *acrD* and *kdpE* were identified in all of the genomes. The aminoglycoside inactivating phosphotransferase gene *aph(3')-Ib* had sequence matches in 21.3% of the genomes, including the genomes from 46% of the DCA isolates, D1K7, D3M9, M5T14, and M5T15. The same genomes had sequence matches for the phosphotransferase gene *aph(6)-Id*, the C1A6, C1A10, C5E6, and C5E7 genomes had 87.57% nucleotide identity. The aminoglycoside inactivating nucleotidyltransferase gene *aadA5* was identified in 16.7% of the genomes, including the genomes from 40% of the DCA isolates and the D1K7 genome. Another aminoglycoside inactivating nucleotidyltransferase gene *ant(3")-IIa* was identified in the genomes from H5J8, M1P7, and M1P10 with 99.59% nucleotide identity . The final aminoglycoside inactivating nucleotidyltransferase gene *aadA2* was only identified in the C5E14 genome. A bacitracin target alteration gene, *bacA*, was identified in all of the genomes. Several β-lactamase genes were identified in all of the genomes; *CRP*, *ampC,* and *ampH*; *ampC1* were identified in all of the genomes except the genomes from D1K4, H3H2, M5T5, M5T8, M5T10, M5T11, and M5T12. *TEM-1* was identified in 40% of the genomes, including the genomes from 67% of the DCA isolates, 37% of the PPD isolates, and 30% of the PRP isolates. *TEM-150* was identified in 2.7% of the genomes,

specifically the genomes from D3M9, H5J8, M5T14, and M5T1. *CARB-3 was* identified in the C5E14 genome only. The fluoroquinolone efflux genes *emrA* and *emrB* were identified in all of the genomes. The lincosamide nucleotidyltransferase gene *lnuD* was identified in the H5J3 genome only with a 94% nucleotide identity . The macrolide phosphotransferase inactivation gene *mphB* was identified in 96.7% of the genomes, only 66% of the genomes from PPL T isolates. Another macrolide phosphotransferase inactivation gene *mphA* was identified in 16.7% of the genomes, including the genomes from 40% of the DCA isolates and one PPD K isolate genome. The macrolide esterase inactivation gene *ereB* was identified in one DCA E isolate genome. Genes for inner membrane RND, MFS, and ABC efflux pumps were identified (e.g., *acrA, mdtA, mdfA, yojI*), outer membrane members of tripartite pumps (*tolC*), and regulatory genes were identified in all the genomes. The CARD ARG database was the only database to identify the *evgA* and *tolC* genes in these genomes. A single DCA E isolate genome was the only genome that did not have a sequence match for *emrB*. A single DCA A isolate genome and 10 genomes from PPL T isolates did not have sequence matches for *mdtM;* 65% of the genomes from DCA isolates had a sequence match for *mdtM* with 98% nucleotide identity . There were *emrE_E. coli* sequence matches in 40% of the genomes; this was a unique variant identified by the CARD ARG database. A single DCA E isolate genome had a sequence match for *kpnH;* this gene was only identified by the CARD ARG database. Every one of the genomes had sequence matches for *marA*. The phenicol inactivating acetyltransferase gene *catI* was identified in 17.3% of the genomes, including the genomes from 60% of the DCA isolates, D1K7, and H5J8. The CARD ARG database was the only

database that identified sequence matches to the *catI* gene. The phosphoethanolamine polymyxin target alteration genes *eptA* and *pmrF* were identified in all of the genomes. Sequence matches for the polymyxin target alteration gene *ugd* were identified in 59% of the genomes, including the genomes from 83% of the PPD isolates, 80% of the PRP isolates, and 73% of the PPL isolates. The sulfonamide target replacement gene *sul2* was identified in 21.3% of the genomes, including the genomes from 70% of the DCA isolates, D1K7, D3M9, M5T14, and M5T15. The other sulfonamide target replacement gene *sul1* was found in 18% of the genomes, including the genomes from 63% of the DCA isolates, D1K7, and H5J8. The trimethoprim target replacement gene *dfra17* was identified in 16.7% of the genomes, including the genomes from 60% of the DCA isolates and the D1K7 genome. The tetracycline efflux genes *ermY* and *ermK* were identified in all of the genomes. Another tetracycline efflux gene, *tet(B),* was identified in 36% of the genomes, including the genomes from 63% of the DCA isolates, 46% of the PPD isolates, and 33% of the PRP isolates. The final tetracycline efflux gene *tet(A)*was identified in 14.7 of the genomes, including the genomes from 18% of the DCA isolates, D1K6, D3M9, D3M14, H3H6, H3H7, H3H9. H3H10, H5J7, H5J8, M5T14, and M5T15. All of the genomes from DCA isolates, PRP isolates, D1K6, and D4M14 had 98% identity matches to the *tet(A)* reference sequence.

Table 5.14: CARD antibiotic resistance genes, mechanism and abundance in the *β E.coli* genomes.

Class	Resistance and mechanism	Gene	Presence (%)
Aminoglycoside	Efflux	acrD	100
		kdpE	100
	Inactivation	APH(3")-Ib	21.3
		APH(6)-Id	21.3
		aadA5	16.7
		ANT(3")-IIa	2
		aadA2	0.7
Bacitracin	Target alteration	bacA	100
β-lactam	Inactivation	CRP	100
		E_coli_ampC	100
		E_coli_ampH	100
		E_coli_ampC1	95.3
		TEM-1	40
		TEM-150	2.7
		CARB-3	0.7
Fluoroquinolone	Efflux	emrA	100
		emrR	100
Lincosamide	Inactivation	lnuD	0.7
Macrolide	Inactivation	mphB	96.7
		mphA	16.7
		EreB	0.7
Multidrug	Efflux	E_coli_acrA	100
		E_coli_mdfA*	100
		H-NS	100
		acrB, acrE, acrF, acrS	100
		baeS,baeR	100
		cpxA	100
		evgA	100
		evgS	100
		gadW, gadX	100
		mdtA, mdtB, mdtC, mdtE, mdtF, mdtG, mdtH, mdtM, mdtN, mdtO, mdtP	100
		msbA	100
		tolC	100
		yojI	100
		emrB	99.3
		mdtM	96
		E_coli_emrE*	40.7
		KpnH	0.7
	Efflux and reduced permeability	marA	100
Phenicol	Inactivation	catI	17.3
	Efflux	floR	2
Polymyxin	Target alteration	eptA	100
		pmrF	100
		ugd	59.3
Sulfonamide /trimethoprim	Target replacement	sul2	21.3
		sul1	18
		dfrA17	16.7
Tetracycline	Efflux	emrK, emrY	100
		tet(B)	36
		tet(A)	14.7

Grey shading highlights a gene only identified by this database, and * highlights a unique variant identified by this database.

5.4.9 Screening the β sample *E. coli* genomes for antibiotic resistance genes in the MEGARes database

ABRicate was used to compare genome contigs against the MEGARes ARG database. The percentage of genomes that contained ARG sequence matches from the MEGARes database are described in this section and presented in Table 5.15 and Table 5.16. There were sequence matches for the aminoglycoside efflux genes *acrD* and *kdpE* in all of the genomes. Of the sequence matches for *kdpE*, 58% were 99% nucleotide identity matches for the reference sequence. Sequence matches for the aminoglycoside phosphotransferase *aph3-D'* and *aph6* genes were identified in 21.3% of the genomes, including the genomes from 47% of the DCA isolates. *aph3-D'* and *aph6* were also identified in the genomes of D1K7, D3M9, M5T14, and M5T15. Sequence matches for the *aph6* reference sequence in the C1A6, C1A10, C5E6, and C5E7 genomes were an 88% identity match to the reference sequence. Sequence matches were identified for the aminoglycoside nucleotidyltransferase *ant3-D'* genes in 19.3% of the genomes, including the genomes from 35% of the DCA isolates, D1K7, H5J8, M1P7, and M1P11. The C5E14 genome had a 98% nucleotide identity match for the *ant3'-D* reference sequence. The bacitracin target modification gene *bacA* and efflux gene *bcr* were found in all of the genomes. All of the genomes had sequence matches for the *ampH* β-lactamase. There were sequence matches for the *blaEC* β-lactamase in 78.7% of the genomes, including the genomes from 53% of the DCA isolates, 93% of the PPD isolates, 100% of the PRP isolates, and 93% of the PPL isolates. Sequence matches with 98% nucleotide identity for the *TEM* β-lactamase were identified in 42.7% of the genomes, including the

genomes from 68% of the DCA isolates, 10% of the PPD isolates, 10% of the PRP isolates, M5T14, and M5T15. Sequence matches for the β-lactamase *ampC* were identified in 21.3% of the genomes, including the genomes from 47% of the DCA isolates, D1K7, D3M9, M5T14, and M5T15. A sequence match for the β-lactamase *CARB* was identified in the C5E14 genome only. Sequence matches for the penicillin-binding protein genes *PBP2* were identified in all of the genomes, and *PBP4B* was identified in 95% of the genomes. The genomes of D1K4, H3H2, M5T5, M5T7, M5T11, M5T12, and M5T13 did not have sequence matches for *PBP4B*. Sequence matches for the fluoroquinolone efflux genes *emrA* and *emrR* were identified in all of the genomes. The H5J3 genome had a 94% nucleotide identity match for the lincosamide nucleotidyltransferase gene *lnuC* reference sequence. Sequence matches for the macrolide phosphotransferase inactivation gene *mphB* were identified in 97% of the genomes. The genomes of M5T5, M5T7, M5T10, M5T11, and M5T12 were the only ones without *mphB* sequence matches. There were sequence matches for the macrolide phosphotransferase inactivation gene *mphA* in 16.7% of the genomes, including the genomes from 40% of the DCA isolates, and D1K7. A sequence match for the macrolide esterase inactivation gene *ereB* was identified in the C5E14 genome. Sequence matches for the inner membrane efflux pump genes (e.g., *acrAB*, *mdfA*, *mdtABC*) as well as regulatory genes for these pumps (e.g., *acrE*, *acrS*, *acrR*, *gadW*, *gadX*, *HNS*, *mdtE-mdtP*) were identified in all of the genomes. The C5E14 genome was the only genome without a sequence match for *emrB*. Sequence matches with 98% nucleotide identity to the *mdtM* gene were identified in 45% of the genomes from DCA isolates, as well as the D1K7

genome. The genomes of M5T5, M5T7, M5T10, M5T11, and M5T12 did not have sequence matches for *mdtM*. Sequence matches for *MVRC* (an analogue of *emrE*) were identified in 40.7% of the genomes, including the genomes from 40% of the DCA isolates, 50% of the PPD isolates, 50% of the PRP isolates, and 6% of the PPL isolates. A sequence match for the *KPN* gene was identified in the C5E14 genome; no literature reference for this gene or its function could be found. Sequence matches for *marA*, *robA*, and *soxS* genes involved in reduced permeability and increased efflux regulators were identified in all genomes. Sequence matches for the phenicol inactivation acetyltransferase gene *catA* were identified in 17.3% of the genomes, including the genomes from 40% of the DCA isolates, D1K7, and H5J9. Sequence matches for the phenicol efflux gene *floR* were identified in 2% of the genomes, specifically in the genomes of D3M9, M5T14, and M5T15. All of the genomes had sequence matches for the outer membrane porin suppressor gene *asmA*. Sequence matches for the polymyxin phosphoethanolamine target modification genes *eptA* and *pmrF* were identified in all of the genomes. There were sequence matches for the phosphoethanolamine *ugd* genes in 59.3% of the genomes, including the genomes from 30% of the DCA isolates, 53% of the PPD isolates, 80% of the PRP isolates, and 73% of the PPL isolates. Sequence matches for the sulfonamide target replacement gene *sul2* were identified in 21.3% of the genomes, including the genomes from 46% of the DCA isolates, D1K7, D3M9, M5T14, and M5T15. There were sequence matches for the sulfonamide target replacement gene *sul1* in 18% of the genomes, including the genomes from 42% of the DCA isolates, D1K7, and H5J8. All of the genomes had sequence

matches for the tetracycline efflux genes *emrK* and *emrY*. Tetracycline efflux genes *tetB* and *tetD* sequence matches were identified in 36% of the genomes, including the genomes from 48% of the DCA isolates, 46% of the PPD isolates, and 33% of the PRP isolates. Sequence matches with 98% nucleotide identity for *tetD* were identified in the genomes of C1A11, C1A12, C1A13, C1A14, C1A15, D1K11, D1K12, D1K13, D1K14, D1K15, D3M1, D3M2, D3M3, D3M4, D3M5, D3M6, D3M7, D3M8, H3H11, H3H12, H3H13, H3H14, H315, H5J11, H5J12, H5J13, H5J14, and H5J15. Sequence matches for the tetracycline efflux gene *tetA* were identified in 14.7% of the genomes, including the genomes from 18% of DCA isolates, D1K6, D3M9, H3H6, H3H7, H3H9, H3H10, H5J8, H5J9, M5T14, and M5T15. There were sequence matches for the transcriptional regulator *CTD* in 18% of the genomes, including the genomes from 42% of the DCA isolates, D1K1, and D1K7. Sequence matches for the iron distributor *FRA* were identified in 16.7% of the genomes, including the genomes from 40% of the DCA isolates, and the D1K7 genome.

Table 5.15: MEGARes antibiotic resistance genes, mechanisms, and abundance identified in *β E. coli* genomes.

Class	Resistance mechanism	Gene	Presence (%)
Aminoglycoside	Efflux	ACRD	100
		KDPE	100
	Inactivation	APH3-D'	21.3
		APH6	21.3
		ANT3-D'	19.3
Bacitracin	Target alteration	BACA	100
	Efflux	BCR	100
β-lactam	Inactivation	AMPH	100
		BLAEC	78.7
		TEM	42.7
		AMPC	21.3
		CARB	0.7
	Penicillin binding protein	PBP2*	100
		PBP4B*	95.3

Grey shading highlights a gene only identified by this database, and * highlights a unique variant identified by this database.

Table 5.16: MEGARes antibiotic resistance genes, mechanisms, and abundance identified in *β E. coli* genomes continued.

Class	Resistance mechanism	Gene	Presence (%)
Fluoroquinolone	Efflux	EMRA	100
		EMRR	100
Lincosamide	Inactivation	LNUC	0.7
Macrolide	Inactivation	MPHB	96.7
		MPHA	16.7
		EREB	0.7
Multidrug	Efflux	ACRA, ACRB, ACRE, ACRF, ARCS	100
		BAER, BAES	100
		CPXAR	100
		CRP	100
		EMRD	100
		EVGS	100
		GADW, GADX	100
		HNS	100
		MARA,	100
		MARR	100
		MDFA	100
		MDTA, MDTB, MDTC, MDTE, MDTF, MDTG, MDTH	100
		MDTI	100
		MDTJ	100
		MDTK	100
		MDTN, MDTO, MDTP	100
		MSBA	100
		YOGI	100
		ROBA	100
		EMRB	99.3
		MDTM	96
	Transporter	MVRC	40.7
	Unknown	KPN	0.7
	Efflux and reduced cell permeability	SOXS	100
Phenicol	Inactivation	CATA	17.3
	Efflux	FLOR	2
Porin	Suppressor	ASMA	100
		KPNO	80
Polymyxin	Target modification	EPTA	100
		PMRF	100
		UGD	59.3
Sulfonamide	Target replacement	SULII	21.3
		SULI	18
Tetracycline	Efflux	EMRK, EMRY	100
		TETB	36
		TETD	36
		TETA	14.7
Global regulators	Transcriptional repressor	CTD	18
	Iron distributor	FRA	16.7

Grey shading highlights a gene only identified by this database, and * highlights a unique variant identified by this database.

5.4.10 Screening the β sample *E. coli* genomes for antibiotic resistance genes in the NCBI ARG database

ABRicate was used to compare contigs against the NCBI ARG database. The percentage of genomes that contained ARG sequence matches from the NCBI ARG database are presented in Table 5.17 and described in this section.

Sequence matches for the aminoglycoside phosphotransferase inactivation genes *aph(3")-Ib* and *aph(6)-Id* were identified in 21.3% of the genomes, including the genomes from 46% of the DCA isolates, D1K7, D3M9, M5T14, and M5T15. The genomes of C1A6, C1A10, C5E6, and C5E7 had sequence matches with 88% nucleotide identity for *aph(6)-Id*. Sequence matches for the aminoglycoside nucleotidyltransferase inactivation gene *aadA5* were identified in 16.7% of the genomes, including the genomes from 40% of the DCA, and D1K7. Only the M1P7 and M1P11 genomes had sequence matches for *aadA22*. A sequence match for *aadA1* was identified in the H5J8 genome. The only sequence match for *aadA2* was identified in the C5E14 genome. A sequence match for the β-lactamase gene *blaEC-18* was identified in 29.3% of the genomes, including the genomes from 36% of the PPD isolates, 36% of the PRP isolates, 56% of the PPL isolates, C1A5, C1A6, C1A10, C5E6, and C5E8 genomes. Sequence matches for *blaEC* were identified in 28% of the genomes, including the genomes from 48% of the DCA isolates, 26% of the PPL isolates, D1K4, D1K7, D3M9, and H5J2. There were sequence matches for the *blaEC-8* in 25.3% of the genomes, including the genomes from 25% of the DCA isolates, 43% of the PPD isolates, and 33% of the PRP isolates. Sequence matches for the *blaTEM-1* were identified in 25.3% of the genomes, including the genomes from 42% of the DCA isolates, 30% of the PPD isolates,

H3H8, H5J6, H5J9, and H5J10. There were sequence matches for the *blaTEM-235* in 25.3% of the genomes, including the genomes from 25% of the DCA isolates, D1K6, D3M14, H3H6, H3H7, H3H19, H3H11, and H5J7. Sequence matches for *blaEC-13* were identified in 12% of the genomes, including the genomes from 18% of the DCA isolates, D1K6, D3M14, H3H6, H3H7, H3H19, H3H11, and H5J7. The genomes of M5T5, M5T7, M5T10, M5T11, and M5T12 had sequence matches for *blaEC-5*. The genomes of D3M9, H5J8, M5T14, and M5T15 had sequence matches for *blaTEM-150*. Sequence matches for *blaEC-15* were identified in the genomes of D1K2, H5J1, and H5J3. The C5E14 genome had a sequence match for *blaCARB-2*. A sequence match with 94% nucleotide identity for the lincosamide nucleotidyltransferase gene *lnu(D)* was found in the H5J3 genome. Sequence matches for the macrolide phosphoesterase gene *mph(A)* were identified in 16.7% of the genomes, including the genomes from 40% of DCA isolates and D1K7. A sequence match for the macrolide esterase gene *ere(B)* was identified in the C5E14 genome. There were sequence matches for the phenicol acetyltransferase gene *catA1* in 17.3% of the genomes, including the genomes from 40% of the DCA isolates, D1K7, and H5J8. Sequence matches for the phenicol efflux gene *floR* were identified in the D3M9, M5T14, and M5T15 genomes. There were sequence matches for the sulfonamide target modification gene *sul2* in 21.3% of the genomes, including the genomes from 47% of the DCA isolates, D1K7, D3M9, M5T14, and M5T15. Sequence matches for *sul1* were identified in 18% of the genomes, including the genomes from 42% of the DCA isolates, D1K7, and H5J8. There were sequence matches for the trimethoprim target modification gene *dfrA17*

identified in 16.7% of all of the genomes, including the genomes from 40% of the DCA isolates. Sequence matches for the tetracycline efflux genes *tet(A)* were identified in 34% of the genomes, including the genomes from 28% of the DCA isolates, 53% of the PPD isolates, 53% of the PRP isolates, M5T14, and M5T15. There were sequence matches for *tet(B)* identified in 16.7% of the genomes, including the genomes from 40% of the DCA isolates, and D1K7.

Table 5.17: NCBI antibiotic resistance genes, mechanisms, and abundance identified in the *β E. coli* genomes.

Class	Resistance mechanism	Gene	Presence (%)
Aminoglycoside	Inactivation	aph(3")-Ib	21.3
		aph(6)-Id	21.3
		aadA5	16.7
		aadA22	1.3
		aadA1	0.7
		aadA2	0.7
β-lactam	Inactivation	blaEC-18	29.3
		blaEC	28
		blaEC-8	25.3
		blaTEM-1	25.3
		blaTEM-235	14.7
		blaEC-13	12
		blaEC-5	3.3
		blaTEM-150	2.7
		blaEC-15	2
		blaCARB-2*	0.7
Lincosamide	Inactivation	lnu(D)	0.7
Macrolide	Inactivation	mph(A)	16.7
		ere(B)	0.7
Phenicol	Inactivation	catA1	17.3
	Efflux	floR	2
Sulfonamide /trimethoprim	Target modification	sul2	21.3
		sul1	18
		dfrA17	16.7
Tetracycline	Efflux	tet(A)	34
		tet(B)	16.7

Grey shading highlights a gene only identified by this database, and * highlights a unique variant identified by this database.

5.4.11 Screening the β sample *E. coli* genomes for antibiotic resistance genes in the ResFinder database

ABRicate was used to compare contigs against the ResFinder database. The percentage of genomes that contained ARGE sequence matches from the ResFinder database are described in this section and presented in Table 5.18. Aminoglycoside phosphoesterase genes *aph(3'')-Ib_5* and *aph(6)-Id_1* were identified in 21.3% of the genomes, including the genomes from 37% of DCA isolates, D1K7, D3M9, M5T14, and M5T15. The genomes of C1A6, C1A10, C5E6, and C5E7 had an 88% identity match for the reference sequence of *aph(6)-Id_1*. Aminoglycoside nucleotidyltransferase gene *aadA5_1* was identified in 16.7% of the genomes, including the genomes from 40% of DCA isolates and D1K7. The genomes from H5J8, M1P7, and M1P11 contained 99.59% identity matches for the *ant(3'')-Ia_1* reference sequence. The C5E14 genome contained a 98% identity match for the *aadA2_1* reference sequence. *blaTEM-1B_1* sequence matches were identified in 25.3% of the genomes, including the genomes from 42% of DCA isolates, 30% of PPD isolates, H5J6, H5J9, and H5J10. Sequence matches for *blaTEM-1C_1* were identified in 14.7% of the genomes, including the genomes from 25% of DCA isolates, D1K7, D3M14, H3H6, H3H7, H3H9, H3H10, and H5J7. The genomes of D3M9, H5J8, M5T14, and M5T15 contained sequence matches for *blaTEM-1A_1*. The genome of C5E14 contained a sequence match for *blaCARB-2_1*. A 94% identity match for the lincosamide nucleotidyltransferase gene *lnu(D)_1* was identified in the H5J3 genome. The macrolide phosphotransferase gene *mph(A)_2* was identified in 16.2% of the genomes, including the genomes from 33% of DCA isolates and D1K7. Sequence matches for the macrolide

esterase gene *ere(B)_2* was identified in the C5E14 genome. The multidrug efflux gene *mdf(A)_1* was identified in all of the genomes. Sequence matches for the phenicol acetyltransferase gene *catA1_1* were identified in 17.3% of the genomes, including the genomes from 40% of DCA isolates, D1K7, and H5J8. Sequence matches with 99.9% identity matches for the phenicol efflux gene *floR_2* were identified in the genomes of D3M9, M5T14, and M5T15. There were sequence matches for the sulfonamide target replacement gene *sul1_5* identified in 18% of the genomes, including the genomes from 42% of DCA isolates, D1K7, and H5J8. Sequence matches for the trimethoprim target replacement gene *dfrA17_1* and sulfonamide target replacement gene *sul2_3* were identified in 16.7% of the genomes, including the genomes from 40% of DCA isolates and D1K7. There were sequence matches for *sul2_2* in 4.7% of the genomes, including the genomes of C1A6, C1A10, C5E6, C5E7, D3M9, M5T14, and M5T15. Sequence matches for the tetracycline efflux gene *tet(B)_2* were identified in 36% of the genomes, including the genomes from 50% of DCA isolates, 47% of PPD isolates, and 33% of PRP isolates. There were sequence matches for *tet(A)_6* in 14.7% of the genomes, including the genomes from 18% of DCA isolates, 20% of PRP isolates, D1K6, D3M9, D3M14, M5T14, and M5T15. All of the sequence matches for *tet(A)_6* in the genomes from DCA isolates. PRP isolates, D1K6, and D3M14 were 98% nucleotide identity matches for the reference sequence.

Table 5.18: ResFinder antibiotic resistance genes, mechanisms, and abundance identified in the *β E. coli* genomes.

Antibiotic Class	Resistance mechanism	Gene	Presence (%)
Aminoglycoside	Inactivation	aph(3'')-Ib_5	21.3
		aph(6)-Id_1	21.3
		aadA5_1	16.7
		ant(3'')-Ia_1	2
		aadA2_1	0.7
β-lactam	Inactivation	blaTEM-1B_1*	25.3
		blaTEM-1C_1*	14.7
		blaTEM-1A_1*	2.7
		blaCARB-2_1*	0.7
Lincosamide	Inactivation	lnu(D)_1	0.7
Macrolide	Inactivation	mph(A)_2*	16.7
		ere(B)_2*	0.7
Multi drug	Efflux	mdf(A)_1	100
Phenicol	Inactivation	catA1_1	17.3
	Efflux	floR_2*	2
Sulfonamide/ trimethoprim	Target modification	sul1_5	18
		dfrA17_1	16.7
		sul2_3*	16.7
		sul2_2*	4.7
Tetracycline	Efflux	tet(B)_2*	36
		tet(A)_6*	14.7

Grey shading highlights a gene only identified by this database, and * highlights a unique variant identified by this database

5.4.12 Number of antibiotic resistance genes and number of unique genes and variants identified across antibiotic resistance gene databases

This thesis has utilised five ARG databases to identify acquired resistance genes by screening contigs in ABRicate. MEGARes identified the most genes at 74, the most unique genes at 17 and two unique variants. CARD identified 65 genes, four unique genes, and two unique variants. NCBI ARG identified 26 genes and six unique genes. ARG-ANNOT identified 26 genes, four unique genes and three unique variants. ResFinder identified 21 genes with 11 unique variants (Table 5.19).

Table 5.19: Number of ARG, unique genes, and unique variants identified in each ARG database

	ARG-ANNOT	CARD	MEGARes	NCBI ARG	ResFinder
Genes identified	25	65	74	26	21
Unique genes	4	4	17	6	0
Unique variants	3	2	2	0	11

5.4.13 Serotyping, sequence typing, and MGE in *E. coli* across bovine developmental stage

To investigates diversity of *E. coli* within and between bovine developmental groups on the SB Farm, 150 isolates were selected for short-read WGS. These genomes were serotyped with EcOH, assigned ST using the Achtman seven gene MLST scheme, and the presence of plasmids were investigated using Plasmidfinder.

5.4.14 *In silico* screening of *E. coli* genomes with the EcOH database to predict serotypes

Serotyping sequence matches were identified in EcOH, all 150 of the genomes had either wzt-O9 (80%) or wzt-O8 (20%) ATP binding protein antigens (Table 5.20). There was more diversity in flaggellin antigens, with a cluster of the PPD genomes containing matching sequences for fliC-H6, a cluster of DCA isoaltes containing matches for fliC-H9, and a cluster of PPL genomes containing matches for fliC-H21. All of the genomes with wzt-O8 ATP binding protein matches also had matches for the wzm-O8 ABC transporter permease. Half of the genomes had matches for the wzx-Onovel14 flippase antigen, the second most common match after wzt-O9 ATP binding protein antigens.

Table 5.20: EcOH database serotype sequence matches, function, and percentage identified in *β E. coli* genomes.

Function	EcOH gene	Presence (%)
ATP binding protein	wzt-O9	80
	wzt-O8	20
Flagellin	fliC-H6	19.3
	fliC-H9	18.7
	fliC-H21	18
	fliC-H4	12
	fliC-H18	4.7
	fliC-H29	4.7
	fliC-H7	4
	fliC-H1	3.3
	fliC-H2	2.7
	fliC-H25	2
	fliC-H43	2
	fliC-H49	2
	fliC-H20	1.3
	fliC-H32	1.3
	fliC-H10	0.7
	fliC-H23	0.7
	fliC-H26	0.7
	fliC-H28	0.7
	fliC-H39	0.7
	fliC-H40	0.7
O antigen ABC transporter permease	wzm-O8	20
	wzm-Onovel32	17.3
	wzm-O9	8.7
O antigen flippase	wzx-Onovel14	50.7
	wzx-O112	8
	wzx-O96	5.3
	wzx-O30	0.7
O antigen polymerase	wzy-O112	8.7
	wzy-O108var1	0.7

Presence (%) is the proportion of all genomes that had a sequence match for an EcOH gene was identified in.

5.4.15 Identifying clusters of *β E. coli* isolates through *in silico* mulitlocus sequence typing

Isolates were assigned a ST and ST complex according to the Achtman seven gene MLST *E. coli* scheme in Enterobase (Table 5.21). The ST and ST complex for each isolate is listed (Appendix 24-Appendix 27). There were 22 STs and nine ST complexes assigned to these *E. coli*. The STs identified in order of abundance were ST405, ST14965, ST88, ST155, ST10, ST345, ST38, ST1204, ST56, ST129, ST706,ST696, ST1039, ST4279, ST5177, ST4054, ST7724, ST5995, ST4093, ST4684, ST69, ST3574, and no ST. The ST complexes identified in order of abundance were ST10 complex, ST405 complex, ST with no ST complex, ST23 complex, ST155 complex, ST38 complex, ST165 complex, ST206 complex, ST446 complex, ST3574 complex, and no ST or ST complex. ST405 was assigned to 28 of the isolates, including five from the DCA samples, ten from the PRP samples, and 13 from the PPD samples. ST14965 was assigned to 25 of the isolates, including 24 from the DCA samples and one from a PPD sample. ST10 was assigned to nine of the isolates, four from the DCA samples, two from the PRP samples, one from a PPD sample, and two from the PPL samples. ST14965 and ST10 are both in the ST10 complex, the 34 isolates assigned to these ST are all clustered within this complex. ST88 was assigned to 18 of the isolates, 11 from the DCA samples, five from the PRP samples, and two from the PPD samples. ST345 was assigned to nine of the isolates, including two from the PRP samples and seven from the PPL samples. The ST23 complex contains ST88 and ST345, the 27 isolates assigned to these STs are all clustered within this complex. ST155 was assigned to 11 of the isolates, including four from the PRP samples

and seven from the PPD samples. ST56 was assigned to six of the isolates from the PPL samples. ST5177 was assigned to one isolate from a PRP sample, ST4054 was assigned to one isolate from a PPD sample, and ST7724 was assigned to one isolate from a DCA sample. The ST155 complex includes ST155, ST56, ST5177, ST4054, and ST7724; 20 of the isolates clustered within this complex. ST38 was the only ST from the ST38 complex and was assigned to nine of the isolates, all collected from the DCA samples. ST1204 was assigned to seven of the isolates, including one from a PRP sample and six from the PPL samples. ST129 was assigned to five of the isolates, including four from the PRP samples and one from a PPD sample. ST129 was identified to five of the isolates, four from the PRP samples and one from a PPD sample. ST706 was assigned to five of the isolates from the PPL samples. ST696 was assigned to four of the isolates from the DCA samples. ST1079 was assigned to three of the isolates from the PPL samples. ST4279 was assigned to two of the isolates, one from a PRP sample and one from a PPD sample. ST5995, in the ST165 complex, was assigned to one isolate from a DCA sample. ST4093, in the ST206 complex, was assigned to one isolate from a PPD sample. ST 4684, in the ST446 complex, was assigned to one isolate from a PPD sample. ST69, in the ST69 complex, was assigned to one isolate from a DCA sample. ST3574 was assigned to one isolate from a PPL sample. The quality of one isolate from a PPD sample was insufficient to determine a ST (Table 5.21).

Table 5.21: Number of β isolates from each sequence type identified by the Achtman seven gene MLST scheme.

ST complex	ST	Total	DCA	PRP	PPD	PPL
10	14965	25	24	0	1	0
10	10	9	4	2	1	2
Cumulative		34	28	2	2	2
405	405	28	5	10	13	0
23	88	18	11	5	2	0
23	345	9	0	2	0	7
Cumulative		27	11	7	2	7
155	155	11	0	4	7	0
155	56	6	0	0	0	6
155	5177	1	0	1	0	0
155	4054	1	0	0	1	0
155	7724	1	1	0	0	0
Cumulative		20	1	5	8	6
38	38	9	9	0	0	0
-	1204	7	0	1	0	6
-	129	5	0	4	1	0
-	706	5	0	0	0	5
-	696	4	4	0	0	0
-	1079	3	0	0	0	3
-	4279	2	0	1	1	0
165	5995	1	1	0	0	0
206	4093	1	0	0	1	0
446	4684	1	0	0	1	0
69	69	1	1	0	0	0
-	3574	1	0	0	0	1
N/A	N/A	1	0	0	1	0

ST155 was identified as the founding ST in these isolates, with each of the three predominant STs placed on different branches of the GrapeTree plot (Figure 5.43). The most prevalent STs identified were ST405, ST 14965, and ST88 with all three placed on the end of separate branches. There were three ST complexes that were shared by multiple different ST. ST155, ST56, ST4054, ST5177, and ST7724 are all in the ST155 complex, and clustered together in the centre of the tree. ST14965 and ST10 are both in the ST10 complex and were closely linked on one branch of the tree. ST88 and ST345 are both in the ST23 complex and clustered on the same branch of the tree (Figure 5.43).

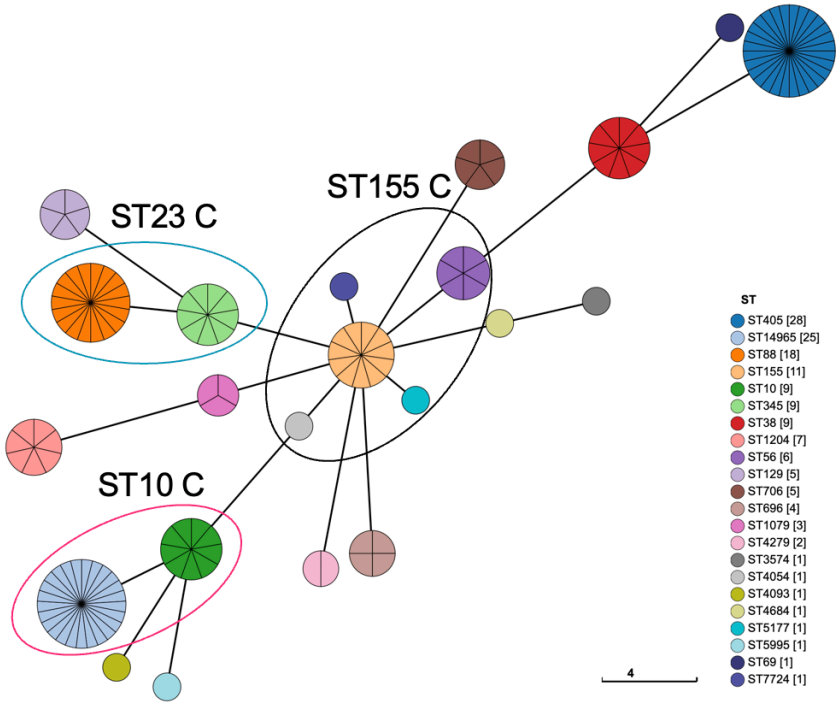


Figure 5.43: MSTree V2 GrapeTree plot of 149 *E. coli* isolates from *β* samples differentiated by the Achtman seven gene MLST scheme. Legend lists the identified STs with the corresponding colour of the node. The number of the isolates per ST is also in the legend and displayed by the number of wedges per node. Major ST complexes are circled and labelled, ST155 C (ST155 Complex) in the black ring (n = 20), ST10C (ST10 Complex) in the pink ring (n = 34), and ST23 C (ST23 Complex) in the blue ring (n = 27).

5.4.15.1 Antibiotic susceptibility of the most prevalent *E. coli* sequence types and sequence type complexes

The antibiotic susceptibility of the major STs and ST complexes were investigated with the ASLs and MASDs. In the 34 ST10 complex *E. coli* isolates, the level of the isolates that were susceptible to cefotaxime (91.2%) was extremely high, to amoxicillin-clavulanate (67.6%) was very high, to kanamycin (26.5%) and ciprofloxacin (23.5%) were high, to ampicillin (11.8%), sulfonamide (14.7%), chloramphenicol (17.6%), and tetracycline (15%) were moderate, and to streptomycin (8.8%) was low (Table 5.22). The level of the isolates that were SIE to kanamycin (73.5%) was extremely high, to streptomycin (14.7%) was moderate, to cefotaxime (8.8%), ciprofloxacin (2.9%), and tetracycline (6%) were low, and to ampicillin (0%), amoxicillin-clavulanate (0%), sulfonamide (0%), and chloramphenicol (0%) were rare. The level of the isolates that were resistant to ampicillin (88.2%), streptomycin (76.5%), sulfonamide (85.3%), chloramphenicol (82.4%), ciprofloxacin (73.5%), and tetracycline (79%) were extremely high, to amoxicillin-clavulanate (32%) was high, to cefotaxime (0%) and kanamycin (0%) were rare in the ST10 complex isolates (Table 5.22).

Table 5.22: Antibiotic susceptibility results and levels of the 34 *E. coli* isolates in the Achtman seven gene MLST ST10 complex.

	AMP	AMC	CTX	S10	K	S300	C	CIP	TE
Susceptible	4	23	31	3	9	5	6	8	5
SIE	0	0	3	5	25	0	0	1	2
Resistant	30	11	0	26	0	29	28	25	27
% Susceptible	11.8	67.6	91.2	8.8	26.5	14.7	17.6	23.5	15
Susceptible level	Moderate	Very high	Extremely high	Low	High	Moderate	Moderate	High	Moderate
% SIE	0	0	8.8	14.7	73.5	0	0	2.9	6
SIE Level	Rare	Rare	Low	Moderate	Extremely high	Rare	Rare	Low	Low
% Resistant	88.2	32	0	76.5	0	85.3	82.4	73.5	79
Resistance level	Extremely high	High	Rare	Extremely high	Rare	Extremely high	Extremely high	Extremely high	Extremely high

SIE = susceptible increased exposure, AMP = ampicillin, AMC = amoxicillin-clavulanate, CTX = cefotaxime, S10 = streptomycin, K = kanamycin, S300 = sulfonamide, C = chloramphenicol, CIP = ciprofloxacin, TE = tetracycline. The level descriptors follow VARSS and EFSA standards defined as rare (<0.1%), very low (0.1-1%), low (1-10%), moderate (10-20%), high (20-50%), very high (50-70%), and extremely high (>70%) (European Food Safety Authority, 2022; Veterinary Medicines Directorate, 2023b).

The median ASRs of these isolates was MDR, with resistance to six antibiotics from six classes (ampicillin, streptomycin, sulfonamide, chloramphenicol, ciprofloxacin, and tetracycline) SIE to kanamycin, and susceptibility to amoxicillin-clavulanate and cefotaxime (Figure 5.44). The median ZOIs for ampicillin, sulfonamide, and chloramphenicol were 6 mm with no interquartile range. The median ZOI for amoxicillin-clavulanate was 19 mm and an interquartile range of 6-20 mm. The median ZOI for cefotaxime was 29 mm and an interquartile range of 28-32 mm. The median ZOIs for streptomycin and tetracycline were 6 mm and an interquartile range of 6-9 mm. The median ZOI for kanamycin was 16 mm and an interquartile range of 15-18 mm. The median ZOI for ciprofloxacin was 12 mm and an interquartile range of 11-26 mm (Figure 5.44).

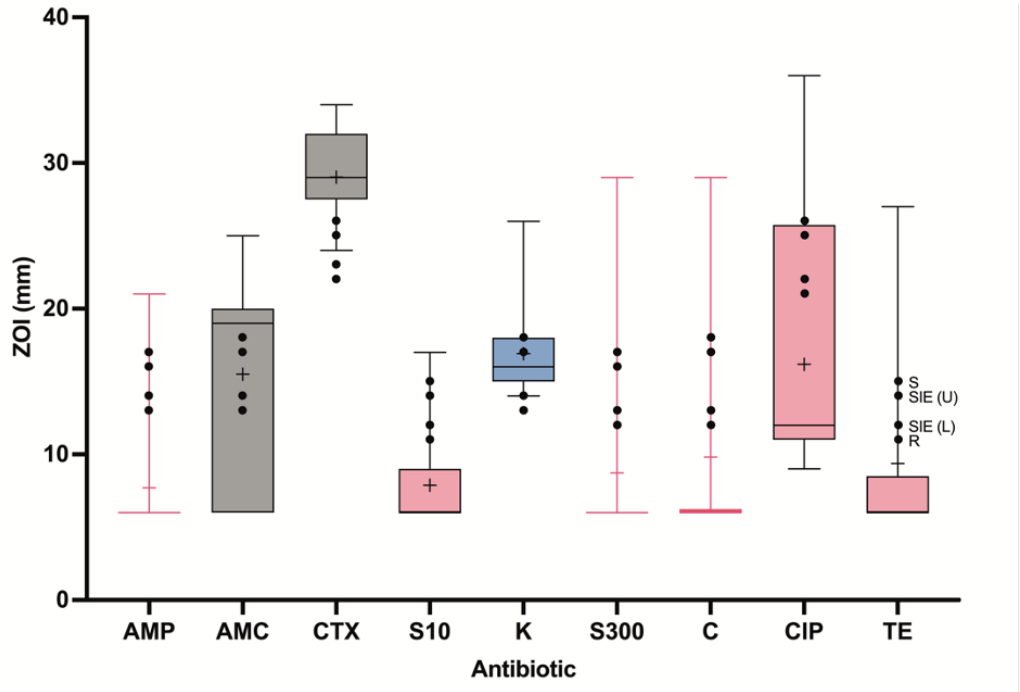


Figure 5.44: Median antibiotic susceptibility distributions of the ST10 complex *E. coli* isolates. The midline represents the median zone of inhibition (ZOI) across all isolates, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown as black dots: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility: grey for susceptible, blue for SIE, and pink for resistant. Abbreviations: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), K (kanamycin), S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), TE (tetracycline).

In the 20 ST155 complex *E. coli* isolates, the levels of susceptibility to amoxicillin-clavulanate (95%), sulfonamide (90%), chloramphenicol (95%), ciprofloxacin (85%), and tetracycline (85%) were extremely high, ampicillin (35%), cefotaxime (25%), streptomycin (20%), and kanamycin (35%) were high. The levels of SIE in these isolates to cefotaxime (75%) and streptomycin (75%) were extremely high, kanamycin (65%) was very high, ciprofloxacin (15%) was moderate, amoxicillin-clavulanate (5%) and tetracycline (5%) were low, and ampicillin (0%) was rare. The levels of resistance to ampicillin (65%) was very high, sulfonamide (10%) and tetracycline (10%) were moderate, streptomycin (5%) and chloramphenicol (5%) were low, amoxicillin-clavulanate (0%), cefotaxime (0%), kanamycin (0%), and ciprofloxacin (0%) were rare (Table 5.23).

Table 5.23: Antibiotic susceptibility results and antibiotic susceptibility levels of the 20 *β E. coli* isolates in the Achtman seven gene MLST ST155 Complex.

	AMP	AMC	CTX	S10	K	S300	C	CIP	TE
Susceptible	7	19	5	4	7	18	19	17	17
SIE	0	1	15	15	13	0	0	3	1
Resistant	13	0	0	1	0	2	1	0	2
% Susceptible	35	95	25	20	35	90	95	85	85
Susceptible level	High	Extremely high	High	High	High	Extremely high	Extremely high	Extremely high	Extremely high
% SIE	0	5	75	75	65	0	0	15	5
SIE Level	Rare	Low	Extremely high	Extremely high	Very high	Rare	Rare	Moderate	Low
% Resistant	65	0	0	5	0	10	5	0	10
Resistance level	Very high	Rare	Rare	Low	Rare	Moderate	Low	Rare	Moderate

SIE = susceptible increased exposure, AMP = ampicillin, AMC = amoxicillin-clavulanate, CTX = cefotaxime, S10 = streptomycin, K = kanamycin, S300 = sulfonamide, C = chloramphenicol, CIP = ciprofloxacin, TE = tetracycline. The level descriptors follow VARSS and EFSA standards defined as rare (<0.1%), very low (0.1-1%), low (1-10%), moderate (10-20%), high (20-50%), very high (50-70%), and extremely high (>70%) (European Food Safety Authority, 2022; Veterinary Medicines Directorate, 2023b).

The median ASP of the ST155 complex *E. coli* isolates was resistance to ampicillin, SIE to three antibiotics from two classes (cefotaxime, streptomycin, and kanamycin), and susceptibility to five antibiotics from five classes (amoxicillin-clavulanate, sulfonamide, chloramphenicol, ciprofloxacin, and tetracycline). The median ZOI for ampicillin was 6 mm with an interquartile range of 6-18 mm. The median ZOI for amoxicillin-clavulanate was 20 mm with an interquartile range of 18-21 mm. The median ZOI for cefotaxime was 25 mm with an interquartile range of 25-26 mm. The median ZOI for streptomycin was 14 mm with an interquartile range of 13-14 mm. The median ZOI for kanamycin was 17 mm with an interquartile range of 16-18 mm. The median ZOI for sulfonamide was 20 mm with an interquartile range of 18-21 mm. The median ZOI for chloramphenicol was 26 mm with an interquartile range of 25-26 mm. The median ZOI for ciprofloxacin was 30 mm with an interquartile range of 26-31 mm. The median ZOI for tetracycline was 26 mm with an interquartile range of 24-26 mm (Figure 5.45).

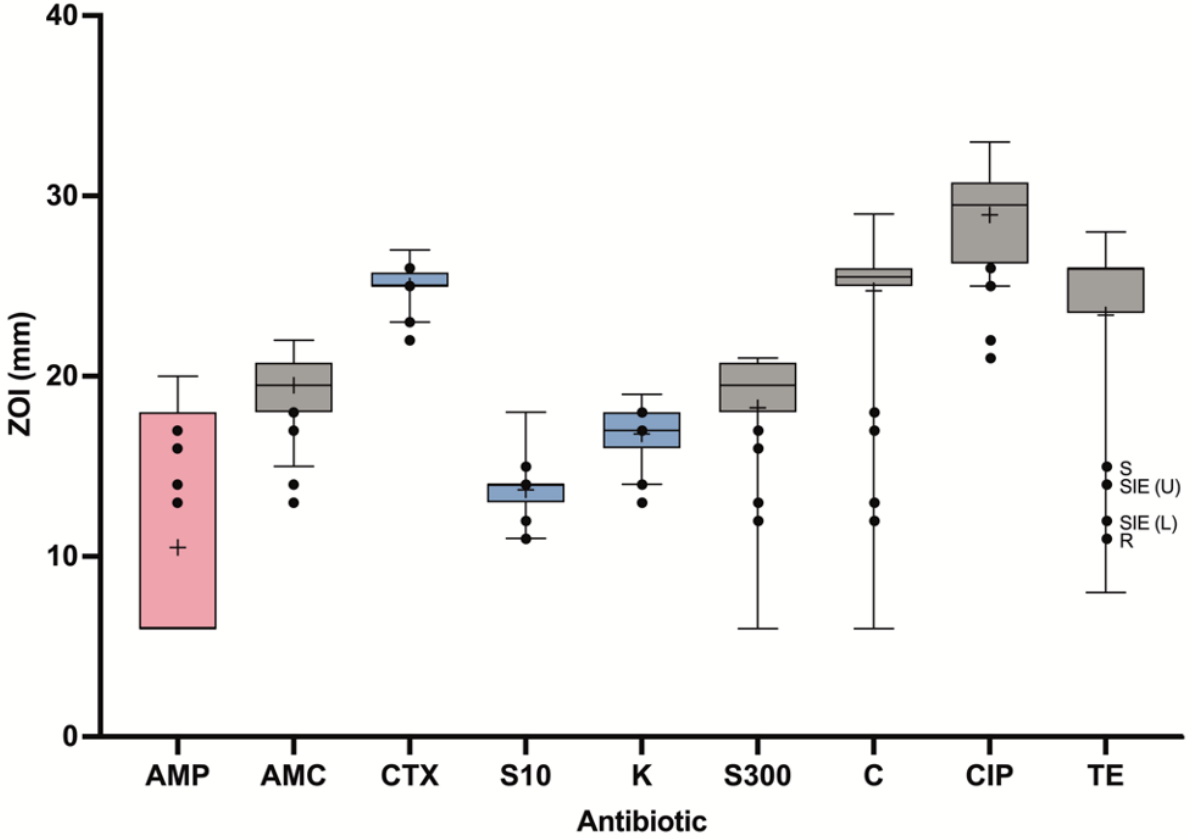


Figure 5.45: Median antibiotic susceptibility distributions of the ST155 complex *E. coli* isolates. The midline represents the median zone of inhibition (ZOI) across all isolates, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown as black dots: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility: grey for susceptible, blue for SIE, and pink for resistant. Abbreviations: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), K (kanamycin). S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), TE (tetracycline).

In the 28 ST405 *E. coli* isolates, the levels of susceptibility to ampicillin (78.6%), amoxicillin-clavulanate (96.4%), sulfonamide (71.4%), chloramphenicol (96.4%), and ciprofloxacin (89.3%) were extremely high, kanamycin (39.3%) was high, cefotaxime (10.7%) and streptomycin (14.3%) were moderate, and tetracycline (0%) was rare. The levels of SIE in these isolates to streptomycin (82.1%) was extremely high, cefotaxime (64.3%) and kanamycin (60.7%) were very high, sulfonamide (17.9%) was moderate, ampicillin (3.6%), amoxicillin-clavulanate (3.6%), and ciprofloxacin (7.1%) were low, chloramphenicol (0%) and tetracycline (0%) were rare. In the ST405 *E. coli* isolates, the levels of resistance to tetracycline (100%) was extremely high, cefotaxime (25%) was high, ampicillin (17.9%) and sulfonamide (10.7%) were moderate, streptomycin (3.6%), chloramphenicol (3.6%) and ciprofloxacin (3.6%) were low, amoxicillin-clavulanate (0%) and kanamycin (0%) were rare (Table 5.24).

Table 5.24: Antibiotic susceptibility results and antibiotic susceptibility levels of the 28 *β E. coli* isolates in the Achtman seven gene MLST ST405.

	AMP	AMC	CTX	S10	K	S300	C	CIP	TE
Susceptible	22	27	3	4	11	20	27	25	0
SIE	1	1	18	23	17	5	0	2	0
Resistant	5	0	7	1	0	3	1	1	28
% Susceptible	78.6	96.4	10.7	14.3	39.3	71.4	96.4	89.3	0
Susceptible level	Extremely high	Extremely high	Moderate	Moderate	High	Extremely high	Extremely high	Extremely high	Rare
% SIE	3.6	3.6	64.3	82.1	60.7	17.9	0	7.1	0
SIE Level	Low	Low	Very high	Extremely high	Very high	Moderate	Rare	Low	Rare
% Resistant	17.9	0	25	3.6	0	10.7	3.6	3.6	100
Resistance level	Moderate	Rare	High	Low	Rare	Moderate	Low	Low	Extremely high

SIE = susceptible increased exposure, AMP = ampicillin, AMC = amoxicillin-clavulanate, CTX = cefotaxime, S10 = streptomycin, K = kanamycin, S300 = sulfonamide, C = chloramphenicol, CIP = ciprofloxacin, TE = tetracycline. The level descriptors follow VARSS and EFSA standards defined as rare (<0.1%), very low (0.1-1%), low (1-10%), moderate (10-20%), high (20-50%), very high (50-70%), and extremely high (>70%) (European Food Safety Authority, 2022; Veterinary Medicines Directorate, 2023b).

The median ASP of the ST405 *E. coli* isolates was resistance to tetracycline, SIE to three antibiotics from two classes (cefotaxime, streptomycin, and kanamycin), and susceptibility to five antibiotics from four classes (ampicillin, amoxicillin-clavulanate, sulfonamide, chloramphenicol, and ciprofloxacin) (Figure 5.46). The median ZOI for ampicillin was 19 mm with an interquartile range of 18-19 mm. The median ZOI for amoxicillin-clavulanate was 21 mm with an interquartile range of 20-22 mm. The median ZOI for cefotaxime was 24 mm with an interquartile range of 22-25 mm. The median ZOI for streptomycin was 12 mm with and interquartile range of 12-13 mm. The median ZOI for kanamycin was 17 mm with an interquartile range of 16-18 mm. The median ZOI for sulfonamide was 18 mm with an interquartile range of 16-18 mm. The median ZOI for chloramphenicol was 26 mm with an interquartile range of 25-27 mm. The median ZOI for ciprofloxacin was 28 mm with an interquartile range of 26-28 mm. The median ZOI for tetracycline was 6 mm with an interquartile range of 6-8 mm (Figure 5.46).

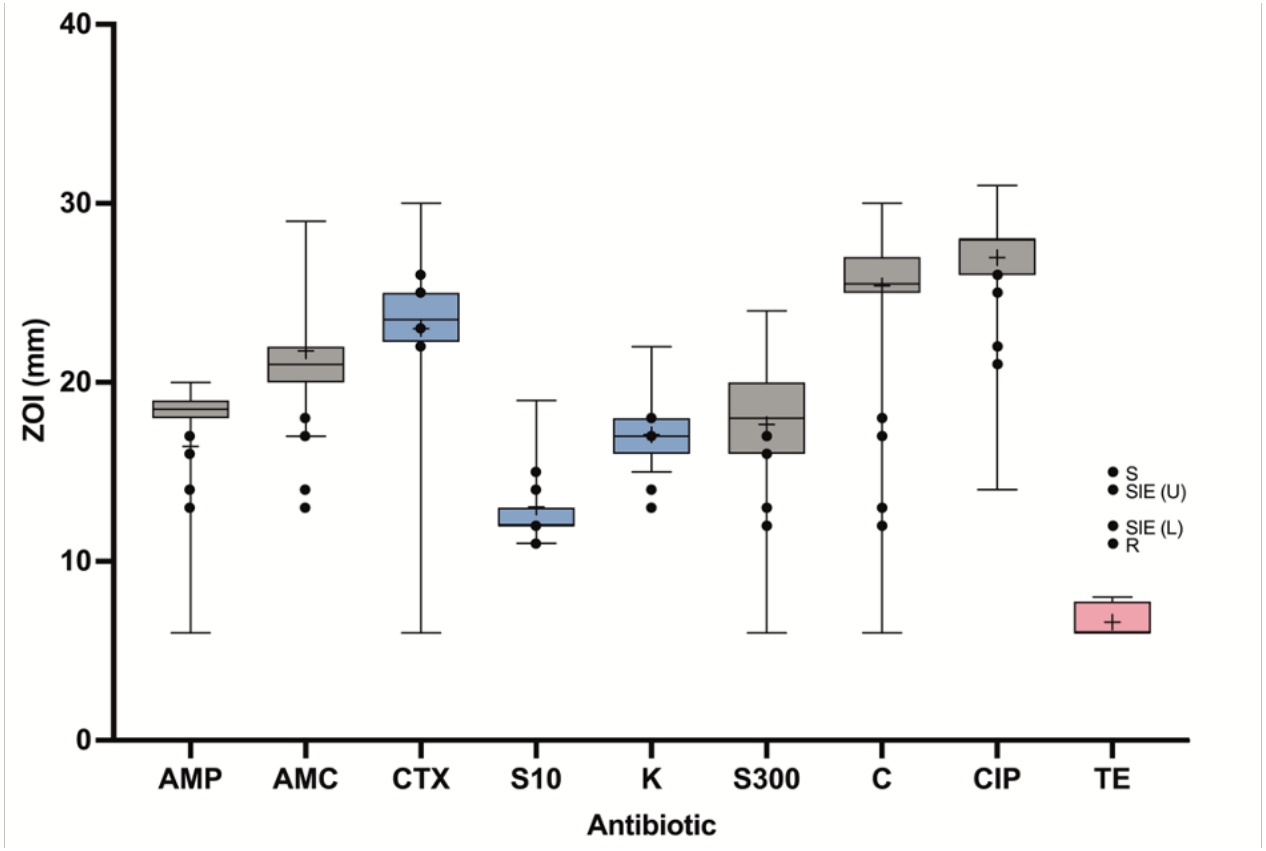


Figure 5.46: Median antibiotic susceptibility distributions of the ST405 *E. coli* isolates. The midline represents the median zone of inhibition (ZOI) across all isolates, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown as black dots: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility: grey for susceptible, blue for SIE, and pink for resistant. Abbreviations: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), K (kanamycin, S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), TE (tetracycline).

In the 27 ST23 Complex *E. coli* isolates, the levels of susceptibility to sulfonamide (92.6%), chloramphenicol (100%), ciprofloxacin (85.2%) was extremely high, to amoxicillin-clavulanate (55.6%) was very high, to ampicillin (33.3%), cefotaxime (29.6%), kanamycin (40.7%), and tetracycline (37%) was high, to streptomycin (18.5%) was moderate. The level of SIE to cefotaxime (63%), streptomycin (66.7%), kanamycin (59.3%) was very high, to amoxicillin-clavulanate (33.3%) was high, to ciprofloxacin (14.8%) and tetracycline (14.8%) was moderate, to sulfonamide (7.4%) was low, to ampicillin (0%) and chloramphenicol (0%) was rare. The level of resistance in to ampicillin (66.7%) was very high, to tetracycline (48.1%) was high, to amoxicillin-clavulanate (11.1%) and streptomycin (14.8%) was moderate, to cefotaxime (7.4%) was low, to kanamycin (0%), sulfonamide (0%), chloramphenicol (0%), and ciprofloxacin (0%) was rare (Table 5.25).

Table 5.25: Antibiotic susceptibility results and antibiotic susceptibility levels of the 27 *β E. coli* isolates in the Achtman seven gene MLST ST23 Complex.

	AMP	AMC	CTX	S10	K	S300	C	CIP	TE
Susceptible	9	15	8	5	11	25	27	23	10
SIE	0	9	17	18	16	2	0	4	4
Resistant	18	3	2	4	0	0	0	0	13
% Susceptible	33.3	55.6	29.6	18.5	40.7	92.6	100	85.2	37
Susceptible level	High	Very high	High	Moderate	High	Extremely high	Extremely high	Extremely high	High
% SIE	0	33.3	63	66.7	59.3	7.4	0	14.8	14.8
SIE Level	Rare	High	Very high	Very high	Very high	Low	Rare	Moderate	Moderate
% Resistant	66.7	11.1	7.4	14.8	0	0	0	0	48.1
Resistance level	Very high	Moderate	Low	Moderate	Rare	Rare	Rare	Rare	High

SIE = susceptible increased exposure, AMP = ampicillin, AMC = amoxicillin-clavulanate, CTX = cefotaxime, S10 = streptomycin, K = kanamycin, S300 = sulfonamide, C = chloramphenicol, CIP = ciprofloxacin, TE = tetracycline. The level descriptors follow VARSS and EFSA standards defined as rare (<0.1%), very low (0.1-1%), low (1-10%), moderate (10-20%), high (20-50%), very high (50-70%), and extremely high (>70%) (European Food Safety Authority, 2022; Veterinary Medicines Directorate, 2023b).

The median ASP of the ST23 complex *E. coli* isolates was resistance to ampicillin, SIE to four antibiotics from three classes (cefotaxime, streptomycin, kanamycin, and tetracycline), and susceptibility to four antibiotics from four classes (amoxicillin-clavulanate, sulfonamide, chloramphenicol, and ciprofloxacin) (Figure 5.47). The median ZOI for ampicillin was 6 mm with an interquartile range of 6-18 mm. The median ZOI for amoxicillin-clavulanate was 18 mm with an interquartile range of 15-20 mm. The median ZOI for cefotaxime was 24 mm with an interquartile range of 23-25 mm. The median ZOI for streptomycin was 12 mm with and interquartile range of 12-14 mm. The median ZOI for kanamycin was 16 mm with an interquartile range of 15-17 mm. The median ZOI for sulfonamide was 20 mm with an interquartile range of 19-21 mm. The median ZOI for chloramphenicol was 26 mm with an interquartile range of 25-27 mm. The median ZOI for ciprofloxacin was 29 mm with an interquartile range of 26-30 mm. The median ZOI for tetracycline was 12mm with an interquartile range of 10-21 mm (Figure 5.47).

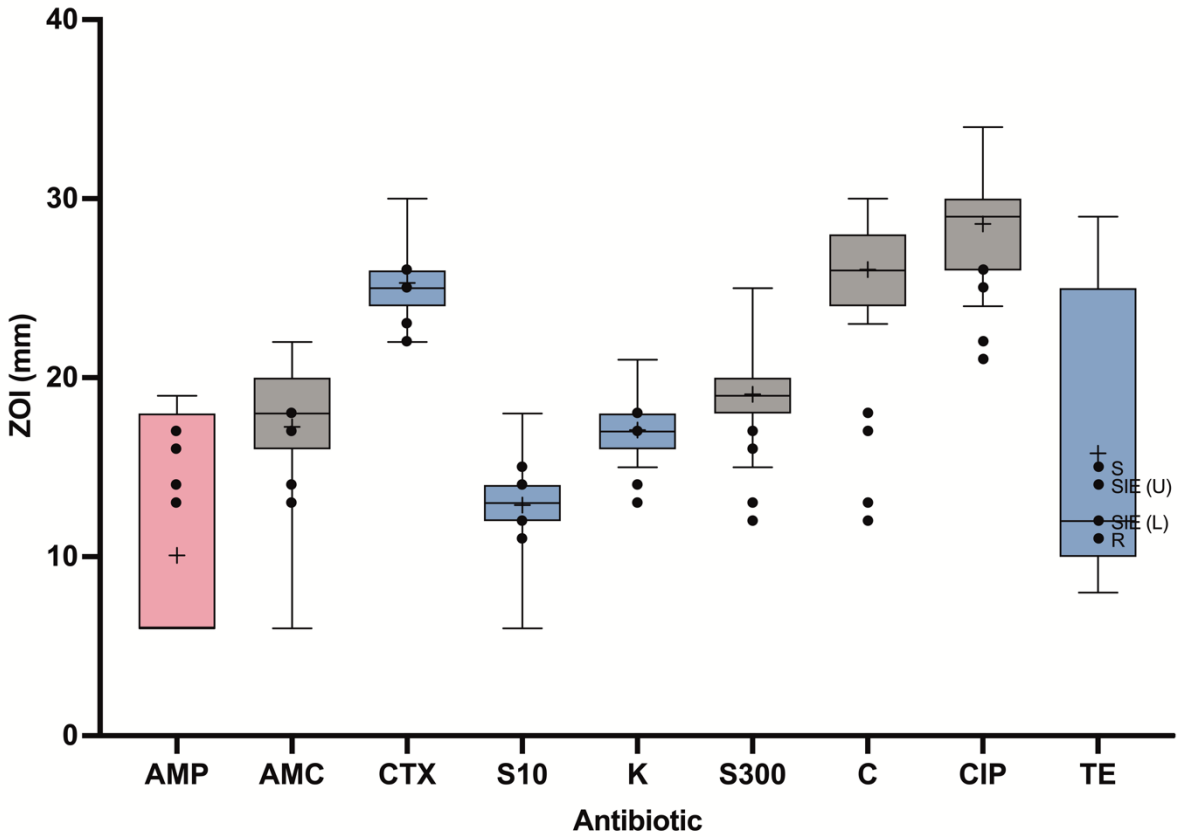


Figure 5.47: Median antibiotic susceptibility distributions of the ST23 Complex *E. coli* isolates. The midline represents the median zone of inhibition (ZOI) across all isolates, with the box indicating the interquartile range (25th to 75th percentile) of ZOI values. Whiskers extend to the minimum and maximum ZOI observed. The mean ZOI is denoted by a cross. EUCAST antibiotic susceptibility cut-offs are shown as black dots: S (susceptible), SIE (U) (susceptible with increased exposure upper limit), SIE (L) (susceptible with increased exposure lower limit), and R (resistant). The box colour indicates the median antibiotic susceptibility: grey for susceptible, blue for SIE, and pink for resistant. Abbreviations: AMP (ampicillin), AMC (amoxicillin-clavulanate), CTX (cefotaxime), S10 (streptomycin), K (kanamycin), S300 (sulfonamide), C (chloramphenicol), CIP (ciprofloxacin), TE (tetracycline).

5.4.16 Plasmid types in *E. coli* across farm groups

ABRicate and Plasmidfinder were used successfully in section 4.6.5.4. In that limited sample set a small range of sequences were identified, in the larger β sample set a greater diversity was found (Table 5.26). Sequences matches were not identified in all genomes, every sample is represented but the proportion of genomes differs. *IncF* was identified in over half of the genomes, with six incompatibility groups identified overall. Several genomes had multiple sequences from the same incompatibility group identified.

Table 5.26: Abundance of sequences matching plasmid incompatibility group sequences in the Plasmidfinder database.

Incompatibility group	PlasmidFinder	Presence (%)	Reference
Colicin V Virulence	ColRNAI_1	27.3	(Waters and Crosa, 1991; Carattoli *et al.*, 2014)
	Col440II_1	15.6	
	Col156_1	10.4	
	Col(MG828)_1	7.8	
	Col8282_1	7.8	
	Col440I_1	5.2	
IncF	IncFIB(AP001918)_1	55.8	(Carattoli *et al.*, 2005, 2014)
	IncFIC(FII)_1	23.4	
	IncFIA_1	14.3	
IncB/O	IncB/O/K/Z_2	5.2	
IncI	IncI1_1_Alpha	3.9	
	IncI2_1_Delta	1.3	
IncX	IncX1_1	16.9	
	IncX3_1	15.6	
	IncX4_1	15.6	
Prophage	IncY_1	24.7	
	pSL483_1	1.3	
	repUS43_1_CDS12738(DOp1)	1.3	

5.5 Discussion

This chapter investigates the differences in *E. coli* collected from the faeces of different bovine developmental groups. Specifically in:

a. Bacterial abundance
b. Antibiotic susceptibility

1. Short-read WGS was completed for select isolates to further investigate the differences in *E. coli* from these development groups. Specifically in:
    a. ARG absence
    b. ST
    c. Plasmid incompatibility groups
2. These results have all been contextualised with national, regional, and global datasets

5.5.1 Isolation of *E. coli* from faecal samples on TBX

E. coli were abundant in the faeces from all developmental groups, with 300 isolates collected across the 20 samples. Collecting reliable bacterial counts was challenging due to the considerable variation in sample compositions impacting the accuracy of pipetting. The ratio of solid to liquid components and the amount of fibrous and gritty aggregates varied significantly across the faecal samples (Appendix 21). For several samples, the bacterial count was recorded as greater than the limit of quantification because every aliquot of the lowest dilution was above the reliably countable range (Appendix 23).

To improve this, the suggestions discussed in section 3.4.2 are also applicable. Diluting the samples further before the serial dilution would

improve the accuracy of all pipetting, and potentially filtering/centrifuging diluted samples before the serial dilution to remove large aggregates.

The bacterial counts of different samples in each group were not normally distributed. Therefore, the KWT was used to confirm significant differences in the ranks of the group median abundance counts.

5.5.1.1 Higher bacterial counts in dairy calf faeces

The DCA faecal samples contained a higher abundance of bacteria than the PRP, PPD, and PPL faecal samples. Compared to the PPL faecal samples, the DCA faecal samples contained a significantly higher count of bacteria on NA with or without antibiotic supplementation and a significantly higher count of *E. coli* on TBX supplemented with antibiotics. There were significantly higher bacterial counts in the DCA samples than in the PPD samples when NA was supplemented with ampicillin. Total bacterial counts (ρ <0.001), counts of ampicillin-resistant *E. coli* (ρ <0.001) and counts of nalidixic acid-resistant *E. coli* (ρ <0.001) decline in the faeces of Scottish beef calves as they aged (Hoyle *et al.*, 2004). Hoyle *et al.,* 2004 sampled the same individuals longitudinally, enabling direct comparison between age, total faecal bacterial count, and resistant proportion. Additionally, Hoyle *et al.,* included control strains to confirm the appropriate concentration of antibiotics in the isolation media; however, this antibiotic susceptibility data is predictive as they did not complete AST on the isolates that grew on selective or non-selective media.

5.5.2 Antibiotic susceptibility of *E. coli* sourced from dairy cow faeces

The levels of ABR matched those of the VARRS 2022 VCD, but differed from those of the α samples presented in Chapter 3. Higher levels of resistance were found in the β samples to sulfonamide, amoxicillin-clavulanate,

chloramphenicol and ciprofloxacin. The very high levels of SIE to the aminoglycosides are consistent across the sample sets, but the β isolates had very high levels of SIE to cefotaxime compared to the high levels in the α isolates. Sections 5.5.2.1 and 5.5.2.2 will discuss the impact of antibiotics in isolation media and the developmental stage of the sampled animal.

5.5.2.1 Antibiotic susceptibility of the isolates picked on TBX supplemented with antibiotics

More isolates were picked from TBX with no additional selective antibiotics, this is the best approach for collecting the greatest number of the isolates without biasing the ASRs. This was intended to investigate the proportions of resistant isolates if they were exceedingly rare. In the older cows, this approach worked to identify these rare isolates shown by the difference in abundance. But in the DCA, this approach showed how much more abundant non-susceptible strains were compared to the older hosts. Isolates from TBX had low levels of resistance to all antibiotics tested, except streptomycin that had a moderate level of resistance; there were also very high levels of SIE (cefotaxime, streptomycin, and kanamycin). Plating on TBX supplemented with ampicillin increased the level of resistance to ampicillin, tetracycline, streptomycin, sulfonamide, chloramphenicol, and amoxicillin-clavulanate. Plating on TBX supplemented with tetracycline reduced the level of resistance to kanamycin and increased the level of resistance to tetracycline, ampicillin, streptomycin, sulfonamide, chloramphenicol, amoxicillin-clavulanate, cefotaxime, and ciprofloxacin.

5.5.2.2 Impact of cow developmental stage group on *E. coli* susceptibility

The isolates from DCA samples had the highest levels of resistance to all classes of antibiotics tested. The only antibiotic that the isolates from DCA didn't have the highest level of resistance to was cefotaxime. Higher proportions of ABR has routinely been identified in DCA (Smith, 1966; Villarroel, 2007; Cao, 2015; Veterinary Medicines Directorate, 2023b). The median ASRs for isolates from the PRP, PPD, and PPL samples were the same for all antibiotics tested except tetracycline; where the median ZOIs were resistant (PRP), SIE (PPD), and susceptible (PPL). The increase in levels of resistance between the α and β samples is driven by the higher proportion of non-susceptible *E. coli* in the DCA samples. Isolates selected from TBX containing antibiotics were included in this chapter because they were isolated in higher numbers from the faeces. The increased abundance showed non-susceptible isolates were a greater proportion of the microflora. The other observed difference was the distribution of ZOI. For isolates from the DCA samples the interquartile range of ZOI around the median ZOI was larger for several antibiotics (amoxicillin-clavulanate, streptomycin, sulfonamide, chloramphenicol, and ciprofloxacin) than in the isolates from the PRP, PPD, and PPL samples. This suggests greater diversity in the antibiotic susceptibility of faecal *E. coli* from DCA. To investigate the diversity further, short-read WGS enabled *in silico* serotyping and identify ARG in the β *E. coli*.

5.5.2.3 Sequencing and assembly quality of the genomes of the β *E. coli*

The number of sequencing reads were high with median contig coverage greater than 100X, therefore, the likelihood of an incorrect base call is low for all of the genomes. The median N50 for all of the genomes was greater than the length of the 400-500 nucleotide loci used in the Achtman seven gene MLST scheme. Therefore, more than half of all contigs should be long enough to contain entire allele sequences and provide a match from the database. The C1A1, C3C5, C5E2, M1P12, M5T5, M5T7, M5T11, and M5T13 genomes had N50 values greater than the average *E. coli* genome length. Therefore, half of the contigs from these assembles are long enough to cover an entire genome providing more confidence for further analysis, especially the wgMLST scheme to call ST. All of these assemblies are sufficient for ABRicate to identify acquired resistance genes.

5.5.3 Antibiotic resistance genes identification and comparison of different reference databases

Across all of the genomes and ARG databases, 86 ARG were identified. The genes that were identified in every genomes suggest resistance mechanisms are present for aminoglycosides, β-lactams, quinolones, polymyxins, tetracyclines and several genes that are involved in multidrug efflux. This high number of ARG, to several different classes of antibiotics increases the likelihood of clinically relevant resistance, resistance does not need to be generated or acquired in this niche because it is already there. Considering that all of the β isolates were not MDR, this suggests that the presence of an ARG does not directly correlate to a resistance phenotype. This could be due to epistasis, interactions between several genes mitigating the impact of

resistance genes. Or due to the lack of additional mutations required to achieve sufficient expression levels for phenotypic resistance. MDR phenotypes have been identified in *E. coli* from the SB Farm previously (Ibrahim *et al.*, 2016; Baker *et al.*, 2022). Transmissible MDR phenotypes were also identified in the α samples in (Chapter 4).

The MEGARes database identified 74 genes, with 17 of these only identified in MEGARes, The majority of these unique genes were transcriptional regulators of efflux and metabolism. The CARD database identified 65 genes with four of these genes unique to this database; A critical gene uniquely identified in the CARD database was *tolC* a crucial component of tripartite efflux pumps combining with the inner membrane and periplasmic components of RND, ABC, and MFS efflux pumps to efflux intracellular compounds across both membranes (Koronakis *et al.*, 1997, 2000; Piddock, 2006). The NCBI ARG database identified 26 genes, with six unique genes, one TEM β-lactamase gene and five *E. coli* specific β-lactamase genes. The ARG-ANNOT database identified 25 genes and four unique genes: one unique aminoglycoside phosphotransferase, two unique TEM β-lactamase, and a repressor of *tetA*. The final database, ResFinder identified 21 genes with no unique genes, but 11 unique variants including resistance determinants for β-lactams, macrolide, phenicols, sulfonamide and tetracycline. These results suggest that if only one database is used, the MEGARes and CARD databases provide the greatest number of genes, but smaller databases such as the NCBI ARG and ResFinder databases provide greater specificity on the exact gene identified. The ARG-ANNOT database has not been updated since 2018, and would not be used in future work. The best database according to

literature is the CARD database with its ability to identify many acquired resistance genes, and mutations that cause resistance (Papp and Solymosi, 2022). Using ABRicate removes the functionality to identify point mutations, but significantly reduced the computational power required making the approach possible for this thesis.

5.5.4 *In silico* identification of sequence type and plasmids incompatibility sequences in *E. coli* across bovine developmental groups

5.5.4.1 *In silico* sequence typing from short-read whole genome sequences

Substantial diversity has been identified in the *E. coli* from these samples. The STs identified in these *E. coli* represent clusters across the global distribution of Bovine *E. coli* STs (Appendix 34).

The ST405 *E. coli* collected in this thesis had concerning levels of resistance to cefotaxime and were all resistant to tetracycline. The narrow MASDs around the SIE cut-offs for cefotaxime, streptomycin, and kanamycin means that treatment with these antibiotics would require an increased dose for effective clinical outcomes. ST405 was assigned to 36 bovine *E. coli* genomes submitted to Enterobase between 2018 and 2024 (0.53%), with 77.8% of these genomes submitted from this thesis. ST405 was not identified in the 523 bovine *E. coli* from cows in Europe (Table 5.1) (Arimizu *et al.*, 2019). This ST appears to be somewhat unique to the SB Farm, identified in the DCA, PRP, and PPD samples. The ASRs of the ST155 *E. coli* collected in this thesis suggest this ST was mostly susceptible, with very high levels of ampicillin resistance, and extremely high levels of SIE to cefotaxime and streptomycin. The MASD to ampicillin for the isolates in this ST suggests a large population of resistant isolates and a small population of very susceptible isolates. ST155

complex was assigned to 551 bovine *E. coli* genomes submitted to Enterobase between 2018 and 2024 (8.17%), with 3.63% of these genomes submitted from this thesis. ST155 complex *E. coli* represented 1.72% of 523 bovine *E. coli* from cows in Europe (Table 5.1) (Arimizu *et al.*, 2019). The ASRs suggests that the ST10 complex *E. coli* collected in this thesis represent a significant risk of resistance to several classes of antibiotics. ST10 complex *E. coli* have been identified frequently in dairy cows, as gastrointestinal commensal strains and as pathogens implicated in mastitis (Leimbach *et al.*, 2017; Alawneh *et al.*, 2020). ST10 complex was assigned to 581 bovine *E. coli* genomes submitted to Enterobase between 2018 and 2024 (8.62%), and the ST10 complex *E. coli* collected in this thesis represent 1.55% of these genomes. The majority of the ST10 complex *E. coli* collected in this thesis were from DCA faeces, with ≥ three isolates assigned ST10 complex from each DCA sample. ST10 complex *E. coli* represented 2.78% of 523 bovine *E. coli* from cows in Europe (Table 5.1) (Arimizu *et al.*, 2019). The ASRs suggest that the ST23 complex *E. coli* identified in this thesis carry some concerning antibiotic resistance, specifically to ampicillin and tetracycline, with concerning levels of non-susceptibility to ampicillin-clavulanate, cefotaxime, and streptomycin. The dispersed MASD for ampicillin and tetracycline suggest large populations of non-susceptible isolates, with smaller populations of susceptible isolates. ST23 was assigned to 229 bovine *E. coli* genomes submitted to Enterobase between 2018 and 2024 (3.4%), with 11.79% of these genomes submitted from this thesis. ST23 *E. coli* represented 1.53% of 523 bovine *E. coli* from cows in Europe (Table 5.1) (Arimizu *et al.*, 2019). ST23 complex *E. coli* have been linked to the spread of ESBL plasmids (Dantas Palmeira and Ferreira,

2020; Veterinary Medicines Directorate, 2023b). Several of the ST23 complex isolates identified in this work may be ESBL positive, future work could investigate the susceptibility of these isolates to more β-lactams. Isolates could also be screened by PCR for specific ESBL associated genes, this approach has previously identified ESBL *E. coli* on the SB Farm (Ibrahim *et al.*, 2016). ST38 was a minor ST identified in the isolates collected from the DCA samples in this thesis, but it was assigned to only 30 bovine *E. coli* Enterobase genomes (0.44%), with 30% of these genomes submitted from this thesis. ST38 *E. coli* represented 0.76% of 522 bovine *E. coli* from cows in Europe (Table 5.1) (Arimizu *et al.*, 2019). The ST and ST complexes identified in the *E.coli* isolates from these samples are spread across a global sample set of bovine *E. coli.* Suggesting that these isolates provide a reliable sample of the the diversity of *E. coli* from the SB Farm. Some notable resistance patterns exist across various STs, specifically MDR ST10 isolates, which were associated with the DCA samples, and ST23 isolates (associated with ESBL-positive plasmids by other investigations) with concerning non-susceptibility to cefotaxime and tetracycline. These observations emphasize the importance of monitoring ABR in bovine *E. coli.* Connections to MGE, such as plasmids identified in these isolates, are the focus of the next section.

5.5.4.2 Plasmids were identified in the majority of *E. coli* genomes

Plasmidfinder found incompatibility group sequence matches in 77 of the β isolate genomes, and 21 of these genomes had sequence matches for different replicons in the same incompatibility group. According to the conventional theory of incompatibility groups, this should not be possible. PlasmidFinder identifies sequence matches when the nucleotide identity of the

query sequence is ≥80% of the reference sequence. Therefore, these issues may be due to unfortunate high similarity between target sequences but further investigations would be required. Screening genomes with plasmids of known incompatibility groups could be used to understand the accuracy and precision of PlasmidFinder. *IncF*-plasmid types were identified in 55.8% of the *β E. coli* genomes, these sequence matches were distributed across the developmental groups, but more were identified in the PPL samples. The isolates from the PPL were mostly susceptible according to the AST, and contained fewer ARG, while *IncF*-plasmid types have been associated with MDR bacteria in humans (Chen *et al.*, 2024). Reinforcing that plasmid incompatibility group is not an indicator of ABR. Other tools for *in silico* plasmid screening are available, such as Plasmer and MOB-suite (Robertson and Nash, 2018; Zhu *et al.*, 2023). The reference databases employed by these tools were not available through ABRicate at the time of completing this thesis. MOB-recon (from MOB-suite) has high sensitivity and specificity (95% and 88% respectively), while PlasmidFinder has high sensitivity but low specificity (99% and 50% respectively) (Robertson and Nash, 2018).

5.6 Limitations and future work

The presence of ARGs is not equivalent to ABR; the fitness costs, epistatic interactions, and the genetic context of many resistance genes dictates the observed phenotype (Vogwill and MacLean, 2015). In environments with low selective pressures, such as farms with low AMU, mutations can occur in an ARG resulting in a pseudogenes (Davis *et al.*, 2011). Pseudogenes are homologous to functional genes, but can no longer be expressed due to random mutations (Lerat and Ochman, 2005). The genomes collected in this

thesis could be analysed for pseudogene sequences using tools such as Pseudofinder, a reference based approach to predict pseudogene presence and infer evolution in closely related genomes (Syberg-Olsen *et al.*, 2022). High quality genomes are required for this approach, and long-read sequencing would provide this quality. Not only is long-read sequencing more expensive, there are concerns about the accuracy of long-read sequencing compared to short-read sequencing (Brown *et al.*, 2021). Additionally, long-read genome assemblers can struggle with plasmids smaller than 10kb in size (Johnson, Soehnlen and Blankenship, 2023). Hybrid assemblies of long-read and the short-read sequencing already collected in this thesis could be a method to obtain the required level of genome resolution.

5.7 Conclusion

The difference in diet and physiology has clearly impacted the diversity of the DCA resistome. Higher proportions of growth on media containing antibiotics and higher levels of resistance to all classes of antibiotics tested provide clear evidence of a greater risk of resistance from younger animals. The treatment records for DCA were sparse, with all male DCA leaving the dairy farm within a few weeks. This needs to be improved before a serious investigation of AMU, and associated AMR can begin. From personal communications with farmers and vets, calves are more likely to receive prophylactic treatment as by the time symptoms are visible it may already be too late. The positive result is that this higher risk is not carried into the older cows and there are no major differences between the older groups due to their mature physiology and microflora. ARG were identified across the age groups to all major classes of antimicrobials including classes with no antibiotics approved for use in

bovines. The number and abundance of ARG suggest more resistance than identified through AST, this approach does not incorporate expression levels of these ARG that will determine their impact on phenotype. Enterobase enabled clustering of these genomes into ST and ST complexes, with several clinically important ST identified. Screening contigs against EcOH and Plasmidfinder in ABRicate was less successful due to multiple conflicting sequence matches, confounding a phenotype prediction. There did appear to be clusters within animals and between animals of the same group, if clusters only existed in one host then this would suggest clonality of the isolates picked from the same plate. However, different hosts were plated separately suggesting there may be some interhost transmission or a common environmental source such as dust, water, or feed.

The next chapter will collect metagenomes from the 20 faecal samples the isolates in this chapter were collected from. Investigating the taxonomic composition of the metagenomes across the bovine development groups, and investigating the taxonomic sources of ARG in the faeces of dairy cattle on the SB Farm.

Chapter 6 Metagenomic identification of ARG to investigate the risk of AMR in the bovine faecal metagenome

To this point, this thesis has investigated *E. coli* isolated from bovine faecal samples, specifically the antibiotic susceptibility and presence of ARG.

These chapters demonstrated there were difference in the ASRs of the isolates from different animals, in samples from treated and untreated PPL, and significant differences between DCA and the older age groups. This chapter will investigate the bovine faecal microbiome in the 20 β faecal samples collected in Chapter 5. The taxonomic composition of these microbiomes will provide context of the environment that the β *E. coli* isolates were sampled from. Metagenome assembled genomes (MAG) will be assembled from metagenomic sequencing reads, enabling the identification of ARG linked to the taxonomical source of a genome. Using metagenome assembled genomes containing antibiotic resistance genes (MARG) enables the identification of the taxonomical source of ARG. This metagenomic context provides greater understanding of the bovine faecal microbiota across developmental stages, and the distribution of ARG in the bovine faecal microbiota.

6.1 Introduction

6.1.1 Metagenomics expands the scope of culture-dependent genomic microbiology

Genomics focuses on the genetic makeup of individual organisms, while metagenomics expands this scope to entire communities or environments (Handelsman *et al.*, 1998) Metagenomics avoids the biases introduced through culturing by extracting total DNA from a sample and attempting to sequence the majority of the genomic information (Roh and Villatte, 2008; Cowan *et al.*, 2015). This approach enables the prediction of new taxa and the reconstruction of genomes from organisms that are difficult or impossible to culture in vitro, providing a more complete representation of the diversity in a sample (Alves *et al.*, 2018). For example, it has been estimated that 99% of the microorganisms in soil are unculturable (Torsvik and Øvreås, 2002). However, another analysis compared 16S sequencing data to sequences of known taxa and determined that far more than 1% of bacteria or taxa have been identified through traditional culture and sequence methods (Martiny, 2019). Across the studied communities, 52% of bacterial sequences and 35% of taxa had related cultured representatives (≥97% 16S rRNA reference sequence similarity) (Martiny, 2019). There were different levels of similarity across communities sourced from humans, air, marine water, marine sediment, freshwater, and soil samples (Martiny, 2019). This suggests that while advances in culturing techniques have improved, some communities are more likely to contain unknown diversity.

6.1.2 Microbial diversity of the bovine gastrointestinal tract

The bovine gastrointestinal tract is an environment that has been explored extensively by culture-dependent and culture-independent methods. The rumen has been a particular area of focus owing to its significant contribution to the health and productivity of ruminant livestock (Cammack *et al.*, 2018). The existence of a core bovine rumen microbiome has been proposed, with a high degree of phylogenetic similarity found in the bovine rumen microbiota of different animals (Li *et al.*, 2009; Jami and Mizrahi, 2012a, 2012b). This similarity in the bovine rumen microbiota was conserved across five rumen compartments sampled at three time points, with some variation between three animals (Li *et al.*, 2009). However, there is still variability across the specific sites of the rumen. It has been reported that 90% of the rumen microbiome is composed of *Firmicutes* and *Bacteroidetes*, but there are distinct populations in the rumen mucosa compared to the lumen (Creevey *et al.*, 2014; Stockler *et al.*, 2022). The abundance of *Firmicutes* and *Bacteroidetes* is also a feature of the mature human gut microbiome (Lozupone *et al.*, 2012).

6.1.3 Variability in the microbiota along the bovine gastrointestinal tract

Digesta samples at ten regions (rumen to rectum) of the gastrointestinal tract of three Simmental x Holstein crossbred 17-month-old PRP were collected, and the taxonomic composition of samples at each site was measured by 16S rRNA sequencing (Wang *et al.*, 2022). *Firmicutes* and *Bacteroidetes* were the most dominant bacterial phyla in the stomach and large intestine. *Firmicutes* were also dominant in the small intestine, while *Bacteroidetes* were rare. Interestingly, there were no significant differences in the species richness,

alpha diversity, or taxonomic profile of the stomach and the large intestine, while the small intestine was significantly different. However, the abundances of *Ruminococcaceae*, *Rikenellaceae*, and *Bacteroidaceae* were higher in the large intestine than in the stomach (Wang *et al.*, 2022). This pattern of similar species richness, alpha diversity, and taxonomic profile has also been observed in the microbiota of the rumen mucosa and the rectum mucosa of PPL Holstein cattle (Mao *et al.*, 2015). However, the microbiota of the rumen digesta and rectum digesta differed significantly, with an increased abundance of *Firmicutes* in the rectum digesta (Mao *et al.*, 2015). A meta-review of 52 studies reported that methanogenic genera were identified in >99.1% of rumen or faecal samples, while *Prevotella* and *Ruminococcus* were identifed in ≥92.9% of samples (Holman and Gzyl, 2019). The same meta review reported *Alistipes*, *Bacteroides*, *Clostridium*, *Faecalibacterium* and *Escherichia/Shigella* were associated with faeces, while *Fibrobacter*, *Prevotella*, *Ruminococcus* and *Succiniclasticum* with the rumen (Holman and Gzyl, 2019). A separation in the microbiome communities of the faeces and rumen was also reported, with significant variation between individual studies in the review (Holman and Gzyl, 2019).

6.1.4 Microbial colonisation of young ruminants

The physiology of DCA differs significantly from that of more developed animals, which impacts the microbiome of these animals. Culture-based investigations of microbial communities in the rumen of young ruminants described rapid colonisation by aerobic and facultative anaerobic taxa, which gradually decreased to a constant level between six to eight weeks old while being replaced by anaerobic taxa (Bryant *et al.*, 1958; Fonty *et al.*, 1987; Minato *et al.*, 1992). Cellulolytic bacteria colonised animals within three to five days after birth, becoming increasingly abundant in the first three weeks (Fonty *et al.*, 1987; Minato *et al.*, 1992). However, the culture-based methodology in these studies described only a fraction of the rumen and gastrointestinal microbiota of young ruminants (Janssen, 2006). Similar patterns of rapid colonisation by aerobic and facultative anaerobic taxa, followed by replacement with anaerobic bacteria, were reported by culture-independent approaches (Jami *et al.*, 2013). *Prevotella* represents the most abundant taxon of these secondary anaerobic colonisers in mature rumen microbiota (Stevenson and Weimer, 2007; Jami and Mizrahi, 2012a).

6.1.5 Impact of antibiotic treatment on bovine gastrointestinal microbiota

Antimicrobial use can alter the microbiota composition and resistome in cattle, swine, and chicken faeces (Looft *et al.*, 2014; Chambers *et al.*, 2015; Xiong *et al.*, 2018). These alterations in the microbiome can either be transient or long-lasting (Dethlefsen and Relman, 2011; Holman, Yang and Alexander, 2019). Generally, broad-spectrum antibiotics reduce a microbiome's bacterial diversity (Dethlefsen and Relman, 2011; Modi, Collins and Relman, 2014). However, the microbiome's resilience and recovery can differ depending on

the antimicrobial used and other host-dependent factors, such as diet (Dethlefsen *et al.*, 2008; Ng *et al.*, 2019). The complex ecological balance of the bovine faecal microbiome is affected by factors other than antimicrobial treatment, such as feed, gastrointestinal disease, and farm management factors such as indoor housing (Auffret *et al.*, 2017; Thomas *et al.*, 2017; Zeineldin, Aldridge and Lowe, 2018; Hagey *et al.*, 2019). The variable impacts of antibiotic treatment on the faecal microbiome of healthy cattle has been reported previously (Chambers *et al.*, 2015; Doster *et al.*, 2018; Holman, Yang and Alexander, 2019; Weinroth *et al.*, 2019). Notably, several have reported reductions in the bovine faecal microbiome richness after antimicrobial treatment, with the time after antimicrobial treatment also identified as an important variable (Thomas *et al.*, 2017; Holman, Yang and Alexander, 2019; F. Chen *et al.*, 2020). Similar trends of decreased bacterial microbiome diversity and richness has been reported in studies of animals receiving antimicrobial treatment, disease severity, specific antimicrobial used and treatment duration, and farm management factors were identified as factors (Collis *et al.*, 2024). Different animals and the composition of their unique microbiomes were identified as the key factor in the impact of antibiotic treatment and the rate of microbiome recovery (Collis *et al.*, 2024).

6.1.6 Measuring species richness and diversity in biology

Rarefaction curves are an ecological method to determine if the number of species identified is likely to reflect the true number of species in an ecological niche (Cayuela, Gotelli and Colwell, 2015). Essentially, how many samples are required to capture rare species. The curve represents the mean curve of thousands of curves drawn showing as many different attempts at counting

the number of species identified for a given sample. Flat curves suggest that new species are still being discovered and the sample size is not sufficient .

The performance of several diversity metrics, including species richness, Simpson index, Shannon index, and Evenness, were evaluated with simulated scenarios (Santini et al., 2016). Species richness identified species extinctions or colonisations well but did not capture other community shifts, making it less sensitive to early signs of biodiversity decline. The Simpson index was responsive to changes in species abundance, particularly when dominant species were affected, but could give misleading signals if common species went extinct. The Shannon index performed poorly in some scenarios, providing inconsistent responses, especially when common species were impacted. Evenness showed strong responses when there were shifts in the distribution of the community but was less reliable under abrupt community changes. Overall, species richness and Simpson index were more consistent, while the Shannon index and evenness were less reliable for detecting biodiversity change (Santini et al., 2016).

6.2 Aims

This chapter will discuss results of metagenomic sequencing carried out on total DNA extracts from the β faecal samples. The aims of this chapter were:

1. Establish quality of sampling, sequencing, and assembly.
2. Determine the taxonomical diversity of faecal metagenomes of DCA, PRP, PPD, and PPL.
3. Identify the taxonomical sources of ARG in the metagenomes and how these differ within and between developmental stages. To contextualise the risk posed by *E. coli* and identify targets for future direct culture and susceptibility testing.

6.3 Methods

Detailed methods for this chapter are described in sections 2.12 and 2.13.

6.3.1 Total DNA extraction from β faecal samples

Total DNA extractions were prepared from the 20 β faecal samples collected in Chapter 5. The manufacturer's instructions were followed for the QIAGEN Powersoil DNA extraction kit. DNA was quantified by Qubit 4 Fluorometer before freezing and shipping on dry ice to preserve the quality of the DNA.

6.3.2 Total DNA short-read metagenomic sequencing

Total DNA extractions were shipped to the University of Leeds Institute of Molecular Medicine (Leeds, UK) for NextSeq 2K 300 cycle lane, 150 bp paired-end metagenomic sequencing (Illumina, USA). Raw sequencing data was provided as the output.

6.3.3 Assembly of metagenomes from short-read sequencing reads

Raw sequencing data was inputted to SqueezeMeta, a bioinformatic pipeline for metagenome assembly and investigation. The data is compiled into a database which can be accessed with SQMtools for analysis. The bioinformatic pipeline used for this analysis incorporated several open-access tools (Table 2.10).

6.3.4 Taxonomic assignment of predicted genes in bovine faecal metagenomes

The SqueezeMeta package contains custom scripts to assign taxonomic ranks to genes identified using the MyTaxa package to categorize to the species level, or the lowest taxonomic rank identifiable (Luo, Rodriguez-R and Konstantinidis, 2014). A gene is likely to occur several times in a metagenome and each occurrence is termed a sequence match. Predicted coding

sequences (CDS) must meet two criteria to be classified as a sequence match. CDS must have ≥ 80% of the bit score of the metagenomic sequence closest to the database type sequence, and CDS must differ by less than 10% in amino acid identity of the database type sequence. Once these sequence matches have been identified, the taxonomical rank is provided by identifying the lowest ranking taxon common to most of the sequence matches, termed the lowest common ancestor (LCA). Each taxon has specific amino acid identity cut offs that must be met for that rank to be assigned (Table 6.1). The taxonomical ranks used in this process were from the NCBI taxonomy database (Schoch *et al.*, 2020).

Table 6.1: Percentage identity cut off values for taonomic assignment of predicted genes.

Taxon	Amino acid identity
Species	85%
Genus	60%
Family	55%
Order	50%
Class	46%
Phylum	42%
Kingdom	40%

Amino acid identity to be termed matches for taxon reference genes in the NCBI GenBank non redundant database.

Unclassified CDS were sequence matches that did not meet the thresholds for taxonomic assignment, unclassified “higher taxonomic level” CDS were sequence matches that did not meet the threshold of the desired taxonomic level but did for higher taxonomic levels, and no CDS were sequencing reads where CDS could not be identified. A reference for the different taxa with corresponding reference species can be found in Appendix 31.

6.3.5 Identifying antibiotic resistance genes in metagenome assembled genomes

MAG are single taxon assembles that are constructed from metagenomic sequencing reads, contigs assigned to a single taxon were binned by MaxBin2 and MetaBAT2 and the contigs in these single taxon bins were combined by

DAS tool (Wu, Simmons and Singer, 2016; Sieber *et al.*, 2018; Kang *et al.*, 2019). ARG are identified in open reading frames (ORF) by Prodigal (Hyatt *et al.*, 2010). Using MARG enables the identification of the taxonomical source of resistance against specific classes of antibiotics.

6.4 Results

6.4.1 Quality of shotgun short-read metagenomic sequencing

Before using metagenomic data to interpret differences in the microbiomes of dairy cows, the quality of the underlying data was assessed.

The total length of the metagenomic sequencing was $1.2x10^9$ nucleotides with 520534 contigs and the N50 length was 2353 nucleotides (range of 1000-1309639 nucleotides). After establishing sequencing quality, the mapping quality dictates the quality of the metagenome assembly. The median mapping percentage of all of the faecal metagenomes was 86.4% of $6x10^7$ reads mapped (Figure 6.1A). The median mapping percentage was highest in the faecal metagenomes from the DCA with 99.2% of $7.4x10^7$ reads mapped (Figure 6.1B), followed by the PPL with 87.3% of $5.6x10^7$ reads mapped (Figure 6.1E), the PPD had 84.9% of $5.6x10^7$ reads mapped (Figure 6.1D), and the PRP had 83.7% of $5.5x10^7$ reads mapped (Figure 6.1C). The median number of nucleotides from the total DNA extracted from all of the samples was $8x10^9$, from the DCA samples was $9.6\ x10^9$, from the PPL samples was $8x10^9$, from the PPD samples was $8x10^9$, and from the PRP samples was $7.9\ x10^9$ (Figure 6.1F). The number of sequencing reads for each metagenome, and the sample group means are in Appendix 28. The number of contigs and percentage of all contigs mapped to each taxonomic rank is presented in Appendix 29.

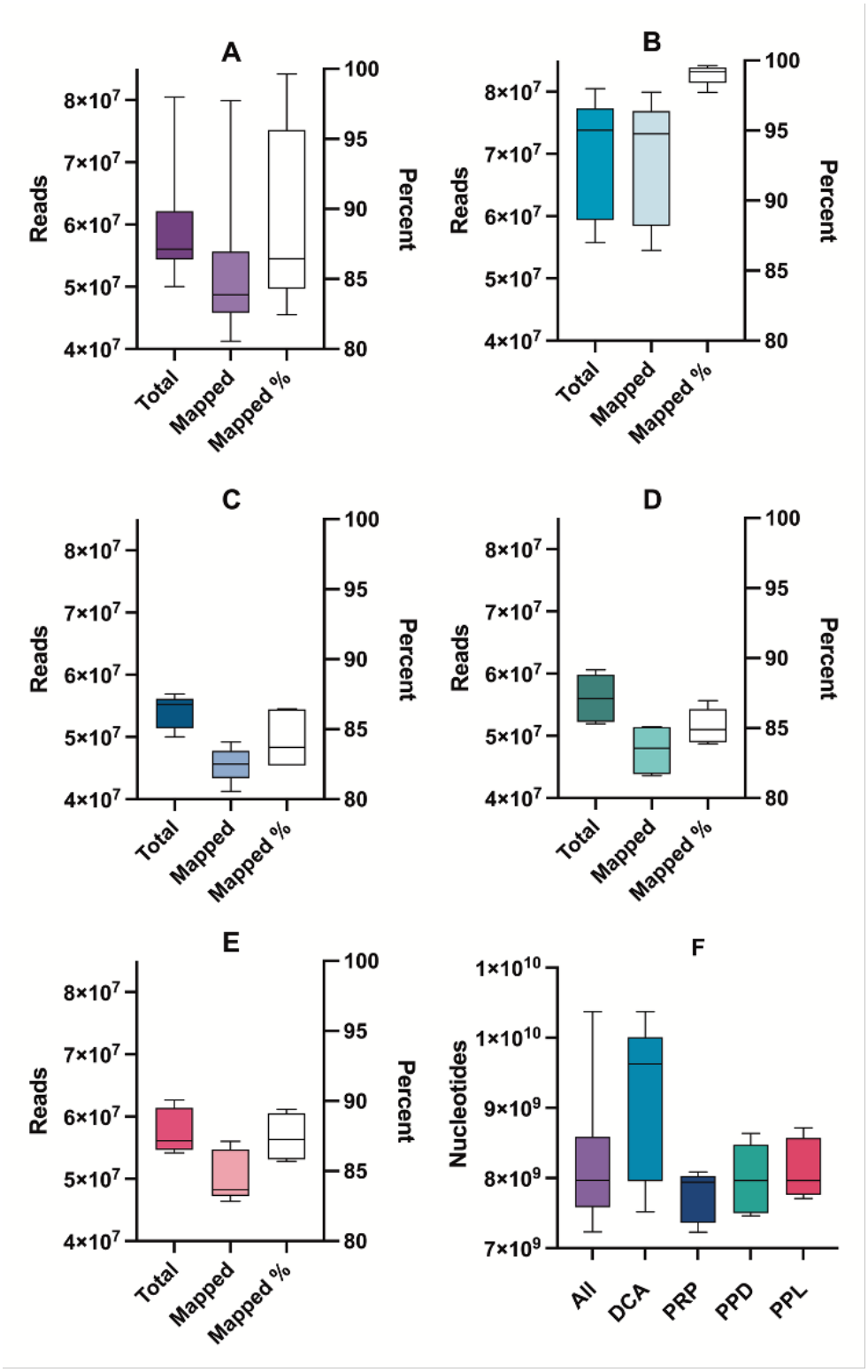


Figure 6.1: Faecal metagenome assembly quality metrics. Across all of the figures the midline is the median, the box displays the 25th to 75th percentile, and the whisker bars extend to the maximum and minimum results. (6.1 A-E) The total number of sequencing reads per metagenome (Dark boxes) and the number of mapped reads (light boxes) on the left y-axis, with the percentage of total reads that were mapped (white box) on the right y-axis. The values are presented separately for the faecal metagenomes of all of the bovines (61.A), the dairy calves (DCA) (6.1B), the pre-parturition cows (PRP) (6.1C), the post-parturition dry cows (PPD) (6.1D), and the post-parturition lactating cows (PPL) (6.1E). The median number of nucleotides in the sequencing data of the faecal metagenomes from all bovines (purple), the DCA (light blue), the PRP (dark blue), the PPD (green), and the PPL (pink) (6.1F).

6.4.1.1 Estimating sampling accuracy

The species number reached the maximum (560) using the tools in the SqueezeMeta pipeline in all of the metagenomes from PRP, PPD, and PPL samples. The metagenomes from the DCA samples were all below the limit of the SqueezeMeta pipeline. The metagenome from the DCA 1 sample had the greatest number of species identified in the DCA samples; followed by the DCA 5 sample, the DCA 4 sample, the DCA 3 sample, and the DCA 2 sample with the lowest number of species in all metagenomes (Figure 6.2).

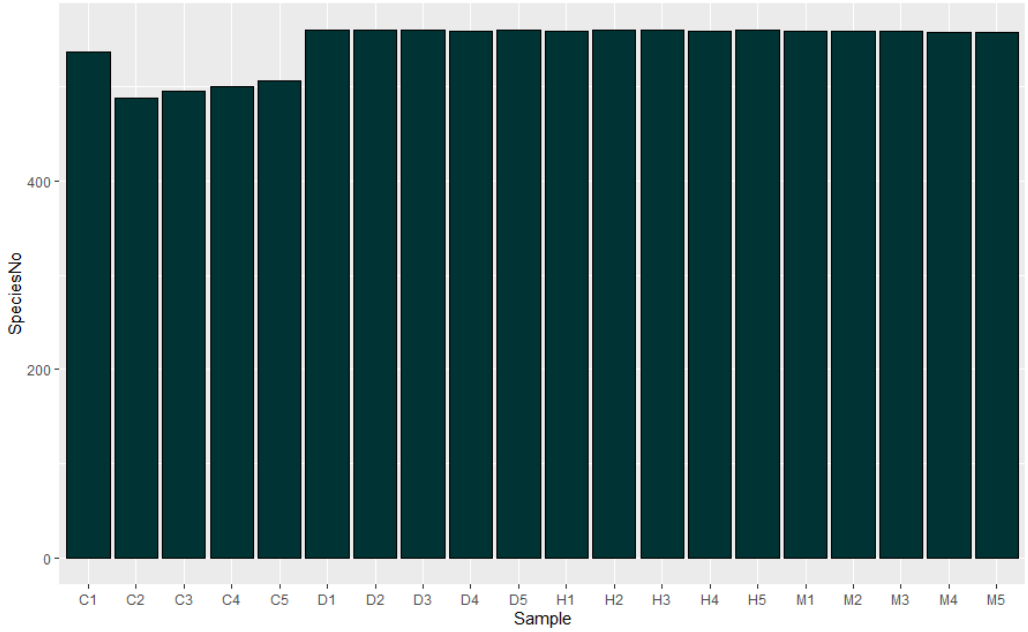


Figure 6.2: Number of bacterial species identified in each faecal metagenome. Sampled from dairy calves (C), post-parturition dry cows (D), pre-parturition cows (H), and post-parturition lactating cows (M). Figure made in R using ggplot2.

The number of species identified was assumed to be true reflection of the actual number according to rarefaction (Figure 6.3). The difference in number of species was visible in the DCA faecal metagenomes, but the shape of the curves indicate this was a true reflection of the number of species. The rarefaction curves of the number of species identified in the PPD, PRP, and PPL metagenomes were indistinguishable (Figure 6.3).

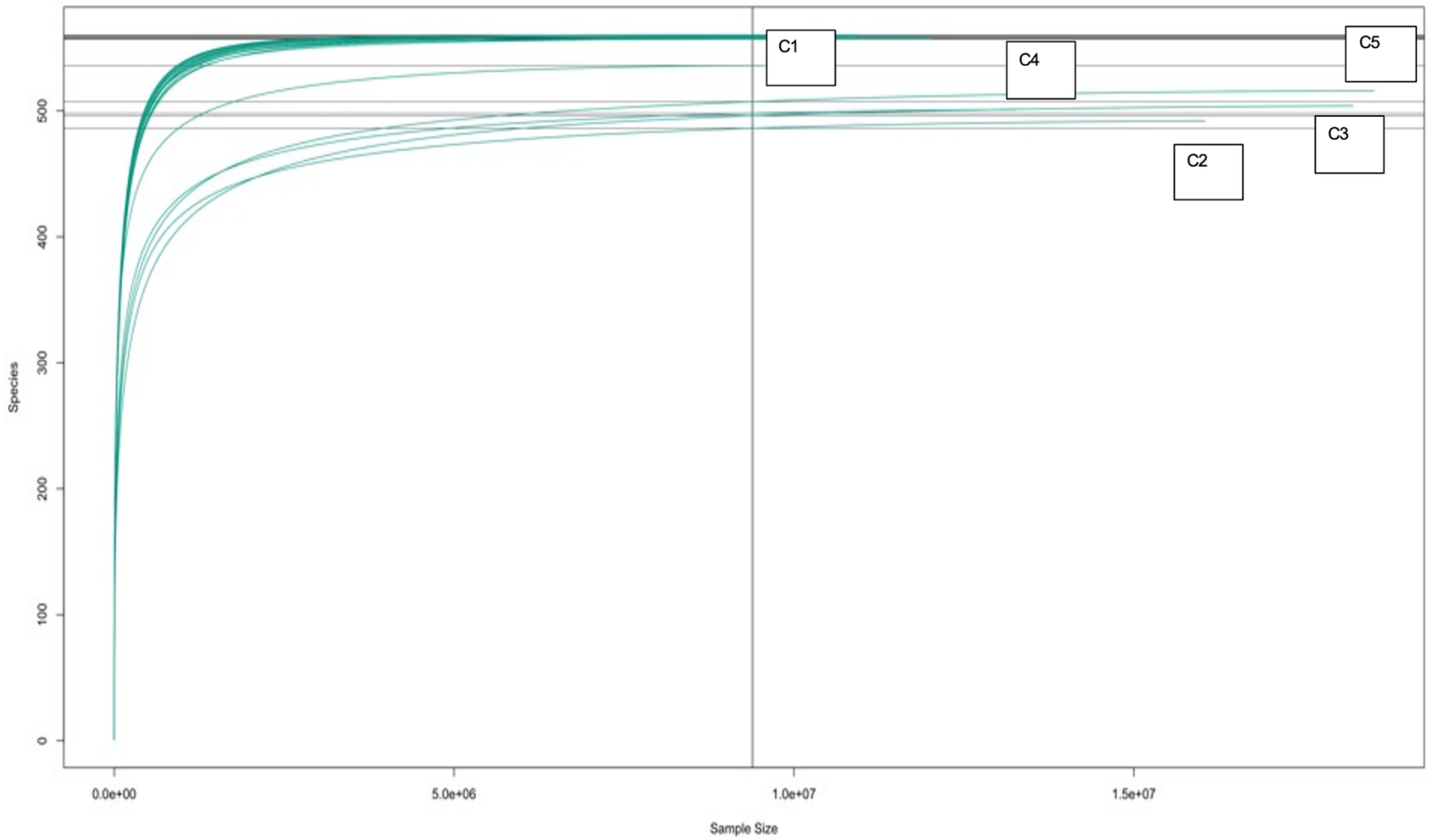


Figure 6.3: Rarefaction curves of species identified for each metagenome. Sample size refers to the number of sequencing reads, all of the metagenomes from the dairy calf (C) samples had fewer species identified than the limit of the SqueezeMeta pipeline and therefore the curves for these metagenomes flattened with a smaller sample size. The curves of the metagenomes from the pre-parturition cows, the post-parturition dry cows, and post-parturition lactating cows were indistinguishable and are not labelled. Figure prepared in R using ggplot2.

6.4.2 Taxonomical diversity of faecal metagenomes

In ecology, diversity is taken as an indication of ecological health, and the same is true for microbiomes (Luz Calle, 2019). This section focused on the alpha diversity of each metagenome (i.e., the diversity within a sample) (Luz Calle, 2019). Several methods were used in this section to understand the species diversity of each metagenome, how similar the metagenomes were within bovine developmental group, and between the different bovine developmental groups.

6.4.2.1 Alpha species diversity of faecal metagenomes

Alpha diversity metrics were calculated for each metagenome using Vegan v2.6-4 (Oksanen *et al.*, 2022). The PRP, PPD, and PPL faecal metagenomes were closely related and clustered together. The DCA faecal metagenomes were distinct from those of the other developmental groups. The DCA 1 faecal metagenome was the most similar, followed by the DCA 4 faecal metagenome. The faecal metagenomes from DCA 3, DCA 2, and DCA 5 were distinct from the others, with the DCA 2 and DCA 5 faecal metagenomes clustering closer together than the DCA 3 faecal metagenome. Fishers Alpha diversity scored the richness of the DCA faecal metagenomes lower than all other metagenomes, and the DCA 1 faecal metagenome was richer than the other DCA faecal metagenomes (Figure 6.4A). The Fishers Alpha diversity score for each faecal metagenome in descending order was 45.79 (richest) from PRP 2, PRP 3, PRP 5, PPD 1, PPD2, PPD 3, and PPD 5; 45.70 (joint eighth richest) from PPD 4, PRP 1, PRP 4, and PPL 1; 45.61 (joint 12th richest) from PPL 2 and PPL 3; from PPL4 and PPL 5 had an alpha score of 45.52 (joint 14th richest); from DCA 1 had an alpha score of 43.65 (16th richest); from

DCA 5 had an alpha score of 41.00 (17th richest); from DCA 4 had an alpha score of 40.47 (18th richest); from DCA 3 had an alpha score of 40.03 (19th richest); and from DCA 2 had an alpha score of 39.33 (least rich) (Figure 6.4B).

The Shannon's index *H* value in descending rank order for the faecal metagenomes was DCA 1 (3.54), PRP 5 (3.29), PRP 3 (3.27), PPD 1 (3.26), PRP 1 (3.22), PPL 3 (3.22), PPD 2 (3.21), PPL 5 (3.20), PPL 4 (3.20), PPL 1 (3.20), PRP 2 (3.18), PPD 4 (3.16), PPD 5 (3.15), PPD 3 (3.15), PRP 4 (3.14), DCA 4 (3.08), PPL 2 (3.07), DCA 5 (2.49), DCA 3 (2.30), and DCA 2 (2.23) (Figure 6.4C).

The Pielou's evenness index *J* score in descending order for the faecal metagenomes was DCA 1 (0.56), PRP 5 (0.52), PRP 3 (0.52), PPD 1 (0.51), PRP 1 (0.51), PPL 3 (0.51), PPD 2 (0.51), PPL 5 (0.51), PPL 4 (0.51), PPL 1 (0.51), PRP 2 (0.50), PPD 4 (0.50), PPD 5 (0.50), PPD 3 (0.50), PRP 4 (0.50), DCA 4 (0.50), PPL 2 (0.49), DCA 5 (0.40), DCA 3 (0.37), and DCA 2 (0.36) (Figure 6.4D).

The Simpson's diversity index *D* value for each faecal metagenome in descending order was 0.93 from DCA 1 (rank = 1st); 0.92 in the faecal metagenomes from PRP 5, PRP 3, PPL 3, PRP 1, PPD 1, PPD 5, and PPL 1 (rank = 8th); 0.91 in the faecal metagenomes from PPL 4, DCA 4, PPD 2, PPD 4, and PPD 5 (rank = 15th); 0.90 in the faecal metagenomes from PPL 2 and PPD 3 (rank = 17th); 0.74 in the faecal metagenomes from DCA 5 and DCA 3 (rank = 19th); and 0.67 in the DCA 2 faecal metagenomes (Figure 6.4E).

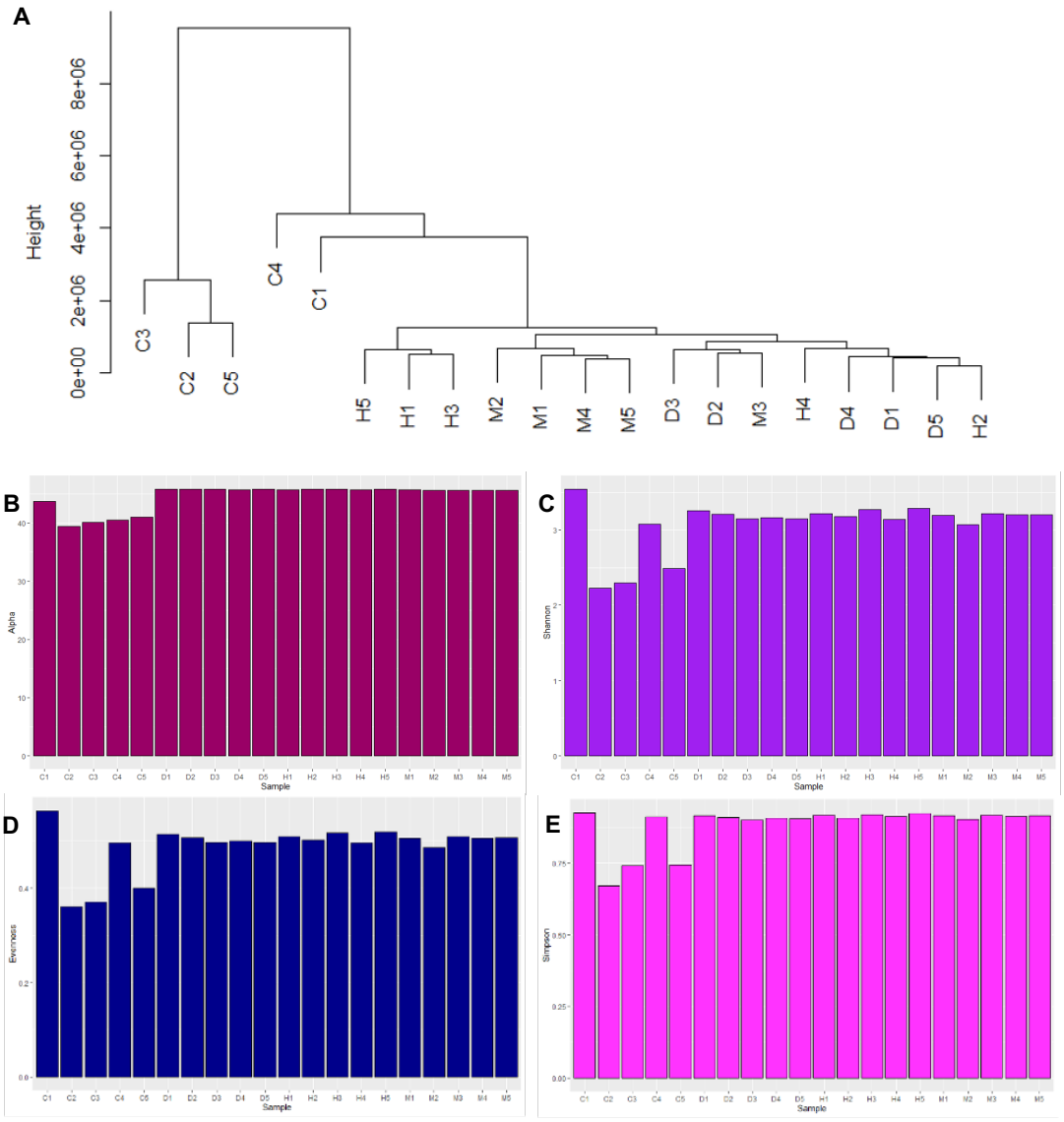


Figure 6.4: Diversity and relatedness of all metagenomes. Metagenomes are from the faecal samples taken from dairy calves (C), post-parturition dry cows (D), pre-parturition cows (H), and post-parturition lactating cows (M). (6.4A) Complete hierarchical clustering of metagenomes to produce a cluster dendrogram. (6.4B) Fishers Alpha diversity of each metagenome, a measure of species richness accounting for total number of taxa and number of individuals in each taxon. (6.4C) Shannon's index of microbial diversity of each metagenome, a higher Shannon's index indicates more taxa, and fewer individuals in each taxon. (6.4D) Pielou's evenness test of microbial diversity of each metagenome, Shannon's index divided by the logarithm of number of taxa present. (6.4E) Simpson's index of microbial diversity of each metagenome, measure for evenness of a community, lower scores indicates that individuals in some taxa are more prevalent than others. Figures were prepared in R with ggplot2.

6.4.2.2 Bacteria were the most abundant kingdom in all of the faecal metagenomes

After assembling the contigs from sequencing reads, predicted genes were identified in the CDS. The lowest common ancestor of these CDS was used to estimate the taxonomical distribution of a metagenome. The mean percentage and standard deviation (SD) of most abundant lowest common kingdom identified in all of the CDS was Bacteria (83.5 ± 7.67%) and less than 1% of all CDS were mapped to Archaea, Eukarya, and Viruses; a significant proportion of reads were unmapped (11.05 ± 6.21%) and unclassified (4.12 ± 1.52%) (Figure 6.5). Bacteria formed a greater proportion of the DCA faecal metagenomes (96.03%) than the faecal metagenomes of the PRP (78.35%), the PPD (78.35%), and the PPL (81.23%). A smaller proportion of CDS were mapped to Archaea in the DCA faecal metagenomes (0.14%) than the faecal metagenomes of the PRP (0.90%), PPD (0.77%), and PPL (0.51%). The DCA 2 faecal metagenome had the highest proportion of CDS mapped to Viruses (0.89%) and the DCA 4 faecal metagenome had the highest proportion of CDS mapped to Eukarya (0.93%).

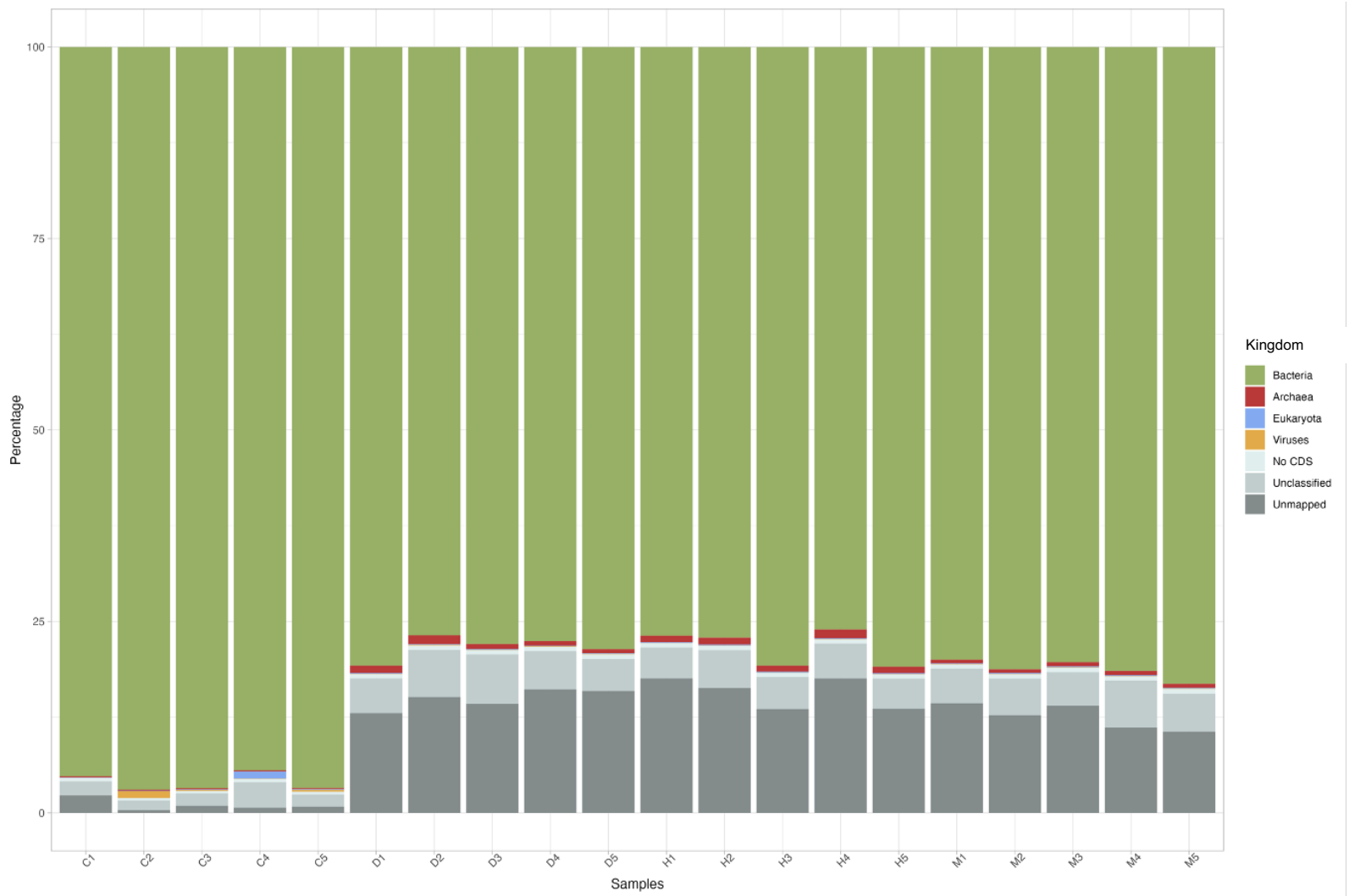


Figure 6.5: Percentage of coding sequences in each faecal metagenome mapped to kingdom. Faecal metagenomes from the dairy calves (C), post-parturition dry cows (D), pre-parturition cows (H), and post-parturition lactating cows (M). No coding sequences (CDS) means no protein coding sequences were identified in these metagenomic sequencing reads, unclassified reads are CDS without kingdom specific sequences, and unmapped are CDS that were not mapped to any genes. Figure was prepared in R with ggplot2.

6.4.2.3 Abundance of bacterial species in the bovine faecal metagenomes

Mapping the CDS from all of the faecal metagenomes to lowest common species identified 1191 taxa. The mean percentage and SD of the most abundant species in descending order of abundance across all of the faecal metagenomes were unclassified *Eubacteriales* (9.15 ± 2.59%), unclassified *Bacteroidales* (8.86 ± 3.05%), unclassified *Clostridia* (5.76 ± 2.72%), unclassified *Oscillospiraceae* (3.66 ± 1.80%), unclassified *Pseudomonas* (3.58 ± 7.60%), unclassified Bacteria (3.54 ± 1.17%), *Bacteroidales bacterium* (2.96 ± 1.41%), unclassified *Acinetobacter* (2.80 ± 3.40%), *E. coli* (2.41 ± 4.54%), unclassified *Prevotella* (2.30 ± 4.65%), unclassified *Faecalibacterium* (2.09 ± 4.58%), unclassified *Bacteroidaceae* (2.02 ± 0.95%), unclassified *Firmicutes* (1.94 ± 0.57%), *Bacteroidaceae bacterium* (1.84 ± 0.88%), and *Oscillospiraceae bacterium* (1.79 ± 0.97%) (Figure 6.6).

6.4.2.3.1 Abundance of bacterial species in the dairy calf faecal metagenomes

The mean percentage and SD of the most prevalent species from all of the DCA faecal metagenomes were unclassified *Pseudomonas* (12.73 ± 13.38%) unclassified *Eubacteriales* (11.29 ± 5.29%), *E. coli* (8.30 ± 6.81%), and unclassified *Faecalibacterium* (7.23 ± 7.68%) (Figure 6.6). The most prevalent taxon mapped to CDS from the DCA 1 faecal metagenome was unclassified *Collinsella* (16.72%). Unclassified *Pseudomonas* was the most prevalent mapped taxon in the faecal metagenomes from DCA 2 (26.80%) and DCA 3 (21.96%). In the DCA 4 faecal metagenome unclassified *Prevotella* was the most prevalent mapped taxon (21.35%). Unclassified *Faecalibacterium* was the most prevalent mapped taxon in the DCA 5 faecal metagenome (13.26%) and the second most prevalent in the DCA 4 faecal metagenome (Figure 6.6).

6.4.2.3.2 Abundance of bacterial species in the post-parturition dry cow faecal metagenomes

The mean percentage and SD of the most abundnnt taxa mapped to CDS from the PPD faecal metagenomes were unclassified *Bacteroidales* (9.90 ± 0.52%), unclassified *Eubacteriales* (7.48 ± 0.46%), unclassified *Clostridia* (7.11 ± 0.88%), unclassified *Acinetobacter* (6.71 ± 3.75%), unclassified Bacteria (3.99 ± 0.40%), *Bacteroidales bacterium* (3.97 ± 0.30%), unclassified *Oscillospiraceae* (3.88 ± 0.22%), unclassified *Bacteroidaceae* (2.47 ± 0.24%), *Bacteroidaceae bacterium* (2.26 ± 0.22%), unclassified *Firmicutes* (2.00 ± 0.16%), *Oscillospiraceae bacterium* (1.99 ± 0.23%), unclassified *Paludibacteraceae* (1.91 ± 0.26%), *Clostridia bacterium* (1.62 ± 0.21%), and unclassified *Rikenellaceae* (1.31 ± 0.22%) (Figure 6.6). Unclassified *Acinetobacter* was the most prevalent mapped taxon in the faecal metagenome of PPD 1 (13.04%), PPD 3 (9.89%) and PPD 5 (10.37%). Unclassified *Bacteroidales* was the most prevalent mapped taxon in the faecal metagenomes of PPD 2 (10.39%) and PPD 4 (10.37%) (Figure 6.6).

6.4.2.3.3 Abundance of bacterial species in the pre-parturition cow faecal metagenomes

The mean percentage and SD of the most abundant taxa mapped to CDS from the PRP faecal metagenomes were unclassified *Bacteroidales* (9.43 ± 0.54%), unclassified *Eubacteriales* (8.46 ± 0.52%), unclassified *Clostridia* (7.28 ± 0.34%), unclassified *Acinetobacter* (3.24 ± 3.08%), unclassified *Oscillospiraceae* (4.52 ± 0.28%), unclassified *Gammaproteobacteria* (1.25 ± 1.49%), unclassified *Bacteria* (3.78 ± 0.25%), *Bacteroidales bacterium* (3.64 ± 0.36%), unclassified *Bacteroidaceae* (2.29 ± 0.17%), *Oscillospiraceae*

bacterium (2.28 ± 0.12%), unclassified *Firmicutes* (2.14 ± 0.09%), *Bacteroidaceae bacterium* (2.07 ± 0.19%), *Sarcina* species (1.37 ± 0.45%), *Clostridia bacterium* (1.67 ± 0.10%), and unclassified *Paludibacteraceae* (1.89 ± 0.33%) (Figure 6.6). The most abundant mapped taxon in the PRP 1 faecal metagenome was unclassified *Eubacteriales* (9.17%). Unclassified *Bacteroidales* was the most abundant mapped taxon in the faecal metagenomes of PRP 2 (10.36%), PRP 3 (9.27%), PRP4 (9.37%), and PRP 5 (9.01%) (Figure 6.6).

6.4.2.3.4 Abundance of bacterial species in the post-parturition lactating cow faecal metagenomes

The mean percentage and SD of the most abundant taxa mapped to CDS from the PPL faecal metagenomes were unclassified *Bacteroidales* (10.76 ± 0.77%), unclassified *Eubacteriales* (9.37 ± 0.40%), unclassified *Clostridia* (7.42 ± 0.22%), unclassified *Oscillospiraceae* (5.43 ± 0.46%), unclassified Bacteria (4.49 ± 0.87%), *Bacteroidales bacterium* (3.61 ± 0.25%), unclassified *Bacteroidaceae* (2.84 ± 0.18%), *Oscillospiraceae bacterium* (2.68 ± 0.21%), *Bacteroidaceae bacterium* (2.57 ± 0.48%), unclassified *Firmicutes* (2.30 ± 0.21%), unclassified *Paludibacteraceae* (2.19 ± 0.24%), unclassified *Bacillales* (1.58 ± 1.83%), *Clostridia bacterium* (1.57 ± 0.14%), unclassified *Rikenellaceae* (1.43 ± 0.14%), and *Paludibacteraceae bacterium* (1.41 ± 0.08%) (Figure 6.6). Unclassified *Bacteroidales* was the most abundant taxon mapped to CDS in the faecal metagenomes of PPL 1 (11.42%), PPL 2 (11.46%), PPL 3 (11.00%), PPL 4 (10.16), and PPL 5 (9.76%). A greater abundance of unclassified *Bacillales* were identified in the PPL 5 faecal metagenome (4.84%) than in all of the other metagenomes (0.88 ± 1.00%).

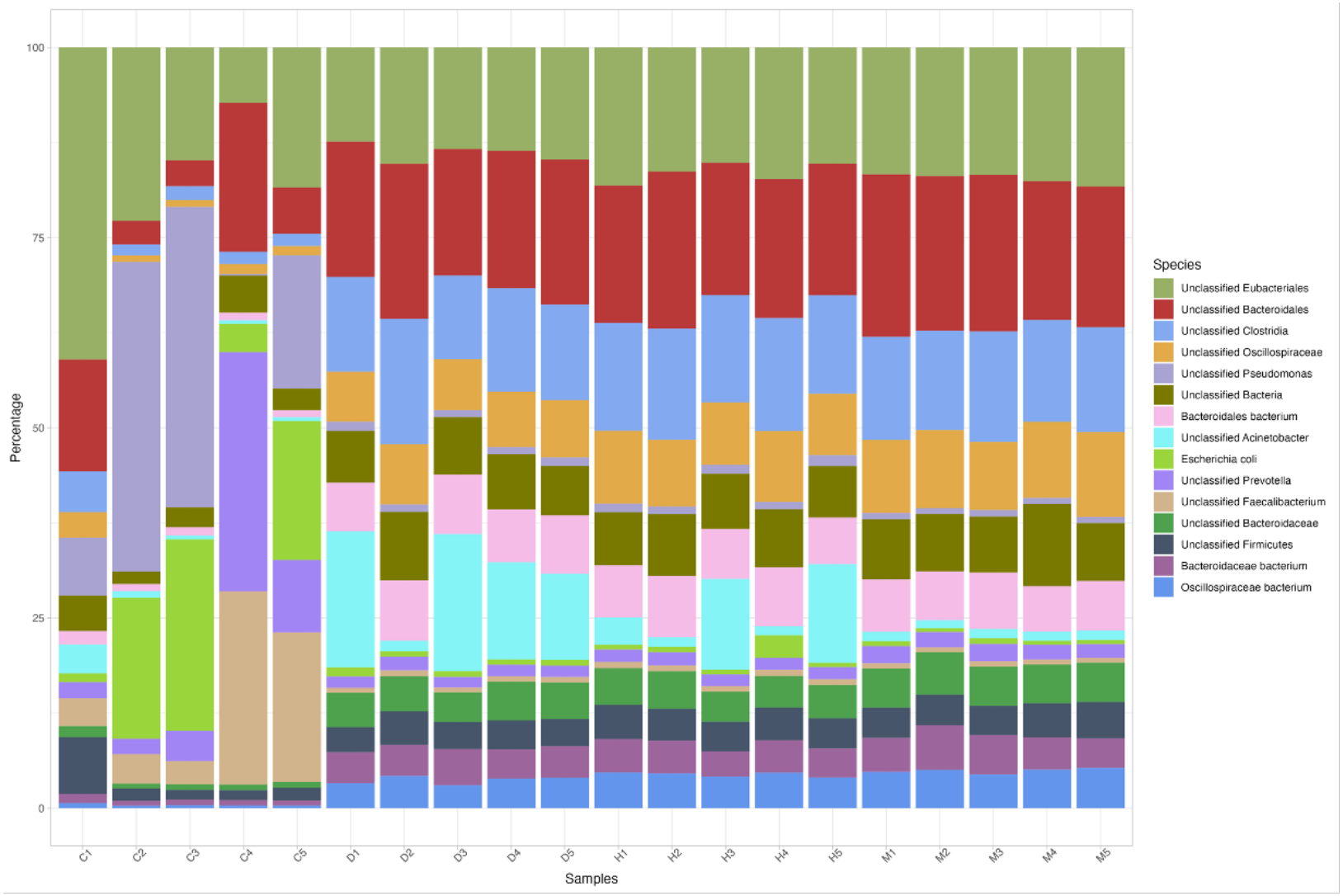


Figure 6.6: Percentage of coding sequences in each faecal metagenome mapped to species. Faecal metagenomes from the dairy calves (C), post-parturition dry cows (D), pre-parturition cows(H), and post-parturition lactating cows (M). Reads with no coding sequences (CDS), unclassified reads, and unmapped reads are not shown here in order to graph less abundant species. Only the 15 most abundant species are presented. Figure was prepared in R with ggplot2.

6.4.3 The overall proportion of antibiotic resistance genes across metagenomes

The mean percentage of MARG to several classes of antibiotics identified in all of the faecal metagenomes are presented in Table 6.2 and described in this section. The mean percentage and SD of MARG identified in all of the MAG from the faecal metagenomes was 1.97 ± 0.54%. The mean percentage of MARG was highest in the MAG from the faecal metagenomes of the DCA (2.78 ± 0.03%), followed by the PPD (1.72 ± 0.008%), the PRP (1.7 ± 0.006%), and the PPL (1.69 ± 0.003%). Genes related to antibiotic efflux were identified in 0.89 ± 0.03% of MAG from all of the faecal metagenomes. The proportion of efflux genes was highest in the faecal metagenomes of the DCA (1.36 ± 0.02%), followed by the PPD (0.77 ± 0.005%), the PRP (0.74 ± 0.004%), and the PPL (0.72 ± 0.001%). Aminoglycoside resistance genes were identified in 0.067 ± 0.001% of MAG from all of the faecal metagenomes. The proportion of aminoglycoside resistance genes was highest in the faecal metagenomes of the PPL (0.07 ± 0.0002%), the PRP (0.067 ± 0.0004%), the PPD (0.066 ± 0.0003%), and the DCA (0.065 ± 0.001%). β-lactam resistance genes were identified in 0.75 ± 0.008% of MAG from all of the faecal metagenomes. β-lactamase genes represented 6.53% of all β-lactam resistance genes and 0.049% of all ARGs identified in the bovine faecal metagenomes. Sulfonamide and trimethoprim resistance genes were identified in 0.24 ± 0.004% of MAG from all of the bovine faecal metagenomes. The proportion of sulfonamide and trimethoprim resistance genes was highest in the faecal metagenomes of the DCA (0.37 ± 0.006%), the PPD (0.2 ± 0.001%), the PRP (0.2 ± 0.001%), and the PPL (0.19 ± 0.006%). Tetracycline resistance genes were identified in 0.069 ± 0.002% of MAG from all of the bovine faecal metagenomes. The

proportion of tetracycline resistance genes was highest in the faecal metagenomes of the DCA (0.11 ± 0.003%), the PPL (0.06 ± 0.0004%), the PRP (0.054 ± 0.0002%), and the PPD (0.05 ± 0.0003%). Quinolone resistance genes were identified in 0.0002 ± 0.0003% of MAG from all of the bovine faecal metagenomes. The proportion of quinolone resistance genes was highest in the faecal metagenomes of the DCA (0.0007 ± 0.0006%), the PPD (0.0001 ± 0.00003%), the PRP (0.00009 ± 0.000009%), and the PPL (0.00008 ± 0.00001%). Phenicol resistance genes were identified in 0.021 ± 0.0009% of MAG from all of the bovine faecal metagenomes. The proportion of phenicol resistance genes was highest in the faecal metagenomes of the DCA (0.034 ± 0.0012%), the PPD (0.018 ± 0.0005%), the PRP (0.016 ± 0.003%), and the PPL (0.013 ± 0.0003%). Polymyxin resistance genes were identified in 0.00014 ± 0.0003% of MAG from all of the bovine faecal metagenomes. The proportion of polymyxin resistance genes was highest in the faecal metagenomes of the DCA (0.00051 ± 0.0004%), the PPD (0.00012 ± 0.000006%), the PRP (0.000023 ± 0.00003%), and the PPL (0.0000073 ± 0.000004%). Macrolide resistance genes were identified in 0.022 ± 0.001% of MAG from all of the bovine faecal metagenomes. The proportion of macrolide resistance genes was highest in the faecal metagenomes of the DCA (0.046 ± 0.002%), the PPD (0.018 ± 0.00009%), the PRP (0.014 ± 0.00007%), and the PPL (0.009 ± 0.00004%).

Table 6.2: Mean prevalence of MARG identified in faecal metagenomes of bovine developmental groups.

Antibiotic class	Faecal metagenomes									
	All		DCA		PPD		PRP		PPL	
	%	SD	%	SD	%	SD	%	SD	%	SD
All	1.97	0.54	2.78	0.032	1.72	0.0088	1.70	0.0068	1.69	0.0034
Efflux	0.89	0.026	1.36	0.018	0.77	0.0052	0.74	0.0043	0.72	0.0015
Aminoglycoside	0.067	0.0011	0.065	0.0013	0.066	0.00034	0.067	0.00039	0.071	0.00022
β-lactams	0.75	0.0083	0.91	0.011	0.70	0.0043	0.72	0.0037	0.68	0.0016
Class A β-lactamase	0.010	0.00046	0.015	0.00086	0.0078	0.00022	0.0078	0.000090	0.0096	0.00017
Class B β-lactamase	0.00073	0.000055	0.00086	0.00012	0.00056	0.000011	0.00074	0.000013	0.00077	0.000027
Class C β-lactamase	0.038	0.0010	0.045	0.0015	0.036	0.00030	0.036	0.00021	0.036	0.00025
Class D β-lactamase	0.00021	0.000041	0.00022	0.000086	0.00032	0.000054	0.00016	0.000027	0.00014	0.0000068
Sul/ tri	0.24	0.0038	0.37	0.0059	0.20	0.0012	0.20	0.0012	0.19	0.00058
Tetracyclines	0.069	0.0019	0.11	0.0027	0.050	0.00025	0.054	0.00020	0.060	0.00040
Quinolones	0.00024	0.00030	0.00071	0.00057	0.00010	0.000025	0.000087	0.0000088	0.000080	0.0000099
Phenicols	0.021	0.00086	0.034	0.0012	0.018	0.00047	0.016	0.00034	0.013	0.00027
Polymyxins	0.00014	0.00029	0.00051	0.00040	0.000012	0.0000057	0.000023	0.000031	0.0000073	0.0000040
Macrolides	0.022	0.0012	0.046	0.0016	0.018	0.00085	0.014	0.00067	0.0090	0.000044

Metagenome assembled genomes containing antibiotic resistance genes (MARG) in the faecal metagenomes of the dairy calves (DCA), post-parturition dry cows (PPD), pre-parturition cows (PRP), and post-parturition lactating cows (PPL), percentage of MARG in all metagenome assembled genomes (%), standard deviation (SD), sulfonamide and trimethoprim (Sul/ tri).

6.4.4 Identifying the taxonomical source of antibiotic resistance genes in faecal metagenomes

The proportions of the most abundant taxonomical sources of MARG in all of the metagenomes are shown in Figure 6.7. The greatest proportion of MARG from all of the faecal metagenomes were from unclassified *Eubacteriales* (0.20 ± 0.04%), unclassified *Bacteroidales* (0.17 ± 0.05%), unclassified *Pseudomonas* (0.13 ± 0.28%), unclassified *Clostridia* (0.13 ± 0.06%), unclassified *Oscillospiraceae* (0.09 ± 0.05%), unclassified *Acinetobacter* (0.09 ± 0.14%), *E. coli* (0.08 ± 0.17%), *Bateroidales bacterium* (0.05 ± 0.03%), *Oscillospiraceae bacterium* (0.05 ± 0.3%), and unclassified *faecalibacterium* (0.05 ± 0.1%). The greatest proportion of MARG in the MAG from the DCA faecal metagenomes were from unclassified *Pseudomonas* (0.47 ± 0.43%), *E. coli* (0.30 ± 0.24), unclassified *Eubacteriales* (0.22 ± 0.090%), and unclassified *Faecalibacterium* (0.15 ± 0.15%). Unclassified *Pseudomonas* MARG represented 0.97% of MAG from the DCA 2 faecal metagenome, and 0.85% of MAG from the DCA 3 faecal metagenome. The greatest proportion of MARG in the MAG from the PPD faecal metagenomes were from unclassified *Acinetobacter* (0.26 ± 0.16%), unclassified *Bacteroidales* (0.19 ± 0.01%), unclassified *Eubacteriales* (0.17 ± 0.02%), and unclassified *Clostridia* (0.16 ± 0.02%). The greatest proportion of MARG in the MAG from the PRP faecal metagenomes were from unclassified *Eubacteriales* (0.19 ± 0.01%), unclassified *Bacteroidales* (0.19 ± 0.01%), unclassified *Clostridia* (0.16 ± 0.01%), and unclassified *Oscillospiraceae* (0.12 ± 0.008). The greatest proportion of MARG in the MAG from the PPL faecal metagenomes were from unclassified *Eubacteriales* (0.22 ± 0.01%), unclassified *Bacteroidales* (0.21 ± 0.02%), unclassified *Clostridia* (0.17 ± 0.004%), and unclassified *Oscillospiraceae* (0.153 ± 0.02%). The taxa of MARG

representing at least 0.1% of the MAG in the DCA 1 metagenome were unclassified *Collinsella* (0.362%), unclassified *Eubacteriales* (0.296%), *Bifidobacterium longum* (0.127%), unclassified *Pseudomonas* (0.127%), unclassified *Bacteroidales* (0.110%), unclassified *Firmicutes* (0.105%), and unclassified *Megamonas* (0.105%). The taxa of MARG representing at least 0.1% of the MAG in the DCA 2 metagenome were unclassified *Pseudomonas* (0.970%), *Escherichia coli* (0.432%), unclassified *Eubacteriales* (0.301%), unclassified *Hafnia* (0.222%), *Pseudomonas lundensis* (0.144%), unclassified *Hafniaceae* (0.140%), and unclassified *Enterobacterales* (0.118%). The taxa of MARG representing at least 0.1% of the MAG in the DCA 3 metagenome were unclassified *Pseudomonas* (0.838%), *Escherichia coli* (0.534%), *Yersinia enterocolitica* (0.172%), *Pseudomonas lundensis* (0.168%), unclassified *Eubacteriales* (0.160%), unclassified *Hafnia* (0.155%), and unclassified *Yersinia* (0.147%). The taxa of MARG representing at least 0.1% of the MAG in the DCA 4 metagenome were unclassified *Prevotella* (0.407%), unclassified *Faecalibacterium* (0.338%), unclassified *Bacteroidales* (0.215%), and *Faecalibacterium prausnitzii* (0.134%). The taxa of MARG representing at least 0.1% of the MAG in the DCA 5 metagenome were *E. coli* (0.450%), unclassified *Pseudomonas* (0.431%), unclassified *Faecalibacterium* (0.301%), unclassified *Eubacteriales* (0.247%), unclassified *Prevotella* (0.113%), and *Faecalibacterium prausnitzii* (0.106%). The taxa of MARG representing at least 0.1% of the MAG in the PPD 1 metagenome were unclassified *Acinetobacter* (0.402%), unclassified *Bacteroidales* (0.200%), unclassified *Eubacteriales* (0.148%), and unclassified *Clostridia* (0.145%). The taxa of MARG representing at least 0.1% of the MAG in the PPD 2 metagenome were unclassified *Bacteroidales* (0.194%), unclassified *Clostridia* (0.174%), unclassified

Eubacteriales (0.167%), unclassified *Oscillospiraceae* (0.104%). The taxa of MARG representing at least 0.1% of the MAG in the PPD 3 metagenome were unclassified *Acinetobacter* (0.402%), unclassified *Bacteroidales* (0.179%), unclassified *Eubacteriales* (0.163%), and unclassified *Clostridia* (0.136%). The taxa of MARG representing at least 0.1% of the MAG in the PPD 4 metagenome were unclassified *Acinetobacter* (0.267%), unclassified *Bacteroidales* (0.183%), unclassified *Eubacteriales* (0.167%), unclassified *Clostridia* (0.166%), and unclassified *Oscillospiraceae* (0.104%). The taxa of MARG representing at least 0.1% of the MAG in the PPD 5 metagenome were unclassified *Acinetobacter* (0.237%), unclassified *Bacteroidales* (0.207%), unclassified *Eubacteriales* (0.189%), unclassified *Clostridia* (0.156%), and unclassified *Oscillospiraceae* (0.113%). The taxa of MARG representing at least 0.1% of the MAG in the PRP 1 metagenome were unclassified *Eubacteriales* (0.202%), unclassified *Bacteroidales* (0.177%), unclassified *Clostridia* (0.163%), and unclassified *Oscillospiraceae* (0.134%). The taxa of MARG representing at least 0.1% of the MAG in the PRP 2 metagenome were unclassified *Bacteroidales* (0.206%), unclassified *Eubacteriales* (0.185%), unclassified *Clostridia* (0.158%), and unclassified *Oscillospiraceae* (0.120%). The taxa of MARG representing at least 0.1% of the MAG in the PRP 3 metagenome were unclassified *Acinetobacter* (0.211%), unclassified *Eubacteriales* (0.184%), unclassified *Bacteroidales* (0.183%), unclassified *Clostridia* (0.167%), and unclassified *Oscillospiraceae* (0.120%). The taxa of MARG representing at least 0.1% of the MAG in the PRP 4 metagenome were unclassified *Eubacteriales* (0.206%), unclassified *Bacteroidales* (0.189%), unclassified *Clostridia* (0.181%), and unclassified *Oscillospiraceae* (0.129%). The taxa of MARG representing at least 0.1% of the MAG in the PRP 5 metagenome were unclassified *Acinetobacter* (0.233%),

unclassified *Eubacteriales* (0.178%), unclassified *Bacteroidales* (0.170%), unclassified *Clostridia* (0.153%), unclassified *Gammaproteobacteria* (0.120%), and unclassified *Oscillospiraceae* (0.115%). The taxa of MARG representing at least 0.1% of the MAG in the PPL 1 metagenome were unclassified *Bacteroidales* (0.214%), unclassified *Eubacteriales* (0.207%), unclassified *Clostridia* (0.165%), and unclassified *Oscillospiraceae* (0.143%). The taxa of MARG representing at least 0.1% of the MAG in the PPL 2 metagenome were unclassified *Eubacteriales* (0.234%), unclassified *Bacteroidales* (0.228%), unclassified *Clostridia* (0.171%), and unclassified *Oscillospiraceae* (0.163%). The taxa of MARG representing at least 0.1% of the MAG in the PPL 3 metagenome were unclassified *Bacteroidales* (0.222%), unclassified *Eubacteriales* (0.210%), unclassified *Clostridia* (0.171%), and unclassified *Oscillospiraceae* (0.132%). The taxa of MARG representing at least 0.1% of the MAG in the PPL 4 metagenome were unclassified *Eubacteriales* (0.224%), unclassified *Bacteroidales* (0.193%), unclassified *Clostridia* (0.163%), and unclassified *Oscillospiraceae* (0.158%). The taxa of MARG representing at least 0.1% of the MAG in the PPL 5 metagenome were unclassified *Eubacteriales* (0.227%), unclassified *Bacteroidales* (0.179%), unclassified *Clostridia* (0.171%), unclassified *Oscillospiraceae* (0.169%), and unclassified *Bacillales* (0.132%).

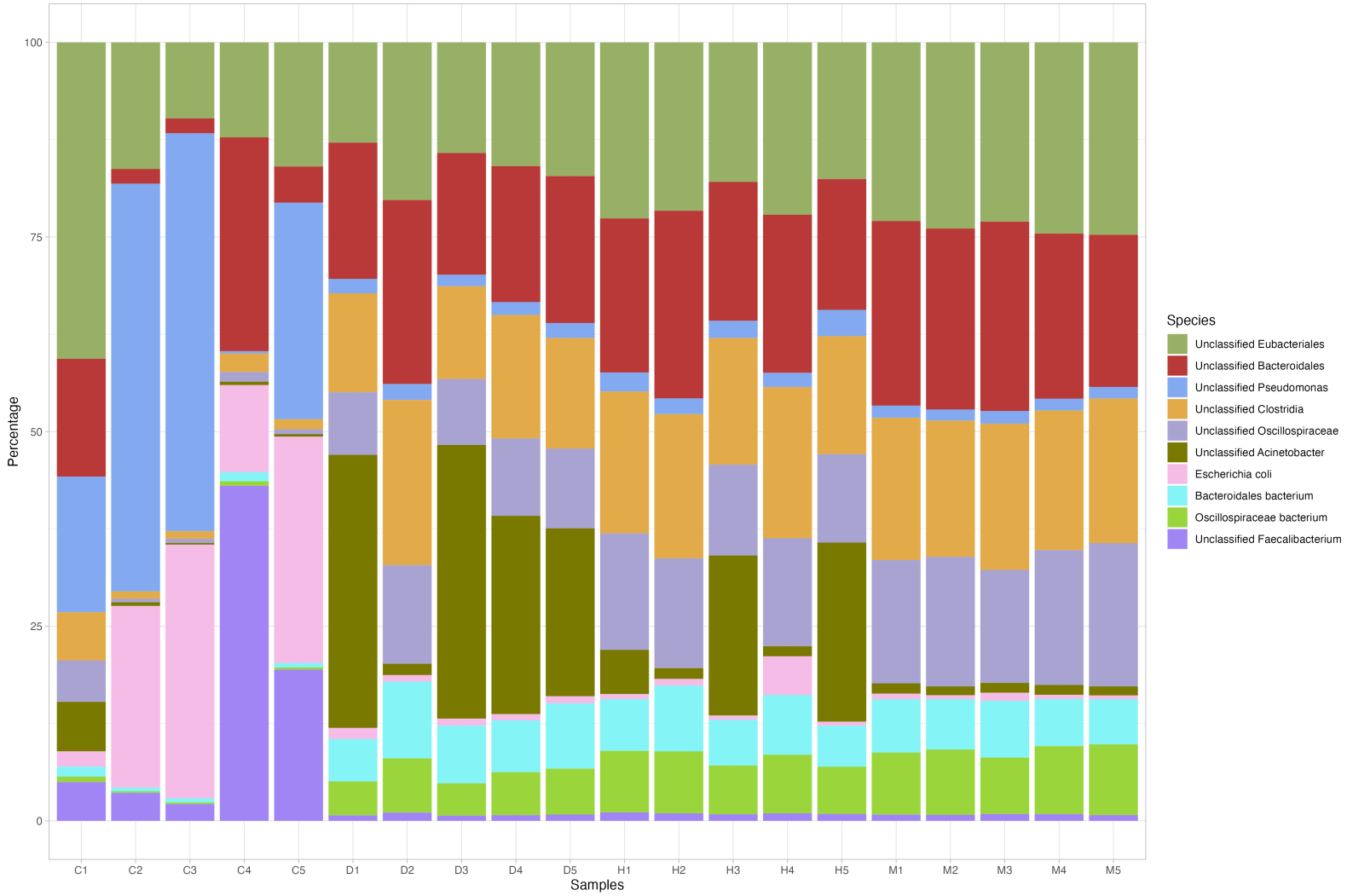


Figure 6.7: Taxonomal source of metagenome assembled genomes containing antibiotic resistance genes. The percentage of metagenome assembled genomes that contained antibiotic resistance genes from the faecal metagenomes of the dairy calves (C), the post-parturition dry cows (D), the pre-parturition cows (H), and the post-parturition lactating cows (M). The y-axis is scaled for each metagenome, this ensures that the percentages of the most abundant taxa across all of the metagenomes are presented. This figure was prepared in R with ggplot2.

6.5 Discussion

How does the faecal microflora differ between the farm groups and is there an explanation for the higher levels of resistance found in the dairy calves? Additionally, can important taxa be identified for future work investigating ARG in the faeces of dairy cows in the future?

6.5.1 Metagenome quality

The total length of the metagenomic sequence is 1167.07 megabase pairs (Mbp) or roughly 2523 *E. coli* genomes (average length = 4.6 Mbp). In metagenomics the N50 is harder to interpret as there is no single target genome. If sequencing data is perfectly distributed across contigs the assumed the N50 value is 2305, therefore the actual median N50 value is higher suggesting better than random assembly quality. The SqueezeMeta pipeline includes quality controls sequencing quality is sufficient for taxonomic profiling and ARG identification. The median mapping percentage was 86.4% linked to the high diversity of unidentifiable reads in the older farm groups that reached the limit of detection for the tools used in this dissertation. The DCA metagenomes had a lower number of species and they had a mapping percentage of 99.2%. If this analysis was repeated now, these values would likely improve as the tools and mapped genomes increase.

6.5.2 Sampling quality

Before investigating the diversity of different genera in the metagenomes, it is important to establish how representative the sampling is of the true population. Rarefaction curves are an ecological method to determine if the number of species identified is likely to reflect the true number of species in an ecological niche. Essentially, how many samples are required to capture

rare species. The curve represents the mean curve of thousands of curves drawn showing as many different attempts at counting the number of species identified for a given sample. Flat curves suggest that new species are still being discovered and the sample size is not sufficient. The shape of the rarefaction curves for all metagenomes indicates that the true species richness of each microbiome has been captured, at least within the scope of the tools available.

6.5.3 Alpha diversity

The alpha diversity was lowest in the DCA metagenomes, these metagenomes are less diverse than the mature metagenomes from the PPD, PRP, and PPL cows. This difference in diversity combined with the difference in susceptibility suggests that an immature microflora, reduces the competition allowing resistant organisms to persist. As the DCA age, and their physiology develop, the diversity of their microflora increases and the fitness cost associated with resistance mechanisms increase. However, DCA 1 was the oldest DCA, while DCA 4 was the joint youngest, therefore, it is surprising these metagenomes both cluster closer to the older cow metagenomes. This may be a reflection of the higher number of species identified in these samples, or an indicator of the increased variability in the DCA metagenomes. External factors may have increased the rate at which the DCA 4 gastro-intestinal tract was colonised.

6.5.4 Taxonomical composition of faecal metagenomes

The difference in the sample group mapping percentages is visible on the kingdom plot, specifically the greater proportion of unmapped CDS in the metagenomes from the PRP, PPD, and PPL samples. To display more of the

mapped taxonomies in the other graphs, unmapped CDS are excluded. Plotting the taxonomical proportion of CDS mapped to species clearly displays the stark difference in the composition of the DCA faecal metagenomes. Other than the differences in the proportions of several taxa in the DCA samples compared to the other samples, there was more variation within the DCA metagenomes. Comparing the faecal metagenomes of the DCA to the PRP, PPD, and PPL resembles a textbook example of early compared to late succession. The DCA faecal microbiota is immature and low in diversity; this results in less competition, allowing generalist, opportunistic organisms to bloom. In comparison, the PRP, PPD, and PPL faecal microbiota are mature and diverse, resulting in increased competition and more rigorously defined ecological niches, an environment for specialists. The significantly greater proportion of *E. coli* mapped to the CDS of the DCA 2, DCA 3, and DCA 4 metagenomes is of interest in the context of this thesis. The abundance of *E. coli* was higher when the DCA2, DC3, and DC4 faecal samples were plated on TBX. However, the abundance of *E. coli* was also high in the faces from PPD K, PPD M, PPL P, PPL R, and PPL T without the corresponding high proportion of CDS in the faecal metagenomes. If the viable counts are taken as evidence that the abundance of *E. coli* in these samples were the same, this highlights how abundant the other taxa identified in the PPD and PPL faecal metagenomes. Their abundance was so great that the proportion of the *E. coli* was significantly reduced. There are some indications that the faecal microbiota in the DCA was in a state of dysbiosis, unclassified *Firmicutes* were mapped to a greater proportion of the PRP, PPD, and PPL metagenomes than the DCA metagenomes, and *Firmicutes* has been reported as important

members of the bovine stomach, large intestine, and faecal microbiomes (Mao *et al.*, 2015; Wang *et al.*, 2022). Interestingly, *Prevotella* was mapped to a proportion of the CDS in DCA 4 8 times higher than the mean mapping proportion of the CDS across all metagenomes, *Prevotella* has been reported as important taxa of the bovine stomach microbiome, while also being identified in the large intestine, and faecal microbiomes (Holman and Gzyl, 2019). Higher proportions of *Prevotella* across the faecal metagenomes might have been expected, if this taxa had not been mapped to such a large proportion of CDS in DCA 4 it might have suggested the approach taken in this thesis has artificially reduced the abundance of this taxon.

6.5.5 Taxonomical sources of ARG in dairy cow faeces

Unclassified *Pseudomonas* MARG represented lower proportions of the MAG from the faecal metagenomes of the PRP, PPD, and PPL than the DCA. Unclassified *Acinetobacter* MARG represented a greater proportion of the MAG from the faecal metagenomes of the PPD and PRP than the DCA and the PPL. *Pseudomonadaceae* and *Coriobacteriaceae* had high proportions of β-lactam (cephalosporin), and tetracycline ARG in the DCA metagenomes. Culturing species such as *P. aeruginosa* could provide additional aspects of the dairy cow faecal resistome not present in *E. coli. P. aeruginosa* is commonly implicated in mastitis in Austria, and ESBL carrying isolates have been identified in bovine faeces in Nigeria (Falodun and Musa, 2020; Schauer *et al.*, 2021). Aminoglycoside ARG were mapped to *Enterobacteriaceae* in all DCA metagenomes and select metagenomes from PRP, PPD, and PPL. *Enterococcus faecalis* is commonly identified in the dairy farm environment, with resistance to aminoglycoside, tetracyclines, lincosamides, and phenicols

(Rózańska *et al.*, 2019). This project intended to investigate resistance in *Enterococcus*, however, the abundance of *Enterococcus* in PPL faecal samples was too low in pilot studies to include in this work.

6.6 Limitations and future work

This investigation used short-read metagenomic sequencing to collect the faecal metagenomes. While this approach has produced significant amounts of data, clearly demonstrating the more variable taxonomic compositions of the DCA faecal metagenomes, which were also significantly different from the PRP, PPD, and PPL faecal metagenomes. It provides limited insights to the most significant taxonomic source of ARG and no insights to the genetic context of ARG in the bovine faecal metagenome. If the workflow was adapted to incorporate long-read sequencing in conjunction with short-read sequencing; it is likely that the number and quality of MAGs would increase, and subsequently the genetic context of ARG in these metagenomes could be investigated more thoroughly (Brown *et al.*, 2021; Chen *et al.*, 2022; Greenman *et al.*, 2024).

The species alpha diversity metrics appear to have some discordance, this is likely due to reaching the upper limit of identifiable species in the faecal metagenomes for the PRP, PPD, and PPL metagenomes and the greater proportion of unclassified and unmapped reads. These factors mean the true diversity of these faecal metagenomes has not been captured by the current method, if this analysis is completed in the future with updated databases then this is likely to improve.

6.7 Conclusion

The DCA faecal metagenomes were more variable across the DCA samples and less diverse than the faecal metagenomes from the more developed bovines. Several taxa were abundant in the faecal metagenomes from the DCA but were rare in the faecal metagenomes of the more developed bovines and vice versa. Literature supports the finding that DCA microbiomes are less diverse but mature quickly, and suggest that a higher diversity in the microbiome (driven by changes in the diet of animals as they develop) increases competition for resistant isolates. These results alone would not be sufficient to argue the level of risk posed by resistance in dairy cow faeces. Combining the ASRs collected from the same faecal samples enrich this dataset. Only 2% of all MAG contained any ARG and *E. coli* MAG were minor proportions of most of these metagenomes. However, there were high levels of resistance to β-lactam and aminoglycoside resistance is high in *E. coli*, highlighting that even small fractions of ARG in the metagenomes can result in concerning levels of ABR identified in targeted approaches with pathogens of interest. This metagenomic approach has increased the breadth of understanding of ABR on the SB dairy farm, but could be further optomised. Future work could incorporate long-read sequencing, to identify more MAG, and increase the genetic context of ARG in a metagenome. Additional approaches could enrich total DNA samples with sequence probes or fluorescently tagged plasmids targeted to ARG of interest (Pinilla-Redondo *et al.*, 2018; Dilthey *et al.*, 2019; Guitor *et al.*, 2019; Greenman *et al.*, 2024).

Chapter 7 General discussion

7.1 Dairy cattle faeces are reservoirs of antibiotic resistant *E. coli*

This thesis has identified high levels of ABR *E. coli*, specifically to ampicillin, streptomycin, and tetracycline in the faeces of dairy cattle. Resistance to these antibiotics is not surprising as they are routinely used on the SB Farm, and across the UK dairy industry. Dairy cattle on the SB Farm and across the globe have been identified as reservoirs of antibiotic resistant bacteria that represent a risk to animal, environmental, and human health (Sawant *et al.*, 2007; Mao *et al.*, 2015; Ibrahim *et al.*, 2016; Madoshi *et al.*, 2016; Thomas *et al.*, 2017; Doster *et al.*, 2018; X. Li *et al.*, 2018; Baker *et al.*, 2022; Barlow *et al.*, 2022; Shoaib *et al.*, 2023). Several reports have linked AMU, specifically β-lactams, in dairy cattle to the prevalence of ABR (Donaldson *et al.*, 2006; Singer, Patterson and Wallace, 2008; Chambers *et al.*, 2015).

7.1.1 The effect of antibiotic supplemented TBX on antibiotic susceptibility

Antibiotics have been included in culture media to capture organisms of interest, namely those with ABR phenotypes. However, when an investigation is attempting to capture as representative a sample as possible it is important to quantify the impact additional selection pressures have on the population of isolates that can be cultured *in vivo*. ABR isolates were picked from selective media in greater proportions, but excluded from further assessment of the ASRs where possible. Therefore, any interpretation of the risk of AMR in these samples is not impacted by potential selection biases artificially increasing the proportion of ABR *E. coli.*

7.1.2 The effect of animal chemotherapeutic treatment on antibiotic susceptibility

The findings of this thesis contribute to the broader investigation regarding the potential impact of AMU on AMR in animals, particularly livestock. The isolates from PPL that had received chemotherapy had lower median ZOIs and significantly different distributions of ZOIs for several antibiotics. This suggests that there has been a selection pressure applied in the general direction of ABR, and the population susceptible and SIE isolates has been shifted down. However, the exact strength of this link is unclear from this thesis. There were no instances where the same PPL was sampled more than once. Therefore, it is unknown if regular subsequent treatments would have an additive, or possibly synergistic effect to reduce the median ZOI.

While this thesis identified reductions in the level of ABR faecal *E. coli* 10 days after PPL chemotherapeutic treatment, others have modelled that reductions in the level of ABR *E. coli* in the slurry tank requires 60 days after faeces are added (Baker *et al.*, 2022). Further investigation is needed to confirm if a reduction in the level of faecal ABR entering the slurry tank can reduce the 60 day storage period.

7.1.3 Antibiotic susceptibility of *E. coli* from different bovine developmental stages

Dairy calves have been identified as a significant source of ABR *E. coli* , and MGE containing ARG, on dairy farms across several regions (Smith, 1966; Wierup, 1975; Khachatryan *et al.*, 2004; Berge, Atwill and Sischo, 2005; X. Li *et al.*, 2018; Salerno *et al.*, 2022). This thesis contributes further evidence, with the ASRs of the isolates from DCA shown to have higher levels of resistance to several important antibiotics. The VCD samples neonates and pre-weaned calves to capture the antibiotic susceptibility of the isolates from these groups, and higher levels of ABR are reported in these younger animals (Veterinary Medicines Directorate, 2023b).

7.2 ERIC-PCR lacks reproducibility for investigations of clonality

To investigate clonality in a population of isolates, small differences are likely to provide crucial segregation. In this thesis, ERIC-PCR could not provide the same electrophoretic banding pattern for triplicate reactions with the same DNA. If the same isolate can be placed on opposite ends of a dendrogram, it is unlikely that the rest of the dendrogram is accurate (Meacham *et al.*, 2003). It has been reported that ERIC-PCR lacks the discriminatory power to differentiate similar isolates, likely due to the non-random distribution in genomes of ERIC sequences, the association with highly expressed genes (which suggests they are evolutionarily conserved), and the underlying variability of PCR techniques (Wilson and Sharp, 2006).

7.3 MDR *E. coli* on the SB Farm

A moderate level (12%) of the *E. coli* from the α samples and a high level (21.3%) of the *E. coli* from the β samples were MDR. In comparison, the VCD reported high levels of MDR *E. coli* from cattle samples in 2020 (43%), 2021 (41%), and 2022 (40%) (Veterinary Medicines Directorate, 2023b). The difference in the proportions of MDR *E. coli* suggests that this phenotype is less prevalent on the SB farm compared to the national population. This thesis investigates additional factors influencing the proportion of MDR isolates on the SB Farm. These factors include the presence of antibiotics in the isolation media, the chemotherapeutic treatment history of the α PPL, and the developmental stage groups of the β samples.

7.3.1 The effect of antibiotic supplemented TBX on MDR proportions

TBX can isolate MDR faecal *E. coli*; however, supplementing TBX with antibiotics increased the proportion of MDR isolates, with spectinomycin and tetracycline having the greatest effect. Adapting the VARSS protocol of supplementing TBX (instead of MacConkey) with cefotaxime inhibited the growth of all *E. coli* in these samples, suggesting that cefotaxime resistance is uncommon in these samples. This result aligns with the low levels of cefotaxime resistance identified in the α and β samples. Considering that this approach has been used previously to identify ESBL *E. coli* on the SB Farm, the susceptibility of the isolates collected in this thesis to cefotaxime may not be representative of all ABR on the SB Farm (Ibrahim *et al.*, 2016).

7.3.2 The effect of chemotherapeutic treatment on MDR levels

The proportion of MDR isolates was higher in the samples from PPL that had received chemotherapeutic treatment before sampling (40.8%) than from the untreated PPL samples (5.8%). The VCD does not report animal treatment history alongside the antibiotic susceptibility results. However, considering that clinical partners provide a significant proportion of the isolates taken from cases of mastitis, it is likely that recent chemotherapy affected the levels of MDR reported in the VCD.

7.3.3 MDR *E. coli* with transmissible phenotypes

Conjugation experiments successfully identified three *E. coli* with MDR phenotypes in the α samples , each conferring resistance to antibiotics that are important in the UK dairy industry. ABR phenotypes that can be shared through MGE are more concerning, with evidence of transmission of ARG between bacteria of the same and different species, as well as transmission of ARG between animals and humans (Rafaï *et al.*, 2015; Madoshi *et al.*, 2016; Salerno *et al.*, 2022; Thuan *et al.*, 2022). MDR *E. coli* were also identified in the β samples, with sequence matches for plasmids identified by ABRicate. These isolates could be screened with conjugation experiments to identify further transmissible MDR phenotype *E. coli*. Alternatively, long-read WGS could be combined with the existing short-read WGS to create hybrid assemblies (Juraschek *et al.*, 2021).

7.3.4 MDR levels from different bovine developmental stages

The developmental stage of the animals also affected the levels of MDR *E. coli*. The proportion of MDR isolates was higher from the DCA samples (58.7%) than the PRP (4%), the PPL (5.3%), and the PPD samples (17.3%). Excluding the isolates from the DCA samples reduced the level of MDR in the β samples from moderate (21.3%) to low (8.8%). The VCD reported that more ABR *E. coli* were sourced from neonates and pre-weaning calves than adult cattle. However, the VCD does not provide a breakdown of MDR proportions by sample group.

7.3.5 Investigating resistance to a larger panel of antibiotics

The VCD determines the antibiotic susceptibility of *E. coli* isolates to more antibiotics than were included in this thesis. In order to more accurately compare the MDR proportions in this thesis with the VCD, the disk diffusion panel could be expanded to include additional antibiotics. However, the disk diffusion panel used in this thesis covers the same antibiotic classes as the VCD. Therefore, it is unlikely that expanding the disk diffusion panel would shift the levels of MDR isolates significantly (e.g. isolates that are susceptible to ciprofloxacin are likely to be susceptible to all fluoroquinolones).

7.3.6 Other investigations of MDR on the SB Farm

Other investigations on the SB Farm have identified very high levels (57.9%) of MDR *E. coli* collected from slurry and faecal samples, with a moderate level (19.8%) of plasmid mediated ESBL-positive *E. coli* (Ibrahim *et al.*, 2016). A high level (37%) of MDR *E. coli* was also reported in the main slurry tank on the SB farm in 2017 (Baker *et al.*, 2022). The results in this thesis combined with these previous studies suggest that the environment, rather than the

animals, is the primary reservoir of MDR phenotypes on the SB Farm. Therefore, measures focusing on the slurry tank may be more effective in reducing the risk of MDR *E. coli* on the SB Farm. However, a delayed link between the dairy cattle faeces and the slurry tank is likely. This is because dairy cattle faeces are the predominant source of bacteria in the SB Farm slurry tank. Therefore, while animal-focused biosecurity measures might not immediately reduce the risk of ABR in the slurry, they are likely to have downstream impacts.

7.4 *In-silico* screening of genomes to understand antibiotic resistance, plasmid carriage, and population structure

7.4.1 Antibiotic resistance genes do not predict antibiotic susceptibility

The presence of an ARG did not consistently predict the observed ASRs in these *E. coli*. This is likely associated with fitness costs and compensatory mechanisms (Andersson and Hughes, 2010). While a gene may be present, its expression level can be crucial to determine its phenotypic impact (Lázár *et al.*, 2014). Therefore, more context is needed than absence or presence of an ARG alone. Previously, this context has been gathered through genome wide association studies (GWAS) in a wide array of pathogenic bacteria (Mosquera-Rendón *et al.*, 2023). The scale required for this investigation is significant, and unfortunately outside the scope of this thesis. For example, evolutionary couplings have been used in 1102 *Neisseria gonorrhoeae* isolates to identify 240 epistatic pairs affecting ABR to 5 antibiotics (Schubert *et al.*, 2019). Evolutionary couplings assumes that functionally related proteins or loci (i.e. loci involved in antibiotic resistance) evolve together due to their interdependence, identifying couples narrows the range of associations that

are assessed in GWAS study, increasing the depth of understanding of the relevant loci (Lapedes *et al.*, 1999; Weinreb *et al.*, 2016; Skwark *et al.*, 2017). While a study of this scope would facilitate predictions of ABR based on WGS data, it would not identify MGE and more complex resistance variants (such as gene insertions or deletions, or transpositions) (Schubert *et al.*, 2019).

7.4.2 Preliminary screens for plasmids could direct further investigations

Screening these *E. coli* genomes in PlasmidFinder has provided as many questions as answers. PlasmidFinder identified *IncF*-plasmid type replicons in two of the transmissible MDR α *E. coli*, and in 55.8% the β *E. coli* across all of the bovine developmental stages. Therefore, *IncF*-plasmid types have been identified in *E. coli* isolates from 23 different animals, including seven different PPL across a period of three years, this might be evidence of similar plasmids circulating in the SB Farm environment. This warrants further investigation as this plasmid type has been associated with MDR bacteria in humans (Chen *et al.*, 2024). However, multiple sequences from this plasmid type were identified, which should not be possible according to the conventional theory of plasmid incompatibility sequences. Additionally, a sequence match for an *Inc1*-plasmid type was identified in a transconjugant isolate, while this plasmid type was not identified in the donor or recipient parent strains. There are alternative *in silico* plasmid screening tools available, such as Plasmer and MOB-suite (Robertson and Nash, 2018; Zhu *et al.*, 2023). The reference databases associated with these tools were not available through ABRicate. MOB-recon (from MOB-suite) specifically could improve the quality of the plasmid screening of the genomes in this these. MOB-recon has 95% sensitivity and 88% specificity, while PlasmidFinder has 99% sensitivity but only 50%

specificity (Robertson and Nash, 2018). This thesis used short-read WGS due to its accessibility, cheaper cost, and established bioinformatic pipelines. However, a significant limitation of this technology is the difficulty in identifying and assembling MGE from short-read data. Long-read sequencing and hybrid assembly with these short-read whole genomes could identify plasmids and other MGE in these genomes while providing additional context to the identified ARG (De Maio *et al.*, 2019; Juraschek *et al.*, 2021). However, it has been reported that long-read genome assemblers can struggle with plasmids smaller than 10kb in size (Johnson, Soehnlen and Blankenship, 2023). Alternatively, isolates that had PlasmidFinder sequence matches could be selected for plasmid DNA extraction with commercially available plasmid extraction kits, and short-read sequencing could be collected on these plasmid-only extractions.

7.4.3 MLST provides a population structure and identified several globally distributed *E. coli* sequence types known to carry antibiotic resistance genes

The Achtman scheme utilises seven highly conserved housekeeping genes, allowing for a robust comparison of genetic variation (Wirth *et al.*, 2006; Jolley and Maiden, 2010; Clermont, Gordon and Denamur, 2015). In this thesis, this scheme has enabled the identification of several ST (ST10 Complex, and ST23) which have been associated with MDR phenotypes. Additionally, three isolates with transmissible MDR phenotypes have been identified in bovine faeces (ST1304, ST93, and ST325). ST1304 and ST93 *E. coli* have been associated with $bla_{CTX\text{-}M\text{-}1}$ in poultry samples (Vogt *et al.*, 2014). Additionally, ST325 *E. coli* have been isolated with resistance to β-lactams,

aminoglycosides, and tetracyclines in Australia (Barlow *et al.*, 2022). Whole genome MLST (wgMLST) schemes expand on the previous MLST schemes, providing a higher resolution for differentiating closely related strains and has been increasingly adopted in studies of *E. coli* (Tré-Hardy *et al.*, 2023). The wgMLST approach is valuable in understanding the genetic basis of ABR, as it encompasses a significantly greater number of loci than other MLST schemes (Tré-Hardy *et al.*, 2023). In this thesis it has demonstrated that the *E. coli* collected in this thesis are diverse, and collected from clusters distributed across a global population of bovine *E. coli* (Figure 1.4).

In addition to providing context to the isolates collected in this thesis, the whole genomes of the *E. coli* have been submitted to the Enterobase database, and are publicly available for other researchers to include in their work. Therefore, this snapshot of *E. coli* collected from a single farm in the East Midlands of the UK can contribute more effectively to the global effort of combatting AMR.

7.5 Investigating the bovine faecal microbiome to understand the resistome

7.5.1 The DCA faecal microbiomes were distinct and more variable

As expected, the DCA faecal metagenomes are distinct from the faecal metagenomes of the PRP, PPD, and PPL. Overall the DCA are more varied and less diverse in their taxonomical composition. The differences in physiology and diet of these groups are the likely explanations for these differences. Additionally, the DCA faecal metagenomes were more varied within their group, highlighting how minor differences at this stage of life can have significant impacts on the faecal microbiome. It is unclear how these differences occurred; colostrum, vaginal, umbilical, and amniotic fluid microbiomes have all been linked to the development of healthy dairy calves

or healthy microbiomes (Dixon, Weigle and Vasquez, 1961; Muller and Ellinger, 1981; Nardone *et al.*, 1997; McGuirk and Collins, 2004; Swan *et al.*, 2007; Chigerwe *et al.*, 2008; Klein-Jöbstl *et al.*, 2019; Zhu *et al.*, 2021). Future studies could investigate the differences across these separate microbiota while also assessing the risk of ARG, and ABR in important pathogens such as *E. coli*.

7.5.2 Identifying taxonomical sources of antibiotic resistance genes

Overall, there were very small proportions of MARG in these faecal metagenomes. Efflux genes were the most abundant, however these genes serve functions other than ABR. ARG against all of the other antibiotic classes that could be identified in these MARG represented just over 1% of all of the MARG. This highlights the importance of targeted approaches, monitoring ABR in the organism of clinical interest. While there is undoubtedly important information across the resistome, approaches which target an investigation provide higher resolution information. The approach taken in this metagenomic sequencing can likely be optimised with techniques such as long-read sequencing, or enrichment of total DNA samples with sequence probes or fluorescently tagged plasmids specific to ARG of interest (Pinilla-Redondo *et al.*, 2018; Dilthey *et al.*, 2019; Guitor *et al.*, 2019; Greenman *et al.*, 2024).

7.5.3 The effect of antibiotic treatment on the microbiome is well understood

In the context of this thesis, it is unfortunate that none of the animals from which the β samples were sourced had any antibiotic treatment in their treatment history. As a result, the effect of antibiotic treatment on the faecal microbiomes of these samples could not be determined. However, AMU in

livestock significantly alters the faecal microbiota and resistome, with effects ranging from transient to long-lasting (Dethlefsen and Relman, 2011; Looft *et al.*, 2014; Chambers *et al.*, 2015; Xiong *et al.*, 2018; Holman, Yang and Alexander, 2019). Broad-spectrum antibiotics generally reduce bacterial diversity, though recovery varies depending on the antimicrobial used, host diet, and microbiome composition prior to treatment (Dethlefsen *et al.*, 2008; Dethlefsen and Relman, 2011; Modi, Collins and Relman, 2014; Ng *et al.*, 2019; Collis *et al.*, 2024). The bovine faecal microbiome is also influenced by feed, gastrointestinal disease, and farm practices such as indoor housing (Auffret *et al.*, 2017; Thomas *et al.*, 2017; Zeineldin, Aldridge and Lowe, 2018; Hagey *et al.*, 2019). Studies consistently report reduced microbiome richness following antimicrobial treatment, with recovery dependent on factors like treatment duration, disease severity, and farm management (Chambers *et al.*, 2015; Thomas *et al.*, 2017; Doster *et al.*, 2018; Holman, Yang and Alexander, 2019; Weinroth *et al.*, 2019; F. Chen *et al.*, 2020; Collis *et al.*, 2024). The animal treatment records for the β samples were collected several months after sample collection from an electronic database, and the records for six samples could not be identified. Therefore, there may have been antibiotic treatment before sampling but unfortunately this data is lost.

7.6 Conclusion

AMR is a significant global challenge affecting multiple sectors. Representing serious risk to public health, as well as modern agriculture. Significant investment and work can be spent trying to control a problem such as AMR, but without sufficient testing it is impossible to monitor progress. The future of antimicrobial stewardship is increased testing and monitoring, however the most effective method to facilitate this monitoring is unknown. Direct culture, short-read WGS, and short-read metagenomics are all tools readily available to microbiologists. No single method is truly the gold standard for resistome investigation as all three are needed in combination. Metagenomics provides low resolution, but broad data across the microflora, short-read sequencing and direct culture increase the resolution significantly, while reducing the scope of an investigation. Combined, these approaches create a full image, a snapshot of the microbiome and resistome. The isolates collected in this thesis had concerning levels of resistance to the predominant antibiotics used in UK food producing animals. Additionally, there is evidence of animal AMU contributing to faecal ABR and transmissible MDR E. coli. This increased level of ABR appears to be transient, with the proportion of ABR *E. coli* significantly reduced 10 days after chemotherapeutic treatment stops. The DCA on the SB Farm have been identified as reservoirs of high levels of AMR, but as these levels are reduced in the PRP, PPD, and PPL samples the risk appears to be mostly contained to the younger animals. The metagenomic aspect of this thesis provides an explanation for this trend, the metagenomes of the DCA were less diverse than the PRP, PPD, and PPL. The DCA faecal microbiota is immature and low in diversity; resulting in less competition, allowing generalist,

opportunistic organisms to bloom. In comparison, the PRP, PPD, and PPL faecal microbiota are mature and diverse, resulting in increased competition and more rigorously defined ecological niches, an environment for specialists. The fitness costs associated with ARG mean that ABR bacteria are fitter and more likely to survive in the low diversity, low competition environment. Therefore, practices that increase the diversity of the DCA faecal metagenome could increase the competition and therefore lower the risk of ABR in the faecal microbiota. To build on the data presented in this thesis, future investigations should focus on the DCA, specifically the interaction between microbial succession and the risk of ABR. Farm practices which can facilitate the succession of a mature microbiome are likely to reduce the risk in the largest reservoir of ABR *E. coli* and ARG in the SB Farm dairy herd. Therefore, providing the greatest improvement in animal health of the most vulnerable animals, and reduce the need for AMU. Additionally, investigating the handling of faeces from animals that have recently received antibiotic treatment, specifically how this material is processed before entering the slurry tank, could provide simple optimisations to reduce the spread of ABR into the environment. A longitudinal study investigating these areas would provide the evidence needed to determine the impact of policy. Incorporating direct culture, targeted metagenomics, and WGS, with additional investment in long-read sequencing and a GWAS would provide even greater resolution and understanding of the mobilome, resistome, and epistatic interactions.

Appendices

Appendix 1: Multidrug efflux pump regulatory genes identified in *E. coli* in this thesis.

Gene	Pump	Reference
acrE	acrAB, acrC	(Lau and Zgurskaya, 2005)
acrF		
acrS		(Hirakawa *et al.*, 2008)
baeR	mdtABC, acrEF	(Nagakubo *et al.*, 2002)
baeS		(Baranova and Nikaido, 2002)
cpxAR	acrD and mdtABC	(Hirakawa, Nishino, Hirata, *et al.*, 2003)
CRP	MdtEF	(Nishino, Senda and Yamaguchi, 2008)
emrR	EmrCAB	(Lomovskaya, Lewis and Matin, 1995)
evgA	EmrKY	(Hirakawa, Nishino, Yamada, *et al.*, 2003)
evgS		(Nishino and Yamaguchi, 2002)
gadW	MdtEF	(Ma *et al.*, 2002)
gadX		
H-NS	AcrEF	(Nishino and Yamaguchi, 2004)
marA	AcrAB	(Cohen, Mcmurry and Levy, 1988)
marR		(Alekshun and Levy, 1997)
robA	AcrAB	(White *et al.*, 1997)
soxS	AcrAB and reduced OmpF expression	(Miller *et al.*, 1994)

Appendix 2: ARG identified in all *E. coli* from bovines submitted to the NDARO database 2019-2023.

		Region	Global	NA	CSA	AUS	Asia	Europe	Africa	None
Class	*E. coli*	Number	8164	7122	30	259	4	645	62	42
	Gene	Present	%							
Aminoglycosides	*aph(3'')-Ib*	1680	20.58	18.58	6.67	10.42	50.00	47.29	33.87	0.00
	aph(6)-Id	1648	20.19	18.20	10.00	10.04	50.00	47.29	25.81	0.00
	aadA1	638	7.81	5.17	23.33	0.00	0.00	40.16	6.45	0.00
	aph(3')-Ia	622	7.62	5.90	6.67	1.16	0.00	30.54	0.00	0.00
	aadA2	260	3.18	2.99	10.00	0.00	0.00	6.67	1.61	0.00
	aadA5	168	2.06	1.49	0.00	0.00	0.00	8.99	6.45	0.00
	aac(3)-VIa	82	1.00	1.14	0.00	0.00	0.00	0.16	0.00	0.00
	aac(3)-IId	56	0.69	0.44	3.33	0.00	50.00	3.41	0.00	0.00
	aac(3)-IIe	48	0.59	0.06	0.00	0.00	0.00	6.82	0.00	0.00
	ant(2'')-Ia	43	0.53	0.00	0.00	0.00	0.00	6.67	0.00	0.00
	aac(3)-IVa	39	0.48	0.01	3.33	0.00	0.00	5.74	0.00	0.00
	aadA25	38	0.47	0.53	0.00	0.00	0.00	0.00	0.00	0.00
	aph(4)-Ia	38	0.47	0.01	3.33	0.00	0.00	5.58	0.00	0.00
	aadA6	30	0.37	0.41	0.00	0.00	0.00	0.16	0.00	0.00
	aac(6')-Ib-cr5	20	0.24	0.01	0.00	0.00	0.00	2.95	0.00	0.00
	aph(3')-IIa	14	0.17	0.17	3.33	0.00	0.00	0.16	0.00	0.00
	aadA7	12	0.15	0.17	0.00	0.00	0.00	0.00	0.00	0.00
	aph(6)-Ic	11	0.13	0.15	0.00	0.00	0.00	0.00	0.00	0.00
	aadA22	10	0.12	0.08	0.00	0.00	0.00	0.62	0.00	0.00
	aac(6')-IIa	5	0.06	0.07	0.00	0.00	0.00	0.00	0.00	0.00
	aac(2')-IIa	3	0.04	0.04	0.00	0.00	0.00	0.00	0.00	0.00
	aadA12	3	0.04	0.04	0.00	0.00	0.00	0.00	0.00	0.00
	aadA13	3	0.04	0.03	0.00	0.00	0.00	0.16	0.00	0.00
	aac(6')-Ib-cr	2	0.02	0.03	0.00	0.00	0.00	0.00	0.00	0.00
	aadA15	2	0.02	0.00	0.00	0.00	0.00	0.31	0.00	0.00
	aadA4	2	0.02	0.01	0.00	0.00	0.00	0.16	0.00	0.00
	aac(6')-Ib4	1	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.00
	aadA9	1	0.01	0.00	0.00	0.00	0.00	0.16	0.00	0.00
β-lactams	*blaEC*	8045	98.54	98.33	100.00	100.00	100.00	100.00	100.00	100.00
	blaTEM-1	563	6.90	4.61	6.67	0.00	50.00	34.11	17.74	0.00
	blaCMY-2	516	6.32	7.16	6.67	0.00	0.00	0.62	0.00	0.00
	blaCTX-M-1	303	3.71	2.20	0.00	0.00	0.00	22.64	0.00	0.00
	ampC_C-42T	157	1.92	1.45	0.00	13.13	0.00	3.10	0.00	0.00
	blaTEM	126	1.54	1.21	33.33	0.00	0.00	4.65	0.00	0.00
	blaCTX-M-14	109	1.34	0.41	0.00	0.00	0.00	12.40	0.00	0.00
	blaCTX-M-15	100	1.22	0.86	3.33	0.00	0.00	5.89	0.00	0.00
	blaCTX-M-27	60	0.73	0.84	0.00	0.00	0.00	0.00	0.00	0.00
	blaOXA-1	59	0.72	0.06	0.00	0.00	0.00	8.53	0.00	0.00
	blaCTX-M-55	54	0.66	0.62	13.33	0.00	0.00	0.93	0.00	0.00
	blaCMY	50	0.61	0.70	0.00	0.00	0.00	0.00	0.00	0.00
	blaTEM-30	16	0.20	0.00	0.00	0.00	0.00	2.48	0.00	0.00
	blaCTX-M	15	0.18	0.15	0.00	0.00	0.00	0.62	0.00	0.00
	blaCTX-M-65	13	0.16	0.14	3.33	0.00	50.00	0.00	0.00	0.00
	blaOXA	9	0.11	0.07	0.00	0.00	0.00	0.62	0.00	0.00
	blaCTX-M-32	7	0.09	0.00	0.00	0.00	0.00	1.09	0.00	0.00
	blaOXA-2	7	0.09	0.07	0.00	0.00	0.00	0.31	0.00	0.00
	blaOXA-10	6	0.07	0.07	0.00	0.00	0.00	0.16	0.00	0.00
	ampC_T-32A	5	0.06	0.03	0.00	0.00	0.00	0.47	0.00	0.00
	blaCARB-2	5	0.06	0.07	0.00	0.00	0.00	0.00	0.00	0.00

Regions: NA (North America), CSA (Central and South America), and AUS (Australasia).

Appendix 2 continues on the next page.

Appendix 2: ARG identified in all *E. coli* from bovines submitted to the NDARO database 2019-2023 continued.

Class		Region	Global	NA	CSA	AUS	Asia	Europe	Africa	None
	E. coli	Number	8164	7122	30	259	4	645	62	42
	Gene	Present	%							
β-lac	*blaCTX-M-2*	5	0.06	0.00	0.00	0.00	0.00	0.78	0.00	0.00
	ampC	2	0.02	0.01	0.00	0.00	0.00	0.16	0.00	0.00
	blaCTX-M-8	2	0.02	0.00	6.67	0.00	0.00	0.00	0.00	0.00
	blaSHV-2A	2	0.02	0.01	3.33	0.00	0.00	0.00	0.00	0.00
	blaCTX-M-137	1	0.01	0.00	0.00	0.00	0.00	0.00	1.61	0.00
	blaTEM-135	1	0.01	0.00	0.00	0.00	0.00	0.16	0.00	0.00
Efflux and permeability	*mdtM*	7862	96.30	97.63	80.00	100.00	100.00	80.31	100.00	100.00
	acrF	6162	75.48	75.95	100.00	59.07	75.00	77.83	100.00	7.14
	emrD	544	6.66	4.91	13.33	27.80	0.00	17.98	3.23	0.00
	marR_S3N	14	0.17	0.20	0.00	0.00	0.00	0.00	0.00	0.00
	ompF_ Q88STOP	5	0.06	0.06	0.00	0.00	0.00	0.16	0.00	0.00
Pho	*glpT_E448K*	7496	91.82	92.78	86.67	100.00	100.00	77.67	90.32	100.00
	cyaA_S352T	360	4.41	4.00	10.00	0.00	0.00	10.85	3.23	0.00
	uhpT_E350Q	145	1.78	1.90	0.00	0.00	0.00	1.24	3.23	0.00
	fosA7.5	84	1.03	1.17	0.00	0.00	0.00	0.16	0.00	0.00
	fosA7	3	0.04	0.04	0.00	0.00	0.00	0.00	0.00	0.00
Mac	*mph(A)*	359	4.40	4.35	3.33	0.00	50.00	6.98	1.61	0.00
	erm(B)	4	0.05	0.00	0.00	0.00	0.00	0.62	0.00	0.00
	mph	3	0.04	0.04	0.00	0.00	0.00	0.00	0.00	0.00
Nit	*nfsA_ Q113STOP*	22	0.27	0.31	0.00	0.00	0.00	0.00	0.00	0.00
	nfsA_R133S	1	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.00
Phe	*catA1*	189	2.32	0.60	0.00	0.00	0.00	22.48	1.61	0.00
	cmlA1	49	0.60	0.27	6.67	0.00	0.00	4.34	0.00	0.00
	cmlA4	8	0.10	0.11	0.00	0.00	0.00	0.00	0.00	0.00
	catA2	7	0.09	0.07	0.00	0.00	0.00	0.16	1.61	0.00
Pol	*pmrB_Y358N*	6429	78.75	80.24	60.00	100.00	75.00	54.11	69.35	100.00
	pmrB_E123D	23	0.28	0.32	0.00	0.00	0.00	0.00	0.00	0.00
	basR_G53R	8	0.10	0.00	0.00	0.00	0.00	1.24	0.00	0.00
	mcr-9.1	5	0.06	0.04	0.00	0.00	0.00	0.31	0.00	0.00
	basR_G53W	1	0.01	0.00	0.00	0.00	0.00	0.16	0.00	0.00
Qui	*floR*	925	11.33	11.49	23.33	0.00	50.00	15.19	0.00	0.00
	gyrA_S83L	329	4.03	2.56	13.33	0.00	25.00	22.02	0.00	0.00
	gyrA_D87N	220	2.69	1.45	16.67	0.00	0.00	17.36	0.00	0.00
	parC_S80I	134	1.64	1.35	3.33	0.00	0.00	5.74	0.00	0.00
	parC_A56T	80	0.98	1.08	3.33	0.00	0.00	0.31	0.00	0.00
	gyrA_D87Y	10	0.12	0.00	0.00	0.39	0.00	1.40	0.00	0.00
Sulfonamide/ trimethoprim	*sul2*	1157	14.17	14.14	0.00	4.25	0.00	18.60	30.65	0.00
	sul1	515	6.31	6.30	0.00	0.00	0.00	8.99	12.90	0.00
	dfrA1	390	4.78	3.78	3.33	0.00	0.00	17.98	6.45	0.00
	dfrA12	207	2.54	2.67	6.67	0.00	0.00	2.17	1.61	0.00
	dfrA17	154	1.89	1.31	3.33	0.00	0.00	8.68	6.45	0.00
	dfrA14	73	0.89	0.69	6.67	0.00	50.00	2.02	11.29	0.00
	dfrA36	72	0.88	0.00	0.00	0.00	0.00	11.16	0.00	0.00
	dfrA5	39	0.48	0.32	0.00	0.00	0.00	2.48	0.00	0.00
	dfrA7	17	0.21	0.13	0.00	0.00	0.00	0.78	4.84	0.00
	sul3	15	0.18	0.10	6.67	0.00	0.00	0.78	1.61	0.00
	dfrA8	12	0.15	0.10	0.00	0.00	0.00	0.78	0.00	0.00
Tet	*tet(A)*	922	11.29	11.81	6.67	2.70	25.00	8.68	24.19	0.00
	tet(B)	631	7.73	8.14	0.00	2.32	0.00	6.67	3.23	0.00
	tet(C)	83	1.02	1.07	0.00	0.00	0.00	1.09	0.00	0.00

Regions: NA (North America), CSA (Central and South America), and AUS (Australasia).

Antibiotic classes: β-lac (β-lactams), Pho (phosphonic acids), Mac (macrolides), Nit (nitrofurans), Phe (phenicols), Pol (polymyxins), Qui (quinolones), Tet (tetracyclines).

Appendix 3: ARG identified in all *E. coli* collected from bovines submitted to the NDARO database 2019-2023 grouped by isolate sample source.

		All	Bovine		Bovine faeces		Calf faeces	
Class	*E. coli*	Number	4434		2634		1096	
	Gene		Number	Percent	Number	Percent	Number	Percent
Aminoglycosides	*aph(3'')-Ib*	1680	1071	24.15	367	13.93	242	22.08
	aph(6)-Id	1648	1040	23.46	368	13.97	240	21.90
	aadA1	638	502	11.32	104	3.95	32	2.92
	aph(3')-Ia	622	535	12.07	26	0.99	61	5.57
	aadA2	260	182	4.10	65	2.47	13	1.19
	aadA5	168	142	3.20	4	0.15	22	2.01
	aac(3)-VIa	82	63	1.42	6	0.23	13	1.19
	aac(3)-IId	56	49	1.11	4	0.15	3	0.27
	aac(3)-IIe	48	47	1.06	0	0.00	1	0.09
	ant(2'')-Ia	43	43	0.97	0	0.00	0	0.00
	aac(3)-IVa	39	39	0.88	0	0.00	0	0.00
	aadA25	38	30	0.68	2	0.08	6	0.55
	aph(4)-Ia	38	38	0.86	0	0.00	0	0.00
	aadA6	30	24	0.54	0	0.00	6	0.55
	aac(6')-Ib-cr5	20	19	0.43	0	0.00	1	0.09
	aph(3')-IIa	14	6	0.14	0	0.00	8	0.73
	aadA7	12	12	0.27	0	0.00	0	0.00
	aph(6)-Ic	11	3	0.07	0	0.00	8	0.73
	aadA22	10	9	0.20	0	0.00	1	0.09
	aac(6')-IIa	5	5	0.11	0	0.00	0	0.00
	aac(2')-IIa	3	0	0.00	0	0.00	3	0.27
	aadA12	3	1	0.02	1	0.04	1	0.09
	aadA13	3	3	0.07	0	0.00	0	0.00
	aac(6')-Ib-cr	2	2	0.05	0	0.00	0	0.00
	aadA15	2	2	0.05	0	0.00	0	0.00
	aadA4	2	2	0.05	0	0.00	0	0.00
	aac(6')-Ib4	1	0	0.00	0	0.00	1	0.09
	aadA9	1	1	0.02	0	0.00	0	0.00
β-lactams	*blaEC*	8045	4394	99.10	2569	97.53	1082	98.72
	blaTEM-1	563	472	10.65	22	0.84	69	6.30
	blaCMY-2	516	481	10.85	4	0.15	31	2.83
	blaCTX-M-1	303	301	6.79	0	0.00	2	0.18
	ampC_C-42T	157	150	3.38	0	0.00	7	0.64
	blaTEM	126	117	2.64	0	0.00	9	0.82
	blaCTX-M-14	109	106	2.39	0	0.00	3	0.27
	blaCTX-M-15	100	93	2.10	0	0.00	7	0.64
	blaCTX-M-27	60	57	1.29	1	0.04	2	0.18
	blaOXA-1	59	56	1.26	0	0.00	3	0.27
	blaCTX-M-55	54	47	1.06	4	0.15	3	0.27
	blaCMY	50	49	1.11	1	0.04	0	0.00
	blaTEM-30	16	16	0.36	0	0.00	0	0.00
	blaCTX-M	15	14	0.32	0	0.00	1	0.09
	blaCTX-M-65	13	12	0.27	0	0.00	1	0.09
	blaOXA	9	9	0.20	0	0.00	0	0.00
	blaCTX-M-32	7	7	0.16	0	0.00	0	0.00
	blaOXA-2	7	5	0.11	1	0.04	1	0.09
	blaOXA-10	6	6	0.14	0	0.00	0	0.00
	ampC_T-32A	5	5	0.11	0	0.00	0	0.00
	blaCARB-2	5	2	0.05	3	0.11	0	0.00
	blaCTX-M-2	5	5	0.11	0	0.00	0	0.00
	ampC	2	1	0.02	0	0.00	1	0.09
	blaCTX-M-8	2	2	0.05	0	0.00	0	0.00
	blaSHV-2A	2	2	0.05	0	0.00	0	0.00
	blaCTX-M-137	1	1	0.02	0	0.00	0	0.00
	blaTEM-135	1	1	0.02	0	0.00	0	0.00

Appendix 3 continues on the next page.

Appendix 3: ARG identified in all *E. coli* collected from bovines submitted to the NDARO database 2019-2023 grouped by isolate sample source continued.

		All	Bovine		Bovine faeces		Calf faeces	
Class	*E. coli*	Number	4434		2634		1096	
	Gene		Number	Percent	Number	Percent	Number	Percent
Efflux and permeability	*mdtM*	7862	4200	94.72	2588	98.25	1074	97.99
	acrF	6162	3395	76.57	1946	73.88	821	74.91
	emrD	544	442	9.97	62	2.35	40	3.65
	marR_S3N	14	9	0.20	2	0.08	3	0.27
	ompF_Q88STOP	5	2	0.05	0	0.00	3	0.27
Pho	*glpT_E448K*	7496	3949	89.06	2531	96.09	1016	92.70
	cyaA_S352T	360	271	6.11	58	2.20	31	2.83
	uhpT_E350Q	145	100	2.26	16	0.61	29	2.65
	fosA7.5	84	18	0.41	44	1.67	22	2.01
	fosA7	3	3	0.07	0	0.00	0	0.00
Mac	*mph(A)*	359	251	5.66	100	3.80	8	0.73
	erm(B)	4	4	0.09	0	0.00	0	0.00
	mph	3	2	0.05	1	0.04	0	0.00
Nit	*nfsA_Q113STOP*	22	9	0.20	13	0.49	0	0.00
	nfsA_R133S	1	0	0.00	0	0.00	1	0.09
Phes	*catA1*	189	179	4.04	1	0.04	9	0.82
	cmlA1	49	46	1.04	1	0.04	2	0.18
	cmlA4	8	8	0.18	0	0.00	0	0.00
	catA2	7	7	0.16	0	0.00	0	0.00
Pol	*pmrB_Y358N*	6429	3222	72.67	2331	88.50	876	79.93
	pmrB_E123D	23	17	0.38	2	0.08	4	0.36
	basR_G53R	8	8	0.18	0	0.00	0	0.00
	mcr-9.1	5	4	0.09	0	0.00	1	0.09
	basR_G53W	1	1	0.02	0	0.00	0	0.00
Qui	*floR*	925	443	9.99	350	13.29	132	12.04
	gyrA_S83L	329	212	4.78	91	3.45	26	2.37
	gyrA_D87N	220	144	3.25	61	2.32	15	1.37
	parC_S80I	134	62	1.40	61	2.32	11	1.00
	parC_A56T	80	65	1.47	10	0.38	5	0.46
	gyrA_D87Y	10	10	0.23	0	0.00	0	0.00
Sulfonamide/ trimethoprim	*sul2*	1157	653	14.73	300	11.39	204	18.61
	sul1	515	344	7.76	132	5.01	39	3.56
	dfrA1	390	258	5.82	118	4.48	14	1.28
	dfrA12	207	136	3.07	61	2.32	10	0.91
	dfrA17	154	128	2.89	6	0.23	20	1.82
	dfrA14	73	67	1.51	5	0.19	1	0.09
	dfrA36	72	72	1.62	0	0.00	0	0.00
	dfrA5	39	35	0.79	1	0.04	3	0.27
	dfrA7	17	17	0.38	0	0.00	0	0.00
	sul3	15	10	0.23	4	0.15	1	0.09
	dfrA8	12	11	0.25	1	0.04	0	0.00
Tet	*tet(A)*	922	482	10.87	296	11.24	144	13.14
	tet(B)	631	432	9.74	83	3.15	116	10.58
	tet(C)	83	54	1.22	16	0.61	13	1.19

Antibiotic classes: Pho (phosphonic acids), Mac (macrolides), Nit (nitrofurans), Phe (phenicols), Pol (polymyxins), Qui (quinolones), and Tet (tetracyclines).

Appendix 4: Overall antibiotic susceptibility and level descriptors for isolates from PPL 1-4.

		AMP	AMC	CTX	S10	S300	C	CIP	TET
1	S	0	39	42	1	1	42	42	1
	I	0	1	0	0	0	1	1	0
	R	43	3	1	42	42	0	0	42
	Total	43	43	43	43	43	43	43	43
	% S	0	90.7	97.7	2.3	2.3	97.7	97.7	2.3
	S level	Rare	E. high	E. high	Low	Low	E. high	E. high	Low
	% SIE	0	2.3	0	0	0	2.3	2.3	0
	SIE Level	Rare	Low	Rare	Rare	Rare	Low	Low	Rare
	% R	100	7	2.3	97.7	97.7	0	0	97.7
	R Level	E. high	Low	Low	E. high	E. high	Rare	Rare	E. high
2	S	3	31	27	1	23	31	24	29
	I	25	0	3	27	6	0	6	0
	R	3	0	1	3	2	0	1	2
	Total	31	31	31	31	31	31	31	31
	% S	9.7	100	87.1	3.2	74.2	100	77.4	93.5
	S level	Low	E. high	E. high	Low	E. high	E. high	E. high	E. high
	% SIE	80.6	0	9.7	87.1	19.4	0	19.4	0
	SIE Level	E. high	Rare	Low	E. high	Moderate	Rare	Moderate	Rare
	% R	9.7	0	3.2	9.7	6.5	0	3.2	6.5
	R Level	Low	Rare	Low	Low	Low	Rare	Low	Low
3	S	19	30	23	2	28	30	28	29
	I	8	0	7	28	2	0	1	0
	R	3	0	0	0	0	0	1	1
	Total	30	30	30	30	30	30	30	30
	% S	63.3	100	76.7	6.7	93.3	100	93.3	96.7
	S level	V. high	E. high	E. high	Low	E. high	E. high	E. high	E. high
	% SIE	26.7	0	23.3	93.3	6.7	0	3.3	0
	SIE Level	High	Rare	High	E. high	Low	Rare	Low	Rare
	% R	10	0	0	0	0	0	3.3	3.3
	R Level	Moderate	Rare	Rare	Rare	Rare	Rare	Low	Low
4	S	0	12	30	19	28	30	29	22
	I	0	8	0	9	0	0	1	1
	R	30	10	0	2	2	0	0	7
	Total	30	30	30	30	30	30	30	30
	% S	0	40	100	63.3	93.3	100	96.7	73.3
	S level	Rare	High	E. high	V. high	E. high	E. high	E. high	E. high
	% SIE	0	26.7	0	30	0	0	3.3	3.3
	SIE Level	Rare	High	Rare	High	Rare	Rare	Low	Low
	% R	100	33.3	0	6.7	6.7	0	0	23.3
	R Level	E. high	High	Rare	Low	Low	Rare	Rare	High

Appendix 5: Overall antibiotic susceptibility and level descriptors for isolates from PPL 5-8.

		AMP	AMC	CTX	S10	S300	C	CIP	TET
5	S	2	29	25	6	25	29	16	29
	I	10	0	3	18	0	0	12	0
	R	17	0	1	5	4	0	1	0
	Total	29	29	29	29	29	29	29	29
	% S	6.9	100	86.2	20.7	86.2	100	55.2	100
	S level	Low	E. high	E. high	High	E. high	E. high	V. high	E. high
	% SIE	34.5	0	10.3	62.1	0	0	41.4	0
	SIE Level	High	Rare	Moderate	V. high	Rare	Rare	High	Rare
	% R	58.6	0	3.4	17.2	13.8	0	3.4	0
	R Level	V. high	Rare	Low	Moderate	Moderate	Rare	Low	Rare
6	S	17	20	21	5	19	21	19	19
	I	1	1	0	15	1	0	1	0
	R	3	0	0	1	1	0	1	2
	Total	21	21	21	21	21	21	21	21
	% S	81	95.2	100	23.8	90.5	100	90.5	90.5
	S level	E. high	E. high	E. high	High	E. high	E. high	E. high	E. high
	% SIE	4.8	4.8	0	71.4	4.8	0	4.8	0
	SIE Level	Low	Low	Rare	E. high	Low	Rare	Low	Rare
	% R	14.3	0	0	4.8	4.8	0	4.8	9.5
	R Level	Moderate	Rare	Rare	Low	Low	Rare	Low	Low
7	S	5	21	16	3	17	26	21	25
	I	9	6	9	23	3	1	5	0
	R	13	0	2	1	7	0	1	2
	Total	27	27	27	27	27	27	27	27
	% S	18.5	77.8	59.3	11.1	63	96.3	77.8	92.6
	S level	Moderate	E. high	V. high	Moderate	V. high	E. high	E. high	E. high
	% SIE	33.3	22.2	33.3	85.2	11.1	3.7	18.5	0
	SIE Level	V. high	High	V. high	Moderate	Moderate	Low	Moderate	Rare
	% R	48.1	0	7.4	3.7	25.9	0	3.7	7.4
	R Level	High	Rare	Low	Low	High	Rare	Low	Low
8	S	0	18	16	0	11	27	26	24
	I	2	1	9	14	11	1	2	0
	R	26	9	3	14	6	0	0	4
	Total	28	28	28	28	28	28	28	28
	% S	0	64.3	57.1	0	39.3	96.4	92.9	85.7
	S level	Rare	V. high	V. high	Rare	High	E. high	E. high	E. high
	% SIE	7.1	3.6	32.1	50	39.3	3.6	7.1	0
	SIE Level	Low	Low	High	High	High	Low	Low	Rare
	% R	92.9	32.1	10.7	50	21.4	0	0	14.3
	R Level	E. high	High	Moderate	High	High	Rare	Rare	Moderate

Appendix 6: Overall antibiotic susceptibility and level descriptors for isolates from PPL 9-12.

		AMP	AMC	CTX	S10	S300	C	CIP	TET
9	S	4	10	11	2	8	24	23	24
	I	4	1	14	14	12	1	2	1
	R	17	14	0	9	5	0	0	0
	Total	25	25	25	25	25	25	25	25
	% S	16	40	44	8	32	96	92	96
	S level	Moderate	High	High	Low	High	E. high	E. high	E. high
	% SIE	16	4	56	56	48	4	8	4
	SIE Level	Moderate	Low	V. high	V. high	High	Low	Low	Low
	% R	68	56	0	36	20	0	0	0
	R Level	V. high	V. high	Rare	High	Moderate	Rare	Rare	Rare
10	S	21	26	24	3	25	26	26	26
	I	3	0	2	22	1	0	0	0
	R	2	0	0	1	0	0	0	0
	Total	26	26	26	26	26	26	26	26
	% S	80.8	100	92.3	11.5	96.2	100	100	100
	S level	E. high	E. high	E. high	Moderate	E. high	E. high	E. high	E. high
	% SIE	11.5	0	7.7	84.6	3.8	0	0	0
	SIE Level	Moderate	Rare	Low	E. high	Low	Rare	Rare	Rare
	% R	7.7	0	0	3.8	0	0	0	0
	R Level	Low	Rare	Rare	Low	Rare	Rare	Rare	Rare
11	S	11	24	20	6	17	23	21	25
	I	11	1	4	17	7	2	4	0
	R	3	0	1	2	1	0	0	0
	Total	25	25	25	25	25	25	25	25
	% S	44	96	80	24	68	92	84	100
	S level	High	E. high	E. high	High	V. high	E. high	E. high	E. high
	% SIE	44	4	16	68	28	8	16	0
	SIE Level	High	Low	Moderate	V. high	High	Low	Moderate	Rare
	% R	12	0	4	8	4	0	0	0
	R Level	Moderate	Rare	Low	Low	Low	Rare	Rare	Rare
12	S	16	24	18	13	16	20	17	24
	I	8	0	5	3	1	0	6	0
	R	0	0	1	8	7	4	1	0
	Total	24	24	24	24	24	24	24	24
	% S	66.7	100	75	54.2	66.7	83.3	70.8	100
	S level	V. high	E. high	E. high	V. high	V. high	E. high	E. high	E. high
	% SIE	33.3	0	20.8	12.5	4.2	0	25	0
	SIE Level	High	Rare	High	Moderate	Low	Rare	High	Rare
	% R	0	0	4.2	33.3	29.2	16.7	4.2	0
	R Level	Rare	Rare	Low	High	High	Moderate	Low	Rare

Appendix 7: Overall antibiotic susceptibility and level descriptors for isolates from PPL 13-16.

		AMP	AMC	CTX	S10	S300	C	CIP	TET
13	S	13	25	22	8	23	25	20	25
	I	12	0	2	17	2	0	4	0
	R	0	0	1	0	0	0	1	0
	Total	25	25	25	25	25	25	25	25
	% S	52	100	88	32	92	100	80	100
	S level	V. high	E. high	E. high	High	E. high	E. high	E. high	E. high
	% SIE	48	0	8	68	8	0	16	0
	SIE Level	High	Rare	Low	V. high	Low	Rare	Moderate	Rare
	% R	0	0	4	0	0	0	4	0
	R Level	Rare	Rare	Low	Rare	Rare	Rare	Low	Rare
14	S	17	25	19	1	18	25	25	25
	I	8	0	5	22	5	0	0	0
	R	0	0	1	2	2	0	0	0
	Total	25	25	25	25	25	25	25	25
	% S	68	100	76	4	72	100	100	100
	S level	V. high	E. high	E. high	Low	E. high	E. high	E. high	E. high
	% SIE	32	0	20	88	20	0	0	0
	SIE Level	High	Rare	Moderate	E. high	Moderate	Rare	Rare	Rare
	% R	0	0	4	8	8	0	0	0
	R Level	Rare	Rare	Low	Low	Low	Rare	Rare	Rare
15	S	13	38	21	8	17	39	37	38
	I	25	1	17	27	19	0	2	0
	R	1	0	1	4	2	0	0	1
	Total	39	39	39	39	38	39	39	39
	% S	33.3	97.4	53.8	20.5	44.7	100	94.9	97.4
	S level	High	E. high	V. high	High	High	E. high	E. high	E. high
	% SIE	64.1	2.6	43.6	69.2	50	0	5.1	0
	SIE Level	V. high	Low	High	V. high	High	Rare	Low	Rare
	% R	2.6	0	2.6	10.3	5.3	0	0	2.6
	R Level	Low	Rare	Low	Moderate	Low	Rare	Rare	Low
16	S	18	24	14	8	16	25	20	25
	I	6	1	7	17	6	0	5	0
	R	1	0	1	0	3	0	0	0
	Total	25	25	22	25	25	25	25	25
	% S	72	96	63.6	32	64	100	80	100
	S level	E. high	E. high	V. high	High	V. high	E. high	E. high	E. high
	% SIE	24	4	31.8	68	24	0	20	0
	SIE Level	High	Low	High	V. high	High	Rare	High	Rare
	% R	4	0	4.5	0	12	0	0	0
	R Level	Low	Rare	Low	Rare	Moderate	Rare	Rare	Rare

Appendix 8: Overall antibiotic susceptibility and level descriptors for isolates from PPL 17 & 18.

		AMP	AMC	CTX	S10	S300	C	CIP	TET
17	S	2	30	17	0	8	25	24	25
	I	22	0	10	22	13	0	5	0
	R	6	0	3	8	9	5	1	5
	Total	30	30	30	30	30	30	30	30
	% S	6.7	100	56.7	0	26.7	83.3	80	83.3
	S level	Low	E. high	V. high	Rare	High	E. high	E. high	E. high
	% SIE	73.3	0	33.3	73.3	43.3	0	16.7	0
	SIE Level	E. high	Rare	High	E. high	High	Rare	Moderate	Rare
	% R	20	0	10	26.7	30	16.7	3.3	16.7
	R Level	Moderate	Rare	Low	High	High	Moderate	Low	Moderate
18	S	11	25	13	1	12	25	24	23
	I	12	0	11	17	7	0	1	0
	R	2	0	1	7	6	0	0	2
	Total	25	25	25	25	25	25	25	25
	% S	44	100	52	4	48	100	96	92
	S level	High	E. high	V. high	Low	High	E. high	E. high	E. high
	% SIE	48	0	44	68	28	0	4	0
	SIE Level	High	Rare	High	V. high	High	Rare	Low	Rare
	% R	8	0	4	28	24	0	0	8
	R Level	Low	Rare	Low	High	High	Rare	Rare	Rare

Appendix 9: Antibiotic susceptibility of all α isolates (329) from untreated PPL.

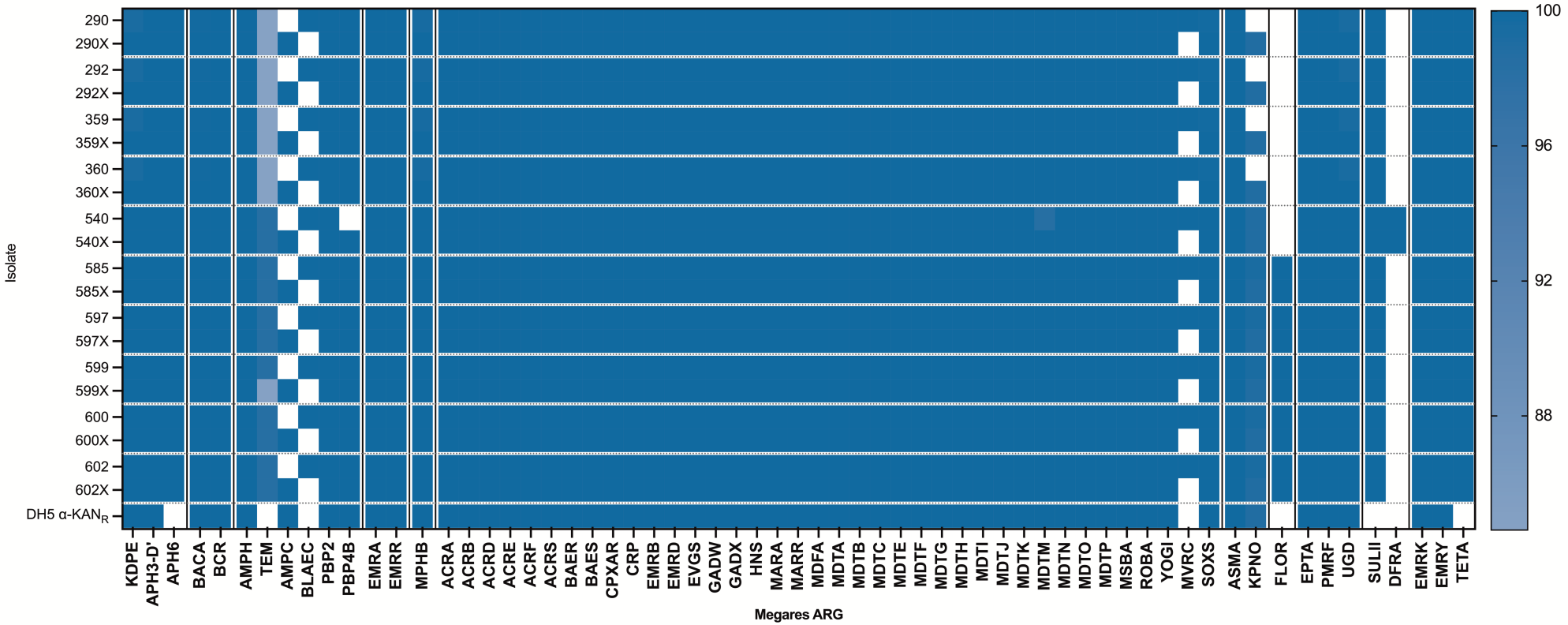
	AMP	AMC	CTX	S10	S300	C	CIP	TE
Susceptible	137	300	233	55	205	316	290	311
SIE	125	6	81	217	82	4	34	1
Resistant	67	23	15	57	42	9	5	17
% Susceptible	41.6	91.2	70.8	16.7	62.3	96.0	88.1	94.5
Susceptible level	High	Extremely high	Extremely high	Moderate	Extremely high	Extremely high	Extremely high	Extremely high
% SIE	38.0	1.8	24.6	66.0	24.9	1.2	10.3	0.3
SIE level	High	Low	High	Very high	High	Low	Moderate	Very low
% Resistant	20.4	7.0	4.6	17.3	12.8	2.7	1.5	5.2
Resistant level	High	Low	Low	Moderate	Moderate	Low	Low	Low

SIE = susceptible increased exposure, AMP = ampicillin, AMC = amoxicillin-clavulanate, CTX = cefotaxime, S10 = streptomycin, K = kanamycin, S300 = sulfonamide, C = chloramphenicol, CIP = ciprofloxacin, TE = tetracycline. The level descriptors follow VARSS and EFSA standards defined as rare (<0.1%), very low (0.1-1%), low (1-10%), moderate (10-20%), high (20-50%), very high (50-70%), and extremely high (>70%) (European Food Safety Authority, 2022; Veterinary Medicines Directorate, 2023b).

Appendix 10: Antibiotic susceptibility of all α isolates (179) from treated PPL.

	AMP	AMC	CTX	S10	S300	C	CIP	TE
Susceptible	37	167	153	19	84	176	153	105
SIE	39	7	19	80	10	3	23	0
Resistant	103	5	7	80	85	0	3	74
% Susceptible	20.7	93.3	85.5	10.6	46.9	98.3	85.5	58.7
Susceptible level	High	Extremely high	Extremely high	Moderate	High	Extremely high	Extremely high	Very high
% SIE	21.8	3.9	10.6	44.7	5.6	1.7	12.8	0.0
SIE level	High	Low	Moderate	High	Low	Low	Moderate	Rare
% Resistant	57.5	2.8	3.9	44.7	47.5	0.0	1.7	41.3
Resistant level	Very high	Low	Low	High	High	Rare	Low	High

SIE = susceptible increased exposure, AMP = ampicillin, AMC = amoxicillin-clavulanate, CTX = cefotaxime, S10 = streptomycin, K = kanamycin, S300 = sulfonamide, C = chloramphenicol, CIP = ciprofloxacin, TE = tetracycline. The level descriptors follow VARSS and EFSA standards defined as rare (<0.1%), very low (0.1-1%), low (1-10%), moderate (10-20%), high (20-50%), very high (50-70%), and extremely high (>70%) (European Food Safety Authority, 2022; Veterinary Medicines Directorate, 2023b).

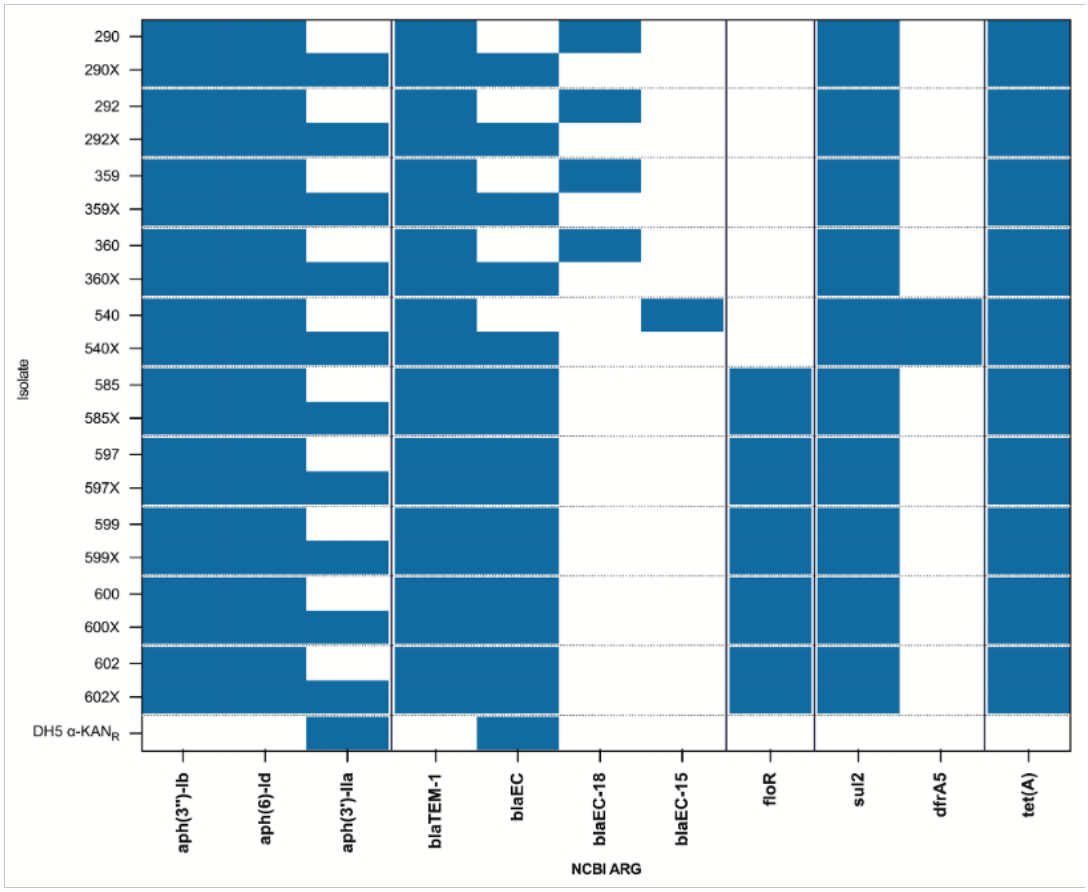


Appendix 11: Absence/presence heatmap of all of the genes in the MEGARes ARG database identified in the genomes of the MDR conjugation panel. The isolates in the conjugation panel are listed on the y-axis, and the black dotted horizontal lines separate the results for each MDR donor isolate and transconjugant pair from the others. ARG in the MEGARes database identified in these isolates are listed on the x-axis, and the black vertical bars separate ARG by antibiotic class. The box colour intensity indicates sequence percent identity (88-100%) between the isolate genome and the database reference sequence, and a white box indicates absence of this gene. The antibiotic resistance genes, associated antibiotic class, resistance mechanism, and abundance are listed in Appendix 12.

Appendix 12: MEGARes database antibiotic resistance genes identified in the MDR conjuagtion panel, associated antibiotic class, resistance mechanism, and abundance.

Antibiotic class	Resistance mechanism	Gene	Presence (%)
Aminoglycoside	Efflux	KDPE	100
	Inactivation	APH3-D'	100
		APH6	95.2
Bacitracin	Target alteration	BACA	100
	Efflux	BCR	100
β-lactam	Inactivation	AMPH	100
		TEM	95.2
		AMPC	52.4
		BLAEC	47.6
	Penicillin binding protein	PBP2	100
		PBP4B	95.2
Fluoroquinolone	Efflux	EMRA, EMRR	100
Macrolide	Inactivation	MPHB	100
Multidrug	Efflux	ACRA, ACRB, ACRD, ACRE, ACRF, ACRS	100
		BAER, BAES	100
		CPXAR	100
		CRP	100
		EMRB, EMRD	100
		EVGS	100
		GADW, GADX	100
		HNS	100
		MARA, MARR	100
		MDFA	100
		MDTA, MDTB, MDTC, MDTE, MDTF, MDTG, MDTH, MDTJ, MDTK, MDTM, MDTN, MDTO, MDTP	100
		MSBA	100
		ROBA	100
		YOGI	100
	Transporter	MVRC	47.6
	Efflux and reduced cell permeability	SOXS	100
Porin	Suppressor	ASMA	100
		KPNO	81
Phenicol	Efflux	FLOR	47.6
Polymyxin	Target modification	EPTA	100
		PMRF	100
		UGD	100
Sulfonamide /trimethoprim	Target replacement	SULII	95.2
		DFRA	9.5
Tetracycline	Efflux	EMRK, EMRY	100
		TETA	95.2

Presence (%) is the percentage of the MDR conjugation panel where a sequence match was identified.

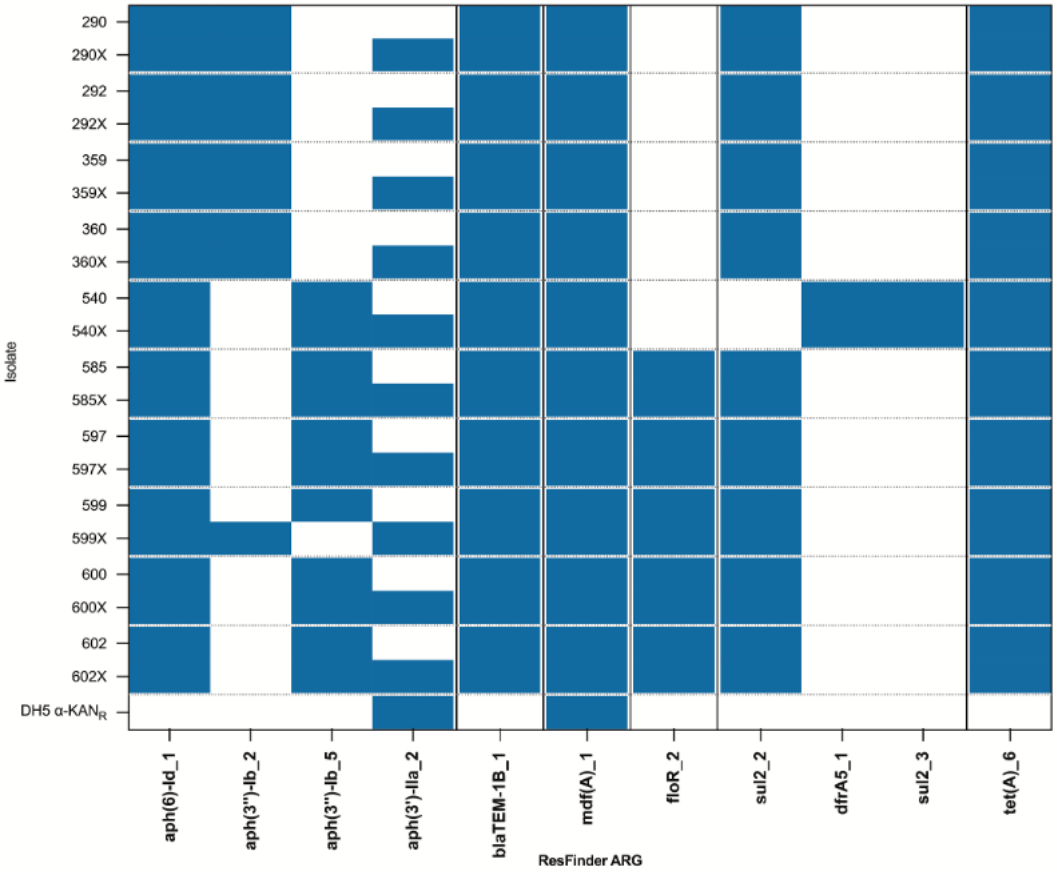


Appendix 13: Absence/presence heatmap of all of the genes in the NCBI ARG database identified in the genomes of the MDR conjugation panel. The isolates in the conjugation panel are listed on the y-axis, and the black dotted horizontal lines separate the results for each MDR donor isolate and transconjugant pair from the others. ARG in the NCBI database identified in these isolates are listed on the x-axis, and the black vertical bars separate ARG by antibiotic class. The box colour intensity indicates sequence percent identity between the isolate genome and the database reference sequence, and a white box indicates absence of this gene. The antibiotic resistance genes, associated antibiotic class, resistance mechanism, and abundance are listed in Appendix 14.

Appendix 14: NCBI ARG database antibiotic resistance genes identified in the MDR conjuagtion panel, associated antibiotic class, resistance mechanism, and abundance.

Antibiotic class	Resistance Mechanism	Gene	Presence (%)
Aminoglycoside	Inactivation	aph(3")-Ib	95.2
		aph(6)-Id	95.2
		aph(3')-IIa	52.4
β-lactam	Inactivation	blaTEM-1	95.2
		blaEC	76.2
		blaEC-18	19
		blaEC-15	4.8
Phenicol	Efflux	floR	47.6
Sulfonamide/trimethoprim	Target replacement	sul2	95.2
		dfrA5	9.5
Tetracycline	Efflux	tet(A)	95.2

Presence (%) is the percentage of the MDR conjugation panel where a sequence match was identified.

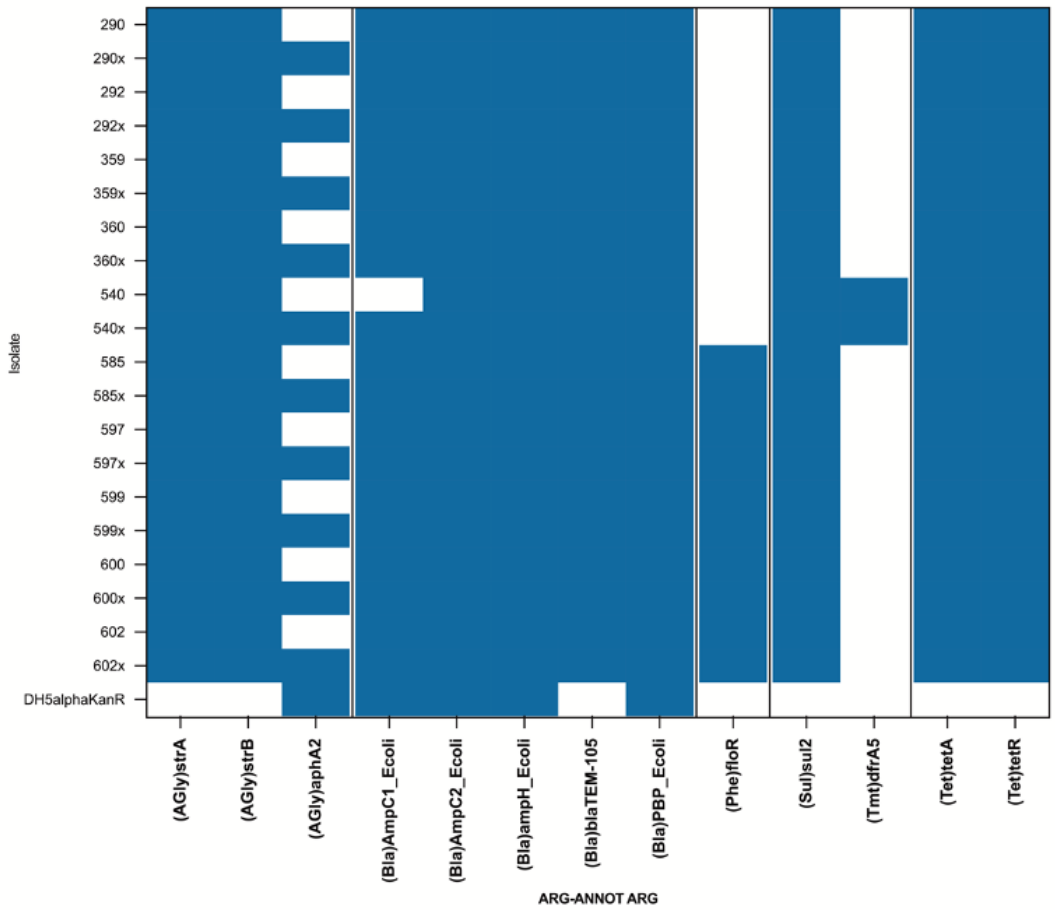


Appendix 15: Absence/presence heatmap of all of the genes in the ResFinder ARG database identified in the genomes of the MDR conjugation panel. The isolates in the conjugation panel are listed on the y-axis and the black dotted horizontal lines separate the results for each MDR donor isolate and transconjugant pair from the others. ARG in the ResFinder database identified in these isolates are listed on the x-axis, and the black vertical bars separate ARG by antibiotic class. The box colour intensity indicates sequence percent identity between the isolate genome and the database reference sequence, and a white box indicates absence of this gene. The antibiotic resistance genes, associated antibiotic class, resistance mechanism, and abundance are listed in Appendix 16.

Appendix 16: ResFinder ARG database antibiotic resistance genes identified in the MDR conjuagtion panel, associated antibiotic class, resistance mechanism, and abundance.

Antibiotic class	Resistance Mechanism	Gene	Presence (%)
Aminoglycoside	Inactivation	aph(6)-Id_1	95.2
		aph(3'')-Ib_5	52.4
		aph(3')-IIa_2	52.4
		aph(3'')-Ib_2	42.9
β-lactam	Inactivation	blaTEM-1B_1	95.2
Multidrug	Efflux	mdf(A)_1	100
Phenicol	Efflux	floR_2	47.6
Sulfonamide/ trimethoprim	Target replacement	sul2_2	85.7
		dfrA5_1	9.5
		sul2_3	9.5
Tetracycline	Efflux	tet(A)_6	95.2

Presence (%) is the percentage of the MDR conjugation panel where a sequence match was identified.

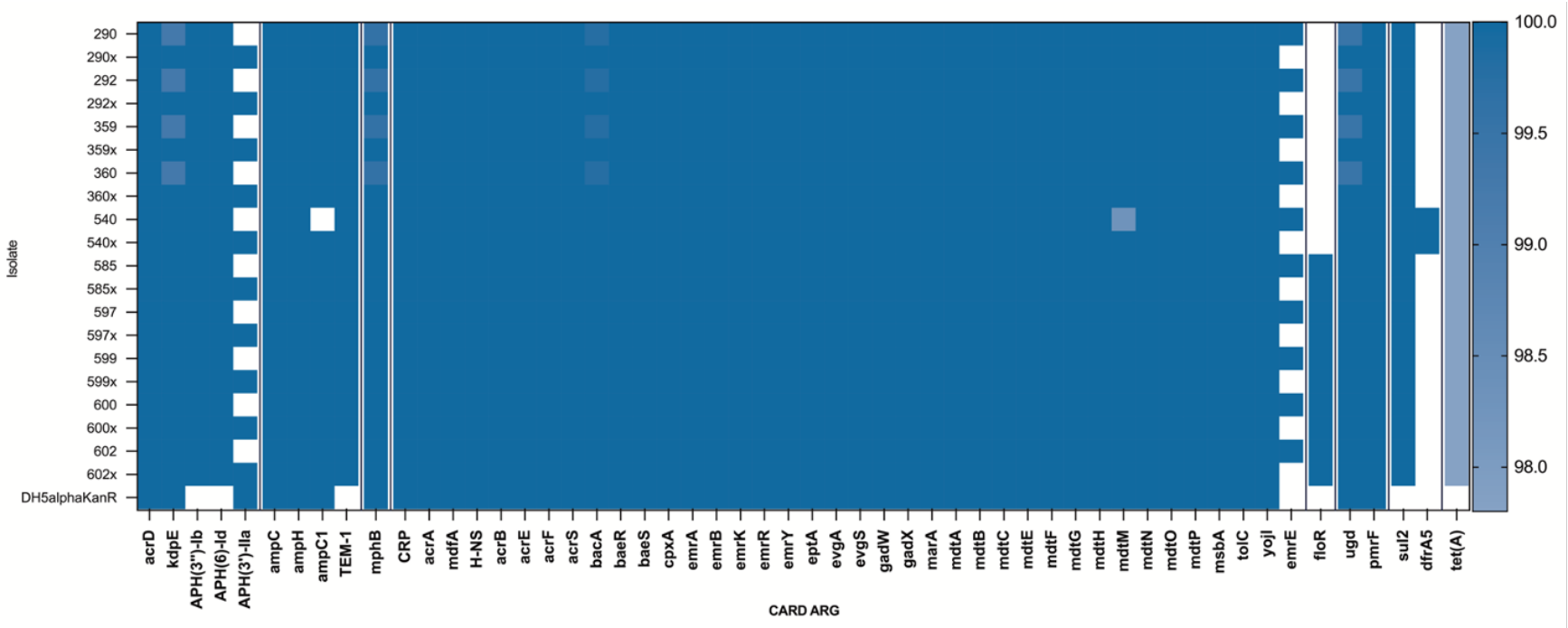


Appendix 17: Absence/presence heatmap of all of the genes in the ARG-ANNOT ARG database identified in the genomes of the MDR conjugation panel. The isolates in the conjugation panel are listed on the y-axis and the black dotted horizontal lines separate the results for each MDR donor isolate and transconjugant pair from the others. ARG in the ARG-ANNOT database identified in these isolates are listed on the x-axis, and the black vertical bars separate ARG by antibiotic class. The box colour intensity indicates sequence percent identity between the isolate genome and the database reference sequence, and a white box indicates absence of this gene. The antibiotic resistance genes, associated antibiotic class, resistance mechanism, and abundance are listed in Appendix 18.

Appendix 18: ARG-ANNOT ARG database antibiotic resistance genes identified in the MDR conjuagtion panel, associated antibiotic class, resistance mechanism, and abundance.

Antibiotic class	Resistance Mechanism	Gene	Presence (%)
Aminoglycoside	Inactivation	(AGly)strA	95.2
		(AGly)strB	95.2
		(AGly)aphA2	52.4
β-lactam	Inactivation	(Bla)AmpC1_Ecoli	95.2
		(Bla)AmpC2_Ecoli	100
		(Bla)ampH_Ecoli	100
		(Bla)blaTEM-105	95.2
	Target replacement	(Bla)PBP_Ecoli	100
Phenicol	Efflux	(Phe)floR	47.6
Sulfonamide/ trimethoprim	Target replacement	(Sul)sul2	95.2
		(Tmt)dfrA5	9.5
Tetracycline	Efflux	(Tet)tetA	95.2
		(Tet)tetR	95.2

Presence (%) is the percentage of the MDR conjugation panel where a sequence match was identified.

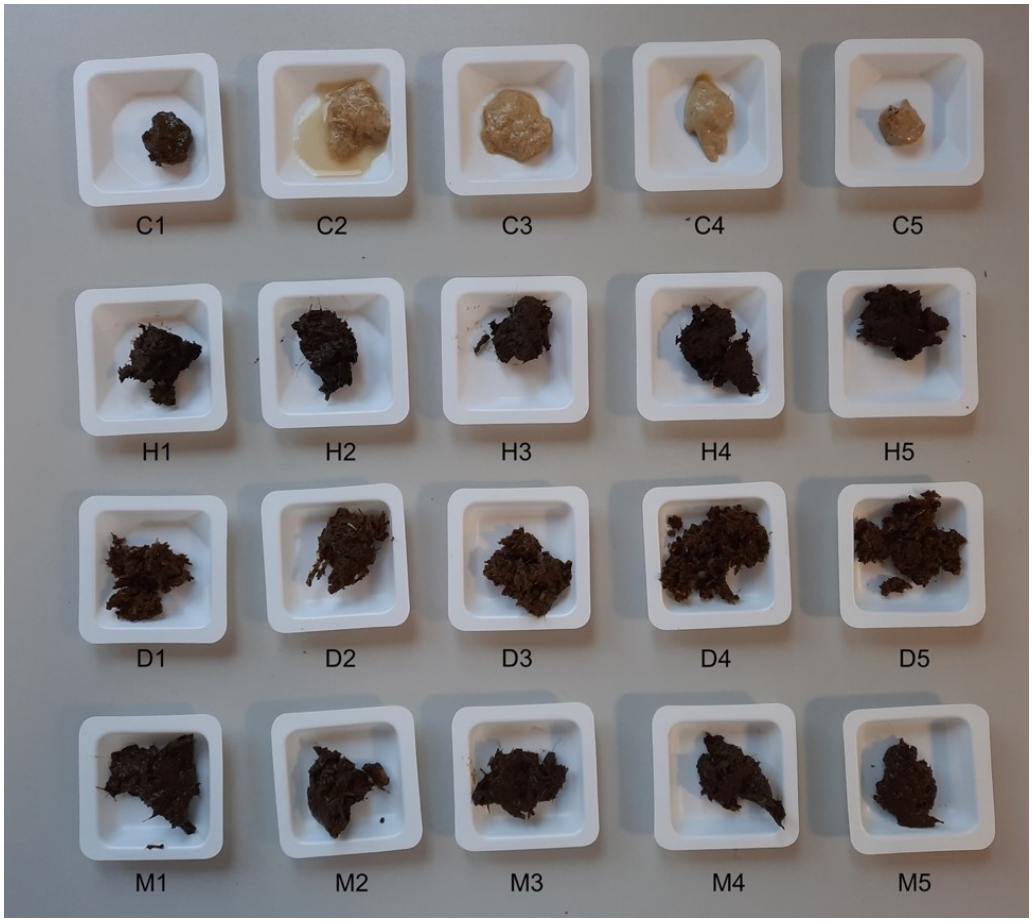


Appendix 19: Absence/presence heatmap of all of the genes in the CARD ARG database identified in the genomes of the MDR conjugation panel. The isolates in the conjugation panel are listed on the y-axis and the black dotted horizontal lines separate the results for each MDR donor isolate and transconjugant pair from the others. ARG in the CARD database identified in these isolates are listed on the x-axis, and the black vertical bars separate ARG by antibiotic class. The box colour intensity indicates sequence percent identity (98-100%) between the isolate genome and the database reference sequence, and a white box indicates absence of this gene. The antibiotic resistance genes, associated antibiotic class, resistance mechanism, and abundance are listed in Appendix 20.

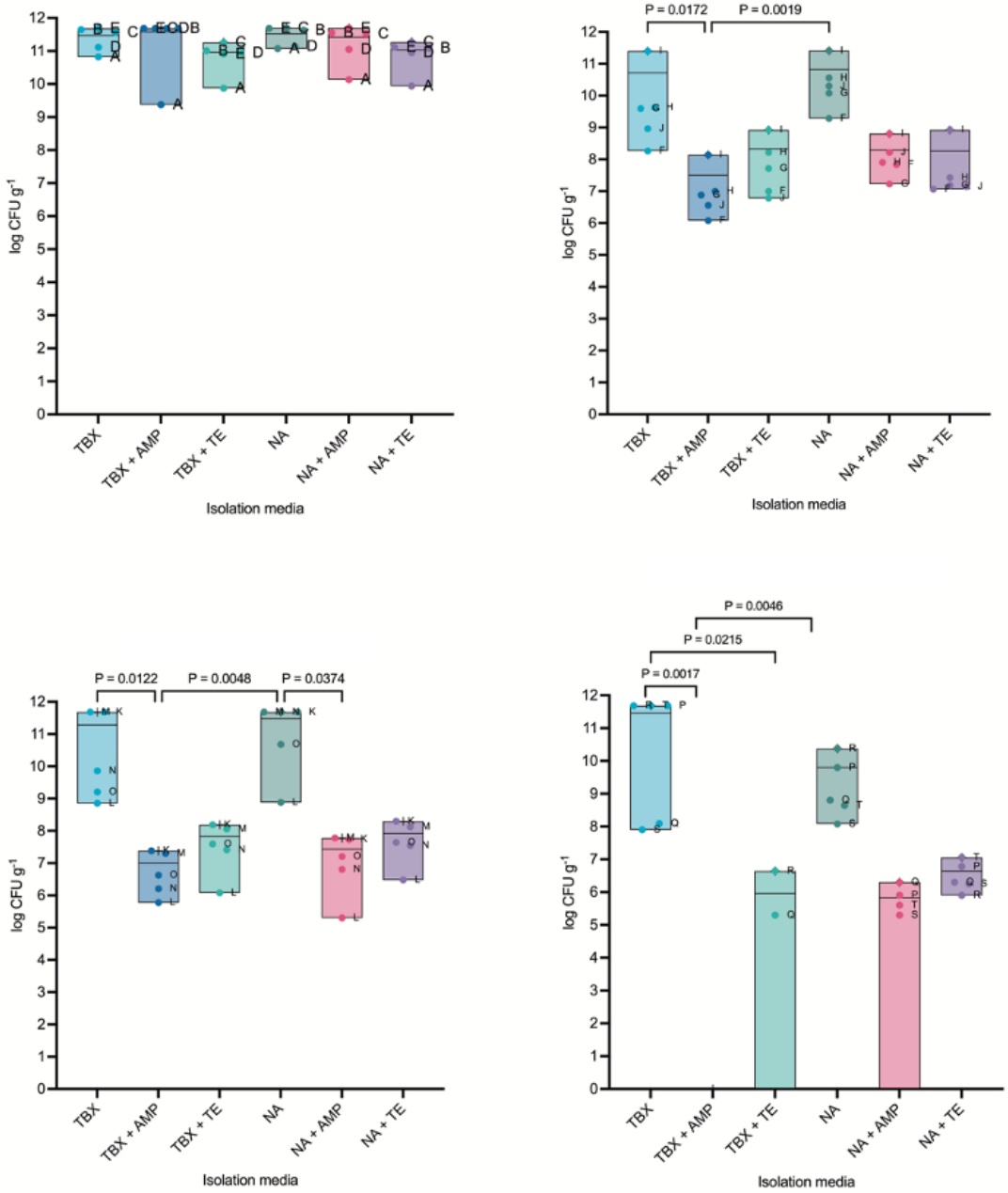
Appendix 20: CARD ARG database antibiotic resistance genes identified in the MDR conjugation panel, associated antibiotic class, resistance mechanism, and abundance.

Antibiotic class	Resistance mechanism	Gene	Presence (%)
Aminoglycoside	Efflux	acrD	100
		kdpE	100
	Inactivation	APH(3")-Ib	95.2
		APH(6)-Id	95.2
		APH(3')-IIa	52.4
β-lactam	Inactivation	ampC	100
		ampH	100
		ampC1	95.2
		TEM-1	95.2
Macrolide	Inactivation	mphB	100
Multidrug	Efflux	CRP	100
		acrA	100
		mdfA	100
		H-NS	100
		acrB, acrE, acrF, acrS	100
		bacA	100
		baeR, baeS	100
		cpxA	100
		ermA, ermB, ermK, ermR, ermY	100
		eptA	100
		evgA, evgS	100
		gadW, gadX	100
		marA	100
		mdtA, mdtB, mdtC, mdtE, mdtG, mdtH, mdtM, mdtN, mdtO, mdtP	100
		msbA	100
		tolC	100
		yojI	100
		emrE	47.6
Phenicol	Efflux	floR	47.6
Polymyxin	Target alteration	ugd	100
		pmrF	100
Sulfonamide/trimethoprim	Target replacement	sul2	95.2
		dfrA5	9.5
Tetracycline	Efflux	tet(A)	95.2

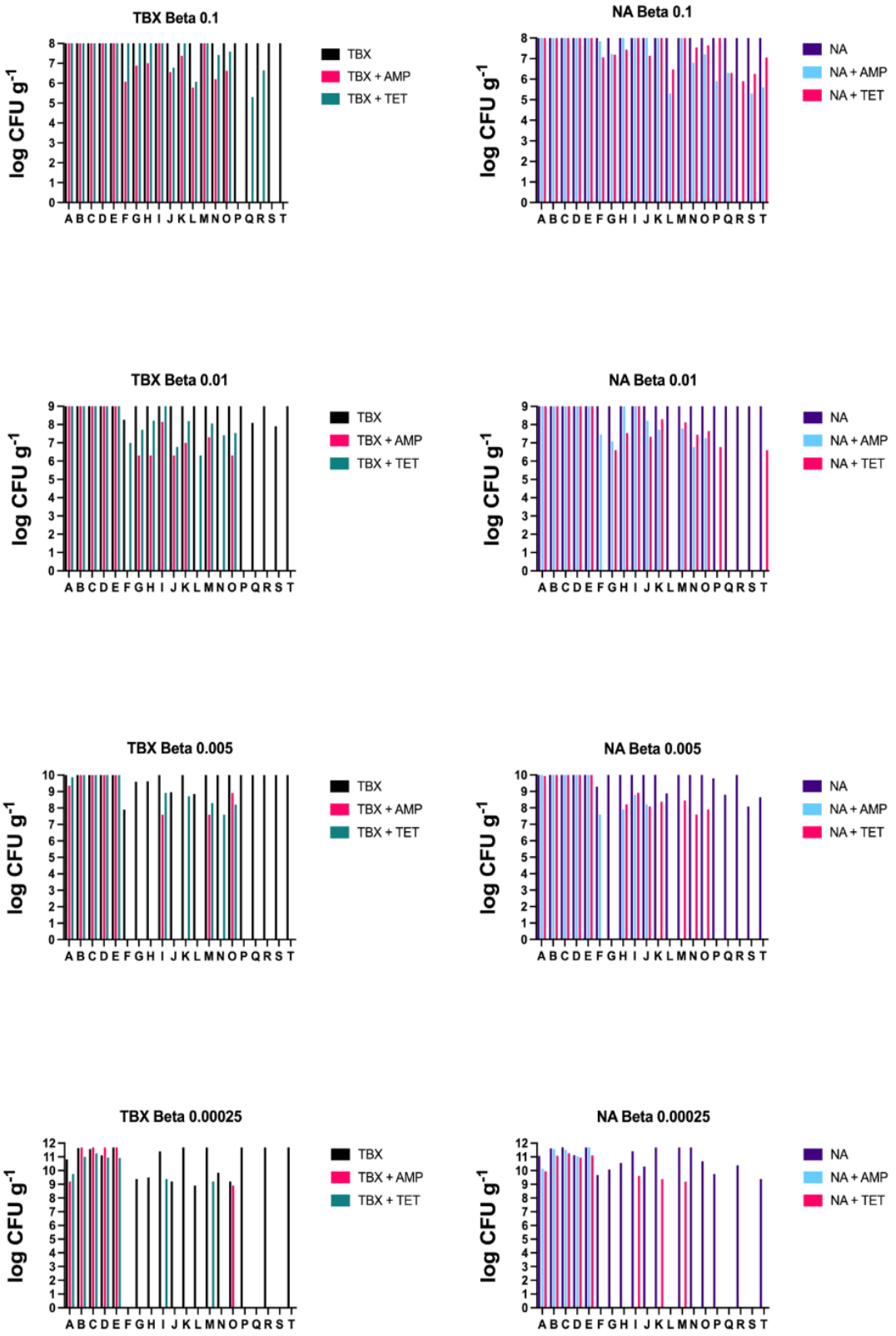
Presence (%) is the percentage of the MDR conjugation panel where a sequence match was identified.



Appendix 21: Variability in the composition of faeces across developmental groups. C1-C5 (DCA), H1-H5 (PRP), D1-D5 (PPD), and M1-M5 (PPL). Image courtesy of Ryan Cook.



Appendix 22: *β* sample bacterial and *E. coli* abundance across sample groups on different media compositions. The light blue box shows the range of counts on tryptone bile X-glucuronide (TBX), the dark blue box shows the range of counts on TBX supplemented with ampicillin (TBX + AMP), the light green box shows the range of counts on TBX supplemented with tetracycline (TBX + TET), the dark green box shows the range of counts on nutrient agar (NA), the pink box shows the range of counts on NA supplemented with ampicillin (NA + AMP), and the purple box shows the range of counts on nutrient agar supplemented with tetracycline (NA + TET). The counts from TBX include colonies with a green/blue morphology only, while the counts from NA include all colony morphologies. The number of colony forming units per gram of faecal samples (CFU g^{-1}) is represented with a logarithmic scale on the y-axis. The midline of the boxes shows the mean colony forming units per gram of faeces (CFU g^{-1}) on that media, and the dots show the CFU g^{-1} of each sample on that media. The top left graph is the counts from the dairy calf samples. The top right graph is the counts from the pre-parturition cow samples. The bottom left graph is the counts from the post-parturition dry cow samples. The bottom right graph is the counts from the post-parturition lactating cow samples. The Kruskal-Wallis ANOVA with Dunn's multiple comparisons 26test was used to determine if significant differences exist between the ranks of the grouped means.



Appendix 23: Bacterial abundance (log CFU g^{-1}) from counts across all serial dilutions. The least diluted CFU for each sample/media was selected for thesis graphs.

Appendix 24: MLST sequence type and sequence complex of β sample DCA isolates determined by Achtman seven gene MLST scheme.

Isolate	Sequence type	Complex	Isolate	Sequence type	Complex	Isolate	Sequence type	Complex	Isolate	Sequence type	Complex
A1	38	ST38	C1	10	ST10	D1	88	ST23	E1	38	ST38
A2	10	ST10	C2	38	ST38	D2	14965	ST10	E2	38	ST38
A3	38	ST38	C3	88	ST23	D3	14965	ST10	E3	88	ST23
A4	10	ST10	C4	38	ST38	D4	5995	ST165	E4	38	ST38
A5	7724	ST155	C5	38	ST38	D5	14965	ST10	E5	38	ST38
A6	696	Unknown	C6	88	ST23	D6	88	ST23	E6	696	Unknown
A7	69	ST69	C7	14965	ST10	D7	14965	ST10	E7	696	Unknown
A8	14965	ST10	C8	14965	ST10	D8	14965	ST10	E8	88	ST23
A9	88	ST23	C9	14965	ST10	D9	88	ST23	E9	88	ST23
A10	696	Unknown	C10	14965	ST10	D10	88	ST23	E10	88	ST23
A11	405	ST405	C11	14965	ST10	D11	14965	ST10	E11	14965	ST10
A12	405	ST405	C12	14965	ST10	D12	14965	ST10	E12	14965	ST10
A13	405	ST405	C13	14965	ST10	D13	14965	ST10	E13	14965	ST10
A14	405	ST405	C14	14965	ST10	D14	14965	ST10	E14	10	ST10
A15	405	ST405	C15	14965	ST10	D15	14965	ST10	E15	14965	ST10
Unique	8	6		4	3		3	3		5	3
Unknown	0	2		0	0		0	0		0	2

Appendix 25: MLST sequence type and sequence complex of β sample PRP isolates determined by Achtman seven gene MLST scheme.

Isolate	Sequence type	Complex	Isolate	Sequence type	Complex
H1	129	Unknown	J1	10	ST10
H2	4279	Unknown	J2	1204	Unknown
H3	129	Unknown	J3	10	ST10
H4	129	Unknown	J4	345	ST23
H5	129	Unknown	J5	345	ST23
H6	88	ST23	J6	155	ST155
H7	88	ST23	J7	88	ST23
H8	155	ST155	J8	5177	ST155
H9	88	ST23	J9	155	ST155
H10	88	ST23	J10	155	ST155
H11	405	ST405	J11	405	ST405
H12	405	ST405	J12	405	ST405
H13	405	ST405	J13	405	ST405
H14	405	ST405	J14	405	ST405
H15	405	ST405	J15	405	ST405
Unique	5	3		8	4
Unknown	0	5		0	1

Appendix 26: MLST sequence type and sequence complex of β sample PPD isolates determined by Achtman seven gene MLST scheme.

Isolate	Sequence type	Complex	Isolate	Sequence type	Complex
K1	4054	ST155	M1	405	ST405
K2	4093	ST206	M2	405	ST405
K3	129	Unknown	M3	405	ST405
K4	4279	Unknown	M4	405	ST405
K5	4684	ST446	M5	405	ST405
K6	88	ST23	M6	405	ST405
K7	14965	ST10	M7	405	ST405
K8	155	ST155	M8	405	ST405
K9	155	ST155	M9	10	ST10
K10	Unknown	Unknown	M10	155	ST155
K11	405	ST405	M11	155	ST155
K12	405	ST405	M12	155	ST155
K13	405	ST405	M13	155	ST155
K14	405	ST405	M14	88	ST23
K15	405	ST405	M15	155	ST155
Unique	9	5		4	4
Unknown	1	3		0	0

Appendix 27: MLST sequence type and sequence complex of β sample PPL isolates determined by Achtman seven gene MLST scheme.

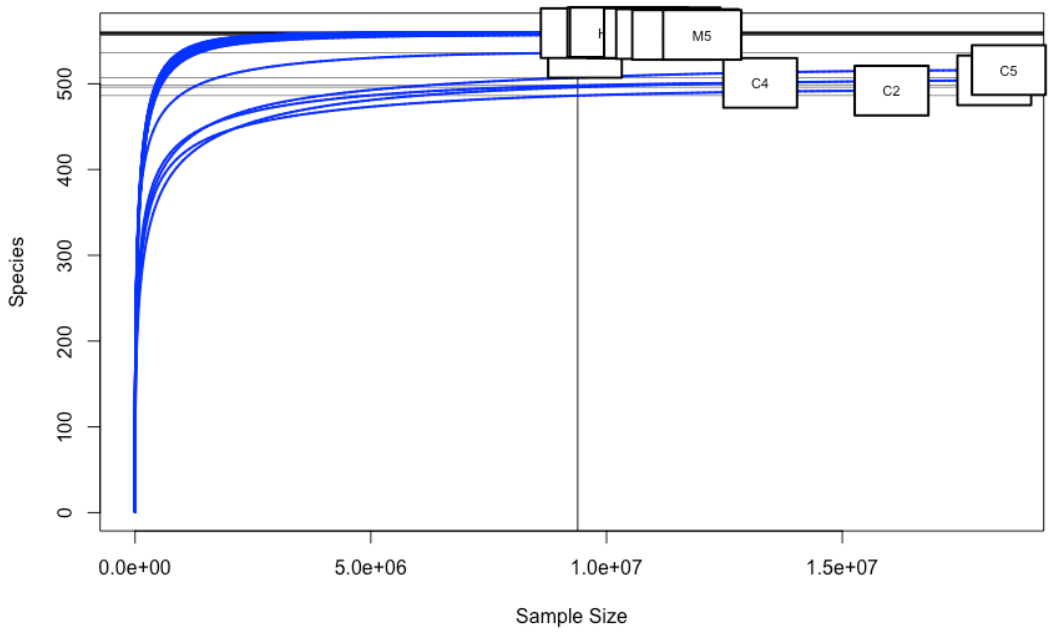
Isolate	Sequence type	Complex	Isolate	Sequence type	Complex
P1	1204	Unknown	T1	1079	Unknown
P2	1204	Unknown	T2	345	ST23
P3	1204	Unknown	T3	1079	Unknown
P4	56	ST155	T4	1079	Unknown
P5	56	ST155	T5	706	Unknown
P6	56	ST155	T6	345	ST23
P7	345	ST23	T7	706	Unknown
P8	1204	Unknown	T8	345	ST23
P9	56	ST155	T9	345	ST23
P10	56	ST155	T10	706	Unknown
P11	345	ST23	T11	706	Unknown
P12	1204	Unknown	T12	706	Unknown
P13	345	ST23	T13	3574	Unknown
P14	56	ST155	T14	10	ST10
P15	1204	Unknown	T15	10	ST10
Unique	3	3		4	2
Unknown	0	6		0	9

Appendix 28: The number of reads across samples from different bovine developmental groups.

Actual read count per metagenome					Group	Mean	SD±
A	B	C	D	E	DCA	69434331	9895158.97
55773367	74105684	62977430	80491074	73824100			
F	G	H	I	J	PRP	54080458.4	2695392.54
55241938	55361568	56933839	50035180	52829767			
K	L	M	N	O	PPD	56020610.8	3848175.52
59044357	60618188	55989168	51952788	52498553			
P	Q	R	S	T	PPL	57648362.4	3615754.97
54140645	55170892	56110805	60146235	62673235			

Appendix 29: Number of contigs and percentage of all contigs mapped to each taxonomic rank.

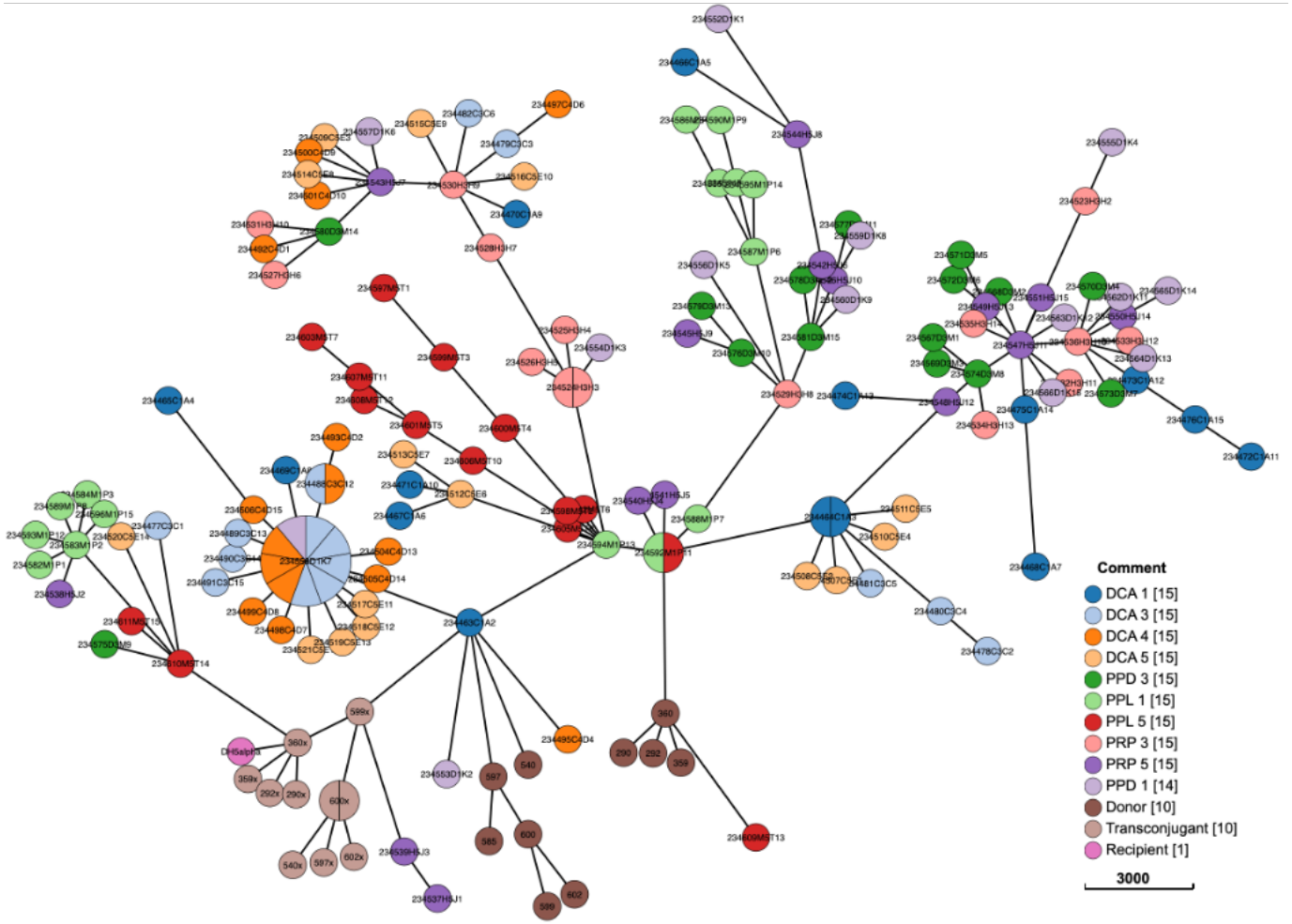
Contigs at kingdom (k) rank	483775 (92.9%), in four kingdoms
Contigs at phylum (p) rank	455799 (87.6%), in 59 phyla
Contigs at class (c) rank	431447 (82.9%), in 62 classes
Contigs at order (o) rank	367664 (70.6%), in 100 orders
Contigs at family (f) rank	222711 (42.8%), in 160 families
Contigs at genus (g) rank	109054 (21%), in 381 genera
Contigs at species (s) rank	127479 (24.5%), in 560 species



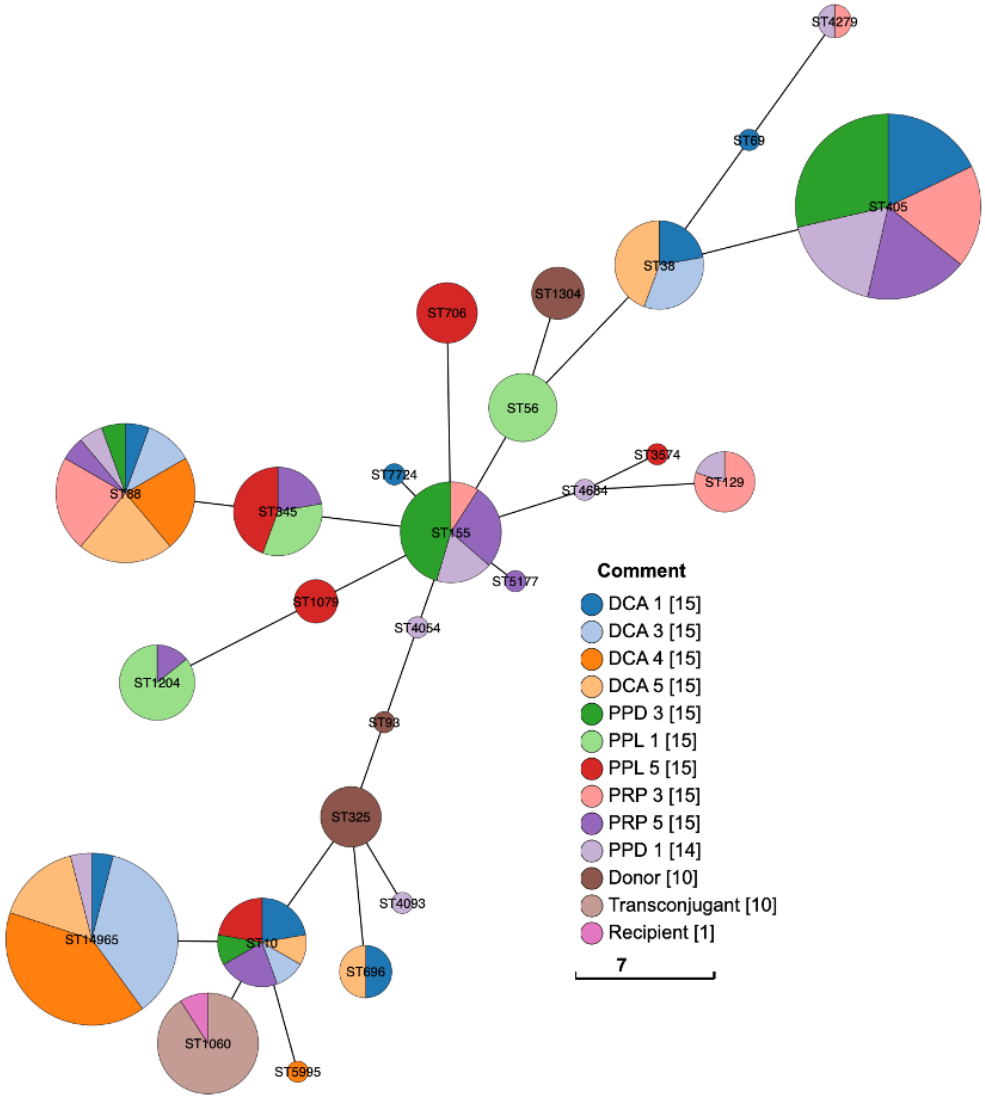
Appendix 30: Rarefaction curves for each metagenome with all labels. C = DCA, H = PRP, M = PPL, and D = PPD.

Appendix 31: Taxonomy of reads not classified to target taxonomic level, with representative species for each.

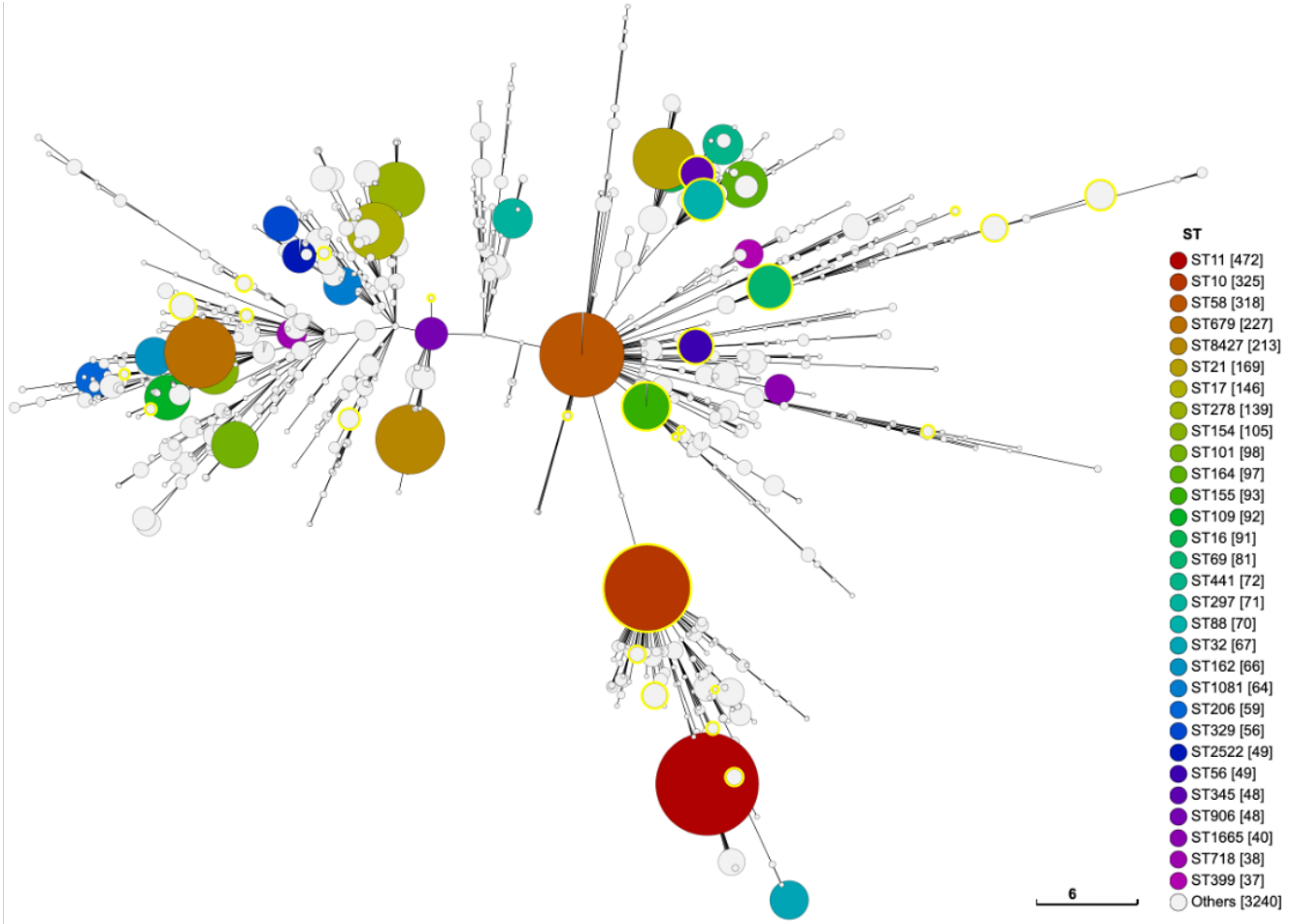
Kingdom	Phylum	Class	Order	Family	Genus	Species
Bacteria	Firmicutes (Bacillota)	Clostridia	*Eubacteriales*	*Oscillospiraceae*	*Ruminococcus*	*flavefaciens*
			Clostridiales	*Ruminococcaceae*	*Faecalibacterium*	*prausnitzii*
				Clostridiaceae	*Clostridium*	*perfringens*
		Bacilli	*Bacillales*	*Staphylococcaceae*	*Staphylococcus*	*aureus*
	Bacteroidetes	Bacteroidia	*Bacteroidales*	*Prevotellaceae*	*Prevotella*	*copri*
				Bacteroidaceae	*Bacteroides*	*fragilis*
		Gammaproteobacteria	*Pasteurellales*	*Pasteurellaceae*	*Pasteurella*	*multocida*
			Pseudomonadales	*Pseudomonadaceae*	*Pseudomonas*	*aeruginosa*
				Moraxellaceae	*Acinetobacter*	*baumanii*
			Enterobacterales	*Enterobacteriaceae*	*Escherichia*	*coli*
	Actinomycetes	Actinomycetia	*Streptomycetales*	*Streptomyceteaceae*	*Streptomyces*	*coelicolor*



Appendix 32: MSTree V2 Minimum spanning tree of all 170 *E. coli* genomes collected in this thesis based on ST determined by whole genome MLST. Genomes are from 10 MDR donor isolates collected from α samples, 10 transconjugant isolates, and DH5 α recipient; and selected isolates from the four DCA (60), two PPD (29), two PPL (30), and the two PRP (30) from the β samples.



Appendix 33: MSTree V2 minimum spanning of all 170 genomes collected in this thesis based on ST determined by Achtman seven gene MLST scheme. Genomes are from 10 MDR donor isolates collected from α samples, 10 transconjugant isolates, and DH5 α recipient; and selected isolates from the four DCA (60), two PPD (29), two PPL (30), and the two PRP (30) from the β samples.



Appendix 34: MSTree V2 GrapeTree plot of 6743 *E. coli* genomes. All isolates are from bovine sources collected between 2018 and 2024 grouped by ST assigned by the Achtman seven gene MLST scheme and coloured by ST. Figure also includes 170 genomes from this thesis, nodes highlighted by yellow circle include these genomes.

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