Metagenomic analysis and culture-based methods to examine the prevalence and distribution of antimicrobial resistance on two New Zealand dairy farms

A thesis presented in partial fulfilment of the requirements for the degree

of

Doctor of Philosophy

in

Veterinary Science

at Massey University, Palmerston North, New Zealand

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2022

Abstract

Antimicrobial resistance (AMR) is a global threat to human and animal health, with the misuse and overuse of antimicrobials being suggested as the main driver of resistance. In a global context, New Zealand (NZ) is a relatively low user of antimicrobials in animal production. However, antimicrobial usage on NZ dairy farms and its potential for driving the spread of AMR within the dairy farm environment is under-researched.

This research addresses the hypothesis that antimicrobial use on NZ dairy farms influences the prevalence of AMR in dairy farm environments, taking into consideration seasonality and contrasting farm management practices. The aims of this study, focused on two NZ dairy farm environments over an 15 month period, were to (i) determine the prevalence and distribution of AmpC- and extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli*, utilising culture-based methods, and (ii) to determine the abundance and diversity of antimicrobial resistance genes (ARGs), utilising a metagenomic approach, and lastly (iii), to assess the impact of systemic antimicrobial treatment on the bovine faecal microbiome.

Overall, the research presented in this thesis has shown a low sample level prevalence of ESBL-producing *E. coli* from two NZ dairy farms (faeces 0%, 1.7%; farm dairy effluent (FDE) 0%, 6.7% from Dairy 1 and Dairy 4, respectively) but AmpC-producing *E. coli* were more frequently isolated across both farms (faeces 3.3%, 8.3%; FDE 38.4%, 6.7% from Dairy 1 and Dairy 4, respectively). AmpC- and ESBL-producing *E. coli* were isolated in spring and summer, during months with varying levels of antimicrobial use. Analysis at the individual animal level showed a decrease in bacterial diversity and richness during systemic antimicrobial treatment and in many cases the microbiome diversity recovered post-treatment when the cow re-entered the milking herd. Compared to overseas data in a similar context, NZ dairy farm environments had a low abundance of ARGs, with the highest abundance detected in soil (0.20 - 0.63 copies of ARG per 16S rRNA gene). However, many of the ARGs identified in soil are not frequently found in human pathogens or acquired genes. FDE had a lower ARG abundance but the ARGs were more diverse (0.03 - 0.37 copies of ARG per 16S rRNA gene). There was no association between the normalised ARG abundance and antimicrobial use or collection date, however the low ARG abundance in the farm samples may have made any associations difficult to detect.

AMR is a burden for human, animal and environmental health and requires a holistic "One Health" approach to address. The outcomes from this research improve our understanding of the current levels of AMR on two NZ dairy farms and identifies areas for future research. Prevention is better than a cure and urgent action is required to slow the development and dissemination of AMR and to improve antimicrobial stewardship in humans and animals.

List of publications

Peer-reviewed:

Collis, R. M., Burgess, S. A., Biggs, P. J., Midwinter, A. C., French, N. P., Toombs-Ruane, L., & Cookson, A. L. (2019). Extended-spectrum beta-lactamase-producing *Enterobacteriaceae* in dairy farm environments: A New Zealand perspective. *Foodborne Pathogens and Disease*, 16(1), 5-22. doi:10.1089/fpd.2018.2524

In preparation:

Collis, R. M., Burgess, S. A., Biggs, P.J., Midwinter, A. C., Brightwell, G., & Cookson, A. L. Prevalence and distribution of extended-spectrum β-lactamase and AmpC-producing *Escherichia coli* in two New Zealand dairy farm environments.

Collis, R. M., Burgess, S. A., Biggs, P.J., Midwinter, A. C., Brightwell, G., & Cookson, A. L. Impact of systemic antimicrobial therapy on the faecal microbiome in dairy cows.

Collis, R. M., Burgess, S. A., Biggs, P.J., Midwinter, A. C., Brightwell, G., & Cookson, A. L. A longitudinal study of the resistome on two New Zealand dairy farms.

List of presentations

2021:

- Collis, R. M., Biggs, P.J., Burgess, S. A., Midwinter, A. C., Brightwell, G., & Cookson, A. L. Metagenomic analysis and culture-based methods to examine the prevalence and distribution of antibiotic resistance on two New Zealand dairy farms. One Health Aotearoa Symposium. December 8th 2021. (Oral) ***Awarded the One Health Aotearoa best student oral presentation.***

2020:

- Collis, R. M., Biggs, P.J., Burgess, S. A., Midwinter, A. C., Brightwell, G., & Cookson, A. L. Metagenomic analysis and culture-based methods to examine the prevalence and distribution of antibiotic resistance on two New Zealand dairy farms. One Health Aotearoa Symposium, Palmerston North, New Zealand. December 8th 2020. (Oral)
- Collis, R. M., Biggs, P.J., Burgess, S. A. , Midwinter, A. C., Brightwell, G., & Cookson, A. L. Metagenomic analysis and culture-based methods to examine the prevalence and distribution of antibiotic resistance on two New Zealand dairy farms. New Zealand Microbiological Society Conference. November 27th 2020. (Oral) ***Awarded 1st place in the student oral competition.***
- Collis, R. M., Biggs, P.J., Burgess, S. A., Midwinter, A. C., Brightwell, G., & Cookson, A. L. Metagenomic analysis and culture-based methods to examine the prevalence and distribution of antibiotic resistance on two New Zealand dairy farms. New Zealand Food Safety Science and Research Centre Spotlight Seminar series. New Zealand. 23rd October 2020. (Oral)
- Collis, R. M., Biggs, P.J., Burgess, S. A., Midwinter, A. C., Brightwell, G., & Cookson, A. L. Metagenomic analysis and culture-based methods to examine the prevalence and distribution of antibiotic resistance on two New Zealand dairy farms. Presented my research to Hon Prime Minister Jacinda Ardern and Hon Minister Grant Robertson, representing the New Zealand Food Safety Science and Research Centre. Food HQ, Palmerston North, New Zealand. September 17th 2020. (Oral)
- Collis, R. M., Biggs, P.J., Burgess, S. A., Midwinter, A. C., Brightwell, G., & Cookson, A. L. Metagenomic analysis and culture-based methods to examine the prevalence and distribution of antibiotic resistance on two New Zealand dairy farms.

Massey University Three Minute Thesis competition. New Zealand. August 2020. (Oral - Three Minute Thesis)

2019:

- Collis, R. M., Biggs, P.J., Burgess, S. A., Midwinter, A. C., Brightwell, G., & Cookson, A. L. Metagenomic analysis and culture-based methods to examine the prevalence and distribution of antibiotic resistance on two New Zealand dairy farms. One Health Aotearoa Symposium, Wellington, New Zealand. December 10th 2019. (Poster) ***Awarded One Health Aotearoa poster prize.***
- Collis, R. M., Biggs, P.J., Burgess, S. A., Midwinter, A. C., Brightwell, G., & Cookson, A. L. Metagenomic analysis and culture-based methods to examine the prevalence and distribution of antibiotic resistance on two New Zealand dairy farms. New Zealand Microbiological Society Conference, Palmerston North, New Zealand. November 26th 2019. (Poster)
- Collis, R. M., Biggs, P.J., Burgess, S. A., Midwinter, A. C., Brightwell, G., & Cookson, A. L. Metagenomic analysis and culture-based methods to examine the prevalence and distribution of antibiotic resistance on two New Zealand dairy farms. Participated in an antimicrobial resistance meeting with a focus on Global One Health. Nanjing Agricultural University, Nanjing, Jiangsu, China. November 2019. (Oral)

2018:

- Collis, R. M., Biggs, P.J., Burgess, S. A., Midwinter, A. C., Brightwell, G., & Cookson, A. L. Metagenomic analysis and culture-based methods to examine the prevalence and distribution of antibiotic resistance on two New Zealand dairy farms. I presented my research to the Primary Production Select Committee, representing the New Zealand Food Safety Science and Research Centre. Parliament, Wellington, New Zealand. September 20th 2018. (Oral)
- Collis, R. M., Biggs, P.J., Burgess, S. A., Midwinter, A. C., Brightwell, G., & Cookson, A. L. Metagenomic analysis and culture-based methods to examine the prevalence and distribution of antibiotic resistance on two New Zealand dairy farms. New Zealand Food Safety Science and Research Centre conference. July 2nd 2018. (Oral - Three Minute Thesis)

Acknowledgements

Firstly, a huge thank you to my four supervisors for their invaluable help, support and encouragement throughout my PhD journey: A/Prof. Adrian Cookson, Prof. Patrick Biggs, Dr Anne Midwinter and Dr Sara Burgess. I am fortunate to have supervisors who are not only excellent scientists and mentors, but are also kind and supportive individuals.

Adrian, thank you for all of the help, support and guidance throughout my PhD. Your enthusiasm, knowledge and ability to think about the 'bigger picture' really helped me to grow as a scientist. You were always happy to answer my many questions and read countless drafts of my work - thank you! Patrick, thank you for helping me to develop my coding skills and for all of your guidance and support analysing the large metagenomic dataset. This was a huge task and I am happy with the final outcome and the skills I have learnt along the way. Thank you for your attention to the small details and helping me to make my plots look as aesthetically pleasing as possible! Sara, thank you for all of help and support, especially for your help in the lab and with the MinION sequencing and with Toastmasters to improve my presentation skills. Anne, thank you for all of your support and guidance in the lab, for helping me to always consider the relevance of my research and for always being there to support me through all aspects of my PhD journey.

Thank you to the many people who have given advice and helped me with various aspects of my PhD project: Dr Jinxin Liu and Prof. David Mills (University of California, Davis) for their guidance on the shotgun metagenomic sequencing and code development and Dan Sun and Alan McCulloch (AgResearch Ltd.) for all of their help using the AgResearch High Performance Computing Servers to analyse my large dataset! Thank you to the Food Integrity AgResearch Ltd. Team Leader Dr Gale Brightwell for her help and support. Thank you to Xiaoxiao Lin (Massey University Genome Service) for his help and advice on the many sequencing components of my project, to Dr Ahmed Fayaz (Massey University) for the database development and maintenance and to Prof. Scott McDougall (Anexa FVC) for his study design advice.

A huge thank you to the Massey University Dairy 1 and Dairy 4 farm staff, especially Fiona Sharland and the farm managers Jolanda Amoore, Hamish Doohan and Joshua Mitchell. Thank you for all of your help with the sample collection and on farm components of my research. Thank to my colleagues who also helped with the sample collection on farm: Amila Nawarathna, Alexis Risson, Eden Esteves and Karina Sakamoto.

I have really enjoyed being a part of the Food System Integrity team at AgResearch Ltd. and the ^{m}EpiLab at Massey University. Thank you to all of my colleagues who I asked questions or bounced ideas off and for the many laughs and chats in the hallway or over a cup of coffee. A special thank you to my fellow PhD students that I shared an office with:

Amila Nawarathna, Alexis Risson, Soundarya Karamcheti, Eden Esteves and Zack Zhang. And thank you to all of the post-graduate students from the ^{m}EpiLab, Olivia Angelin-Bonnet for her friendship and R knowledge and my car-pooling friends Ellie Bradley and Holly Gray. No words can express how grateful I am for your friendship, memories and support over the last three years.

I would like to acknowledge the financial support of the New Zealand Food Safety Science and Research Centre, the AgResearch Ltd. Food Integrity SSIF programme, the Massey University School of Veterinary Science post-graduate research fund and the Eric Ojala Trust post-graduate scholarship in agricultural policy research for funding components of this project. I am grateful for the support of One Health Aotearoa and the New Zealand Microbiological Society through the oral presentation and poster prizes I was awarded.

I would like to thank the following Trusts for the post-graduate scholarships I have received throughout my studies: C. Alma Baker Trust post-graduate scholarship, Sydney Campbell Memorial Trust post-graduate scholarship, Catherine Baxter post-graduate scholarship, Freemasons post-graduate scholarship and the Tararua Provincial Rural Women NZ tertiary resource/book grant.

Lastly, thank you to my amazing family for their support throughout my PhD. Thank you to my parents, Tracey and Mike, who have always encouraged my passion for science and learning from a young age and have inspired me to work hard and to always do my best. Thank you to my three sisters Deana, Grace and Lucy and my brother-in-law Ash, for supporting me over the last three years and always making me smile. Thank you to my mother- and father-in-law, Viv and Graeme, for their support and to Viv for cooking us dinner every Thursday. And of course, thank you to my kind and supportive husband, Louis. You have always supported me throughout my studies and encouraged me to follow my dreams. Your love and support throughout the good and bad times has helped get me through – thank you!

Contents

List of Figures

List of Tables

Abbreviations

AA	Amino acid
AMR	Antimicrobial resistance
AMU	Antimicrobial usage
ARG	Antimicrobial resistance gene
ASTs	Antimicrobial susceptibility tests
ASV	Amplicon sequence variant
BPW	Buffered peptone water
CAZ	Ceftazidime
CLSI	Clinical and Laboratory Standards Institute
CTX	Cefotaxime
DCT	Dry cow therapy
ESAC	Extended-spectrum AmpC β-lactamases
ESBL	Extended-spectrum β-lactamase enzymes
ESBL-E	Extended-spectrum β-lactamase-producing *Enterobacteriaceae*
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FDE	Farm dairy effluent
HGT	Horizontal gene transfer
MALDI-TOF MS	Matrix-assisted laser desorption/ionization-time of flight mass spectrometry
MAGs	Metagenome-assembled genomes
MC	MacConkey agar
MIC	Minimum inhibitory concentration
MLST	Multi-locus sequence type
NMDS	Non-metric multidimensional scaling
NZ	New Zealand
NZVA	New Zealand Veterinary Association
pAmpC	Plasmid-mediated AmpC β-lactamase
PBS	Phosphate buffered saline
PCA	Principal component analysis
PMA	Propidium monoazide
PCR	Polymerase chain reaction
PCU	Population correction unit
rRNA	Ribosomal ribonucleic acid
SCC	Somatic cell count
SNP	Single nucleotide polymorphism
ST	Sequence type
WGS	Whole genome sequencing
WHO	World Health Organization

Chapter 1

Introduction

1.1 General background

Antimicrobial resistance (AMR) is growing global public and animal health concern. The misuse and over-use of antimicrobials in both human and animal health have been suggested as the main drivers of AMR [1]. However, the development and dissemination of AMR is complex [2] and other factors such as heavy metal use may co-select for resistance [3]. Of particular concern are antimicrobial resistance genes (ARGs) which can be encoded on mobile genetic elements and shared to bacteria via horizontal gene transfer (HGT) [4]. The number of antimicrobial resistant infections in humans has been increasing and at current rates, it is estimated that by 2050, 10 million human deaths per year will be attributable to resistant infections [5].

Antimicrobial resistant bacteria and ARGs have been detected globally in agricultural environments including dairy and beef cattle and calves [6, 7, 8, 9, 10, 11, 12, 13]. Antimicrobial usage (AMU) in food-producing animals has become an established consumer concern [14]. A survey of 1,000 participants in the USA found that 90.7% of respondents were concerned that AMU on dairy farms posed a threat to human health [15]. NZ is a comparatively low user of antimicrobials in animal production [16, 17], however given the prominence of the NZ dairy industry in the local and export markets, it is important to consider the role of the NZ dairy farm environment in the development and transmission of AMR. NZ dairy farm systems are largely pasture-based [18] and have a lower prevalence of some cattle diseases such as coliform mastitis [19] and compared with more intensive farming systems such as poultry and swine, dairy farming is less reliant on antimicrobials in NZ [20]. The majority of antibiotics used on NZ dairy farms are for the treatment and prevention of mastitis [21].

Traditionally, AMR surveillance and research has focused on specific target organisms and genes using culture-based methods and molecular techniques. With the development and increased availability of next-generation sequencing methods, the focus of AMR prevalence studies has shifted to also include whole genome sequencing (WGS) which allows a comparison of the genomic epidemiology of isolates and a high-resolution analysis of ARGs. Shotgun metagenomic sequencing enables a broader analysis of ARGs present in a sample. If the sequencing depth is sufficient, the genomic context and bacterial host identification

of ARGs can also be inferred. A combination of targeted culture-based analysis and next-generation sequencing methods, coupled with shotgun metagenomic sequencing, allows a deep and holistic understanding of AMR in the NZ dairy farm environment.

1.2 Antimicrobial resistance on New Zealand dairy farms

Studies researching AMR in NZ dairy farm environments have predominantly focussed on mastitis-associated pathogens such as *Staphylococcus aureus*, *Streptococcus dysgalactiae* and *Streptococcus uberis* [22, 23, 24] as well on the AmpC- and extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* (ESBL-E) [25, 26]. Of the the limited studies conducted in NZ dairy farm environments, the prevalence of ESBL- and plasmid-mediated AmpC-producing *E. coli* was low in dairy cattle faeces [25, 26] and they were not isolated from calf faecal enrichments (n=40) [27]. Farms which were positive for chromosomal mediated AmpC-hyperproducing *E. coli* (7 of 26 farms) were associated with a higher use of injectable amoxicillin antibiotics [26]. AMU and farm management practices likely drive the prevalence of AmpC- and ESBL-E in agricultural environments and associated food products. Most studies investigating the prevalence of AmpC- and ESBL-E on dairy farms have been conducted in the northern hemisphere or are cross-sectional studies [25, 26, 28]. Therefore, additional studies investigating the prevalence of AmpC- and ESBL-E in NZ dairy farm environments are required, particularly those using a longitudinal study design. Such studies would provide useful insight into any effects farm management practices and seasonal variation could have on AMR in agricultural environments. Few longitudinal studies globally, and to the best of my knowledge none in NZ, have been undertaken using culture-independent metagenomic approaches to investigate the abundance and diversity of ARGs in dairy cattle and the dairy farm environment. Such studies would be beneficial to examine the transmission pathways and reservoirs of AMR by providing a high level of detection of ARGs in these environments, and this would aid AMR surveillance. Further research is required to understand the role of dairy farms in the development and transmission of AMR in NZ, with a particular focus on pathogens of concern such as AmpC- and ESBL-E as well as a broader approach to investigate the abundance of ARGs in the wider dairy farm environment.

1.3 Thesis aims

This research, using a longitudinal approach, addresses the hypothesis that antimicrobial use on NZ dairy farms influences the prevalence of AMR in dairy farm environments and takes into consideration seasonality and contrasting farm management practices. This research utilises a combination of phenotypic, molecular and next-generation sequencing techniques to study AMR in NZ dairy farm environments.

The specific research objectives of this study were:

1. To investigate the sample level prevalence of AmpC- and ESBL-producing *Escherichia coli* from two NZ dairy farm environments.
2. To characterise AmpC- and ESBL-producing *E. coli* isolated from the two dairy farm environments and compare the genomic epidemiology (with a particular focus on ARGs) of a subset of strains.
3. Assess the impact of systemic antimicrobial treatment on the bovine faecal microbiome composition and diversity, as well as the microbiome recovery post antimicrobial treatment.
4. To identify the abundance and diversity of antimicrobial, heavy metal and biocide resistance genes from farm environmental samples.
5. To infer the bacterial host of acquired ARGs identified in draft bacterial contigs assembled from farm environmental samples and infer the ARG risk.

Therefore, a three-fold approach was used to achieve these key research objectives in this study:

1. Targeting specific antibiotic resistant *E. coli* using culture-based methods **(Chapter 3)**.
2. Investigating the impact of systemic antimicrobial therapy on the bovine faecal microbiome and the emergence of antimicrobial resistant bacteria at the individual animal level **(Chapter 4)**.
3. An extensive approach utilising shotgun metagenomic sequencing to estimate the abundance and diversity of antimicrobial, heavy metal and biocide resistance genes in farm environmental samples collected over a 15 month period and to identify the bacterial host and genomic context of acquired ARGs **(Chapter 5)**.

1.4 Thesis structure

Chapter 1 provides a general background and information on AMR in the NZ dairy farm environment and outlines the structure of this thesis. The literature review in **Chapter 2** highlights and critically evaluates research relevant to this PhD project. Sections of the literature review were previously published in *Foodborne Pathogens and Disease* in 2018 [28] and have been updated in this thesis, as well as including additional sections relevant to the research. In **Chapter 3**, the isolation of AmpC- and ESBL-producing *E. coli* from farm environmental samples collected during the longitudinal study is presented along with the molecular characterisation of isolates, antimicrobial resistance profiles and the genomic epidemiology of a subset of strains (Figure 1.1). **Chapter 4** presents the research of the microbiome study investigating the impact of systemic antimicrobial therapy on the bovine faecal microbiome. **Chapter 5** presents the results from the longitudinal study looking at antimicrobial, heavy metal and biocide resistance genes in two NZ dairy farm environments and inferring the bacterial host of acquired ARGs. **Chapters 3 - 5** are presented in publication style, with a separate introduction, methods and discussion section per chapter. As a result, there will be some overlap of concepts as each chapter is intended to be understood as an individual piece of work. The final thesis chapter, **Chapter 6**, provides a holistic general discussion of the overall thesis themes and highlights research gaps and recommends future work to study AMR in NZ.

This thesis improves the understanding of the prevalence and genomic epidemiology of AmpC- and ESBL-producing *E. coli* from NZ dairy farms, whilst taking into consideration fluctuations in AMU and changes in seasonality and farm management practices. This research investigated the impact of a range of systemic antimicrobial treatments on the bovine faecal microbiome and demonstrated an overall reduction within sample taxonomic diversity during treatment and that perturbations in the bovine faecal microbiome generally recovered post-treatment. To the best of my knowledge, this research provides the first comprehensive understanding of the on-farm abundance and diversity of ARGs and improves our overall understanding AMR in NZ dairy farm environments.

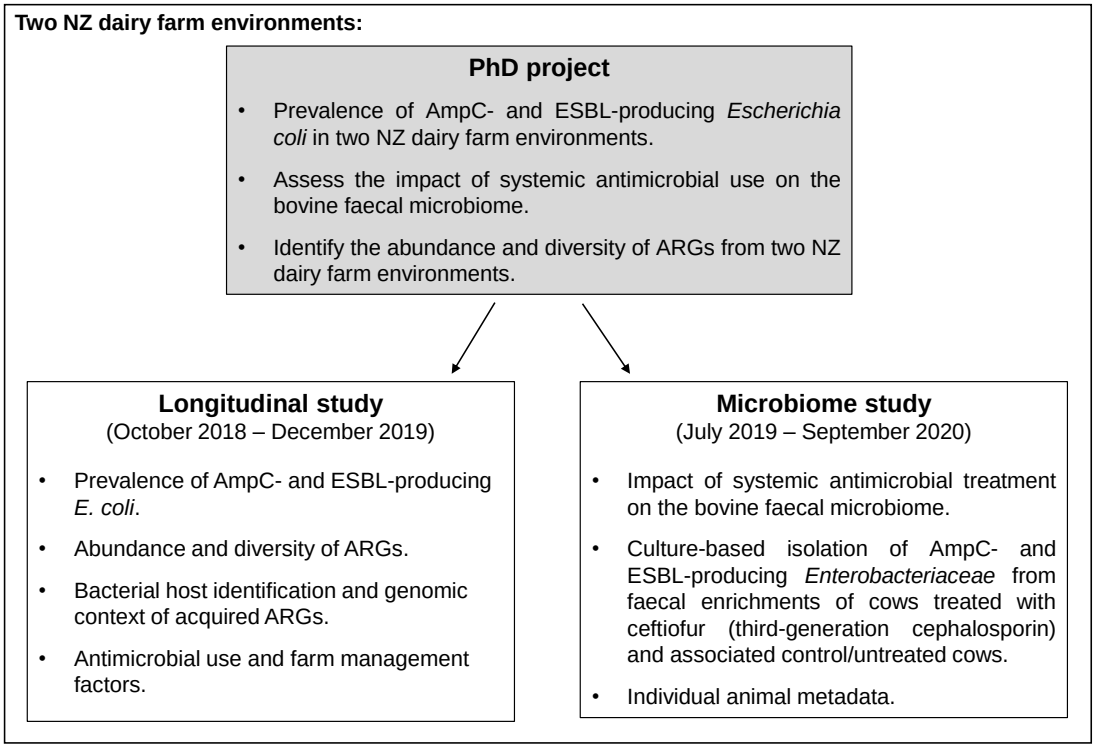

Figure 1.1: General outline of the PhD research showing the two on-farm studies and how they relate to the research objectives

Chapter 2

Literature review

This chapter is partially based on the publication by Collis *et al.*, [28], as described previously.

2.1 Introduction

Antibiotics are a subclass of antimicrobials, along with antifungals, antivirals and antiparasitics [29]. Antibiotics are molecules which can either inhibit (bacteriostatic, prevents growth) or kill (bactericidal) bacteria [30, 31]. Antimicrobial resistance (AMR) occurs when bacteria acquire, or inherently have, the ability to circumvent a drug's mechanism of action [32]. AMR is a global public and animal health concern, primarily due to infections caused by antimicrobial resistant bacteria being difficult to treat and often associated with increased disease severity [33]. AMR has been described as a "slow-burning" major public health concern [34, 35]. If left unchecked and at current rates it is estimated that by 2050, 10 million human deaths per year will be attributable to AMR globally [5]. A recent study estimated the AMR burden across 204 countries and territories and found that globally in 2019, 4.95 million deaths and 1.27 millions deaths were associated with or attributable to bacterial AMR, respectively [36]. Of the deaths attributable to AMR in 2019, *E. coli* was the bacterial pathogen associated with the most deaths [36]. Among the bacterial pathogens, resistance to fluoroquinolones and β-lactam antibiotics (carbapenems, cephalosporins and penicillins) accounted for more than 70% of human deaths attributable to AMR [36].

The misuse and over-use of antimicrobials in both human and animal health has been suggested as the main drivers of resistance [1, 2], however other factors such as heavy metal, biocide or disinfectant use may co-select for AMR [3, 37, 38]. The emergence, persistence and spread of AMR is often more intricate than a single driver [2] and antimicrobial resistance genes (ARGs) evolved in natural environments prior to the use of antibiotics in clinical settings [39]. ARGs in human pathogens are hypothesised to originate from environmental organisms [40] and the misuse and over-use of antimicrobials in human and animal health has exacerbated the selection pressures for resistant phenotypes.

In New Zealand (NZ) animal production, prophylactic use of antimicrobials is only permitted with a veterinary prescription, and the use of antimicrobials for growth promotion is prohibited [41]. A target has been set by the NZ Veterinary Association (NZVA), that

by 2030, NZ will no longer rely on antimicrobials for the maintenance of animal health and wellness [42]. Reports analysing antimicrobial sales data from 30 countries identified NZ as the third lowest user of antimicrobials in animal production [16, 17], using approximately 12.9 times less than in human medicine [16]. Compared to poultry and swine, dairy farming is less dependent on antimicrobials, and in comparison to international standards, a study found NZ dairy herds (n=477) were generally low users of antimicrobials, however some high-use herds were identified [43]. On NZ dairy farms, the majority of antibiotics are used for the treatment and prevention of mastitis [21], such as for dry cow therapy, which is the prophylactic use of antibiotics to reduce further mastitis events. The NZ dairy production system is largely pasture-based, which may contribute to the low antimicrobial use in this industry.

The development and transmission of antimicrobial resistant bacteria between humans, animals and the environment is multi-factorial and complex, and several potential transmission pathways of resistant bacteria between humans, the dairy farm environment and vice versa have been proposed [28]. The extensive use of antimicrobials in food-producing animals globally has become a consumer concern [14]. Compared to other farming systems, little is known about the contribution of dairy farming as a source of antimicrobial resistant bacteria and ARGs, however epidemiological relationships between genetically similar antimicrobial resistant bacteria found in both humans and livestock have been proposed [44, 45, 46].

2.2 Antimicrobial resistance mechanisms

Key mechanisms of AMR include (i) target site modification or protection, (ii) efflux pumps, (iii) degradation or modification of the antibiotic, (iv) reduced permeability and (v) expression changes [30, 32]. AMR can either be intrinsic, acquired or adaptive. Intrinsic resistance is the inherent characteristic of a resistant phenotype in a given bacterial species [40]. For example, Gram-negative bacteria are intrinsically resistant to glycopeptides due to their inability to permeate the outer membrane of Gram-negative bacteria [32]. In contrast, acquired resistance occurs when a previously susceptible bacteria acquires resistance towards an antibiotic through either mutation or acquisition of new genetic material via horizontal gene transfer (HGT) [32]. For example, acquisition of the $bla_{\text{CTX-M}}$ genes in *E. coli*. HGT can occur through either conjugation, transduction or transformation [2]. Plasmids play a key role in the transfer of ARGs [47] and they may harbour multiple resistance genes, resulting in multi-drug resistant phenotype which is of particular concern [48, 49, 50]. Mobile genetic elements such as transposons, integrons and insertion sequences are involved in the transfer and acquisition of ARGs on plasmids [32]. Thirdly, adaptive resistance occurs in response to environmental conditions and is not the result of genetic changes [32].

Natural environments are a key source of antibiotics and thus are locations where resistance has evolved [51], however some ARGs may have functions other than resistance in the natural environment. For example multi-drug efflux pumps are also involved in the detoxification of intracellular metabolites, cell homeostasis and bacterial virulence within plant and animal hosts [52]. Therefore, to understand AMR risk, particularly in sequence-based studies, it is essential to determine the bacterial host of an ARG (e.g. a human pathogen) and the genetic context (e.g. intrinsic or acquired resistance) [53].

2.3 Bacterial pathogens of concern

The World Health Organisation (WHO) has defined a priority list for 20 bacterial pathogens of concern to human health, and categorised them as critical, high and medium, according to ten criteria relating to health and resistance [54]. This list includes several pathogens of concern which are also associated with dairy cattle including ESBL-E, fluoroquinolone-resistant *Salmonella* spp., fluoroquinolone resistant *Campylobacter* spp., methicillin-resistant and vancomycin intermediate and resistant *Staphylococcus aureus* [55, 56, 57].

2.3.1 Mastitis-associated pathogens

Numerous bacterial pathogens may cause bovine mastitis and can be categorised into two groups according to their main reservoir: contagious or environmental pathogens [58]. Contagious mastitis-causing pathogens generally include *S. aureus*, *Streptococcus agalactiae* and *Streptococcus dysgalactiae* [58], although *Strep. dysgalactiae* can also be considered as an environmental pathogen [59]. Environmental pathogens also include members of the *Enterobacteriaceae* family where *Escherichia coli*, *Klebsiella pneumoniae* and *Klebsiella oxytoca* are of particular importance, as are *Streptococcus uberis*, coagulase negative staphylococci [60, 61, 58], *Serratia* spp., *Enterococcus* spp. [59] and *Pseudomonas aeruginosa* [62]. Coliforms are not a common cause of mastitis in NZ [19].

The prevalence and diversity of mastitis-associated pathogens varies between studies, geographic regions, can be herd-specific [59, 63, 64, 65, 66, 67] and may be due to different methods used for the detection of these pathogens. Variations may also occur in case definitions of clinical mastitis as well as national and local policies regarding AMU in livestock animals and farming practices. The wide range of bacterial pathogens that may trigger bovine mastitis highlights the difficulty in identifying the causative organism(s) and incorrect treatment (either antibiotics to which the aetiological agent is resistant to or using third-generation cephalosporins when penicillin would suffice) could potentially contribute to the development and spread of AMR in dairy farm environments. The use of on-farm bacterial culture and subsequent selective antimicrobial therapy was associated with a decrease in AMU compared to blanket antimicrobial treatment for clinical mastitis

cases in a study on NZ dairy farms (n=7) [68]. In addition, a network meta-analysis suggested that critically important antimicrobials are not required to treat nonsevere clinical cases of mastitis caused by *E. coli* or *Klebsiella* spp. [69] and that the treatment efficacy of clinical mastitis with both critically important and not critically important antimicrobials was comparable for the most prevalent mastitis-associated pathogens including *S. aureus*, *E. coli*, *Klebsiella* spp., non-*aureus* staphylococci and non-*agalactiae* streptococci [69]. NZ veterinary clinical pathology laboratories had reported an increase in β-lactam resistance among *Strep. uberis* bovine clinical isolates and a recent study found that this resistance was mediated by mutations in penicillin binding proteins [24]. A study of *S. aureus* (n=57) isolated from bovine mastitis cases in NZ found that AMU patterns were not associated with ARGs in the *S. aureus* isolated across the dairy farms studied (n=17), although some genes were over-represented among herds using ampicillin/cloxacillin dry cow therapy, however these genes were not all associated with AMR [23].

2.3.2 *Enterobacteriaceae*

Enterobacteriaceae are part of the normal microflora in the intestinal tract of many mammals including humans and dairy cows and can survive in variable environmental conditions. *E. coli*, which belong to the *Enterobacteriaceae* family, are a diverse species which are naturally found as commensals in the intestinal tract of healthy humans and animals, however a range of *E. coli* pathotypes such as Shiga toxin-producing *E. coli* (STEC) and enteropathogenic *E. coli* (EPEC) can also cause severe human disease [70]. *E. coli* are classified serologically according to O (O-specific polysaccharide component of lipopolysaccharide), H (flagellar) and K (capsular) antigens [71, 72]. Currently, 188 O-serogroups and 53 H antigens are included in the *E. coli* serotyping scheme [73]. Specific combinations of certain O and H antigens are defined as a serotype [72]. *E. coli* can also be classified using multi-locus sequence typing (MLST) [74] and by phylogroups [75, 76], which is useful to help understand the phylogenetic relationship between isolates and for epidemiological studies. *E. coli* can be divided into eight *E. coli* phylogroups (A, B1, B2, C, D, E, F and G) [76, 77, 78] that can be identified using PCR or by *in silico* analysis of whole genome sequencing (WGS) data [76, 78, 77]. It has been proposed that certain *E. coli* phylogroups may be more abundant in different niches [79, 80], although such associations have been debated and may be complicated by the genetic fluidity of *E. coli* and their ability to adapt to different ecological niches. In addition, *E. coli* can be characterised by pathotypes such as STEC and EPEC according to the symptoms they cause and pathotype-specific virulence factors [72, 81]. These pathotypes can be difficult to distinguish due to the lack of definitive defining features between pathotypes [81] which is further complicated by the potential for acquisition of virulence factors via HGT.

Enterobacteriaceae can develop resistance to a range of antimicrobials including colistin [46], tetracycline [82] and carbapenems [83]. *E. coli* isolated from dairy cattle and calves in NZ were resistant to β-lactams including third-generation cephalosporins, tetracycline,

streptomycin and a small number to ciprofloxacin [25, 27]. However one of the most widespread mechanisms of AMR in *Enterobacteriaceae* is the production of AmpC and ESBL β-lactamase enzymes. A study assessing the global burden of AMR in human populations found that 59,900 and 50,100 AMR attributable deaths in 2019 were caused by third-generation cephalosporin resistant *E. coli* and *K. pneumoniae*, respectively [36]. AmpC-, ESBL-E and mastitis-associated pathogens are both significant bacterial pathogens from dairy farm environments when considering human and animal health and the development and transmission of AMR. AmpC- and ESBL-E are discussed in sections 2.4.1 and 2.4.2, with a particular focus on *E. coli*.

2.4 β-lactamases

β-lactamase enzymes are found in both Gram-negative and Gram-positive bacteria [84] and they existed prior to the widespread use of β-lactam antibiotics [85]. The production of β-lactamase enzymes encoded by the *bla* genes is a common mechanism of resistance to β-lactams in Gram-negative bacteria [86], which is concerning as the β-lactams are commonly used as first-line therapy to treat infections caused by Gram-negative bacteria [87]. β-lactamase enzymes inactivate β-lactam antibiotics by hydrolysis [88], specifically by cleaving the β-lactam amide bond [89]. β-lactam enzymes have been classified using either amino acid (AA) sequence similarity in the Ambler classification system (A to D [84]) or using functional classification (substrate spectrum and inhibitor profiles) in the Bush-Jacoby-Medeiros scheme (1 to 4 with subgroups [90, 86]). The characteristics of β-lactamase enzymes, including the substrate spectrum, is shown in Table 2.1. A large number of variants within the β-lactamase families have been described [85].

Table 2.1: Characteristics of β-lactamase enzymes

Enzyme	**Ambler classification**	**Bush-Jacoby-Medeiros group**	**Examples**	**Substrate spectrum**	**Inhibitors**
Broad spectrum penicillinases	Class A	2b	TEM-1, TEM-2, SHV-1	Penicillins, narrow-spectrum cephalosporins	Clavulanic acid
ESBLs	Class A	2be	CTX-M, TEM and SHV (not parent type enzymes)	Penicillins, monobactams, cephalosporins (1st- and 3rd-gen.)	Clavulanic acid, tazobactam, sulbactam
KPC carbapenemases	Class A	2f	KPC	Penicillins, cephalosporins, carbapenems	Clavulanic acid (weak), tazobactam, boronic acid
Metallo-β-lactamases	Class B	3	IMP, VIM, NDM	Penicillins, cephalosporins, cephamycins, carbapenems	Metal chelators
AmpC β-lactamases	Class C	1	CMY, FOX, ACT MOX, ACC, DHA	Penicillins, monobactams, cephalosporins (1st, 2nd and 3rd-gen.), cephamycins	Cloxacillin, boronic acid
Oxacillinases	Class D	2d	OXA-1	Penicillins, narrow-spectrum cephalosporins, increased hydrolysis of cloxacillin or oxacillin	NaCl
Oxacillinases	Class D	2df	OXA-48	Penicillins, cloxacillin or oxacillin, cephalosporins, carbapenems[b]	NaCl

[a] Information in the table is adapted from Pitout (2012), Rubin and Pitout (2014), Bush and Jacoby (2010), Jacoby and Munoz-Price (2005) [91, 92, 86, 93].

[b] Substrate spectrum varies. Some variants also hydrolyse expanded-spectrum oxyimino-cephalosporins [94].

2.4.1 ESBL enzymes

Extended-spectrum β-lactamase (ESBL) enzymes belong to the Amber Class A and Bush-Jacoby-Medeiros group 2be [86]. ESBLs confer resistance to a broad range of β-lactam antibiotics, including penicillins, monobactams and first- and third-generation cephalosporins (Table 2.1) [92, 95] and have variable levels of resistance to fourth-generation cephalosporins [96]. Third- and fourth-generation cephalosporins have been identified as 'critically important' antimicrobials for use in human medicine as they are the antibiotics of last resort to treat serious human infections and pathogens causing these infections could be acquired via non-human sources [97, 98]. Resistance to third-generation cephalosporins in human clinical infections caused by *E. coli* and *K. pneumoniae* enhances the reliance on the remaining antimicrobials available to treat such infections (e.g. carbapenems) [99].

The most common ESBL variants include the sulfhydryl reagent variable (SHV), Temoneira (TEM) (excluding the parent type) enzymes and the CTX-M variants [91]. Less frequently identified ESBL variants include the SFO, PER, BES, BEL, TLA, GES and VEB types [100]. The TEM- and SHV-type enzymes are derived from the narrow spectrum parent-type enzymes (TEM-1, TEM-2, TEM-13 and SHV-1) by AA substitutions, however these changes can result in an extended hydrolytic spectrum [86, 92, 96]. CTX-M type ESBLs were named based on their increased activity against cefotaxime compared to ceftazidime [101], however some CTX-M types such as CTX-M-15 confer higher levels of resistance to ceftazidime [96]. CTX-M type ESBLs likely emerged from chromosomal β-lactamase genes from *Kluyvera* spp. which were mobilised onto plasmids [102]. Various genetic elements, particularly IS*Ecp1* and IS*CR1*, have been associated with the mobilisation of the $bla_{\text{CTX-M}}$ genes onto plasmids, likely on multiple occasions [96, 101, 102]. Over 172 CTX-M types have been identified [103] and are classified into five main groups (CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25) according to their AA sequence [96]. Each cluster was named after the first member of each group that was identified [103]. Changes in the AA sequence can give rise to CTX-M variants within each group [104, 105]. AA changes in the parent OXA-type enzymes has given rise to some variants with an extended-spectrum of hydrolysis and are thus classified as ESBLs [106]. The dissemination of ESBLs has largely been driven by the spread of plasmids harbouring ARGs between related bacteria [107] and by the spread of epidemic clones harbouring ESBLs such as sequence type 131 (ST131) in humans [108, 109].

The predominant ESBL variants differ geographically and can exhibit temporal shifts [110, 111, 112], however CTX-M-14 and CTX-M-15 (which belong to CTX-M-9 and CTX-M-1 groups, respectively) are the predominant genotypes in most geographic regions [110]. In livestock and animal sectors, CTX-M-1, CTX-M-14 and CTX-M-15 ESBL types have frequently been detected [113, 114, 115, 116, 117]. ESBLs have been identified in numerous *Enterobacteriaceae*, such as *K. pneumoniae*, *E. coli*, *Enterobacter* spp., *Serratia* spp., *Proteus* spp., *Providencia* spp., *Salmonella* spp. and *Morganella morganii* [95], but they are most frequently detected in *E. coli* isolates [33], which is of high concern to public health.

ESBL-E can cause urinary tract and bloodstream infections [33] and are associated with both hospital-acquired [118] and community-acquired infections [119]. Recently there has been an increase in the number of community-acquired infections associated with ESBL-E [120]. In NZ, the national period-prevalence rate of ESBL-E (from a clinical sample) was 11.1 people per 100,000 population in 2016, according to a one month survey [121]. The majority of ESBL-E identified from clinical samples during the study were *E. coli* (74.1%, 386 of 521) and the infections were mostly categorised as community-related (60.8%)[121].

ESBLs may be located on the bacterial chromosome but are often encoded on plasmids [102], the latter of which is of particular concern as such plasmids can be transferred via HGT and these plasmids may harbour additional ARGs, resulting in a multi-drug resistant phenotype [48]. Many ESBL-E are resistant to multiple classes of antibiotics [122, 123, 124] and of particular concern are those that are also resistant to carbapenems. The WHO bacterial priority list used this resistance class as a marker for multi-resistant and pan-resistant (resistance to agents in all antimicrobial classes) bacteria [54] and carbapenems are often the 'last line of defence' for treating serious infections [98]. Another 'critically important' antimicrobial is colistin which is a polymyxin antibiotic [125] that is often used to treat infections caused by multi-drug resistant Gram-negative bacteria. Worryingly, three ESBL-producing *E. coli* isolated from dairy farms in China also harboured the plasmid-mediated colistin resistance gene, *mcr-1* [126].

ESBL-E may cause disease in dairy cattle and have been isolated from milk from cows with mastitis in France [127], Turkey [128], Greece [124], Japan [129] and China [55]. Although less frequent, ESBL-E abundance may be underestimated if sampling of apparently healthy dairy cattle with subclinical levels of mastitis does not occur [55]. In NZ, coliform mastitis (caused by members of the *Enterobacteriaceae* family) is rare [19, 130, 68]. The prevalence of ESBL-producing *E. coli* was low in recent cross-sectional surveys of dairy farms in NZ [25, 26] and was not detected from recto-anal mucosal swabs from calves fed waste milk in NZ [27].

2.4.2 AmpC β-lactamase enzymes

Class C AmpC β-lactamase enzymes utilise serine for β-lactam hydrolysis and belong to the Bush group 1 β-lactamases [86]. These enzymes are located in the bacterial periplasm [131] and confer resistance to penicillins, monobactams, first-, second- and third-generation cephalosporins and cephamycins (Table 2.1).

2.4.2.1 Chromosomal *ampC* genes

Enterobacteriaceae with chromosomally-encoded AmpC β-lactamases include *Enterobacter* spp., *Escherichia albertii*, *Escherichia fergusonii*, *E. coli*, *Hafnia alvei* and *Serratia* spp. [131]. The chromosomal *ampC* gene (synonym for the bla_{EC} gene [132]) is absent in *K.*

pneumoniae, *K. oxytoca* and *Salmonella* spp. [131, 133]. Inducible β-lactamases, which are usually produced at low levels but are inducible when exposed to certain β-lactam antibiotics, are found in *Enterobacter* spp., *M. morganii*, *Citrobacter freundii*, *Serratia marcescens* and *Providencia* spp. [134]. However, the chromosomal *ampC* gene in *E. coli* is non-inducible as *E. coli* lacks the transcriptional regulator *ampR* gene [135] and it is instead regulated by promoter and attenuator mechanisms [136] and therefore is weakly expressed [91]. Mutations in the promoter and attenuator regions can result in over-expression of the *ampC* gene, increased production of the AmpC β-lactamase and thus resistance [137]. Common positions for mutations in the promoter region of the *ampC* gene include -42, -18, -1, +58, +70 and +81 [138, 139]. Mutations which are important in strengthening the *ampC* promoter include creating a consensus -35 box (transversions at either position -32 [T $\rightarrow$ A] or -42 [C $\rightarrow$ T] and base pair insertions that result in a promoter which more closely resembles the *E. coli* consensus sigma promoter sequence (insertions which increase the distance between the -35 and -10 boxes from 16 bp to either 17 or 18 bp) [140, 139]. Mutations can also arise in the attenuator region and are thought to destabilise the mRNA hairpin structure, leading to increased transcription of the *ampC* gene [139]. No association was identified between specific mutations found in the *ampC* promoter region and the level of AmpC β-lactamase production [138, 140]. Other factors such as porin expression and the rate of substrate delivery to the enzyme can also influence the spectrum of resistance [131].

2.4.2.2 Plasmid-mediated AmpC-producing *E. coli*

Plasmid-borne AmpC β-lactamase genes are derived from bacteria which have a chromosomal *ampC* that has been mobilised onto plasmids (Table 2.2) [133]. According to AA sequence similarity, the plasmid AmpC β-lactamase variants are classified into five main groups (Table 2.2) [131]. In addition to the AmpC β-lactamase variant groups listed in Table 2.2, a novel plasmid-mediated AmpC β-lactamase CFE-1 was detected in Japan in a clinical *E. coli* isolate [141] and more recently in 2018, CFE-2 was characterised from a clinical isolate of *C. freundii* [142]. The nomenclature for AmpC β-lactamase enzymes is inconsistent, with some variants named according to their preferred substrate, acronyms for Amber Class C and even abbreviations of the location where the variant was first identified [133, 143]. The majority of the pAmpC genes are constitutively expressed [143], with the exception of DHA-1 and ACT-1 which are inducible by β-lactam antibiotics [144, 145]. Surprisingly, the ACC-1 AmpC β-lactamase variant is susceptible to cephamycins, including cefoxitin [146] which is often used to screen for AmpC-producing *Enterobacteriaceae*. This complicates the isolation of *Enterobacteriaceae* producing this AmpC β-lactamase variant and may result in its true prevalence being under-reported. However, CMY-2 is the most prevalent plasmid-mediated AmpC β-lactamase globally [131].

Table 2.2: Plasmid AmpC β-lactamase variant groups

Group	**AmpC β-lactamase types**	**Likely origin of the *ampC* gene**	**GC content (%)**	**Ref.**
CIT	CMY-2 to CMY-7, CMY-12 to CMY-18, CMY-21 to CMY-23, LAT-1 to LAT-3, BIL-1	*Citrobacter freundii*	52	[147]
EBC	ACT-1, MIR-1	*Enterobacter* spp.	55	[148, 149]
DHA	DHA-1, DHA-2	*Morganella morganii*	52	[144, 150]
ACC	ACC-1, ACC-2	*Hafnia alvei*	46	[146]
MOX/FOX	MOX-1, MOX-2, CMY-1, CMY-8 to CMY-11, CMY-19, FOX-1 to FOX-5	*Aeromonas* spp.	63	[151, 152]

[a] Information in the table adapted from Jacoby (2009) [131].

2.4.2.3 Extended-spectrum AmpC β-lactamases

Extended-spectrum AmpC (ESAC) β-lactamases can arise from insertions, deletions and substitutions of AA in the AmpC β-lactamase, specifically in the 10 or 11 helix, R2 loop or Ω loop [153]. This can result in more flexibility in the enzymes active site and thus a broadened hydrolysis spectrum, including against fourth-generation cephalosporins such as cefepime [153, 154]. ESAC β-lactamases can arise from both chromosomal-mediated and plasmid-mediated *ampC* genes. Worryingly, some ESAC β-lactamases have an increased efficiency towards the carbapenem imipenem [153], which is a critically important antimicrobial in human medicine [155]. ESAC-producing *E. coli* have been isolated from human clinical samples [132] as well as from bovine milk and faeces in Brazil [154], *E. coli* isolated from faecally contaminated environments on UK dairy farms [156] and from healthy and diseased cattle in France [157]. ESAC β-lactamases have also been reported in other *Enterobacteriaceae* including *Enterobacter* spp. [158, 159] and *S. marcescens* [160].

2.5 Methods for detecting AmpC- and ESBL-producing *Enterobacteriaceae*

Traditionally, bacteria have been classified as 'susceptible' or 'resistant' according to the likelihood of therapeutic treatment success or failure respectively, with a given antibiotic [40]. Minimum inhibitory concentration (MIC) breakpoints and disc diffusion tests with zone diameter (mm) breakpoints have been defined using the pharmacodynamics and pharmacokinetics of the antibiotic, information regarding the wild-type distribution of MICs as well as clinical outcomes of infections that have been treated with the specific antibiotics [161]. In addition, isolates with zone diameters which fall in-between 'susceptible' or 'resistant' are classified as 'intermediate', where the therapeutic outcome is unclear due to an intermediate level of antimicrobial activity. Recently, the European Union Committee

on Antimicrobial Susceptibility Testing (EUCAST) guidelines reclassified this category as 'susceptible, increased exposure', to reflect the uncertainty and that changes to various factors such as the dosing regimen or antibiotic concentration at the infection site may result in an increased likelihood of therapeutic success [162]. Such breakpoints should be reviewed after the antibiotic has been used in clinical settings to take into consideration resistance mechanisms which may have developed [161]. Published guidelines and standards for antimicrobial susceptibility testing (AST) interpretation of breakpoints and cutoffs have been provided by the Clinical and Laboratory Standards Institute (CLSI) [163] and EUCAST [164, 165]. More recently, epidemiological cutoff (ECOFF) values have been proposed which can be used to determine whether a strain is categorised as fully susceptible (wild-type) or less susceptible (non wild-type) within a specific bacterial species [166]. The use of ECOFF values removes the biases associated with using clinical breakpoints according to the likelihood of treatment failure or success, although current ECOFF estimations rely on databases which are likely to be biased towards bacteria of importance for human health [167]. Normalised resistance interpretation (NRI) methods can be used to define ECOFF values within a specific bacterial population [168], thus removing these biases. These methods are particularly useful for non-human associated or environmental bacterial populations. For example, these methods have been used to assess AMR in *E. coli* and *Salmonella* spp. from wild ungulates in Portugal [169]. The use of NRI defined ECOFFs may be limited when the number of wild-type (fully susceptible) isolates for a given population is low [168].

2.5.1 ESBL-producing *Enterobacteriaceae*

Published guidelines are available from CLSI and EUCAST for the isolation and detection of ESBL-E [163, 164, 170]. Detection of ESBL-E is based on resistance to oxyimino-cephalosporins in a screening test, followed by subsequent phenotypic confirmation [170] which has been shown to be highly sensitive to detect ESBL-E [171]. Cefpodoxime is hydrolysed by TEM, SHV and CTX-M type ESBLs and is the most sensitive individual cephalosporin indicator [96, 170]. However, screening with only cefpodoxime may lead to a high number of false-positives as mechanisms other than ESBL production can result in cefpodoxime resistance, such as the production of AmpC β-lactamases. Ceftazidime is an effective substrate for TEM and SHV ESBLs [96]. The hydrolytic activity of ESBL variants towards some β-lactams may differ [172], therefore it is recommended to use a combination of indicator third-generation cephalosporins, such as cefotaxime (or ceftriaxone) and ceftazidime for screening [170, 171], which is more specific than testing cefpodoxime alone. According to EUCAST and CLSI guidelines, a screening breakpoint of >1 mg/L is recommended for cefotaxime, ceftriaxone, ceftazidime and cefpodoxime for ESBL-E [170, 163]. ESBL production can be confirmed using either the double-disc synergy, ESBL gradient or the broth microdilution tests [170]. Double-disc synergy tests are commonly used for ESBL confirmation and they aim to demonstrate a synergistic effect between a third-generation cephalosporin and an ESBL inhibitor [172]. For example, double-disc synergy tests that

compare the zone diameters for cefotaxime and ceftazidime with/without clavulanic acid is recommended for phenotypic confirmation of *Enterobacteriaceae* with no chromosomal de-repressed or inducible AmpC (e.g. *E. coli*, *Klebsiella* spp.). For *Enterobacteriaceae* with inducible AmpC (e.g. *Enterobacter* spp., *Serratia* spp.) it is recommended to use cefepime, which is stable to AmpC hydrolysis [170]. The latter method is also recommended for indeterminate results using the cefotaxime/ceftazidime and clavulanic acid confirmation method [170]. Phenotypic confirmation tests are essential to reduce false positive results. AmpC β-lactamases can complicate ESBL confirmation tests as they are not inhibited by ESBL inhibitors and are resistant to third-generation cephalosporins (Table 2.1) [143].

Detection of ESBL genes using either PCR [173, 7] or WGS is useful for both AMR surveillance and epidemiological studies. As next-generation sequencing is becoming cheaper and more widely available, prevalence studies are more frequently utilising these methods [25, 26, 174] which allows for a higher resolution analysis of β-lactam resistance genes as well as additional ARGs and enables a comparison of the genomic epidemiology of ESBL-E. WGS may be particularly useful when phenotypic confirmation methods are inconclusive [175].

2.5.2 AmpC-producing *Enterobacteriaceae*

Similarly, a two-step method can be used for the screening and confirmation of AmpC-producing *Enterobacteriaceae*. Resistance to cefoxitin can be used to differentiate AmpC production from other β-lactamases [131, 157], therefore reduced susceptibility to cefotaxime and/or ceftazidime and cefoxitin can be used in the screening process, followed by confirmation with a synergy test containing either cloxacillin or boronic acid [170]. A three-disc comparison assay can also be used for AmpC β-lactamase confirmation in which the zone diameters are compared between discs containing (i) cefpodoxime and an AmpC inducer, (ii) cefpodoxime, AmpC inducer and an ESBL inhibitor and (iii) cefpodoxime, an AmpC inducer, an ESBL inhibitor and AmpC inhibitors (D69C AmpC disc test, Mast Group Ltd., Liverpool, United Kingdom). Plasmid-mediated ACC-1 AmpC β-lactamases are susceptible to cephamycins however [146] and therefore will not be included in any subsequent analyses if reduced susceptibility to cefoxitin is a requirement for AmpC confirmation. After AmpC confirmation, it is important for AMR surveillance to distinguish between plasmid-mediated and chromosomal AmpC, which cannot be distinguished using phenotypic methods [176]. Instead, in *E. coli* they can be differentiated using either a multiplex PCR for the pAmpC genes [177, 178], mutations in the *ampC* β-lactamase gene promoter region can be amplified using PCR and subsequent Sanger sequencing of the PCR product [138] or the resistance mechanism can be identified through WGS. Mechanisms other than AmpC β-lactamase production can result in resistance to cefoxitin in *Enterobacteriaceae*, such as porin loss [179], that may lead to false positive results after the screening test and highlights the importance of confirmatory tests. Isolates showing false positive results should be further analysed to identify the resistance mechanism for

cefoxitin and third-generation cephalosporins, which is important for AMR surveillance. Reduced susceptibility to cefoxitin and cefepime in *E. coli* may be indicative of ESAC β-lactamase production [163, 154] or isolates co-producing plasmid-mediated AmpC and ESBL β-lactamases [153]. ESBL confirmation tests and identification of pAmpC genes should be carried out for *E. coli* with suspected ESAC β-lactamase production. Once these resistance mechanisms are excluded, the *ampC* gene should be sequenced in these isolates and examined to confirm ESAC production [154].

New diagnostic methods to detect AmpC- and ESBL-E have been developed, such as the matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS) based direct-on-target microdroplet growth assay which relies on the synergistic effect of indicator cephalosporins and inhibitors [175] and a similar method has also been developed for the detection of carbapenemases in *Enterobacterales* [180]. These methods provide fast detection of AmpC- and ESBL-E which enables prompt patient treatment, however further validation and standardisation of such methods is required before they can be routinely used [175].

2.6 Prevalence of AmpC- and ESBL-E in the dairy farm environment

2.6.1 Prevalence of AmpC- and ESBL-E in dairy cattle, calves and farm dairy effluent

Many studies have investigated the prevalence and incidence of AmpC- and ESBL-E in dairy cattle, calves and effluent and of those, the prevalence and incidence varies widely (Table 2.3). Study comparisons are often confounded due to the study-specific sampling and detection methods used as well as the different sample types, which often results in data from study populations that cannot be extrapolated [7]. The culture-based selection methods and enrichment protocols may differ in several studies (Table 2.3) resulting in varying screening sensitivities. For example, many studies use non-selective enrichment of faecal samples or effluent followed by plating on selective agar (Table 2.3), whereas some studies do not use a pre-enrichment step [181] and others use indicator antibiotics in the pre-enrichment step followed by plating on selective agar [7, 182, 183, 184]. In addition, numerous types of selective and chromogenic agar plates are available for the isolation of AmpC- and ESBL-E (Table 2.3). A study comparing AmpC- and/or ESBL-producing *E. coli* isolated from pig caeca samples on either MacConkey (MC) agar supplemented with cefotaxime (1 mg/L) or *Brilliance*^{TM} ESBL agar found that in 2013 and 2017 the recovery of isolates with a multi-drug resistant phenotype was higher on *Brilliance*^{TM} ESBL agar and the proportions of multi-drug resistant isolates from both agars was similar in 2015 [185]. This finding may be partially due to *Enterobacteriaceae* producing either carbapenemases, ESBLs or AmpC β-lactamases, being able to grow on MC agar with cefotaxime,

whereas *Brilliance*^{TM} ESBL agar selects for ESBLs only and the genes encoding ESBLs are often associated with additional ARGs, which can result in a multi-drug resistance phenotype [50, 48, 49]. ESBL-producing *E. coli* are also commonly found as contaminants in both Bolton broth and modified charcoal cefoperazone deoxycholate agar used for the isolation of *Campylobacter* spp. [186, 187], which both contain the third-generation cephalosporin cefoperazone and other antimicrobial agents, and may be brought about by the presence of additional ARGs co-harboured by ESBL-E. Therefore, multiple selective agars should be used in parallel for AMR surveillance studies. Such variations in the culture-based detection methods can complicate comparisons between prevalence studies and lead to estimates that differ from the actual prevalence. Farm selection criteria and smaller sample sizes may also influence the prevalence estimates.

In some prevalence studies AmpC- and ESBL-E are not differentiated [188, 189, 190] which is an important step in AMR surveillance and epidemiological studies. Ideally, more than one isolate per sample should be analysed, as distinct ESBL-producing *E. coli* can be isolated from the same sample [126]. In some studies only one presumptive isolate was analysed per plate [182, 183, 191] which could potentially reduce the diversity observed in prevalence studies if further characterisation of the isolates is not completed. A combination of both culture-based isolation methods and WGS are advantageous for surveillance of AmpC- and ESBL-producing *E. coli* [185]. WGS, particularly both short- and long-read sequencing, enables the detection and genomic context of a range of ARGs, high-resolution analysis of plasmids and can be used to help identify novel ARGs in conjunction with culture-based methods [185]. Generally the phenotypic and genotypic data are concordant, however for a small number of antimicrobial classes, the presence of a gene does not always correlate with a resistant phenotype [185] which highlights the importance of using both methods in parallel.

In NZ, few studies have investigated the prevalence of antimicrobial resistant bacteria, including AmpC- and ESBL-E, in animal-derived food products, dairy cattle and their environment. One regional cross-sectional study of dairy farms in NZ found a low prevalence of ESBL-producing *E. coli* (1 of 116; 0.9%) in pooled faecal samples [25]. No *E. coli* with pAmpC genes were isolated but 7.89% (9 of 114) of faecal samples were positive for putative AmpC hyperproducers with mutations in the promoter region of the *ampC* gene [25]. Similarly, a nationwide cross-sectional study (n=26 farms) in NZ did not detect any ESBL-producing *E. coli* [26] but chromosomal AmpC-producing *E. coli* were isolated from 14% (11 of 78) of pooled faecal enrichments originating from seven farms [26]. The lower prevalence of AmpC- and ESBL-E on NZ dairy farms compared to overseas studies may be due to the comparatively low use of antimicrobials in NZ animal production systems [16, 17] or the generally less intensive pasture-based farming system. A cross-sectional study of ruminant farming systems in Spain found beef cattle herds and sheep flocks, which have a semi-intensive pasture-based farming system, had significantly lower herd level prevalence of cefotaxime-resistant *E. coli* compared to dairy cattle, which are generally housed inside pens [9]. The prevalence of ESBL-E has been demonstrated to be

lower on organic dairy farms than on conventional dairy farms within the same country [7, 192]. The detection of ESBL-E on organic farms which have reduced AMU compared to conventional farms suggests that factors other than AMU may also contribute to the development of antimicrobial resistant bacteria [192].

The prevalence of antimicrobial resistant bacteria shed in faeces is typically highest in young calves compared to adult dairy cattle [193]. The age distribution of antimicrobial resistant *E. coli* from calves and dairy cattle in the USA was examined and tetracycline resistant *E. coli* were more prevalent in calves compared to lactating cows [193]. Removing oxytetracycline from the diet of calves did not reduce the proportion of tetracycline resistant *E. coli* shed in calf faeces [193], which suggests that antimicrobial exposure may not be required for the persistence and shedding of antimicrobial resistant *E. coli* in calf faeces. *E. coli* isolated from calves in the study population were frequently resistant to streptomycin, sulfadiazine and tetracycline (SSuT) [193]. To understand the factors driving the high prevalence of this resistant phenotype in young calves, experimental oral inoculation studies were performed in young calves and heifers (12 to 14 months) [193]. The shedding of SSuT resistant *E. coli* compared to susceptible strains was significantly higher in neonatal calves, whereas no difference was observed for heifers. In neonatal calves, the SSuT resistant *E. coli* out-competed susceptible *E. coli*, whereas in heifers no competitive advantage in the resistant isolates was observed [193]. These findings suggest that the SSuT resistant *E. coli* have a competitive advantage in the calf gut, which may be partially related to their diet as the prevalence of SSuT resistant *E. coli* was highest when the calves were fed a milk diet [193].

A higher prevalence of AmpC- and/or ESBL-E has been identified in calves compared to adult dairy cattle [10, 194, 195]. A study analysing recto-anal mucosal swabs from waste milk fed dairy calves (n=40) in NZ did not identify any ESBL-producing *E. coli*, however 25% of enrichments (10 of 40) were putative AmpC hyperproducing *E. coli* [27]. Additionally, an NZ study between 2009 - 2010 found no ESBL-producing *E. coli* (0 of 300) from bobby calf carcasses (4 to 10 days old) [196]. However, the detection of antimicrobial resistant bacteria from veal calf faecal samples and bobby veal in published studies overseas (Table 2.3) suggest that carcass contamination and transfer to the food chain may represent a public health concern if good hygienic slaughter practices are not adhered to. This food safety concern may be enhanced if non-optimal transport, cooking or storage conditions occurs.

Calf faeces may also contain a number of ARGs. A shotgun metagenomic study comparing the faecal resistome in preweaned calves compared to lactating dairy cows across 17 commercial farms in the USA found that the abundance of ARGs was significantly higher in preweaned calves (0.43 - 2.9 ARGs per copy of 16S rRNA gene) compared to lactating dairy cattle (0.11 - 0.6 ARGs per copy of 16S rRNA gene) [13]. Antimicrobial resistant bacteria shed in calf faeces may contaminate the environment, especially in calf pens, and this may be a potential transmission route for antimicrobial resistant bacteria and ARGs

between calves and within the farm environment. A study utilising shotgun metagenomic sequencing found that the faecal resistome in young dairy calves significantly changed over time and the total abundance of ARGs decreased significantly with increasing age [11]. The researchers found that changes in the diet and the age of calves contributed to the gut microbiome composition, with the faecal microbiota diversity increasing over time as the calf diets became more diverse, which subsequently influenced the faecal resistome [11]. To determine whether colostrum is a source of ARGs to young calves, the resistome between calf faeces and colostrum was compared. The faecal resistome structure of early-life calves and colostrum was similar, as 90.1% of the ARGs detected in faeces (73 of 81) were also found in the colostrum [11]. Therefore early-life colostrum feeding may provide a mechanism of transmission where ARGs are seeded in the developing calf gut.

Table 2.3: Prevalence of AmpC- and ESBL-E in dairy cattle, calves and farm dairy effluent

Sample type	**Year**[a]	**Country**	**Detection method**[b]	**Prevalence**[c]	**Study population**	**Ref.**
Pooled paddock faecal samples	Spring 2016 autumn 201	NZ	Enriched in BPW and plated on MC agar, MC + CTX, MC + CAZ and CHROMagar^{TM} ESBL.	0.9% samples positive for ESBL-producing *E. coli* (1 of 116) and 7.9% (9 of 114 samples; samples from three farms) were positive for putative chromosomal AmpC hyperproducers.	Regional (Manawatū) cross-sectional study of 15 randomly selected dairy farms.	[25]
Pooled paddock faecal samples	2017	NZ	Enriched in BPW and plated on MC agar, MC + CTX, MC + CAZ and CHROMagar^{TM} ESBL.	14% of pooled faecal enrichments (11 of 78) from seven farms were positive for putative chromosomal AmpC hyperproducers. No ESBL- or plasmid-mediated AmpC-producing *E. coli* were detected.	Nationwide cross-sectional study of 26 dairy farms.	[26]
Recto-anal mucosal swabs from waste milk fed dairy calves	2014	NZ	Swabs enriched with modified tryptone soy broth (mTSB) and enrichments plated on MC agar, MC + CTX, MC + CAZ and CHROMagar^{TM} ESBL.	25% of calves (10 of 40) were positive for AmpC-producing *E. coli* (putative AmpC hyperproducers).	Four farms from the Canterbury region which used waste milk were randomly selected from a larger cross-sectional study of dairy farms (n=102) across six regions in NZ [197].	[27]
Faecal samples from calves, young stock, dairy cattle and pooled faecal pat samples from the floor where dairy cows were housed.	2013	Netherlands	The faeces were plated on MC agar No. 3 with 1mg/L CTX and Luria-Bertani (LB) broth with 1mg/L CTX.	59.6% (109 of 183) of dairy herds had at least one sample positive for AmpC and/or ESBL-producing *E. coli*	Randomly selected conventional dairy herds (n=196).	[8]

Sample type	Year[a]	Country	Detection method[b]	Prevalence[c]	Study population	Ref.
Faecal contaminated environments and pastureland (adult dairy cows, dry cows, heifers, calves and samples where no animals were present) and publicly accessible farmland (footpaths).	2017-18	England	Faeces were plated on tryptone bile X-glucuronide agar (TBX) supplemented with 16 mg/L cephalexin. *E. coli* from these plates were sub-cultured on TBX agar containing 2 mg/L CTX.	9.3% of samples (384 of 4,145) representing 88.7% (47 of 53) dairy farms were positive for *E. coli* growth on agar containing CTX. The sample level prevalence was 5.4% (224 of 4,145) for $bla_{\text{CTX-M}}$ positive *E. coli*.	A convenience sample from 53 dairy farms. Monthly sampling over 12 months.	[195]
Pooled faecal samples from calves, dairy cows and the manure pit.	2017	Canada	Samples enriched in BPW and plated on MC + CTX.	85% of farms (86 of 101) had at least one sample positive for AmpC-/ESBL-producing *E. coli*. The likelihood of isolating AmpC-/ESBL-producing *E. coli* was higher from calves compared to adult dairy cows or the manure pit.	Cross-sectional study on 101 randomly selected dairy farms.	[194]
Rectal faecal samples	2014-16	Spain	Faeces enriched in mTSB with novobiocine, followed by enrichment in MC broth + CTX and plated on MC + CTX.	9.6% (10 of 104) of beef cattle, 32.9% (27 of 82) of dairy cattle and 7.0% (8 of 114) of dairy sheep were positive for third-generation cephalosporin resistant *E. coli*.	Cross-sectional study of dairy cattle (n=82), beef cattle (n=104) and dairy sheep (n=114) herds.	[9]
Pooled slurry samples	2011-12	Netherlands	Enriched in LB broth with 1 mg/L CTX and plated on MC + CTX.	41% (41/100) herds positive for ESBL-producing *E. coli*.	Cross-sectional study of 100 randomly selected conventional dairy farms.	[7]
Slurry samples from organic farms	2011	Netherlands	Faecal samples streaked on MC + CTX and the swab also inoculated in LB broth supplemented with 1 mg/L CTX.	13% (12/90) of slurry samples from 90 herds were positive for ESBL- and AmpC-producing *E. coli*.	Cross sectional study of 90 randomly selected dairy herds.	[192]

Sample type	Year[a]	Country	Detection method[b]	Prevalence[c]	Study population	Ref.
Floor faecal samples	2008-09	United Kingdom	Enriched in BPW and plated on Chromagar CTX.	Within herd proportion of CTX-M *E. coli* was 17% (219/1292), with a higher proportion of milking cows positive (30.3%, 200/660) compared to non-milking cows (3.0%, 19/632).	One CTX-M-15 positive dairy farm was sampled over two years.	[198]
Pooled faecal samples from dairy and beef cattle	2012	Germany	Enriched in BPW and plated on *Brilliance*^{TM} ESBL agar.	54.5% (6/11) of dairy farms were positive for ESBL-producing *Escherichia* spp.	Cross-sectional study of 11 dairy farms. Farms were selected according to herd size criteria and convenience sampling.	[6]
Pooled faecal samples from dairy and beef cattle	2011-12	Germany	Enriched in LB broth and plated on MC + CTX.	86.7% (39/45) farms were positive for ESBL-producing *E. coli*.	Randomly selected 45 dairy farms and 10 control farms. The control group was farms which reported no antimicrobial usage in the previous six months before the study commenced.	[199]
Individual faecal samples	2007, 2009	Japan	Faeces streaked on BTB-lactose agar supplemented with 2 mg/L CTX and 8 mg/L vancomycin.	5.2% (20/381) of herds were positive for CTX-M-type ESBL-E.	Herds and samples included in the study were farms which sent diarrhoea samples from cattle and calves to a clinical diagnostic laboratory.	[200]
Rectal faecal swabs	2014	Egypt	Enriched in BPW and plated on *Brilliance*^{TM} ESBL agar.	46.6% (98/210) of samples were positive for ESBL-producing *E. coli*.	Four dairy farms sampled.	[201]
Faecal swabs	2009, 2010	France	Faeces plated on ESBL screening agar plates containing Drigalsky medium supplemented with either 4 mg/L CTX or 4 mg/L CAZ.	CTX-M type ESBL-producing *E. coli* were detected in 5% (13/271) of faecal swabs.	182 dairy farms sampled, investigating prevalence in healthy cattle, calves with enteric disease and healthy cattle linked to the sick calves.	[202]

Sample type	Year[a]	Country	Detection method[b]	Prevalence[c]	Study population	Ref.
Swab rinsates from very young calf carcasses.	2009-10	NZ	ASTs were carried out on *E. coli* isolated from swab rinsates on petrifilms.	Prevalence of ESBL-producing *E. coli* was 0% (0/300) from isolates obtained from swab rinsates of young calf carcasses.	Samples were collected as part of the National Microbiological Database programme, in which samples from processing plants were collected for a one month period in 2009 and a three month period in 2010.	[196]
Faecal samples from veal calves.	2012	France	Faeces diluted in PBS and plated on MC agar and ChromID ESBL agar.	Prevalence of ESBL-E was 29.4% (144/489) in veal faecal samples.	Faecal samples (n=491) were collected from 12 slaughter houses over a six month period.	[203]
Faecal swabs from veal calves.	2011	Netherlands	Samples enriched in LB broth with 1 mg/L CTX and plated on MC + CTX.	66% of herds (66/100) were positive for CTX resistant *E. coli*.	Ten calf faecal swabs were taken post-slaughter from 100 slaughter batches (from five slaughter houses), which originated from different herds.	[182]
Pooled faecal samples from veal calves.	1997 - 2010	Netherlands	Samples enriched in LB broth with 1 mg/L CTX and plated on MC + CTX.	The prevalence of CTX resistant *E. coli* showed an increasing trend in the study population, ranging from 6% (3/49) in 1997 to 39% (71/182) in 2010.	Samples were stored from a longitudinal study (1997-2010) from randomly selected farms which were participating in a study of foodborne zoonotic pathogens.	[183]
Rectal faecal swabs from veal calves.	2009	Netherlands	Plated on MC + CTX and in LB broth supplemented with 1 mg/L CTX.	When the calves arrived at the farms, the prevalence of calves positive for ESBL- and AmpC-producing *E. coli* was 18-26%. However, this prevalence decreased during the study period.	Three veal-calf farms were studied over a ten week period in 2009. Farms were selected according to criteria set by the authors.	[204]

Sample type	Year[a]	Country	Detection method[b]	Prevalence[c]	Study population	Ref.
A combination of both farm and harvest faecal samples, pre-evisceration swabs and carcass swabs.	Unknown	USA	Enrichment in MC broth with 2 mg/L CTX and subsequently plated on MC agar supplemented with either 8 mg/L cefoxitin or 4 mg/L cefepime.	*E. coli* resistant to CTX and cefepime were: in farm faecal samples 91% (127/140) and 24% (33/140); pre-evisceration swabs 34% (55/164) and 6.7% (11/164) and final carcass swabs 19% (23/122) and 0.8% (1/122), respectively.	A prospective cohort study following four veal calf cohorts on separate farms.	[184]
Faecal samples from calves and their respective mothers.	2014	Czech Republic	Selective media: MC agar with 2 mg/L CTX	No ESBL-producing *E. coli* were identified and 94% (n=87) CTX resistant *E. coli* were AmpC positive. CTX resistant *E. coli* were isolated from all calves (n=13) and only two dairy cows (15.3%, 2 of 13).	One dairy farm (it is unclear how the farm was selected). Faecal samples were collected from calves (n=13) 1-63 days old periodically and their respective mothers (n=13) prior to calving.	[10]
Rectal swabs from calves	2016	Latvia	Faecal swab was mixed with saline and plated on MC + CTX.	11.1% of samples (20 of 180) were positive for AmpC/ESBL-producing *E. coli*.	Conventional dairy farms (n=18). The calves were 3 days - 2 months old.	[189]
Pooled faecal samples (n=3) from stable floor, dust sample and boot swabs from the feed alley.	2011-12	Germany	LB broth suspension prepared for faeces and boot swabs and dust swabs mixed with PBS. The samples were inoculated in LB broth overnight. Enriched samples were plated on MC + CTX.	At least one sample was positive for CTX resistant *E. coli* from 70% beef cattle units (42 of 60) compared to 85% of dairy cattle production units (44 of 52).	Cross-sectional study on dairy (n=52) and beef (n=60) cattle production units.	[190]
Rectal swabs	2014-15	Canada	Swabs enriched in BPW followed by *E. coli* broth which was subsequently plated on tryptic soy agar with 5% sheep blood containing vancomycin (6 mg/L), CAZ (2 mg/L), amphotericin B (2 mg/L), and clindamycin (1 mg/L).	81.2% of calves (396 of 488) had *E. coli* with reduced susceptibility to extended-spectrum cephalosporins.	Calves on dairy farms (n=8). Farms were recruited by convenience sampling.	[205]

Sample type	Year[a]	Country	Detection method[b]	Prevalence[c]	Study population	Ref.
Faecal samples	2018-19	Germany	Faecal samples were mixed with NaCl-Trytpone broth, diluted and plated on *Brilliance*^{TM} ESBL agar.	96.5% of calves (193 of 200) were positive for ESBL-E.	Ten dairy farms visited twice during the study. Calves were less than two weeks old.	[181]
Farm slurry	2014	UK, Scotland and Wales	Slurry enriched in BPW and plated on CHROMagar ESBL and CHROMagar CTX.	28% (27 of 97) slurry samples were positive for presumptive ESBL-producing *E. coli*.	Slurry samples collected from dairy farms after milking and before cleaning and were collected from five sites on each farm.	[206]
Untreated farm waste and effluent samples	2019-20	Ireland	Samples were filtered and the filter enriched in BPW. Enrichments were plated on *Brilliance*^{TM} ESBL agar.	Of the antibiotic resistant bacteria isolated, 36% on dairy and 4.5% on dairy and poultry farms were ESBLs. Across the different farms, a higher proportion of antibiotic resistant bacteria were isolated from influent compared to effluent samples.	Monthly sampling over 12 months of effluent samples collected before (influent) and after treatment (Integrated Constructed Wetland) from four different farm production systems (suckler, dairy farm, dairy and poultry and pig).	[188]
Faecal samples	2016	China	Faeces enriched in LB broth and plated on ChromID ESBL agar plates.	43.6% cows (284 of 651) were positive for ESBL-producing *E. coli*.	Ten randomly selected dairy farms.	[126]

[a] The year(s) the samples were collected.
[b] BPW, Buffered peptone water. MC, MacConkey agar. MC + CTX, MacConkey agar supplemented with 1 mg/L cefotaxime. MC + CAZ, MacConkey agar + 1 mg/L ceftazidime. TSB, Tryptic Soy Broth. TBX, Tryptone Bile X-Glucuronide medium. LB, Luria-Bertani Broth. PBS, Phosphate Buffered Saline.
[c] ESBL, Extended-spectrum β-lactamase. ESBL-E, ESBL-producing *Enterobacteriaceae*.

2.6.2 Prevalence of AmpC- and ESBL-E in milk

The prevalence of ESBL-E in bulk tank milk and milk from mastitic cows has not been investigated in NZ, but varies widely between studies undertaken overseas (Table 2.4). The prevalence in milk from mastitic cows was low (1.5%) in a study in Switzerland [207] and high in milk samples (25.53%) from Chinese dairy farms [55]. The prevalence of ESBL-producing *E. coli* from milk samples from individual healthy lactating cows in Malaysia was also comparatively high (16.9%) [208] and this may be due to environmental contamination on the udder prior to milk collection. In addition, the milk samples analysed were hand-milked from individual lactating cows [208], whereas bulk tank milk is often used in other studies. The prevalence of ESBL-E in bulk tank milk was relatively low (0-22.6%; Table 2.4), and although pasteurisation of milk using established heat-treatment processes will inactivate *Enterobacteriaceae* [209] these data indicate that raw milk consumption may pose a public health risk. Ceftazidime resistant *E. coli* were also isolated from retail milk samples in the USA, however some of these products for sale were unpasteurised [210]. Another study identified a higher proportion of *E. coli* from bulk tank milk samples (34.4%) in Northern China, some of which carried β-lactamase genes (Table 2.4) [211], however, culture-based selection methods for AmpC- and ESBL-E were not utilised in this study and therefore the prevalence in these samples may be under-reported.

Table 2.4: Prevalence of AmpC- and ESBL-producing *Enterobacteriaceae* in milk

Sample type	**Year**[a]	**Country**	**Detection method**[b]	**Prevalence**	**Study population**	**Ref.**
Milk from cows with mastitis	Jan. 2015 - May 2016	China	Direct plating (10 µL) onto MC + CTX.	Of 153 *E. coli* isolates from 1,252 mastitis milk samples, 23.53% (36/153) were ESBL-producers.	A total of 1,252 mastitis milk samples were collected when animals were ill from 61 commercial dairy herds.	[55]
Bulk tank milk and milk from cows with mastitis	Oct. 2009, Nov. 2010 to March 2011	Switzerland	Enriched in *Enterobacteriaceae* enrichment broth and plated on *Brilliance*^{TM} ESBL agar.	Zero out of 100 bulk tank milk samples were positive for ESBL-E and 1.5% (1/67) *E. coli* isolates from mastitis milk displayed an ESBL phenotype.	Bulk tank milk from a dairy manufacturing plant representing 100 dairy farms was collected and 67 *E. coli* isolates from mastitis milk were analysed.	[207]
Bulk tank milk	2010-13	Czech Republic	Enrichment in BPW and plated on MC agar supplemented with 2 mg/L CTX and TBX agar.	An ESBL phenotype was displayed in 0.7% (2/270) of *E. coli* isolates.	40 randomly selected dairy farms were sampled, including 35 conventional and 5 organic farms	[212]
Bulk tank milk	Nov/Dec. 2011, Aug. 2012	Germany	Enriched with 1 mg/L CTX and plated on MC + CTX.	9.5% (82/866) of bulk tank milk samples on farms were positive for ESBL-E.	Bulk tank milk samples were obtained from a pre-established and mandatory milk sampling program.	[209]
Bulk tank milk	2011	Indonesia	Enriched with 1 mg/L CTX and plated on MC + CTX.	8.75% (7/80) of bulk milk tank samples were positive for ESBL-producing *K. pneumoniae*.	80 randomly selected dairy farms.	[213]
Milk samples were hand-milked from an individual lactating cow.	NA	Malaysia	Selective isolation using Chromocult® Coliform Agar and subsequently CHROMagar^{TM} ESBL.	16.9% (12 of 71) of milk samples from individual healthy cows were positive for ESBL-producing *E. coli*.	Ten dairy farms.	[208]
Bulk tank milk	2016	Turkey	Enriched in BPW and plated on ChromID ESBL plates.	22.6% of milk samples were positive for ESBL-producing *E. coli*	The samples were collected from 62 bulk tanks across 1,252 farms.	[191]

Sample type	Year[a]	Country	Detection method[a]	Prevalence	Study population	Ref.
Retail milk samples: unpasteurised milk, pasteurised milk via vat pasteurisation, high-temperature-short-time pasteurisation, and ultra-pasteurisation milk.	2017	USA	Presumptive *E. coli* isolated during bacterial quantification of samples and ASTs completed for these isolates.	60% of *E. coli* displayed a CAZ resistant phenotype (57 of 95).	2,034 retail milk samples purchased.	[210]
Bulk tank milk	2015	China	Enriched in tryptic soy broth and plated on Eosin Methylene Blue agar plates.	34.4% (67 of 195) of milk samples were positive for *E. coli* and β-lactamase genes were identified in 34.3% of *E. coli*, specifically the bla_{SHV}(1.5%), bla_{TEM}(20.9%), bla_{CMY}(10.4%), $bla_{\text{CTX-M}}$(1.5%) genes.	195 dairy farms in Northern China	[211]

[a] The year(s) the samples were collected. NA, Not available.
[b] BPW, Buffered peptone water. MC, MacConkey agar. MC + CTX, MacConkey agar supplemented with 1 mg/L cefotaxime. MC + CAZ, MacConkey agar + 1 mg/L ceftazidime.

2.7 Shotgun metagenomics for the detection of ARGs

Culture-independent metagenomic methods targeting total DNA from an environmental sample provides a holistic approach for the detection of antimicrobial resistant bacteria and their genetic determinants. Culture-independent methods include quantitative real-time PCR for targeted amplification of ARGs and shotgun metagenomics involving sequencing of the collective genetic material in an environment [214]. Specific ARG databases such as the Comprehensive Antibiotic Resistance Database (CARD) [215] and ResFinder [216] are available to assign reads to DNA sequences to infer resistance. Databases such as PointFinder [217] can be used to identify chromosomal point mutations associated with AMR, however due to sequencing errors, ARGs identified in metagenomic sequencing data due to point mutations should be further validated. Metagenomic methods allow for community analysis to determine the abundance and diversity of microbes present in a sample, in contrast to culture-based methods which are often limited to specific bacteria according to the isolation methods used.

Culture-independent methods may be limited by the presence of low read numbers of ARGs sequences [218] and databases may only classify ARG to certain phyla, rather than to the species level [218], however shotgun metagenomics provides improved taxonomical classification at the genus level [219]. The classification method and reference database used can influence the taxonomic classification of short-read shotgun metagenomic sequencing data, especially at the species level. For example, the number of faecal samples classified as positive for *S. enterica* from feedlot cattle was discordant between culture and PCR methods compared to metagenomic sequencing and it was found that plasmids which were associated with *S. enterica* genomes in the reference database were influencing the taxonomic results. One limitation of culture-independent methods for resistome analysis is that ARG detection relies on gene homology and often does not consider flanking sequences important for gene function such as promoters [220], and this may overestimate the burden of AMR. In addition, culture-independent methods detect DNA and therefore it is not known whether a specific ARG originated from live or dead bacteria. Lastly, even though a gene is known to confer resistance to antimicrobials in one organism, it may not be functional in another [214]. Thus, the complementary use of both culture-based and metagenomic methods in parallel may provide a more thorough AMR surveillance approach.

Several factors require consideration for ensuring the accuracy of the sequencing data compared to the true microbial population composition during metagenomic studies. Such factors include sample storage, DNA extraction, read length, sequencing depth and bioinformatic analysis [30, 221, 222]. Consistent sample storage conditions within a study are crucial to minimise changes to the microbial population prior to DNA extraction. Various storage methods may be used, however it is recommended that faecal samples should not be stored for more than 3 hours at room temperature prior to low-temperature storage [223]. Storage of soil, human skin and faeces at -80°C prior to DNA extraction has been

shown to have a minimal effect on the microbiota composition [223, 224], however in one of these studies, a relatively low sequencing depth per sample was used [224]. In comparison, storage temperature and time has been shown to affect bacterial community composition in soil samples [225]. Similarly, the repeated freeze-thawing of samples should also be avoided to prevent DNA degradation [223].

Across all sample matrices, the same DNA extraction kit should be used due to possible kit reagent contamination [226] and the effect kits may have on the downstream sequencing results [227, 228]. Numerous studies have compared the efficacy of DNA extraction kits across specific sample matrices [227, 229, 230, 228, 231, 232, 233], however no study has compared all matrices used in this study. To mitigate the effect of contamination introduced via DNA extraction kits, blank extraction controls should be concurrently sequenced which is especially important for low biomass samples [234, 235, 226]. In addition, the use of mock communities (samples with a known composition) provides a good control to standardise analyses and/or determine any biases [221].

Sequencing depth is another crucial step when designing a shotgun metagenomic study. The effect of sequencing depth on the characterisation of the microbiome and resistome has been investigated in composite faecal samples from beef feedlot cattle [236]. The proportion of read assignments to phyla and antimicrobial classes was relatively unaffected by sequencing depth, however a significant increase in the number of sequencing reads assigned to ARGs and microbial taxa was correlated with increasing sequencing depth [236]. A sequencing depth of 59 million sequence reads per sample was suggested as suitable to accurately describe the cattle faecal microbiome and resistome [236]. Subsampling of deep shotgun metagenomic sequencing data from the Human Microbiome Project [237] across multiple sample matrices found that a depth of 0.5 million sequences was sufficient to identify the same trends in species-level α- and β-diversity as was observed in the full dataset [238]. However, the Human Microbiome Project did not compare the detection of ARGs, which would likely be present at a lower proportion in the samples. A study of human effluent and pig caeca samples found that the number of AMR gene families stabilised at 80 million reads per sample [239]. However, the human effluent sample had the most diverse range of ARGs and the detection of ARG allelic variants had not stabilised at a sequencing depth of 200 million reads per sample [239]. In summary, the required sequencing depth depends on the study objectives.

The proportion of contaminating host DNA (e.g. bovine DNA from a milk sample) versus microbial DNA can also affect the desired sequencing depth. For example, if a large proportion of sequencing read pairs are host DNA and thus removed, the total sequencing depth is reduced. Treatment of DNA with propidium monoazide (PMA) has been suggested as an effective method to remove host DNA from human saliva samples [240], and PMA treatment has been used on cattle effluent samples for subsequent quantitative PCR and 16S rRNA sequencing [241]. A recent study found that pre-extraction methods were the most effective for host depletion of *Bos taurus* reads from bulk tank milk and in-line

milk filter homogenate samples, compared to post-extraction methods [242]. Regardless of sequencing depth and treatment, the bulk tank milk samples (82.5 - 99.4%) had a higher proportion of host contamination compared to the milk filter homogenate samples (24.7 - 91.5%) and the authors therefore suggested that milk filters may be a suitable sample matrix to study as a proxy for bulk tank milk [242]. The detection of ARGs in milk filters may infer the presence in bulk tank milk, however this can not be confirmed. Reducing the number of contaminating host reads resulted in a higher detection of microbial taxa and ARGs [242]. Due to the potentially high level of host contamination in bulk tank milk samples, a high sequencing depth is required to detect ARGs which are hypothesised to be present in a low abundance in these samples [242]. A low percentage (average <1%) of contaminating *Bos taurus* DNA has been identified in cattle faeces [243], effluent and soil [244] compared to a higher proportion in bulk tank milk or milk filter homogenate samples (24.7 - 99.4%) [242]. Free DNA may also be encountered in soil samples [245].

Metagenomic studies assessing AMR often focus on ARG abundance, however there is growing evidence to suggest that the risk associated with ARGs in the resistome should also be considered [53]. Key considerations in an ARG risk ranking framework include (i) whether the ARG is part of the intrinsic resistome, (ii) fitness cost of the resistance gene and (iii) the environment in which the ARG was detected; for example, high risk ARGs found in humans poses a higher risk than if the gene was detected in a remote environment [53]. ARGs classified as the highest risk are those which can be identified on mobile genetic elements, are known to contribute to the failure of antibiotic treatment and have previously been found in human bacterial pathogens [53]. Therefore, to estimate the burden of AMR in an environment or ecosystem, metagenomic studies should evaluate not only the abundance of ARGs but they should also assess the risk each ARG poses. A similar risk framework approach has been applied to assess ARGs from human gut microbiome genomes and only a small proportion of ARGs were ranked as the highest risk group in the study (3.6%) [246]. A limitation of the current proposed ARG risk frameworks is that they are often human centric [53, 247, 246] with the risk described in relation to human health. Future modifications to risk frameworks should take a "One Health" approach and include risk assessments which collectively consider human, animal and environmental health.

2.8 AMU on dairy farms

There has been a global movement to reduce AMU in livestock animals to prevent the development and transmission of AMR, to improve AMR surveillance and to encourage prudent AMU and develop and adopt antimicrobial alternatives [248, 249, 250]. For example, to encourage both prudent use and the reduction of antimicrobials in animal husbandry, the NZVA has aspired that by 2030 NZ will not require antimicrobials for the maintenance of animal health and wellness [42] but rather reserve their use for the treatment of disease. A sample of dairy herds in NZ (n=477 herds) demonstrated that the antibiotic use rate was

generally low compared to international standards, but herd-to-herd variation occurred, with some high-use herds identified [43]. The ubiquity of a largely pasture-based system may be a contributing factor to the low antimicrobial use in NZ dairy cattle. A phone survey of 1,000 randomly selected adults in the USA found that 90.7% of participants (892 of 983) were concerned that AMU on dairy farms posed a threat to human health and 71.5% (580 of 811) of consumers were prepared to pay more for milk produced on a farm without antibiotics [15]. The participant responses were associated with demographic factors and worryingly only 51.5% of survey respondents were aware that antibiotics were used to treat bacteria only [15]. This suggests that increased education for the general public regarding prudent antimicrobial stewardship and the development of AMR is required.

Dairy farm management practices involving AMU to maintain animal welfare and reduce the prevalence of foodborne pathogens, and antimicrobial resistant bacteria, are interrelated [251]. Regulations for AMU in dairy cattle vary considerably, with some countries (e.g. USA and China) permitting the use of antimicrobials for growth promotion in addition to prophylactic use for the treatment, control and prevention of animal disease without a veterinary prescription [252]. In comparison the use of antimicrobials for growth promotion is not permitted in NZ [41] and European Union (EU) countries, and their prophylactic use is only permitted in animals with a veterinary prescription [252]. There is currently a lack of reliable surveillance data on AMU in livestock globally [253] and in many developing countries AMU is unregulated in food-producing animals [252].

AMU also differs between conventional and organic farm management systems. In contrast to conventional dairy farms, organic farms must adhere to AMU regulations aimed at improved environmental based management practices. For example antimicrobial therapy is banned in dairy cattle on USA organic farms; if an animal requires treatment they must be removed from the herd [251, 254]. In the EU however, organic dairy farms are permitted to use antimicrobials for disease treatment with an increased withholding milk period compared to conventional farms [251]. In NZ the regulations are designed to meet export market requirements, therefore organic dairy farms are banned from using antimicrobials (with some specified exceptions) to enable organic milk products to be exported to countries including the USA and the EU members [255, 256].

The range of antimicrobials approved for veterinary use differs between countries and there is some overlap between those approved for use in both human and animal health. In dairy cattle, antimicrobials are used to treat diseases such as mastitis, endometritis, neonatal diarrhoea and for dry cow therapy (DCT) [21]. Treatment with DCT (a long-lasting intra-mammary antibiotic preparation) at the end of lactation is intended to treat both existing mastitis infections and prevent new cases during the dry period. When DCT is applied to the whole herd, it is a farm management practice which may contribute to the development of antimicrobial resistant bacteria [257]. In NZ, antimicrobial DCT was not economically viable until the introduction of somatic cell count (SCC) penalties (as SCC is an indicator for intra-mammary infections) in 1993 [258, 259]. According to

antibiotic sales data from the dairying Waikato region of NZ (n=477 herds), 86% of the total antibiotics used in June 2009 - 2012 (measured as daily doses/animal/year) were for DCT and mastitis treatment [21]. Analysis of antimicrobial sales data for 2018 in NZ found that almost half of the antibiotics sold (47%) were registered for use in pigs and poultry, compared to 14% registered for use in cattle [20]. However, some products are registered for multiple species (production and companion animals) and multiple production animals, therefore the use of antibiotics in these categories cannot be distinguished using sales data [20]. In 2018, antibiotics for DCT represented 9.5% of overall sales and this represented a 16% decrease in antibiotic mass sold for DCT compared to 2017 [20]. The sale of critically important antibiotics such as third- and fourth-generation cephalosporins (23% and 27%, respectively), macrolides (15%) and fluoroquinolones (4%) decreased in 2018 [20]. Colistin, which belongs to the polymyxin class and is a critically important antibiotic, is not registered for use in NZ dairy cattle [20]. The reported antimicrobial use on 26 NZ dairy farms was 4.39 - 20.92 mg/population correction unit (PCU, which accounts for the animal population and estimated animal weight at the time of treatment) according to sales data, and β-lactams were the most frequently used antibiotic group [26]. A study of dairy farms across four regions in NZ also found that penicillins were the most commonly used antibiotic [260]. A convenience sample of dairy farms (n=358) in Great Britain found that the AMU ranged from 0.36 - 97.79 mg/PCU (mean 20.62 mg/PCU) according to antimicrobial sales data, and some high use farms were identified [261].

Caution should be used when comparing AMU between studies and countries as the methods used to assess AMU may differ [261]. This is of particular concern when comparing mg/PCU between countries as the population structure (e.g. animal weight) and recommended dosing regimes can differ [262]. It is essential to also consider the use of specific antimicrobials, as some critically important antimicrobials such as the third- and fourth-generation cephalosporins and fluoroquinolones are used at low doses [262, 261], therefore the use of these antimicrobials (compared to other antibiotics recommended at a higher dosage) would reduce the total amount of antimicrobials used but would increase the use of critically important antimicrobials. This could lead to a misinterpretation of AMU on farm. AMU calculated based on farm records (e.g. individual animal treatments) may potentially under-report the actual usage due to reporting bias or errors in updating records. Using antibiotic sales data as a proxy for true antimicrobial use can also be unreliable as farmers may receive bulk antibiotic prescriptions during a season, therefore it is possible the total antibiotics sold are not always used in one season. Standardised reporting protocols and metrics are essential for national AMU monitoring and comparison between farms and countries [261]. Further research is required to gather more accurate AMU data across dairy farms, including detailed information regarding treatments, frequency and time of use during the farming season. This detailed AMU data would help determine the baseline levels of AMU which could be used as targets to reduce AMU and it would be useful for programmes targeting improved antimicrobial stewardship. A systematic review analysed the association between antimicrobial restriction and the prevalence of ARGs and found that generally restricted use of antimicrobials in food animals was associated with a lower

or equal prevalence of ARGs in the study population, however the specific ARG, host species and antibiotic all influenced this effect [263]. For example, the prevalence of the $bla_{\text{CTX-M}}$ gene was lower in intervention groups, however no effects were observed for other β-lactamases [263]. These data suggest mitigation strategies aimed at preventing mastitis and targeted antimicrobial use for mastitis treatment may be the most effective measure to reduce AMU on NZ dairy farms.

The NZVA has set high aspirations to reduce the reliance on antimicrobials for the maintenance of animal health and wellness in NZ by 2030 [42]. Veterinarians play a vital role in promoting prudent antimicrobial stewardship on NZ dairy farms and are a trusted source of information for farmers. A survey of NZ farmers and veterinarians determined that AMU was influenced by veterinarian advice and personal experience, with farmers generally having limited knowledge of the development and spread of AMR [264]. A study of Swedish dairy farms (n=7) also found that advice from the local veterinarian was valued and important [265]. Increased awareness and education provided to dairy farmers by their local veterinarian regarding prudent antimicrobial stewardship and the development of AMR in agricultural environments will likely have a beneficial role on the use of critically important antimicrobials in NZ dairy farms. The identification of some farms with higher AMU [43, 261] suggests that involving them in AMU reduction strategies will help reduce AMU across the dairy farm production system.

In the dairy industry alternatives to antimicrobials for the treatment and prevention of mastitis have been recommended including good hygienic practice and the use of teat sealants for the prevention of new mastitis infections during the non-lactation period [266]. Whole herd application of teat sealants during the dry off period has been shown to have a protective effect against the development of ESBL-E positive herd-level status [7]. Other alternatives to antimicrobials for mastitis treatment and prevention [58] include the use of phage therapy and vaccines. These methods are more commonly utilised in the more intensive farming systems of the northern hemisphere where there are higher rates of mastitis. These alternatives have limitations due to the complexity and wide range of aetiological agents causing mastitis in dairy cattle.

2.9 Other farm management practices which may influence AMR on dairy farms

In addition to AMU, a number of other farm management practices are likely to impact the prevalence of foodborne pathogens, antimicrobial resistant bacteria and ARGs in agricultural environments [251]. Other farm management practices (Table 2.5) such as the use of biocides or disinfectants (e.g. quaternary ammonium compounds, triclosan) and the presence of heavy metals (e.g. copper, zinc) may influence the prevalence of AMR by co-selecting for ARGs. Co-selection for resistance genes can occur via co-resistance which

occurs when the selection of one gene aids the maintenance of another resistance gene, or through cross-resistance, which occurs when one gene confers resistance across multiple toxic compounds such as antibiotics, biocides or heavy metals [267]. A study analysing publicly available bacterial genomes and plasmids identified heavy metal and biocide resistance genes in 86% of the genomes and 17% of these co-harboured ARGs [29]. Interestingly, plasmids hosted by *Escherichia*, *Staphylococcus*, *Salmonella* and *Klebsiella*, which are often associated with human and animal pathogens, were more likely to co-harbour antimicrobial, heavy metal and biocide resistance genes compared to other bacteria in the study [29]. Plasmids co-harbouring ARGs and heavy metal/biocide resistance genes were uncommon from external environments (<0.7%) but were more common in plasmids from either human (5%) or domestic animal (7%) origins [29], suggesting that drivers in human and animal populations (e.g. antibiotics) may be co-selecting for these resistance genes.

Other farm management factors, such as feed type and herd size may influence the prevalence of diseases such as clinical mastitis, as a higher prevalence of certain pathogens have been associated with these more intensive farming practices. Intensive farming practices (e.g. supplementary feed and indoor housing) within northern hemisphere farming systems have been associated with an increase in livestock pathogens including coliform mastitis which may result in increased AMU to prevent and treat such infections [251, 268]. Cows which were confined in a more contaminated area were observed to have a higher incidence of coliform mastitis compared to pasture-based cows [269]. Northern hemisphere dairy farming systems often employ indoor barns, such as tie-stall or freestall barns to house cattle during the winter, with limited access to pasture [251, 254, 270]. In comparison, NZ dairy farming systems employ a majority pasture-based system where cattle are routinely grazed on ryegrass/clover [18], however there are examples of NZ farms where off pasture systems and open feed-pads are becoming more common. Feed regimes may also influence teat infections and consequently the use of antimicrobials to treat such infections; cows fed a total mixed ration (silage and concentrates) diet had a higher incidence of clinical mastitis compared to all-pasture based diets [269]. In contrast, a metagenomic study of the rumen gut microbiome found no correlation between the prevalence of ARGs in feedlot steers fed antimicrobial feed additives compared to steers where antimicrobial additives were withheld [219]. Farms operating an open herd policy (bringing new animals into the farm) may also increase the possibility of animals carrying antimicrobial resistant bacteria entering the farm. However, the role and significance each management factor has on driving AMR in the dairy farm environment remains to be fully elucidated.

Climate change may also have an impact on AMR in agricultural environments. Climate change is likely to increase the frequency of extreme weather events, heavy rainfall, heat stress in animals, result in changes to annual pasture and crop yields and potentially increase the incidence of some disease such as mastitis, which would necessitate further AMU and the persistence of pathogenic and antimicrobial resistant bacteria in the dairy farm envrionment [271, 272, 273]. All of these impacts of climate change would negatively influence agricultural sectors and cause challenges to these production systems. Some

proposed mitigation strategies to limit climate change would also have a beneficial impact on AMR. For example, smaller dairy cattle herd sizes to reduce methane emissions (in conjunction with other approaches such as feed management and selective breeding to increase animal production) would result in less intensive farming practices [274], that have been associated with a lower odds of detecting specific antimicrobial resistant bacteria [275, 189].

Table 2.5: Factors which may influence AMR in agricultural environments

Factor	**Country**[a]	**Importance**	**Bacterial group studied**	**Study type**	**Ref.**
Total AMU	Canada	Herd-level associations between AMU and AMR in *E. coli* were observed for certain antimicrobials, although no associations were observed for *Klebsiella* spp.	*E. coli* and *Klebsiella* spp.	On-farm cohort study.	[276]
Specific AMU (third- and fourth-generation cephalosporins)	Netherlands	The use of third- and fourth-generation cephalosporins is a risk factor for a dairy herd testing positive for ESBL and/or AmpC-producing *E. coli*.	*E. coli*	On-farm cross-sectional study.	[7]
Specific AMU (amoxicillin)	England, NA	Higher amoxicillin/clavulanate use on dairy farms has been associated with an increased risk of detection AmpC-producing *E. coli* with mutations in the promoter region of the *ampC* gene. *In vitro* studies also showed an association between amoxicillin use and AmpC-producing *E. coli* arising from mutations in the promoter region of the *ampC* gene.	*E. coli*	On-farm longitudinal study and an *in vitro* model.	[156, 277]
Post-milking teat disinfectants	NZ	Post-milking disinfection using a teat spray reduces both the incidence of new intra-mammary infections caused by specific bacterial pathogens and the somatic cell count in bulk tank milk.	Intra-mammary infections caused by *S. aureus*, *Strep. uberis*, *Corynebacterium* spp. and coagulase negative staphylococci were reduced.	On-farm randomized control trial.	[278]
Heavy metals (e.g. zinc, copper, mercury)	China, Scotland, USA	A significant correlation between some heavy metals and ARGs has been observed in agricultural soil and manure. A correlation between low levels of heavy metals and ARGs from soil microbes has also been detected. Network inference modelling found a strong correlation between ARGs and heavy metal and biocide resistance genes from shotgun metagenomic sequencing data, suggesting co-selection of these resistance genes in dairy calves.	Culture-independent methods (PCR) detecting specific ARGs and shotgun metagenomic sequencing.	On-farm cross-sectional studies.	[279, 280, 11]

Factor	Country[a]	Importance	Bacterial group studied	Study type	Ref.
Use of biocides and disinfectants	Spain, NA	A significant positive correlation between biocide tolerance and AMR to certain antimicrobials was observed in a study of bacteria isolated from seafood, suggesting that the use of biocides may co-select for AMR. A review of current evidence found a number of laboratory based studies suggested the use of biocides can co-select for AMR. Field studies (e.g. on farm) providing sufficient evidence for this correlation are currently lacking.	A number of bacterial genera were investigated including *Pseudomonas*, *Acinetobacter*, *Listeria* and *Shewanella*.	Laboratory analysis of bacterial isolates from seafood products and a review study.	[281, 282]
Open herd policy	England/ Wales, Latvia, Great Britain	Operating an open herd policy such as buying in calves, bulls or fattening cattle has been identified as a risk factor for third-generation cephalosporin resistant *E. coli*.	*E. coli*, *Enterobacteriaceae*, *Enterococcus* spp.	On-farm cross-sectional studies.	[283, 189, 284]
Waste milk disposal	USA	A PCR-based study found a higher number of faecal *E. coli* isolates from calves fed pasteurized waste milk were phenotypically resistant to some β-lactams including the third-generation cephalosporin ceftiofur, however resistance to other antimicrobials including sulphonamides, tetracyclines and aminoglycosides was high in calves fed both feed types.	PCR-based methods.	On-farm cohort study.	[285]
Herd size	USA, Latvia	Larger farming operations (>500 cattle) were associated with a 58% higher likelihood of detecting cefotaxime resistant bacteria from faecal samples in a cross-sectional of grazing beef cattle farms. A study of calves from dairy farms in Latvia found significantly higher levels of AMR to penicillins, cephalosporins and fluoroquinolones in *E. coli* and resistance to fluoroquinolones and macrolides in *Enterococcus* spp. in large farms (>201 milking cows).	*E. coli* and *Enterococcus* spp.	On-farm cross-sectional studies.	[275, 189]

Factor	Country[a]	Importance	Bacterial group studied	Study type	Ref.
Nutrition and feed type	UK, USA, NZ	A metagenomics study found that generally, the relative abundance and diversity ARGs was significantly increased in beef cattle fed concentrates. A metagenomics study found that the faecal resistome structure of early-life calves and colostrum was similar, and therefore colostrum may help seed ARGs in the developing calf gut. A study comparing feed type (mixed ration of silage and concentrates compared to pasture-based) in dairy cattle found cows fed a mixed ration had a higher incidence of clinical and sub-clinical mastitis, which may lead to an increase in AMU for mastitis treatment and prevention.	Shotgun metagenomics and culture-based methods	On-farm cohort studies.	[286, 11, 269]

[a] NA, Not applicable.

2.10 "One Health" approach

The One Health High Level Expert Panel recently formed a definition of "One Health" as being "an integrated, unifying approach that aims to sustainably balance and optimize the health of people, animals and ecosystems" [287]. "One Health" often uses multi- and trans-disciplinary methods. A "One Health" approach is required to tackle complex issues such as AMR, as humans, animals and ecosystems are intertwined and the development and dissemination of AMR within each sector is interdependent and is not distinct [99, 287]. Thus, selection pressures and the development of AMR in one sector may be reflected across other sectors [99]. Recent prevalence studies of ESBL-producing *E. coli* [206, 288] and AMR source attribution studies [289] have utilised a "One Health" approach and analysed *E. coli* from human, animal and retail food sources. However, these studies often have not included environmental samples nor samples from other vectors (e.g. birds, rodents, mammalian pests); the role of these sources in the development and dissemination of AMR remains largely unknown. Future studies focusing on the development and transmission of AMR should utilise a holistic "One Health" approach to fully understand the transmission pathways and reservoirs between humans, animals and the environment.

2.11 Transmission of antimicrobial resistant bacteria and ARGs in the dairy farm environment

Several potential pathways for the transmission of AMR within the dairy farm environment and humans have been identified (Figure 2.1) including contact with faeces-contaminated environments, via direct contact (for example, farmers may have a high risk during close contact with animals during milking or calving) or through the food chain from animal-derived products including raw milk and veal [290]. However, the actual contribution and direction of each pathway is undetermined.

Within agricultural environments, the ruminant digestive tract has been suggested as a reservoir for the development and spread of AMR due to the diverse range of bacterial species and ARGs present in both the bovine and ovine ruminant gut [286, 291]. Antimicrobial resistant bacteria and resistance genes can be excreted in cattle faeces [292, 293] which can subsequently contaminate the dairy farm environment, such as faecal contamination of pasture/soil during the application of manure or dairy effluent as a fertilizer to pastures [294].

Evidence for AmpC and ESBL-E and ARG transfer from food-producing animals to humans remains equivocal. A German study assessing the risk of animal-human direct contact for the cross-transmission of ESBL-E identified two isolates, one from dairy cattle and the other from a farm worker, that shared identical multi-locus sequence types (MLST) and CTX-M alleles, potentially indicating zoonotic transfer [6]. Whereas a comparison of

ESBL- and AmpC-producing *E. coli* isolated from animal-derived food products, animal gut microbiota and human clinical cases such as urinary tract infections, found little similarity between animal and human isolates at the level of MLST and virulence and AMR gene profiles [45]. Only 1.2% (3 of 258) of the animal isolates were ≥70% similar to the human isolates in relation to MLST clonal complex and microarray gene profiles [45]. Specific cases were observed however in which animal and human derived isolates were similar, with a UK cattle isolate and Dutch human isolate sharing >90% similarity in virulence and AMR microarray gene profiles, however such similarities only indicate genetic similarity and do not provide sufficient epidemiological evidence supporting transfer between humans and cattle [45]. A systematic review of the role of food-producing animals as a source of human infections caused by expanded-spectrum cephalosporin resistant extra-intestinal *E. coli* described studies both supporting and rejecting food-producing animals as a source of infection [295]. Where there are associations between resistance in food-producing animals and humans this appears to be sporadic and limited to specific strains. For example, WGS identified the recent emergence of a distinct lineage of third-generation cephalosporin resistant *Salmonella enterica* serovar Typhimurium in Australia, from both human infections and dairy cattle, suggesting transmission between dairy cattle and humans [296]. A large study comparing plasmid replicon types and ESBL/pAmpC genes combinations from farm animals, food and humans in Sweden found there was limited dissemination of ESBL/pAmpC genes and plasmids carrying these genes between sectors [297], but a limited number of bovine isolates (n=9 from preweaned calves) were included in the study. Currently, there are no published studies investigating the transmission and zoonotic transfer of AmpC- and ESBL-E in NZ environments. The evidence for direct contact as a transmission route of antimicrobial resistant bacteria from cattle to humans is inconsistent partly due to the number of potential transmission pathways, the methodologies used to infer transmission as well as the difficulty proving the direction of transmission.

AmpC- and ESBL-E have been detected in numerous animal-derived food products overseas including in bulk tank milk from dairy farms [209, 213, 298], raw milk and cheese during the production process [298] and veal calves and carcasses [203, 182, 184] indicating their occurrence along the food chain. A recent study of veal calves on-farm and pre- and post-slaughter isolated antimicrobial resistant *E. coli* from both farm faecal and pre-evisceration samples sharing the same pulsed-field gel electrophoresis patterns and resistance phenotypes [184], highlighting a potential pathway for the transfer of antimicrobial resistant bacteria to the human food production chain.

Human-human spread is also an important route of transmission of AmpC- and ESBL-E [206, 299]. Using a source attribution model, it was identified that most community acquired carriage of plasmid-mediated AmpC- or ESBL-producing *E. coli* in the Netherlands were attributed to human to human transmission [299]. A recent study utilised a "One Health" approach to investigate source attribution of ESBL-producing *E. coli* isolated from human faecal samples and bloodstream infections, human sewage, food, dairy farm slurry and veterinary diagnostic samples from food-producing animals across England, Wales

and Scotland [206]. Across human-associated samples (faeces, bloodstream infections and sewage) the predominant sequences types were ST131 followed by ST38 and ST648 with $bla_{\text{CTX-M-15}}$ being the most common ESBL variant [206]. In the food products and veterinary isolates, the most common sequence types were ST602, ST23 and ST117 with $bla_{\text{CTX-M-1}}$ being the most common ESBL variant. ST10 *E. coli* were isolated from both humans and animal-associated samples, however the ST10 isolates belonged to a diverse range of serotypes [206]. *E. coli* isolated from a cross-sectional study of livestock (cattle, pig and poultry farms) and retail meat (beef, chicken, pork, venison, veal and turkey) were compared with *E. coli* from human bloodstream infections in the UK [288]. WGS and core genome comparisons demonstrated that the livestock associated and human isolates were distinct (median 41,658 SNPs), however genetically similar *E. coli* (0-5 SNPs) were isolated from animals on different farms [288], perhaps suggesting animal to animal transmission. There was a small number of human and livestock associated isolates which were highly similar, with two human isolates being within 15 SNPs of two pig and one turkey isolate [288]. The predominant sequence types from the study in humans were ST73, ST131 and ST95 compared to ST10, ST117 and ST602 in livestock. These findings suggested that it was unlikely that livestock and retail meat are a major source of the *E. coli* causing bloodstream infections in the UK [288]. Recently, a study utilised shotgun metagenomic sequencing and Random Forests models for AMR source attribution in pigs, veal calves, broilers, turkey and humans occupationally exposed to livestock in Europe and identified some country-specific and reservoir-specific resistome markers which could potentially be used for source attribution studies [289]. However, additional sampling of the general human population, collecting human samples from more countries and including additional livestock species will strengthen these models and potential markers [289].

Numerous potential AMR transmission pathways have been identified, however the importance of each of these routes still remains unclear. Variation in the food production chains, farm management practices and policy regarding AMU in agricultural environments may influence the transmission pathways of antimicrobial resistant bacteria from animals to humans and vice versa [295]. Future work using short- and long-read WGS and shotgun metagenomics are required to determine the transmission pathways of antimicrobial resistant bacteria and ARGs at the dairy farm, human and environmental interface.

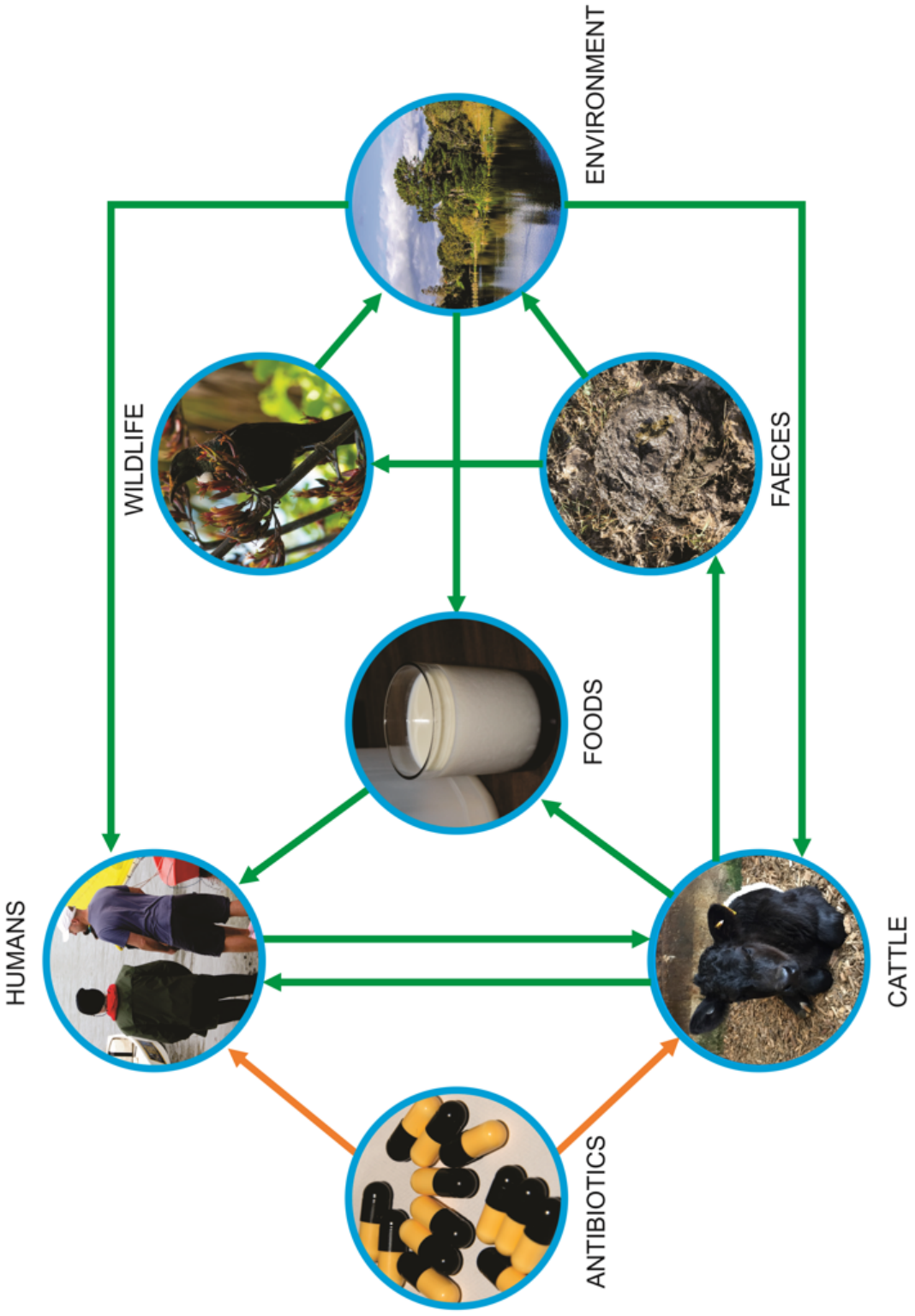

Figure 2.1: Potential transmission pathways of ESBL-producing *Enterobacteriaceae* and antimicrobial resistant bacteria in dairy farm environments. Orange arrows indicate drivers of AMR, green arrows indicate potential transmission pathways and arrow orientation indicates the direction of the relationship. Figure adapted from Collis *et al.*, (2019)

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2.12 Conclusion

AMU in dairy farm environments and the consequent emergence and transmission of antimicrobial resistant bacteria and ARGs is a concern for both public, animal and environmental health. Antimicrobial resistant bacteria of concern to human health such as AmpC- and ESBL-E have been detected both overseas and in the NZ dairy farm environment. The prevalence of AmpC- and ESBL-E in calves and cattle, and associated food products and the wider farm environment varies between studies and is likely due to contrasting farm management practices, national and local veterinary AMU regulations, food processing standards, pathogen surveillance and the sensitivity and specificity of detection methods to identify antimicrobial resistant bacteria. Therefore, caution should be used when comparing data between studies.

Evidence of transmission of antimicrobial resistant bacteria from livestock to humans and vice versa remains equivocal, but some studies focusing on ESBL-producing *E. coli* in Europe found that the predominant sequence types of *E. coli* from human bloodstream infections and livestock were genetically distinct. However, further sampling of livestock animals of various ages, environmental sources, vectors (e.g. birds, rodents and mammalian pests) and healthy humans are required to further assess any potential transmission pathways. Enhanced knowledge of the prevalence, distribution and transmission of antimicrobial resistant bacteria and ARGs in the dairy farm environment, including pasture-based farms and those with low AMU such as in NZ, is required to understand the transmission pathways and risk factors for subsequent development of mitigation strategies to reduce antimicrobial use and AMR in agricultural environments. Additional high-resolution WGS (e.g. short- and long-read sequencing) or metagenomic studies would provide important transmission and prevalence data. Studies using shotgun metagenomic sequencing to assess ARG abundance should also apply an ARG risk framework approach to consider the bacterial host, genomic context and risk each ARG poses to fully understand the burden of AMR in agricultural environments.

Lastly, NZ has policies and guidelines for the judicious use of antimicrobials in animal health and is a comparatively low user of such antimicrobials. However, education, outreach and stakeholder engagement (by both veterinary professionals and farmers) is required to meet the targets set by the NZVA and to promote prudent antimicrobial stewardship. Addressing the rising threat of AMR requires a holistic "One Health" approach across multiple sectors and further studies are required to understand the role of the dairy farm environment in the development, persistence and transmission of antimicrobial resistant bacteria and ARGs.

Studies investigating AMR in NZ dairy farm environments have generally taken a culture-based approach, focusing on specific target organisms and resistant phenotypes [22] while utilising a cross-sectional study design [25, 26]. This research, using a longitudinal approach, addresses the hypothesis that antimicrobial use on NZ dairy farms influences the

prevalence of AMR in dairy farm environments, taking into consideration seasonality and contrasting farm management practices. This study utilises a combination of phenotypic, molecular and next-generation sequencing techniques to study AMR in two NZ dairy farm environments.

Chapter 3

Prevalence and distribution of extended-spectrum β-lactamase and AmpC-producing *Escherichia coli* in two New Zealand dairy farm environments

3.1 Introduction

Antimicrobial resistance (AMR) is a complex "One Health" issue which affects human, animal and environmental health [300]. Antimicrobial exposure, particularly the misuse and over-use of antimicrobials, in both human and animal health has been suggested as the main driver of AMR [1, 2]. However, other factors including heavy metal [280, 279, 301, 302] and biocide use [281], and in the dairy farm environment, management practices such as waste milk disposal and feed type, may also influence the development and dissemination of AMR [28].

Multi-drug resistant *Enterobacteriaceae*, particularly AmpC- and extended-spectrum β-lactamase-producing *Enterobacteriaceae* (ESBL-E) are of concern to human health. AmpC and ESBLs are β-lactamase enzymes which confer resistance to first- and third-generation cephalosporins, penicillins and monobactams. The AmpC β-lactamase enzymes also confer resistance to the second-generation cephalosporins and cephamycins [92]. ESBL-E can cause both hospital- [118] and community-acquired infections [119] and have been classified as critical on the World Health Organisation's "Priority Pathogens List" [54]. ESBL genes are often encoded on plasmids which can carry multiple antimicrobial resistance genes (ARGs), resulting in a multi-drug resistance phenotype [50, 48]. In *E. coli*, an AmpC phenotype can arise from mutations in the promoter region of the chromosomal *ampC* gene resulting in putative AmpC hyperproducers [138], or from plasmid-mediated AmpC β-lactamase (pAmpC) genes [177]. Plasmid-mediated resistance is of particular concern as this phenotype can be shared by horizontal gene transfer (HGT) between bacteria.

Globally, AmpC- and ESBL-E have been detected in various agricultural environments

including dairy [7, 192, 194, 156], poultry [303, 304, 305], swine [306, 307] and aquaculture [308]. The prevalence of AmpC- and ESBL-E varies between studies which could partially be due to variation in selection methods, farm management practices and antimicrobial use. Risk factors for AmpC- and ESBL-E on dairy farms include third- and fourth-generation cephalosporin use, increased antimicrobial use in calves [7] and amoxicillin use for AmpC-producing *E. coli* [156]. AmpC- and ESBL-E have also been detected in organic dairy herds with low antimicrobial usage (AMU) [192]. A study using mixed effects logistic regression and population-attributable fractions found that AMU could only explain 22 - 36% of the AmpC/ESBL positive samples on dairy farms in the Netherlands, although this study investigated the selection of AMR rather than introduction [309]. These findings highlight the complexity and multitude of factors involved in the development and transmission of AMR in agricultural environments.

Few studies have investigated the prevalence of AmpC and ESBL-E in pasture-based dairy farm environments such as those found in New Zealand (NZ) where there is relatively low use of antimicrobials in food-producing animals [16, 17]. One regional cross-sectional study of dairy farms in NZ found a low prevalence of ESBL-producing *E. coli* (1 of 116; 0.9%) in pooled faecal samples [25]. No *E. coli* with pAmpC genes were isolated but 7.9% (9 of 114) of faecal samples were positive for putative AmpC hyperproducers with mutations in the promoter region of the *ampC* gene [25]. Similarly, a nationwide cross-sectional study (n=26 farms) in NZ did not detect any ESBL-producing *E. coli* [26] and chromosomal AmpC-producing *E. coli* were isolated from 14% (11 of 78) of pooled faecal enrichments originating from seven farms [26]. Additionally, an NZ study between 2009 - 2010 found no ESBL-producing *E. coli* (0 of 300) from bobby calf carcasses (4 to 10 days old) [196]. Hence, the aims of this study were to utilise culture-based methods to investigate the prevalence of AmpC and ESBL-E from two NZ dairy farm environments over a 15 month period, taking into consideration seasonal variation and farm management practices.

3.2 Methods

3.2.1 Study population and sample collection

The Massey University research farms No. 1 Dairy Farm (referred to hereafter as Dairy 1) and No. 4 Dairy Farm (referred to hereafter as Dairy 4) were recruited for inclusion in this study. The two dairy farms are located in Palmerston North, New Zealand, are <5 kilometres apart and both operate a closed dairy farm system (animals are not introduced into the herd). Dairy 1, located next to the Manawatū River, is a small-scale 142.7 hectares (ha) farm with 261 cows during the study period. Dairy 4 is a larger scale farm of 250 ha and a herd size of 584 cows during the study period. The two farms are pasture-based, with the use of supplementary feed such as silage (pickled pasture) and baleage when required. Both farms have stand-off pads (Dairy 1 with a 200 cow capacity, compared to Dairy 4

which has two, of 150 and 200 cow capacities) and Dairy 4 has a freestall barn (200 cow capacity). The predominant breeds on both farms are Friesian, Jersey and Friesian/Jersey cross breeds. Both dairy farms have a spring calving system, and Dairy 1 uses once a day milking whereas Dairy 4 milks twice a day (morning and afternoon). The two farms use selective dry cow therapy (DCT) and teat sealants are applied to the whole herd. On Dairy 4, farm dairy effluent (FDE) is applied to the paddocks using a traveling irrigator system, which is a common practice on NZ dairy farms. The FDE management strategy on Dairy 1 changed during the study period. From October 2018, the FDE was filtered using the dairy effluent recycling system by Forsi Innovations [310] and applied to paddocks. Due to technical issues, from July 2019 onwards the FDE was not applied to paddocks and was discharged into the Palmerston North sewage system.

Collection of farm environmental samples was on a monthly basis from October 2018 to December 2019 inclusive, spanning a 15 month period. Both farms were sampled on the same day. On each sampling occasion, soil, composite cow faeces from cow pats in a recently grazed paddock, FDE, bulk tank milk (referred to hereafter as milk), milk filters and supplementary feed were collected. Samples were transported to the Hopkirk Research Institute (Massey University, Palmerston North, New Zealand) in a chilly-bin with ice-bricks. Samples were stored at 4°C until processing and were processed within eight hours of collection. The collection method for each sampling type is described in Table 3.1. Farm management practice metadata such as the use of supplementary feed and hygiene practices were collected during sampling visits. AMU was reported as individual animal antimicrobial treatments recorded on farm. The total amount administered for each treatment (mg) was calculated according to the concentration of the product (mg/mL), number of doses and volume (mL). AMU was reported as mg/population correction unit (PCU) calculated using the total active ingredient weight (mg) / herd size / average cow size in NZ (453 kg).

3.2.2 Sample processing

To aid in the growth of *Enterobacteriaceae*, the environmental samples were enriched in buffered peptone water (BPW) (BD Difco^{TM}, Fort Richard Laboratories, Auckland, New Zealand). The enrichment methods are detailed in Table 3.2. Once inoculated in BPW, each sample was mixed by vortex and incubated at 35°C for 18 hours. After incubation, enrichments were mixed by vortex and 1 mL was mixed with glycerol (30% [v/v]) and stored at -80°C.

Table 3.1: Sample collection methods

Sample type	**Source**	**Collection method**
Soil	Recently grazed paddock (grazed the night prior to sampling).	Four soil cores (10 cm depth; 3 cm diameter) collected from near the centre of the paddock. Each soil core taken in a transect line, approximately 1 m apart. The collection of pasture and faeces was avoided.
Faeces	Recently grazed paddock (grazed the night prior to sampling).	Sixteen fresh faecal samples randomly collected from the paddock and pooled as groups of four.
FDE	Dairy 1, two sample collection points were used. Collection point one was the effluent sump by the cow-shed (October 2018 - May 2019) and collection point two was from a grate in the cow-shed (July 2019 - December 2019). For Dairy 4, the FDE was collected from the effluent pond.	Approximately 500 mL FDE collected in two sterile 250 mL Schott bottles attached to the end of a pole.
Milk	Milk vat.	Approximately 1 L collected from the milking vat. On Dairy 4, milk collected from the morning milking. The milk sample collected in October 2019 from Dairy 1 was waste milk (DF0167), rather than bulk tank milk. The sample collection issue was discovered during the analysis of the shotgun metagenomic sequencing data from this sample.
Milk filter	Cow-shed.	Collected by farm staff prior to vat cleaning and stored in a clean bag until collection.
Silage	Silage pit.	Approximately 30 g collected.

Table 3.2: Sample processing methods

Sample type	**Sample volume or weight**	**BPW[a] (mL)**	**Additional steps**
Soil	1 g	9	Soil samples homogenised by hand in the WhirlPak bag.
Faeces	Pea-size amount per sample	15	The prevalence of ESBL-producing *E. coli* was expected to be low, therefore four faecal samples were pooled. A pea-size amount of each faecal sample was transferred using a sterile cotton swab into BPW.
FDE	100 µL re-suspended pellet	9.9	100 µL of the re-suspended pellet from the DNA extraction (refer to section 5.2.1).
Milk	100 µL re-suspended pellet	9.9	100 µL of the re-suspended pellet from the DNA extraction (refer to section 5.2.1).
Milk filter	Sponge swab	25	The milk filters were cut open with flame sterilised scissors. The filter was swabbed with a sponge swab, then stomached for 2 min to extract and wash any microbes present into the BPW.
Silage	10 g	50	Samples stomached for 2 min in BPW.

[a] BPW, Buffered peptone water.

3.2.3 Microbiological methods

Stored frozen enrichments were removed from -80℃ and kept on ice. A loopful of frozen enrichment was directly plated on (i) MacConkey (MC) Agar plates (Fort Richard Laboratories, Auckland, New Zealand) as a positive control to ensure growth of *Enterobacteriaceae*, (ii) MC agar (BD Difco™, Fort Richard Laboratories, Auckland, New Zealand) with 1 µg/mL cefotaxime sodium (Sigma-Aldrich, St. Louis, MO, USA), (iii) MC agar (BD Difco™) with 1 µg/mL ceftazidime pentahydrate (Sigma-Aldrich) and (iv) CHROMagar™ ESBL (CHROMagar, Paris, France). Agar plates were incubated aerobically at 35℃ for 18 hours. After incubation, a minimum of two presumptive *Enterobacteriaceae* representing all distinct colony morphologies from plates (i-iii) were sub-cultured onto Columbia Sheep Blood agar (5% blood) (Fort Richard Laboratories). From CHROMagar™ ESBL, if present a minimum of two presumptive *E. coli* (dark pink to reddish) and *Klebsiella* (metallic blue) colonies were sub-cultured onto fresh CHROMagar™ ESBL to ensure purity using colour differentiation as a guide. The colonies were subsequently sub-cultured onto Columbia Sheep Blood agar (5% blood). The AmpC-producing *E. coli* NZRM4402, the ESBL-producing *Klebsiella pneumoniae* NZRM3681 and the susceptible *E. coli* NZRM916 were used as reference strains for quality control of the selective MC agar and CHROMagar™ ESBL plates. Presumptive *Enterobacteriaceae* pure isolates were stored in brain heart

infusion broth (Oxoid, Hampshire, United Kingdom) containing glycerol (30% [v/v]) at -80℃. Isolates were identified by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS) (Bruker Daltonics, Billerica, CA, USA) using the previously described "on slide formic acid extraction" method [311]. For MALDI-TOF MS analysis, pure cultures were streaked on Columbia Sheep Blood agar (5% blood) either from glycerol stocks stored at -80℃ or culture plates stored at 4℃ and incubated at 35℃ for 18 hours. Briefly, using a sterile toothpick, a sample of a single colony was collected and smeared on the MALDI-TOF MS target plate. Next, 1 µL of 70% formic acid was applied to each spot on the target plate and once dried, 1 µL HCCA matrix was added. The target plate was dried at room temperature and the preparation was checked to ensure a homogeneous appearance for each spot. The target plate was read using MALDI-TOF MS using Bruker Daltonics flexControl (v3.4) and Bruker Daltonics MALDI Biotyper Compass (v4.1.100). Each sample was analysed in duplicate and a score value $\geq$2 indicated species identification.

3.2.4 Antimicrobial susceptibility tests

Confirmed *E. coli* strains isolated from selective agars (ii - iv) outlined above were subjected to Kirby-Bauer disc diffusion antimicrobial susceptibility tests (ASTs) for six antimicrobials of interest and interpreted according to either CLSI [312] or EUCAST [165] guidelines (Table 3.3). Bacterial cultures stored in glycerol broth (section 3.2.3) were inoculated on Columbia Sheep Blood agar (5% blood) and incubated at 35℃ for 18 hours. After incubation, an individual colony was subsequently streaked onto Columbia Sheep Blood agar and incubated at 35℃ for 18 hours. A bacterial suspension was prepared in sterile saline equivalent to a 0.5 McFarland standard by mixing pure colonies with a cotton swab. The bacterial suspension was mixed with a new cotton swab, and a Mueller Hinton agar plate (Fort Richard Laboratories) was swabbed in three directions to achieve a lawn of growth. Antibiotic discs were applied within 15 min and the plates incubated at 35℃ for 16 - 20 hours. An AmpC and ESBL positive phenotype was confirmed for isolates resistant to either cefoxitin and cefotaxime and/or cefpodoxime (Figure 3.1) using either a three-disc (D69C AmpC disc test, Mast Group Ltd., Liverpool, United Kingdom) or double-disc comparison assay (D62C cefotaxime and D64C ceftazidime ESBL disc tests, Mast Group Ltd., Liverpool, United Kingdom), respectively. AmpC β-lactamase enzymes can hydrolyse cefotaxime and ceftazidime and are less effected by ESBL inhibitors such as clavulanic acid [131] which is used in the D62C/D64C ESBL double-disc comparison assays. Therefore, isolates which were AmpC positive and had a zone size which could not be differentiated as either positive or negative ($\geq$2 - $\leq$5mm) for either ESBL double-disc comparison assay were further tested using an assay containing cefepime, which is stable in the presence of *Enterobacteriaceae* with chromosomal *ampC* expression (D63C cefepime ESBL disc test, Mast Group Ltd., Liverpool, United Kingdom). The AmpC-producing *E. coli* NZRM4402 and the ESBL-producing *Klebsiella pneumoniae* NZRM3681 were used as positive controls in the AmpC and ESBL confirmatory disc assays, respectively and the

susceptible *E. coli* NZRM916 was used as a negative control for both assays.

Table 3.3: Discs for antimicrobial susceptibility tests.

Antibiotic disc	**Antibiotic class**	**Con.[a] (µg)**	**Guideline**	**Susceptible zone size (mm)**	**Resistant zone size (mm)**
Cefotaxime (CTX)	β-lactam	30	CLSI	$\geq$26	$\leq$22
Cefoxitin (FOX)	β-lactam	30	CLSI	$\geq$21	$\leq$17
Cefpodoxime (CPD)	β-lactam	10	CLSI	$\geq$18	$\leq$14
Tetracycline (TET)	Tetracycline	30	CLSI	$\geq$15	$\leq$11
Streptomycin (STR)	Aminoglycoside	10	CLSI	$\geq$15	$\leq$11
Ciprofloxacin (CIP)	Fluoroquinolone	5	CLSI	$\geq$26	$\leq$21

[a] Con, Concentration.

3.2.5 Molecular characterisation

Crude DNA was extracted from pure isolates by adding 3-4 colonies to 400 µL sterile molecular biology-grade water. The bacteria were heated at 100℃ for 10 min in a heating block and stored at -20℃. The supernatant was used for subsequent PCR reactions. PCR reactions, control strains and gel electrophoresis conditions are detailed in Appendix A. Briefly, the *E. coli* phylogroup was determined using the Clermont quadruplex PCR Typing method [76]. *E. coli* with an AmpC phenotype were tested for pAmpC gene families using a multiplex PCR [177] and a PCR targeting the bla_{CMY} gene family [313] was used on boiled DNA preparations from isolates positive for the CITM primer set (Table 3.4), indicative of CMY-positive *E. coli*. *E. coli* in which no pAmpC genes were identified were further analysed to identify mutations in the promoter region of the *ampC* gene [138]. The ESBL $bla_{\text{CTX-M}}$ group was confirmed using the CTX-M-1-group PCR [7] for the ESBL-producing *E. coli* which did not undergo whole genome sequencing (WGS).

PCR products were purified using the QIAquick PCR purification kit (QIAGEN, Hilden, Germany) and primers corresponding to the PCR product being sequenced were used in the sequencing reaction (Appendix A). Briefly, separate sequencing reactions were performed in 10 µL reaction volumes, using the same forward and reverse primers, with 1.75 µL buffer, 0.5 µL Big Dye™ Terminator v3.1 (ThermoFisher Scientific Inc., Waltham, MA, USA) and 1 µL primer (3.2 pmol/µL). For sequencing of the *ampC* gene promoter region PCR product (191 bp), 5.75 µL sterile molecular biology-grade water and 1 µL DNA template (3 ng) was used. For sequencing of $bla_{\text{CMY-2}}$ and $bla_{\text{CTX-M-1}}$ PCR products (1,138 and >1000 bp, respectively), 4.75 µL sterile molecular biology-grade water and 2 µL DNA template (12 ng) was used. The sequencing PCR reactions were undertaken on a Bio-Rad T100 Thermal Cycler (Bio-Rad, Waltham, MA, USA) using the following conditions: 95℃ for 1 min, then 25 cycles of 95℃ for 10 sec, 50℃ for 10 sec, and 60℃ for 90 sec. Capillary separation of sequencing reactions was undertaken by the Massey Genome Service using an ABI3730 DNA analyser (Massey University, Palmerston North, New Zealand). DNA sequence chromatograms were trimmed at the proximal 5' and distal 3' end to remove poor

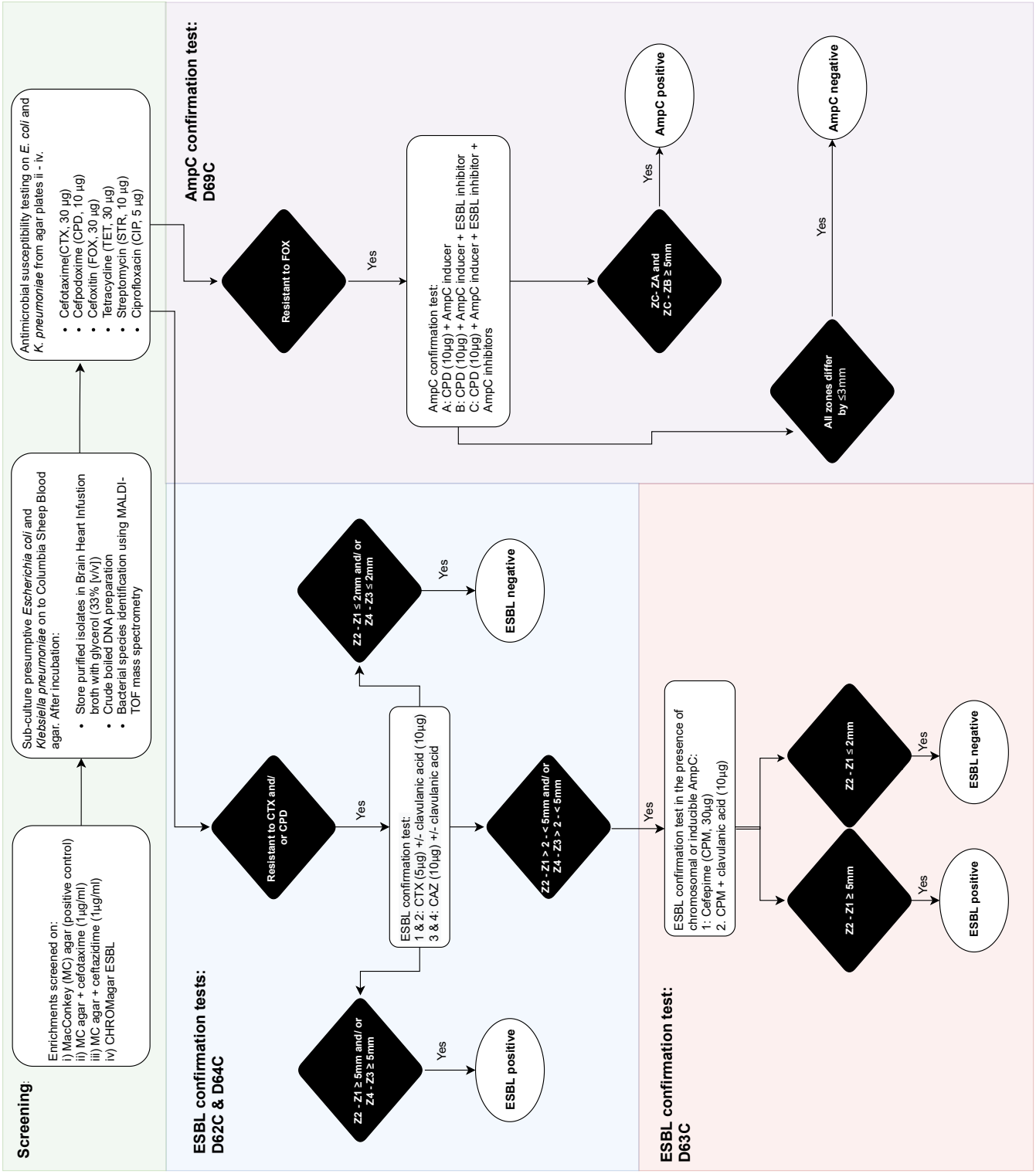

Figure 3.1: Workflow of sample screening, antimicrobial susceptibility testing and ESBL/AmpC confirmation tests

quality sequence using Geneious Prime v2019.1.1 [314, 315] and forward and reverse traces were aligned to form a contiguous DNA sequence representing the complete PCR product. The PCR products were analysed using BLASTN [316].

Table 3.4: PCR primer sequences and resulting amplicon lengths

PCR reaction	**Target**	**Primer set**	**Primer sequence (5' → 3')**	**Product size (bp)**	**Reference**
AmpC promoter region	Chromosomal *ampC*	AmpC1[71] AmpC2[120]	AATGGGTTTTCTACGGTCTG GGGCAGCAAATGTGGAGCAA	191	[138]
pAmpC	MOX-1, MOX-2, CMY-1, CMY-8 to CMY-11	MOXMF MOXMR	GCTGCTCAAGGAGCACAGGAT CACATTGACATAGGTGTGGTG	520	[177]
pAmpC	LAT-1 to LAT-4, CMY-2 to CMY-7, BIL-1	CITMF CITMR	TGGCCAGAACTGACAGGCAAA TTTCTCCTGAACGTGGCTGGC	462	[177]
pAmpC	DHA-1, DHA-2	DHAMF DHAMR	CCGTACGCATACTGGCTTTGC AACAGCCTCAGCAGCCGGTTA	405	[177]
pAmpC	ACC	ACCMF ACCMR	AACAGCCTCAGCAGCCGGTTA TTCGCCGCAATCATCCCTAGC	346	[177]
pAmpC	FOX-1 to FOX-5b	FOXMF FOXMR	CAAAGCGCGTAACCGGATTGG AACATGGGGTATCAGGGAGATG	190	[177]
CMY-group	CMY-group	CMY-2-F CMY-2-R	ATGATGAAAAAATCGTTATGCTGC GCTTTTCAAGAATGCGCCAGG	1,138	[313]
CTX-M-1	CTX-M-1-group	CTX-1-SEQ-F CTX-1-SEQ-R	CCCATGGTTAAAAAATCACTGC CAGCGCTTTTGCCGTCTAAG	>1,000	[313]
Quadruplex	*chuA*	chuA.1b chuA.2	ATGGTACCGGACGAACCAAC TGCCGCCAGTACCAAAGACA	288	[76, 75]
Quadruplex	*yjaA*	yjaA.1b yjaA.2b	CAAACGTGAAGTGTCAGGAG AATGCGTTCCTCAACCTGTG	211	[76]
Quadruplex	TspE4.C2	TspE4C2.1b TspE4C2.2b	CACTATTCGTAAGGTCATCC AGTTTATCGCTGCGGGTCGC	152	[76]
Quadruplex	*arpA*	AceK.f ArpA1.r	AACGCTATTCGCCAGCTTGC TCTCCCCATACCGTACGCTA	400	[76, 317]
Group C	*trpA*	trpAgpC.1 trpAgpC.2	AGTTTTATGCCCAGTGCGAG TCTGCGCCGGTCACGCCC	219	[318]
Group E	*arpA*	ArpAgpE.f ArpAgpE.r	GATTCCATCTTGTCAAAATATGCC GAAAAGAAAAAGAATTCCCAAGAG	301	[318]

3.2.6 DNA extraction, library preparation, and whole genome sequencing

A subset of AmpC- and ESBL-producing *E. coli* were selected for WGS according to phenotype, resistance profile, *E. coli* phylogroup and metadata (including farm, source and collection date). For samples where multiple isolates were identified, only one isolate per molecular and resistance profile combination was included. Bacterial isolates from glycerol broths stored at -80℃ (section 3.2.3) were inoculated on Columbia Sheep Blood agar (5% blood) and incubated for 18 hours at 35℃. An individual colony was subsequently sub-cultured onto a fresh Columbia Sheep Blood agar plate (5% blood) and incubated at 35℃ for 18 hours. An individual colony was inoculated in 4 mL Luria-Bertani Miller broth (Fort Richard Laboratories) and incubated at 35℃ for 15 hours at 200 rpm. Genomic DNA (for both Illumina and MinION sequencing methods) was extracted using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA), according to the manufacturer's instructions for gram negative bacteria. Several modifications were included to optimise the protocol for *E. coli*. Briefly, 2 mL overnight culture was centrifuged at 13,000 x *g* for 2 min to pellet the cells which were treated with RNAse at 37℃ for 1 hour. After the Protein Precipitation Solution was added to the cell lysate and incubated on ice for 5 min, the sample was centrifuged at 13,000 x *g* for 3 min, the supernatant transferred to a clean tube and centrifuged again at 13,000 x *g* to reduce any residual protein contamination. The DNA was re-hydrated overnight at 4℃ in 100µl 10mM Tris-HCl (Geneaid Biotech Ltd, New Taipei City, Taiwan).

The DNA concentration was quantified using a Qubit 4.0 fluorometer (ThermoFisher Scientific Inc., Waltham, MA, USA) and $A_{260/280}$ and $A_{260/230}$ ratios determined using the Nanodrop microvolume spectrophotometer (Nanodrop 2000c, ThermoFisher Scientific Inc., Waltham, MA, USA). DNA integrity and size was visualised on a 0.8% [w/v] agarose gel using a high molecular weight Lambda DNA/*Hin*d III ladder (ThermoFisher Scientific Inc., Waltham, MA, USA). The libraries were prepared using the Nextera XT DNA library preparation kit (Illumina, San Diego, CA, USA), and sequencing performed using an Illumina MiSeq v3 with 2 x 300 bp paired-end reads (Massey Genome Service, Massey University, Palmerston North, New Zealand). The Nanopore MinION sequencing was performed at the Molecular Epidemiology and Public Health Laboratory (Massey University, Palmerston North, New Zealand) using a R9.4.1 flow cell (Oxford Nanopore Technologies, UK). The libraries were prepared using the Rapid Barcoding Sequencing kit (SQK-RBK004; Oxford Nanopore Technologies, UK) according to the manufacturer's instructions ("Library Preparation"), with minor modifications for *E. coli*. Briefly, 600ng DNA template was used as input. After the beads were washed twice with 70% ethanol, the residual ethanol was removed and the pellet dried for 15 min. The pellet was then re-suspended in 10 µL 10mM Tris-HCl/50mM NaCl and the solution containing the beads and DNA was then incubated at 50℃ for 10 min. The flow cell was primed and loaded according to the manufacturer's instructions and run for 24 - 48 hours (SQK-RBK004,

"Priming and loading the SpotON flow cell", Oxford Nanopore Technologies, UK).

3.2.7 Bioinformatic analysis

Due to a high sequencing depth, the Illumina MiSeq sequencing reads were randomly subsampled down to a 100X genome coverage using Rasusa v0.6.0 [319]. The Illumina MiSeq sequencing reads were subsequently processed using the Nullarbor v2.0 [320] pipeline with default parameters. In summary, adapters were removed from raw reads using Trimmomatic v0.39 [321], species identification by *k*-mer analysis performed using the Kraken v1.1.1 database [322], the genomes were assembled using SKESA v2.4.0 [323] and annotated with Prokka v1.14.6 [324]. The sequence type was determined using mlst v2.19.0 [325] with information downloaded from PubMLST [326], the resistome profile identified with ABRicate v1.0.1 [327] using the Resfinder 4.0 database [216, 328], and the Centre for Genomic Epidemiology website [329] was used to detect the virulence genes and serotype in assembled genomes using the VirulenceFinder 2.0.3 database (v2020-05-29) [73] and the SerotypeFinder 2.0.1 database (v1.0.0), respectively. The presence/absence data from 37 virulence associated genes identified in the whole genome sequences (n=12) was used to construct a hierarchical cluster tree using Jaccard distances and the tree was further annotated using the Interactive Tree of Life webserver [330]. The core single nucleotide polymorphism (SNP) variation was assessed using Snippy v4.4.3 [331] with a ST131 ESBL-producing *E. coli* JJ1887 as the reference sequence (Genbank accession: CP014316). A maximum-likelihood tree was generated from the core SNP alignment using a general time-reversible model with the Randomised Axelerated Maximum Likelihood (RAxML) Next-Generation tool [332] and visualised in GrapeTree [333].

For long-read data, the MinION fast5 sequencing read files were basecalled using Guppy v4.2.2. The reads were de-multiplexed using qcat v1.1.0 [334] and adapters removed with Porechop v0.2.4 [335] using default settings. Filtlong v0.2.0 [336] was used to trim the reads with a minimum length of 1 kb, the lowest quality bases filtered (10%) and the best reads kept up to 500 Mb. Hybrid assemblies were generated using Unicycler v0.4.9b [337] with default settings. Plasmids from hybrid assemblies were re-constructed and typed using MOB-suite v1.4.9.1 [338, 339] and annotated with Prokka v1.14.6 [324] using a custom database consisting of the best-match "nearest neighbour" plasmids as identified by MOB-suite v1.4.9.1 (Genbank accessions: CP009566, CP015997, CP018107, CP016585 and KF362121). For pMLST, variants within IncI group plasmids and Inc groups for short-read data were identified using PlasmidFinder v2.1 [340]. Plasmid core genome variation for selected isolates was assessed using Snippy v4.6.0 [331] with pDF0049.2e_1 as the reference (Table 3.7). Clusters of Orthologous Groups (COG) were identified using eggNOG-mapper v2 [341, 342, 343] and plasmid annotations visualised using Mauve [344] and Geneious Prime v2019.1.1 [314, 315]. The plasmid *oriC* region was identified using DoriC v10.0 [345] and the figures were edited using Inkscape v0.92.1 [346].

3.2.8 Statistical tests

Statistical tests were performed in Minitab® 19.1.1 [347] using a one-way analysis of variance (ANOVA) with a 95% confidence interval for sample prevalence comparisons.

3.3 Results

Of the two dairy farms visited, 101 enrichments from Dairy 1 and 103 enrichments from Dairy 4 were resuscitated on four agar plates: MC agar, MC supplemented with either 1 µg/mL cefotaxime or 1 µg/mL ceftazidime and CHROMagar™ ESBL plates. From the selective agar plates, 52 putative third-generation cephalosporin resistant *Enterobacteriaceae* (n=24 Dairy 1; n=28 Dairy 4) were isolated and the species confirmed as *E. coli* using MALDI-TOF MS (Appendix B). No *Klebsiella pneumoniae* were isolated from the selective agar plates.

3.3.1 Antimicrobial resistance profiles

The susceptibility of *E. coli* isolated in this study (n=52) to six clinically relevant antimicrobials (Table 3.3), including three β-lactams, was examined (Figure 3.2; Appendix C). All of the *E. coli* (n=52) were resistant to cefpodoxime, 46 of 52 (89%) to cefoxitin and 34 of 52 (65%) were resistant or intermediate (15 of 52, 29%) to cefotaxime. The high levels of resistance to the second- and third-generation cephalosporins reflects the selective methods used to isolate AmpC- and ESBL-E in this study. Numerous isolates were resistant to streptomycin (41 of 52; 79%) and tetracycline (33 of 52, 64%) and all isolates (n=52) were susceptible to ciprofloxacin, which is a critically important antimicrobial in human medicine [155]. According to phenotypic testing, 33 of 52 *E. coli* (64%) were multi-drug resistant, but phenotypic testing was only performed using antibiotics representing four classes (β-lactams, tetracyclines, aminoglycosides and fluoroquinolones).

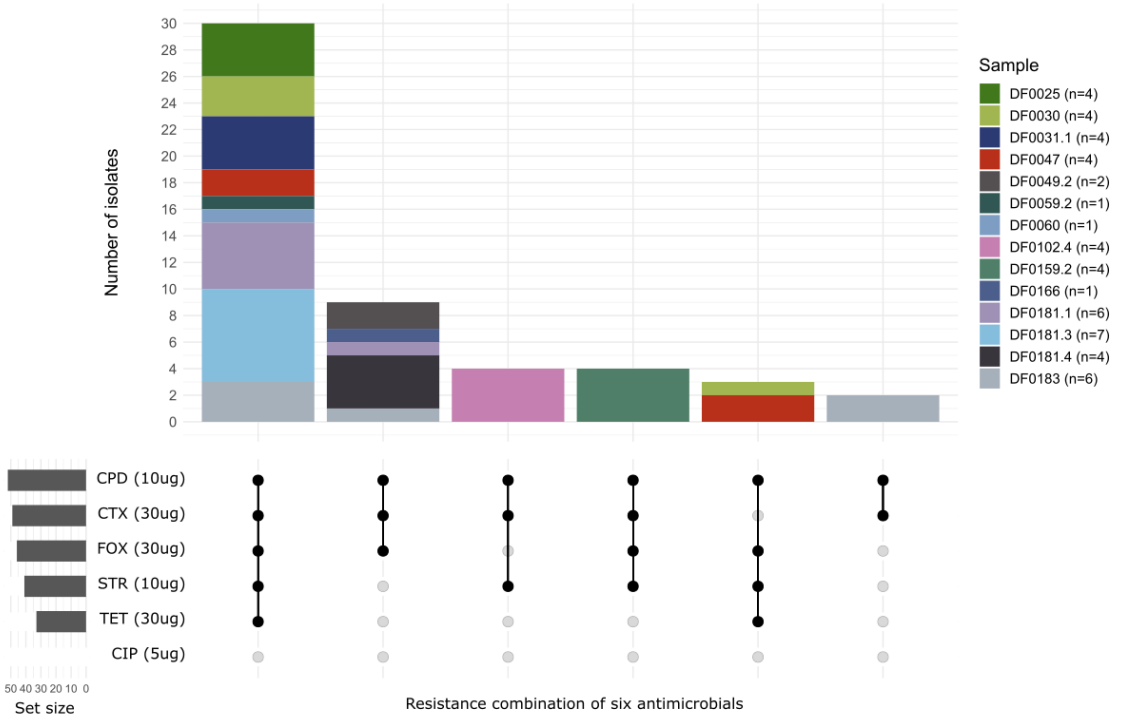

Figure 3.2: Resistance profiles of *E. coli* (n=52) isolated across 14 farm samples. Isolates with intermediate resistance to cefotaxime (n=15) were grouped as resistant in the Upset plot.

[a] CPD, cefpodoxime; FOX, cefoxitin; STR, streptomycin; CTX, cefotaxime; TET, tetracycline; CIP, ciprofloxacin

All 52 *E. coli* were tested for ESBL production (Figure 3.1) and two strains isolated from the same FDE sample were confirmed as ESBL producers (DF0183c and DF0183g). All 52 *E. coli* were tested for AmpC production and 46 AmpC-producing *E. coli* were identified (46 of 52, 88.5%). The two ESBL-producing *E. coli* were AmpC negative and four isolates from the same sample (DF0102.4e-h) were both ESBL and AmpC negative according to the phenotypic confirmation tests.

3.3.2 Molecular characterisation of AmpC- and ESBL-producing *E. coli*

E. coli isolates (n=52) were differentiated into phylogroups using the Clermont Typing method [76]. The predominant phylogroups were B1 (18 of 52, 35%) and C (17 of 52, 33%), followed by E (6 of 52, 12%), D (5 of 52, 10%), A (4 of 52, 8%) and F (2 of 52, 4%). The $bla_{\text{CTX-M-15}}$ gene was identified in the ESBL-producing *E. coli* (DF0183c and DF0183g). *E. coli* with an AmpC phenotype (n=46) were investigated for pAmpC genes, and the $bla_{\text{CMY-2}}$ gene was detected in 28 isolates (28 of 46, 61%). For the remaining 18 AmpC-producing *E. coli*, mutations were identified in the promoter region of the *ampC* gene. Interestingly, 27 of 28 (96%) isolates with the $bla_{\text{CMY-2}}$ gene were isolated from

Dairy 4. All putative AmpC hyperproducers (n=18) were isolated from Dairy 1. The 18 isolates were all phylogroup C and compared to a susceptible *E. coli* reference strain (ATCC® 25922™), had mutations in the promoter region of the *ampC* gene at positions -42 (C → T), -18 (G → A), -1 (C → T) and +58 (C → T), excluding DF0025c in which position -42 could not be determined (positions relative to the *E. coli* K12 transcriptional start base (+1) [348]). No pAmpC or ESBL genes were detected in the four *E. coli* which were resistant to cefotaxime and cefpodoxime but were AmpC and ESBL negative in the phenotypic testing (DF0102.4e-h). Therefore, the mechanism for resistance to third-generation cephalosporins was unassigned for these isolates (designated as unknown in subsequent figures).

Interestingly, the 15 strains with intermediate resistance to cefotaxime were all putative AmpC hyperproducers, with mutations in the promoter region of the *ampC* gene. The remaining isolates shared identical mutations (n=3) yet were all susceptible to cefotaxime. In comparison, all AmpC-producing *E. coli* with pAmpC genes (n=28) were resistant to cefotaxime. The difference in AST cefotaxime zone sizes (mm) from 15 of 46 distinct AmpC-producing *E. coli* clones (excluding *E. coli* with an identical phylogroup and AST profile which were isolated from the same sample) were compared and plasmid-mediated AmpC-producing *E. coli* had larger zone sizes (Appendix D), however a larger sample size is required to perform any statistical comparisons.

3.3.3 Population structure and comparative genomics

A subset of AmpC- and ESBL-producing *E. coli* were analysed using WGS to understand the genomic epidemiology and transmission dynamics of these bacteria on farm (Table 3.5). The *E. coli* were selected for WGS according to phenotype, resistance profile (focusing on isolates with pAmpC genes and ESBL producers), *E. coli* phylogroup and metadata (including farm, source and collection date). *E. coli* isolates with a plasmid-mediated AmpC-producing phenotype (n=5), ESBL-producing (n=1) and an unassigned mechanism for resistance to third-generation cephalosporins (n=1) were also selected for MinION long-read sequencing, with a focus on generating complete genomes and examining plasmids in detail. For samples where multiple isolates were identified, only one isolate per molecular and resistance profile combination was selected for WGS.

The population structure of the AmpC- and ESBL-producing *E. coli* which were sequenced in this project (n=12) was diverse and the isolates belonged to eight sequence types including ST56 (3 of 12, 25%), ST57 (2 of 12, 17%), ST88 (2 of 12, 17%) and singletons for ST442, ST973, ST2541, ST4553 and ST5135 (1 of 12, 8%) (Table 3.5). The three ST56 and two ST88 strains had identical serotypes (O8/O40:H21 and O8/O32:H19, respectively). The remaining isolates (n=7), including the two ST57 strains, had different serotypes. According to SerotypeFinder, the O serogroup was non-typeable for three strains. All isolates had a genome size ranging from 4,839,855 - 5,431,661 bp and a GC content between 51.7

- 52.0%.

Core genome SNP analysis separated the strains into eight clusters, each representing a different ST (Figure 3.3). The separation of clusters according to ST was reflected in the large number of SNPs identified in the core genome of these strains (102,841 SNPs), accounting for approximately 2.0% of the *E. coli* genome. However, within two clusters genetically similar isolates representing two STs were identified. Three *E. coli* with the $bla_{\text{CMY-2}}$ gene were isolated in December 2019 on Dairy 4 from different samples and all belonged to ST56, were serotype O8/O40:H21 and were genetically similar (58 - 65 SNPs; isolates DF0181.1c, DF0181.3c and DF0183e). Similarly, two putative AmpC hyperproducers had the same serotype, were isolated in December 2018 and January 2019 from Dairy 1 and differed by 82 SNPs (DF0031.1c and DF0047c). In each case, the *E. coli* were isolated from both faeces (ST56 n=2; ST88 n=1) and FDE (ST56 n=1; ST88 n=1), but the number of SNPs is too high to infer recent within farm transmission. In contrast, the two isolates belonging to ST57 were isolated from separate farms and were genetically distinct (6,750 SNPs).

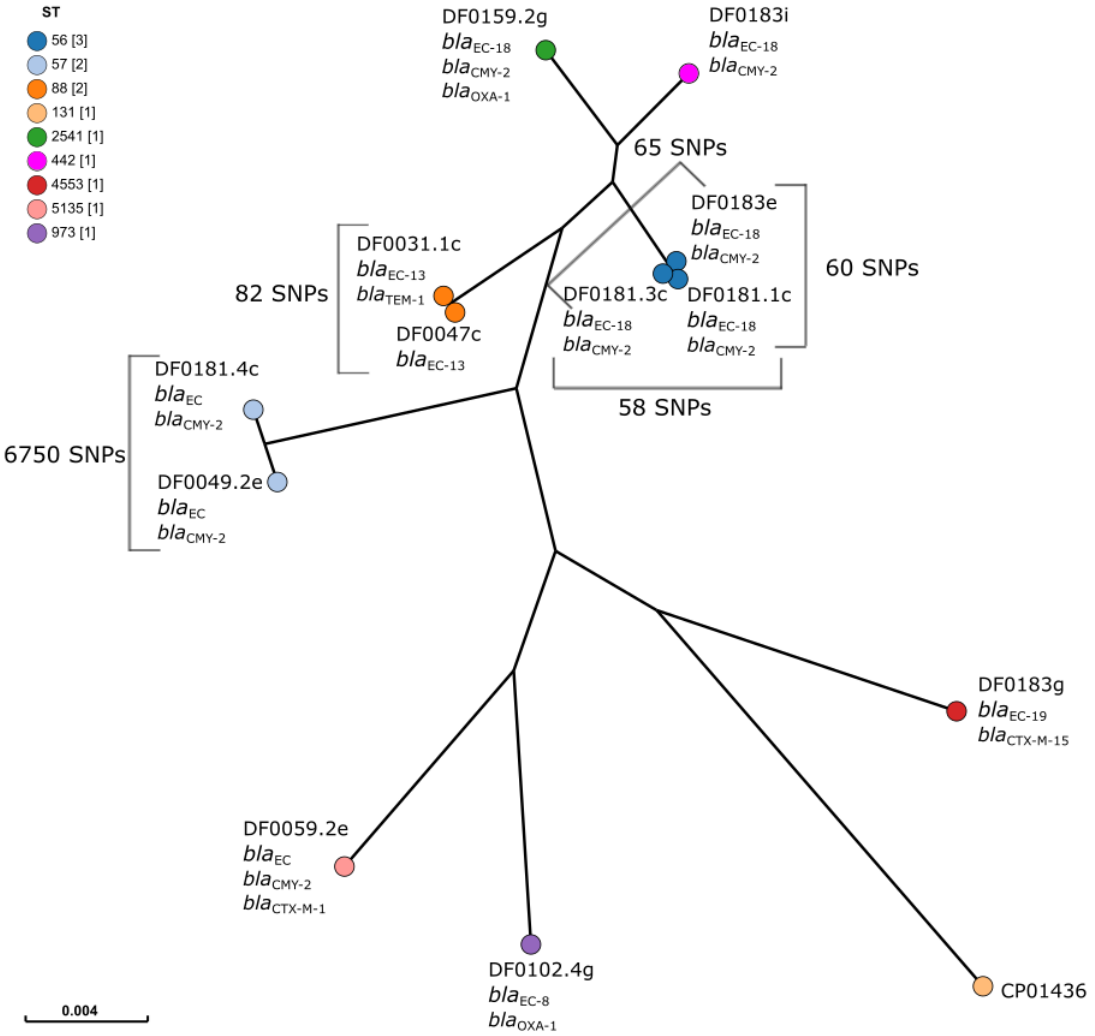

Figure 3.3: Maximum-likelihood tree of core genome single nucleotide polymorphism (SNP) analysis of AmpC and ESBL-producing *E. coli* (n=12). *E. coli* CP014316 (ST131, $bla_{\text{CTX-M-15}}$ positive) was used as the reference and nodes are coloured by sequence type, as indicated in the figure legend. The scale bar indicates the proportion of the core genome alignment over which core SNPs have been calculated. The number of SNPs between isolates in the same cluster is indicated on the figure.

Table 3.5: Genome composition of *E. coli* (n=12) sequenced in this study

Isolate[a]	**Farm**	**Source**	**Collection date**	**Phylo-group**	**ST**	**Serotype**[b]	**Phenotype**[c]	**Genome size (bp)**	**Contigs**[d]	**GC** (%)	**Plasmids**
DF0031.1c	1	Faeces	12/2018	C	88	O8/O32:H19	AmpC	5,180,929	91	51.9	IncFIB, IncFII, IncQ1
DF0047c	1	FDE	01/2019	C	88	O8/O32:H19	AmpC	5,157,783	90	51.9	IncFIB, IncFII, IncQ1
DF0049.2e[a]	1	Faeces	01/2019	E	57	O27:H18	pAmpC	5,047,883	86 (2*)	51.8	IncI1
DF0059.2e[a]	4	Faeces	02/2019	D	5135	ONT:H26	pAmpC/ESBL	5,437,661	99 (4*)	51.7	IncI1, IncY, IncFIA/IncQ1
DF0102.4g[a]	1	Faeces	05/2019	D	973	ONT:H15	Unknown	5,041,356	81 (4)	51.7	IncI1, IncI1/IncFII
DF0159.2g[a]	4	Faeces	10/2019	A	2541	ONT:H7	pAmpC	4,869,780	80 (5)	51.9	IncI1
DF0181.1c[a]	4	Faeces	12/2019	B1	56	O8/O40:H21	pAmpC	5,199,529	192 (3)	51.9	IncI1
DF0181.3c	4	Faeces	12/2019	B1	56	O8/O40:H21	pAmpC	5,202,415	194	52.0	IncI1
DF0181.4c	4	Faeces	12/2019	E	57	O124/O164:H25	pAmpC	4,961,348	83	51.9	IncI1
DF0183e[a]	4	FDE	12/2019	B1	56	O8/O40:H21	pAmpC	5,201,499	194 (2*)	51.8	IncI1
DF0183g[a]	4	FDE	12/2019	F	4553	O83:H42	ESBL	5,001,896	70 (1*)	51.8	None
DF0183i	4	FDE	12/2019	B1	442	O146:H21	pAmpC	4,839,955	69	51.8	IncI1/ IncFIB

[a] Isolates sequenced using both Illumina MiSeq and Oxford Nanopore Technologies.
[b] ONT, O non-typeable.
[c] pAmpC, Plasmid-mediated AmpC β-lactamase; AmpC, *E. coli* with mutations in the promoter region of the *ampC* gene.
[d] Hybrid assembly contig numbers in brackets. Closed genomes indicated by an asterisk.

3.3.4 Antimicrobial resistance genes

Analysis of WGS data of 12 *E. coli* confirmed the presence of the β-lactam resistance genes $bla_{\text{CMY-2}}$ (8 of 12, 66.7%), $bla_{\text{OXA-1}}$ (2 of 12, 16.7%), $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-15}}$ (1 of 12 each, 8.3%) (Table 3.6). Surprisingly, the $bla_{\text{CTX-M-1}}$ gene was detected in the sequence data for isolate DF0059.2e, which was negative using the double-disc ESBL confirmation test (D62C cefotaxime and D64C ceftazidime ESBL disc tests) as described in sections 3.2.4 and 3.3.1. However, DF0059.2e was AmpC positive and harboured the $bla_{\text{CMY-2}}$ gene. AmpC β-lactamases are not inhibited by ESBL inhibitors such as clavulanic acid (Table 2.1), which may explain the negative ESBL confirmation test. Subsequent ESBL confirmation tests using an assay containing cefepime, which is stable to hydrolysis by AmpC β-lactamases, were carried out for isolate DF0059.2e and other AmpC-producing *E. coli* that had a zone size which could not be differentiated as either positive or negative using the ESBL double-disc tests (n=3) (Figure 3.1). The other three *E. coli* were confirmed as ESBL negative, whereas DF0059.2e was ESBL positive. Therefore, isolate DF0059.2e was confirmed as both AmpC and ESBL positive using phenotypic and genotypic testing and co-harboured the $bla_{\text{CMY-2}}$ and $bla_{\text{CTX-M-1}}$ genes. The remaining genotypic results were in agreement with the confirmed ESBL and AmpC phenotypes. All isolates carried the bla_{EC} gene (a synonym for the *ampC* gene [132]) and gene-specific variation was observed which was broadly associated with the different *E. coli* phylogroups.

Other ARGs including *mph(A)*, *catA1*, *dfrA5*, *dfrA17*, *sul1* and *sul2* were identified during analysis of the assembled genomes but representative phenotypes were not established. Initial PCR methods failed to identify any ESBL and AmpC genetic determinants associated with the four DF0102.4e-h isolates that were resistant to third-generation cephalosporins (cefotaxime and cefpodoxime). Further analysis of DF0102.4g using WGS also failed to determine the genetic basis for the AMR phenotype, therefore the resistance mechanisms is currently unassigned, but this isolate carried the $bla_{\text{OXA-1}}$ gene which encodes a narrow-spectrum Class D β-lactamase.

Table 3.6: Resistance profiles of AmpC- and ESBL-producing *E. coli* (n=12)

Isolate	**ST**[a]	**AST**[b] **phenotype**	**β-lactam resistance genes**[c]	**Other ARGs**[d]
DF0031.1c	88	CPD, FOX, TET, STR	$bla_{\text{EC-13}}$, $bla_{\text{TEM-1}}$	*aph(3")-Ib*, *aph(3')-Ia*, *aph(6)-Id*, *dfrA5*, *sul1*, *sul2*, *tet(A)*
DF0047c	88	CPD, FOX, TET, STR	$bla_{\text{EC-13}}$	*aph(3")-Ib*, *aph(3')-Ia*, *aph(6)-Id*, *sul2*, *tet(A)*
DF0049.2e	57	CTX, CPD, FOX	bla_{EC}, $bla_{\text{CMY-2}}$	None
DF0059.2e	5135	CTX, CPD, FOX, TET, STR	bla_{EC}, $bla_{\text{CMY-2}}$, $bla_{\text{CTX-M-1}}$	*aac(3)-IId*, *aadA5*, *aph(3")-Ib*, *aph(3')-Ia*, *aph(6)-Id*, *catA1*, *dfrA17*, *mph(A)*, *sul1*, *sul2*, *tet(B)*
DF0102.4g	973	CTX, CPD, STR	$bla_{\text{EC-8}}$, $bla_{\text{OXA-1}}$	*aadA1*, *sul1*
DF0159.2g	2541	CTX, CPD, FOX, STR	$bla_{\text{EC-18}}$, $bla_{\text{CMY-2}}$, $bla_{\text{OXA-1}}$	*aadA1*, *sul1*
DF0181.1c	56	CTX, CPD, FOX, TET, STR	$bla_{\text{EC-18}}$, $bla_{\text{CMY-2}}$	*aph(3")-Ib*, *aph(6)-Id*, *sul2*, *tet(B)*
DF0181.3c	56	CTX, CPD, FOX, TET, STR	$bla_{\text{EC-18}}$, $bla_{\text{CMY-2}}$	*aph(3")-Ib*, *aph(6)-Id*, *sul2*, *tet(B)*
DF0181.4c	57	CTX, CPD, FOX	bla_{EC}, $bla_{\text{CMY-2}}$	None
DF0183e	56	CTX, CPD, FOX, TET, STR	$bla_{\text{EC-18}}$, $bla_{\text{CMY-2}}$	*aph(3")-Ib*, *aph(6)-Id*, *sul2*, *tet(B)*
DF0183g	4553	CTX, CPD	$bla_{\text{EC-19}}$, $bla_{\text{CTX-M-15}}$	None
DF0183i	442	CTX, CPD, FOX	$bla_{\text{EC-18}}$, $bla_{\text{CMY-2}}$	None

[a] Sequence type.

[b] Antimicrobial susceptibility testing; CPD, cefpodoxime; FOX, cefoxitin; STR, streptomycin; CTX, cefotaxime; TET, tetracycline.

[c] bla_{EC} is a synonym for the *ampC* gene [132].

[d] Antimicrobial resistance genes defined in Appendix E.

3.3.5 Virulence factors and *E. coli* pathotypes

The *E. coli* sequenced in this study carried a range of virulence factors identified with VirulenceFinder [73], which were mainly involved in adhesion, protection/serum resistance, iron uptake and toxins, hemolysins, proteases or autotransporters (Figure 3.4). *E. coli* strains of the same ST clustered together. The number of virulence factors each *E. coli* harboured varied, with strains DF0049.2e and DF0183i carrying the fewest virulence factors (n=4 each) and DF0031.1c and DF0047c carrying the most (n=27 each). All *E. coli* harboured *terC*, which is involved in tellurite resistance [349].

Five *E. coli* strains were putatively classified as avian pathogenic *E. coli* (APEC), a subgroup of extraintestinal pathogenic *E. coli* due to the presence of the following virulence factors: salmochelin siderophore receptor (*iroN*), outer membrane protease (*ompT*) and increased serum survival (*iss*) (in DF0181.1c, DF0181.3c and DF0183e) as well as ferric aerobactin receptor (*iutA*) and avian hemolysin (*hlyF*) which were also detected in strains DF0031.1c and DF0047c. These virulence factors have been associated with APEC and are often encoded on plasmids [350]. No *E. coli* harboured the Shiga toxin genes (*stx1* or *stx2*) or the locus of enterocyte effacement pathogenicity island, indicative of Shiga-toxin producing *E. coli* (STEC) or enteropathogenic *E. coli* (EPEC), respectively [81]. Three strains carried the gene for the enteroaggregative immunoglobulin repeat protein Air (*air*) which is commonly found in enteroaggregative *E. coli* (EAEC), however other virulence genes associated with this pathotype were not detected (*EAST-1* and *pic*) [350]. Five strains harboured enterotoxigenic *E. coli* (ETEC) associated fimbriae F17 (*f17A* and *f17G*) encoding genes, although no other ETEC-related toxin or fimbriae genes were detected in these strains; two of these strains, DF0031.1c and DF047c, were previously identified as APEC.

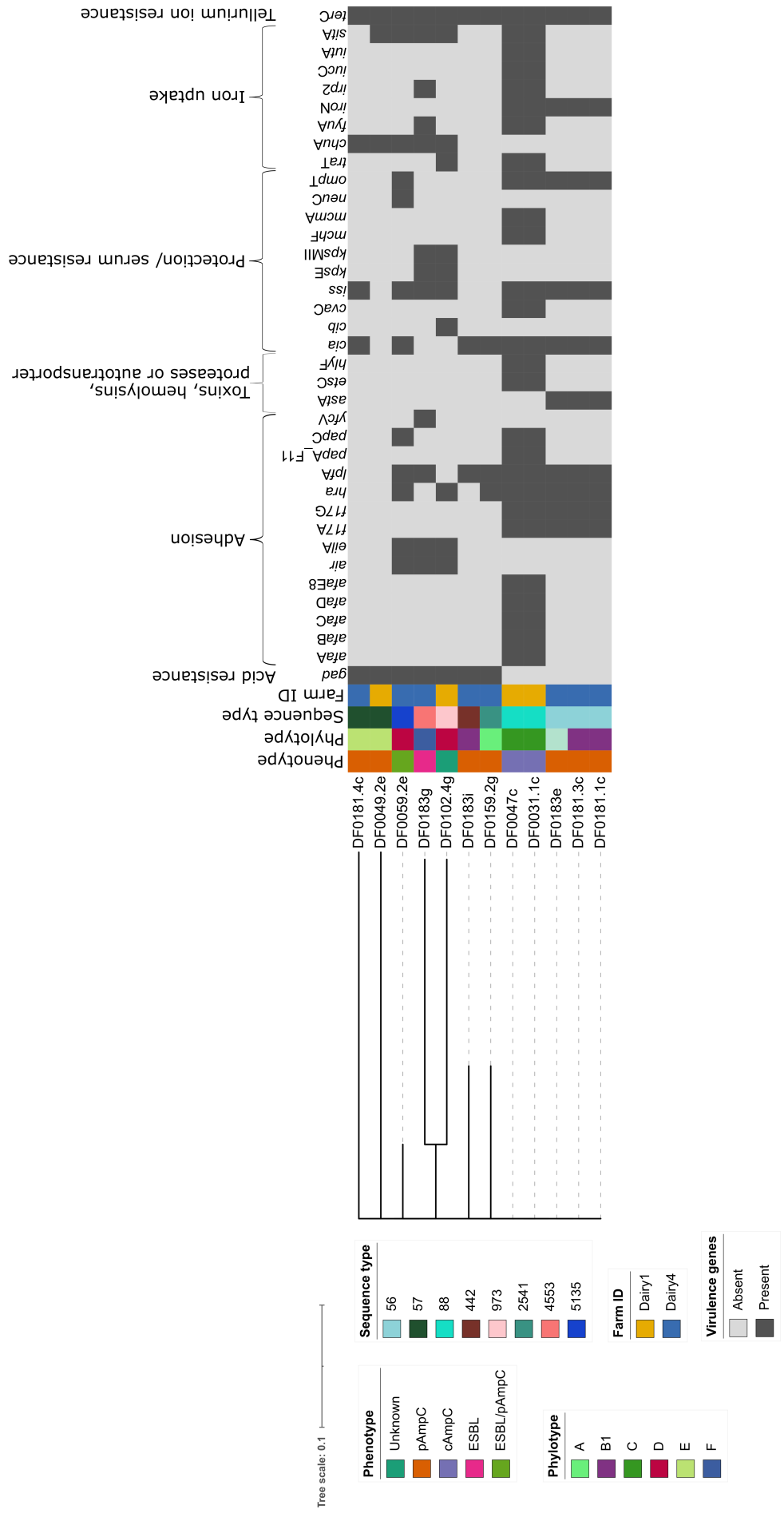

Figure 3.4: Hierarchical cluster tree constructed using Jaccard distances for the presence or absence data from 37 virulence genes identified using VirulenceFinder. The tree was edited using the Interactive Tree of Life webserver. Isolate metadata is included for AmpC or ESBL phenotype, farm and sequence type, as indicated by the colour keys. The virulence genes are grouped and annotated by general function.

3.3.6 Plasmid characteristics

A large proportion of the AmpC- (8 of 10, 80%) and ESBL-encoding (1 of 2, 50%) genes identified from *E. coli* isolated within this study are plasmid-associated. To examine the plasmids in more detail, seven isolates were also sequenced using MinION long-read sequencing technology (Table 3.5). If present, the plasmids were re-constructed and analysed from *E. coli* hybrid assemblies (n=7) using MOB-suite [338, 339].

Four isolates carried one plasmid, one isolate carried two and one isolate carried three plasmids (Table 3.7). No plasmids were identified from the assembled sequence data of DF0183g, instead the $bla_{\text{CTX-M-15}}$ gene was chromosomally encoded. All plasmids contained a relaxase and mate-pair formation marker, and were therefore classified as conjugative except for plasmid pDF0059.2e_2 which belonged to the IncY group and was non-mobilisable. DF0059.2e also carried an IncFIA/IncQ1 plasmid of unknown pMLST, which was 244,307 bp in size (the nearest plasmid neighbour was the *E. coli* strain T23 multi-drug resistant plasmid pEQ1; Genbank accession: KF362121). The pDF0059.2e_3 plasmid was annotated and is included in Appendix F. This plasmid carried multiple ARGs (n=12), potentially conferring resistance to aminoglycoside, β-lactam, phenicol, trimethoprim, macrolide, sulfonamide and tetracycline antibiotic classes (Table 3.7). Physical linkage of ARGs and the location of mobile genetic elements on pDF0059.2e_3 is shown in Figure 3.6. In addition, a partial copy of the $bla_{\text{TEM-105}}$ gene (279 bp; 31.82% coverage), the *catB4* gene (106 bp; 19.49% coverage) and the *aadA1* gene (166 bp; 17.18% coverage) were detected in this plasmid using ABRicate v1.0.1 [327] and the Resfinder 4.0 database [216, 328]. However, the partial copy of the *catB4* and *aadA1* genes could not be confirmed by visual inspection of the annotated plasmid. The genetic region surrounding the partial $bla_{\text{TEM-105}}$ gene and the upstream IS26 transposase was extracted (2,438 bp) from the pDF0059.2e_3 plasmid and aligned to the complete $bla_{\text{TEM-105}}$ reference gene sequence (Genbank accession number: NG_050150, 966 bp). Pairwise alignment showed that 274/279 bp of the partial $bla_{\text{TEM-105}}$ gene was an identical match to the distal end of the reference gene sequence, with the IS26 transposase disrupting the upstream region of the $bla_{\text{TEM-105}}$ gene (Figure 3.5). We can hypothesise that plasmid pDF0059.2e_3 once harboured a complete copy of the $bla_{\text{TEM-105}}$ gene and a recombination event involving IS26 transposase disrupted this gene sequence.

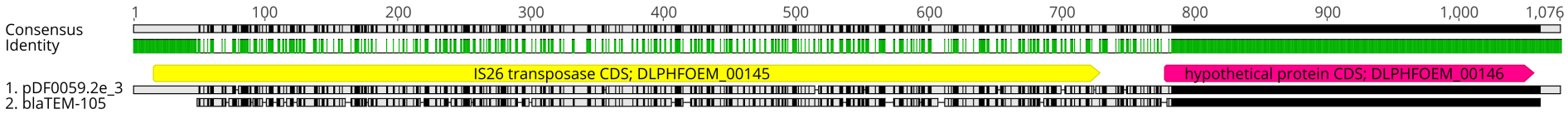

Figure 3.5: The 279 bp partial region of the $bla_{\text{TEM-105}}$ gene was located directly downstream of a IS26 transposase. The complete $bla_{\text{TEM-105}}$ gene reference sequence (accession number: NG_050150; 966 bp) was aligned to the genetic region surrounding the partial $bla_{\text{TEM-105}}$ gene in the pDF0059.2e_3 plasmid. The extracted region was 2,483 bp in size and encompassed the partial copy of the $bla_{\text{TEM-105}}$ gene and the IS26 transposase directly upstream. The bases are shaded according to nucleotide sequence similarity, with black indicating a perfect match.

Five strains carried IncI1 plasmids (also called IncIα) that were pMLST 23, had a similar number of coding sequences (101-109), encoded the *bla*$_{\text{CMY-2}}$ gene and ranged in size from 89,859 - 94,357 bp. Using the MOB-suite database (consisting of 17,779 complete plasmids) the most similar reference to the IncI1 plasmids identified using Mash distances, was the *Salmonella enterica* subspecies enterica serovar Newport plasmid pCVM22462 (Genbank accession number: CP009566.1). Core genome SNP analysis of these five plasmids, using pDF0049.2e_1 as the reference, suggested they were genetically similar (Table 3.8). Strikingly, there were no SNP differences between the plasmid-core genome of DF0181.1c and DF0183e, which were isolated from composite faeces and FDE respectively on Dairy 4 in December 2019 (Table 3.5). The core genome of these isolates was also genetically similar, differing by only 65 SNPs (Figure 3.3). *E. coli* DF0181.3c was isolated from the same sampling month as DF0181.1c and DF0183e and had an identical serotype and resistance profile. IncI1 plasmid incompatibility factors were detected in the draft genome for DF0181.3c, and therefore this isolate may carry a similar plasmid as the aforementioned strains, however this plasmid could not be reconstructed and compared in detail in the absence of long-read sequencing data (Figure 3.5). Plasmid DF0049.2e_1 was annotated as a representative of the IncI1 plasmids and is included in Appendix G and the location of the *bla*$_{\text{CMY-2}}$ gene, mobile genetic elements and the *tra* genes involved in the conjugal transfer system is shown in Figure 3.6.

According to the plasmid core genome, plasmid pDF0059.2e_1 was the most genetically dissimilar to the other four plasmids (19 - 25 SNPs); this plasmid is a different pMLST, size and Inc group compared to the other plasmids analysed in this study (Table 3.7). Plasmid pDF0049.2e_1 was associated with an *E. coli* isolated from Dairy 1 whereas the remaining plasmids originated from *E. coli* isolated on Dairy 4. Interestingly, pDF0049.2e_1 was genetically similar (1 - 26 SNPs) to the other four *bla*$_{\text{CMY-2}}$ positive pMLST 23 plasmids (Table 3.8). Of the plasmid core SNPs with annotations (n=7), five resulted in missense mutations in either the conjugal transfer proteins (n=2), pilus assembly protein PilO (n=2) or in a methyltransferase (n=1) (Appendix H). One synonymous SNP was detected in a hypothetical protein and plasmid pDF0059.2e_1 had a complex mutation (mixture of SNPs and multiple nucleotide polymorphisms) resulting in an absent start site in a DNA primase CDS. Annotated comparison of the five plasmids (Appendix I) reflects the similarity and gene synteny among these plasmids. All plasmids showed variation near the shufflon protein (Appendix J), a genetic region involved in conjugation of IncI1 plasmids [351].

Illumina WGS of two chromosomal mediated AmpC-producing *E. coli* identified additional ARGs which potentially confer resistance to aminoglycoside, trimethoprim, sulfonamide and tetracycline antibiotics (trimethoprim and sulfonamide resistance was not phenotypically confirmed) as well as plasmid incompatibility factors. These findings suggest that these isolates harbour additional ARGs, some of which are likely encoded on plasmids due to the co-location of ARGs (*aph(3")-Ib*, *aph(6)-Id*, *sul2*) and plasmid incompatibility factors (IncQ1) on the same contig and therefore could potentially spread via HGT. IncQ is a group of mobilisable plasmids with a broad host range that are not often reported in

the literature [47]. Interestingly, IncQ plasmids harbouring the *sul2-strA-strB* gene cluster (synonyms for *aph(3")-Ib* and *aph(6)-Id*, respectively) were detected in *S. enterica* serovar Typhimurium phage type 9 isolates from bovine and human sources in Australia [352]. The plasmids and ARGs in isolates DF0031.1c and DF0047c could be further elucidated by the addition of long-read sequencing data which would enable a hybrid assembly to be generated, allowing for a high resolution analysis and clarification of this issue.

VirulenceFinder was used to detect virulence genes carried on the plasmids. The five IncI1 plasmids harboured virulence genes encoding colicins, namely *cia* (n=4, $bla_{\text{CMY-2}}$ positive plasmids) and *cib* (n=1, pDF0102.4g_1). Plasmid pDF0102.4g_2 harboured the *traT* gene, which encodes an outer membrane protein involved in complement resistance, although the *traT* gene is also a part of the transfer operon in conjugative plasmids and is involved in surface exclusion between identical or closely related plasmids by preventing stable mating aggregates [353]. Plasmids pDF0059.2e_2 and pDF0059.2e_3 did not carry any known virulence genes.

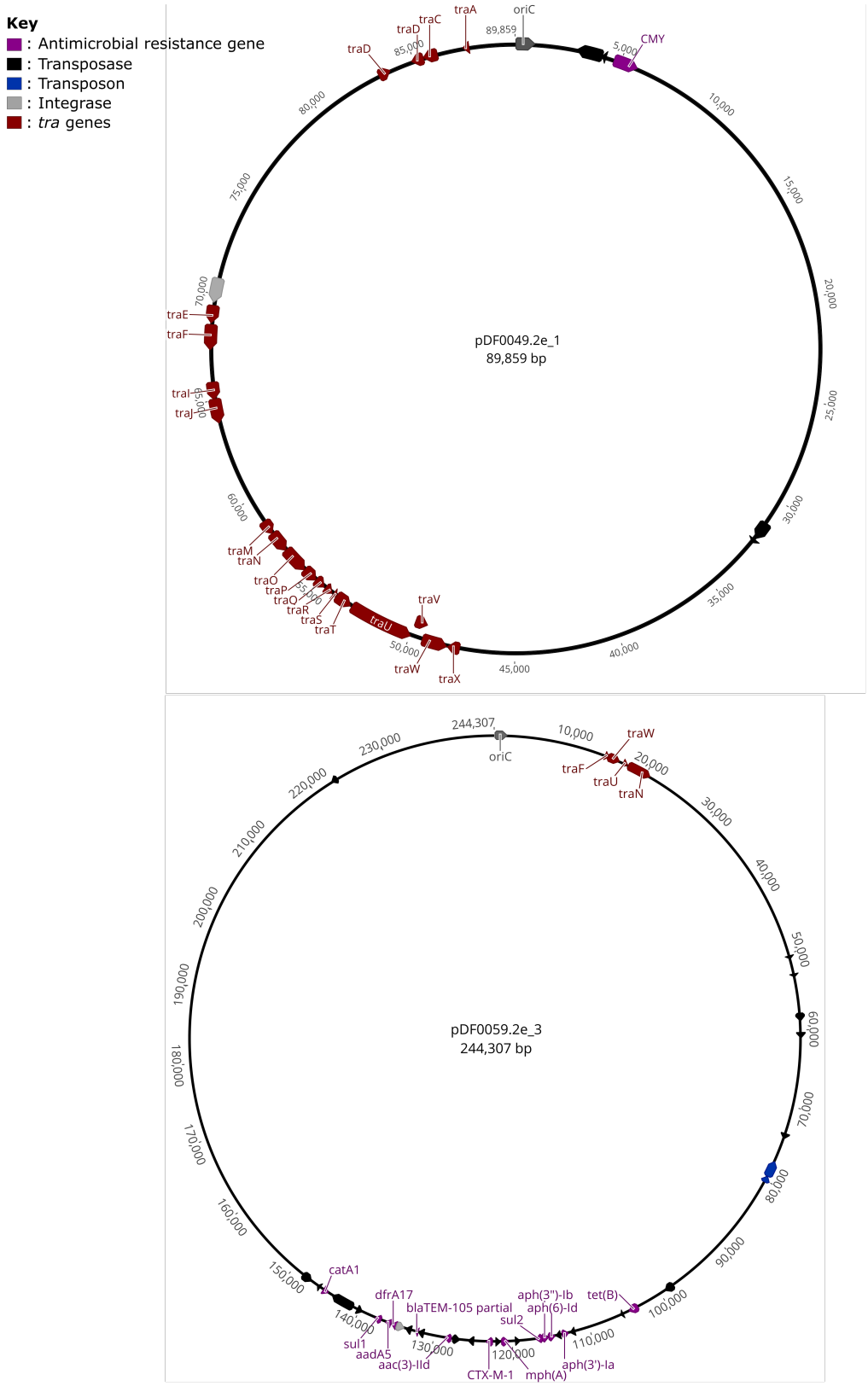

Figure 3.6: Representative IncI1 complete circular plasmid pDF0049.2e_1 and IncFI-A/IncQ1 plasmid pDF0059.2e_3 assembled from MinION and Illumina sequencing data showing physical linkages of antimicrobial resistance genes and mobile elements. The *tra* genes are involved in the conjugal transfer system.

Table 3.7: Characteristics of plasmids from *E. coli* sequenced with short-read Illumina and long-read MinION methods

Isolate	**Plasmid**	**Size (bp)**	**pMLST**	**Inc group**	**CDS[a]**	**ARGs[b]**	**Partial ARGs[c]**
DF0049.2e	pDF0049.2e_1	89,859	23	IncI1	102	$bla_{\text{CMY-2}}$	None
DF0059.2e	pDF0059.2e_1	94,357	23	IncI1	109	$bla_{\text{CMY-2}}$	None
DF0059.2e	pDF0059.2e_2	97,734	-	IncY	108	None	None
DF0059.2e	pDF0059.2e_3	244,307	$[F- : A8 : B-]$	IncFIA/IncQ1	277	*aac(3)-IId*, *aadA5*, *aph(3")-Ib*, *aph(3')-Ia*, *aph(6)-Id*, *catA1*, $bla_{\text{CTX-M-1}}$, *dfrA17*, *mph(A)*, *sul1*, *sul2*, *tet(B)*	*aadA1* (17.2%, 96.0%) *catB4* (19.5%, 100%) $bla_{\text{TEM-105}}$ (31.8%, 100%)
DF0102.4g	pDF0102.4g_1	97,225	26	IncI1	109	$bla_{\text{OXA-1}}$, *sul1*, *aadA1*	*aadA1* (17.4%, 97.1%) *catB4* (19.5%, 100%)
DF0102.4g	pDF0102.4g_2	58,773	$[F35 : A- : B-]$	IncI1/IncFII	64	None	None
DF0159.2g	pDF0159.2g_1	89,920	23	IncI1	101	$bla_{\text{CMY-2}}$	None
DF0181.1c	pDF0181.1c_1	90,151	23	IncI1	103	$bla_{\text{CMY-2}}$	None
DF0183e	pDF0183e_1	92,691	23	IncI1	106	$bla_{\text{CMY-2}}$	None

[a] CDS, Coding sequence.

[b] ARGs, Antimicrobial resistance genes defined in Appendix E.

[c] Partial ARGs, ARGs in which the coverage is less than 80%. Numbers in brackets represent the percentage of coverage and identity, respectively.

Table 3.8: Core genome single nucleotide polymorphism analysis of IncI1 plasmids carrying the $bla_{\text{CMY-2}}$ gene identified in this study

Plasmid	**pDF0049.2e_1**	**pDF0059.2e_1**	**pDF0159.2g_1**	**pDF0181.1c_1**	**pDF0183e_1**
pDF0049.2e_1	0	26	1	9	9
pDF0059.2e_1	26	0	25	19	19
pDF0159.2g_1	1	25	0	8	8
pDF0181.1c_1	9	19	8	0	0
pDF0183e_1	9	19	8	0	0

3.3.7 Prevalence of AmpC- and ESBL-producing *E. coli* from farm environmental samples

Pooled faecal samples were investigated for AmpC- and ESBL-producing *E. coli* (Table 3.9). One out of 60 (1.7%) pooled faecal and one out of 15 (6.7%) FDE samples from Dairy 4 were positive for ESBL-producing *E. coli*, with one and two isolates cultured from these samples, respectively. No ESBL-producing *E. coli* were isolated from pooled faeces and FDE from Dairy 1 nor from soil or milk samples from either farm. AmpC-producing *E. coli* were isolated from faeces (2 of 60, 3.3%; 5 of 60, 8.3%) and FDE (5 of 13, 38.5%; 1 of 15, 6.7%) on Dairy 1 and Dairy 4, respectively and none were isolated from soil (0 of 30) or milk (0 of 26) from either farm. The sample level prevalence of AmpC-producing *E. coli* isolated from Dairy 1 and Dairy 4 ($p = 0.526$) and between sample types (faeces or FDE; $p = 0.408$) was not statistically significant. Across the study, multiple isolates were obtained from the same sample on the selective agar plates and some of which were examined in more detail to determine if they were clonal.

Table 3.9: Number of positive AmpC- and ESBL-producing *E. coli* samples and isolates

Farm	**Sample type**[a]	**ESBL-producing *E. coli*[b]**		**AmpC-producing *E. coli***	
		No. samples (%)	**No. isolates**	**No. samples (%)**	**No. isolates**
Dairy 1	FDE	0/13	0	5/13 (38.5%)	14
	Faeces	0/60	0	2/60 (3.3%)	6
	Milk	0/13	0	0/13	0
	Soil	0/15	0	0/15	0
Dairy 4	FDE	1/15 (6.7%)	2	1/15 (6.7%)	4
	Faeces	1/60 (1.7%)	1	5/60 (8.3%)	22
	Milk	0/13	0	0/13	0
	Soil	0/15	0	0/15	0

[a] FDE, Farm dairy effluent.
[b] One isolate was both AmpC and ESBL positive (DF0059.2e) and has been included in both columns.

Low numbers of ESBL-producing *E. coli* were isolated from Dairy 4 and none were isolated from Dairy 1. Dairy 4 had a higher prevalence of AmpC-producing *E. coli* from faeces (8.3%), whereas Dairy 1 had a higher prevalence in FDE (38.5%). The effluent management strategy on Dairy 1 changed during the study period (section 3.1) and compared to the first collection point where four samples were positive for AmpC-producing *E. coli* (4 of 7; 57.1%), only one sample (1 of 6; 16.7%) from the second collection point was positive.

3.3.8 Estimated antimicrobial use on dairy farms between October 2018 and December 2019

Between October 2018 and December 2019 the estimated AMU on Dairy 1 and Dairy 4 was 17.09 mg/PCU and 5.36 mg/PCU, respectively (Appendices K and L). The antimicrobial products used on Dairy 1 and Dairy 4 are detailed in Appendix M. The predominant route of antibiotic administration for both Dairy 1 and Dairy 4 was parenteral other/other (Appendix N). Months with higher AMU are consistent with spring calving in NZ. The NZVA has classified antimicrobials as green, yellow or red tier according to the World Health Organisation classes [42]. Of the total AMU used on Dairy 1 and Dairy 4, the majority of antimicrobials were green tier (91.1% and 30.5%), followed by yellow tier (6.2% and 67.9%) and red tier antimicrobials were infrequently used (2.75% and 1.6%), respectively.

Table 3.10: Amount of the antimicrobial classes used on Dairy 1 and Dairy 4 between October 2018 and December 2019

Antimicrobial class	**Dairy 1[a] (mg/PCU)**	**Dairy 4 (mg/PCU)**
Aminoglycosides	0.68 (4.0%)	0.19 (3.5%)
Aminopenicillins	0.05 (0.3%)	0.02 (0.4%)
First-generation cephalosporins	0.23 (1.3%)	2.83 (52.9%)
Third-generation cephalosporins	0.12 (0.7%)	0.04 (0.8%)
Penicillins	15.50 (90.7%)	2.18 (40.7%)
Quinolones	0.35 (2.1%)	0.03 (0.6%)
Tetracyclines	0.17 (1.0%)	0.04 (0.7%)
Macrolides	0	0.02 (0.4%)
Multiple classes	0	<0.01 (0.02%)

[a] mg/PCU, mg per active ingredient per population correction unit (% total PCU).

As shown in Figure 3.7, AmpC-producing *E. coli* were isolated after months of both high and low AMU. On Dairy 1, the highest AMU occurred in October 2018 (12.3%) and August to November 2019 (8.7 - 18.6%); whereas on Dairy 4 the highest AMU predominantly occurred in September 2019 (56.7%) (Figure 3.7 and Appendices K - L). AMU treatments varied between farms, with the predominant reasons for treatment on Dairy 1 including

clinical or subclinical mastitis (65.5%), DCT (9.7%) and between claw/footrot (8.7%). On Dairy 1, 70 cows were treated for mastitis during the study period and half of the cows (35 of 70; 50%) received treatment on multiple occasions which may account for the higher AMU related to mastitis. In comparison, less than 20% of cows on Dairy 4 were treated multiple times for mastitis (7 of 36; 19.4%). The predominant reasons for treatment on Dairy 4 was for a metri-check (used for early identification of cases of endometritis; 35.2%), clinical or subclinical mastitis (22.5%) and DCT (19.7%). Further work is required, including comparing antimicrobial sales data and discussions with the farmers and veterinarians, to further understand the AMU patterns on farm.

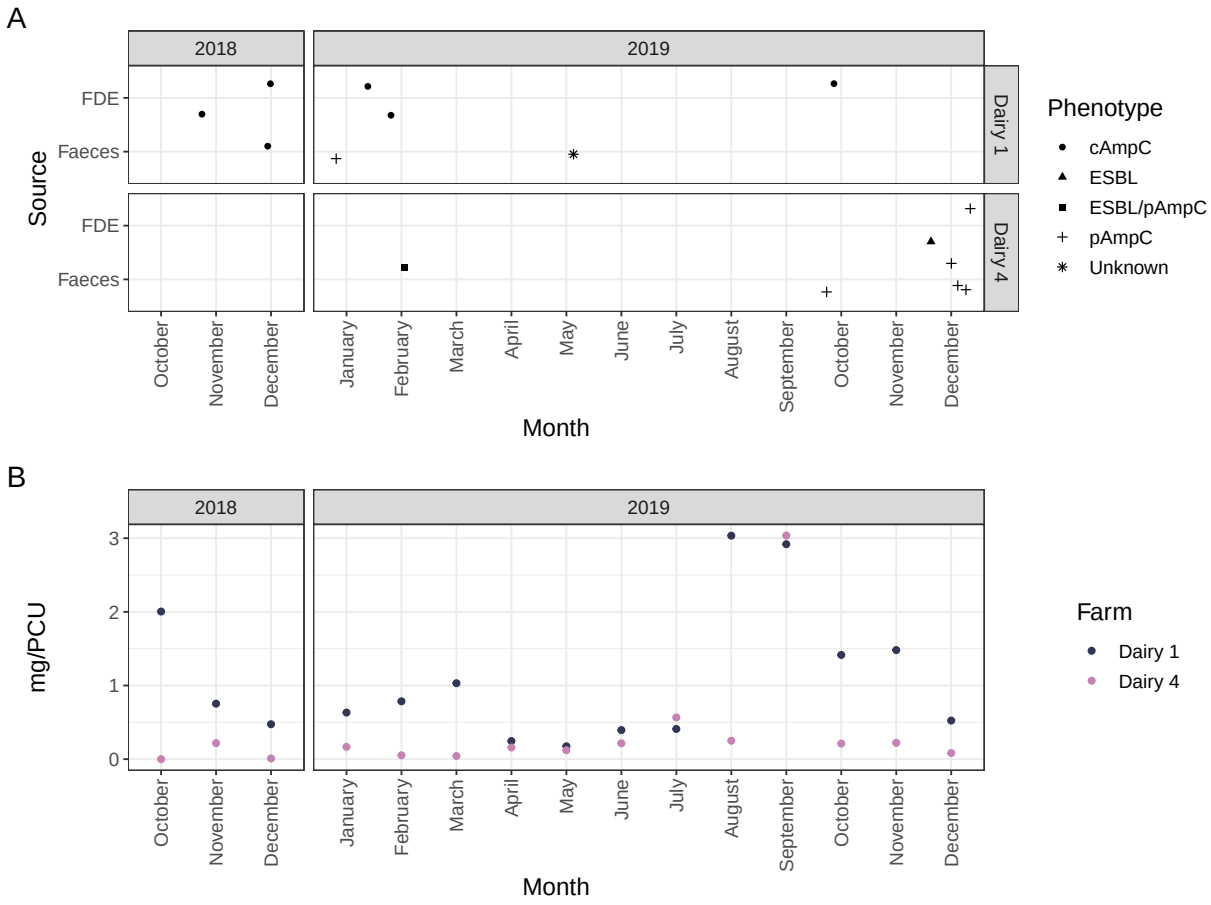

Figure 3.7: **(A)** Collection dates of samples positive for AmpC- and/or ESBL-producing *E. coli* over the 15 month study period. The phenotype is represented by shape as indicated in the figure legend. **(B)** Antimicrobial use per month (mg active ingredient per population correction unit) on Dairy 1 and Dairy 4 during the 15 month study period.

3.4 Discussion

The sample level prevalence of ESBL-producing *E. coli* from faeces and FDE was low on Dairy 4 and they were not detected on Dairy 1 (Table 3.9). These results are consistent with a previous regional-based cross-sectional study of NZ dairy farms, which found a low

prevalence of ESBL-producing *E. coli* in pooled faecal samples and no *E. coli* with pAmpC genes were identified [25]. Similarly, a nationwide cross-sectional study did not detect any ESBL- or plasmid-mediated AmpC-producing *E. coli* [26]. Overseas studies have found a higher prevalence of AmpC- and/or ESBL-producing *E. coli* from dairy farms, with herd level prevalence estimates ranging from 13% on organic dairy farms in the Netherlands [192] to 5.2 - 86.7% on conventional farms [7, 200, 6, 199, 8]. A large study across 53 UK dairy farms sampled a range of faecal contaminated environments and pastureland and found the sample level prevalence to be 5.4% (224 of 4,145) for $bla_{\text{CTX-M}}$ positive *E. coli* [195]. In this study, ESBL-producing *E. coli* were isolated from faeces and FDE samples collected in February and December 2019 respectively, which is during summer in NZ. A recent study suggested that lower monthly ambient temperatures were associated with a lower odds of identifying $bla_{\text{CTX-M}}$ positive *E. coli* samples [195]. Despite this finding, the small number of samples (n=2) positive for ESBL-producing *E. coli* in this study does not allow for any associations between seasonality and ESBL positive samples to be observed.

The sample level prevalence of AmpC-producing *E. coli* in this study was also relatively low (Table 3.9). AmpC-producing *E. coli* were isolated after months of both high and low AMU, highlighting that additional factors other than total AMU may also play a role in the development and transmission of antimicrobial resistant bacteria in the dairy farm environment. *E. coli* with pAmpC genes were infrequently identified on Dairy 1 (1 of 18; 5.6%) and all AmpC-producing *E. coli* isolated from Dairy 4 were plasmid-mediated (27 of 27; 100%; $bla_{\text{CMY-2}}$). This suggests that although Dairy 1 had a higher sample level prevalence of AmpC-producing *E. coli*, particularly in FDE, these putative AmpC hyperproducers predominantly spread by vertical transmission. This is less concerning compared to resistance genes located on mobile genetic elements that can spread via HGT. The differences in sample prevalence from FDE may be due to different effluent management strategies between farms, with Dairy 4 storing FDE in a large pond prior to spraying onto paddocks which likely results in the dilution of bacteria. Univariable analysis on British beef farms found that spreading of farm manure was significantly associated with an animal testing positive for AmpC-producing *E. coli* [284]. The FDE management strategy on Dairy 1 changed during the study period and therefore two FDE sample collection points were used (Table 3.1). On Dairy 1, the majority of the AmpC-producing *E. coli* were isolated from samples collected from the first sampling site (4 of 5 AmpC positive samples; 80%), which were collected from the FDE sump that is smaller in size and may allow for concentration of bacteria. In addition, there may have been pump issues feeding into the sump. On Dairy 1, raw FDE is not applied to paddocks which reduces the risk of transmission of these putative AmpC hyperproducers within the farm environment.

Of the of AmpC-producing *E. coli* isolated during this study (n=46), 18 (39.1%) were putative AmpC hyperproducers. A study of 2,304 extended-spectrum cephalosporin resistant *E. coli* from livestock in the Netherlands found that mutations in the promoter region of the *ampC* gene accounted for a small proportion of the total resistant *E. coli* (217 of 2,034; 9.4%) [354]. However the proportion of putative *ampC* hyperproducing *E. coli* may depend

on the study population as a higher proportion (46.2% of cefotaxime-resistant *E. coli*) was identified across 53 farms in the UK [156, 195]. Higher use of amoxicillin/clavulanate and sampling of faecal samples from the environment of young calves has been associated with an increased risk of identifying putative *ampC* hyperproducers on dairy farms [156] and *in vitro* studies have shown an association between amoxicillin use and AmpC-producing *E. coli* arising from mutations in the promoter region of the *ampC* gene [277, 355]. Amoxicillin/clavulanate are classified as yellow tier antimicrobials by the NZVA, indicating their use should be restricted in NZ veterinary practices [42]. Amoxicillin/clavulanate was not used on Dairy 1 and Dairy 4 during the study period and this study did not include samples from the calf environment. Cephalosporin use (including third-generation cephalosporins) was not identified as a risk factor for putative *ampC* hyperproducing *E. coli* [156] and third-generation cephalosporin use on Dairy 1 and Dairy 4 was very low during the 15 month study period (<1% total mg/PCU). The lack of known risk factors for putative AmpC hyperproducers on Dairy 1 highlights the complexity of factors involved in the development of AMR and suggests additional studies are required to identify risk factors for AmpC hyperproducers, particularly in pasture-based dairy farms.

Infections caused by AmpC/ESBL positive *Enterobacteriaceae* pose significant treatment option challenges for clinicians, with cefepime (a fourth-generation cephalosporin) or carbapenems being suggested as the main treatment options [356], both of which are critically important antimicrobials for human medicine [98]. The co-occurrence of plasmid-mediated AmpC and ESBL positive *Enterobacterales* has been reported to be relatively uncommon in clinical studies [356], although the prevalence likely differs between study populations. For example, a higher proportion of ESBL-producing *E. coli* and *Klebsiella* spp. were also AmpC positive (30%; 69 of 230) from human clinical samples in Iran [357]. One *E. coli* isolated from faeces in this study (1.9%, 1 of 52) was both AmpC and ESBL positive, carrying the $bla_{\text{CMY-2}}$ and $bla_{\text{CTX-M-1}}$ genes on two distinct plasmids (Table 3.7). A low proportion of *E. coli* isolates displayed an AmpC/ESBL phenotype in a study of dairy and beef cattle and sheep farms in Spain (5.2%, 7 of 135 isolates) [9]. One AmpC/ESBL positive isolate was sequenced in the aforementioned study, and this isolate co-harboured the $bla_{\text{CTX-M-14}}$ gene and a mutation in the *ampC* gene promoter region, confirming the AmpC/ESBL phenotype [9]. A cross-sectional study of dairy farms in Canada (n=102) also found a low proportion of AmpC/ESBL positive *E. coli* (2%) in comparison to AmpC (51%) and ESBL (46%) phenotypes [194]. These findings suggest that *E. coli* displaying both an AmpC and ESBL phenotype are infrequently isolated from dairy farm environments and that the AmpC/ESBL genetic determinants co-harboured by such *E. coli* may differ.

The ESBL phenotype for isolate DF0059.2e was not detected in the initial ESBL confirmation tests which is likely due to the production of AmpC β-lactamases which is less affected by ESBL inhibitors such as clavulanic acid. The ESBL gene was identified using WGS, and the subsequent ESBL phenotype confirmed using cefepime (fourth-generation cephalosporin) which is stable to AmpC hydrolysis. This highlights the importance of

using both culture-based and culture-independent methods in parallel, in particular using genotypic methods to confirm a resistant phenotype and vice versa. These findings suggest that although relatively uncommon in dairy farm environments, additional ESBL confirmation tests using a fourth-generation cephalosporin may be required for *E. coli* with plasmid-mediated AmpC β-lactamases which may mask ESBL production in confirmation tests that rely on third-generation cephalosporin and clavulanic acid combinations.

AmpC- and ESBL-producing *E. coli* were not detected in soil or bulk tank milk samples on either farm. Despite a small sample size, the lack of detection from these matrices over a 15 month period indicates that they are less likely sources of AmpC- and ESBL-producing *E. coli* in the NZ dairy farm environment, whereas faeces and FDE are more probable sources. At the sample level, $bla_{\text{CTX-M}}$ or $bla_{\text{CMY-2}}$ positive *E. coli* were infrequently detected from soil samples across 17 commercial beef farms in the USA (3.89%; 3 of 77) [174]. The prevalence of AmpC- and ESBL-E from bulk tank milk was more varied. A study of bulk tank milk samples representing 100 dairy farms in Switzerland found no ESBL-E positive samples (0%; 0 of 100) [207], and bulk tank milk samples from conventional and organic dairy farms in the Czech Republic found that 0.7% of *E. coli* (2 of 270) displayed an ESBL phenotype [212]. Some studies have reported ESBL-E at a higher prevalence in bulk tank milk; analysis of a large number of bulk tank milk samples in Germany found 9.5% (82 of 866) were positive for ESBL-E [209], 8.75% (7 of 80) bulk tank milk samples were positive for ESBL-producing *K. pneumoniae* in Indonesia [213] and 16.9% (12 of 71) of milk samples from individual healthy cows were positive for ESBL-producing *E. coli* in Malaysia [208]. In the Malaysian study, 229 faecal samples collected from cattle of various ages across ten farms identified only one faecal sample (1 of 229; 0.4%) which was positive for ESBL-producing *E. coli*, indicating a higher prevalence in milk samples compared to faeces in this study population. The increased detection of ESBL-producing *E. coli* from milk than faeces is unexpected, and may be due to environmental contamination on the udder prior to milk collection. In addition, the milk samples analysed were hand-milked from individual lactating cows [208], whereas bulk tank milk is often used in other studies. Kamaruzzaman *et al.*, [208] also isolated ESBL-producing *E. coli* from the farm environment including drinking water, house flies and water sources but not floor, feed or water trough swabs or supplementary feed. A recent study analysing raw bulk tank milk from dairy farms (n=195) in Northern China found 34.4% (67 of 195) of milk samples were positive for *E. coli* and β-lactamase genes were identified in 34.3% of *E .coli*, specifically the bla_{SHV} (1.5%), bla_{TEM} (20.9%), bla_{CMY} (10.4%) and $bla_{\text{CTX-M}}$ (1.5%) genes [211], however only a limited number of β-lactamase resistance genes were tested. In addition, *E. coli* with the bla_{CMY} and $bla_{\text{CTX-M}}$ genes were not phenotypically confirmed as AmpC- or ESBL-producing *E. coli*, respectively. The lack of detection of ESBL-E in bulk tank milk in NZ is not unexpected due to the stringent hygiene and food safety standards for dairy farming and milk storage in NZ [358].

It is important to consider variations in study design (sample size and animal age/health status), sample matrices and culture selection methods when comparing between prevalence

studies. For example, this study did not use a pre-enrichment step for AmpC- and ESBL-producing *E. coli* prior to plating on selective agar, which is a technique used in some prevalence studies. The age of the study population is also a crucial factor to consider. A study of 101 dairy farms in Canada detected AmpC- or ESBL-producing *E. coli* at least once during the study in 85% of farms, although the majority were isolates from calves [194]. A longitudinal study of a dairy farm previously identified as ESBL positive in the UK found a higher proportion of *E. coli* positive for the $bla_{\text{CTX-M}}$ gene in milking cows (30.3%) compared to non-milking cows (3.0%) [198], although this study did not look for other ESBL enzyme types. Antimicrobial use, including products containing third- and fourth-generation cephalosporins, was higher in the site used to house the lactating and calving cows, however, other management factors also differed between the cattle groups studied including location on the farm [198]. This study focused only on adult dairy cattle and did not examine the prevalence in young calves or non-milking cows. It is hypothesised that a higher prevalence of AmpC- and ESBL-producing *E. coli* would be present in faeces from young calves compared to adult dairy cows. A study analysing recto-anal mucosal swabs from waste milk fed dairy calves (n=40) in NZ did not identify any ESBL-producing *E. coli* and 25% of enrichments (10 of 40) were positive for AmpC-producing *E. coli*, all of which were putative AmpC hyperproducers [27]. Given the higher prevalence of AmpC- and ESBL-E in young calves globally, this could be an area of further research in NZ.

Variation in farming systems between countries is also important when comparing studies. Intensive farming systems, particularly indoor housing, have been associated with a higher prevalence of mastitis [269], which can lead to higher AMU and subsequently increased levels of AMR. It has been proposed that the NZ pasture-based farming system, in conjunction with low AMU in food-producing animals, may contribute to lower levels of AMR [28]. The low sample level prevalence of AmpC- and ESBL-producing *E. coli* from Dairy 1 and Dairy 4 supported this hypothesis. A cross-sectional study of ruminant farming systems in Spain found beef cattle herds and sheep flocks, which have a semi-intensive pasture-based farming system, had significantly lower herd level prevalence of cefotaxime-resistant *E. coli* compared to dairy cattle, which are generally housed inside pens [9]. Similarly, a cross-sectional study of 60 beef and 52 dairy cattle herds in Germany found a lower sample level prevalence of cefotaxime-resistant *E. coli* in beef cattle (35%, 161 of 455) compared to dairy cattle (48%, 156 of 323) [190]. Factors linked to less intensive farming practices were associated with fewer positive samples for both beef and dairy cattle [190]. A cross-sectional study of grazing beef cattle farms (n=17) in the USA found that larger farming operations (>500 cattle) were associated with a 58% higher likelihood of detecting cefotaxime resistant bacteria from faecal samples [275]. Interestingly, soil, water (ponds and water troughs) and forage samples had a higher prevalence of cefotaxime resistant bacteria compared to faecal samples, although bacteria isolated from MC agar containing 4 µg/mL cefotaxime were classified as cefotaxime resistant bacteria and the cefotaxime minimum inhibitory concentration of only 87 of 3,175 isolates was confirmed [275]. In addition, the bacteria were not identified to the species level [275], therefore a high abundance of bacteria with intrinsic resistance to cefotaxime, such as *M. morganii* and *S. marcescens*

which have an inducible chromosomal AmpC β-lactamase [134], may have accounted for the higher prevalence from environmental samples. Another study found samples collected from pasture (collected by traversing pasture areas where animals were grazing or publicly accessible farmland with sterile over-boot socks) had a lower odds of *E. coli* positive for $bla_{\text{CTX-M}}$, compared to samples collected from the rest of the farm environment (collected by traversing various faeces contaminated environments of lactating cows, dry cows, heifers and calves with sterile over-boot socks) [195].

Both farms in this present study operated a closed herd system, which may reduce the risk of introductions of antimicrobial resistant bacteria into the herd by not introducing animals from outside sources to the farm. For example, on British beef farms, buying bulls or fattening cattle have been identified as risk factors for $bla_{\text{CTX-M}}$ positive *Enterobacteriaceae* or AmpC-producing *E. coli*, respectively [284]. However, the impact that introductions of antimicrobial resistant bacteria has compared to AMR selection in agricultural environments is unknown.

Interestingly, Dairy 1 had a higher total AMU during the study period, yet no ESBL-producing *E. coli* were detected on Dairy 1 (Appendices K and L). However, a significant proportion of the total AMU on Dairy 1 was classified as green tier antimicrobials by the NZVA with Dairy 4 using a higher proportion of yellow tier antimicrobials. The variation, predominantly green on Dairy 1 and yellow tier classes on Dairy 4, is likely associated with the main treatments per farm. For example, Dairy 4 had a higher proportion of endometritis treatments in which cephapirin (a first-generation cephalosporin) was used (yellow tier; Appendix M). Other factors may have an impact on the AMU between farms, such as the average age of the cows as the incidence of clinical mastitis is higher in older cows [359]. The estimated use of antimicrobials in all food-producing animals in NZ in 2018 was 10.21 mg/PCU [17]. The representative total AMU during the study period on Dairy 1 and Dairy 4 was slightly higher (17.09 mg/PCU) and lower (5.36 mg/PCU) than this estimate, respectively (Figure 3.7). The AMU on D1 and D4 was also within the range reported in a cross-sectional study of 26 dairy farms across NZ (4.39 - 20.92 mg/PCU) [26], albeit at the higher and lower end of the spectrum, respectively. Although the total AMU was estimated using sales data in the aforementioned study, individual antimicrobial treatments were used for calculating the total AMU in this study, which makes comparisons difficult. The use of third-generation cephalosporins (<1% total mg/PCU), which have been identified as a risk factor for ESBL-E [7], and NZVA red tier classified antimicrobials was very low on both farms during the study period (Table 3.10 and Appendices M - L). AMU between farms likely differs due to a number of management factors including average age of the herd, disease outbreaks, hygiene practices, the use of teat sealants as well as the farmers perception towards antimicrobial stewardship.

Multiple distinct *E. coli* clones were isolated from one composite faecal sample (DF0181.1) and one FDE sample (DF0183), both collected in December 2019 from Dairy 4. From sample DF0181.1, one *E. coli* isolate had a different AST profile compared to the other

isolates (1 of 6 *E. coli*) and was susceptible to tetracycline and streptomycin (Appendix C), suggesting that two distinct *E. coli* clones were isolated from this sample. Two ESBL- and four AmpC-producing *E. coli* were isolated from the FDE sample DF0183. However, the AST profile of one AmpC-producing *E. coli* differed compared to the other *E. coli* (n=3) as this strain was susceptible to tetracycline and streptomycin whereas the other strains were resistant to these antimicrobials, indicating three distinct *E. coli* clones were isolated from sample DF0183. These findings highlight the importance of using multiple selective agar plates to enrich and isolate AmpC and ESBL-producing *E. coli* and suggests that multiple colonies from each agar plate should be isolated and analysed in prevalence studies. Analysing only one presumptive AmpC- and ESBL-E per sample may underestimate the true prevalence.

The AmpC- and ESBL-producing *E. coli* which were sequenced in this study belonged to a diverse range of STs and serotypes. Similar findings have been reported for $bla_{\text{CMY-2}}$-positive *E. coli* from human clinical cases, livestock and food matrices in which a diverse range of STs were reported [360]. In contrast *E. coli* ST131, which are frequently multi-drug resistant and harbour $bla_{\text{CTX-M}}$ genes, are widely disseminated in humans globally [361, 362]. The two sequenced putative AmpC hyperproducers belonged to ST88 (phylogroup C), which is consistent with previous findings that AmpC hyperproducers predominantly belong to this sequence type [156, 203]. In contrast, three putative AmpC hyperproducing *E. coli* isolated from dairy cattle in NZ belonged to ST1148 (n=2) and ST298 (n=1) [25]. In comparison to previous studies in which AmpC hyperproducers often belonged to *E. coli* phylogroup A [363, 157], the 18 *E. coli* in this study with mutations in the promoter region of *ampC* gene were phylogroup B1 (n=1) or C (n=17). AmpC hyperproducing *E. coli* belonging to phylogroups B1 and C have also been reported from faecal samples on dairy farms in the UK [156]. Identical mutations in the promoter region of the *ampC* gene to those seen in the AmpC-producing *E. coli* in this study (n=18) have been previously described [348, 138]. The mutations at positions -42 and -18 change the secondary structure of the -35 and -10 boxes, respectively and increase the separation distance between the two boxes to 17 bp, resulting in an alternative displaced stronger promoter and thus putative AmpC β-lactamase hyperproduction and increased resistance [140, 139]. The mutation at the -42 position (C $\rightarrow$ T) has been shown to be homoplastic and was identified in 24 *E. coli* across five distinct phylogroups [364]. This mutation was also significantly associated with cefotaxime resistance and almost exclusively identified in the absence of plasmid-mediated *ampC* genes [364, 139]. The two mutations at positions -1 and +58 are commonly found together, and have been proposed to represent strain-specific polymorphisms [139].

E. coli which have been phenotypically confirmed as AmpC positive should undergo further molecular characterisation to deduce whether this phenotype is a result of a mutation in the promoter region of the *ampC* gene (chromosomal) or plasmid-mediated *ampC* genes, which has implications for the dissemination of AMR. Cefoxitin resistance combined with resistance to cefotaxime and/or ceftazidime is recommended for the detection of AmpC-producing *Enterobacteriaceae* [170]. In this study, differences in the AST zone

sizes (mm) for cefoxitin and cefotaxime differed between putative AmpC hyperproducers and plasmid-mediated AmpC-producing *E. coli*, however the sample size is small which limits any statistical comparisons. These results suggest that cefoxitin and/or cefotaxime zone size may be a useful preliminary method to distinguish between putative AmpC hyperproducers and plasmid-mediated AmpC-producing *E. coli*, followed by subsequent molecular confirmation using PCR and sequencing. However, the resistance spectrum of an isolate is complex and other factors such as porins and efflux pumps will contribute to the level of resistance [131]. A study of clinical AmpC-producing *E. coli* isolated from a French hospital found that phenotypic testing (broth microdilution and minimum inhibitory concentration) could not distinguish between putative AmpC hyperproducers and plasmid-mediated AmpC-producing *E. coli*. However discrepancies in putative AmpC hyperproducers susceptibility to some β-lactams varied, which may be due to differences in mutations in the *ampC* gene promoter region and the varying effects on promoter strength [132]. Despite the small sample size in this study (n=14 distinct AmpC-producing *E. coli* clones), these results warrant further investigation of the cefoxitin and cefotaxime zone sizes of a larger number of AmpC-producing *E. coli* isolated from various sources and a range of resistance mechanisms (including different mutations in the *ampC* gene promoter region as well as numerous pAmpC types).

ESBL-producing *E. coli* were isolated from two samples in this study (DF0059.2e and DF0183). Isolate DF0183g, belonging to ST4553, harboured a chromosomally encoded $bla_{\text{CTX-M-15}}$ and *ampC* gene (no mutations in the promoter region), but did not harbour any additional ARGs. Four ST4553 *E. coli* positive for the $bla_{\text{CTX-M-15}}$ gene have been detected in dog faeces (n=1) [365] and storm water (n=3) in NZ [366]. Three isolates did not harbour any other ARGs, and one isolate from storm water co-harboured the $bla_{\text{CMY-2}}$ gene. *E. coli* DF0059.2e belongs to ST5135 and to the best of our knowledge, this is the first *E. coli* ST5135 $bla_{\text{CTX-M-1}}$ to be reported. Few *E. coli* ST5135 have been reported on Enterobase, and those identified were isolated from human, livestock and poultry samples (accessed 27th July 2021). *E. coli* ST57 have been reported from chickens and pigs ($bla_{\text{CMY-2}}$ positive) [360] and from veal calves ($bla_{\text{CTX-M-14}}$ positive) [309]. The remaining STs identified in this study (ST56, ST442, ST973, ST2541) have been infrequently associated with AmpC or ESBL-producing *E. coli* in the published literature.

It is important to assess the virulence potential of AmpC- and ESBL-producing *E. coli* to ascertain the public and animal health risk posed. The majority of the virulence factors identified in the AmpC- and ESBL-producing *E. coli* sequenced in this study were involved in adhesion and protection/serum resistance (Figure 3.4). Two isolates (DF0031.1c and DF0047c) carried more virulence factors compared to the other *E. coli* sequenced and the remaining strains carried much fewer virulence genes (4 - 13; average 9). Consistent with this study, genes associated with the diarrheagenic *E. coli* pathotypes EIEC, ETEC (toxin related genes) and STEC have not been identified in other studies investigating AmpC- and ESBL-producing *E. coli* isolated from food animals [117], suggesting these pathotypes infrequently confer AmpC and ESBL phenotypes in healthy adult cattle.

Five *E. coli* sequenced in this study were identified as APEC, harbouring a number of virulence factors associated with this *E. coli* pathotype. According to core genome SNP analysis, these five strains formed two clusters: (i) DF0031.1c and DF0047c and (ii) DF0181.1c, DF0181.3c and DF0183e (Figure 3.3). Unsurprisingly, within these clusters the strains are epidemiologically related, genetically similar and are identical STs and genotypes (Table 3.5). These five *E. coli* strains were all serogroup O8, which has been frequently detected in APEC strains [367]. However one O serogroup may be associated with multiple *E. coli* pathotypes [72]. The assignment of strains to specific *E. coli* pathotypes can also be complicated due to the genetic fluidity of *E. coli*, their ability to readily acquire new genetic material via HGT and some genetic markers for specific pathotypes being non-exclusive [81]. Despite APEC being associated with avian sources, these *E. coli* were isolated from bovine faeces (n=3) and FDE (n=2). A global comparison of AmpC- and ESBL-producing *E. coli* from healthy food animals (broiler chickens, beef cattle and pigs) across Europe found that of the 99 APEC identified, 19.6% were from non-avian sources and consisted of a diverse range of STs [117]. Plasmids harbouring the $bla_{\text{CMY-2}}$ gene were reconstructed for three APEC strains in this study (pDF0181.1c_1, pDF0181.3c_1 and pDF0183e_1) and no APEC associated virulence genes were identified on these plasmids. The remaining two APEC strains were putative AmpC hyperproducers and hybrid assemblies were not generated, although plasmid incompatibility factors IncFIB, IncFII and IncQ1 were detected in these whole genome sequencing data.

Bacteria with the bla_{CMY} and $bla_{\text{CTX-M}}$ genes have been isolated from migratory and non-migratory birds on Ohio dairy farms, suggesting that birds may represent vectors for transmission of third-generation cephalosporin resistant *Enterobacteriaceae* [368]. It is plausible that birds may also be vectors for pathogenic and/or antimicrobial resistant *E. coli* on NZ dairy farms. Bird faecal samples were collected from the two dairy farms in this study, however, the samples have not yet undergone culture-based analysis for AmpC- and ESBL-E. These enrichments could be examined to determine whether birds on NZ dairy farms may be a vector of AmpC- or ESBL-E in NZ dairy farm environments.

The resistance mechanism for third-generation cephalosporins is currently unassigned in four *E. coli* (DF0102.4e-h) as they were both AmpC and ESBL negative. The *E. coli* (DF0102.4e-h) were isolated from a composite faecal enrichment on MC agar with 1 µg/mL cefotaxime (DF0102.4e-f) and CHROMagar$^{\text{TM}}$ ESBL plates (DF0102.4g-h). These *E. coli* were resistant to cefotaxime, cefpodoxime and streptomycin and intermediate to cefoxitin (Table 3.6). Resistance to cefepime was confirmed in isolates DF0102.4f and DF0102.4h, but resistance to cefepime has not been assessed for DF0102.4e or DF0102.4g. *E. coli* DF0102.4g underwent whole genome sequencing using both Illumina and MinION sequencing methods. Two plasmids were identified and one plasmid harboured the $bla_{\text{OXA-1}}$, *sul1* and *aadA1* genes as well as partial copies of the *aadA1* and *catB4* gene (Table 3.7) and this plasmid was predicted to be mobile. The $bla_{\text{OXA-1}}$ gene encodes a narrow-spectrum β-lactamase which traditionally confers resistance to aminopenicillins, carboxypenicillins

and ureidopenicllins [369], however overexpression of this gene has been shown to confer reduced susceptibility to fourth-generation cephalosporins such as cefepime and susceptibility to cefotaxime and ceftazidime when coupled with porin loss (OmpC and/or OmpF) [370]. In addition, other resistance mechanisms such as decreased expression of porins, overexpression of efflux pumps, mutations in the *mar*R gene and the presence of the β-lactamase TEM-1 may also be involved in conferring resistance to third- and/or fourth-generation cephalosporins among *Enterobacteriaceae* [369, 156]. Three AmpC-producing *E. coli* isolated from UK dairy farms also carried the $bla_{\text{OXA-1}}$ gene and showed reduced susceptibility to cefepime [156]. *Salmonella enterica* (serovar Typhimurium) isolated from human clinical samples in Kenya, Malawi and Ireland carried the $bla_{\text{OXA-1}}$ gene and were shown to have elevated cefepime MIC values [371]. OXA-1-like β-lactamase enzymes do not confer resistance to ceftazidime [153] and no third-generation cephalosporin resistant *E. coli* were isolated from MC agar with 1 µg/mL ceftazidime from the faecal enrichment DF0102.4, compared to *E. coli* isolated from both MC agar with 1 µg/mL cefotaxime (n=2) and CHROMagar$^{\text{TM}}$ ESBL plates (n=2). This further strengthens the hypothesis that the overexpression of the $bla_{\text{OXA-1}}$ gene and/or porin modification is involved in the resistance of *E. coli* isolates DF0102.4e-h to third- and fourth-generation cephalosporins. Further work is required to identify the resistance mechanism of *E. coli* DF0102.4g to third- and fourth-generation cephalosporins (confirmation of resistance to fourth-generation cephalosporins is still required). Possible resistance mechanisms warranting further investigation include the overexpression of the $bla_{\text{OXA-1}}$ gene coupled with porin loss assessed using expression studies and proteomics, respectively or modification and mutations in other genes such as the *mar* genes. The identification of the mechanism conferring resistance to third- and fourth-generation cephalosporins within an isolate is essential for AMR surveillance and risk assessment as well as for epidemiological studies.

AmpC-producing *E. coli* were not routinely screened for reduced susceptibility to cefepime in this study, therefore, further work is required to investigate whether any AmpC-producing *E. coli* isolated in this study produced ESAC β-lactamases by amplifying and sequencing the *ampC* gene to identify putative ESAC associated AA changes [154] and/or performing cloning experiments where feasible [203]. In particular, any ESBL negative *E. coli* isolated from CHROMagar$^{\text{TM}}$ ESBL plates (e.g. DF0159.2g) should be investigated as ESAC β-lactamase activity may enable these isolates to grow on this medium. The exact composition of CHROMagar$^{\text{TM}}$ ESBL plates is unknown which makes it difficult to ascertain how the ESBL negative *E. coli* were able to grow on these agar plates. ESAC β-lactamase activity, overexpression of the $bla_{\text{OXA-1}}$ gene and/or porin loss or modification [372] or the overexpression of efflux pumps [373] may have enabled these isolates to grow on CHROMagar$^{\text{TM}}$ ESBL plates in the absence of ESBL enzymes. The isolation of ESBL negative *E. coli* from CHROMagar$^{\text{TM}}$ ESBL plates highlights the importance of confirming putative ESBL producers using phenotypic tests to reduce false positive results.

The detection of ARGs on plasmids is of particular concern due to the potential for dissemination within bacterial populations, particularly to pathogenic bacteria. Within plasmid groups, plasmid backbones are comparatively conserved but the gene content can be variable [374]. Plasmids play a major role in the dissemination of AMR in *Enterobacteriaceae* [47]. ESBL genes, often encoded on plasmids, can carry several ARGs, resulting in a multi-drug resistance phenotype [50, 48]. Isolate DF0059.2e harboured three plasmids, two of which encoded ARGs including pDF0059.2e_2 which carried the $bla_{\text{CMY-2}}$ gene. pDF0059.2e_1 belonged to the IncY group, which are prophages that autonomously replicate and have been reported to be co-associated with other plasmid types including IncF and/or IncI [47], as was seen in this isolate. Although no ARGs were encoded on pDF0059.2e_1, IncY plasmids have been reported to carry resistance genes including $bla_{\text{CTX-M-15}}$ in *Klebsiella pneumoniae* [375] and *mcr-1* in an *E. coli* isolated from a pig farm in China [376].

The third plasmid, pDF0059.2e_3, harboured 12 ARGs including $bla_{\text{CTX-M-1}}$. All 12 of these genes were physically linked on the plasmid and mobile genetic elements were identified surrounding many of these genes (Appendix F). Interestingly, pDF0059.2e_3 also harboured partial copies of three ARGs: $bla_{\text{TEM-105}}$, *catB4* and the *aadA1*, although partial copies of the *catB4* and *aadA1* genes could not be confirmed in the annotated plasmid, perhaps due to the low coverage (19.49% and 17.18%, respectively) and length (106 bp and 166 bp, respectively) of these partial genes. The 279 bp partial region of the $bla_{\text{TEM-105}}$ gene was located directly downstream of an IS26 transposase (Figure 3.5).

Several copies of transposases surrounding ARGs including transposon Tn7 transposition proteins TnsA and TnsB were detected in pDF0059.2e_3. The "core machinery" of the Tn7 trasposon includes proteins TnsA, TnsB and TnsC. Target-site selecting proteins TnsD and TnsE are involved in preferential insertion into the bacterial chromosome (vertical transmission) and conjugable plasmids (horizontal transfer), respectively [377]. Tn7-like transposons containing ARGs have also been been identified in *Enterobacterales* isolated from samples collected on chicken and swine farms in China [378]. Detection of Tn7 transposition proteins TnsA and TnsB in pDF0059.e_3 suggests that this plasmid has undergone recombination events, potentially contributing to the development of a multi-drug resistant plasmid in this isolate. Such recombination events can make plasmid replicon or MOB-typing difficult [374] and may explain why the scheme could not assign a pMLST type for pDF0059.2e_3. Multi-drug resistance plasmids, such as pDF0059.2e_3, pose a significant public and animal health concern.

Plasmid associated β-lactamase gene types have been shown to have a highly homologous genetic environment, regardless of source [174]. In this study, five strains harboured highly similar IncI1 plasmids which encoded the $bla_{\text{CMY-2}}$ gene (Table 3.8). IncI plasmids are conjugative, have a low copy number and a narrow host range [47]. A high proportion of AmpC and ESBL genetic determinants have also been found on IncI1 plasmids in other studies [9, 117], with IncI1 plasmids being frequently identified from extended-spectrum

cephalosporin resistant *E. coli* isolated from healthy livestock in the Netherlands over a ten year period [354]. Although the plasmid and AmpC/ESBL gene combinations in *E. coli* isolated from dairy cattle and veal calves was more variable compared to the plasmid and gene combinations identified in *E. coli* isolated from other livestock sectors [354]. Gene synteny for the five IncI1 plasmids was highly similar, excluding the Shufflon A gene region where there was variation between the strains (Appendix J). The shufflon gene region is involved in conjugation of IncI1 plasmids [351]. IncK plasmids harbouring the $bla_{\text{CMY-2}}$ gene originating from human and poultry sources showed high nucleotide sequence identity when the shufflon region was excluded [360]. In the same study, IncI1 plasmid backbones also showed a high level of sequence similarity, but were a different pST to the plasmids characterised in this study [360]. Genetically similar *E. coli* harbouring IncI1 plasmids were isolated from multiple samples collected during the same sampling month on one farm in this study (faeces n=2; FDE n=1). This finding suggests that these *E. coli* may have been circulating within the farm environment. Composite faeces were randomly collected from faecal pats deposited throughout the paddock, and it cannot be discounted that the faeces were excreted from the same animal. Highly similar plasmids have also been detected in multiple isolates across other farm studies. For example, genetically similar plasmids harbouring identical co-located ARGs ($bla_{\text{CTX-M-1}}$, *sul2* and *tetA*) were identified in six *E. coli* isolated from the same beef cattle farm [284].

Plasmids and HGT likely play a key role in the dissemination of third- and fourth-generation cephalosporin resistance in *Enterobacteriaceae*. Core genome SNP analysis of $bla_{\text{CTX-M}}$ or $bla_{\text{CMY-2}}$ positive *E. coli* (n=59) showed clustering according to farm location, however, there was some incidences where isolates from different farms and sources (forage and cattle faeces) clustered together [174]. SNP-based analysis of $bla_{\text{CMY-2}}$ positive *E. coli* isolated from human clinical cases, livestock and food samples identified examples where *E. coli* isolates of the same ST but different sample origin were genetically very similar and were separated by a small number of SNPs [360]. For example, two human clinical ST131 isolates were genetically similar to a chicken isolate and differed by 27-28 SNPs [360]. However within a ST, generally livestock and food isolates were less genetically similar (according to SNP distances) compared to human clinical isolates [360]. A diverse range of STs and phylogenetic groups were detected among third-generation cephalosporin resistant *Escherichia* spp. isolated from healthy cattle, pigs and chickens. This suggested that the clonal spread of single lineages is unlikely [117]. Together, these results and the results of this study highlight the importance of horizontal plasmid transfer in the dissemination of third-generation cephalosporin resistance among *Enterobacteriaceae*.

3.5 Conclusion

In conclusion, this study assessed the sample level prevalence of AmpC- and ESBL-producing *E. coli* on two NZ dairy farms over a 15 month period. No AmpC- or ESBL-producing *E. coli* were isolated from bulk tank milk or soil in this study, suggesting they are infrequent reservoirs in the NZ dairy farm environment. ESBL-producing *E. coli* were infrequently identified during the study, suggesting they are present at a low prevalence in these two dairy farms. Plasmid-mediated AmpC-producing *E. coli* were isolated at a low prevalence in faeces and FDE, but were isolated at a higher frequency on the higher intensity farm (Dairy 4) compared to Dairy 1. In contrast, AmpC-producing *E. coli* with mutations in the promoter region of the *ampC* gene were detected at a higher prevalence, particularly in FDE, but only on Dairy 1. The detection of AmpC-producing *E. coli* with mutations in the promoter region of the *ampC* gene is less concerning for human and animal health, as the chromosomal *ampC* gene cannot be transferred by HGT. These findings highlight the necessity to confirm the genotype of an AmpC phenotype in *E. coli*.

Both AmpC- and ESBL-producing *E. coli* were isolated at various time points throughout the 15 month study period and their detection was not associated with periods of elevated AMU on NZ dairy farms. Dairy 1 had higher AMU (mg/PCU) than Dairy 4 during this study period, however both farms used a low proportion of third- and fourth-generation cephalosporins which are known risk factors for ESBL-E. In addition, the farms used low levels of antimicrobials classed as red tier by the NZVA and these results support the hypothesis that AMU on NZ dairy farms influences the prevalence of AMR in the dairy farm environment. Additional studies should focus on high risk animals such as young calves and a larger number of dairy farms throughout NZ, including farms with known risk factors or management practices which may influence AMR such as high use of third- and fourth-generation cephalosporins, an open herd system, feeding waste milk to calves and the use of blanket dry cow therapy.

Chapter 4

Impact of systemic antimicrobial therapy on the faecal microbiome in dairy cows

4.1 Introduction

Antimicrobial therapy, either for the treatment of disease or prophylactically as a feed additive, can alter both the bacterial community composition and faecal resistome in cattle, swine and chicken faeces [292, 379, 380]. Such perturbations in the microbiome can either be transient and the bacterial composition recovers, or microbiome changes may be long-lasting [381]. Broad-spectrum antibiotics generally reduce the microbiome diversity [381], however the recovery and resilience of the microbiome can differ depending on the spectrum of the antimicrobial used, the treatment duration [382] and due to other host-dependent factors [383]. The ecological balance of the bovine faecal microbiome is complex and can also be affected by factors other than antimicrobial therapy such as feed type and concentrates [219, 286], gastrointestinal disease [384] and other farm management factors such as housing (e.g. freestalls or indoor barns) [385].

There is growing concern that antimicrobial use in food-producing animals may increase the prevalence of antimicrobial resistant bacteria and ARGs, as well as increasing the potential for HGT of such resistance genes to pathogenic bacteria [381]. Treatment with subtherapeutic levels of antimicrobials has been associated with an increase in the prevalence of antimicrobial resistant *E. coli* in cattle faeces [243]. Third-generation cephalosporins, such as ceftiofur, have a broad spectrum of activity including against a variety of Gram-negative pathogens [386]. Ceftiofur is the active ingredient in a number of products such as Excede® LA and Excenel® RTU which are used to treat infections on dairy farms, particularly infections other than mastitis such as metritis [387]. However, the use of third- and fourth-generation cephalosporins is a known risk factor for detecting ESBL- and AmpC-producing *E. coli* on dairy farms [7]. Ceftiofur is also classified as red tier by the NZVA [388], therefore its use should be limited to the treatment of specific susceptible bacteria or resistant infections. Using metagenomic sequencing it has been demonstrated that faeces from cows (n=3) injected with ceftiofur had a higher proportion of β-lactam resistance genes compared to control cow faeces (n=3), but this was admittedly based on

a small sample size. This finding was not replicated with ARGs belonging to all antibiotic classes [292]. Another study found no significant changes in the abundance of β-lactam resistance genes in the faeces from cows injected with ceftiofur [243], however there was a significant increase in the abundance of tetracycline resistance genes in the faeces of cattle fed chlortetracycline [243]. Cows treated with ceftiofur also had an increased proportion of gene sequences associated with mobile genetic elements (plasmids, phage, prophage and transposable elements) [292] which suggests that the treatment with ceftiofur may increase the potential for of HGT and thus, the spread of ARGs. Similarly, the use of in-feed antibiotics in swine has been associated with an increase in prophage induction [389], which may mediate gene transfer.

The diversity and composition of the bacterial microbiome can influence the resistome. For example, some bacteria have higher levels of intrinsic resistance whilst others, such as clinically relevant bacteria from the phylum Proteobacteria, have been associated with a number of ARGs [390]. Therefore, in order to understand the impact of antimicrobial therapy on the faecal microbiome in cattle, it is important to first investigate the changes to the microbiome composition and diversity pre-, during and post-treatment, which can have an important impact on the resistome structure. For example, in the study by Chambers *et al.*, [292] the increase in β-lactam resistance genes may be partially attributed to an increase in *Bacteroidia* in faeces from treated cows compared to control cows, as the most abundant genes within the β-lactam class, *cfxA2* and *cfxA3* encode class A β-lactamases that are often found in *Prevotella* spp. [391] and *Capnocytophaga* spp. [392].

Of the limited studies conducted in food-producing animals, including cattle, there has been a focus on the use of specific antimicrobial treatments or feed additives in controlled environments or experiments, often in the absence of disease. Therefore, the aim of this study was to assess the impact of systemic antimicrobial therapy on the faecal microbiome in dairy cows in the natural farm environment, by analysing faecal samples from cattle impacted by a number of different clinically-defined conditions with corresponding antimicrobial treatments.

4.2 Methods

4.2.1 Study population and sample collection

Animals were recruited in the study and faecal samples were collected over a 14 month period from 1st July 2019 – 30th September 2020, however samples were not collected between 23rd March 2020 – 13th May 2020 due to the Covid-19 pandemic restrictions in NZ. Dairy cows requiring systemic antimicrobial treatment from Massey University Dairy 1 and Dairy 4 farms were recruited in the study on an opportunistic basis at the farm managers' discretion. Faecal samples were collected from cows receiving systemic

antimicrobial therapy pre- (S1), during (S2) and post-treatment (S3) (Figure 4.1). S1 samples were collected directly after diagnosis and before receiving the first antimicrobial treatment. Sample S2 was collected during treatment and was dependent on the prescribed treatment course (median one day after first treatment) and sample S3 was collected post-treatment once the cow re-entered the milking herd (median six days). Samples from healthy cows within the herd were also collected at the same sampling period as a untreated control to account for temporal variation in farm management factors such as feed type and breed as well as any farm-specific variation throughout the duration of the experiment. Where possible, untreated control cows were matched with more than one treated cow if the treatment was started on the same day. Cows which had received systemic antimicrobial therapy within 6 months prior to sample collection were excluded from the study. Each treated cow was assigned a number (1 - 30) and the respective control cow was designated using the same numbering system (1C - 30C). Sample collection was performed by the farm staff. Briefly, dairy cows were safely restrained and using a disposable glove and lubricant, faeces were collected and the first portion discarded due to potential contamination and approximately 5 g of faeces was collected and stored in a sterile pottle. Samples were stored at 4℃ and processed within 24 hours of collection. A questionnaire regarding animal health, prescribed treatment course and antimicrobial treatment history was completed by farm staff per sick cow and healthy control (Appendix O). This research was approved by the Massey University Animal Ethics Committee (Protocol number 18/123).

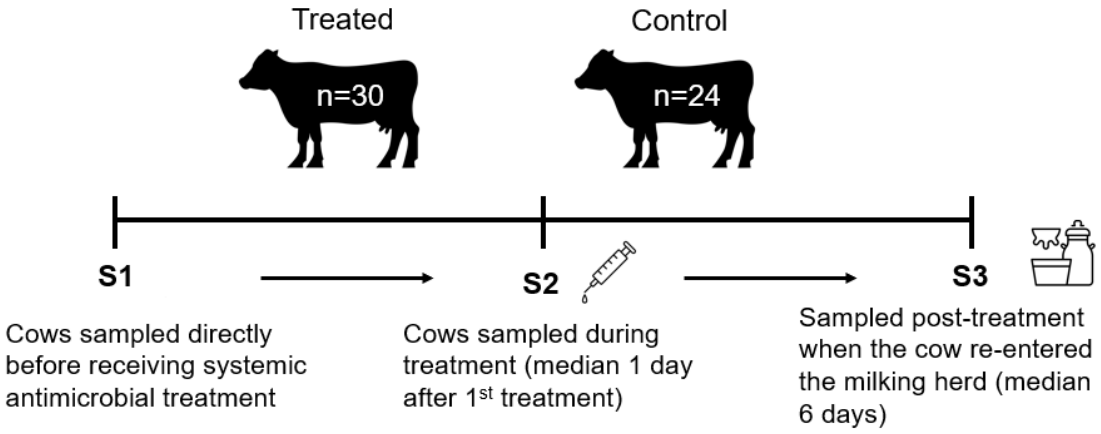

Figure 4.1: Timeline for faecal sampling. Experimental sampling days are indicated on the figure (S1, S2 and S3) and the number of treated and control (untreated) cows is shown at the top of the figure. Where possible control cows were matched with more than one treated cow if treatment was started on the same day, hence the differing numbers between treatment groups. Antimicrobial treatment is indicated by the syringe and the cows re-entered the milking herd at sample S3. Healthy control cow samples were collected at the same time as the treated cows.

4.2.2 Sample processing

Faecal samples were homogenised and 0.2 g was stored in triplicate in cyrovials (Greiner Bio-One, Austria) at -80℃ until DNA extraction. A pea-sized amount of homogenised faecal material was collected with a cotton swab and vortexed and enriched in 10 mL phosphate-buffered peptone water (BD Difco^{TM}, Fort Richard Laboratories, Auckland, New Zealand) at 35℃ for 18 hours. After incubation, enrichments were mixed by vortex and 1 mL of the enrichment was mixed with 429 µL of glycerol (30%[v/v]) and stored at -80℃. In addition, 1 mL of the enrichment was pelleted at 17,000 x *g*. The supernatant was removed and the final pellet re-suspended and washed twice with phosphate-buffered saline (PBS) (10 mM). The remaining pellet was re-suspended in 1 mL molecular biology grade water, vortexed and heated in a heating block at 100℃ for 10 min. The boiled DNA lysate preparations were stored at -20℃ and used as a template for subsequent PCR reactions.

4.2.3 16S rRNA V3-V4 sequencing

4.2.3.1 DNA extraction and sequencing

Faecal samples for DNA extraction were removed from the -80℃ freezer, defrosted on ice and transferred to a bead-beating tube for DNA extraction. Genomic DNA was extracted using the Presto Stool DNA Extraction Kit (Geneaid Biotech Ltd, New Taipei City, Taiwan) according to the manufacturers instructions with minor modifications. Briefly, 0.2 g of faeces was used for the DNA extraction. For sample lysis, 800 µL of ST1 buffer was added, mixed by vortexing and incubated at 70℃ for 5 min. The sample was vortexed at maximum speed for 7 min (Vortex Mixer, Labnet International, New Jersey, USA) and then centrifuged at 8,000 x *g* for 2 min. Next, 500 µL of supernatant was transferred to a new tube and 5 µL RNAse A (100 mg/mL, QIAGEN, Hilden, Germany) was added and incubated for 10 min at 37℃. Subsequently, 150 µL of ST2 buffer was added and mixed briefly by vortexing. The sample was incubated at 0 - 4℃ for 5 min and centrifuged at 16,000 x *g* for 3 min to remove PCR inhibitors. Next, 500 µL of clear supernatant was transferred to a PCR inhibitor removal column, centrifuged at 16,000 x *g* for 1 min and the column discarded. For DNA binding, 800 µL of ST3 buffer was added to the flow through, mixed vigorously by hand for 5 sec and 700 µL transferred to a GD column. The sample was centrifuged at 16,000 x *g* for 1 min, the flow through was discarded and the remaining sample mixture transferred to the GD column. The GD column and sample was again centrifuged at 16,000 x *g* for 1 min and the flow through discarded. To wash the DNA, 400 µL of ST3 buffer was added to the GD column and centrifuged at 16,000 x *g* for 30 sec. The flow through was discarded, 600 µL of wash buffer was added and the sample centrifuged at 16,000 x *g* for 30 sec. The flow through was discarded and the wash step was repeated. After the final wash step, the GD column was centrifuged at 16,000 x *g* for

3 min to dry the column.

To elute the DNA, the GD column was transferred to a new tube and 30 µL of elution buffer pre-heated to 60℃ was added to the centre of the column and left to stand for 2 min. The sample was then centrifuged at 16,000 x *g* for 2 min and the elution step repeated using the elution buffer containing the DNA. The DNA was transferred to a new microcentrifuge tube and stored at -20℃. All centrifuge steps were carried out at room temperature. The DNA concentration was quantified using a Qubit 4.0 fluorometer (ThermoFisher Scientific Inc., USA) and the $A_{260/280}$ and $A_{260/230}$ ratios were determined using a Nanodrop microvolume spectrophotometer (Nanodrop 2000c, ThermoFisher Scientific Inc., USA). The DNA integrity and size was visualised on a 0.8% [w/v] agarose gel which was prepared using 0.8g agarose (Invitrogen, Auckland, New Zealand) and 100 mL of 0.5x Tris-borate-EDTA buffer. The gel was stained with RedSafe (iNtRON Biotechnology, South Korea) and run at 100V for 4 hours. A high molecular weight *Hin*d III/λ ladder (ThermoFisher Scientific Inc., USA) was used for size comparison and the gel images were captured and stored using the GelDoc XR^+ system (BioRad, New Zealand).

Next-generation sequencing was conducted using an Illumina MiSeq v2 platform with 2 x 250 base paired-end sequencing reads (Massey Genome Service, Massey University, Palmerston North, New Zealand). The total bacterial community was targeted using dual index primers which flank the V3-V4 hypervariable region of the 16S rRNA gene (16SF V3 and 16SR V4 primers, as detailed in [393]) and the 161 samples were run on a single Illumina MiSeq v2 lane.

4.2.3.2 Bioinformatics

Adapter sequences and PhiX control library reads were removed from the demultiplexed fastq sequencing reads using FASTQ-MCF [394] by the Massey Genome Service (Massey University, Palmerston North, New Zealand) and the reads were quality filtered to a Phred score of Q20 (Massey Genome Service, Palmerston North, Massey University, New Zealand). The 16S rRNA data was analysed using the dada2 package v1.16.0 [395] in R v4.0.2 [396]. Briefly, the demultiplexed reads were quality filtered and 10 bp and 40 bp trimmed from forward and reverse reads, respectively. The denoised reads were merged, the chimeras removed and the reads remaining post-processing were used to construct an amplicon sequence variant (ASV) table. Taxonomy was assigned to the ASV table using the Silva v138.1 database [397, 398, 399]. The ASV table, sample metadata (Appendix P) and taxonomy table were combined to construct a phyloseq object using the R package Phyloseq v1.32.0 [400]. Diversity metrics were calculated using the vegan [401] and microbiome [402] R packages. The data was normalised to the median sequencing depth prior to calculating the diversity metrics. Data visualisation was conducted in R using a range of packages including ggplot2 [403], tidyverse [404], dplyr [405], Manu [406], RColorBrewer [407] and the website Colorgorical [408].

4.2.4 Statistical tests

The α-diversity of the faecal samples was compared using a generalised linear mixed effect model in R using the lme4 package [409]. Animal health status (treated or untreated control) and sample collection order (S1, S2 or S3) were included as fixed effects in the model as well as the interaction of both health status and sample order. The individual animal was included as a random effect. Pairwise comparison of health status and sample order was tested using the emmeans package and p-values were adjusted using the Tukey method [410].

4.2.5 Microbiological methods and molecular characterisation

Faecal enrichments were removed from storage at -80℃ and kept on ice. Culture-based methods (detailed in Chapter 3, sections 3.2.3 and 3.2.4) were used to enrich and isolate third-generation cephalosporin resistant *Enterobacteriaceae* from the faecal enrichments. Briefly, the faecal enrichments from the ceftiofur treated cows (n=3) and associated control cows (n=3) were plated on MC agar as a positive control to confirm *E. coli* detection as well as MC agar supplemented with cefotaxime (1 µg/mL), MC agar supplemented with ceftazidime (1 µg/mL) and CHROMagar^{TM} ESBL plates to select for third-generation cephalosporin resistant *Enterobacteriaceae*, particularly *E. coli* or *Klebsiella* spp. Presumptive *Enterobacteriaceae* were identified using MALDI-TOF MS. Kirby-Bauer disc diffusion ASTs were undertaken on *E. coli* isolates for six antimicrobials (Table 3.3) and AmpC and ESBL phenotypes were confirmed using double-disc and three-disc comparison assays respectively, as outlined in section 3.2.4. For genotypic confirmation, *E. coli* with an AmpC phenotype were tested for pAmpC gene families using a multiplex PCR [177]. A PCR targeting the bla_{CMY} gene family [313] was performed on isolates positive for the CITM primer set (Table 3.4), indicative of CMY-positive *E. coli* (using boiled DNA lysate preparations as DNA templates). The presence of pAmpC gene families in boiled DNA lysate preparations from enriched faecal samples was also analysed using the multiplex PCR. The PCR reaction and gel electrophoresis conditions are detailed in Appendix A.

4.2.6 Whole genome sequencing

4.2.6.1 DNA extraction

Bacterial isolates from glycerol broths stored at -80℃ (section 3.2.3) were inoculated on Columbia Sheep Blood agar (5% blood) (Fort Richard Laboratories, Auckland, New Zealand) and incubated for 18 hours at 35℃. An individual colony was subsequently subcultured onto a fresh Columbia Sheep Blood agar plate (5% blood) and incubated at 35℃ for 18 hours. Pure cultures were sent on LB-agar slopes to The Institute of Environmental Science and Research (Christchurch, New Zealand) where the DNA was extracted with

the Qiagen DNeasy Blood and Tissue kit (QIAGEN, Hilden, Germany) according to the manufacturers instructions, using a QIAcube (QIAGEN, Hilden, Germany). The DNA libraries were prepared using the Nextera XT v2 Library Preparation Kit (Illumina, San Diego, USA) and sequenced on a NextSeq 550 sequencer (Illumina, San Diego, USA) by The Institute of Environmental Science and Research (Christchurch, New Zealand).

4.2.6.2 Genome analysis

The Illumina MiSeq sequencing reads were processed using the Nullarbor v2.0 [320] pipeline with default parameters. In summary, the adapters were removed from raw reads using Trimmomatic v0.39 [321], the species identification by k-mer analysis was performed using the Kraken v1.1.1 database [322], the genomes were assembled using SKESA v2.4.0 [323] and annotated with Prokka v1.14.6 [324]. The sequence type was determined using mlst v2.19.0 [325] with information downloaded from PubMLST [326], the resistome profiles were assessed with ABRicate v1.0.1 [327] using the ResFinder database (downloaded 19/04/2020) [328] and the Centre for Genomic Epidemiology website [329] was used to detect the virulence genes and serotype in assembled genomes using the VirulenceFinder 2.0.3 database (v2020-05-29) [73] and the SerotypeFinder 2.0.1 database (v1.0.0), respectively. The SNP variation was assessed using Snippy v4.4.3 [331] with isolate DF0183e (ST56) as the reference genome (Table 3.5).

4.3 Results

A total of 30 treated and 24 healthy (untreated) cows were recruited into the study (Appendix P). A further 15 treated cows were initially recruited in the study but were not included in the final analysis due to missing untreated control cow samples (n=6) or incomplete sampling (i.e. three faecal samples not collected, n=9) due to various reasons including animal death or welfare. Sample collection was at the farm manager's discretion. For one control cow, only two faecal samples were collected. Therefore, 161 faecal samples were analysed and from these, 15,263 unique ASVs were observed. Of these, 118 were singletons and 1,687 were doubletons, highlighting the high diversity and variation of ASVs within the faecal samples. The minimum, maximum and mean number of reads across the faecal samples was 16,100, 88,841 and 48,002 reads, respectively. Metadata for the faecal samples is detailed in Appendix P.

4.3.1 Microbiome diversity

Comparison of the Shannon diversity from the faecal microbiomes of treated and control cows suggested that the microbiota was diverse and species-rich, as the diversity index was relatively high (4.09 - 6.59) which is consistent with the large number of unique ASVs

detected across the samples (Figure 4.2). For the treated cows, the Shannon diversity decreased during treatment (S2) compared to pre- (S1) and post-treatment (S3) samples ($p = 0.0029$), which suggests that the microbiome composition of these samples was less diverse with fewer abundant ASVs. Interestingly, the Shannon diversity from the treated cow faecal samples collected post-treatment (S3) was higher compared to S2 (during) and was more comparable with pre-treatment (S1) samples. No statistically significant changes in α-diversity for control cows were observed. The Chao1 diversity index indicated that the community richness was relatively even across the samples (Figure 4.2), suggesting a similar number of ASVs in the faecal microbiomes, except from S2 for treated cows ($p = 0.0124$) which had a decreased Chao1 diversity index.

The microbiome profiles did not cluster according to health status or sample order (pre- (S1), during (S2) and post-treatment (S3)), with a low amount of variance explained in the Principal Component Analysis (Figure 4.3, PC1: 5.36%, PC2: 3.50%). Although the S2 faecal microbiomes from treated cows did not cluster together, they were generally more dispersed compared to the S1 and S3 samples. The dispersion of the faecal microbiome samples in the PCA plot and the low variance explained may be attributed to the variety of factors which can influence the microbiome composition such as variance at the individual animal level as well as treatment with specific antimicrobials and illnesses. Therefore, the faecal microbiomes from individual cows were further analysed as case studies differentiated by antimicrobial treatment type.

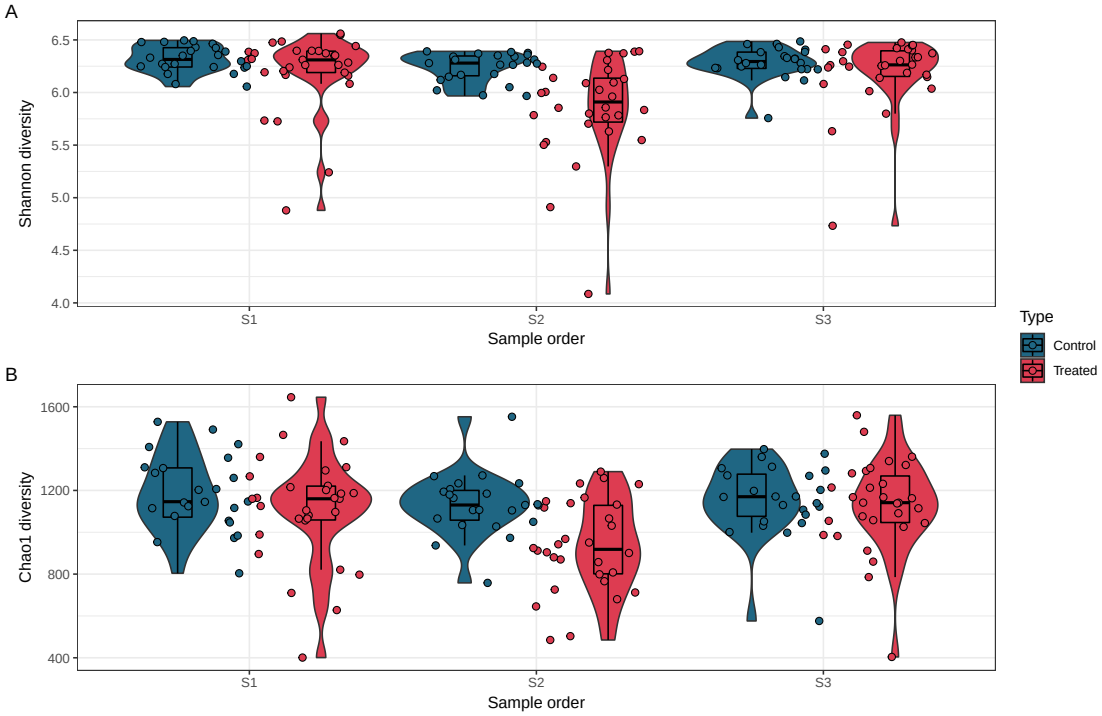

Figure 4.2: Diversity between treated (n=30) and control (n=24) cows for **A**: Shannon diversity and **B**: Chao1 diversity

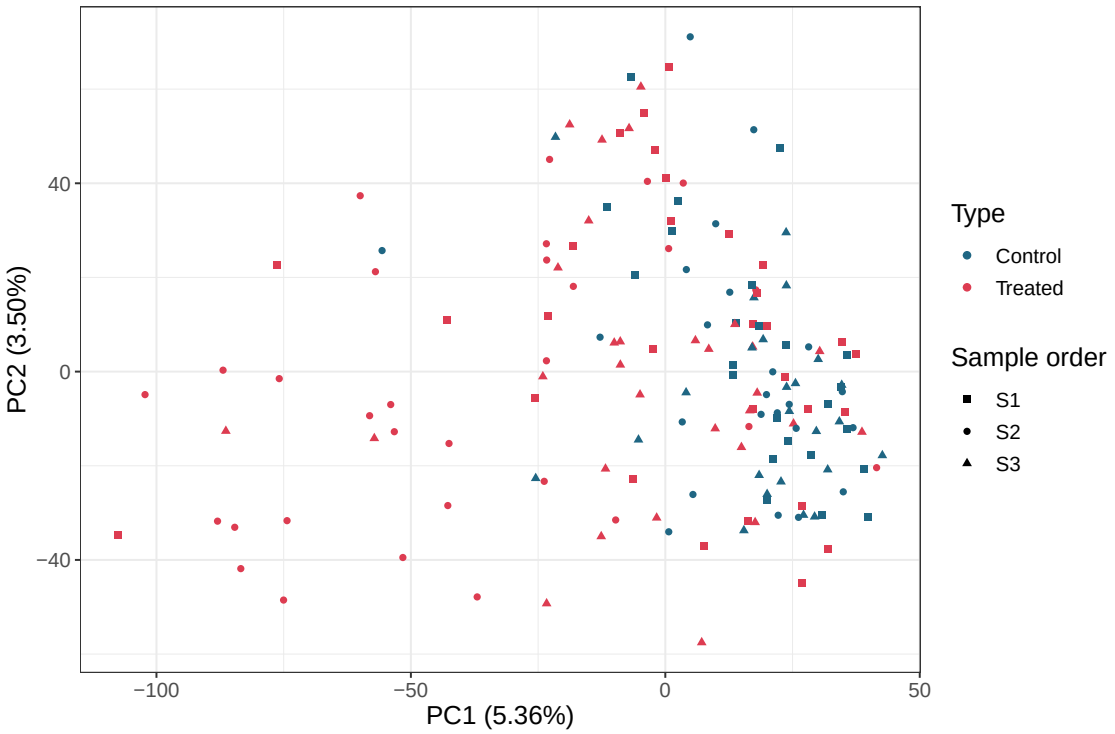

Figure 4.3: Principal Component Analysis for the faecal microbiome in treated animals (red) and control animals (blue) over time (shapes). The percentage of variation explained in the Principal Component Analysis is indicated on the axis labels.

4.3.2 Case studies

The animals were grouped and analysed by antimicrobial treatment. The systemic antimicrobial treatments included in this study were procaine penicillin G (n=23 cows), ceftiofur (n=3 cows) and penethamate hydriodide (n=3 cows) which are all β-lactam antibiotics, and a mixture of marbofloxacin/penethamate hydriodide (n=1 cow) which is a quinolone and β-lactam antibiotic, respectively. The untreated control cows associated with each treated cow were analysed in the respective case study.

4.3.2.1 Procaine penicillin G treated cows

Procaine penicillin G was the active ingredient in a number of products used on the farms during the study including Intracillin® 300 and Phoenix Pharmacillin 300 (Appendix M). These products were used to treat numerous conditions including between claw/footrot, a penetration on the leg, left displacement of the abomasum, a swollen vulva and a retained membrane. Procaine penicillin G is a β-lactam antibiotic and is classified as green tier by the NZVA [388]. Penicillin G has *in vitro* activity against Gram-positive and

Gram-negative bacteria, however it is usually used to treat infections caused by susceptible Gram-positive bacteria such as *Streptococcus* spp [387]. Therefore, it was hypothesised that systemic antimicrobial therapy with procaine penicillin G would reduce the relative abundance of Gram-positive bacteria in the faecal microbiome. There was a high level of variation in the microbiome composition of the 23 cows treated with procaine penicillin G (Appendix Q) as well as variation at the individual animal level. Therefore, four of these cows and their associated controls were analysed in more detail as a case study. The following cows were included in the case study and represented a range of treatments: (i) cow 9: treated for between claw/footrot, (ii) cow 10: treated for left displacement of the abomasum, (iii) cow 16: treated for a swollen vulva and (iv) cow 24: treated for a retained membrane post-calving.

Compared to the post-treatment faecal sample (S3), the Shannon and Chao1 α-diversity was lower for samples S1 and S2 for cow 10 (Figure 4.4A-B). Compared to the other treated and control cows, cow 24 had a lower Shannon and Chao1 α-diversity across all faecal samples collected (S1, S2, S3), except for S2 collected from cow 10. The faecal samples from treated cows 9 and 16 had a similar Shannon α-diversity compared to the control cows, however, the Chao1 α-diversity was lower for the S2 samples for all four treated cows, suggesting that there were fewer unique ASVs from these faecal samples. The Chao1 α-diversity of S3 samples from cows 9, 10 and 16 was similar compared to the control cows (S1, S2 and S3), whereas the S3 Chao1 α-diversity for cow 24 was the lowest across all faecal microbiomes in this case study. The faecal microbiomes from each control cow clustered together in the PCA plot (Figure 4.4D) compared to the cows treated with procaine penicillin G (n=4). The faecal microbiomes from individual animals (S1, S2 and S3) generally clustered together, suggesting that the faecal microbiomes from the same animal were more similar (demonstrating that animal is a major driver of variation).

The faecal microbiome composition across samples S1, S2 and S3 from cow 16 was relatively stable (Figure 4.4C). Faecal sample S1 for cow 9 had a higher abundance of an ASV belonging to *Romboutsia* compared to samples S2 and S3, whereas the remaining microbiome composition at the genus level was relatively consistent. In comparison, perturbations were observed in the faecal microbiome of treated cows 10 and 24. For cow 10, an ASV classified as *Escherichia-Shigella* was present in S1, with a higher abundance in S2 and was absent in S3. Sample S2 also had a higher abundance of an ASV belonging to the order *Bacteroidales*, which is labelled as "NA" in the plot as it could not be classified at the genus level. The microbiome composition of faecal samples S1, S2 and S3 for treated cow 24 varied, with *Prevotellaceae* UCG-003 present in a higher abundance in samples S1 (pre-) compared to S2 (during) and S3 (post-treatment). Interestingly, six ASVs belonging to *Prevotellaceae* UCG-003 were present in S1 compared to two in S2 and only one in S3. An ASV classified as *Bacteroides* was more abundant in S2 compared to S1 or S3 from cow 24. For the control cows in this case study (n=3), the faecal microbiome composition was relatively consistent for each animal during the study period (S1, S2 and S3) compared to the treated cows, although there was some perturbations in the relative abundance of

specific ASVs (Figure 4.4C).

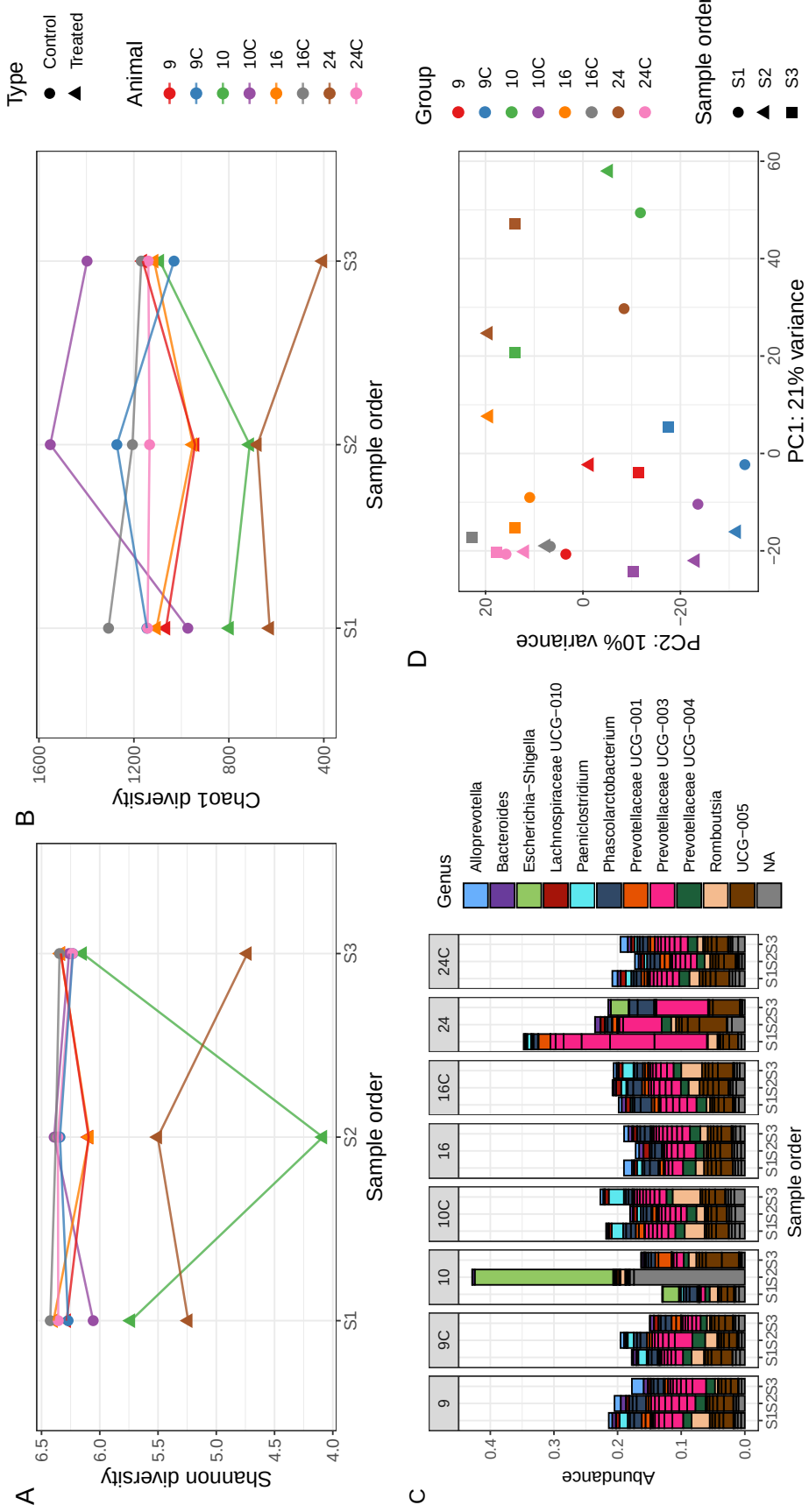

Figure 4.4: Shannon (**A**) and Chao1 (**B**) α-diversity, and abundance of the top 30 ASVs at the genus level (**C**) of faecal samples from cows treated with procaine penicillin G (n=3) and respective control cows (n=3). Principal Component Analysis (**D**) for the faecal microbiome of treated and control cows at the individual animal level (colours) over the sampling period (shapes). The percentage of variation explained in the Principal Component Analysis is indicated on the axis labels.

4.3.2.2 Penethamate hydriodide treated cows

One cow from Dairy 1 and two cows from Dairy 4 farm were treated for mastitis with penethamate hydriodide (Penethaject) during the study period. These mastitis cases were diagnosed by positive culture and/or had culture susceptibility testing undertaken. Penethamate hydriodide is a benzyl penicillin prodrug [65] and is classed as green tier by the NZVA [388]. The spectrum of activity for penethamate hydriodide includes a number of Gram-positive mastitis causing pathogens such as *Staphylococcus aureus*, *Streptococcus uberis* and *Streptococcus dysgalactiae* subsp. *dysgalactiae* [65, 411]. One cow was used as the untreated control for two treated cows as they started antimicrobial treatment on the same day (control cow 18C/19C).

The Shannon diversity was comparatively lower from the pre-treatment and during treatment samples (S1 and S2) from cow 19 and the S2 sample from cow 18 (Figure 4.5A). The α-diversity of post-treatment samples (S3) from cows 18 and 19 were similar to control cows. In comparison, both the Shannon and Chao1 diversity of all three faecal samples from cow 6 (S1, S2, S3) was consistently similar to the diversity observed in control cows (Figure 4.5B), whereas sample S3 from control cow 6C showed a decrease in both the Shannon and Chao1 α-diversity. The faecal microbiome samples clustered at the individual animal level for control cows 6C and 18C/19C and treated cow 6 (Figure 4.5D). In comparison, pre- (S1) and post-treatment (S3) samples for cows 18 and 19 were clustered together, however the during treatment samples (S2) were distinct (Figure 4.5D).

As suggested by the diversity metrics, the bacterial microbiome composition of samples S1, S2 and S3 from cow 6 remained relatively stable during the study (Figure 4.5C), with small perturbations in the abundance of key ASVs. The microbiome composition for control cow 6C was also stable during the study period, despite a reduction of α-diversity from sample S3, which suggests there was a reduction in ASVs present at a lower relative abundance post-treatment. At the individual animal level, the faecal samples collected during treatment (S2) from cows 18 and 19 differed from samples S1 and S3, with ASVs belonging to *Escherichia-Shigella* being more abundant in S2 for both cows. ASVs classified as *Alloprevotella* were more abundant in S2 from cow 18 and S1 from cow 19. In addition, ASVs belonging to *Paeniclostridium* were in low abundance in S2 for both cows and ASVs classified as *Romboutsia* were reduced in S2 compared to S1 and S3 for cow 19. The most abundant ASVs from faecal samples of control cow 18C/19C were relatively stable during the sampling period (S1, S2 and S3).

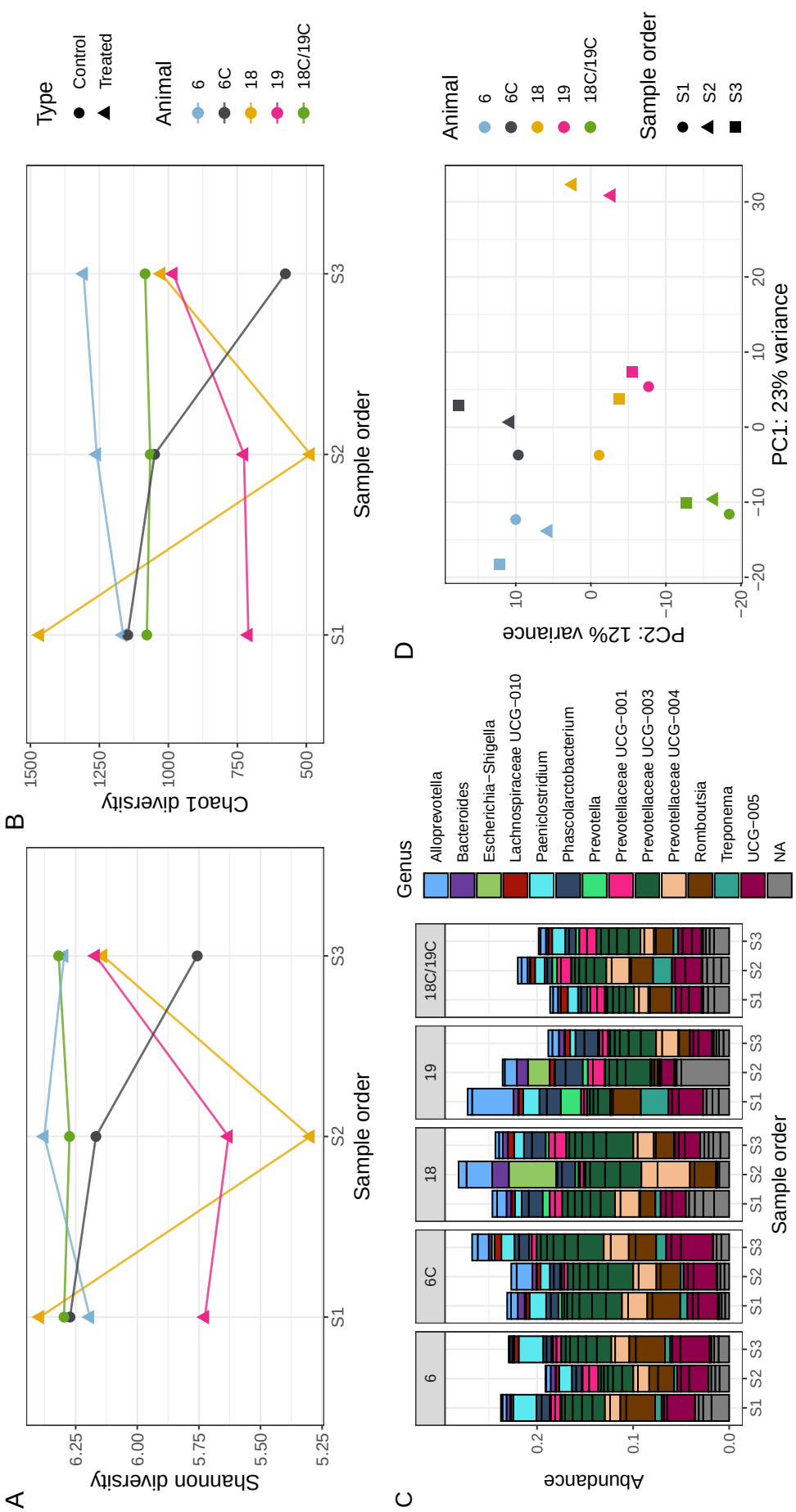

Figure 4.5: Shannon (**A**) and Chao1 (**B**) α-diversity, and abundance of the top 30 ASVs at the genus level (**C**) of faecal samples from cows treated with penethamate hydriodide (n=3) and respective control cows (n=3). Principal Component Analysis (**D**) for the faecal microbiome of treated and control cows at the individual animal level (colours) over the sampling period (shapes). The percentage of variation explained in the Principal Component Analysis is indicated on the axis labels.

4.3.2.3 Marbofloxacin/penethamate hydriodide treated cow

One cow from Dairy 4 (n=1) was treated for mastitis with Marbocyl® 10% and Penethaject, which is a combination of marbofloxacin and penethamate hydriodide belonging to the quinolone and β-lactam antibiotic classes, respectively. In addition to penethamate hydriodide which is classified as green tier by the NZVA, marbofloxacin is classified as red tier [388]. Cow 14 was treated with the intramammary antibiotic Mastalone® (oxytetracycline, oleandomycin and neomycin) 24 hours before the S1 sample was collected.

The Shannon and Chao1 α-diversity of the faecal microbiome of the untreated control cow in this case study (14C) was relatively high and consistent across all sample periods (S1, S2 and S3) which indicates a high level of bacterial diversity. In comparison, pre (S1) and during treatment (S2) samples collected from treated cow 14 had a comparatively lower Shannon and Chao1 diversity compared to the post-treatment sample (S3) and this is likely due treatment with penethamate hydriodide and marbofloxacin. This combination of antibiotics would likely have a significant effect on the faecal microbiome diversity and richness by reducing the abundance of susceptible populations. The α-diversity of sample S3 appeared to have recovered post-treatment and was more similar to the diversity observed in the control cow faecal microbiomes (S1, S2 and S3) than to S1 or S2 from treated cow 14.

For treated cow 14, the microbiome composition of S1 was unique, with a number of ASVs belonging to genera which were absent or in low abundance in S2 and S3 such as *Burkholderia/Caballeronia/Paraburkholderia* (could not be differentiated at a higher resolution), *Clostridium sensu stricto* 1, *Succiniclasticum* and *Terrisporobacter*. In cow 14, the microbiome composition was altered during treatment (S2), with a higher abundance of ASVs belonging to *Lachnoclostridium*, *Prevotellaceae* UCG-001 and UCG-005. ASVs belonging to *Escherichia-Shigella* were more abundant in S1 and S2 of treated cow 14, but were absent in S3 from cow 14 as well as from the control cow faecal microbiomes (14C: S1, S2 and S3). The post-treatment sample (S3) for cow 14 was more comparable to the faecal microbiome of the control cow (14C) compared to the pre- and during treatment samples for cow 14. ASVs belonging to *Paeniclostridium* and *Romboutsia* were more abundant in S3 samples from both the treated and control cows.

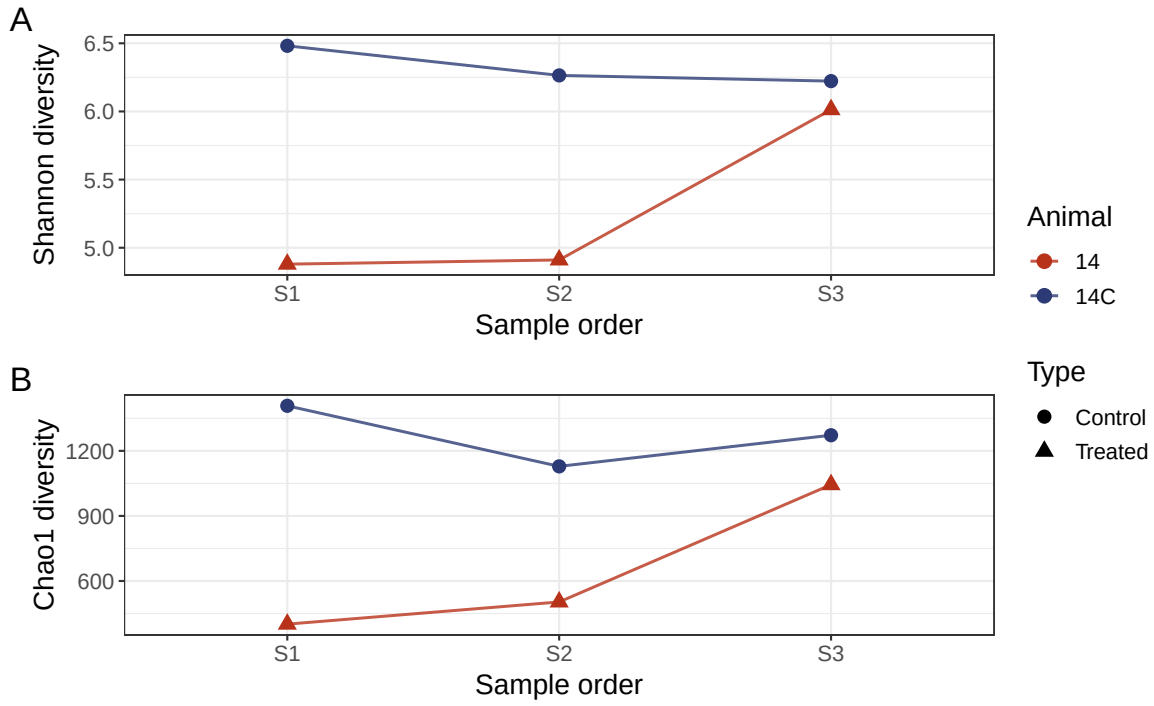

Figure 4.6: Shannon (**A**) and Chao1 (**B**) α-diversity of faecal samples from a cow treated with marbofloxacin/penethamate hydriodide (n=1) and the respective control cow (n=1).

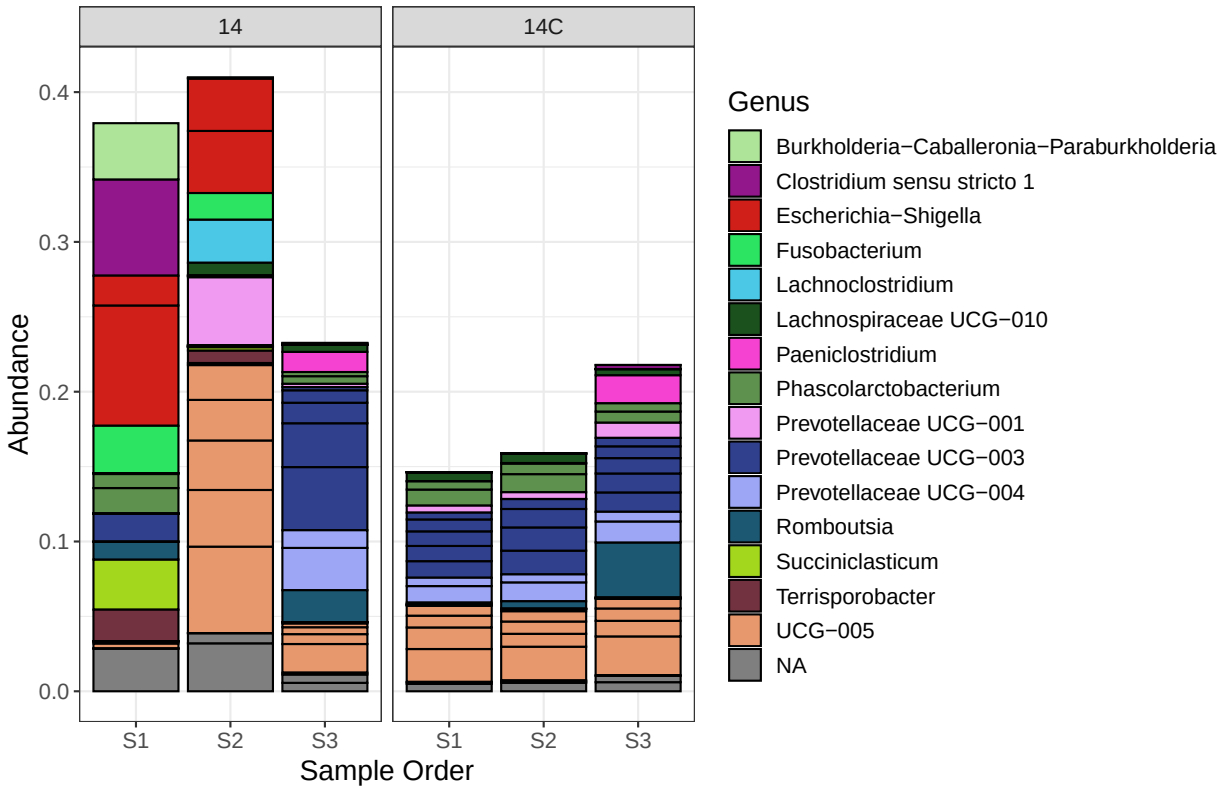

Figure 4.7: Abundance of the top 30 ASVs at the genus level of faecal samples from a cow treated with marbofloxacin/penethamate hydriodide (n=1) and the respective control cow (n=1).

4.3.2.4 Ceftiofur treated cows

Ceftiofur is a third-generation cephalosporin which has a broad spectrum of activity against a variety of Gram-negative pathogens [386]. Ceftiofur is the active ingredient in a number of products used to treat infections on dairy farms, particularly infections other than mastitis such as metritis [387]. Ceftiofur is classified as red tier by the NZVA [388] and therefore its use should be limited to specific organisms or resistant infections. Three cows were treated with ceftiofur for caesarean calf deliveries during the study period (Excede LA; Appendix P).

Both the Shannon and Chao1 α-diversity was similar across samples S1 for both treated and control cows (Figures 4.8A-B) and the α-diversity remained relatively consistent across the treated and control cows, except for sample S2 from control cow 11C which increased and samples S2 and S3 from treated cow 12 which was reduced during and post-treatment compared to pre-treatment (S1) levels. Perturbations in the microbiome composition were observed at the individual animal level during treatment (Figure 4.8C). For cow 11, ASVs belonging to the genera *Paeniclostridium*, *Romboutsia* and *Prevotellaceae* UCG-001 were in low abundance from sample S2 and *Prevotellaceae* UCG-004 associated ASVs were reduced during treatment (S2). In comparison, the microbiome composition in the associated control cow (11C) was relatively stable, with some perturbations in ASV abundance. For example, ASVs belonging to the genus *Alloprevotella* were more abundant in S3 compared to S1 and S2. For treated cow 12, *Paeniclostridium* classified ASVs were in low abundance during treatment (S2) and a higher abundance of an ASV belonging to the family *Bacteroidales* RF16 group was detected during treatment (this ASV could not be classified at the genus level and is listed as "NA" in Figure 4.8C). The microbiome composition of the associated control cow 12C differed in S1, with ASVs belonging to *Alloprevotella* and *Paeniclostridium* either present in low abundance or were absent. The microbiome composition for treated cow 13 varied during the study period. During treatment (S2), ASVs belonging to *Prevotellaceae* UCG-001 were in low abundance and there was a higher abundance of two ASVs listed as "NA" which corresponded to the family *Bacteroidales* RF16 group and p-251-o5 groups. The microbiome composition of key genera in control cow 13C was stable during the study period. According to PCA analysis, the three faecal microbiomes (S1, S2 and S3) from control cows were clustered together (cows 11C, 12C and 13C) whereas the S2 (during) samples for treated cows were distinct compared to S1 and S3, except for cow 12 in which samples S2 and S3 clustered together (Figure 4.8D).

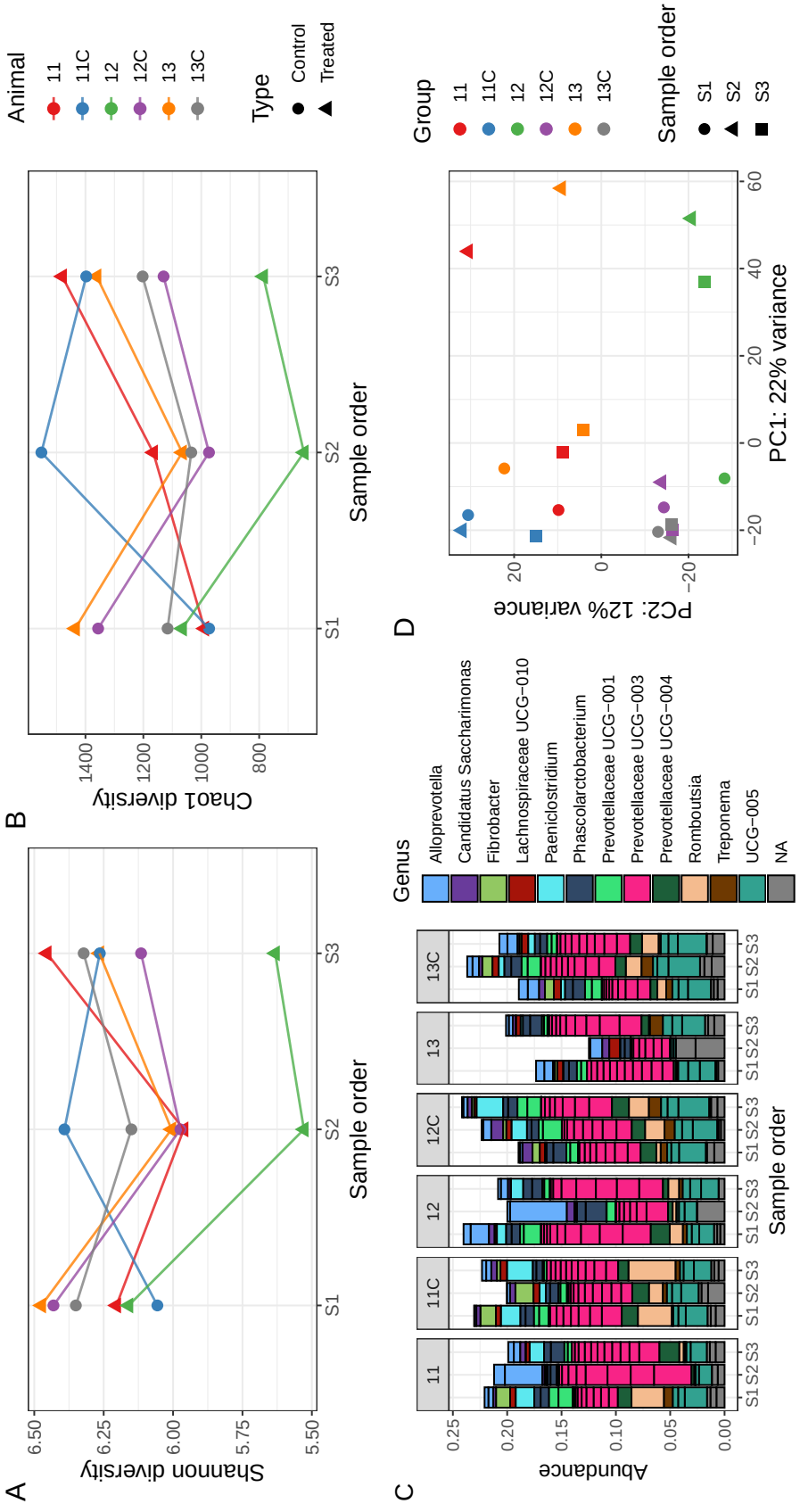

Figure 4.8: Shannon (**A**) and Chao1 (**B**) α-diversity, and abundance of the top 30 ASVs at the genus level (**C**) of faecal samples from cows treated with ceftiofur (n=3) and respective control cows (n=3). Principal Component Analysis (**D**) for the faecal microbiome of treated and control cows at the individual animal level (colours) over the sampling period (shapes). The percentage of variation explained in the Principal Component Analysis is indicated on the axis labels.

4.3.3 AMR in action

The use of third- and fourth-generation cephalosporins is a known risk factor for AmpC and/or ESBL-E being detected on a dairy farm [7]. Three cows were treated with ceftiofur during the study period (cows 11, 12 and 13) and despite no obvious change in abundance of ASVs belonging to *Escherichia-Shigella* during or post-treatment (Appendix R), culture enrichments from faecal samples obtained from treated and control animals (S1, S2 and S3) were investigated further to determine the presence and emergence of third-generation cephalosporin resistant *Enterobacteriaceae*. *Enterobacteriaceae* were identified from all enrichments on the positive control plates (MC agar). Presumptive third-generation cephalosporin resistant *E. coli* were isolated on selective agar plates from samples S2 and S3 collected from the treated cow 12 (Table 4.1). No *E. coli* were isolated from the selective agar plates for sample S1 from cow 12 nor from the other seven treated or nine control cow enrichments.

Boiled DNA lysates from the faecal enrichments were screened using a multiplex PCR to identify pAmpC genes (Table 3.4). PCR of boiled lysate preparations from enrichments DG079 (S2) and DG082 (S3) from cow 12 demonstrated the presence of the $bla_{\text{CMY-2}}$ type gene, whereas enrichment S1 did not yield a PCR product. Faecal sample enrichments from control cow 12C (S1, S2 and S3) had a faint band which did not match any expected amplicon PCR product sizes (380 bp) and may have been due to non-specific priming. Faecal enrichment boiled lysates from the remaining ceftiofur treated cows (n=2) and associated control cows (n=2) did not yield any PCR products from the multiplex PCR.

Table 4.1: Isolates identified from faecal enrichments of ceftiofur treated cows (n=3) and associated control cows (n=3)

Animal	**Lab ID**	**Farm**	**Type**	**Sample order**[a]	**No. isolates**	**MALDI-TOF ID**
11	DG067	Dairy 4	Treated	S1	0	-
11	DG073	Dairy 4	Treated	S2	0	-
11	DG087	Dairy 4	Treated	S3	0	-
11C	DG069	Dairy 4	Control	S1	0	-
11C	DG074	Dairy 4	Control	S2	0	-
11C	DG088	Dairy 4	Control	S3	0	-
12	DG070	Dairy 1	Treated	S1	0	-
12	DG079	Dairy 1	Treated	S2	6	*E. coli*
12	DG082	Dairy 1	Treated	S3	4	*E. coli*
12C	DG071	Dairy 1	Control	S1	4	*Morganella morganii*
12C	DG080	Dairy 1	Control	S2	0	-
12C	DG083	Dairy 1	Control	S3	0	-
13	DG075	Dairy 4	Treated	S1	0	-
13	DG077	Dairy 4	Treated	S2	0	-
13	DG084	Dairy 4	Treated	S3	0	-
13C	DG076	Dairy 4	Control	S1	0	-
13C	DG078	Dairy 4	Control	S2	0	-
13C	DG085	Dairy 4	Control	S3	0	-

[a] Faecal samples were collected pre- (S1), during (S2) and post-treatment (S3) from sick dairy cows. Healthy control (untreated) cow samples were collected during the same sampling period.

Species identification of presumptive *Enterobacteriaceae* was determined using MALDI-TOF MS. Isolates from the MC agar plates, which were used as a positive control, were not analysed further nor were any *Morganella morganii* isolates. *E. coli* which were isolated from selective agar plates (n=10) were subjected to Kirby-Bauer disc diffusion ASTs for six antimicrobials (Table 3.3). All isolates were resistant to cefotaxime, cefpodoxime and cefoxitin and susceptible to tetracycline, streptomycin and ciprofloxacin; except for isolate DG079e which was intermediate for cefoxitin (Table 4.2). All isolates were confirmed as AmpC positive and ESBL negative using the three-disc or double-disc comparison assays, respectively (Figure 3.1). The AmpC-producing *E. coli* were investigated using a multiplex PCR (Table 3.4) to identify pAmpC genes. All *E. coli* isolates (n=10) were positive for the CITM primer set (Table 3.4), indicative of a CMY-2 type β-lactamase. The majority

of isolates had the same resistance profile, however the AST zone sizes varied between isolates. Comparing sample metadata, resistance profiles and AST zone sizes suggested that three groups of *E. coli* isolated from the S2 and S3 faecal samples, indicated by the bold lines in Table 4.2. Group 1 isolates (DG079c-f) were isolated from the faecal sample collected during treatment (S2) on either MC agar supplemented with cefotaxime (1 µg/mL) or ceftazidime (1 µg/mL). Group 2 isolates (DG079g-h) were isolated on CHROMagar^{TM} ESBL plates from sample S2 and in general had smaller AST zone sizes for both cefotaxime and cefoxitin compared to the other isolates (Table 4.2). Group 3 consisted of isolates DG082c-f isolated on either MC agar supplemented with cefotaxime (1 µg/mL) or ceftazidime (1 µg/mL) from the post-treatment faecal sample (S3). *E. coli* isolates from group 1 and group 3 had similar AST zone sizes. One isolate from each group was further analysed using WGS.

Table 4.2: Antimicrobial susceptibility testing and molecular characterisation of the *E. coli* isolates

Isolate	**Phenotype**	***ampC* gene**	**CTX[a]**	**CPD**	**FOX**	**TET**	**STR**	**CIP**
DG079c	AmpC	$bla_{\text{CMY-2}}$ type	20.3	6.6	14.0	23.7	15.3	33.5
DG079d	AmpC	$bla_{\text{CMY-2}}$ type	19.3	6.6	13.9	21.0	15.1	32.5
DG079e	AmpC	$bla_{\text{CMY-2}}$ type	19.3	6.6	15.0	23.7	16.7	33.7
DG079f	AmpC	$bla_{\text{CMY-2}}$ type	18.8	6.6	14.0	23.5	15.3	33.3
DG079g	AmpC	$bla_{\text{CMY-2}}$ type	9.6	6.6	6.6	22.4	16.0	30.6
DG079h	AmpC	$bla_{\text{CMY-2}}$ type	12.0	6.6	6.6	23.3	15.9	31.2
DG082c	AmpC	$bla_{\text{CMY-2}}$ type	19.3	6.6	14.0	22.4	15.0	34.5
DG082d	AmpC	$bla_{\text{CMY-2}}$ type	19.0	6.6	13.5	23.1	15.7	34.9
DG082e	AmpC	$bla_{\text{CMY-2}}$ type	20.2	6.6	13.7	22.2	15.1	31.2
DG082f	AmpC	$bla_{\text{CMY-2}}$ type	18.8	6.6	13.9	22.6	15.5	33.1

[a] Zone sizes in millimetres. CTX, cefotaxime (30µg); CPD, cefpodoxime (10µg); FOX, cefoxitin (30µg); TET, tetracycline (30µg); STR, streptomycin (10µg); CIP, ciprofloxacin (5µg).

4.3.3.1 Whole genome sequencing

Whole genome sequence reads were obtained from DNA extracts of three *E. coli* to understand the molecular epidemiology and genetic context of ARGs from these strains. The three *E. coli* were all isolated from cow 12, two during treatment from MC agar supplemented with ceftazidime (DG079c) and CHROMagar^{TM} ESBL (DG079h), and one *E. coli* isolated from the post-treatment sample on MC agar supplemented with cefotaxime (DG082f). The three *E. coli* isolates were the same serotype (O160:H32) and ST (ST5514) and were genetically similar, differing by 53 - 170 SNPs (Table 4.4). Interestingly, according to the core SNP genome, isolates DG079c and DG082f (isolated from S2 and S3,

respectively) were the most similar differing by 53 SNPs. Isolate DF0049.2e, which is a ST57 CMY-2 producing *E. coli* isolated from Dairy 1 during the longitudinal study (Table 3.5), was included as the reference genome as four isolates are required to carry out the core genome SNP analysis. DF0049e differed from the other isolates by 59,783 - 62,032 SNPs.

Table 4.3: Genome composition of three *E. coli* isolates

Isolate	**Contigs**	**Genome size (bp)**	**GC (%)**	**Depth**	**N50**	**ST**	**Serotype**	**Plasmids**
DG079c	112	4,880,186	50.3	120	110,495	5514	O160:H32	IncFIB_1, IncI1
DG079h	308	4,789,972	50.3	82	37,402	5514	O160:H32	IncFIB_1, IncI1
DG082f	128	4,875,060	50.8	89	91,090	5514	O160:H32	IncFIB_1, IncI1

The ARGs $bla_{\text{CMY-2}}$, chromosomal *ampC*, *aph(6)-Id*, *dfrA14* and *sul2* were detected in the three *E. coli* genomes, which potentially confer resistance to the β-lactam, aminoglycoside, trimethoprim and sulfonamide classes, respectively. The detection of the $bla_{\text{CMY-2}}$ gene was consistent with the AST data and resistance to the third-generation cephalosporins. In contrast, all three isolates carried the *aph(6)-Id* gene which hypothetically confers resistance to streptomycin [412], yet the *E. coli* were susceptible in the AST data highlighting the necessity to confirm a resistance phenotype. Phenotypic testing for trimethoprim and sulfonamide resistance was not undertaken. All three *E. coli* carried the two plasmid incompatibility factors IncFIB_1 and IncI1_1α. According to VirulenceFinder [73], isolates DG079c and DG082f harboured seven and DG079h harboured eight virulence factors (Appendix S). Compared to the other two isolates, DG079h also carried the *gad* gene (glutamate decarboxylase). All of the *E. coli* harboured the *f17*A and *f17*G fimbriae genes. No STEC or EPEC associated virulence genes were identified among the three *E. coli* isolates.

Table 4.4: Core genome single nucleotide polymorphism analysis of three AmpC-producing *E. coli* from in this study

Isolate	**DF0049.2e**	**DG079c**	**DG079h**	**DG082f**
DF0049.2e (reference)	0	61,753	59,783	62,032
DG079c	61,753	0	153	53
DG079h	59,783	153	0	170
DG082f	62,032	53	170	0

4.4 Discussion

It was hypothesised that systemic antimicrobial treatment would impact the bovine faecal microbiome by decreasing species diversity and reducing the abundance of susceptible bacterial populations which could allow non-susceptible bacteria to flourish. The impact of treatment with various antimicrobials in diseased animals on working dairy farms in NZ is unknown. Therefore, faecal samples were collected pre- (S1), during (S2) and post-treatment (S3) from cows receiving systemic antimicrobial therapy across two working dairy farms. Faecal samples were collected at the same time from healthy control/untreated cows on the same farm to account for temporal variation in farm management factors such as feed type and breed as well as any farm-specific variation throughout the duration of the experiment. The results of this study demonstrate that systemic antimicrobial treatment with either procaine penicillin G, ceftiofur, penethamate or marbofloxacin/penethamate reduced the bacterial diversity and richness during treatment (S2) in the bovine faecal microbiome, which was likely a result of the antimicrobials targeting susceptible bacteria. Generally the α-diversity increased post-antimcirobial treatment (S3) when the cow re-entered the milking herd and perturbations in the bovine faecal microbiome were more pronounced in cows with severe disease (for example, cows with left displacement of the abomasum compared to footrot).

Other studies have also demonstrated a reduction in the bovine faecal microbiome richness post antimicrobial treatment. The bovine faecal microbiome OTU richness was reduced two days post-treatment in beef cattle that received a single oxytetracycline injection (n=12) two days after being transported to a feedlot [382]. The rumen microbiota was also reduced after treatment with doxycycline (n=3) in beef cattle [413] and rumen, colon and caecum digesta samples from steers fed a diet supplemented with monesin and tylosin showed decreased bacterial diversity and richness compared to control steers [219]. In comparison, another study found the difference in α-diversity between cattle fed a diet supplemented with tylosin compared to control cattle at feedlots in the USA was not statistically significant [243], although treated cattle had a lower α-diversity compared to that of controls. No significant difference in microbiome diversity or richness was also observed between beef cattle treated with tulathromycin (n=15) or controls (n=15) at a commercial feedlot [414]. Such findings highlight that numerous factors can affect the microbiome richness and diversity, such as the spectrum of antimicrobial activity as well as host-dependent factors. In many cases in this study, the bacterial diversity and richness recovered post-treatment (median 6 days) when the cow re-entered the milking herd (Figures 4.4, 4.5, 4.6, 4.8). However, the microbiome was analysed at the ASV level and the abundance of ASVs were grouped by genus for the top 30 ASVs in the case studies, therefore further work is required to determine whether the same ASVs are present in the pre- and post-treatment samples, or whether different ASVs belonging to the same genus are flourishing post-treatment.

The shared changes in bacterial microbiome composition between treated and control animals could not be investigated due to the study being set in a natural farm environment, with variation in animal health status, prescribed treatment product and duration in individual animals as well as temporal changes in seasonality and feed at the farm level. Therefore, alterations in the bovine faecal microbiome during systemic antimicrobial treatment (S1, S2 and S3) was analysed at the individual animal level. In general, perturbations in the bacterial microbiome composition were observed during treatment (S2) compared to pre- (S1) and post-treatment (S3) samples for treated cows.

Changes in the relative abundance of specific ASVs during treatment differed at the individual animal level. For example, cow 10 treated for a left displaced abomasum with procaine penicillin G displayed major perturbations in bacterial diversity, composition and richness during treatment (Figure 4.4), whereas in comparison the bacterial diversity and richness was lower in cow 12 treated with ceftiofur during and post-treatment, however changes in the microbiome composition of the top 30 ASVs was less apparent (Figure 4.8). This may be due to only the top 30 ASVs across the animals in the case study being analysed and therefore there may have been more apparent changes in ASVs which were present in a lower abundance. Perturbations in the microbiome for cows with the same illness and treatment regime was also animal specific. For example, the diversity, richness and taxonomic composition was relatively stable in the faecal microbiome from cow 6 treated for mastitis with penethamate hydriodide, whereas the α-diversity metrics and bacterial community composition changed for the other two cows treated with penethamate hydriodide (cows 18 and 19; Figure 4.5). These findings highlight that a multitude of factors other than antimicrobial treatment and disease can drive the diversity and composition of the bovine faecal microbiome, such as animal-level variation, diet [286, 415], housing and geographical location [414]. A study of oxytetracycline (tetracycline class) or tulathromycin (macrolide) single injection treatment in feedlot cattle found that the treatments altered the faecal microbiome at days two and five post-treatment, however the microbiome community composition was more affected by transport to the feedlot and time (day 2 to 34 in the study) compared to antimicrobial treatment [382]. Another study analysing pooled faecal samples from pen floors of control cattle or cattle fed a diet supplemented with tylosin did not detect any changes in microbiome composition among treated or control cattle, however geographic location of the feedlot had a significant association with microbiome composition [416]. Both studies demonstrate that environmental and farm management factors can also have a significant effect on the bovine faecal microbiome.

Procaine penicillin G is the active ingredient in a number of products used to treat infections susceptible to penicillin on NZ dairy farms [387]. Penicillin G has *in vitro* activity against Gram-positive and Gram-negative bacteria, however it is usually used to treat infections caused by susceptible Gram-positive bacteria such as *Streptococcus* spp. Therefore, it was hypothesised that systemic antimicrobial therapy with procaine penicillin G would reduce the relative abundance of Gram-positive bacteria in the faecal microbiome.

There was higher variation of microbiome composition at the individual animal level across cows treated with procaine penicillin G (n=23) compared to other treatment groups, with some treated cows showing large perturbations in community composition (e.g. cows 10 and 24) and other cows in which microbiota composition was comparatively stable during treatment (e.g. cows 9 and 16). This may be reflective of the larger sample size for animals treated with procaine penicillin G, which is classified as green tier by the NZVA [388] and is therefore is routinely used to treat infections caused by bacteria susceptible to penicillin. The relative abundance of ASVs classified as *Paeniclostridium* and *Romboutsia*, which are both Gram-positive bacteria, was reduced during treatment in cows 9, 16 and 24 and may be a result the activity of procaine penicillin G against Gram-positive bacteria. The impact of procaine penicillin G on *Lactobacillales*, which includes *Streptococcus* spp., could not be assessed as they were present in low abundance and were not included in the top 30 ASVs detected from faeces in this case study.

In addition, ASVs belonging to the *Bacteroidales* order, which are Gram-negative bacteria, were more abundant in faecal samples collected during treatment (S2) for cows 10 and 24 (Figure 4.5C). Treated cow 10 had an increased abundance of *Escherichia-Shigella* pre- and during treatment and an increase of a ASV belonging to the *Bacteroidales*. The increase in Gram-negative bacteria during treatment may be due to reduced competition with Gram-positive bacteria, which are generally susceptible to procaine penicillin G. In contrast, *Escherichia-Shigella* were higher in abundance post-treatment for cow 24 (Figure 4.4). A decrease in Gram-positive bacteria at the genus level was also observed in cattle fed feed supplemented with monesin and tylosin, which have activity against Gram-positive bacteria [219], yet no changes in Gram-positive bacterial population structure was observed at the phylum level in another study analysing tylosin as a feed additive in beef feedlot cattle [416]. The family *Prevotellaceae* was identified as a core member of the faecal microbiota in cattle [385, 415] and are metabolically diverse [417], with some members of *Prevotella* being identified as more abundant in animals fed concentrates [415]. Interestingly, six ASVs classified as *Prevotellaceae* UCG-003, a genus which was detected a higher relative abundance pre- and during treatment for cow 24, were detected in sample S1 compared to two in S2 and only one in S3 (Figure 4.4), suggesting some *Prevotellaceae* UCG-003 ASVs were more persistent after treatment with procaine penicillin G.

A higher number of animals were treated with procaine penicillin G in this study (23 of 30, 76.7%), which may account for the higher animal level variation seen in this treatment group. Disease severity can also impact on the faecal microbiome, for example more severe disease such as left displacement of the abomasum (cow 10) compared to between claw/-footrot (cow 9) are likely to have an increased impact on overall animal health. Treated animals within other case studies were all treated for the same illness (e.g. ceftiofur for a caesarean section and penethamate hydriodide for mastitis), therefore the impact of illness on the faecal microbiome could not be assessed. It should be noted their perturbations in bacterial community composition were less common in control (untreated) cow faecal samples where the control cows were sampled at the same time as treated cows. The treated

and control cows had access to the same feed sources on farm. Therefore, one hypothesis is that antimicrobial therapy, illness and behavioural changes such as reduced feed intake in ill cattle may have a significant impact on the microbiome.

Penethamate hydriodide (Penethaject) is recommended for the treatment of mastitis caused by Gram-positive bacteria as well as for metritis, foot-rot and respiratory infections [387]. As such, the spectrum of activity of penethamate hydriodide includes a number of Gram-positive mastitis causing pathogens such as *S. aureus*, *Strep. uberis* and *Strep. dysgalactiae* subsp. *dysgalactiae* [411, 65]. Therefore, it was hypothesised that the relative abundance of Gram-positive bacteria will be reduced post-treatment with penethamate hydriodide. A small number of cows were treated for mastitis with penethamate hydriodide during the study period (n=3) and all cows were diagnosed by positive culture and/or susceptibility testing undertaken. The majority of mastitis cases on farm were treated with procaine penicillin G and both of these antimicrobials are classified as green tier by the NZVA, which reflects the prudent antimicrobial stewardship on Dairy 1 and Dairy 4. The bacterial microbiome composition was altered in two of three cows treated with penethamate hydriodide (Figure 4.5). The relative abundance of *Escherichia-Shigella* and *Alloprevotella* was higher in faecal samples collected during treatment (S1 and S2) for cows 18 and 19. This increase in *Escherichia-Shigella* and *Alloprevotella*, which are both Gram-negative bacteria, may be due to a reduction in Gram-positive bacteria which are susceptible to penethamate hydriodide allowing for Gram-negative bacteria to persist and increase in overall abundance. ASVs classified as *Paeniclostridium* and *Romboutsia* were less abundant in some during treatment faecal samples (S2), which may be due to the susceptibility of Gram-positive bacteria to penethamate hydriodide.

Marbofloxacin is a third-generation fluoroquinolone developed for veterinary treatment and has a broad spectrum of activity against most Gram-negative and some Gram-positive bacteria [418]. The label use of Marbocyl® 10%, containing marbofloxacin as the active ingredient, is for the treatment of respiratory or Gram-negative infections [387], with limits on its use for severe or resistant infections as it is classified as red tier by the NZVA [388]. Penethamate hydriodide is active against a number of Gram-positive mastitis causing pathogens such as *S. aureus*, *Strep. uberis* and *Strep. dysgalactiae* subsp. *dysgalactiae* [411, 65], as discussed above. Therefore, it is hypothesised that simultaneous treatment with both marbofloxacin and penethamate hydriodide would alter the faecal microbiome during and post-treatment by reducing the abundance of susceptible bacteria, due to the combined wide spectrum of activity of these two antimicrobials.

The sample S1 faecal microbiome for cow 14 was unique (Figure 4.7), as this cow was also treated 24 hours prior to initial sample collection with the intramammary treatment Mastalone, which is used for severe cases of mastitis and consists of a combination of the three antibiotics oxytetracycline, oleandomycin and neomycin [387]. A number of ASVs were abundant in S1 and either absent or low in abundance from samples S2 and S3

from cow 14 as well as from the top 30 ASVs detected in the other case studies, in particular ASVs classified as *Burkholderia/Caballeronia/Paraburkholderia*, *Clostridium sensu stricto* 1, *Succiniclasticum* and *Terrisporobacter*, which includes a mixture of Gram-positive and Gram-negative bacteria. Some bacterial species within these genera, for example *Burkholderia* spp. [419], have high levels of acquired or intrinsic resistance which may account for the increased abundance of ASVs in this sample post-treatment with oxytetracycline, oleandomycin and neomycin compared to other faecal samples in this study. Interestingly, network analysis of swine manure inoculated with mature compost (high temperature compost containing mesophilic and thermophilic microorganisms) identified *Romboutsia*, *Clostridium sensu stricto* 1 and *Terrisporobacter* as harbouring a number of ARGs and mobile genetic elements [420]. These ASVs were not highly abundant in faecal samples collected during (S2) and post antimicrobial treatment (S3) for cow 14.

The relative abundance of *Escherichia-Shigella* was higher in S1 and S2 for treated cow 14. An increased ratio of Proteobacteria compared to Firmicutes and Bacteroidetes has been associated with rumen dysbiosis identified in cattle mainly fed a concentrate-based diet [286], which could potentially increase the prevalence of ARGs, which are often found in Proteobacteria [390]. Proteobacteria were present at a higher abundance in cattle treated with doxycycline (n=3) compared to healthy controls (n=3) [413], however other external factors such as feed may also affect their abundance as Proteobacteria were more abundant in animals fed a concentrate diet, compared to those with a forage-based diet [286]. Therefore, the higher relative abundance of *Escherichia-Shigella* in samples S1 and S2 may have been due to illness or external factors such as feed. ASVs classified as *Paeniclostridium* and *Romboutsia* were more abundant in samples S3 from both the treated and control cows (14 and 14C, respectively). The relative abundance of such ASVs is variable in some animals from the case studies described, although the relative abundance was higher in both the treated and control (untreated) cow within this case study and therefore may be due to factors other than antimicrobial treatment.

Ceftiofur is a third-generation cephalosporin which has a broad spectrum of activity against a variety of Gram-negative pathogens [386], including susceptible *E. coli*. Across the ceftiofur treated cows (n=3), ASVs belonging to *Alloprevotella* and *Bacteroidales* (Gram-negative bacteria) were enriched during treatment. Interestingly, alterations in the faecal microbiome community structure in cattle treated with ceftiofur (n=3) compared to controls (n=3) was largely due to an increase in the relative abundance of *Bacteroidia* and a decrease in *Actinobacteria* in treated cows [292]. In the same study, in the faeces of ceftiofur treated cows (n=3) the most abundant genes within the β-lactam class were *cfxA2* and *cfxA3* [292], which encode class A β-lactamases that are often found in *Prevotella* spp. [391] and *Capnocytophaga* spp. [392]. The *cfx*A gene has also been detected in *Alloprevotella* spp. isolated from patients with periodontitis in Spain [421]. As seen in other case studies (Figures 4.4 and 4.5), ASVs classified as *Paeniclostridium* and *Romboutsia* (Gram-positive) were absent or in low abundance from the S2 sample from one and two cows, respectively. ASVs belonging to these two genera varied across treatment groups

within this study.

It was hypothesised that treatment with a third-generation cephalosporin (ceftiofur) would increase the prevalence of AmpC and/or ESBL-producing *E. coli* in bovine faeces. Therefore, faecal enrichments from cows treated with ceftiofur (n=3) and associated control cows (n=3) were plated on agar selective for cephalosporin-resistant *Enterobacteriaceae*. *M. morganii* were isolated from the pre-treatment sample for control cow 12C (Table 4.1). Inducible AmpC-β-lactamases are frequently found in *M. morganii* and confer resistance to third-generation cephalosporins [134]. Interestingly, emergent AmpC-producing *E. coli* were isolated during treatment (n=6, 48 hours after ceftiofur treatment) and post-treatment (n=4, 4 days after treatment) for cow 12. Molecular characterisation confirmed that the AmpC-producing *E. coli* harboured the plasmid-mediated $bla_{\text{CMY-2}}$ gene, which is the most prevalent pAmpC gene globally [131] and were isolated from seven samples during the longitudinal study (Chapter 3, Table 3.9).

The genomes of three *E. coli* were sequenced and all were identified as the same serotype and ST (O160:H32 and ST5514). No AmpC-producing *E. coli* of this serotype or ST were isolated from the longitudinal study (Chapter 2). To the best of my knowledge this is the first report of an ST5514 AmpC-producing *E. coli* and only one *E. coli* with this sequence type has been reported on Enterobase (https://enterobase.warwick.ac.uk/species/index/ecoli; accessed 06 December 2021). The plasmid incompatibility factors IncFIB and IncI1 were detected in the three AmpC-producing *E. coli*. IncFIB was found in two chromosomal AmpC-producing *E. coli* isolated from Dairy 1 in the longitudinal study and all plasmid-mediated AmpC-producing *E. coli* had the IncI1 plasmid incompatibility factor (Chapter 3, Table 3.5). The $bla_{\text{CMY-2}}$ gene has been identified on IncI1 plasmids in *E. coli* isolated from humans, pigs, turkey and various meats (chicken, turkey and pork) [360]. The two *E. coli* identified as the most genetically similar according to the core genome SNP analysis (Table 4.4; isolates DG079c and DG082f) were isolated from samples S2 and S3, and were more similar compared to the two *E. coli* isolated from sample S2. This finding was consistent with the AST zone sizes (Table 4.2) and suggests that genetically similar AmpC-producing *E. coli* could be isolated from bovine faeces two and four days post-treatment with ceftiofur. Additional samples were not collected post-treatment, therefore it is not known how long the emergent AmpC-producing *E. coli* persisted and were shed in the faeces of cow 12. There was an increased risk of detecting AmpC-producing *E. coli* in the faeces of dogs treated with cefalexin (first-generation cephalosporin) or cefovecin (third-generation cephalosporin) in a clinical setting immediately after treatment, however one-month post-treatment the risk of detection was not significant [422].

An increase in third-generation cephalosporin resistant *E. coli* in cattle faeces has been identified during and post-treatment with ceftiofur in previous culture-based studies. A pair-matched longitudinal study (n=124 pairs) of cows treated with two-doses of ceftiofur crystalline-free acid across USA dairy farms (n=3) found that third-generation

cephalosporin resistant *E. coli* were higher in the treated group compared to to the untreated group (1.5 $\log_{10}$ colony forming units [CFU]) by day six of the study, and remained higher at day 16 (0.5 $\log_{10}$ CFU) [423]. By days 28 and 56 the population of third-generation cephalosporin resistant *E. coli* in the treated group had decreased and was comparable with the untreated group [423]. The molecular mechanisms for the third-generation cephalosporin resistance was not investigated. Interestingly, a higher abundance of third-generation cephalosporin resistant *E. coli* was identified in cows with a lactation number >3 (compared to first lactation) [423], which may be due to pre-exposure to antimicrobials. *E. coli* with reduced susceptibility to ceftriaxone (third-generation cephalosporin) were more likely to be isolated from farms that reported using ceftiofur in the USA (n=18 farms), however no associations were identified at the individual animal level in cows treated with ceftiofur within six months of sampling [424]. In comparison, no association was identified between ceftiofur use and cephalosporin resistance in *E. coli* across 42 farms across the USA [425]. However, the aforementioned study only examined ceftiofur use on farm [425], and the total AMU and other antimicrobials were not included in the analysis.

Similar to this study, faecal samples from ceftiofur treated (n=5) and untreated (n=5) cattle on a dairy farm in the USA were analysed for ceftiofur resistant *E. coli* [426]. Cows were housed in a barn and treated for infertility due to *Leptospira borgpetersenii* serovar Hardjo-bovis infection. No ceftiofur resistant *E. coli* were isolated from faecal samples collected from untreated cows (0 of 265 *E. coli*) or before treatment for treated cows (days -1 and 0), and were only isolated from faecal samples collected during and directly post-treatment from treated cows (12 of 203 *E. coli*) using MC agar without antibiotics [426]. The $bla_{\text{CMY-2}}$ gene was detected in all 12 ceftiofur resistant *E. coli* and was not detected after day 6 (cows sampled post-treatment at days 5 - 11, 14, 18, 25 and 32). In addition, total *E. coli* counts decreased during treatment in treated cows, however the number of resistant *E. coli* did not increase in treated cows and was not persistent post-treatment [426].

Genome sequencing revealed the *E. coli* (n=3) carried the *aph(6)-Id* gene, which encodes a streptomycin phosphotransferase that has been shown to confer resistance to streptomycin [412], yet all ten of the *E. coli* were susceptible to streptomycin in phenotypic testing (Table 4.2). Studies have shown that APH(6) enzymes are less efficient at inactivating streptomycin [412] and the *aph(6)-Id* gene is often found in combination with other aminoglycoside resistance genes such as *aph(6)-Id*, *aph(3")*, and *ant(3")*, suggesting that additional 3'-phosphotransferase or 3'-adenylyltransferase activity may be required for sufficient streptomycin inactivation in the presence of *aph(6)-Id* [412]. This finding highlights the necessity to confirm a resistance phenotype and caution should be used when reporting a resistance phenotype predicted from genotypic information.

Isolation of plasmid-mediated AmpC-producing *E. coli* post-treatment with ceftiofur is a public and animal health concern due to the health impacts of resistant infections and the potential dissemination of pathogenic bacteria harbouring clinically relevant ARGs. The

lack of detection of AmpC-producing *E. coli* pre-treatment, and culture-based isolation and molecular detection from enrichment broths during and post-treatment potentially represents an interesting case of "AMR in action" and corroborates the recommendation to reduce the use of critically important antimicrobials such as third- and fourth-generation cephalosporins [155].

4.5 Conclusion

This research assessed the impact of systemic antimicrobial treatment on the bovine faecal microbiome in a natural environment by studying diseased animals treated with a range of antimicrobials, providing important information in a natural or "uncontrolled" setting. A decrease in bacterial microbiome diversity and richness was observed (measured as Shannon and Chao1 α-diversity, respectively) during systemic antimicrobial treatment, but the α-diversity had often recovered post-treatment (S3) when the cow re-entered the milking herd. In general, the faecal microbiome of control cows was more stable compared to those which were treated with systemic antimicrobials, with more perturbations observed during treatment (S2) compared to samples S1 and S3 for treated cows. Although perturbations in the microbiome composition and the ability of microbiomes to recover was specific on a case by case basis, highlighting that the individual animal is the main driver of variation, additional factors such as disease severity, the prescribed antimicrobial treatment and duration and changes in farm management factors such as feed may also impact the bovine faecal microbiome.

Emergent AmpC-producing *E. coli* were isolated from faecal enrichments collected during (S2) and post (S3) treatment with ceftiofur for one cow (1 of 3 cows; 33.3%) and no third-generation cephalosporin resistant *E. coli* were isolated from pre-treatment nor from any untreated control cow samples. This finding represents an interesting case of "AMR in action" and indicated that either a resistant *E. coli* population was present in low abundance pre-treatment at undetectable levels using culture-methods and was able to flourish post ceftiofur treatment or the antimicrobial resistant *E. coli* strain was able to successfully colonise the bovine gut in the 48 hours between the S1 (pre-treatment) and S2 (during treatment) sample occasions due to reduced competing bacterial microbiota. The isolation of plasmid-mediated AmpC-producing *E. coli* which were genetically similar during and post-treatment has implications for the development and dissemination of clinically relevant ARGs as well as supporting the need to limit the use of critically important antimicrobials to reduce the development and dissemination of AMR. Future research should focus on any changes in the abundance of ARGs or antimicrobial resistant bacteria during (S2) and post-antimicrobial treatment (S3) by analysing the abundance of specific ARGs in boiled DNA lysate preparations from enriched faecal samples S1, S2 and S3 from treated and control cows using real-time PCR and plating faecal enrichments on various selective agars, respectively. In addition, this work and the example of "AMR in action"

should be communicated through a publication and by discussion with veterinarians and farmers' to encourage prudent antimicrobial stewardship in NZ dairy farming, particularly reducing the use of critically important antimicrobials.

Chapter 5

A longitudinal study of the resistome on two New Zealand dairy farms

5.1 Introduction

The development and transmission of antimicrobial resistance (AMR) is a serious global public and animal health concern. Antimicrobial resistance genes (ARGs) have been identified in varying abundance across numerous environments, including urban sewage [427, 428], hospital wastewater [429], agricultural environments such as dairy farms [430, 431] and soil samples with low anthropogenic impact [432]. Traditional methods for AMR surveillance have focused on culture-based screening of specific bacterial pathogens such as *Escherichia coli* [194], or polymerase chain reaction (PCR) of a limited number of target ARGs [433]. Advances in next-generation sequencing technologies and a reduction in their costs [30] has led to an increase in the number of studies utilising shotgun metagenomic sequencing to study AMR. This has enabled a deeper understanding of the bacterial taxa and ARGs present in various ecosystems. An advantage of shotgun metagenomic sequencing methods is the depth of sequencing data which can be achieved, allowing for detection of genes of interest that may be present in low abundances [236]. If the sequencing depth is sufficient, it can allow for the genomic context of ARGs to be determined. Acquired ARGs can be transferred via horizontal gene transfer (HGT) and are of particular concern due to their ability to disseminate within bacterial populations. This contextual inference is crucial as it is important to assess (i) whether the ARGs are transferable and (ii) which bacterial species harbour the resistance gene(s) to assess the public and animal risk associated with each specific ARG [246].

Globally, ARGs have been detected in the dairy farm environment including in faeces, farm dairy effluent (FDE) and soil [430, 431, 218, 244] as well as raw bulk tank milk [434]. Although the misuse and overuse of antimicrobials in human and animal health have been suggested as the main drivers of AMR [1], the human, animal and environment AMR nexus is complex and numerous factors are involved. For example, heavy metal [280, 279, 301, 302] and biocide use [281] may co-select for AMR, and farm management factors such as buying in cattle or bulls [284] and feeding waste milk to calves [435, 436] may influence the prevalence and shedding of antimicrobial resistant bacteria in the dairy farm environment. The factors which may influence AMR are not constant, such as seasonality

changes, farm management practices and antimicrobial use. For example, the majority of antimicrobial usage (AMU) on New Zealand (NZ) dairy farms is for mastitis treatment and prevention [21], and therefore AMU is likely to be higher post-lactation. A recent study suggested that single time point sampling or collecting samples from a limited number of locations on a farm may be insufficient to accurately estimate the prevalence of antibiotic resistant *E. coli* [195]. Therefore, longitudinal study designs may be more suitable for AMR surveillance in agricultural environments, as they account for changes in AMR prevalence, seasonality, AMU and farm management practices.

NZ dairy farm management practices differ compared to international systems in that they are largely pasture-based [437], have a larger average herd size (431 cows) [438] and a low prevalence of diseases such as coliform mastitis [19]. AMU for growth promotion is banned in NZ [41]. The New Zealand Veterinary Association (NZVA) has aspired that by 2030 antimicrobials will not be required for the maintenance of animal health and welfare [439] and instead their use will be reserved for the treatment of disease. As such, the NZVA have categorised antimicrobials used in animal medicine with a tier traffic light system [388], with the aim of reducing the use of antimicrobials which are critical in treating refractory conditions in human and animal health. Compared to many international systems, NZ uses a low amount of antimicrobials in food-producing animals [16, 17].

It can therefore be hypothesised that the prevalence of ARGs in the NZ dairy farm environment will be low. The aim of this study was to assess the abundance and diversity of ARGs in two NZ dairy farm environments, taking into account changes in seasonality, AMU and farm management practices. Few longitudinal studies have investigated ARGs in NZ dairy farm environments, and to the best of my knowledge this is the first study to utilise shotgun metagenomic sequencing technology for ARG surveillance in NZ dairy farm systems.

5.2 Methods

5.2.1 Study population, sample collection and processing

The study population and sample collection methods are outlined in section 3.2.1. Environmental samples for DNA extraction were promptly stored at -80℃ to prevent DNA degradation and changes to the bacterial community composition. For faeces, a pea-size amount of each faecal sample collected per sampling visit (n=16) was transferred and homogenised in a 15 mL centrifuge tube (Greiner, Sigma-Aldrich, St. Louis, Missouri, USA). Composite faecal material was weighed and 0.25 g stored in triplicate at -80℃. Soil samples were homogenised by hand in the Whirl-Pak® bag (Nasco, Fort Atkinson, Wisconsin, USA), and 0.25 g was stored in triplicate at -80℃. In addition, 1 g of both faecal and soil samples were stored in a cryovial at -80℃. To concentrate the microbial DNA and reduce

the protein and fat content, approximately 400 mL of milk was centrifuged at 10,000 x *g* (Sorvall LYNX 4000 Superspeed Centrifuge, Thermo Fisher Scientific, Waltham, Massachusetts, United States) for 45 min at 4℃. The fat was scooped off and 95% of the supernatant was decanted and the pellet was re-suspended in the remainder of the supernatant. The re-suspended pellet mix was transferred to a 50 mL centrifuge tube (Greiner, Sigma-Aldrich, St. Louis, Missouri, USA) and centrifuged at 10,000 x *g* for 10 min at 4℃. The supernatant was discarded, and the pellet washed twice in 5 mL PBS (pH 7.4) (Thermo Fisher Scientific, Waltham, Massachusetts, United States) at 10,000 x *g* for 10 min at 4℃. The re-suspended pellet was transferred and stored in aliquots at -80℃. To concentrate the FDE (based on the method described in [244]), approximately 400 mL of FDE was centrifuged at 10,000 x *g* (Sorvall LYNX 4000 Superspeed Centrifuge, Thermo Fisher Scientific, Waltham, Massachusetts, United States) for 20 min at 4℃. After, 95% of the supernatant was decanted and the pellet was re-suspended in the remainder of the supernatant and stored at -80℃.

5.2.2 DNA extraction

Samples for DNA extraction were removed from the -80℃ freezer, defrosted on ice and transferred to a bead-beating tube for DNA extraction. Genomic DNA was extracted using the Presto Stool DNA Extraction Kit (Geneaid Biotech Ltd, New Taipei City, Taiwan) according to the manufacturer's instructions with minor modifications. Briefly, 0.25 g faeces and soil and 200 µL of milk and FDE was used for the DNA extraction. For sample lysis, 800 µL of ST1 buffer was added, mixed by vortexing and incubated at 70℃ for 5 min. The sample was vortexed at maximum speed for 7 min (Vortex Mixer, Labnet International, New Jersey, USA) and then centrifuged at 8,000 x *g* for 2 min. Next, 500 µL of supernatant was transferred to a new tube and 5 µL RNAse A (100 mg/mL; QIAGEN, Hilden, Germany) added and incubated for 10 min at 37℃. To remove PCR inhibitors, 150 µL of ST2 buffer was added and mixed briefly by vortex. The sample was incubated at 0 - 4℃ for 5 min and subsequently centrifuged at 16,000 x *g* for 3 min. Next, 500 µL of clear supernatant was transferred to a PCR inhibitor removal column, centrifuged at 16,000 x *g* for 1 min and the column discarded. For DNA binding, 800 µL of ST3 buffer was added to the flow through, mixed vigorously by hand for 5 sec and 700 µL transferred to a GD column. The sample was centrifuged at 16,000 x *g* for 1 min, the flow through discarded and the remaining sample mixture transferred to the GD column. The GD column and sample was again centrifuged at 16,000 x *g* for 1 min and flow through discarded. To wash the DNA, 400 µL of ST3 buffer was added to the GD column and centrifuged at 16,000 x *g* for 30 sec. The flow through was discarded, 600 µL of wash buffer added and centrifuged at 16,000 x *g* for 30 sec. The flow through was discarded and the wash step was repeated. After the final wash step, the GD column was centrifuged at 16,000 x *g* for 3 min to dry the column.

To elute the DNA, the GD column was transferred to a new tube, 30 µL of pre-heated (60℃)

elution buffer was added to the centre of the column and left to stand for 2 min. The sample was then centrifuged at 16,000 x *g* for 2 min and the elution step repeated using the elution buffer containing the eluted DNA. The DNA was transferred to a new microcentrifuge tube and stored at -20℃. All centrifuge steps were carried out at room temperature. The DNA concentration was quantified using a Qubit 4.0 fluorometer (ThermoFisher Scientific Inc., USA) and $A_{260/280}$ and $A_{260/230}$ ratios determined using the Nanodrop microvolume spectrophotometer (Nanodrop 2000c, ThermoFisher Scientific Inc., USA). DNA integrity and size was visualised on a 0.8% [w/v] agarose gel which was prepared using 0.8 g agarose (Invitrogen, Auckland, New Zealand) and 100 mL of 0.5x Tris-borate-EDTA buffer. The gel was stained with RedSafe (Invitrogen, Auckland, New Zealand) and run at 100V for 4 hours. A high molecular weight *Hin*d III/λ ladder was used for size comparison (ThermoFisher Scientific Inc., USA) and gel images were captured and stored using the GelDoc XR^+ system (BioRad, New Zealand).

5.2.3 Shotgun metagenomic sequencing

The metagenomic sequencing run (faeces n=30; soil n=30; FDE n=28; milk n=25 and waste milk n=1) was performed on an Illumina Novaseq S4 platform with 2 x 150 paired-end reads (≥40 million paired-end reads or ≈13 Gb per sample). The libraries were prepared using the NEBNext® DNA Library Preparation Kit (New England Biolabs, Inc, Ipswich, Massachusetts, USA) and sequenced by Novogene, Singapore. The following controls (n=10) were included in this study: (i) blank reagent control for each DNA extraction kit batch (n=2), (ii) phosphate buffered saline (PBS) (n=2) used for preparation of the milk samples and (iii) ZymoBIOMICS™ Microbial Community DNA Standard (D6305; n=3) [440] and ZymoBIOMICS™ Microbial Community DNA Standard II (log distribution) (D6311; n=3) [441].

5.2.4 Bioinformatic analysis

The shotgun metagenomic sequencing analysis was based on a previously described workflow by Liu *et al.*, [11] and the analysis was run on the AgResearch Ltd. High Performance Computing servers. The key steps of the workflow are outlined in Figure 5.1 and the bioinformatic pipeline is detailed here. Briefly, TrimGalore v0.6.6 [442] was used for raw read trimming and quality assessment, using Cutadapt v1.18 [443] and FastQC v0.11.9 [444], respectively. A Phred quality score threshold of 20 was used. Host contamination (*Bos taurus*) was removed by aligning the reads to the bovine genome UMD3.1.1 (accession number: AAFC00000000.3) using BMTagger in bmtools v3.101 [445]. For the ARG normalisation calculations, the number of 16S rRNA genes per sample were identified using METAXA2 v2.2 [446]. The average genome size was estimated using MicrobeCensus v1.1.1 [447], with the number of reads set to 100,000,000 to utilise the maximum reads in each sample. The processed sequencing reads were taxonomically classified using Kraken2

v2.1.1 [448] with the Kraken2 standard database v20200919. The relative abundance of taxa within the samples was estimated at various classification levels using Bracken v2.6.0 [449] and phyla present in ≤5% total abundance were grouped together as "Other" for subsequent analyses. The processed reads were assembled into contigs using MEGAHIT v1.2.9 [450] with default parameters.

5.2.5 Resistance gene analysis

The resistome (collection of all ARGs) in the processed sequencing reads was analysed following the AMR++ pipeline (https://megares.meglab.org/amrplusplus/latest/html/index.html) with minor modifications. Briefly, the antimicrobial, biocide, and heavy metal resistance genes were identified by mapping the processed sequencing reads to the ARG MEGARes database v2.0 [451] using BWA v0.7.17 with default settings [452]. The SAM formatted alignment files were analysed using ResistomeAnalyzer v2018.09.06 (https://github.com/cdeanj/resistomeanalyzer) to generate the sample resistome for each level of the database hierarchy (gene, group, mechanism and class). For gene-level analysis, a gene fraction threshold of 80% was used to reduce false positive hits. Rarefaction analysis was performed per sample to determine whether the sequencing depth used in this study was sufficient to detect the antimicrobial, biocide and heavy metal resistance genes present. The SAM formatted alignment file was used as input for RarefactionAnalyzer v2018.09.06 (https://github.com/cdeanj/rarefactionanalyzer), with sub-sampling of sequencing reads at 5% increments and a gene fraction threshold of 80%.

To allow for more accurate comparisons, ARG gene-level data was normalised to avoid bias associated with ARG size and the microbial load per sample. ARG abundance was expressed as "copy of ARG per copy of 16S rRNA gene" and normalisation calculations performed as previously described by Li *et al.*, [453] using the formula described below. In equation 5.1, ^{n} represents each ARG, $N_{\text{ARG-like sequence}}$ indicates the number of reads annotated as a specific ARG; $L_{\text{ARG reference sequence}}$ is the sequence length (bp) of the corresponding ARG sequence in the MEGARes database v2.0; $N_{\text{16S rRNA sequence}}$ is the number of reads mapping to the 16S rRNA gene in the sample as identified by METAXA2; $L_{\text{16S rRNA sequence}}$ was set to be 1,432 bp which corresponds to the average length of 16S rRNA gene in the Greengenes database [454] and L_{reads} is the length of the sequencing reads used in this study (150 bp).

$$\text{Abundance} = \sum_{1}^{n} \frac{N_{\text{ARG-like sequence}} \times L_{\text{reads}} \ / \ L_{\text{ARG reference sequence}}}{N_{\text{16S rRNA sequence}} \times L_{\text{reads}} / L_{\text{16S rRNA sequence}}} \tag{5.1}$$

Acquired ARGs were identified with Abricate v1.0.1 [https://github.com/tseemann/abricate] [327] using the MEGARes database v2.0 [451]. Contigs containing acquired ARGs were taxonomically classified with the Contig Annotation Tool (CAT) v5.2.1 [455] using NCBI taxonomy files and the NCBI nr database (generated 2021-01-07) with default settings.

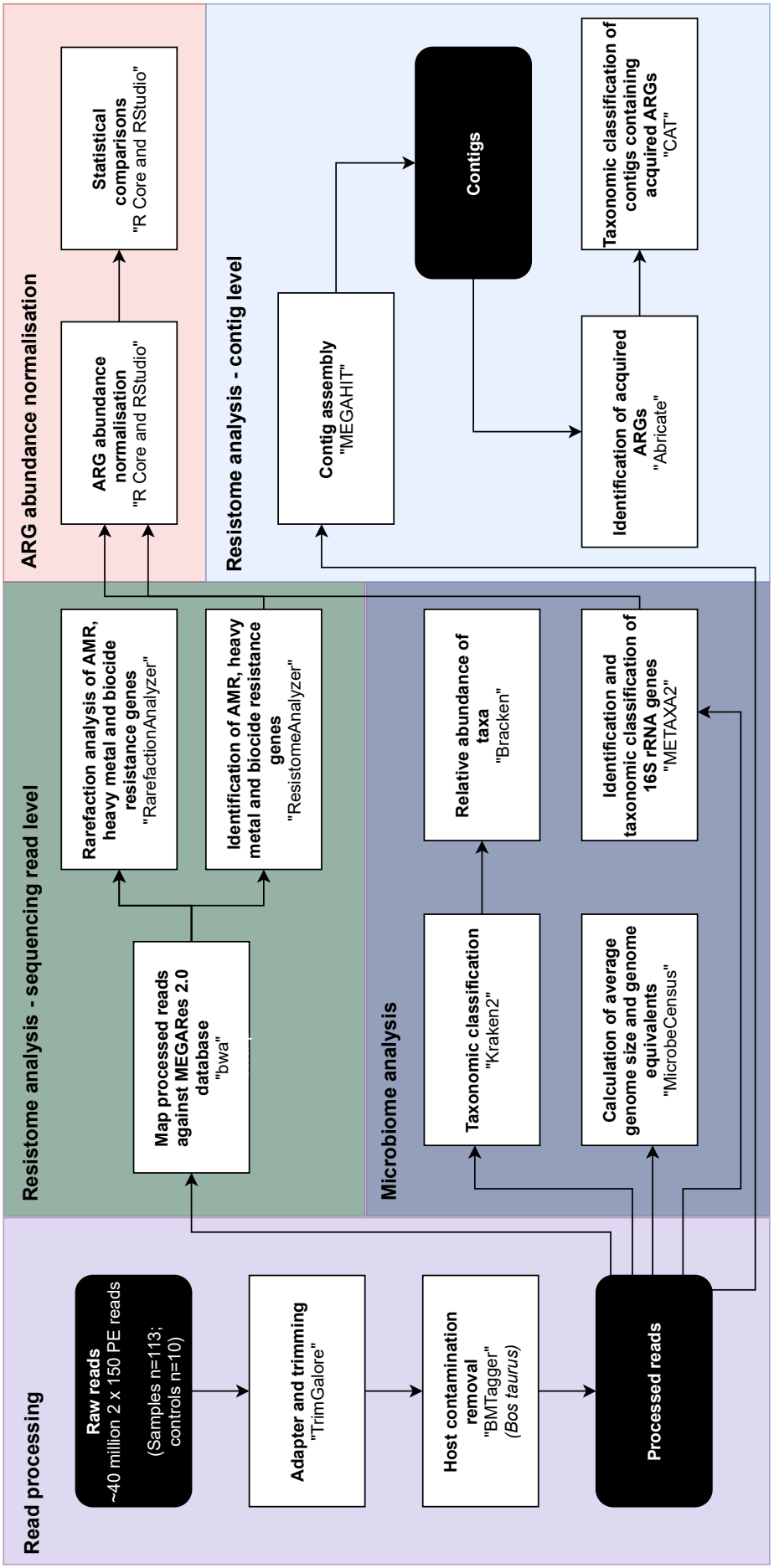

Figure 5.1: Workflow for bioinformatic analysis of shotgun metagenomic sequencing reads. Black rounded-edge rectangles stipulate the sample input for each analysis. White rectangles indicate the analysis steps and the tools used are shown in quotation marks. Arrows show the direction of analysis.

5.2.6 Statistical analysis and data visualisation

Statistical tests and data visualisation was conducted in R Core v4.0.2 [396] and RStudio v1.3.959 [456]. Permutational multivariate analysis of variance test (PERMANOVA) was implemented in the vegan package in R [401] and was used to test the effects of farm (Dairy 1 or Dairy 4) and AMU (mg/PCU per month) and the interactions of these variables on normalised ARGs (copies of ARG per 16S rRNA gene) in each sample using a generalised mixed effect linear model. Sample type (faeces, FDE, milk and soil) was included in the model as a random effect. Only genes in which the normalised ARG value was $>$ 0 for at least one sample was included in the analysis. Post-hoc analysis was calculated using the R package emmeans [410] and pairwise comparisons adjusted using the Tukey method. Statistical tests for the resistome analysis were performed in Minitab® 19.1.1 [347] using a generalised linear model with post-hoc analysis using the Tukey method and a 95% confidence interval. Differences in microbiome profiles based on Bray-Curtis distance measures were analysed using non-metric multidimensional scaling (NMDS) in the vegan package [401]. Data visualisation was conducted in R using a range of packages including ggplot2 [403], tidyverse [404], dplyr [405], Manu [406], Colorgorical [408] and RColorBrewer [407].

5.3 Results

In this study, 123 metagenomic libraries were sequenced consisting of faeces (n=30), soil (n=30), FDE (n=28), milk (n=25) and waste milk (n=1) samples collected over a 15 month period (sample metadata here). The following controls were also included: a mock community DNA standard (n=3), mock community log distribution standard (n=3), PBS (n=2) and negative blank reagent (n=2) controls. The milk sample collected in October 2019 from Dairy 1 was waste milk (DF0167), rather than bulk tank milk. The sample collection issue was discovered post-sequencing, when anomalies were detected in this sample such as a high abundance of ARGs as well as multiple assembled contigs belonging to mastitis-associated pathogens including *Streptococcus uberis*. Therefore, 25 bulk tank milk (referred to hereafter as milk) and one waste milk sample were included in the study. The shotgun metagenomic libraries were analysed to determine their microbiome composition, abundance and distribution of ARGs within the farm environment as well as identifying the bacterial hosts carrying acquired ARGs. A comparison of farm management practices and AMU between the two farms included in this study is detailed in sections 3.2.1 and 3.10.

5.3.1 Microbial community composition

At the phylum level, the microbiome composition was similar across the faecal samples (n=30) from both farms, with 23 phyla identified, excluding phyla present in ≤5% total abundance which were grouped together as "Other" (Appendix T). The phylum Firmicutes (45.2%; range 42.5% - 48.5%), Bacteroidetes (20.4%; range 18.4% - 23.9%), Proteobacteria (17.7%; range 15.6% - 19.7%) and Actinobacteria (8.6%; range 6.9% - 11.8%) were dominant in total abundance across the samples. The same four phyla were also the most abundant in the FDE samples, however the total abundances varied (Proteobacteria [45.1%; range 23.7% - 87.57%], Actinobacteria [17.4%; range 2.7% - 47.4%], Firmicutes [16.6%; range 2.4% - 31.2%] and Bacteroidetes [11.9%; range 2.9% - 18.9%]) and the taxonomic composition was more diverse across the different FDE samples with 31 distinct phyla identified (Appendix T). In particular, the total abundance of Proteobacteria in FDE was the most variable over the 15 month sampling period (23.7 - 87.5%).

A total of 18 different phyla were identified across the soil samples (n=30) (excluding the phyla which were present in ≤5% total abundance grouped together as "Other" (Appendix T)). The microbial community composition was relatively similar across the soil samples at the phylum level, with the two dominant phyla Proteobacteria and Actinobacteria accounting for on average 57.7% (52.3% - 61.0%) and 27.8% (24.3% - 33.8%) of the total abundance, respectively. The predominant bacterial phyla in the milk samples were Proteobacteria and Firmicutes (Appendix T) and the total abundance of these phyla varied across the milk samples collected during this study. In general, either Proteobacteria

(54.2%; range 0.6% - 89.5%) or Firmicutes (21.7%; range 0.7% - 68.6%) was dominant in each sample, which reflects the wide range in total abundance of these two phyla across the samples. The waste milk sample had a comparatively high total abundance of both Proteobacteria (59.8%) and Firmicutes (33.9%). The milk samples also had a high total abundance of the phylum Chordata (21.0%; range 6.2% - 32.5%), which is likely remnants of bovine host contamination still present after read processing.

The microbiome profiles clustered by sample type, irrespective of farm (Figure 5.2). The faecal and soil microbiomes were homogeneous between the farms. According to the microbiome composition, the milk samples clustered in two groups, which was independent of farm or collection date. The two clusters were separated by the most abundant phyla in the samples (Appendix T), with Firmicutes being the predominant phyla in cluster one (n=7) and Proteobacteria in cluster two (n=18). The waste milk microbiome profile clustered separately from the other sample types. The FDE microbiome profiles were more diverse across both farms, reflecting the complex composition of FDE. On Dairy 1, the clustering of FDE microbiome profiles was independent of collection site (Table 3.1). Surprisingly, the microbiome profile of one FDE sample collected from Dairy 1 was more similar to the soil microbiomes compared to the other FDE samples. This was the last sample from the first collection site on Dairy 1, which was collected when there was issues with the FDE filtration system pump. The FDE in the sump during this collection period was visibly dryer than usual and therefore the FDE was likely more stagnant. Hence, a different microbiome composition was observed compared to the other FDE samples.

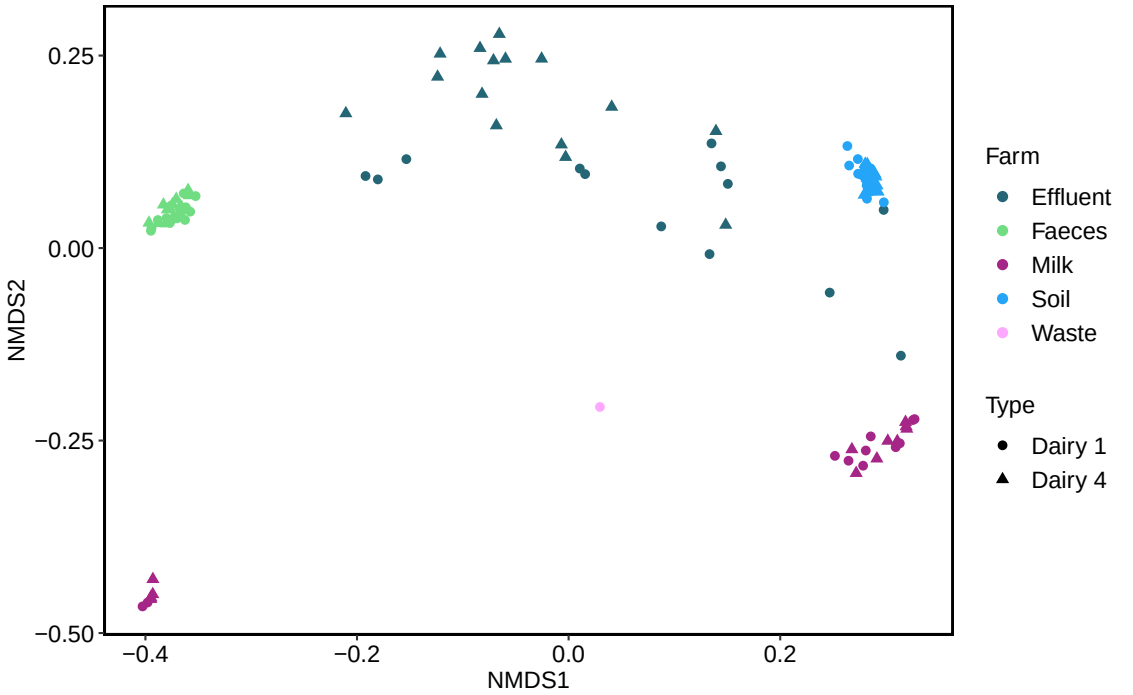

Figure 5.2: NMDS of microbiome profiles at the phylum level of farm samples based on Bray-Curtis dissimilarity matrix (k=3; stress=0.023). Samples are coloured by type and shapes represent farm, as indicated in the figure key.

5.3.2 Resistome analysis

Shotgun metagenomic sequencing of faeces, FDE, soil, milk and waste milk samples from Dairy 1 and Dairy 4 identified 372 unique ARGs, representing 37 resistance classes belonging to the drugs, biocide, metal and multi-compound classes, as classified by the MEGARes2.0 database. Focusing on ARGs belonging to the drug and multi-compound classes (excluding biocide and heavy metal classes), the copies of ARG per 16S rRNA gene per sample ranged from 0.03 - 0.37 in effluent, 0.08 - 0.17 in faeces and the lowest and highest abundance in milk and soil, respectively (0.0 - 0.12; 0.20 - 0.63) (Figure 5.3). ARG abundance in soil was higher compared to FDE and faeces, despite harbouring fewer unique ARGs. Three FDE samples were identified as outliers in the boxplots, with a higher ARG abundance, two collected from Dairy 1 in in October 2018 (DF0009) and December 2019 (DF0188) and one from Dairy 4 in July 2019 (DF0115). The copies of ARG per 16S rRNA gene was not statistically significant between Dairy 1 and Dairy 4 over the 15 month period ($p= 0.321$). ARG abundance between the FDE samples on Dairy 1 and Dairy 4 was statistically significant ($p= 0.010$) but the ARG abundance between soil ($p= 0.271$), faeces ($p= 0.145$) and milk ($p= 0.872$) samples from Dairy 1 and Dairy 4 was not statistically significant. Between all sample types, ARG abundance was statistically significant ($p= <0.001$), except for the FDE and faeces resistomes ($p= 0.954$). Compared to all other sample types, the waste milk sample (n=1) had a higher abundance of antimicrobial, heavy metal and biocide resistance genes, with 0.0 - 3.05 copies of resistance genes per 16S rRNA gene. The antimicrobial, heavy metal and biocide resistance gene abundance (copies of ARG per 16S rRNA gene) at the class level is shown in Figures 5.5 and 5.6.

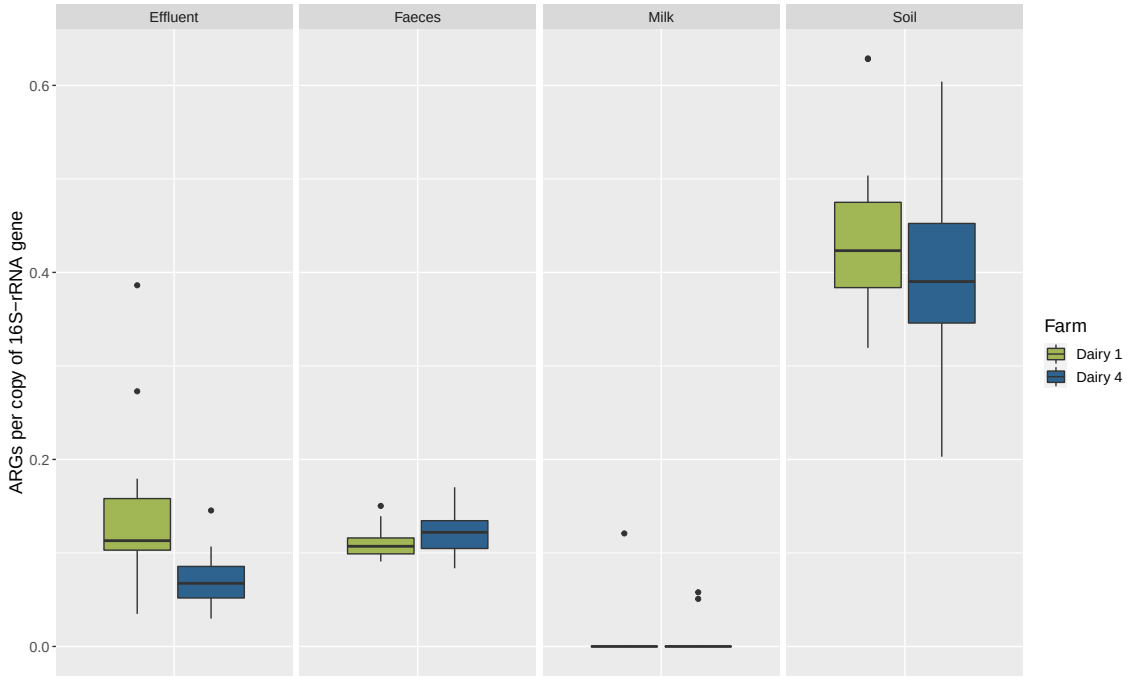

Figure 5.3: Normalised antimicrobial resistance gene abundance (copies of ARG per 16S rRNA gene) in farm dairy effluent, faeces, soil and milk samples collected over a 15 month period on Dairy 1 and Dairy 4. The boxes show the median and upper and lower quartiles. The whiskers show the minimum and maximum values within the interquartile range and the outliers are indicated by a black dot.

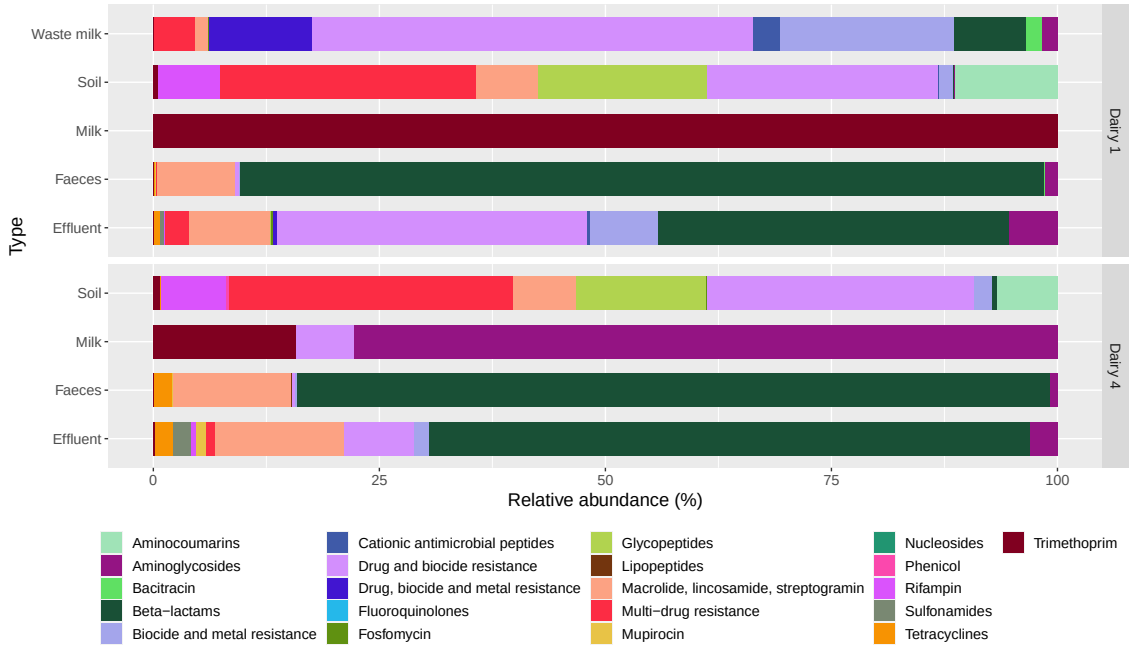

Figure 5.4: Relative abundance of antimicrobial resistance genes in farm dairy effluent, faeces, soil and milk samples on Dairy 1 and Dairy 4. ARGs are classified by antibiotic class (including any ARG conferring resistance across multiple classes but excluding genes only involved in biocide and heavy metal resistance) as indicated in the figure legend.

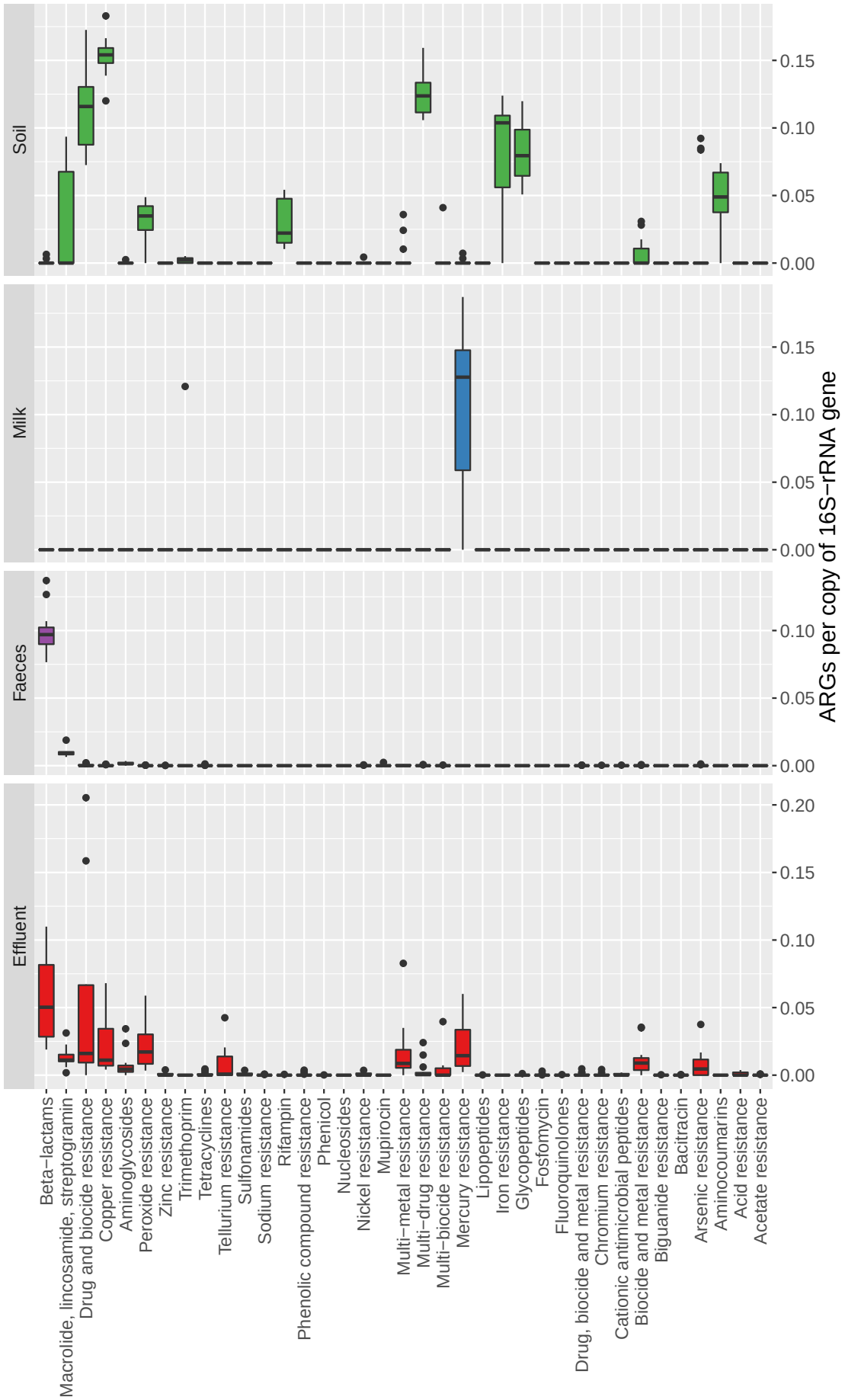

Figure 5.5: Antimicrobial, heavy metal and biocide resistance gene abundance (copies of ARG per 16S rRNA gene) across farm dairy effluent, faeces, milk and soil samples on Dairy 1. Resistance genes are classified at the class level. The boxes show the median and upper and lower quartiles. The whiskers show the minimum and maximum values within the interquartile range and the outliers are indicated by the small circles.

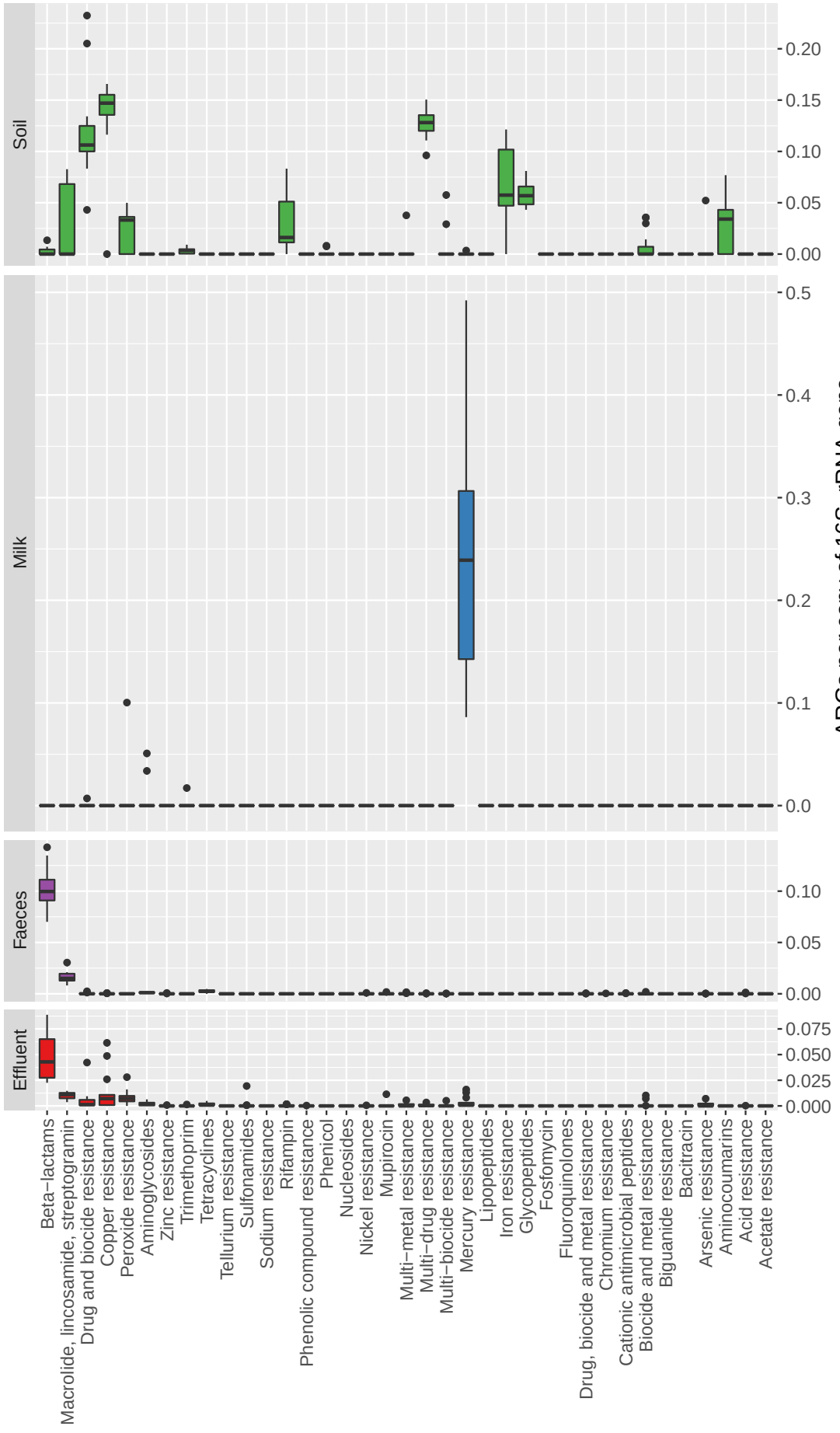

Figure 5.6: Antimicrobial, heavy metal and biocide resistance gene abundance (copies of ARG per 16S rRNA gene) across farm dairy effluent, faeces, milk and soil samples on Dairy 4. Resistance genes are classified at the class level. The boxes show the median and upper and lower quartiles. The whiskers show the minimum and maximum values within the interquartile range and the outliers are indicated by the small circles.

The number of unique ARGs identified in the five sample types varied (Figure 5.4); FDE samples harboured the most diverse range of ARGs, with 164 unique ARGs representing 19 resistance classes identified, followed by faeces and soil with 51 and 30 ARGs, representing 10 and 11 resistance classes, respectively. The fewest unique ARGs (n=3) were identified in the milk, representing only three resistance classes. In the waste milk sample, 81 unique ARGs belonging to 14 resistance classes were identified (Figure 5.9).

The ARGs detected in faeces on both farms predominantly belonged to the β-lactam or macrolide, lincosamide and streptogramin (MLS) antibiotic classes. The predominance of ARGs belonging to the β-lactam or MLS antibiotic classes is likely due to the presence of the *cfxA* and *lnuC* genes respectively, which were identified in all faecal samples (n=30; Appendix U). The *cfxA* gene encodes a class A β-lactamase found on a mobilisable transposon detected in *Bacteroides* species [457] and the abundance of the *cfxA* gene ranged from 0.07 - 0.14 copies of ARG per 16S rRNA gene. The *lnuC* gene encodes a lincosamide nucleotidyltransferase, which was first characterised in a clinical *Streptococcus agalactiae* on a transposon-like element [458]. In comparison to the *cfxA* gene, the *lnuC* gene was detected at a lower abundance ranging from 0.01 - 0.03 copies of ARG per 16S rRNA gene.

The FDE samples contained the most diverse range of ARGs and the predominant antibiotic classes included the β-lactams, MLS and aminoglycoside classes. Resistance genes potentially conferring resistance to multi-compounds were also identified (drug/biocide resistance and biocide/metal resistance). Although the ARGs identified in FDE were more diverse, they were generally detected at a relatively low abundance (compared to the soil resistome which had the highest ARG abundance in this study). The *cfxA* gene group was identified in 27 of 28 of FDE samples (0.0 - 0.10 copies of ARG per 16S rRNA gene), and the *lnuC* and *sodB* genes belonging to MLS and peroxide resistance classes, were identified in 27 of 28 and 25 of 28 of FDE samples respectively, albeit at a low abundance (0.0 - 0.01 and 0.0 - 0.06 copies of ARG per 16S rRNA gene, respectively; Appendix U).

In soil samples, the resistance classes identified were comparatively similar between Dairy 1 and Dairy 4, with ARGs predominantly belonging to multi-drug resistance, drug/biocide resistance, glycopeptides, aminocoumarins, MLS and rifampin resistance classes. ARGs were detected in a limited number of milk samples (1 of 11 on Dairy 1; 2 of 13 on Dairy 4) and they belonged to trimethoprim, aminoglycoside and drug/biocide resistance classes and the abundance of these genes was very low (0.00 - 0.12, 0.00 - 0.05, 0.00 - 0.01 copies of ARG per 16S rRNA gene, respectively). The gene *dfrB* potentially conferring trimethoprim resistance, was identified from a single milk sample from both Dairy 1 (DF0012) and Dairy 4 (DF0001). The same milk sample from Dairy 4 also harboured the *ant6* and *qac*$\triangle$1 genes, belonging to aminoglycosides and drug/biocide resistance classes, respectively. The *ant6* gene was also identified from another single milk sample from Dairy 4 (DF0184).

The abundance of heavy metal and biocide resistance genes between Dairy 1 and Dairy 4 (Figure 5.7) was not statistically significant (p= 0.712). However, heavy metal and biocide resistance gene abundance between the four sample types was statistically significant

(faeces and effluent (p= 0.028), soil and milk (p= 0.005) and all other combinations (p= <0.001)). Heavy metal and biocide resistance gene abundance in FDE was statistically significant between the farms (p= 0.003), with a higher abundance detected in Dairy 1 (0.01 - 0.33 copies of ARG per 16S rRNA gene) compared to Dairy 4 (0.00 - 0.08 copies of ARG per 16S rRNA gene). Across the FDE samples, 149 unique heavy metal and biocide resistance genes were detected representing 14 resistance classes (Figure 5.8). The *qac* gene encoding resistance to quaternary ammonium compounds which are commonly used in disinfectants, were identified in FDE sporadically on both farms (Appendix U).

In the milk samples, a higher abundance of heavy metal and biocide resistance genes were identified on Dairy 4 (0.09 - 0.59 copies of ARG per 16S rRNA gene) compared to Dairy 1 (0.00 - 0.19), which was statistically significant (p= 0.012). Interestingly, one peroxide resistance gene was detected (*sodB*) on Dairy 4 and the remaining resistance genes all conferred mercury resistance (*mer*ABCDEFPRT, Appendix U). The abundance of heavy metal and biocide resistance genes in faeces was very low on both farms (0.00 - 0.01 copies of ARG per 16S rRNA gene; p= 0.972). Heavy metal and biocide resistance gene abundance was highest in soil samples compared to the other sample types and the abundance between soil on Dairy 1 (0.18 - 0.43 copies of ARG per 16S rRNA gene) and Dairy 4 (0.00 - 0.37 copies of ARG per 16S rRNA gene) was not statistically significant (p=0.093). Genes representing a diverse range of resistance classes were identified from the soil samples, with the most resistance genes belonging including copper, peroxide and iron resistance classes.

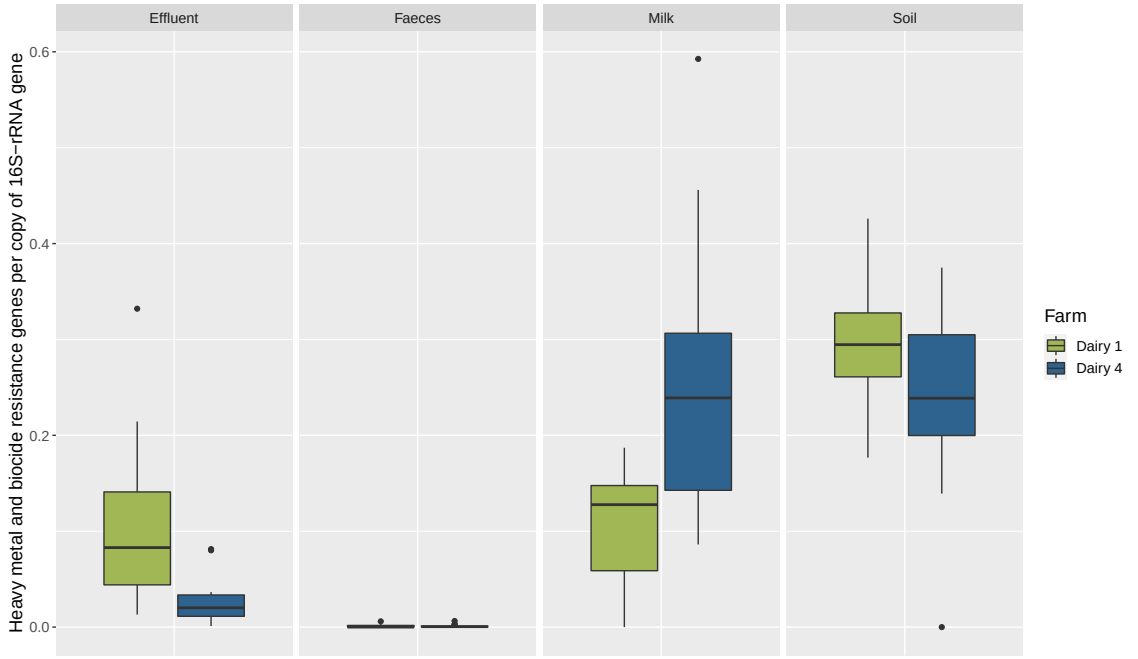

Figure 5.7: Normalised heavy metal and biocide resistance gene abundance (copies of ARG per 16S rRNA gene) in farm dairy effluent, faeces, soil and milk samples collected over a 15 month period on Dairy 1 and Dairy 4. The boxes show the median and upper and lower quartiles. The whiskers show the minimum and maximum values within the interquartile range and the outliers are indicated by a black dot.

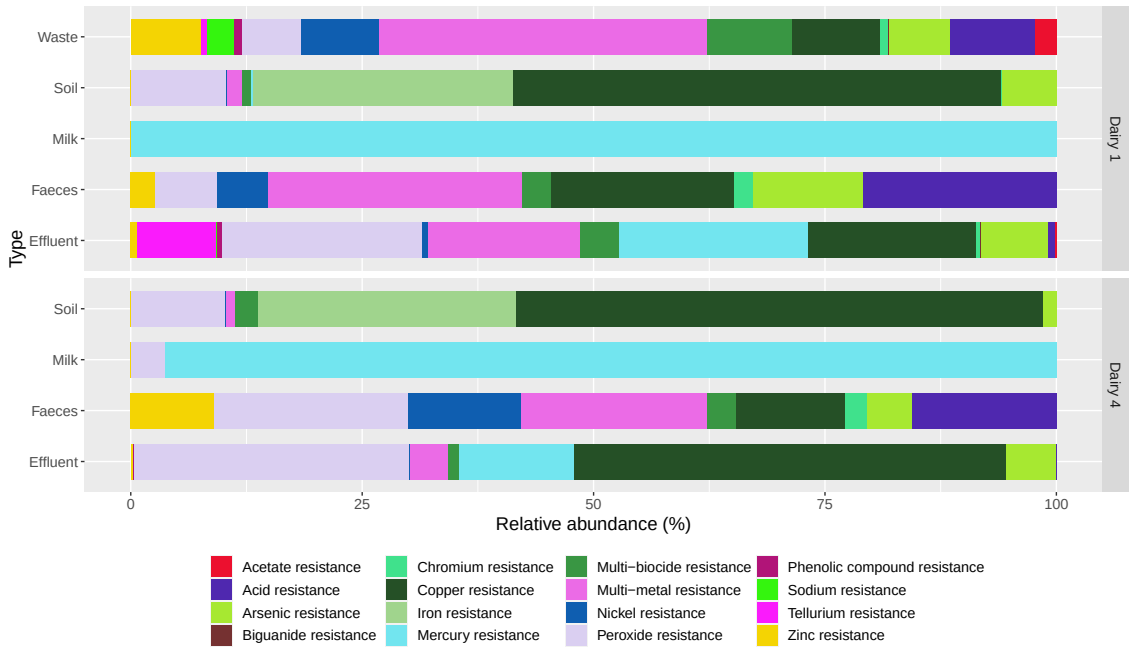

Figure 5.8: Relative abundance of heavy metal and biocide resistance genes in farm dairy effluent, faeces, soil and milk samples on Dairy 1 and Dairy 4. Resistance genes are classified by resistance class as indicated in the figure legend.

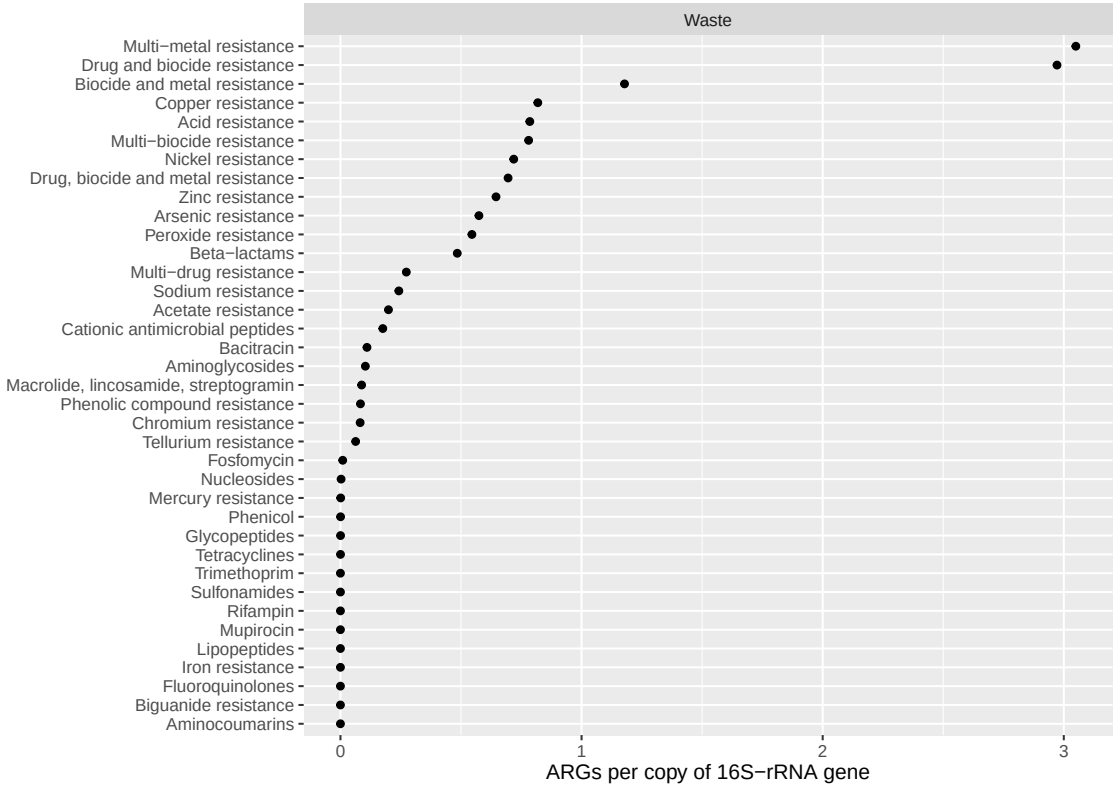

Figure 5.9: Antimicrobial, heavy metal and biocide resistance gene abundance (copies of ARG per 16S rRNA gene) identified in the waste milk sample. Resistance genes are classified at the class level.

From the waste milk sample, 103 unique antimicrobial, heavy metal or biocide resistance genes were identified representing 14 resistance classes including copper, acid, nickel, zinc, arsenic and peroxide (Figure 5.9). Interestingly, the most abundant resistant classes in the waste milk were often multi-compound such as multi-metal (3.05 copies of ARG per 16S rRNA gene), drug/biocide (2.97 copies of ARG per 16S rRNA gene), biocide/metal (1.18 copies of ARG per 16S rRNA gene) and multi-biocide (0.78 copies of ARG per 16S rRNA gene), drug/biocide/metal (0.70 copies of ARG per 16S rRNA gene) and multi-drug (0.27 copies of ARG per 16S rRNA gene) classes were also relatively abundant.

No significant difference in antimicrobial, heavy metal or biocide resistance gene abundance was observed between farms and differences in AMU (p= 0.6264), taking into account the random effect of sample type (faeces, FDE, soil or milk). PERMANOVA analysis suggested that AMU did not account for the total variation in ARG abundance between the samples.

5.3.3 Bacterial host range harbouring contigs containing acquired ARGs

The detection of acquired ARGs is a concern due to their ability to spread via HGT and thus the potential for the development of multi-drug resistant phenotypes to emerge. Across the sequenced farm samples (n=113), 147,822,721 contigs were assembled. Of these, 1,014 contigs (0.0007%) harboured at least one acquired ARG and were assembled from 88 metagenomic samples (88 of 113; 78.8%). A total of 200 contigs could be classified at the phylum level (200 of 1,014; 19.7%). The number of contigs which were taxonomically assigned reduced as the classification levels decreased down to the class (140 of 200; 70.0%), order (67 of 200; 6.6%), family (58 of 200; 29.0%), genus (42 of 200; 21.0%) and species (12 of 200; 6.0%) levels. The predicted taxonomic ranks of contigs harbouring acquired ARGs at the class, order, genus and species level is shown in Appendix V. The remaining contigs could not be taxonomically classified at a lower taxonomic rank with the classification parameters used (section 5.2.5).

Within the 200 contigs originating from 70 metagenomic samples (Figure 5.10), 57 unique ARGs were identified, representing 14 antibiotic classes. Seven contigs (7 of 200; 3.5%) co-harboured multiple ARGs (Table 5.1). Interestingly, contigs co-harbouring the *aph(3'')* and *aph6* gene groups belonging to Proteobacteria were assembled from FDE samples on both Dairy 1 and Dairy 4 in November 2018 and March 2019, respectively. Three contigs co-harbouring the bla_{PER} and *fosA* genes belonging to *Gammaproteobacteria* were assembled from FDE samples (n=2 samples) from November and December 2019. The bla_{ACC} and *crp* genes were identified on a contig assembled from the waste milk sample and belonged to the bacterial order *Enterobacterales*. The bla_{ACC} gene encodes a plasmid-mediated Class C β-lactamase.

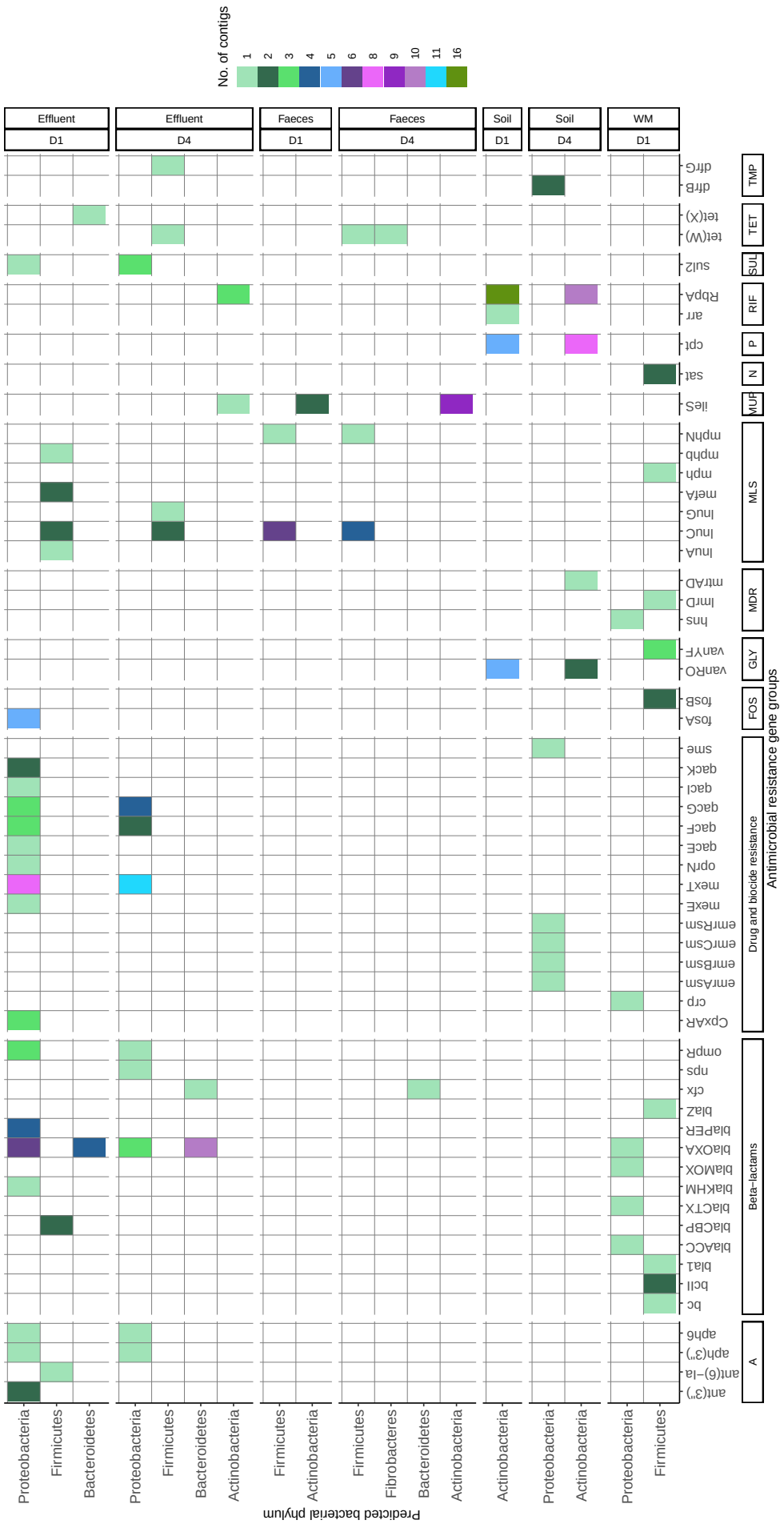

Figure 5.10: The predicted bacterial phyla of 200 contigs harbouring acquired antimicrobial resistance genes (ARGs). ARG groups were grouped per class of antibiotics and the contigs grouped by sample type and farm. The number of contigs with the unique phyla and ARG combination are indicated by the colour key on the Figure legend. A, Aminoglycoside; FOS, Fosfomycin; GLY, Glycopeptides; MDR, Multi-drug resistance; MLS, Macrolide, lincosamide and streptogramin; MUP, Mupirocin; N, Nucleosides; P, Phenicol; RIF, Rifampin; SUL, Sulfonamides; TET, Tetracycline; TMP, Trimethoprim; WM, Waste milk.

Most of the acquired ARGs were on contigs that belonged to the Proteobacteria (30 genes) and Firmicutes (19 genes) phylum, followed by the Actinobacteria (6 genes), Bacteroidetes (3 genes) and Fibrobacteres (1 gene) phylum. At the phylum level, the majority of the contigs harbouring acquired ARGs were assembled from FDE samples (n=102 contigs), with a higher number of contigs assembled from Dairy 1 (n=57) compared to Dairy 4 (n=46). ARGs identified in contigs assembled from FDE samples potentially confer resistance across ten antimicrobial classes, with the highest number of gene groups belonging to drug and biocide resistance (n=9), β-lactams (n=7), MLS (n=5) and aminoglycosides (n=4). These genes were predominantly found in Proteobacteria but were detected in contigs belonging to Actinobacteria, Bacteroidetes and Firmicutes. In addition, a number of *qac* genes were found in contigs belonging to Proteobacteria from both farms. The second highest number of contigs harbouring acquired ARGs were assembled from soil samples (n=52 contigs) and the number of contigs from each farm was similar (Dairy 1 n=27; Dairy 4 n=25). Relatively fewer contigs with ARGs were assembled from faeces samples (n=26), with a higher number from Dairy 4 (n=17) compared to Dairy 1 (n=9). No contigs containing acquired ARGs were assembled from milk samples. From the waste milk sample, 19 contigs containing ARGs were assembled, with one contig co-harbouring two ARGs. The ARGs identified in the waste milk sample potentially confer resistance to the β-lactam, drug and biocide resistance and MLS classes from contigs belonging to the phylum Proteobacteria, as well as β-lactam, fosfomycin, glycopeptide, multi-drug, MLS and nucleoside classes detected in contigs belonging to the Firmicutes phylum.

Table 5.1: Taxonomic classification of contigs co-harbouring two acquired resistance genes

Sample	**Farm**[a]	**Type**[b]	**Gene groups**	**Class**	**Classification**[c]
DF0025	D1	FDE	*aph(3")*, *aph6*	Aminoglycosides	Proteobacteria
DF0068	D4	FDE	*aph(3")*, *aph6*	Aminoglycosides	Proteobacteria
DF0097	D4	Soil	*emrAsm, emrBsm emrCsm, emrRsm*	Drug and biocide	*Stenotrophomonas*
DF0167	D1	WM	bla_{ACC}, *crp*	β-lactams Drug and biocide	*Enterobacterales*
DF0176	D1	FDE	bla_{PER}, *fos*A	β-lactams Fosfomycin	*Gammaproteobacteria*
DF0188	D1	FDE	bla_{PER}, *fos*A	β-lactams Fosfomycin	*Gammaproteobacteria*
DF0188	D1	FDE	bla_{PER}, *fos*A	β-lactams Fosfomycin	*Gammaproteobacteria*

[a] D1, Dairy 1; D4, Dairy 4.
[b] FDE, Farm dairy effluent; WM, Waste milk.
[c] Classification recorded for the lowest taxonomic rank identified.

The phylum Proteobacteria, which includes clinically relevant pathogens such as *E. coli*, *Klebsiella pneumoniae* and *Acinetobacter baumannii*, harbours the most diverse range of ARGs across the sample types. Proteobacteria was one of the predominant phyla found in the FDE samples (average: 45.1%, Appendix T), and the number of contigs containing

ARGs classified as Proteobacteria across FDE samples from both farms was high (Dairy 1 n=43; Dairy 4 n=26). Acquired ARGs from contigs belonging to the Proteobacteria phylum were found in the waste milk sample (n=4 contigs) and soil samples (n=4 contigs), although the number of contigs was lower from these sources. Samples which had a high ARG abundance at the sequencing read level (Figure 5.12) also had a higher number of contigs with acquired ARGs belonging to Proteobacteria (FDE: DF0176 and DF0188; waste milk: DF0167). Despite being a predominant phylum detected in the faecal microbiome composition analysis, no Proteobacteria contigs containing ARGs were detected in faecal samples from either farm during the study. Acquired ARGs were detected in contigs assembled from faecal samples belonged to Firmicutes (n=13 contigs), Actinobacteria (n=11 contigs), Bacteroidetes (n=1 contig) and Fibrobacteres (n=1 contig) phyla.

The phylum Firmicutes also harboured a diverse range of ARGs (n=19) and contigs were assembled from FDE (n=14 contigs), faeces (n=13 contigs) and waste milk (n=14 contigs) samples. The *lnuC* gene, which encodes a lincosamide nucleotidyltransferase and was identified from all faecal samples (n=30) and most FDE samples (n=27; 96.4%), was identified in 14 contigs across FDE (n=4) and faeces (n=10) samples. Four of these contigs could be classified at the family level, and belonged to the *Lachnospiraceae*, *Ruminococcaceae* and *Sporomusaceae* families (Figure 5.11). The identification of *lnuC* across multiple bacteria families may explain the high detection of this gene from faeces and FDE. Acquired ARGs were identified in contigs belonging to Bacteroidetes (n=17 contigs) from FDE (n=13 samples) and faeces samples (n=1 sample). Of these, 14 contigs harboured the bla_{OXA} gene and five contigs could be classified at the family level, belonging to *Flavobacteriaceae*. One contig harboured the *tetX* gene and belonged to the *Sphingobacteriaceae* family. Two contigs assembled from faeces and FDE (n=1 each) harboured the bla_{CFX} gene and the *cfxA* gene was identified in all faecal samples (n=30) and most FDE samples (n=27; 96.4%) at the sequencing read level. These two contigs could not be classified at a lower taxonomic rank.

The phylum Actinobacteria, which was prevalent in the soil and FDE microbiomes (Appendix T), had a high number of contigs containing acquired ARGs across the soil samples on both farms (Dairy 1: 13 of 15 samples; 27 contigs; Dairy 4: 11 of 15, 21 contigs), as well as in faeces (Dairy 1: 2 of 15, 2 contigs; Dairy 4: 9 of 15, 9 contigs) and only FDE on Dairy 4 (4 of 15, 4 contigs). A range of acquired ARGs (six genes) were carried by contigs belonging to Actinobacteria, with *ileS* (encoding an isoleucyl-tRNA synthetase which confers resistance to mupirocin) found in faeces and FDE and *rbpA* (encoding an RNA-polymerase binding protein which confers resistance to rifampin) found in soil and FDE being the most prevalent. FDE samples from both farms harboured the most diverse host range of contigs with contigs belonging to *Gammaproteobacteria*, Actinobacteria, *Bacilli* and *Clostridia* which were also identified in other sample types, as well as *Betaproteobacteria*, *Flavobacteriia*, *Alphaproteobacteria*, *Epsilonproteobacteria*, *Negativicutes* and *Sphingobacteriia*.

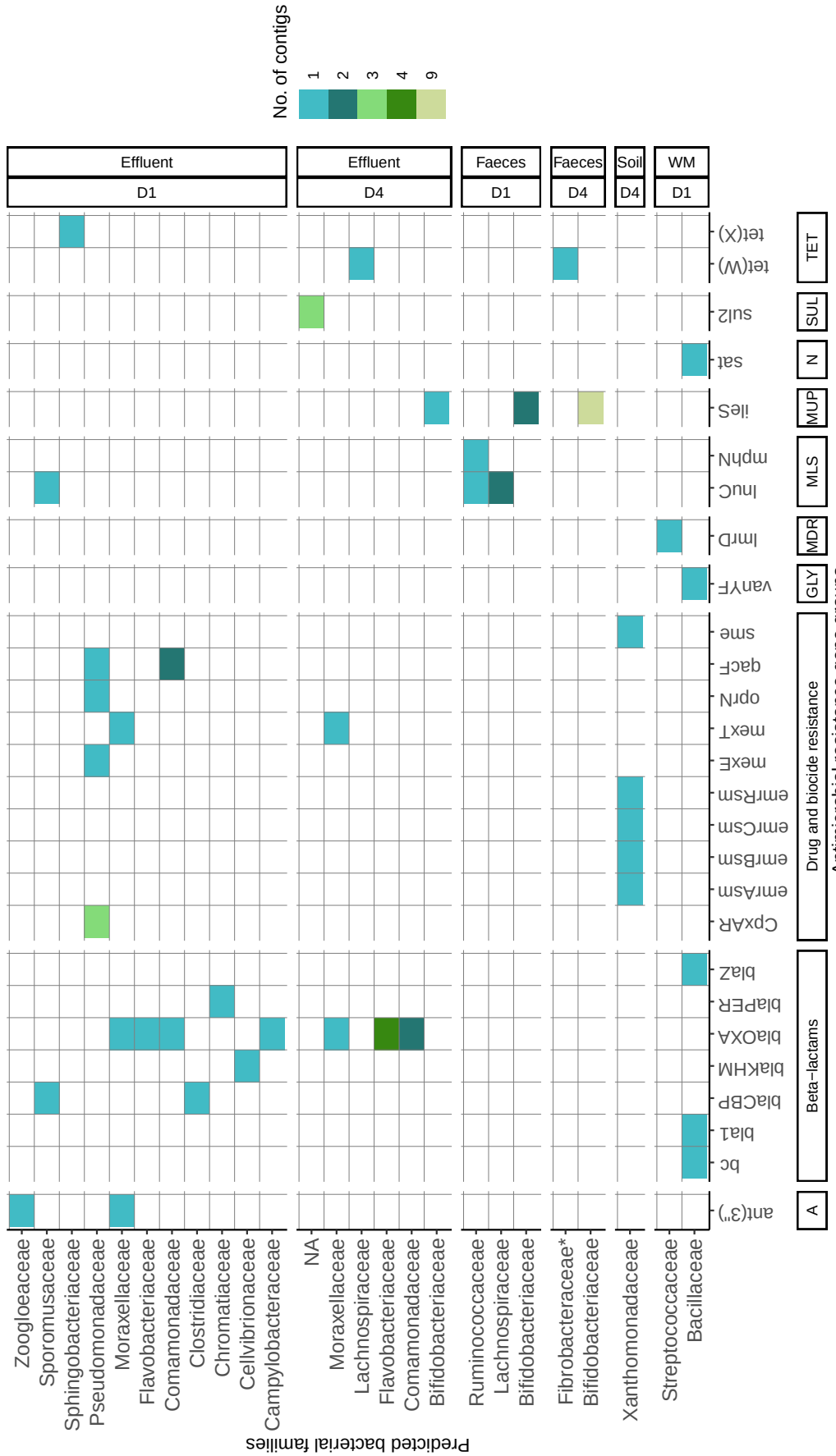

Figure 5.11: The predicted bacterial family of 58 contigs harbouring acquired antimicrobial resistance genes (ARGs). ARG groups were grouped per class of antibiotics and the contigs grouped by sample type and farm. The number of contigs with the unique family and ARG combination are indicated by the colour key on the Figure legend. A, Aminoglycoside; GLY, Glycopeptides; MDR, Multi-drug resistance; MLS, Macrolide, lincosamide and streptogramin; MUP, Mupirocin; N, Nucleosides; SUL, Sulfonamides; TET, Tetracycline; WM, Waste milk. The *Fibrobacteraceae* family is marked with an asterisk as this was the only family from the order *Fibrobacterales* that was present in the database. NA, Bacterial family not identified by contig classified as *Betaproteobacteria*.

5.3.4 Examination of sequencing depth for the detection of antimicrobial, heavy metal and biocide resistance genes

To determine whether the sequencing depth used in this study was sufficient to detect the resistome in each sample, including resistance genes present in low abundance, rarefaction analysis was used which identifies the number of unique genes while by sub-sampling the sequencing reads at 5% increments. If the depth was sufficient to detect the total resistome, the number of unique genes should plateau after 100% of the reads have been sampled. Previous research has shown that the relative proportion of reads assigned to specific ARG classes was relatively consistent across various sampling depths in bovine faeces (average read counts used in the study: 26, 59 and 117 million), however increasing the sequencing depth resulted in higher numbers of ARG assigned reads [236]. An average sample read count of 59 million was suggested as suitable to sufficiently describe the resistome in bovine faeces [236]. Given that the abundance of ARGs will vary depending on sample type as well as population level factors, such as a hypothesised low abundance of ARGs in NZ dairy farm environments, the sequencing depth required to analyse the resistome is study-specific.

This study used a sequencing depth of $\geq$40 million read pairs per sample and rarefaction analysis suggested that this depth was sufficient to study the resistome in the FDE, faeces, milk and soil samples sequenced in this study. However the relative abundance of ARGs was higher in three FDE samples (DF0145, DF0176, DF0188) and the waste milk sample (DF0167) and the number of unique genes was still increasing after all of the reads had been sub-sampled (Figure 5.12). Compared to the other samples analysed in this study, the number of resistance genes was much higher (>200 genes) in these four samples. The three FDE samples identified as outliers compared to the other FDE samples were collected from Dairy 1 in September, November and December 2019 from the second collection point in the study which was from a grate in the cowshed compared to the effluent sump (see section 3.1). Interestingly, the samples collected in July, August and October 2019 from this location had lower antimicrobial, heavy metal and biocide resistance genes identified (<200 genes) and the number of unique genes appeared to plateau off, suggesting the sequencing depth was sufficient to detect the resistome within these samples. The waste milk sample contained a high number of ARGs, even when a low proportion of reads were sub-sampled.

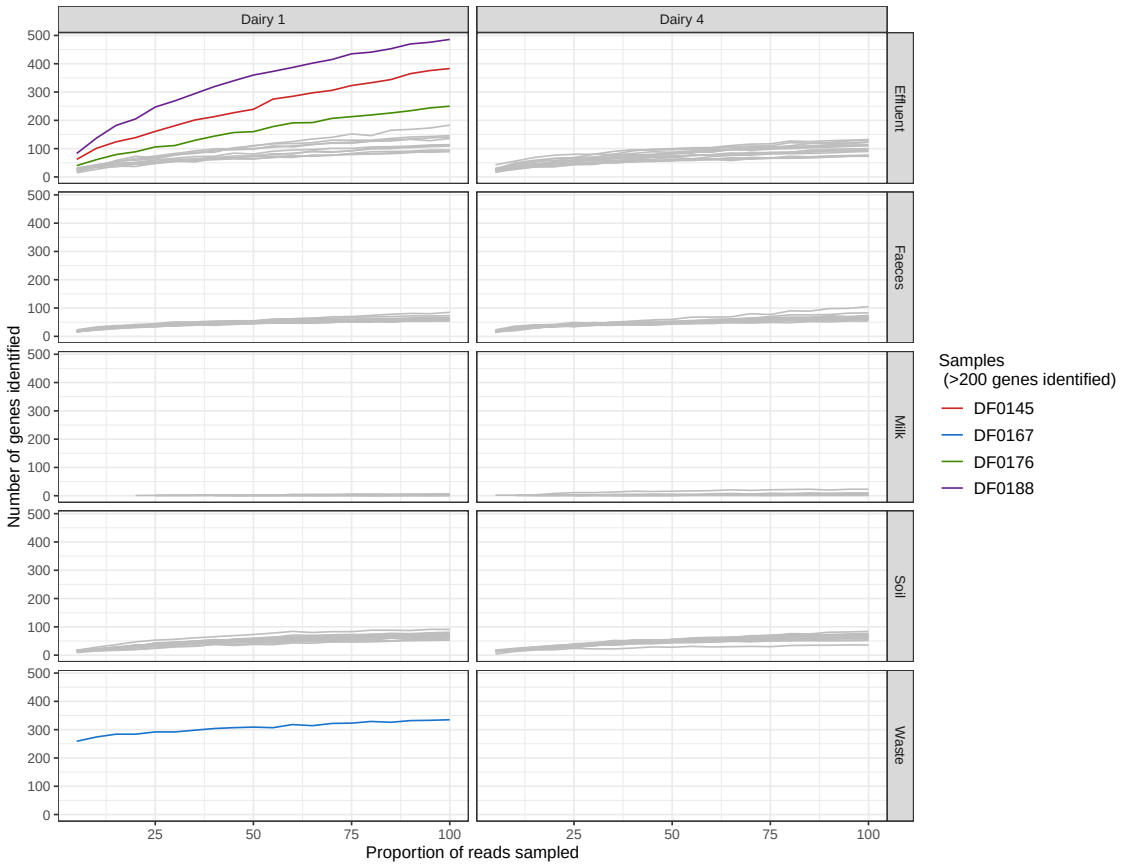

Figure 5.12: Rarefaction analysis of antimicrobial, heavy metal and biocide resistance genes detected in sequencing reads. Samples with >200 genes detected are highlighted in colour as defined in the legend.

5.3.5 Sequencing controls

Controls were included in the shotgun metagenomic sequencing runs in this project including a mock community DNA control to identify any biases introduced during the library preparation process and quantification of any sequencing errors and a mock community log distribution control which was included to assess the detection limits of the bioinformatics workflow. These two controls were run in triplicate across the two sequencing lanes. Blank reagent controls (n=2) of the two batches of DNA extraction kits and PBS controls (n=2) were included to identify reagent contamination associated with the DNA extraction kits and the PBS used to wash the milk pellet, respectively (section 5.2.1).

Sequencing of the microbial community mock DNA standard identified all eight bacteria (five Gram-positive, three Gram-negative) included in the control and the proportion of reads assigned to each bacterial species was similar to the expected values (Table 5.2). Reads classified as *Pseudomonas aeruginosa* and *S. enterica* (both Gram negative) were over-represented in the reads from the microbial community mock DNA standard compared to the expected proportion and the reads classified as *Bacillus subtilis* were much lower than expected (6.6% compared to 12%). However, 4.7% of reads in the mock community

control were classified as *Bacillus intestinalis* and these two species combined account for 11.3% of reads in the sample which is similar to the overall expected value for *B. subtilis*. The proportion of reads belonging to the remaining five bacteria were relatively similar (9.1 - 11.2%) to the expected proportion (12%). Fungi were not included in the database used to taxonomically classify reads in this study, therefore the expected and actual proportion of reads classified as *Saccharomyces cerevisiae* and *Cryptococcus neoformans* was not assessed. *Lactobacillus fermentum* reads were taxonomically classified as *Limosilactobacillus fermentum* in the database, as the *Lactobacillus* taxonomy has recently undergone changes [459]. These results suggest a slight bias towards *P. aeruginosa* and *S. enterica* during the library preparation process and that the Gram-positives were detected at a lower proportion than expected. There were classification issues within the database used for *B. subtilis* and instead a proportion of the reads were likely incorrectly classified as *B. intestinalis*.

Table 5.2: The expected and actual proportion of reads taxonomically classified in the DNA mock community sequencing control

Species	**Expected (%)**	**Reads[a] (%)**
Pseudomonas aeruginosa	12	17.1
Escherichia coli	12	11.2
Salmonella enterica	12	17.7
Bacillus subtilis	12	6.6
Lactobacillus fermentum	12	9.1
Enterococcus faecalis	12	10.0
Listeria monocytogenes	12	10.2
Staphylococcus aureus	12	9.8
Saccharomyces cerevisiae	2	NA
Cryptococcus neoformans	2	NA

[a] NA, Not assessed.

Generally, the proportion of reads taxonomically classified from the microbial community log distribution standard was similar expected distributions (Table 5.3). As with the microbial community DNA standard, *P. aeruginosa* and *S. enterica* were detected at a higher proportion than expected. In addition, the proportion of reads taxonomically classified as *B. subtilis* was lower than expected, however some reads from this sample were classified as *B. intestinalis* (0.3%) and *Bacillus cereus* (0.2%) which may account for the under-representation of reads classified as *B. subtilis*. These findings suggest that there was taxonomic classification issues within the *Bacillus* genus. Both *L. fermentum* and *S. aureus*, which were present at low concentrations, could not be detected in the microbial community log distribution standard using the bioinformatics pipeline used in this study.

At the genus level, the nine most abundant contaminants in both the PBS (n=2) and blank reagent controls (n=2) were similar (Table 5.4), suggesting that these bacteria are common contaminants in the laboratory and/or in the DNA extraction kits used in this study. Contaminating human DNA sequences were infrequently identified (<1%) in the PBS and negative blank reagent controls.

Table 5.3: Genomic DNA content and the proportion of reads taxonomically classified in the microbial community log distribution standard

Species	**Genomic DNA**	**Reads[a] (%)**
Listeria monocytogenes	89.1	85.4
Pseudomonas aeruginosa	8.9	12.6
Bacillus subtilis	0.89	0.4
Saccharomyces cerevisiae	0.89	NA
Escherichia coli	0.089	0.1
Salmonella enterica	0.089	0.1
Lactobacillus fermentum	0.0089	0
Enterococcus faecalis	0.00089	0.04
Cryptococcus neoformans	0.00089	NA
Staphylococcus aureus	0.000089	0

[a] NA, Not assessed.

Table 5.4: The most abundant contaminants in the PBS and blank reagent controls

Genus	**Reads (%) PBS**		**Reads(%) Blank reagent**	
	C01	**C02**	**C03**	**C04**
Rhizobium	10.8	11.4	11.3	10.8
Aquabacterium	9.3	9.8	9.4	10.8
Brevundimonas	11.5	11.3	11.6	10.7
Azospira	8.7	8.0	7.7	8.9
Acidovorax	7.7	7.4	7.7	6.7
Pseudomonas	4.2	4.1	4.2	4.4
Diaphorobacter	3.4	3.2	3.4	2.9
Sphingomonas	3.0	2.9	3.0	2.8
Agrobacterium	2.5	2.6	2.6	2.5

5.4 Discussion

The role of the NZ dairy farm environment in the development and spread of AMR is not fully understood. Due to the comparatively low use of antimicrobials in food-producing animals in NZ and the largely pasture-based dairy farm system, it was hypothesised that the abundance of ARGs in the dairy farm environment would be low. To test this hypothesis, shotgun metagenomic sequencing was utilised to examine and compare the resistome of environmental samples (faeces, FDE, soil and milk) collected over a 15 month period from two NZ dairy farms with contrasting farm management practices, taking into consideration seasonal variation and AMU. ARG abundance from FDE, faeces and milk collected over a 15 month period on Dairy 1 and Dairy 4 was relatively low compared to levels found in more intensive farming systems overseas, and was similar between the two farms (Figure 5.3). Interestingly, a higher number of unique ARGs were detected in FDE compared to soil, however they were detected at a lower abundance. FDE harbours a diverse range of ARGs

[433, 460] and applying FDE to pasture has been associated with higher detection of samples positive for specific antimicrobial resistant bacteria, for example, AmpC-producing *E. coli* on beef farms in the UK [284]. Similarly, a study of a USA dairy farm using pasture-based grazing (eight months of the year and an indoor barn in winter) used qPCR targeting 113 ARGs to assess the dairy farm resistome. The most diverse ARGs and highest ARG abundance was identified in the stagnant lagoon, compared to cow faeces, the agitated lagoon, compost, soil, corn silage, animal drinking water or environmental water [433]. Congruent with this study, these findings suggest that FDE is likely a reservoir of ARGs and applying FDE to pasture may be a transmission pathway for the dissemination of ARGs within the dairy farm environment. The effluent management strategy differed between the two farms: Dairy 1 had two effluent management strategies during the study period including storage in a small sump which may allow for concentration of bacteria and/or ARGs prior to being filtered and the treated FDE being applied to paddocks, or being discharged into the sewage system (Table 3.1), therefore Dairy 1 does not apply raw FDE to pasture. On Dairy 4, the FDE is stored in a large pond prior to being applied to pasture as a source of nitrogen. Therefore, the on-farm FDE strategy is a farm management practice which may influence the levels on AMR within a farm. The ARG abundance in FDE from Dairy 4 was relatively low, and due to the large FDE pond size, any ARGs present would be diluted prior to being applied to pasture. It is also recommended that paddocks are rested for 10 - 14 days between FDE application and grazing [461]. The soil samples in this study were collected from recently grazed paddocks, therefore FDE would not have recently been applied to these paddocks prior to the sample collection periods (>10 days). There may also be regional specific compliance requirements for on-farm FDE management.

The diverse number of ARGs detected in FDE may reflect the complex composition and microbiota of this sample type, consisting of faeces and run-off from the cowshed. These results suggest that factors other than on-farm FDE management are likely to have a role in the variation in ARG abundance in FDE between Dairy 1 and Dairy 4. Factors which may influence the diversity or abundance of ARGs in effluent include the bacterial community composition [462], temperature [462, 463] or storage conditions [464]. Variation in ARG abundance has also been observed for FDE collected at different sampling depths [464], which may be due to the different nutrient and pH levels as well as the microbiome composition at different depths.

The phyla Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria were predominant in the faecal samples from both farms in this study. Consistent with this finding, the most abundant bacterial phyla from faecal samples from Canadian feedlots were Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Spirochaetes and Euryarchaeota [12]. ARG abundance in faeces ranged from 0.08 - 0.17 copies of ARG per 16S rRNA gene, which was lower compared to the abundance of ARGs in 145 animal-associated metagenomes including from faeces, manure, agricultural soil and water [465] and the total ARG abundance in young calf faeces (0.77 - 5.14 copies of ARG per 16S rRNA gene) [11]. A study

comparing the faecal resistome of preweaned calves compared to lactating dairy cows on 17 commercial farms in the USA found a significantly higher ARG abundance in calves (0.43 - 2.9 copies of ARG per 16S rRNA gene) compared to dairy cows (0.11 - 0.6 copies of ARG per 16S rRNA gene) [13]. ARG abundance in the lactating dairy cow faeces was higher compared to this study, but was more comparable than ARG abundances found in calf faeces [13].

ARGs belonging to the β-lactam resistance class were the most abundant in faeces which was due to the presence of the *cfxA* gene. The *cfxA* gene encodes a class A β-lactamase, which has been associated with the non-autonomous conjugative transposon Tn*4555* [457, 466] and it is proposed that this transposon is involved in the horizontal transfer of the *cfxA* gene between *Bacteroides* and *Prevotella* species [466, 467, 468]. Therefore the high abundance of this gene in bovine faeces may be due to Bacteroidetes being a predominant phylum detected in faecal microbiome of lactating dairy cows [385]. Similar to this study, a high abundance of β-lactam resistance genes were identified in a dried faecal sample (n=1) from a USA dairy farm, which was largely driven by the presence of the *cfxA* gene [433]. The aforementioned study used targeted qPCR for 113 ARGs, of which only 17 belonged to the β-lactam resistance class. The average number of ARGs was also lower in faeces compared to stagnant/agitated lagoon and compost samples in a targeted qPCR based study of a USA dairy farm [433], although admittedly only one sample of each type was analysed. The abundance of heavy metal and biocide resistance genes in faeces was extremely low on both farms (0.00 - 0.01 copies of resistance genes per 16S rRNA gene), suggesting these genes are not prevalent in the faecal microbes from healthy dairy cattle.

The abundance of ARGs in milk was very low (0.00 - 0.12 copies of ARG per 16S rRNA gene) on both farms during the study period. Normalised ARGs were only detected in three milk samples which were collected in October 2018 (n=2) and December 2019 (n=1). Raw retail milk had a higher abundance of ARGs compared to pasteurised milk and using the HiSeq 4000 sequencing platform, ARGs were not detected from raw retail milk at the first time point (0 hours) but were detected after 24 hour room temperature incubation [210]. In the same study, additional raw retail milk samples collected across states in the USA were analysed using the NovaSeq S4 sequencing platform and ARGs were detected across most samples at an abundance of 0.0 - 1.0 copies of ARG per 16S rRNA gene [210]. The aforementioned study used a sequencing depth of approximately 40 million reads (150 bp paired-end) on the NovaSeq S4 platform, which is similar to the depth used in this study. These findings suggest that the abundance of ARGs in raw milk is low and that a high sequencing depth is required to detect any ARGs if they are present. In contrast, the milk from both Dairy 1 and Dairy 4 had a higher abundance of heavy metal resistance genes (0.00 - 0.59 copies of resistance genes per 16S rRNA). All of the metal resistance genes detected in milk potentially conferred mercury resistance and only one biocide resistance gene (*sod*B) was detected in one milk sample (0.10 copies of resistance genes per 16S rRNA gene). Other studies utilising shotgun metagenomics to study the resistome of retail raw milk have not investigated the abundance of heavy metal and biocide resistance genes

[210, 434]. The abundance of mercury resistance genes in soil (0.003 - 0.007 copies of ARG per 16S rRNA gene across three samples), waste milk (0.001) and faeces (0.00) were comparatively lower compared to milk samples. Mercury resistance genes were detected in FDE in four samples at a similar abundance compared to milk (0.03 - 0.06 copies of resistance genes per 16S rRNA gene). However, the abundance was lower across the majority of FDE samples (average: 0.01 copies of resistance genes per 16S rRNA gene). Genes conferring mercury resistance (*mer*ABCDEFPRT) have been identified in numerous bacteria, including among members of Proteobacteria and Firmicutes [469], which were the two predominant phyla across the milk samples. The milk samples collected in this study were raw bulk tank milk, and therefore would be pasteurised prior to human consumption.

The milk samples clustered into two distinct groups according to milk microbiome composition (Figure 5.2 and Appendix T). The predominant bacterial phyla differed between the two clusters. Cluster one contained two samples from Dairy 1 and five from Dairy 4, which had a high abundance of Firmicutes. Except for samples collected in December 2018 (n=2), the remaining five samples did not overlap in collection date (Dairy 1: December 2018 and February 2019; Dairy 4: October - December 2018, August - September 2019). The variation in microbiome compositional changes may be due to changes in supplementary feed type on farm (Appendix W). No single feed type was only fed during the months where the milk microbiome composition was dominated by Firmicutes, although the amount of each feed type fed may have fluctuated per month and this data was not recorded. The remaining 18 milk samples clustered together and the predominant phylum was Proteobacteria. Two retail raw milk samples collected from markets in two cities in Hungary showed a large amount of diversity in microbiome composition, with *Bacilli* the predominant class in the first sample and *Gammaproteobacteria* being predominant in the second sample, followed by Actinobacteria and *Bacilli* classes [434]. Although the sequencing depth of used in the aforementioned study was much lower compared to this study (17,773,004 and 8,425,326 paired-end reads from the two samples). The microbiome composition of retail raw milk samples across states in the USA also varied [210]. To the best of my knowledge, this is the first longitudinal study to utilise shotgun metagenomic sequencing to analyse the microbiome and resistome of raw bulk tank milk (prior to pasteurisation).

Waste milk (sometimes referred to as discard milk) is any form of unsaleable milk produced on farm. The composition of waste milk varies and may consist of (i) milk from dairy cows receiving antimicrobial treatment (either systemic or intramammary) that has a withholding period, (ii) milk from cows receiving non-antimicrobial drugs, (iii) colostrum from cows shortly after calving and (iv) milk from ill cows or milk with a somatic cell count exceeding the saleable limit [470, 436]. The waste milk disposal strategy on farm depends on the volume of waste milk as well as farm management practices. Disposal of waste milk generally includes either disposal into the effluent pond (or similar storage area) or drains or may be fed to calves [470]. Both disposal strategies may facilitate the dissemination of antimicrobial, heavy metal and biocide resistance genes.

Feeding waste milk, which may contain low concentrations of antimicrobials [471], to young calves has been suggested as a risk factor for shedding and transmission of antimicrobial resistant bacteria [472]. This is also a concern if waste milk contains pathogenic bacteria. Antimicrobial resistant bacteria, including multi-drug resistant *E. coli* have been isolated from waste milk samples on USA dairy farms (2 of 10 *E. coli* isolated from waste milk samples) [471]. A higher prevalence of *E. coli* resistant to antimicrobials including cefotaxime, nalidixic acid, trimethoprim/sulfamethoxazole and cephalothin were isolated from the faeces of calves that were fed waste milk (pasteurised and unpasteurised) compared to calves fed bulk tank milk [436] on a commercial dairy farm in Germany. Other factors may also be associated with a higher prevalence of antimicrobial resistant bacteria detected in calf faeces, such as the use of specific antimicrobials on farm and calf housing [472]. The aforementioned studies utilised culture-based methods and focused on specific AMR phenotypes particularly in *E. coli*. The abundance of specific ARGs was compared using quantitative PCR between calves pre- and post-weaning which were fed milk replacer with or without antibiotics (oxytetracycline and neomycin). Generally, feeding milk replacer with antibiotics was not associated with an increased absolute abundance of ARGs, however the relative abundance of *tet*O was higher in calves fed with waste milk containing a higher dose of the antibiotics (therapeutic compared to sub-therapeutic levels) [473]. These findings also suggested feeding waste milk to calves may increase ARG abundance in faeces.

The inclusion of waste milk in this study was unplanned, yet provided useful insights into the ARG abundance in waste milk in NZ. The waste milk sample was collected from Dairy 1 in October 2019. According to individual antimicrobial animal treatments recorded on farm, five cows were receiving antimicrobial treatment within six days prior to the October sampling date. The illnesses treated were mastitis and/or between claw/footrot (Appendix M). The active antimicrobial compounds in the products used were β-lactams, namely procaine penicillin G and penethamate. Therefore, milk from these cows may have been present in the waste milk sample (DF0167). Smaller volumes of waste milk on Dairy 1 and Dairy 4 are disposed of in accordance with the effluent management strategy on farm (sewage system and FDE pond, respectively). The waste milk sample (n=1) had a higher abundance of ARGs as well as heavy metal and biocide resistance genes (0.0 - 3.05 copies of resistance genes per 16S rRNA gene) compared to soil, FDE, faeces and milk samples. Interestingly, the most abundant resistance mechanisms identified were ARGs belonging to multi-compound classes, particularly multi-metal, drug/biocide and biocide/metal classes including a number of ARGs encoding efflux pumps or regulators, highlighting that waste milk is not only a source of ARGs but also genes conferring resistance to other compounds such as heavy metals and biocides. Acquired ARGs were identified in 46 contigs assembled from the waste milk sample, however, only 19 contigs could be taxonomically classified at the phylum level. The detection of clinically relevant ARGs, such as $bla_{\text{CTX-M}}$ which encodes an ESBL enzyme in *Enterobacterales* from waste milk is a public and animal health concern.

Soil, including those with low anthropogenic impact, has been shown to harbour a diverse range and abundance of ARGs [474, 475] and many antimicrobial compounds are naturally produced by soil microorganisms [475]. ARG abundance as well as heavy metal and biocide resistance gene abundance was highest in the soil samples throughout the 15 month study period (0.20 - 0.63 copies of ARG per 16S rRNA gene and 0.00 - 0.43 copies of resistance genes per 16S rRNA gene) compared to the FDE, faeces and milk sample types. Oddly, one soil sample collected in October 2018 on Dairy 4 (DF0006) did not contain any heavy metal or biocide resistance genes and this sample also had the lowest ARG abundance among soil samples from Dairy 4 (0.20 copies of ARG per 16S rRNA gene). At the time of sampling, it was noted that this paddock had poor drainage, although no changes were observed in the microbiome composition of this soil sample compared to the other soil samples (Appendix T). Further soil samples could be analysed from this paddock to determine whether the observed ARG abundance was consistently low or whether the previous ARG abundance may have been affected by poor drainage at the time of sampling. The high abundance of antimicrobial, heavy metal and biocide resistance genes identified in soil compared to FDE, faeces and milk and highlights that the soil resistome is abundant on both farms throughout the 15 month study period.

Soil microbiome composition can be highly complex [476]. Both ARG and bacterial diversity were significantly correlated in a study comparing soil from three distinct ecosystems (tundra, temperate and tropical) [474], which may suggest that microbiome sample variation drives ARG diversity. The microbiome from soil samples collected on Dairy 1 and Dairy 4 were relatively homogeneous, with the two predominant phyla being Proteobacteria and Actinobacteria, which is congruent with previous studies of soil from feedlots (n=4) in Canada [12] and soil samples collected from five USA dairy farms [218], although the soil samples in the aforementioned study were collected at a depth of 5 cm (compared to 10 cm in this study). However, soil communities were shown to be similar in composition and diversity at various sampling depths (samples compared at depths of 15.24 and 30.48 cm) [464]. There was no statistical difference (p= 0.271) in ARG abundance in the soil samples between the two dairy farms, which is not unexpected given the close geographical proximity of the farms (<5km) and the similarity in microbiome between the soil samples. The soil composition on Dairy 1 consists of a mixture of Rangitikei or Karapoti Brown sandy loam with some Manawatu sandy loam/gravelly phase and Manawatu mottled silt loam. On Dairy 1, the soil is free-draining alluvial soil. The soil on Dairy 4 is predominantly Tokomaru silt loam, with some compact clay loam and Ohakea silt loam. The soil on Dairy 4 has poor natural drainage and is artificially drained. ARG abundance in soil samples collected in this study (0.20 - 0.63 copies of ARG per 16S rRNA gene) was comparatively low compared to soil microcosms with/without compost manure collected from cows treated with specific antibiotics [477] and ARG abundance in soil collected from pens housing untreated (n=3) and florfenicol treated calves (n=3) [478] which ranged from 0.62 - 4.53 copies of ARG per 16S rRNA gene. ARG abundance in soil defined as pristine with little anthropogenic impact had an ARG abundance of 0.05 - 0.28 copies of ARG per 16S rRNA gene [432], which is less than the highly curated soils in this study.

The soil samples had the highest ARG abundance and was the third most diverse sample type (after FDE and faeces), with 30 ARG groups belonging to 11 resistance classes (Figures 5.3 and 5.4). The most abundant resistance classes for ARGs were glycopeptides, multi-drug resistance and drug/biocide resistance followed by aminocoumarins and MLS resistance classes and copper, iron and peroxide resistance classes were the most abundant for heavy metal and biocide resistance. Actinomycetes belong to the phylum Actinobacteria, which was the second most abundant phylum detected in soil samples in this study. Interestingly, soil samples (n=4) from feedlots in Canada had a smaller number of unique ARG groups identified compared to this study, with only nine ARG groups belonging to six classes detected [12], although the sample size was considerably smaller compared to this study. The ARGs from soil belonging to the multi-drug resistance class encoded multi-drug efflux pumps (*muxB* and *tap*) or regulators (*mtrad*). Multi-drug efflux pumps are also important for functions other than AMR such as detoxification of intracellular metabolites, cell homeostasis and bacterial virulence within plant and animal hosts [52]. A number of drug/biocide resistance genes were identified in soil (Appendix T), all of which encode efflux pumps or regulators. Despite ARGs detected at a higher abundance in soil, some ARGs identified in soil have been shown to share relatively low sequence similarity to the corresponding ARG in clinical pathogens [474], although a limited number of clinical ARGs were analysed and the phenotypic consequence of this finding is unknown. A threshold of >80% similarity at the nucleotide level was used to identify ARGs in this study to reduce false positive results. Individual ARGs identified in soil samples, particularly those of clinical significance, could be examined in closer detail and the genetic similarity compared to the corresponding ARG from clinical pathogens.

The bacterial host and genetic context of ARGs is crucial to assess the health risk posed. In addition, some ARGs such as efflux pumps, can have alternative functions and be involved in physiological processes unrelated to AMR. Mobile ARGs which were associated with humans were identified as the highest risk in a recent risk framework study [246]. Analysis of the ARGs used in the risk framework found that 70% of ARGs analysed (1,816 of 2,579) were not human-associated (enriched in environments with anthropogenic impact) and were classified as the lowest risk. The majority of these ARGs were not found on mobile genetic elements [246]. A diverse range of ARGs (n=372) belonging to the drugs, biocide, metal and multi-compound classes were identified in this study, many of which were identified in FDE. Despite a large number of unique ARGs being identified, the majority of these were not classified as the highest risk gene families [246]. At the sequencing read level, the high risk ARGs *aac6'* were identified in faeces, *dfrB* from one milk sample (*dfrB1* highest risk ARG) and bla_{OXA}, bla_{CTX} and *lnuA* from FDE (Appendix T). The soil samples had the highest ARG abundance in this study, yet no high risk ARGs were identified in soil, suggesting that although the overall ARG abundance in these samples was high compared to faeces, FDE and milk, the public and animal health risk associated with these ARGs is low.

ARGs potentially conferring resistance to critically important antimicrobials in human

medicine [98] including plasmid-mediated *mcr* genes conferring resistance to colistin [46], carbapenem resistance genes [479] or the resistance gene *mecA* from methicillin-resistant *S. aureus* [480] were not identified in this study. Of the acquired ARGs detected in contigs assembled from the metagenomic samples, some high risk ARGs were identified. Of particular concern, the bla_{OXA} gene was detected in a contig belonging to the *Campylobacteraceae* family from FDE, and *bla*Z and the extended-spectrum β-lactamase resistance gene $bla_{\text{CTX-M}}$ were identified in *Bacillaceae* and *Enterobacterales* from waste milk, respectively. The *tet(W)* gene, which is classified as high risk rank II gene (which includes resistance emerging in non-pathogens) was detected in *Fibrobacteraceae* and *Lachnospiraceae* from faeces and FDE samples on Dairy 4, respectively. ARGs were analysed at the gene family level compared to gene sequence, as was used in the risk framework [246], which is potentially a limitation of this study. These findings suggest that although a diverse range of ARGs were detected across the faeces, FDE, soil and milk samples, albeit at a comparatively low abundance, the majority of the ARGs detected do not pose a high public and animal health risk. These results highlight the importance of determining the bacterial host and mobility of ARGs to assess the relevant public and animal health risk posed.

Comparison of ARG abundance between shotgun metagenomic studies can be difficult due to the various bioinformatic methods and databases to identify ARGs, as well as the normalisation used for reporting ARG abundance. For example, some ARG databases contain only acquired resistance genes (e.g. ResFinder [216]), whereas other such as MEGARes2.0 [451] contain both acquired genes as well as chromosomal gene mutations. Therefore the ARG abundances reported in studies focusing on acquired resistance may be lower when compared to similar study populations in which resistance databases containing both acquired genes as well as chromosomal gene mutations were used. The normalisation methods may also differ between studies. For example, Zaheer *et al.*, uses the Cumulative Sum Scaling normalisation method for microbiome and resistome analyses [12], compared to the method by Li *et al.*, [453] using ARG sequencing hits and gene length, read length as well as the number of 16S rRNA genes identified per sample which was utilised in this study. Normalisation by ARG gene length was shown to have an important effect on the distribution of ARG allelic variants from pig caeca and human treated effluent samples [239]. ARG abundance in this study was normalised and expressed as "ARG per copy of 16S rRNA gene" as this method adjusts for bias relating to sequencing length, the microbial load per sample which is important for low biomass samples such as milk and this method is well-utilised in this field and thus allows for a comparison of the results from this study with the current literature.

The sequencing depth across studies can vary and the depth required to analyse the resistome is study-specific. Previous research has shown that the relative proportion of reads assigned to specific ARG classes was relatively consistent across various sampling depths in bovine faeces (average read counts used in the study: 26, 59 and 117 million), however, increasing the sequencing depth resulted in higher numbers of ARG assigned reads [236]. Studies with low sequencing depth may not be able to analyse the total number of unique

ARGs in a resistome, or examine the genetic context of specific ARGs. The sequencing depth in this study (Figure 5.12) was sufficient to analyse the resistome structure for the majority of samples. Although the rarefaction curves for three FDE and the waste milk sample did not plateau after 100% of reads were sub-sampled, suggesting that an increased sequencing depth may have identified additional unique ARGs. The sequencing depth can be specific to the sample matrix and dependent on the proportion of ARGs within a sample. For example, deep sequencing (at least 80 million reads per sample) of pooled pig caeca, treated human effluent and river sediment upstream from sewage treatment found that a low proportion of sequencing reads belonged to ARGs (<0.05%) and that the sequencing depth was not sufficient to detect the full allelic diversity in the treated effluent sample [239]. Even at a high sequencing depth, some ARGs could not be assigned to specific allelic variants, although a stringent threshold of 100% identity was used in the aforementioned study. However, the sequencing depth was sufficient for the treated effluent and pig caeca when analysing ARG families [239].

Analysis of the mock community DNA standard highlighted that taxonomic classification at the species level between related bacteria may lead to classification errors, such as those identified with *B. subtilis* and *B. intestinalis* (Table 5.2). Sequencing of the negative blank reagent (n=2) and PBS controls (n=2) identified that the nine most abundant genera were contaminants of both the negative blank reagent and PBS controls, suggesting that these bacteria are common contaminants in the laboratory and/or in the DNA extraction kits. The majority of these genera, except for *Agrobacterium*, have previously been reported as contaminants in sequenced negative blank controls [226, 481].

The high proportion of host reads (*Bos taurus*) in milk samples can complicate shotgun metagenomic sequencing analysis [242] and a higher sequencing depth is required to obtain sufficient coverage of the microbial DNA [482]. A high proportion of the sequencing reads across the milk samples was host DNA and was removed from subsequent analyses (metagenomic sequence read numbers can be accessed here). High proportions of host DNA has been shown to reduce microbiome profile sensitivity [482, 242] and this finding most likely can be extrapolated to include ARG detection sensitivity. The high amount of host DNA in milk samples drastically reduced the sequencing depth in these samples and may reduce the taxonomic classification sensitivity. To overcome this issue in future studies, host-depletion methods could be utilised prior to shotgun metagenomic sequencing, such as methods which utilise differential lysis of microbial and mammalian cells or post-extraction methods that selectively bind and remove CpG-methylated host DNA [242]. Alternatively, sequencing of milk filters have been proposed as a representative sample to analyse ARGs in bulk tank milk [242]. However, the milk is filtered through milk filters prior to storage in the bulk tank vat and although the pore size is large enough for bacteria to pass through, any ARGs or bacteria detected in the milk filter may not necessarily be present in the bulk tank milk.

5.5 Conclusion

ARG abundance in faeces, FDE, soil and milk samples across two NZ dairy farms over 15 months was comparatively low compared to overseas studies. No statistically significant difference in overall antimicrobial, heavy metal and biocide resistance genes was observed between Dairy 1 and Dairy 4. However, ARG abundance between FDE from the two farms was statistically significant, with a higher ARG abundance in FDE from Dairy 1. Dairy 1 also had greater AMU (17.09 mg/PCU) between October 2018 and December 2019 compared to Dairy 4 (5.36 mg/PCU). FDE samples harboured the most diverse range of ARGs, some of which are classified as a high risk to public health. Compared to FDE, faeces samples had lower diversity of ARGs and the faecal resistome on both farms was dominated by genes potentially conferring β-lactam resistance, in particular the *cfxA* gene. Milk samples had a low abundance of ARGs, but had a higher abundance of heavy metal, particularly mercury resistance genes. Soil samples had the highest ARG abundance (excluding the waste milk sample), however the ARGs in soil were not classified as high risk to public and animal health, highlighting that abundance as well as genetic context and risk (human-associated and mobility) should be considered when analysing resistomes using shotgun metagenomic sequencing. The waste milk sample had a high abundance of antimicrobial, heavy metal and biocide resistance genes. The detection of clinically significant ARGs, as well as contigs from mastitis-associated pathogens in the waste milk sample suggests that waste milk may be a source of pathogens and ARGs in the dairy farm environment. Due to the numerous ARG identification and normalisation methods, as well as variation in sequencing depth, caution should be used when comparing between studies using shotgun metagenomic sequencing methods. No association was identified between normalised ARG abundance and AMU or seasonality (assessed as sample collection period). Although the low ARG abundances and AMU on the farms during the study period may have made any associations difficult to detect. Future research on dairy farms with contrasting AMU are needed to address these research hypotheses.

Chapter 6

General discussion

6.1 Overview

Antimicrobial resistance (AMR) poses a significant public and animal health concern worldwide. The development and transmission of AMR between humans, animals and the environment is multi-factorial [28] and requires a holistic "One Health" approach to address. New Zealand (NZ) is a comparatively low user of antimicrobials in animal production [16, 17] and dairy farming uses less antimicrobials compared to both the poultry and swine industries [17]. In comparison to international standards, a study found NZ dairy herds (n=477) were generally low users of antimicrobials, however, some high-use herds were also identified [43]. The majority of antibiotic use on NZ dairy farms is for the treatment and prevention of mastitis [21].

Studies investigating AMR in NZ dairy farm environments have generally taken a culture-based approach, focusing on specific target organisms and resistant phenotypes [22] while utilising a cross-sectional study design [25, 26]. The research in this thesis using a longitudinal approach addresses the hypothesis that antimicrobial use on NZ dairy farms influences the prevalence of AMR in dairy farm environments, taking into consideration seasonality and contrasting farm management practices and utilises a combination of phenotypic, molecular and next-generation sequencing techniques to study AMR in NZ dairy farm production systems (Figure 6.1). Therefore, a three-fold approach was used to assess AMR in the NZ dairy farm environment, (i) targeting specific antibiotic resistant *E. coli* using culture-based methods (Chapter 3), (ii) investigating the impact of systemic antimicrobial therapy on the bovine faecal microbiome and the emergence of antimicrobial resistant bacteria at the individual animal level (Chapter 4) and (iii) an extensive approach utilising shotgun metagenomic sequencing to estimate the abundance of antimicrobial, heavy metal and biocide resistance genes in farm environmental samples collected over a 15 month period and to identify the bacterial host and genomic context of acquired antimicrobial resistance genes (ARGs) (Chapter 5).

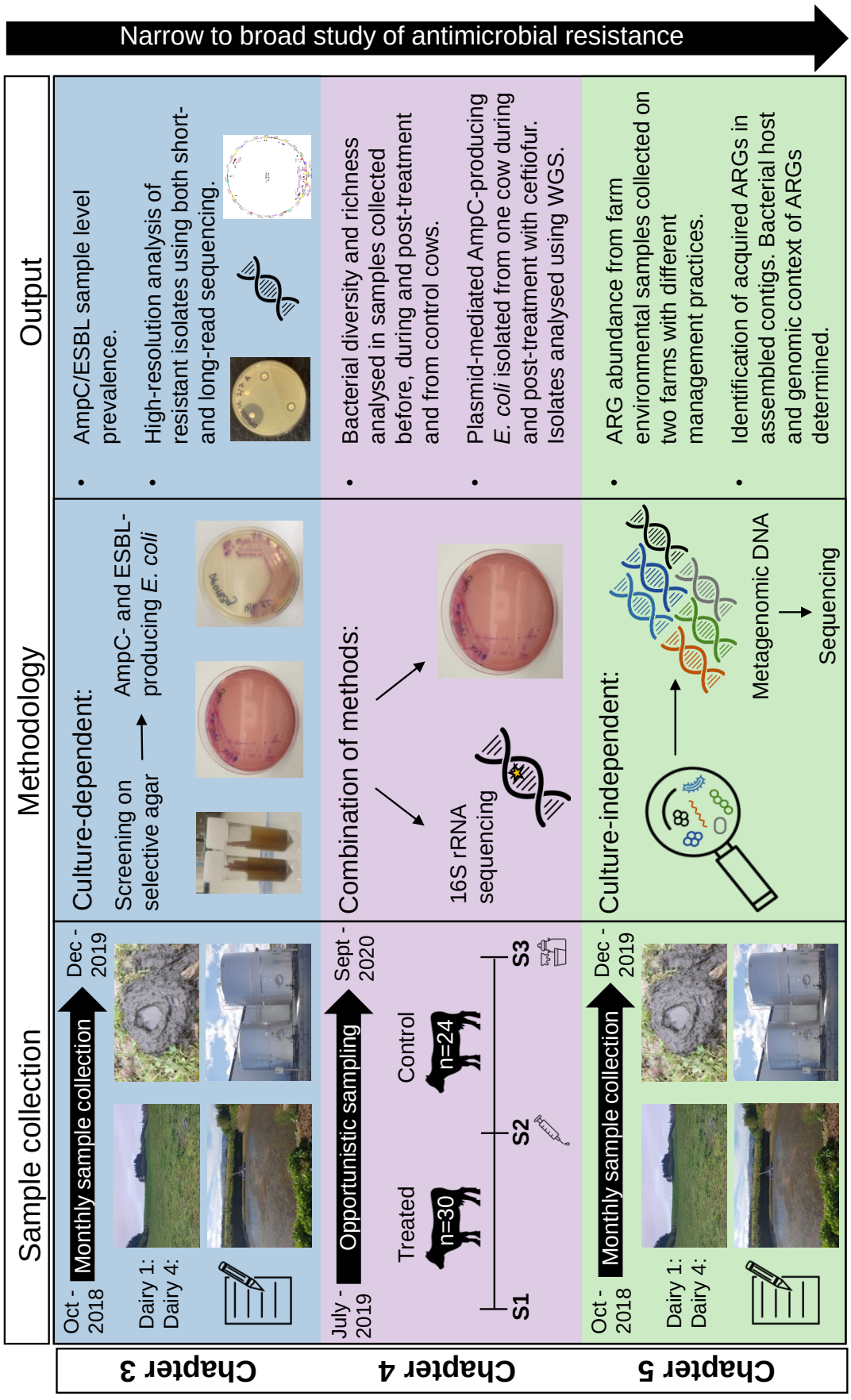

Figure 6.1: Summary of sample collection, methodology and outputs to address the research objectives in this study.

6.2 Findings, implications and general discussion

Overall, the research presented in this thesis has shown a low sample level prevalence of ESBL-producing *E. coli* from two NZ dairy farms with AmpC-producing *E. coli* being more frequently isolated across both farms (Chapter 3). AmpC- and ESBL-producing *E. coli* were isolated in spring and summer, during months with varying levels of antimicrobial usage (AMU). Analysis at the individual animal level showed a decrease in bacterial diversity, richness and composition during systemic antimicrobial treatment, and in many cases the microbiome structure recovered post-treatment when the cow re-entered the milking herd (median 6 days; Chapter 4). Changes to the faecal microbiome varied at the individual animal level which was likely due to a multitude of factors such as illness, disease severity, prescribed antimicrobial and treatment duration as well as feed type. Shotgun metagenomic sequencing of farm environmental samples provided a broader approach to study AMR in the dairy farm environment, examining a diverse range of antimicrobial, heavy metal and biocide resistance genes. Compared to overseas data in a similar context [478, 477, 465, 13, 210], NZ dairy farm environments had a low abundance of ARGs (normalised to per copy of the 16S rRNA gene), with the highest abundance detected in soil (Chapter 5). However, many of the ARGs identified in soil were not classified as high risk [246]. FDE samples had a lower abundance of ARGs but these were more diverse, suggesting that FDE may be a reservoir for ARGs in the dairy farm environment. Acquired resistance genes were predominantly found in Firmicutes and Proteobacteria. Proteobacteria contains a number of human bacterial pathogens, however, only a small number of contigs could be classified at the species level.

6.2.1 Antimicrobial resistance in the New Zealand dairy farm environment

ESBL-producing *E. coli* are hypothesised to be at a low prevalence in NZ dairy herds due to NZ's predominantly pasture-based dairy farm production system, coupled with the low AMU in animal production. The sample level prevalence of ESBL-producing *E. coli* in this study was low on Dairy 4 (1 of 60 (1.7%) from pooled faeces; 1 of 15 (6.7%) from FDE) compared to overseas studies [7, 6, 199] and no ESBL-producing *E. coli* were isolated from Dairy 1. A low prevalence of ESBL-producing *E. coli* was also detected in a regional cross-sectional study (1 of 15, 6.7%) [25] and a nationwide cross-sectional study (n=26 farms) in NZ did not detect any ESBL-producing *E. coli* [26]. These findings are consistent with overseas studies of pasture-based farming systems, for example, a cross-sectional study of ruminant farms in Spain found beef cattle herds and sheep flocks, which have a semi-intensive pasture-based farming system, had significantly lower herd level prevalence of cefotaxime-resistant *E. coli* compared to dairy cattle, which are generally housed inside pens [9]. A study across 40 cattle farms in Israel found that pasture-based farms had the lowest number of ESBL-producing *Enterobacteriaceae* compared to quarantine stations (housing calves only) and fattening farms (intensive farming without grazing) [483], but pasture-based farms did not include calves <4 months of age [483], which may confound the results as studies have shown a higher prevalence of AmpC- and ESBL-producing *E. coli* in calves compared to adult dairy cows [483, 194]. Few studies have investigated the prevalence of AmpC- and ESBL-producing *E. coli* on pasture-based dairy farms and further longitudinal studies across a larger number of farms are required to identify the risk factors for AmpC- and ESBL-producing *E. coli* in these farm production systems.

In contrast to ESBL-producing *E. coli*, the sample level prevalence of AmpC-producing *E. coli* was higher in this study and they were isolated from pooled faecal and FDE enrichments on both the Dairy 1 and Dairy 4 farms (Table 3.9). A nationwide cross-sectional study in NZ (n=26 dairy farms) detected chromosomal AmpC-producing *E. coli* from 14% (11 of 78) of pooled faecal enrichments originating from seven farms [26]. In comparison, a cross-sectional study in the Manawatū region (the same region as this study) found 7.8% (9 of 116) of pooled faecal samples were positive for chromosomal AmpC-producing *E. coli* and no *E. coli* with pAmpC genes were identified [26]. In this present study, the AmpC-positive sample level prevalence from pooled faecal enrichments was comparable to other cross-sectional studies undertaken in NZ, however the sample level prevalence was higher in FDE (which was not assessed in the other studies). This suggests that FDE may be a reservoir of AmpC-producing *E. coli* and would therefore represent a good proxy to assess antimicrobial resistant bacteria in the dairy farm environment.

In NZ, veterinarians and farmers are being encouraged to reduce the use of antimicrobials classified as orange and red tier by the NZVA, which will help facilitate improved antibiotic stewardship in agricultural production systems. The aforementioned study [26] across NZ dairy farms (n=26) found that a larger herd size and greater total AMU was associated

with AmpC positive farms. Dairy 4 has a larger herd size compared to Dairy 1, with 584 and 261 cows in each herd respectively, yet Dairy 1 had a higher AMU during the study period (D1, 17.09 mg/PCU; D4, 5.36 mg/PCU). Further research is required to elucidate these differences in AMU between the two farms. For example, in depth interviews could be conducted with the farm managers and consulting veterinarians from the two dairy farms to identify factors which contribute to the AMU on both Dairy 1 and Dairy 4. The AMU on Dairy 1 and Dairy 4 was within the range reported in a cross-sectional study of 26 dairy farms across NZ (4.39 - 20.92 mg/PCU) [26], albeit at the higher and lower end of the scale, respectively. However, the AMU in this study was calculated using individual antimicrobial treatments recorded on farm which were provided by the farmer, in contrast to antimicrobial sales data in the study by Burgess *et al.*, [26]. Antimicrobial sales data from Dairy 1 and Dairy 4 could be collected and compared with individual farm records as well as published AMU data from dairy farms throughout NZ.

Despite differences in herd size and AMU between the farms, the sample level prevalence of AmpC-producing *E. coli* in pooled faeces was comparable (Dairy 1: 3.3%; Dairy 4: 8.3%). The sample level prevalence was higher in FDE from Dairy 1, which may be due to contrasting effluent management strategies between the farms with Dairy 1 having a smaller storage sump, potentially allowing for an increased bacterial concentration. Despite the higher sample level prevalence of AmpC-producing *E. coli* in FDE, the resistance was mediated through chromosomal point mutations in the promoter region of the *ampC* gene. This mechanism of resistance is of concern for the relevant bacterium that harbours it and for vertical transmission, but is less likely to spread horizontally within bacterial populations.

The use of injectable amoxicillin [156, 26] and third- and fourth-generation cephalosporins [7] have been associated with the detection of AmpC- and/or ESBL-producing *E. coli* on dairy farms and *in vitro* studies have shown an association between amoxicillin use and AmpC-producing *E. coli* arising from mutations in the promoter region of the *ampC* gene [277, 355]. Both farms in this study did not report any injectable amoxicillin-clavulanic acid use during the study period (Appendix M). However, a low level of third-generation cephalosporins were used on both farms (Appendices K - L) and this research has demonstrated that plasmid-mediated AmpC-producing *E. coli* were isolated from an individual cow during and post-treatment with ceftiofur (Chapter 4, section 4.3.3 and Appendix P), suggesting that treatment with third-generation cephalosporins may be associated with the enrichment of AmpC-producing *E. coli*. Interestingly the plasmid-mediated AmpC-producing *E. coli* isolated during the microbiome study (Chapter 4) was from an individual animal on Dairy 1, yet during the longitudinal study in which only pooled faeces samples were examined, only one plasmid-mediated AmpC-producing *E. coli* was isolated from this farm (Chapter 3, Table 3.9). The virulence factors found in the *E. coli* (n=3) isolated from an individual animal (Appendix S) were also found in AmpC-producing *E. coli* isolated during the longitudinal study (Figure 3.4). This finding reflects the high resolution analysis which can be undertaken when sampling at the individual animal level, although

such study designs are not always feasible. Additional studies with a larger sample size utilising both culture-based and molecular methods (e.g. WGS of antimicrobial resistant isolates and resistome analysis of faecal samples using shotgun metagenomic sequencing or targeted qPCR) are required to assess any potential associations. The total amount of amoxicillin and ceftiofur used on Dairy 1 and Dairy 4 during the study period was low (Appendices K and L), and this may have contributed to the low sample level prevalence of ESBL-producing *E. coli* on these two dairy farms.

ARGs have been found in a wide range of environments including human sewage [427], agricultural settings [13, 244, 484] and pristine soil with low anthropogenic impact [432]. Metagenomic studies assessing AMR often focus on the abundance of ARGs, however there is growing evidence to suggest that the risk associated with ARGs in the resistome should also be considered [53]. A framework for ARG risk ranking has been proposed and the key considerations include: (i) whether the ARG is part of the intrinsic resistome, (ii) fitness cost of the resistance and (iii) the environment in which the ARG was detected, for example high risk genes found in humans pose a higher risk than if the gene was detected in a remote environment [53].

Intrinsic resistance or resistance caused by mutations in chromosomal genes is relevant for the bacterium that harbours it and for vertical transmission, however ARGs which are transferable are of higher concern as they may result in ARG dissemination horizontally within a bacterial population. For example, the *cfxA* gene, encoding a class A β-lactamase often found on a mobilisable transposon detected in *Bacteroides* species [457], was found in all faecal and most FDE samples in this study (Chapter 5). Thus, the identification of this gene in faeces and FDE may therefore be indicative of the predominance of Bacteroidetes in the faecal microbiome of lactating dairy cows [385] rather than suggesting the detection of the *cfxA* gene in other bacterial species. ARGs classified as the highest risk (defined as resistance readiness condition 1 as described in [53]) are those which can be identified on mobile genetic elements, are known to contribute to the failure of antibiotic treatment and have previously been found in human bacterial pathogens [53]. An example of a high risk ARG is the $bla_{\text{CTX-M}}$ gene, which was identified in a contig belonging to the *Enterobacterales* order that was assembled from the waste milk sample (Chapter 5), suggesting that clinically relevant antimicrobial resistant bacteria may be present in waste milk. Therefore, to estimate the burden of AMR in an environment or ecosystem, metagenomic studies should evaluate not only the abundance of ARGs but they should also assess the risk each ARG poses. A similar risk framework approach has been applied to assess ARGs from human gut microbiome genomes and only a small proportion of ARGs were ranked as the highest risk group in the study (3.6%) [246]. A limitation of the current proposed ARG risk frameworks is that they are often anthropocentric [53, 247, 246] with the risk described in relation to human health. Future modifications to risk frameworks should take a "One Health" approach and include risk assessments which collectively consider human, animal and environmental health.

ARG abundance measured using shotgun metagenomic sequencing was highest in soil samples during the study period (Chapter 5, Figure 5.3), however, many of the ARGs identified were not classified as high risk [246] suggesting that soil poses a lower public and animal health risk. Instead, the ARGs present in soil may be due to the natural production of antimicrobials by soil microorganisms [475], which acts as a selection pressure within the soil environment. Therefore, the high abundance of ARGs in the soil from the two dairy farm environments (collected as soil cores from the paddock) is more likely due to the natural environment and microbial populations rather than the anthropogenic impact of dairy farming. Further work analysing soil samples collected from sites with various levels of anthropogenic impact, such as higher impacted sites on dairy farms including around calf pens or water troughs and low impact sites such as native forest, is required to address this hypothesis. Despite being identified in a natural environment such as soil, some ARGs may pose a health risk if they are identified in bacterial pathogens, or if they are encoded on mobile genetic elements that could be transferred to pathogenic bacteria [51]. Thus, it is important to understand the genomic context of ARGs to assess risk (e.g. bacterial host, mobility), rather than only focusing on abundance.

The ARG abundance was low and no cephalosporin resistant *Enterobacteriaceae* were isolated from bulk tank milk samples in this study (Chapter 5, Figure 5.3). FDE harboured the most diverse range of ARGs, albeit at a lower abundance (Chapter 5, Figure 5.3). Consistent with this finding, multiple AmpC and/or ESBL-producing *E. coli* clonal types were isolated from FDE samples (Chapter 3, Figure 3.7). The diverse range of ARGs along with the presence of AmpC- and/or ESBL-producing *E. coli* provides further support that FDE is a reservoir for a diverse range of antimicrobial resistant bacteria and ARGs in the dairy farm environment. The composition of FDE and effluent management strategy will differ between farms, however FDE is a complex matrix of faeces, urine, waste milk (if present on farm and/or not fed to calves) and wastewater from the cow shed environment. As such, FDE/manure is a nutrient rich matrix with a high abundance and diversity of microbial populations which may contain antibiotic and biocide/disinfectant residues. Therefore, FDE provides a suitable environment for HGT and the dissemination of antimicrobial resistant bacteria and ARGs [460, 485]. Sampling FDE for the detection of antimicrobial resistant bacteria and ARGs could be used as a proxy for an entire farm. Urban sewage has previously been used to monitor ARGs globally [427] as well as for other pathogens such as testing wastewater to monitor SARS-CoV-2 in the NZ community [486]. Composite samples, compared to a grab sample, should be used for surveillance studies. Future work should also assess the limit of detection of both ARGs and AmpC- and ESBL-E from FDE, as well as investigating how representative a sample of FDE would be of the entire dairy herd.

Seasonality and months following increased AMU were not associated with an increased abundance of ARGs (Chapter 3, Figure 3.7). However, any correlations may have been difficult to identify due to the low ARG abundance across farm environmental samples in this study. Three FDE samples were identified as outliers and had a higher ARG abundance

(Chapter 5, Figure 5.3) but no AmpC- or ESBL-producing *E. coli* were isolated from these samples. This was not unexpected as the relative abundance of the genes associated with these resistant phenotypes were infrequently identified in the metagenomic sequence data (Appendix U). Interestingly, AmpC- and/or ESBL-producing *E. coli* were only identified during spring and summer, which correlates with calving/the start of lactation and the dry-off period, respectively which is a time period associated with increased AMU and mastitis cases. The third-generation cephalosporin resistant *E. coli* (DF0102.4e-h; AmpC and ESBL negative) was isolated in autumn (Chapter 3, Figure 3.7). No AmpC- and ESBL-producing *E. coli* were isolated during winter. It is important to note however that the longitudinal sample collection was carried out over 15 months and there was uneven sampling during some seasons which may exaggerate the number of AmpC or ESBL positive samples (Figure 3.7; summer: 4 visits; autumn: 3 visits; winter: 3 and spring: 5 visits). There was also a low sample level prevalence for some resistance phenotypes (e.g. ESBL producers) which makes any associations between AMU, seasonality and sample level prevalence difficult to determine. Further longitudinal studies with a larger sample size and number of farms are required to assess any potential associations between seasonality and greater AMU with ARG abundance and the prevalence of of antimicrobial resistant bacteria.

ESBL-producing *E. coli* were only isolated from one farm (1 of 15) during spring in NZ [25] which was consistent with this study. Detection of ESBL-positive faecal samples on dairy farms (n=3) in France varied across sampling months and was highest during spring and summer on one farm which was positive for ESBL-producing *E. coli* [487]. Increased temperatures have also been associated with a higher likelihood of detecting $bla_{\text{CTX-M}}$ positive *E. coli* samples in the UK [195]. Season significantly affected *E. coli* persistence in cow pat faeces, with higher counts detected in summer and autumn and the most rapid decline in *E. coli* during winter [488], suggesting that seasonal trends are also associated with other faecal bacteria and not limited to AmpC- and ESBL-producing *E. coli*. These findings as well as the isolation of AmpC- and ESBL-producing *E. coli* from dairy farms in spring and summer only in this study, suggests that prevalence studies in NZ should have more intensive sampling during spring and summer. Further studies with a larger sample size are required to assess seasonal trends in the prevalence of AmpC- and ESBL-producing *E. coli* in NZ dairy farm environments. Climate change may have a negative impact on dairy farm production systems and animal welfare; for example, increased frequency of heavy rainfall or extreme weather events, heat stress in animals, changes in annual pasture yield and increased incidence of mastitis could lead to increased AMU and persistence of pathogenic and antimicrobial resistant bacteria in the dairy farm envrionment [271, 272, 273].

6.2.2 Selection of antimicrobial resistant bacteria

Analysis of AMR at the individual animal level and targeted culture-based methods allowed for high resolution analysis at the isolate level compared to the broader culture-based

and culture-independent techniques used for the farm environmental samples. This analysis provided an example of the emergence of antimicrobial resistant bacteria, whereby plasmid-mediated AmpC-producing *E. coli* were isolated during and post ceftiofur treatment in an individual animal (Chapter 4, Table 4.1). No cephalosporin resistant *E. coli* were isolated from the pre-treatment sample for this cow nor from any of the associated control cow samples (S1, S2 and S3), which suggests that treatment with ceftiofur may have enriched and selected for AmpC-producing *E. coli*. Interestingly, third-generation cephalosporin resistant *E. coli* were only isolated from one of the three cows treated with ceftiofur (Chapter 4, Table 4.1), which indicates that antimicrobial treatment does not always enrich for resistant bacteria, or they were enriched but at numbers not detectable using culture-based methods. However, a PCR targeting the $bla_{\text{CMY-2}}$ gene from the crude boiled lysates from the faecal enrichments of ceftiofur treated cows was concordant with the culture-based methods. Further work is required to assess the abundance of additional ARGs from the faecal samples from individual animals in this study to see whether other antimicrobial resistant phenotypes are also enriched.

6.3 Potential limitations and future work

6.3.1 Farm choice

The rationale for recruiting the Massey University dairy farms (Dairy 1 and Dairy 4) in this study was (i) contrasting farm management practices, (ii) they are research farms and therefore have good farm records and farm staff who are able to assist with research projects and (iii) are in close proximity to the Hopkirk Research Institute (the latter two were required for the research outlined in Chapter 4, Figure 4.1). Some on farm processes may differ between research and conventional dairy farms which is a potential limitation of this study. For example, the Massey University dairy farms were more likely to consult with veterinarians in the diagnosis and treatment of sick dairy cows compared to conventional farms (personal communication with farm managers). In addition, access to veterinary consultation was not a barrier on the research farms and therefore even dairy cows with severe illness may have been treated rather than being culled.

The Massey University dairy farms are also used for veterinary teaching purposes which may result in scenarios that would not typically occur on conventional farms e.g. the caesarean cows that were treated with ceftiofur (Appendix P). A high level of veterinarian involvement on the two Massey University dairy farms may have contributed to the low use of antimicrobials classified as red tier by the NZVA and the good antimicrobial stewardship on these farms, such as the low use of third- and fourth-generation cephalosporins and marbofloxacin (Appendices K - L). Despite these differences between the Massey University research farms and conventional dairy farms, the inclusion of these farms was appropriate to answer the research aims and meet the requirements of this study design. Across NZ

dairy farms there is also regional variation in the topography, soil type, rainfall and climate as well as differences in farm management practices such as feed type, housing and waste milk disposal. In addition to between farm variation, there is variation within farm in regards to seasonality, farming practices (e.g. calving), feed sources and antimicrobial use. To account for within farm variation, a longitudinal study design was utilised. Although the Massey University research farms are not representative of all NZ dairy farms, the conclusions drawn from this research are still applicable as the core farm production system and management is typical of NZ dairy farms to ensure that they adhere to the food quality, health and safety, animal welfare and environmental regulations set out by the governing organisations and/or local government. Thus, a representative sample is not always a requirement for scientific research and is instead dependent on the research aims [489].

6.3.2 AMR risk factors

This research has assessed AMR on two NZ dairy farm environments, taking into consideration seasonality and farm management practices. Additional research is required to assess whether any AmpC-producing *E. coli* isolated in this study are resistant to fourth-generation cephalosporins, and subsequently have ESAC β-lactamase activity (Chapter 3). Additionally the resistance mechanism of isolates DF0102.4e-h to third-generation cephalosporins should be further elucidated (Chapter 3). *In silico* analysis suggested that all plasmids characterised in this study were conjugative, except for plasmid pDF0059.2e_2 which belonged to the IncY group (Chapter 3, section 3.3.6). Conjugation experiments are required to determine whether these plasmids can be transferred under laboratory conditions. Further studies with a larger sample size are also required across NZ dairy farms to understand the risk factors for AmpC- and ESBL-producing *Enterobacteriaceae* and higher ARG abundance across NZ dairy farms. Samples should be collected at multiple time points to account for within farm variation and potentially during spring and summer when an increased number of AmpC or ESBL positive samples have previously been detected (Chapter 3, Figure 3.9). Future work should also focus on understanding ARG abundance and the prevalence of AmpC- and ESBL-producing *Enterobacteriaceae* in higher risk animals (e.g. calves, including a comparison between calves fed waste milk compared to milk replacer) and higher-risk farms (e.g. those with higher total AMU and/or use of antimicrobials which are risk factors for AmpC- and ESBL-producing *E. coli* such as amoxicillin/clavulanic acid and third- and fourth-generation cephalosporins). Calves were not included in this study as more intensive sampling at shorter intervals would be required (compared to once a month sampling), which was outside the scope of this research. However, on dairy farms calves have been associated with a high ARG abundance [13] and prevalence of AmpC- and ESBL-producing *E. coli* [194]. Therefore, it is hypothesised that NZ calf faeces would have a higher prevalence of AmpC- and ESBL-producing *E. coli* compared to other farm environmental samples analysed in this study. In addition, further studies using WGS are required to determine the transmission pathways of antimicrobial resistant bacteria at the dairy farm, human and environmental interface, utilising

a broader "One Health" approach encompassing human, animal, environmental (e.g. farm drains and nearby waterways) and wildlife (e.g. birds and mammalian pests within the farm environment) sources.

6.3.3 Sample variables

The faecal samples collected from sick dairy cows receiving systemic antimicrobial therapy (Chapter 4) were collected from working dairy farms (Dairy 1 and Dairy 4). As such, the study variables were not all controlled and there was variation in illness type and severity at the individual animal level as well as temporal variation in farm management factors. Although the illness for each cow was described in the sampling questionnaire (Appendix O), no measurement of disease severity was included as this is subjective and multiple vet/farm staff were involved in the faecal sample collection across the two farms, making a consistent comparison difficult. To overcome the latter study limitation, healthy untreated cows were included as controls to account for temporal variation in season as well as differences in management factors. A farm level comparison of the impact of antimicrobial therapy on the faecal microbiome could not be undertaken due to the difference in sample number across the two farms, with 28 and 2 treated cows recruited in the study from Dairy 4 and Dairy 1, respectively (Appendix P). Both farms were recruited in the study for the same period of time and data collected from the longitudinal study indicated that AMU was higher on Dairy 1 (Chapter 3), however the smaller sample size on Dairy 1 may have been due to fewer farm staff working and available for sample collection or differences between the number of cows treated with systemic antimicrobials during the microbiome study (only six months of the longitudinal (Chapters 3 and 5) and microbiome (Chapter 4) study periods overlapped). Despite these limitations, the results of this research shed light on the impact on the bovine faecal microbiome of a range of systemic antimicrobial treatments in diseased cows from working NZ dairy farms, providing "real life" scenarios.

6.3.4 Sequencing methodologies

This research assessed the impact of antimicrobial treatment on the faecal microbiome using 16S rRNA amplicon sequencing which is a cost-effective, high throughput method to study the microbiome [221]. However, limitations of this method include potential primer biases [221] and in comparison, shotgun metagenomic methods allow deeper sequencing and have been shown to identify taxa present at a lower abundance [490]. Shotgun metagenomic sequencing also allows for functional analysis and detection of the resistome, including antimicrobial, heavy metal and biocide resistance genes [30]. However, 16S rRNA amplicon sequencing was suitable to answer the specific research objectives in this study, allowing a high resolution comparison of the bovine faecal microbiome between sampling periods at the ASV level (Chapter 4) [491]. This study did not focus on archaea or investigate changes in fungal populations in the faecal microbiome pre-, during and post-treatment, the latter

of which could be additionally analysed using internal transcribed spacer marker gene analysis [221]. Due to the high amount of variation at the individual animal level, treated animals within a case study could not be grouped at the treatment level. Therefore, robust statistical tests to compare changes in the relative abundance of specific ASVs between samples S1, S2 and S3 could not be used. Future studies should collect replicate faecal samples from individual cows for such analyses. The data also suggested that generally the bacterial diversity, richness and composition had recovered post-treatment when the cow re-entered the milking herd (median 6 days). Therefore, additional studies should focus on a smaller number of cows and collect samples more frequently between treatment and when the cow re-enters the milking herd to examine the stages of microbiome recovery in more detail e.g. the sequence which ASVs return to pre-treatment levels.

6.3.5 Computational limitations

The bioinformatic tools and databases used for AMR research are constantly evolving. As such, additional modifications to improve the pipeline used for the analysis of shotgun metagenomic sequencing reads (Chapter 5, Figure 5.1) should be routinely undertaken. Specific modifications to include are secondary functional validation of ARGs which were identified as requiring SNP confirmation using the Resistance Gene Identifier [492]. This secondary validation is used to reduce any false positive results in ARGs which confer resistance due to SNPs, such as mutations in the *gyrA* gene which may lead to reduced susceptibility to fluoroquinolones. Additionally, the sequencing reads and contigs were taxonomically classified using Kraken and CAT, respectively (Chapter 5, sections 5.2.4 and 5.2.5). The databases used were not customised to include additional metagenome-assembled genomes (MAGs). Inclusion of such sequences, for example a collection of 4,941 rumen MAGs [493], could be added to create a customised database which may improve the number of reads and contigs taxonomically classified in this study.

6.3.6 Analysis of faecal samples enriched in media containing specific antibiotics

High resolution analysis of the impact of systemic antimicrobial treatment on the faecal microbiome and resistome is crucial to understand the potential selection pressures driving AMR in the dairy farm environment with an increased focus at the individual animal level. Further research in this area could include enriching the faecal samples in media containing specific antibiotics to select for functional resistant phenotypes and analysing the samples using shotgun metagenomic sequencing. These enriched faecal samples could be plated on selective media to isolate antimicrobial resistant bacteria of interest. Antimicrobial susceptibility testing and WGS, particularly long-read sequencing (e.g. PacBio or MinION sequencing platforms), of resistant isolates would enable high resolution analysis of the molecular mechanisms for resistance and mobility, including the genomic context of ARGs.

In addition, shotgun metagenomic sequencing of the enriched faecal sample would allow an in depth analysis of antimicrobial, heavy metal and biocide resistance genes enriched during antimicrobial treatment and assembly of MAGs would enable host identification, genomic context and gene linkage for resistance genes to be assessed. Analysing pre-enriched faecal samples from cows receiving systemic antimicrobial treatment would provide a comparison of the selective pressures and impact of treatment in the "natural" farm environment as well as *in vitro* enrichment under laboratory conditions.

6.3.7 Waste milk

Waste milk has been linked to the development of antimicrobial resistant bacteria [436, 494] and it is not recommended to feed waste milk to calves in NZ (https://www.dairynz.co.nz/news/waste-milk-is-it-calf-feed/), although some calves are fed waste milk in NZ. Shotgun metagenomic sequencing analysis of one waste milk sample revealed a number of contigs belonging to mastitis-associated pathogens such as *S. uberis* as well as a plethora of antimicrobial, heavy metal and biocide resistance genes (Chapter 5, Figure 5.9), many of which potentially confer resistance across a number of compounds. A small proportion of contigs assembled from the waste milk harboured acquired ARGs (Chapter 5, Figure 5.10), however MAGs were not assembled from the samples in this study. Future work should focus on assembling MAGs from the farm environmental samples and identifying MAGs harbouring antimicrobial, heavy metal or biocide resistance genes to elucidate the genomic context and the bacterial hosts harbouring such genes. If the ARGs are present on plasmids, additional Hi-C analysis may be required to link the plasmid with the bacterial host [495]. These findings suggests that waste milk may be a source of pathogenic bacteria and ARGs within the dairy farm environment. Further research is required to understand how many farms in NZ feed waste milk to calves, any risks this practice poses as well as providing clear industry guidelines on the use of waste milk as calf feed in NZ.

6.3.8 Environmental vectors

AmpC- and ESBL-producing *E. coli* have been identified from cloacal and surface swabs from migratory and wild birds near Ohio dairy farms [368] as well as from wild birds in the Netherlands [496] and in Spain [497]. AmpC- and ESBL-producing *E. coli* have also been isolated from other non-avian vectors including wildlife such as rats (*Rattus* spp.), mice (*Mus musculus*) and hedgehogs (for example, *Erinaceus europaeus*) [498, 499], all of which are pests that can be found on NZ dairy farms. White stork (*Ciconia ciconia*) colonies, which are migratory birds, were closer in distance to landfills with human waste were more likely to be positive for third-generation cephalosporin resistant *E. coli* [500] and the isolation rate of AmpC- and ESBL-producing *E. coli* was significantly higher in birds associated with aquatic environments [496]. The environment and feed may be a source of antimicrobial resistant bacteria carried by wild birds and therefore birds may be

a potential vector for the dissemination of antimicrobial resistant bacteria.

The Dairy 1 and Dairy 4 farms are located near the city of Palmerston North, as well as in close proximity to other dairy farms. Therefore, birds and wildlife could potentially mix between urban and agricultural environments. If present, bird faecal samples were collected from the dairy farm environment at each sampling visit in this study. The samples were processed in accordance with the methods detailed in Chapter 3, section 3.2.3 and the enrichments stored with glycerol (30% [v/v]) at -80℃. In addition, supplementary feed was collected during sampling visits if it was being used (Appendix W) and processed as described above. Enrichment samples from bird faeces and supplementary feed could be plated on selective agar plates to select for AmpC- and ESBL-producing *Enterobacteriaceae* to identify whether birds or supplementary feed may be a vector for these resistant bacteria in the NZ dairy farm environment. To the best of the author's knowledge, no studies have investigated the prevalence of AmpC- and ESBL-producing *Enterobacteriaceae* from birds or wildlife in the NZ dairy farm environment.

6.4 Conclusion

This research used multiple approaches to study AMR in the NZ dairy farm environment; (i) targeted culture-based methods to examine the sample level prevalence of AmpC- and ESBL-producing *E. coli*, (ii) 16S rRNA sequencing to examine the impact of systemic antimicrobial therapy on the bovine faecal microbiome coupled with culture-based isolation of specific antimicrobial resistant bacteria at the individual animal level, and (iii) using shotgun metagenomics to assess the abundance of antimicrobial, heavy metal and biocide resistance genes as well as identifying the bacterial host and genomic context of ARGs where possible.

The sample level prevalence of ESBL-producing *E. coli*, which are listed as "critical" on the WHO Priority Pathogens List [54], was low in the two dairy farms included in this study. This low prevalence may be attributed to NZ's predominantly pasture-based dairy farm production system and the comparatively low AMU in animal production, particularly on dairy farms. The recommendation to reduce the use of antimicrobials classified as red tier by the NZVA may also influence AMU and the low sample level prevalence of ESBL-producing *E. coli*. The AMU on both farms was comparable to other dairy farms throughout NZ and interestingly Dairy 1, which has a smaller herd size, had a higher AMU during the study period, although both farms used a low amount of antimicrobials classified as red tier by the NZVA. Compared to ESBL-producing *E. coli*, the sample level prevalence was higher for AmpC-producing *E. coli*. All AmpC-producers on the larger farm (Dairy 4) were plasmid-mediated, compared to Dairy 1 in which most of the AmpC-producers were chromosomally mediated (14 of 16 *E. coli*). Further studies are required to assess the risk factors between plasmid and chromosomally mediated AmpC-producing *E. coli* and to explore any seasonal effects on the prevalence of AmpC- and ESBL-producing *E. coli* as they were only isolated during spring and summer in this study.

This research demonstrated that systemic antimicrobial therapy reduced the diversity and richness of the bovine faecal microbiome during treatment and that this perturbation in bacterial community structure generally recovered post-treatment when the cow re-entered the milking herd. However, the animal level variation was high and cows with more serious conditions showed greater perturbations in the faecal microbiome composition (e.g. cows with left displacement of the abomasum compared to footrot). Treatment with ceftiofur resulted in the emergence of plasmid-mediated AmpC-producing *E. coli* in one of the three animals treated, which highlights the need for prudent antimicrobial stewardship in both animal and human health to reduce the development and spread of AMR. The abundance of ARGs across the NZ dairy farm environment was comparatively low compared to overseas data [478, 477, 465, 13, 210]. There was no statistical difference in the ARG abundance between either farm. Overall, the highest ARG abundance was in soil, yet the most diverse range of ARGs was in FDE. There was no association between the normalised ARG abundance and AMU or seasonality, although the low ARG abundances in farm

environmental samples may have made any associations difficult to detect.

At current rates and left unchecked it is estimated that by 2050, 10 million human deaths per year will be attributable to AMR worldwide [5]. In 2019 it was estimated that globally 4.95 million deaths and 1.27 millions deaths were associated with or attributable to bacterial AMR, respectively [36]. Therefore, AMR is a global burden for human, animal and environmental health and requires a holistic "One Health" approach to address [501]. The "New Zealand Antimicrobial Resistance Action Plan" was published in 2017 and priority areas for action were identified including (i) increasing awareness and understanding of AMR, (ii) strengthening AMR surveillance and research, (iii) improving infection prevention and control measures to prevent the transmission of antibiotic reistant bacteria, (iv) improving antimicrobial stewardship in human health, animal health and agriculture and (v) establishing clear governance, collaboration and investment for approaches to counter AMR [502]. This PhD research contributes to objective two of NZ's AMR action plan. To encourage prudent antimicrobial stewardship in animal husbandry, the NZVA has aspired that by 2030 NZ will not require antimicrobials for the maintenance of animal health and wellness [42], instead their use will be reserved for the treatment of disease. The SARS-CoV-2 pandemic has demonstrated how globally connected the world is, thus efforts to combat AMR need to include both national and global initiatives which encompass a "One Health" approach. Prevention is better than a cure and urgent action is required to slow the development and dissemination of AMR worldwide as well as encouraging prudent antimicrobial stewardship and continuing the research and development of new antimicrobial treatments.

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Appendix A

PCR reaction and gel electrophoresis conditions

PCR reaction	Primer set(s)	Reaction conditions[a]	Gel conditions[b]	Controls	Ref
ampC promoter region	AmpC1[71]/AmpC2[120]	Initial denaturation at 94℃ for 90 sec, then 30 cycles of 94℃ for 30 sec, 57℃ for 30 sec, and 72℃ for 1 min, with a final extension step at 72℃ for 10 min.	2% agarose gel run at 90V for 40 min.	LT9031a (positive *E. coli*), FS192 (negative *Staphylococcus aureus*) and non-template control.	[138]
pAmpC	MOXMF/MOXMR CITMF/CITMR DHAMF/DHAMR ACCMF/ACCMR FOXMF/FOXMR CMY-F/CMY-R	Initial denaturation at 94℃ for 3 min, then 25 cycles of 94℃ for 30 sec, 64℃ for 30 sec, and 72℃ for 1 min, with a final extension step at 72℃ for 7 min.	2% agarose gel run at 80V for 90 min.	NZRM4402 (CMY-positive *E. coli*), NZRM4464 (FOX-5-positive *K. pneumoniae*), NZRM4403 (DHA-1-positive *E. coli*), ATCC25922 (negative *E. coli*) and non-template control.	[177]
CMY group	CMY-2-F/CMY-2-R	Initial denaturation at 94℃ for 3 min, then 30 cycles of 94℃ for 30 sec, 58℃ for 30 sec, and 72℃ for 1 min, with a final extension step at 72℃ for 10 min.	2% agarose gel run at 80V for 40 min.	NZRM4402 (CMY-positive *E. coli*), ATCC25922 (negative *E. coli*) and and non-template control.	[313]
CTX-M-1	CTX-1-SEQ-F/CTX-1-SEQ-R	Initial denaturation at 95℃ for 5 min, then 30 cycles of 95℃ for 30 sec, 60℃ for 30 sec, and 72℃ for 1 min, with a final extension step at 72℃ for 1 min.	0.8% agarose gel run at 80V for 40 min.	DF0183g (CTX-M-15 positive *E. coli*), DF0025d (CTX-M negative *E. coli*) and non-template control.	[313]

PCR reaction	Primer set(s)	Reaction conditions[a]	Gel conditions[b]	Controls	Ref
Clermont quadruplex PCR typing method	chuA.1b/chuA.2 yjaA.1b/yjaA.2b TspE4C2.1b/ TspE4C2.2b AceK.f/ArpA1.r	The PCR was undertaken in a Bio-Rad T100 Thermal Cycler (Bio-Rad, USA) using KAPA HiFi HotStart ReadyMix (KAPA BioSystems, Wilmington, USA) under the following conditions: initial denaturation at 94℃ for 4 min, then 30 cycles of 98℃ for 20 sec, 61℃ for 20 sec, and 72℃ for 5 min.	2% agarose gel run at 80V for 90 min.	AGR3560 (Clade III, IV or V positive *E. coli*), AGR3575 (mixed *E. coli* DNA positive for *arpA*, *chuA*, *yjaA* and TspE4C2 PCR products) and non-template control.	[76, 75]
Clermont typing method: Group C	trpAgpC.1/ trpAgpC.2	PCR was undertaken in a Bio-Rad T100 Thermal Cycler (Bio-Rad, USA) using KAPA HiFi HotStart ReadyMix (KAPA BioSystems, Wilmington, USA) under the following conditions: initial denaturation at 94℃ for 4 min, then 30 cycles of 98℃ for 20 sec, 58℃ for 20 sec, and 72℃ for 5 min.	2% agarose gel run at 80V for 90 min.	AGR4288 (*E. coli* phylogroup C positive control) and non-template control.	[318]
Clermont typing method: Group E	ArpAgpE.f/ ArpAgpE.r	PCR was undertaken in a Bio-Rad T100 Thermal Cycler (Bio-Rad, USA) using KAPA HiFi HotStart ReadyMix (KAPA BioSystems, Wilmington, USA) under the following conditions: initial denaturation at 94℃ for 4 min, then 30 cycles of 98℃ for 20 sec, 58℃ for 20 sec, and 72℃ for 5 min.	2% agarose gel run at 80V for 90 min.	*E. coli* O157 (phylogroup E positive control) and non-template control.	[318]

[a]PCR was undertaken in a SensoQuest Lightcycler machine (SensoQuest, Germany) using HOT FIREPol® Blend Master Mix (10mM Mg) (Solis BioDyne, Tartu, Estonia) unless stated otherwise. [b]PCR products (2 µL) were mixed with 1 µL gel loading buffer (BlueJuice™, ThermoFisher Scientific Inc., USA). Electrophoresis agarose gels were stained with RedSafe (Invitrogen, Auckland, New Zealand) and a 1kb+ ladder (Invitrogen, Auckland, New Zealand) (1.5 µL) was electrophoresed as a size standard. Gel images were captured and stored using a GelDoc XR+ (BioRad, New Zealand).

Appendix B

Bacterial strains isolated in this study

Strain	Source	Farm	Collection date	Isolation agar[a]	Phylogroup	Phenotype[b]	WGS	β-lactam resistance genes[c]
DF0025c	FDE	1	05/11/2018	MCAZ	C	cAmpC	No	ND
DF0025d	FDE	1	05/11/2018	MCAZ	C	cAmpC	No	ND
DF0025e	FDE	1	05/11/2018	MCTX	C	cAmpC	No	ND
DF0025f	FDE	1	05/11/2018	MCTX	C	cAmpC	No	ND
DF0030c	FDE	1	03/12/2018	MCAZ	C	cAmpC	No	ND
DF0030d	FDE	1	03/12/2018	MCAZ	C	cAmpC	No	ND
DF0030e	FDE	1	03/12/2018	MCTX	C	cAmpC	No	ND
DF0030f	FDE	1	03/12/2018	MCTX	C	cAmpC	No	ND
DF0031.1c	Faeces	1	03/12/2018	MCAZ	C	cAmpC	Yes	$bla_{\text{EC-13}}$, $bla_{\text{TEM-1}}$
DF0031.1d	Faeces	1	03/12/2018	MCAZ	C	cAmpC	No	ND
DF0031.1e	Faeces	1	03/12/2018	MCTX	C	cAmpC	No	ND
DF0031.1f	Faeces	1	03/12/2018	MCTX	C	cAmpC	No	ND
DF0047c	FDE	1	29/01/2019	MCAZ	C	cAmpC	Yes	$bla_{\text{EC-13}}$
DF0047d	FDE	1	29/01/2019	MCAZ	C	cAmpC	No	ND
DF0047e	FDE	1	29/01/2019	MCTX	C	cAmpC	No	ND
DF0047f	FDE	1	29/01/2019	MCTX	C	cAmpC	No	ND
DF0049.2c	Faeces	1	29/01/2019	MCAZ	E	pAmpC	No	ND
DF0049.2e	Faeces	1	29/01/2019	MCTX	E	pAmpC	Yes	bla_{EC}, $bla_{\text{CMY-2}}$
DF0059.2e	Faeces	4	04/02/2019	MCTX	D	pAmpC/ESBL	Yes	bla_{EC}, $bla_{\text{CMY-2}}$, $bla_{\text{CTX-M-1}}$
DF0060c	FDE	1	04/02/2019	MCAZ	C	cAmpC	No	ND
DF0102.4e	Faeces	1	06/05/2019	MCTX	D	Unknown	No	ND
DF0102.4f	Faeces	1	06/05/2019	MCTX	D	Unknown	No	ND
DF0102.4g	Faeces	1	06/05/2019	CHRO	D	Unknown	Yes	$bla_{\text{EC-8}}$, $bla_{\text{OXA-1}}$

Strain	Source	Farm	Collection date	Isolation agar[a]	Phylogroup	Phenotype[b]	WGS	β-lactam resistance genes[c]
DF0102.4h	Faeces	1	06/05/2019	CHRO	D	Unknown	No	ND
DF0159.2d	Faeces	4	07/10/2019	MCAZ	A	pAmpC	No	ND
DF0159.2e	Faeces	4	07/10/2019	MCTX	A	pAmpC	No	ND
DF0159.2g	Faeces	4	07/10/2019	CHRO	A	pAmpC	Yes	$bla_{\text{EC-18}}$, $bla_{\text{CMY-2}}$, $bla_{\text{OXA-1}}$
DF0159.2h	Faeces	4	07/10/2019	CHRO	A	pAmpC	No	ND
DF0166c	FDE	1	07/10/2019	MCAZ	B1	cAmpC	No	ND
DF0181.1c	Faeces	4	02/12/2019	MCAZ	B1	pAmpC	Yes	$bla_{\text{EC-18}}$, $bla_{\text{CMY-2}}$
DF0181.1d	Faeces	4	02/12/2019	MCAZ	B1	pAmpC	No	ND
DF0181.1e	Faeces	4	02/12/2019	MCTX	B1	pAmpC	No	ND
DF0181.1f	Faeces	4	02/12/2019	MCTX	B1	pAmpC	No	ND
DF0181.1j	Faeces	4	02/12/2019	MCAZ	B1	pAmpC	No	ND
DF0181.1k	Faeces	4	02/12/2019	MCTX	B1	pAmpC	No	ND
DF0181.3c	Faeces	4	02/12/2019	MCAZ	B1	pAmpC	Yes	$bla_{\text{EC-18}}$, $bla_{\text{CMY-2}}$
DF0181.3d	Faeces	4	02/12/2019	MCAZ	B1	pAmpC	No	ND
DF0181.3e	Faeces	4	02/12/2019	MCTX	B1	pAmpC	No	ND
DF0181.3f	Faeces	4	02/12/2019	MCTX	B1	pAmpC	No	ND
DF0181.3i	Faeces	4	02/12/2019	MCAZ	B1	pAmpC	No	ND
DF0181.3j	Faeces	4	02/12/2019	MCTX	B1	pAmpC	No	ND
DF0181.3k	Faeces	4	02/12/2019	MCTX	B1	pAmpC	No	ND
DF0181.4c	Faeces	4	02/12/2019	MCAZ	E	pAmpC	Yes	bla_{EC}, $bla_{\text{CMY-2}}$
DF0181.4d	Faeces	4	02/12/2019	MCAZ	E	pAmpC	No	ND
DF0181.4e	Faeces	4	02/12/2019	MCTX	E	pAmpC	No	ND
DF0181.4f	Faeces	4	02/12/2019	MCTX	E	pAmpC	No	ND
DF0183c	FDE	4	02/12/2019	MCAZ	F	ESBL	No	ND
DF0183d	FDE	4	02/12/2019	MCAZ	B1	pAmpC	No	ND
DF0183e	FDE	4	02/12/2019	MCTX	B1	pAmpC	Yes	$bla_{\text{EC-18}}$, $bla_{\text{CMY-2}}$
DF0183f	FDE	4	02/12/2019	MCTX	B1	pAmpC	No	ND
DF0183g	FDE	4	02/12/2019	CHRO	F	ESBL	Yes	$bla_{\text{EC-19}}$, $bla_{\text{CTX-M-15}}$
DF0183i	FDE	4	02/12/2019	MCAZ	B1	pAmpC	Yes	$bla_{\text{EC-18}}$, $bla_{\text{CMY-2}}$

[a] MCAZ, MacConkey (MC) agar + 1 µg/ml ceftazidime; MCTX, MC agar + 1 µg/ml cefotaxime; CHRO, CHROMagar$^{\text{TM}}$ ESBL.

[b] cAmpC, Putative Ampc hyperproducers; pAmpC, Plasmid-mediated AmpC phenotype (CMY-2); ESBL, ESBL phenotype.
[c] ND, Not done (β-lactam resistance genes identified only from isolates that were sequenced in this study).

Appendix C

E. coli AST zone sizes (mm)

Strain	CTX[a]	CPD	FOX	TET	STR	CIP
DF0025c	24.6	7.2	12.9	6.6	6.6	33.5
DF0025d	24.0	6.6	13.2	6.3	6.6	31.4
DF0025e	24.7	7.0	13.5	6.6	6.6	32.2
DF0025f	23.8	7.2	13.5	6.6	6.6	32.1
DF0030c	26.1	7.2	13.5	6.9	6.6	32.2
DF0030d	23.7	7.3	13	6.6	6.6	31.2
DF0030e	24.7	7.0	12.3	6.6	6.6	31.8
DF0030f	24.2	6.6	12.1	6.5	6.6	34.7
DF0031.1c	24.9	7.4	13.3	6.6	6.6	30.8
DF0031.1d	24.5	6.8	12.7	6.6	6.6	30.9
DF0031.1e	25.9	6.66	13.4	6.4	6.6	33.7
DF0031.1f	25.6	6.2	13.5	6.5	6.6	32.3
DF0047c	24.8	6.6	13.6	6.3	6.6	30.1
DF0047d	26.4	6.6	13.9	6.4	6.6	32.8
DF0047e	26.1	7.3	13.2	6.6	6.6	32.0
DF0047f	23.7	6.3	12.4	6.4	6.6	31.5
DF0049.2c	16.7	6.6	6.9	23.6	17.0	32.1
DF0049.2e	15.9	6.6	7.3	23.2	16.5	31.9
DF0059.2e	9.9	6.6	6.6	6.6	6.6	32.5
DF0060c	25.6	6.6	10.8	6.5	6.6	32.6
DF0102.4e	17.9	12.4	16.0	22.7	6.6	31.7
DF0102.4f	19.1	12.8	16.8	22.4	6.5	33.6
DF0102.4g	18.4	12.1	16.3	22.7	6.6	33.7
DF0102.4h	17.9	12.1	16.1	21.6	6.3	32.3
DF0159.2d	15.7	6.6	7.7	23.1	7.8	29.3
DF0159.2e	15.6	6.6	7.1	25.0	8.2	30.4
DF0159.2g	16.0	6.6	8.4	23.0	8.6	32.6
DF0159.2h	13.8	6.6	6.9	21.4	7.6	29.4
DF0166c	23.9	6.9	12.8	24.0	18.1	36.0
DF0181.1c	18.4	6.6	11.5	6.6	6.4	33.3
DF0181.1d	16.3	6.6	7.7	21.8	17.4	32.7
DF0181.1e	19.5	6.6	12.4	6.6	7.1	33.5
DF0181.1f	18.9	6.6	12.2	6.6	7.2	35.2
DF0181.1j	17.1	6.6	11.5	6.6	6.9	36.1
DF0181.1k	17.1	6.6	11.6	6.6	6.5	34.4
DF0181.3c	17.2	6.6	11.3	6.6	6.7	35.8
DF0181.3d	17.8	6.6	12.3	6.6	7.7	35.8
DF0181.3e	16.3	6.6	10.4	6.6	7.1	34.9
DF0181.3f	18.9	6.6	11.6	6.6	7.6	35.6
DF0181.3i	16.8	6.6	10.6	6.6	6.7	34.7
DF0181.3j	17.6	6.6	10.6	6.6	7.5	35.1
DF0181.3k	16.7	6.6	11.2	6.6	7.1	34.0
DF0181.4c	19.0	6.8	10.2	25.1	17.7	34.0
DF0181.4d	16.1	6.6	9.7	22.2	17.3	32.8
DF0181.4e	18.3	6.6	9.0	23.9	17.0	32.3

Strain	CTX[a]	CPD	FOX	TET	STR	CIP
DF0181.4f	16.4	6.6	9.2	23.0	16.3	32.4
DF0183c	7.4	6.6	17.9	22.5	16.3	29.2
DF0183d	17.8	6.6	11.2	6.6	8.2	36.5
DF0183e	17.3	6.6	11.9	6.6	7.0	36.0
DF0183f	19.6	6.6	12.4	6.6	7.5	34.5
DF0183g	8.1	6.6	18.0	22.3	16.3	30.3
DF0183i	16.2	6.6	6.7	21.4	16.6	30.5

[a] CTX, cefotaxime (30µg); CPD, cefpodoxime (10µg); FOX, cefoxitin (30µg); TET, tetracycline (30µg); STR, streptomycin (10µg); CIP, ciprofloxacin (5µg). Zone sizes in mm.

Appendix D

AmpC-producing *E. coli* AST zone sizes for selected antibiotics

Isolate metadata and antimicrobial susceptibility testing zone size (mm) for cefotaxime, cefpodoxime and cefoxitin.

Isolate	**CTX**[a]	**CPD**	**FOX**	**AmpC**	**Phylogroup**	**Phenotype**
DF0025c	24.6	7.2	12.9	Chrom	C	Intermediate
DF0030c	26.1	7.2	13.5	Chrom	C	Intermediate
DF0031.1c	24.9	7.4	13.3	Chrom	C	Intermediate
DF0047c	24.8	6.6	13.6	Chrom	C	Intermediate
DF0049.2c	16.7	6.6	6.9	CMY-2	E	Resistant
DF0059.2e	9.9	6.6	6.2	CMY-2	D	Resistant
DF0060c	25.6	6.6	10.8	Chrom	C	Intermediate
DF0159.2d	15.7	6.6	7.7	CMY-2	A	Resistant
DF0166c	23.9	6.9	12.8	Chrom	B1	Intermediate
DF0181.1c	18.4	6.6	11.5	CMY-2	B1	Resistant
DF0181.1d	16.3	6.6	7.7	CMY-2	B1	Resistant
DF0181.3c	17.2	6.6	11.3	CMY-2	B1	Resistant
DF0181.4c	19.0	6.8	10.2	CMY-2	E	Resistant
DF0183d	17.8	6.6	11.2	CMY-2	B1	Resistant
DF0183i	16.2	6.6	6.7	CMY-2	B1	Resistant

[a] CTX, cefotaxime (30µg); FOX, cefoxitin (30µg); CPD, cefpodoxime (10µg).

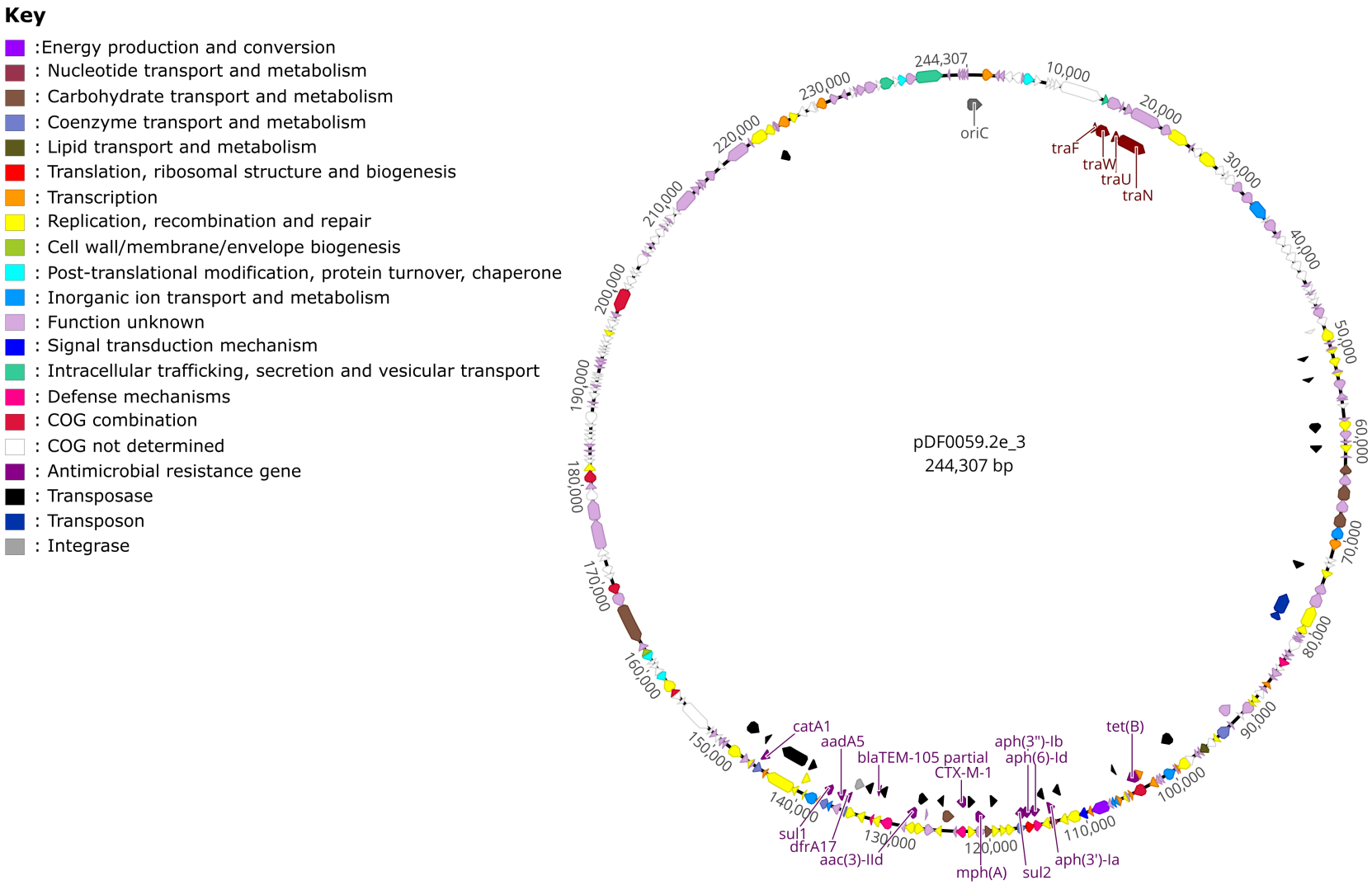
Appendix E

Antimicrobial resistance genes identified in *E. coli* genome sequences

Gene	AMR gene family	Drug class	Resistance mechanism
aac(3)-IId	AAC(3)	Aminoglycoside	Antibiotic inactivation
aadA1	ANT(3")	Aminoglycoside	Antibiotic inactivation
aadA5	ANT(3")	Aminoglycoside	Antibiotic inactivation
aph(3')-Ia	APH(3')	Aminoglycoside	Antibiotic inactivation
aph(3")-Ib	APH(3")	Aminoglycoside	Antibiotic inactivation
aph(6)-Id	APH(6)	Aminoglycoside	Antibiotic inactivation
catA1	Chloramphenicol acetyltransferase (CAT)	Phenicol	Antibiotic inactivation
dfrA17	Trimethoprim resistant dihydrofolate reductase dfr	Diaminopyrimidine	Antibiotic target replacement
dfrA5	Trimethoprim resistant dihydrofolate reductase dfr	Diaminopyrimidine	Antibiotic target replacement
mph(A)	Macrolide phosphotransferase (MPH)	Macrolide	Antibiotic inactivation
sul1	Sulfonamide resistant sul	Sulfonamide	Antibiotic target replacement
sul2	Sulfonamide resistant sul	Sulfonamide	Antibiotic target replacement
tet(A)	Major facilitator superfamily (MFS) antibiotic efflux pump	Tetracycline	Antibiotic efflux
tet(B)	Major facilitator superfamily (MFS) antibiotic efflux pump	Tetracycline	Antibiotic efflux

Appendix F

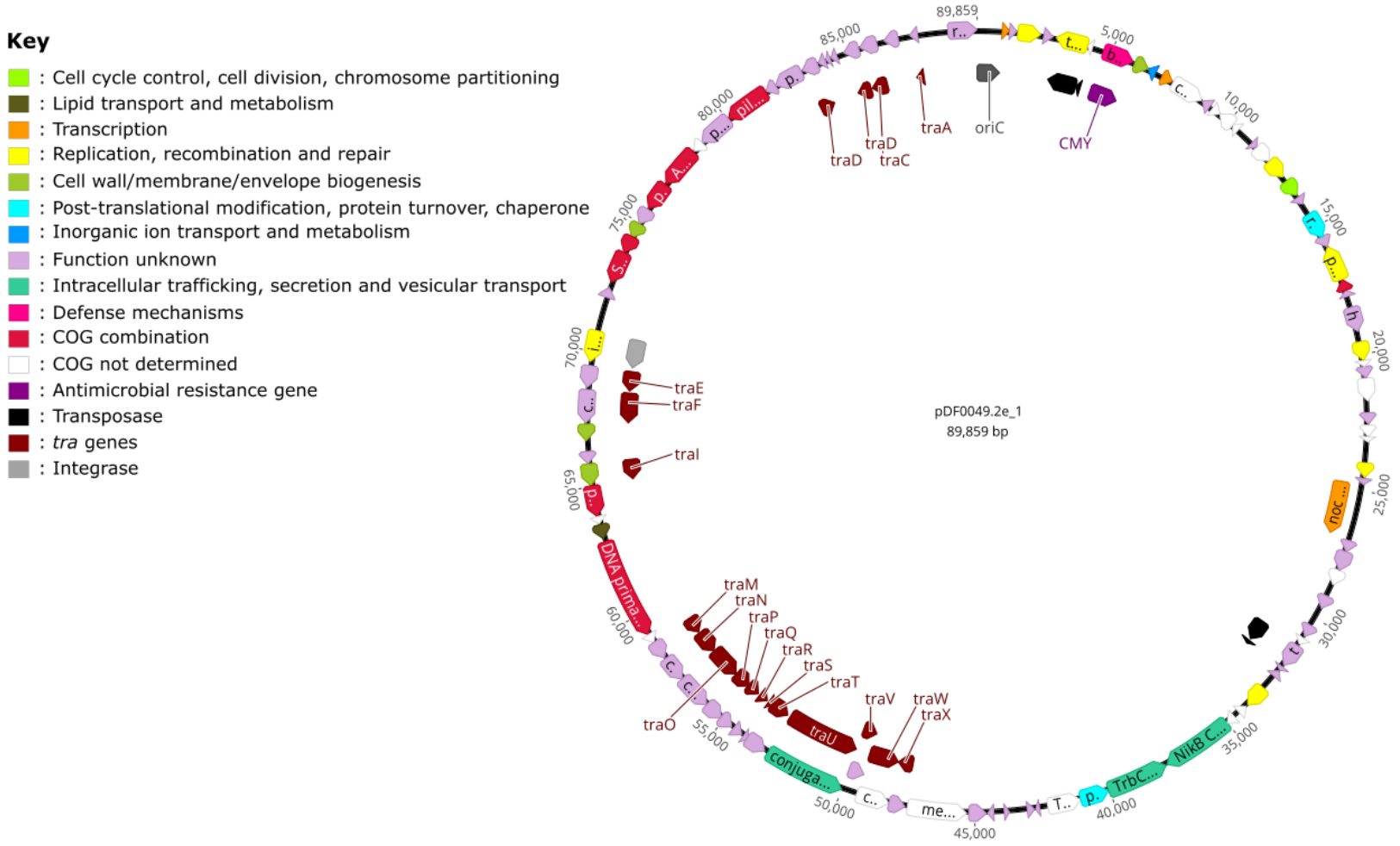
Complete circular plasmid pDF0059.2e_3



Complete circular plasmid pDF0059.2e_3 assembled from Nanopore and Illumina data showing cluster of orthologous groups on the outer circle and physical linkages of ARGs and mobile elements on the inner circle.

Appendix G

Representative IncI1 complete circular plasmid pDF0049.2e 1



Representative IncI1 complete circular plasmid pDF0049.2e_1 assembled from Nanopore and Illumina data showing cluster of orthologous groups on the outer circle and ARGs and mobile elements on the inner circle.

Appendix H

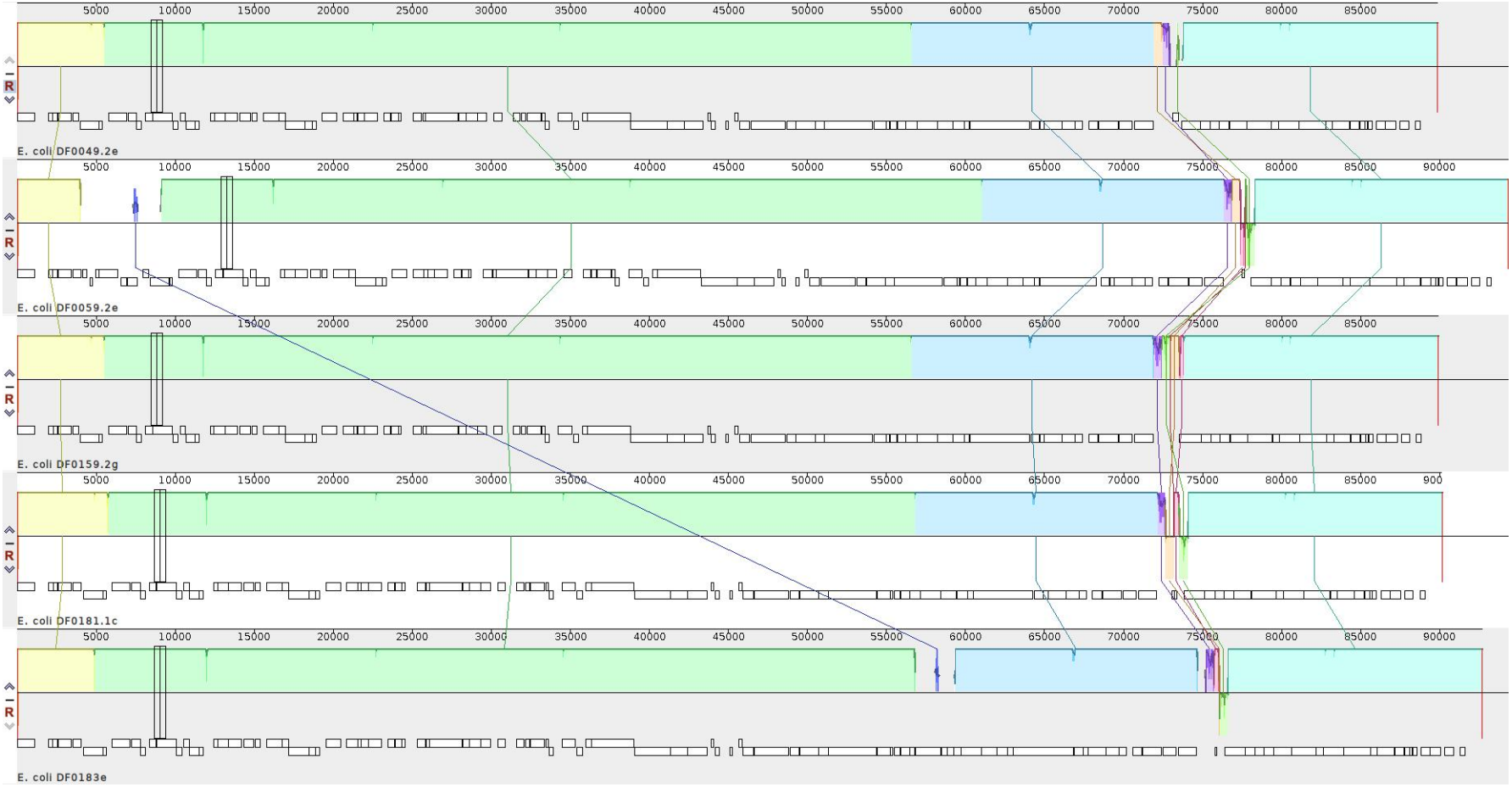
Details of the SNP annotations (n=7) identified in the core genome of five IncI1 plasmids

Reference	Position	Ref	49.2e[a]	59.2e	159.2g	181.1c	183e	Product	Effect	Locus tag
DF0183e	22701	T	C	T	C	T	T	Hypothetical protein	Synonymous variant c.513T>C p.Thr171Thr	IPPFNOJB_00031
DF0183e	34544	G	C	C	C	G	G	Methyltransferase	Missense variant c.76G>C p.Gly26Arg	IPPFNOJB_00047
DF0183e	66791	G	G	T	G	G	G	DNA primase	Start lost c.2_5 del TGCC ins GGCA p.MetPro1	IPPFNOJB_00077
DF0183e	66891	T	T	A	T	T	T	Conjugal transfer protein	Missense variant c.537_546 del AGACTGGAAA ins CGACGGTAAT p.TrpLys181GlyAsn	IPPFNOJB_00078
DF0183e	66906	C	C	A	C	C	C	Conjugal transfer protein	Missense variant c.531G>T p.Lys177Asn	IPPFNOJB_00078
DF0183e	82769	C	A	C	C	C	C	Pilus assembly protein PilO	Missense variant c.1152G>T p.Gln384His	IPPFNOJB_00095
DF0183e	83326	A	C	A	C	A	A	Pilus assembly protein PilO	Missense variant c.595T>G p.Ser199Ala	IPPFNOJB_00095

[a] 49.2e, pDF0049.2e_1; 59.2e, pDF0059.2e_1; 159.2g, pDF0159.2g_1; 181.1c, pDF0181.1c_1; 183e, pDF0183e_1.

Appendix I

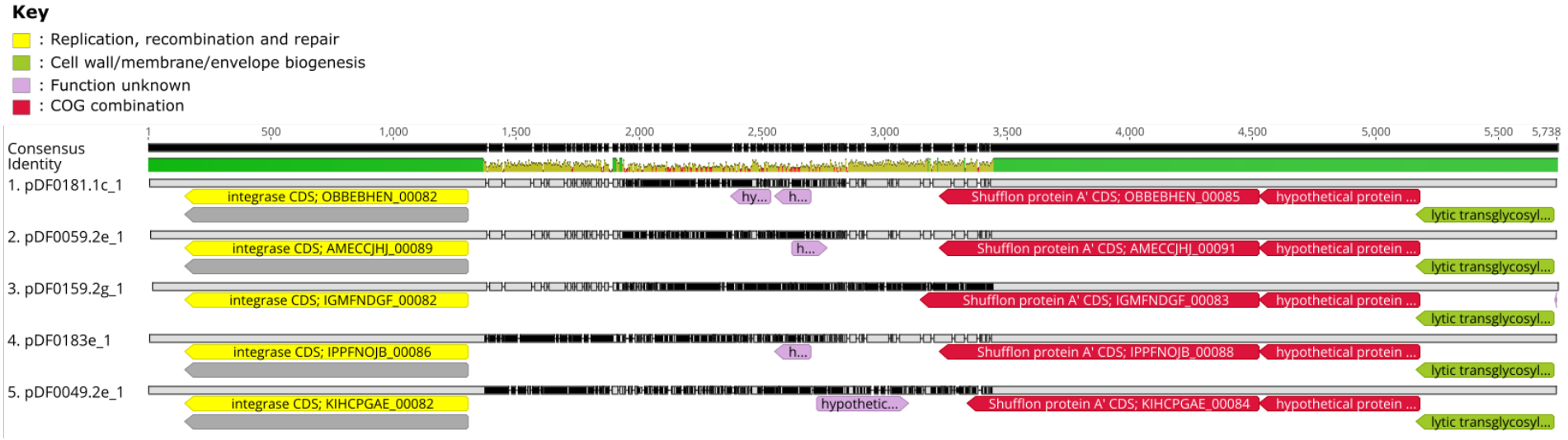
Gene synteny comparison of five IncI1 plasmids



Gene synteny comparison of five IncI1 plasmids carrying the $bla_{\text{CMY-2}}$ gene.

Appendix J

Surrounding genetic region of the Shufflon A coding sequence



Surrounding genetic regions of the Shufflon A coding sequence in the five *E. coli* IncI1 plasmids. The cluster of orthologous groups are colour coded as indicated in the figure key.

Appendix K

Antimicrobial use on Dairy 1

Amount (mg) of antimicrobial products used on Dairy 1 between October 2018 and December 2019.

Antimicrobial product	Amount (mg)	Administration route[a]	Active ingredient	Class
Betamox LA	6,000	Parenteral other	Amoxycillin	Aminopenicillins
Bivatop® 200	20,000	LCT par	Oxytetracycline	Tetracyclines
Bomacure	20,000	Parenteral other	Cephapirin	First-generation cephalosporins
Metri-Clean™ or Metricure®	7,000	Parenteral other	Cephapirin	First-generation cephalosporins
Penethaject	966,570	Parenteral other	Penethamate	Penicillins
Vibrostrep™	80,000	Parenteral other	Streptomycin	Aminoglycosides
Orbenin DC	12,000	DCT	Cloxacillin	Penicillins
Intracillin® 1000 Milking Cow	336,000	Parenteral other	Procaine penicillin G	Penicillins
Excede LA	14,000	LCT par	Ceftiofur	Third-generation cephalosporins
Intracillin® 300	495,000	Other	Procaine penicillin G	Penicillins
Marbocyl 10%	1,200	Other	Marbofloxacin	Quinolones
Phoenix Pharmacillin 300	22,500	Other	Procaine penicillin G	Penicillins
Forcyl	40,320	Other	Marbofloxacin	Quinolones
Total	2,020,590			

[a]DCT, dry cow therapy; LCT imam, lactating cow therapy intramammary; LCT par, lactating cow therapy parenteral.

Amount (mg) of antimicrobial per class used on Dairy 1

Class	Amount (mg)	PCU[a]	% of total
Aminoglycoside	80,000	0.68	3.96
Aminopenicillin	6,000	0.05	0.30
First-generation cephalosporin	27,000	0.23	1.34
Third-generation cephalosporin	14,000	0.12	0.69
Penicillin	1,832,070	15.50	90.67
Quinolone	41,520	0.35	2.05
Tetracycline	20,000	0.17	0.99
Total	2,020,590	17.09	100.00

[a]PCU, Population correction unit.

Antimicrobial use per month on Dairy 1

Date	Amount (mg)	PCU[a]
October 2018	248,782	2.10
November 2018	93,494	0.79
December 2018	58,830	0.50
January 2019	78,496	0.66
February 2019	97,492	0.82
March 2019	127,996	1.08
April 2019	30,500	0.26
May 2019	21,698	0.18
June 2019	48,996	0.41
July 2019	50,898	0.43
August 2019	376,648	3.19
September 2019	362,268	3.06
October 2019	175,656	1.49
November 2019	183,840	1.55
December 2019	64,996	0.55
Total	2,020,590	17.09

[a]PCU, Population correction unit.

Appendix L

Antimicrobial use on Dairy 4

Amount (mg) of antimicrobial products used on Dairy 4 between October 2018 and December 2019.

Antimicrobial product	Amount (mg)	Administration route[a]	Antimicrobial ingredient	Class
Betamox LA	6,000	Parenteral other	Amoxycillin	Aminopenicillins
Bivatop® 200	10,000	LCT par	Oxytetracycline	Tetracyclines
Bomacure	750,000	Parenteral other	Cephapirin	First-generation cephalosporins
Mastalone®	49.19	Parenteral other	Oxytetracycline, oleandomycin, neomycin	Multiple classes
Penethaject	43,329	Parenteral other	Penethamate	Penicillins
Tylo 200	5,000	Parenteral other	Tylosin	Macrolides
Vibrostrep™	50,000	Parenteral other	Streptomycin	Aminoglycosides
Orbenin DC	84,000	DCT	Cloxacillin	Penicillins
Intracillin® 1000 Milking Cow	1,000	Parenteral other	Procaine penicillin G	Penicillins
Penclox 1200™	72,000	Parenteral other	Penicillin G and cloxacillin	Penicillins
Excede LA	10,600	LCT par	Ceftiofur	Third-generation cephalosporins
Intracillin® 300	121,800	Other	Procaine penicillin G	Penicillins
Marbocyl 10%	8,300	Other	Marbofloxacin	Quinolones
Phoenix Pharmacillin 300	255,000	Other	Procaine penicillin G	Penicillins
Total	1,417,078.19			

[a]DCT, dry cow therapy; LCT imam, lactating cow therapy intramammary; LCT par, lactating cow therapy parenteral.

Amount (mg) of antimicrobial per class used on Dairy 4

Class	Amount (mg)	PCU[a]	% of total
Aminoglycoside	50,000	0.19	3.53
Aminopenicillin	6,000	0.02	0.42
First-generation cephalosporin	750,000	2.83	52.93
Third-generation cephalosporin	10,600	0.04	0.75
Penicillin	577,129	2.18	40.73
Quinolone	8,300	0.03	0.59
Tetracycline	10,000	0.04	0.71
Multiple classes	49.19	0.00	0.00
Macrolide	5,000	0.02	0.35
Total	1,417,078.19	5.36	100.00

Antimicrobial use per month on Dairy 4

Date	Amount (mg)	PCU[a]
November 2018	57,399	0.22
December 2018	2,400	0.01
January 2019	44,100	0.17
February 2019	13,800	0.05
March 2019	11,200	0.04
April 2019	42,000	0.16
May 2019	32,000	0.12
June 2019	57,000	0.22
July 2019	149,800	0.57
August 2019	66,200	0.25
September 2019	804,447.19	3.04
October 2019	56,000	0.21
November 2019	58,632	0.22
December 2019	22,100	0.08
Total	1,417,078.19	5.36

[a]PCU, Population correction unit.

Appendix M

Antimicrobial products used on Dairy 1 and Dairy 4

Product	**Condition**	**Antimicrobial ingredient**	**Class**	**NZVA classification[a]**	**Administration route[b]**
Mastalone	Mastitis - clinical	Oxytetracycline, Oleandomycin, Neomycin	Multiple classes	Green (oxytetracycline), Red (oleandomycin), Yellow (neomycin)	Parenteral other
Bivatop® 200	Sick - undiagnosed, theileria	Oxytetracycline	Tetracyclines	Green	LCT par
Penethaject	Mastitis – clinical or subclinical	Penethamate	Penicillins	Green	Parenteral other
Intracillin® 1000 Milking Cow	Mastitis – clinical or subclinical	Procaine penicillin G	Penicillins	Green	Parenteral other
Intracillin® 300	Calving disorder - unspecified, white line disease, uterine infection, between claw/footrot, severe injury, retained membranes, sick - undiagnosed	Procaine penicillin G	Penicillins	Green	Other
Phoenix Pharmacillin 300	Between claw/footrot, abomassal disorder - unspecified, pneumonia or respiratory disease, other lameness, calving trouble, retained membranes	Procaine penicillin G	Penicillins	Green	Other
Betamox LA	Between claw/footrot	Amoxycillin	Aminopenicillins	Yellow	Parenteral other
Bomacure	Metri-check (treatment of endometritis)	Cephapirin	First Generation Cephalosporins	Yellow	Parenteral other
Metri-Clean™ or Metricure®	Metri-check (treatment of endometritis)	Cephapirin	First-generation Cephalosporins	Yellow	Parenteral other

Product	Condition	Antimicrobial ingredient	Class	NZVA classification[a]	Administration route[b]
Orbenin DC	Mastitis and reduces risk of new infections during dry period	Cloxacillin	Penicillins	Yellow	DCT
Penclox^{TM} 1200	Mastitis – clinical or subclinical, teat injury	Penicillin G and cloxacillin	Penicillins	Yellow	Parenteral other
Vibrostrep	Wooden tongue, sick - undiagnosed	Streptomycin	Aminoglycosides	Yellow	Parenteral other
Excede LA	Pneumonia or respiratory illness, calving disorder	Ceftiofur	Third-generation Cephalosporins	Red	LCT par
Marbocyl 10%	Pneumonia or respiratory illness, mastitis - clinical	Marbofloxacin	Quinolones	Red	Other
Forcyl	Mastitis - clinical, pneumonia or respiratory illness, sick - undiagnosed	Marbofloxacin	Quinolones	Red	Other
Tylo 200	Mastitis - clinical	Tylosin	Macrolides	Red	Parenteral other

[a]NZVA, New Zealand Veterinary Association. [b]LCT imam, lactating cow therapy intramammary; DCT, dry cow therapy; LCT par, lactating cow therapy parenteral.

Appendix N

Antimicrobial use per route of administration

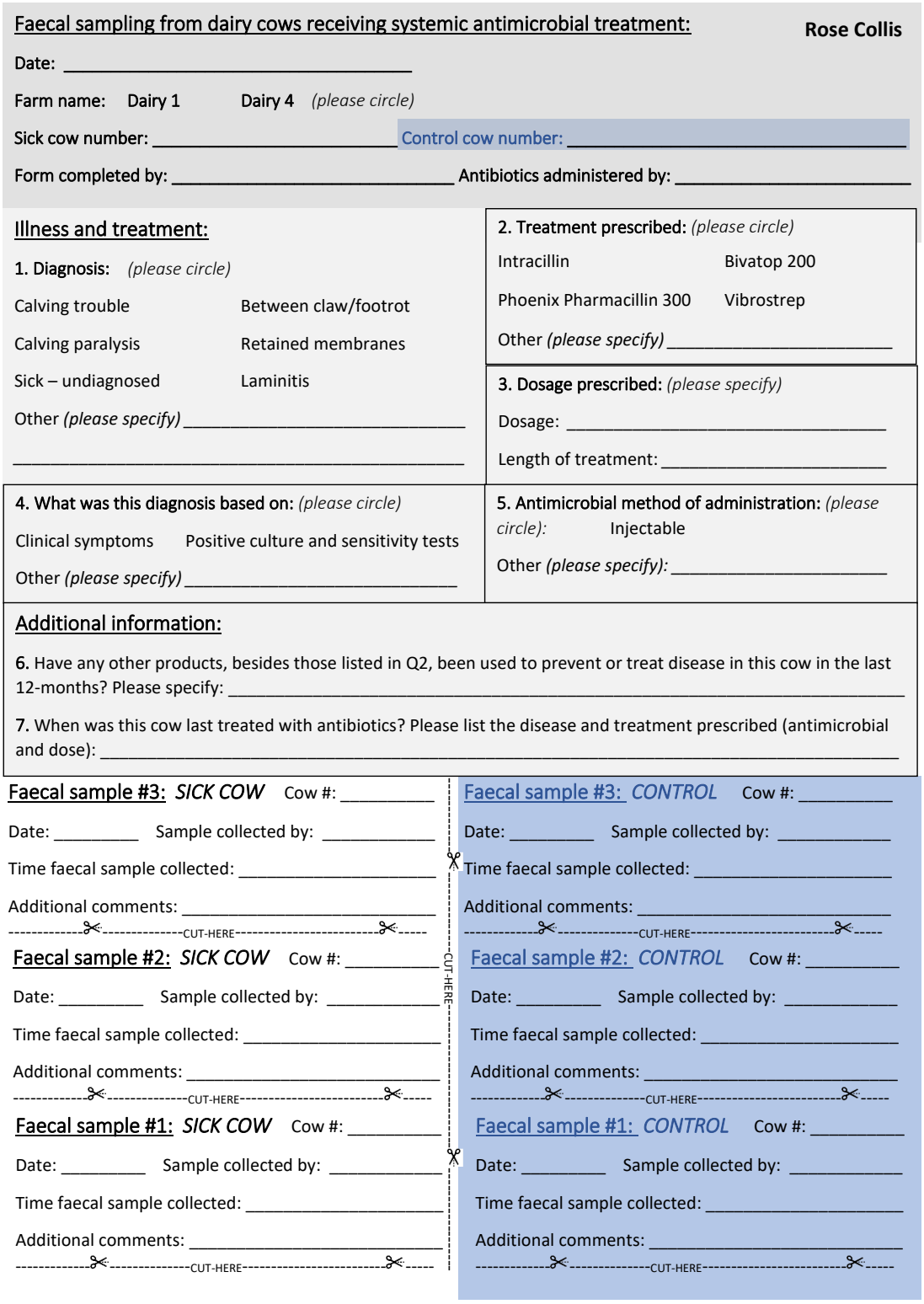
Amount (mg per active ingredient per population correction unit (% total PCU)) of antimicrobials per route of administration on Dairy 1 and Dairy 4 between October 2018 and December 2019.

Route[a]	**Dairy 1**	**Dairy 4**
Dry cow therapy	0.10 (0.59)	0.32 (5.93)
LCT parenteral	0.29 (1.70)	0.08 (1.49)
Parenteral other	11.97 (70.00)	3.51 (65.36)
Other	4.73 (27.67)	1.46 (27.18)

[a]LCT, lactating cow therapy.

Appendix O

Faecal sample collection questionnaire



Faecal sampling from dairy cows receiving systemic antimicrobial treatment: **Rose Collis**

Date: _____

Farm name: Dairy 1 Dairy 4 *(please circle)*

Sick cow number: _____ Control cow number: _____

Form completed by: _____ Antibiotics administered by: _____

Illness and treatment:

1. Diagnosis: *(please circle)*

Calving trouble | Between claw/footrot

Calving paralysis | Retained membranes

Sick – undiagnosed | Laminitis

Other *(please specify)* _____

_____

2. Treatment prescribed: *(please circle)*

Intracillin | Bivatop 200

Phoenix Pharmacillin 300 | Vibrostrep

Other *(please specify)* _____

3. Dosage prescribed: *(please specify)*

Dosage: _____

Length of treatment: _____

4. What was this diagnosis based on: *(please circle)*

Clinical symptoms | Positive culture and sensitivity tests

Other *(please specify)* _____

5. Antimicrobial method of administration: *(please circle):* Injectable

Other *(please specify):* _____

Additional information:

6. Have any other products, besides those listed in Q2, been used to prevent or treat disease in this cow in the last 12-months? Please specify: _____

7. When was this cow last treated with antibiotics? Please list the disease and treatment prescribed (antimicrobial and dose): _____

Faecal sample #3: *SICK COW* Cow #: _____

Date: _____ Sample collected by: _____

Time faecal sample collected: _____

Additional comments: _____

----------CUT-HERE----------

Faecal sample #2: *SICK COW* Cow #: _____

Date: _____ Sample collected by: _____

Time faecal sample collected: _____

Additional comments: _____

----------CUT-HERE----------

Faecal sample #1: *SICK COW* Cow #: _____

Date: _____ Sample collected by: _____

Time faecal sample collected: _____

Additional comments: _____

----------CUT-HERE----------

CUT-HERE

Faecal sample #3: *CONTROL* Cow #: _____

Date: _____ Sample collected by: _____

Time faecal sample collected: _____

Additional comments: _____

----------CUT-HERE----------

Faecal sample #2: *CONTROL* Cow #: _____

Date: _____ Sample collected by: _____

Time faecal sample collected: _____

Additional comments: _____

----------CUT-HERE----------

Faecal sample #1: *CONTROL* Cow #: _____

Date: _____ Sample collected by: _____

Time faecal sample collected: _____

Additional comments: _____

----------CUT-HERE----------

Faecal sample collection from dairy cows receiving systemic antimicrobial treatment

Appendix P

Sample metadata of faecal samples collected for 16S rRNA V3-V4 amplicon sequencing

Lab ID	Type	Animal	Sample order	Farm	Treatment	Antimicrobial	Class	Illness	Collection date
processed_DG020	Treated	1	S1	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	Footrot	9/08/2019
processed_DG021	Treated	2	S1	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	Footrot	9/08/2019
processed_DG022	Control	1C/2C	S1	Dairy 4	NA	NA	NA	Control	9/08/2019
processed_DG023	Treated	2	S2	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	Footrot	11/08/2019
processed_DG024	Treated	1	S2	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	Footrot	11/08/2019
processed_DG025	Treated	3	S1	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	Footrot	11/08/2019
processed_DG026	Treated	4	S1	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	Footrot	11/08/2019
processed_DG027	Control	3C/4C	S1	Dairy 4	NA	NA	NA	Control	11/08/2019
processed_DG028	Control	1C/2C	S2	Dairy 4	NA	NA	NA	Control	11/08/2019
processed_DG029	Treated	3	S2	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	Footrot	13/08/2019
processed_DG030	Treated	4	S2	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	Footrot	13/08/2019
processed_DG031	Control	3C/4C	S2	Dairy 4	NA	NA	NA	Control	13/08/2019
processed_DG032	Control	5C	S1	Dairy 4	NA	NA	NA	Control	16/08/2019
processed_DG033	Treated	5	S1	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	Footrot	16/08/2019
processed_DG034	Treated	2	S3	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	Footrot	17/08/2019
processed_DG035	Treated	1	S3	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	Footrot	17/08/2019
processed_DG036	Control	1C/2C	S3	Dairy 4	NA	NA	NA	Control	17/08/2019
processed_DG037	Treated	5	S2	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	Footrot	18/08/2019
processed_DG038	Control	5C	S2	Dairy 4	NA	NA	NA	Control	18/08/2019
processed_DG039	Treated	4	S3	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	Footrot	19/08/2019
processed_DG040	Control	3C/4C	S3	Dairy 4	NA	NA	NA	Control	19/08/2019
processed_DG041	Treated	3	S3	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	Footrot	19/08/2019
processed_DG042	Treated	5	S3	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	Footrot	26/08/2019
processed_DG043	Control	5C	S3	Dairy 4	NA	NA	NA	Control	26/08/2019
processed_DG046	Treated	6	S1	Dairy 1	Penethaject	Penethamate hydriodide	Beta-lactam	Mastitis	1/09/2019
processed_DG047	Control	6C	S1	Dairy 1	NA	NA	NA	Control	1/09/2019
processed_DG048	Treated	6	S2	Dairy 1	Penethaject	Penethamate hydriodide	Beta-lactam	Mastitis	4/09/2019
processed_DG049	Control	6C	S2	Dairy 1	NA	NA	NA	Control	4/09/2019
processed_DG050	Treated	7	S1	Dairy 4	Pharmacillin 300	Procaine penicillin G	Beta-lactam	LDA	7/09/2019
processed_DG051	Control	7C	S1	Dairy 4	NA	NA	NA	Control	7/09/2019

Lab ID	Type	Animal	Sample order	Farm	Treatment	Antimicrobial	Class	Illness	Collection date
processed_DG052	Treated	7	S2	Dairy 4	Pharmacillin 300	Procaine penicillin G	Beta-lactam	LDA	8/09/2019
processed_DG053	Control	7C	S2	Dairy 4	NA	NA	NA	Control	8/09/2019
processed_DG054	Treated	8	S1	Dairy 4	Intracillin 300	Procaine penicillin G	Beta-lactam	Footrot	8/09/2019
processed_DG055	Treated	9	S1	Dairy 4	Intracillin 300	Procaine penicillin G	Beta-lactam	Footrot	8/09/2019
processed_DG056	Control	8C/9C	S1	Dairy 4	NA	NA	NA	Control	8/09/2019
processed_DG057	Treated	9	S2	Dairy 4	Intracillin 300	Procaine penicillin G	Beta-lactam	Footrot	9/09/2019
processed_DG058	Treated	8	S2	Dairy 4	Intracillin 300	Procaine penicillin G	Beta-lactam	Footrot	9/09/2019
processed_DG059	Control	8C/9C	S2	Dairy 4	NA	NA	NA	Control	9/09/2019
processed_DG060	Treated	6	S3	Dairy 1	Penethaject	Penethamate hydriodide	Beta-lactam	Mastitis	13/09/2019
processed_DG061	Control	6C	S3	Dairy 1	NA	NA	NA	Control	13/09/2019
processed_DG062	Treated	9	S3	Dairy 4	Intracillin 300	Procaine penicillin G	Beta-lactam	Footrot	14/09/2019
processed_DG063	Treated	8	S3	Dairy 4	Intracillin 300	Procaine penicillin G	Beta-lactam	Footrot	14/09/2019
processed_DG064	Control	8C/9C	S3	Dairy 4	NA	NA	NA	Control	14/09/2019
processed_DG065	Treated	7	S3	Dairy 4	Pharmacillin 300	Procaine penicillin G	Beta-lactam	LDA	16/09/2019
processed_DG066	Control	7C	S3	Dairy 4	NA	NA	NA	Control	16/09/2019
processed_DG067	Treated	11	S1	Dairy 4	Excede LA	Ceftiofur	Beta-lactam	Caesarean	17/09/2019
processed_DG068	Treated	10	S1	Dairy 4	Intracillin 300	Procaine penicillin G	Beta-lactam	LDA	17/09/2019
processed_DG069	Control	10C/11C	S1	Dairy 4	NA	NA	NA	Control	17/09/2019
processed_DG070	Treated	12	S1	Dairy 1	Excede LA	Ceftiofur	Beta-lactam	Caesarean	18/09/2019
processed_DG071	Control	12C	S1	Dairy 1	NA	NA	NA	Control	18/09/2019
processed_DG072	Treated	10	S2	Dairy 4	Intracillin 300	Procaine penicillin G	Beta-lactam	LDA	18/09/2019
processed_DG073	Treated	11	S2	Dairy 4	Excede LA	Ceftiofur	Beta-lactam	Caesarean	18/09/2019
processed_DG074	Control	10C/11C	S2	Dairy 4	NA	NA	NA	Control	18/09/2019
processed_DG075	Treated	13	S1	Dairy 4	Excede LA	Ceftiofur	Beta-lactam	Caesarean	19/09/2019
processed_DG076	Control	13C	S1	Dairy 4	NA	NA	NA	Control	19/09/2019
processed_DG077	Treated	13	S2	Dairy 4	Excede LA	Ceftiofur	Beta-lactam	Caesarean	20/09/2019
processed_DG078	Control	13C	S2	Dairy 4	NA	NA	NA	Control	20/09/2019
processed_DG079	Treated	12	S2	Dairy 1	Excede LA	Ceftiofur	Beta-lactam	Caesarean	20/09/2019
processed_DG080	Control	12C	S2	Dairy 1	NA	NA	NA	Control	20/09/2019
processed_DG082	Treated	12	S3	Dairy 1	Excede LA	Ceftiofur	Beta-lactam	Caesarean	22/09/2019
processed_DG083	Control	12C	S3	Dairy 1	NA	NA	NA	Control	22/09/2019

Lab ID	Type	Animal	Sample order	Farm	Treatment	Antimicrobial	Class	Illness	Collection date
processed_DG084	Treated	13	S3	Dairy 4	Excede LA	Ceftiofur	Beta-lactam	Caesarean	26/09/2019
processed_DG085	Control	13C	S3	Dairy 4	NA	NA	NA	Control	26/09/2019
processed_DG086	Treated	10	S3	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	LDA	26/09/2019
processed_DG087	Treated	11	S3	Dairy 4	Excede LA	Ceftiofur	Beta-lactam	Caesarean	26/09/2019
processed_DG088	Control	10C/11C	S3	Dairy 4	NA	NA	NA	Control	26/09/2019
processed_DG089	Treated	14	S1	Dairy 4	Marbocyl 10%/Penetha-ject	Marbofloxacin/Penetha hydriodide	Quinolone/Beta-lactam	Mastitis	29/09/2019
processed_DG090	Control	14C	S1	Dairy 4	NA	NA	NA	Control	29/09/2019
processed_DG091	Treated	14	S2	Dairy 4	Marbocyl 10%/Penetha-ject	Marbofloxacin/Penetha hydriodide	Quinolone/Beta-lactam	Mastitis	30/09/2019
processed_DG092	Control	14C	S2	Dairy 4	NA	NA	NA	Control	30/09/2019
processed_DG093	Treated	14	S3	Dairy 4	Marbocyl 10%/Penetha-ject	Marbofloxacin/Penetha hydriodide	Quinolone/Beta-lactam	Mastitis	9/10/2019
processed_DG094	Control	14C	S3	Dairy 4	NA	NA	NA	Control	9/10/2019
processed_DG095	Treated	15	S1	Dairy 4	Pharmacillin	Procaine penicillin G	Beta-lactam	Footrot	11/10/2019
processed_DG096	Control	15C	S1	Dairy 4	NA	NA	NA	Control	11/10/2019
processed_DG097	Treated	15	S2	Dairy 4	Pharmacillin	Procaine penicillin G	Beta-lactam	Footrot	12/10/2019
processed_DG098	Control	15C	S2	Dairy 4	NA	NA	NA	Control	12/10/2019
processed_DG099	Treated	15	S3	Dairy 4	Pharmacillin	Procaine penicillin G	Beta-lactam	Footrot	17/10/2019
processed_DG100	Control	15C	S3	Dairy 4	NA	NA	NA	Control	17/10/2019
processed_DG101	Treated	16	S1	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	Swollen vulva	21/10/2019
processed_DG102	Control	16C	S1	Dairy 4	NA	NA	NA	Control	21/10/2019
processed_DG103	Treated	16	S2	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	Swollen vulva	23/10/2019
processed_DG104	Control	16C	S2	Dairy 4	NA	NA	NA	Control	23/10/2019
processed_DG105	Treated	17	S1	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	Footrot	23/10/2019
processed_DG106	Control	17C	S1	Dairy 4	NA	NA	NA	Control	23/10/2019
processed_DG107	Treated	17	S2	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	Footrot	23/10/2019
processed_DG108	Control	17C	S2	Dairy 4	NA	NA	NA	Control	23/10/2019
processed_DG113	Treated	17	S3	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	Footrot	30/10/2019

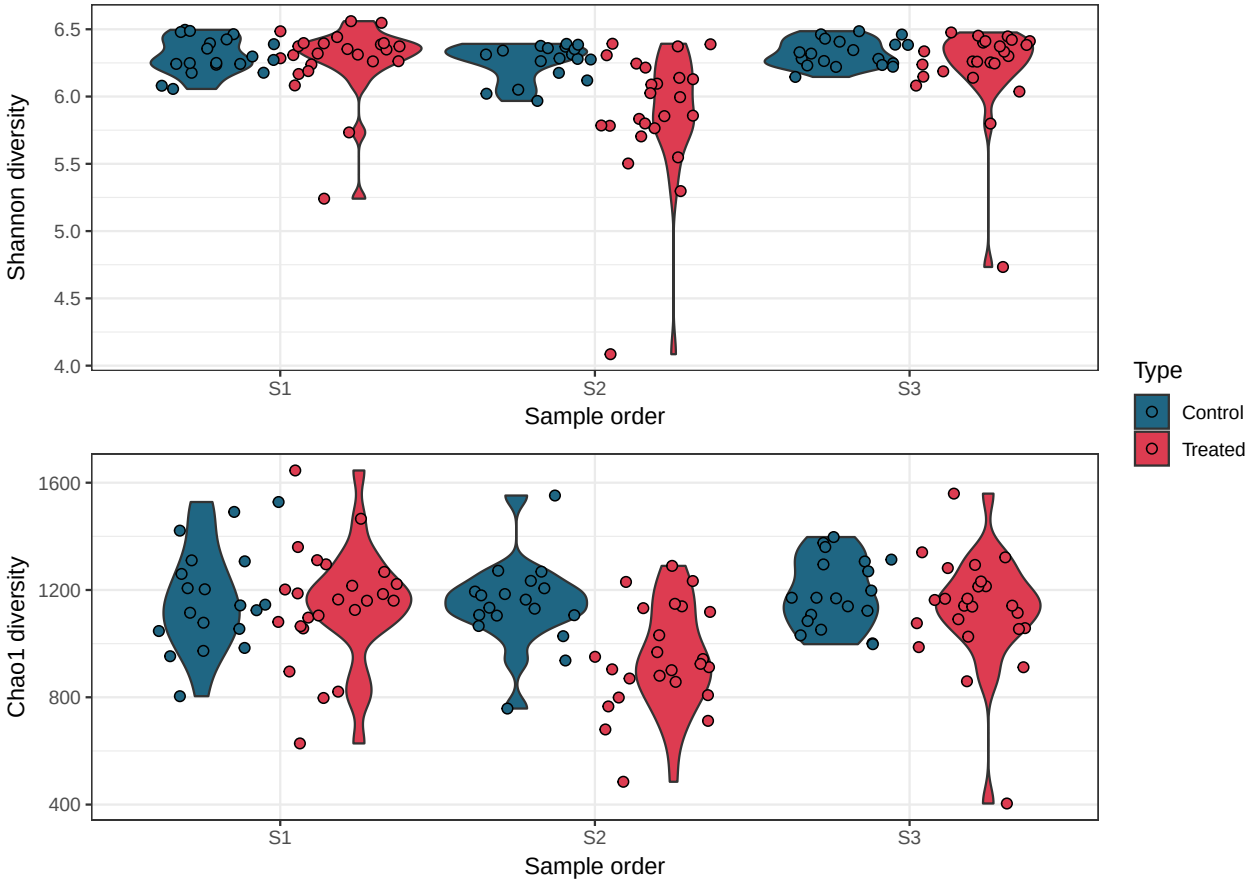
Lab ID	Type	Animal	Sample order	Farm	Treatment	Antimicrobial	Class	Illness	Collection date
processed_DG114	Control	17C	S3	Dairy 4	NA	NA	NA	Control	30/10/2019
processed_DG115	Treated	16	S3	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	Swollen vulva	30/10/2019
processed_DG116	Control	16C	S3	Dairy 4	NA	NA	NA	Control	30/10/2019
processed_DG117	Control	18C/19C	S1	Dairy 4	NA	NA	NA	Control	22/11/2019
processed_DG118	Treated	18	S1	Dairy 4	Penethaject	Penethamate hydriodide	Beta-lactam	Mastitis	22/11/2019
processed_DG119	Treated	20	S1	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	Footrot	22/11/2019
processed_DG120	Treated	19	S1	Dairy 4	Penethaject	Penethamate hydriodide	Beta-lactam	Mastitis	22/11/2019
processed_DG121	Control	18C/19C	S2	Dairy 4	NA	NA	NA	Control	23/11/2019
processed_DG122	Treated	20	S2	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	Footrot	23/11/2019
processed_DG123	Treated	18	S2	Dairy 4	Penethaject	Penethamate hydriodide	Beta-lactam	Mastitis	23/11/2019
processed_DG124	Treated	19	S2	Dairy 4	Penethaject	Penethamate hydriodide	Beta-lactam	Mastitis	23/11/2019
processed_DG125	Treated	21	S1	Dairy 4	Intracillin	300 Procaine penicillin G	Beta-lactam	Footrot	27/11/2019
processed_DG126	Control	21C	S1	Dairy 4	NA	NA	NA	Control	27/11/2019
processed_DG127	Treated	21	S2	Dairy 4	Intracillin	300 Procaine penicillin G	Beta-lactam	Footrot	28/11/2019
processed_DG128	Control	21C	S2	Dairy 4	NA	NA	NA	Control	28/11/2019
processed_DG129	Treated	21	S3	Dairy 4	Intracillin	300 Procaine penicillin G	Beta-lactam	Footrot	2/12/2019
processed_DG130	Control	21C	S3	Dairy 4	NA	NA	NA	Control	2/12/2019
processed_DG131	Treated	18	S3	Dairy 4	Penethaject	Penethamate hydriodide	Beta-lactam	Mastitis	2/12/2019
processed_DG132	Control	18C/19C	S3	Dairy 4	NA	NA	NA	Control	2/12/2019
processed_DG133	Treated	20	S3	Dairy 4	Intracillin	300 Procaine penicillin G	Beta-lactam	Footrot	2/12/2019
processed_DG134	Treated	19	S3	Dairy 4	Penethaject	Penethamate hydriodide	Beta-lactam	Mastitis	2/12/2019
processed_DG135	Control	22C	S1	Dairy 4	NA	NA	NA	Control	20/07/20

Lab ID	Type	Animal	Sample order	Farm	Treatment	Antimicrobial	Class	Illness	Collection date
processed_DG136	Treated	22	S1	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	Penetration on leg	20/07/20
processed_DG137	Control	22C	S2	Dairy 4	NA	NA	NA	Control	21/07/20
processed_DG138	Treated	22	S2	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	Penetration on leg	21/07/20
processed_DG143	Treated	22	S3	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	Penetration on leg	4/08/20
processed_DG144	Control	22C	S3	Dairy 4	NA	NA	NA	Control	4/08/20
processed_DG145	Treated	23	S1	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	Footrot	5/08/20
processed_DG146	Treated	23	S2	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	Footrot	6/08/20
processed_DG147	Control	23C	S1	Dairy 4	NA	NA	NA	Control	6/08/20
processed_DG148	Control	23C	S3	Dairy 4	NA	NA	NA	Control	12/08/20
processed_DG149	Treated	23	S3	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	Footrot	12/08/20
processed_DG150	Control	24C	S1	Dairy 4	NA	NA	NA	Control	12/08/20
processed_DG151	Treated	24	S1	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	Retained membrane	12/08/20
processed_DG152	Control	25C	S1	Dairy 4	NA	NA	NA	Control	13/08/20
processed_DG153	Treated	25	S1	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	Footrot	13/08/20
processed_DG154	Treated	25	S2	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	Footrot	14/08/20
processed_DG155	Control	25C	S2	Dairy 4	NA	NA	NA	Control	14/08/20
processed_DG156	Treated	24	S2	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	Retained membrane	14/08/20
processed_DG157	Control	24C	S2	Dairy 4	NA	NA	NA	Control	14/08/20
processed_DG158	Treated	26	S1	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	Footrot	14/08/20
processed_DG159	Control	26C	S1	Dairy 4	NA	NA	NA	Control	14/08/20
processed_DG160	Treated	26	S2	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	Footrot	15/08/20
processed_DG161	Control	26C	S2	Dairy 4	NA	NA	NA	Control	15/08/20
processed_DG162	Treated	24	S3	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	Retained membrane	20/08/20
processed_DG163	Control	24C	S3	Dairy 4	NA	NA	NA	Control	20/08/20
processed_DG164	Treated	26	S3	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	Footrot	20/08/20
processed_DG165	Control	26C	S3	Dairy 4	NA	NA	NA	Control	20/08/20

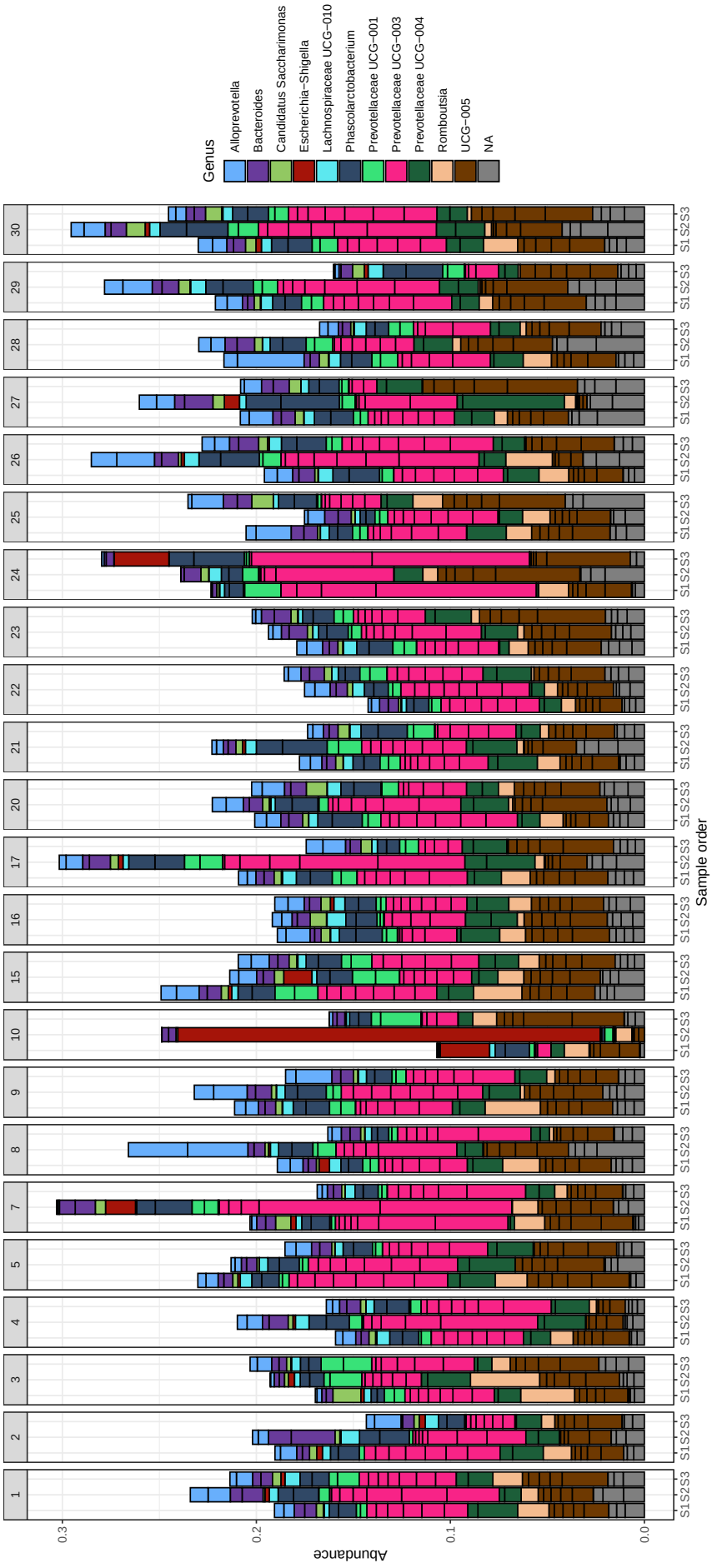
Lab ID	Type	Animal	Sample order	Farm	Treatment	Antimicrobial	Class	Illness	Collection date
processed_DG167	Treated	25	S3	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	Footrot	20/08/20
processed_DG168	Control	25C	S3	Dairy 4	NA	NA	NA	Control	21/08/20
processed_DG169	Treated	27	S1	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	Footrot	4/09/20
processed_DG170	Control	27C	S1	Dairy 4	NA	NA	NA	Control	4/09/20
processed_DG171	Treated	27	S2	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	Footrot	5/09/20
processed_DG172	Control	27C	S2	Dairy 4	NA	NA	NA	Control	5/09/20
processed_DG173	Treated	28	S1	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	Footrot	7/09/20
processed_DG174	Control	28C	S1	Dairy 4	NA	NA	NA	Control	7/09/20
processed_DG175	Treated	28	S2	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	Footrot	8/09/20
processed_DG176	Control	28C	S2	Dairy 4	NA	NA	NA	Control	8/09/20
processed_DG177	Treated	27	S3	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	Footrot	11/09/20
processed_DG178	Control	27C	S3	Dairy 4	NA	NA	NA	Control	11/09/20
processed_DG179	Control	28C	S3	Dairy 4	NA	NA	NA	Control	15/09/20
processed_DG180	Treated	28	S3	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	Footrot	16/09/20
processed_DG181	Treated	29	S1	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	Footrot	21/09/20
processed_DG182	Control	29C	S1	Dairy 4	NA	NA	NA	Control	21/09/20
processed_DG183	Treated	29	S2	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	Footrot	22/09/20
processed_DG184	Control	29C	S2	Dairy 4	NA	NA	NA	Control	22/09/20
processed_DG185	Treated	30	S1	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	Footrot	23/09/20
processed_DG186	Control	30C	S1	Dairy 4	NA	NA	NA	Control	23/09/20
processed_DG187	Treated	30	S2	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	Footrot	24/09/20
processed_DG188	Control	30C	S2	Dairy 4	NA	NA	NA	Control	24/09/20
processed_DG189	Treated	30	S3	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	Footrot	29/09/20
processed_DG190	Control	30C	S3	Dairy 4	NA	NA	NA	Control	29/09/20
processed_DG191	Treated	29	S3	Dairy 4	Intracillin	Procaine penicillin G	Beta-lactam	Footrot	29/09/20
processed_DG192	Control	29C	S3	Dairy 4	NA	NA	NA	Control	29/09/20

Appendix Q

Case study of 23 cows treated with procaine penicillin G



Shannon (**A**) and Chao1 (**B**) α diversity of faecal samples from cows treated with procaine penicillin G (n=23).



Abundance of the top 30 amplicon sequence variants (ASVs) at the genus level of faecal samples from cows treated with procaine penicillin G (n=23).

Appendix R

Ceftiofur case study: *Enterobacteriaceae* abundance

Counts and relative abundance of ASVs belonging to *Enterobacteriaceae* identified in the faecal samples from ceftiofur treated (n=3) and control cows (n=3).

			Counts				**Relative abundance**			
			ASV30	ASV5332	ASV10841	ASV13004	ASV30	ASV5332	ASV10841	ASV13004
Type	**Grp**[a]	**Ord**[b]	***Escherichia-Shigella***	***Salmonella***	***Escherichia-Shigella***	***Escherichia-Shigella***	***Escherichia-Shigella***	***Salmonella***	***Escherichia-Shigella***	***Escherichia-Shigella***
Treated	11	S1	13	0	0	0	0.000272794	0	0	0
Treated	11	S2	0	0	5	0	0	0	0.00010498	0
Treated	11	S3	23	0	0	0	0.000482555	0	0	0
Control	11C	S1	12	0	0	0	0.00025181	0	0	0
Control	11C	S2	32	0	0	0	0.000671324	0	0	0
Control	11C	S3	0	0	0	0	0	0	0	0
Treated	12	S1	11	0	0	0	0.000231068	0	0	0
Treated	12	S2	0	0	0	0	0	0	0	0
Treated	12	S3	14	0	0	0	0.000293914	0	0	0
Control	12C	S1	19	0	0	0	0.000398732	0	0	0
Control	12C	S2	0	0	0	0	0	0	0	0
Control	12C	S3	3	0	0	0	6.29736E-05	0	0	0
Treated	13	S1	44	43	0	0	0.000923535	0.000902546	0	0
Treated	13	S2	9	0	0	0	0.000188857	0	0	0
Treated	13	S3	0	0	0	2	0	0	0	4.19956E-05
Control	13C	S1	5	0	0	0	0.000104875	0	0	0
Control	13C	S2	21	0	0	0	0.000440815	0	0	0
Control	13C	S3	8	0	0	0	0.000167817	0	0	0

[a] Group [b] Sample order.

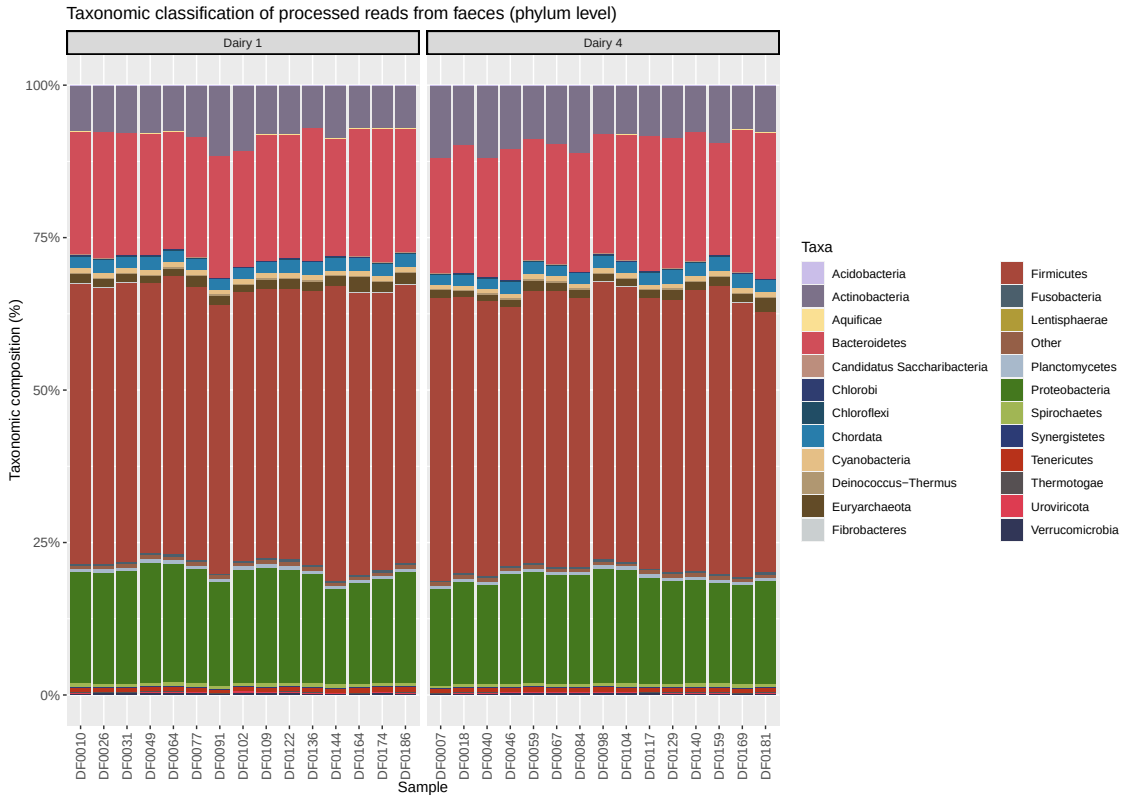
Appendix S

Virulence factors from *E. coli* genomes

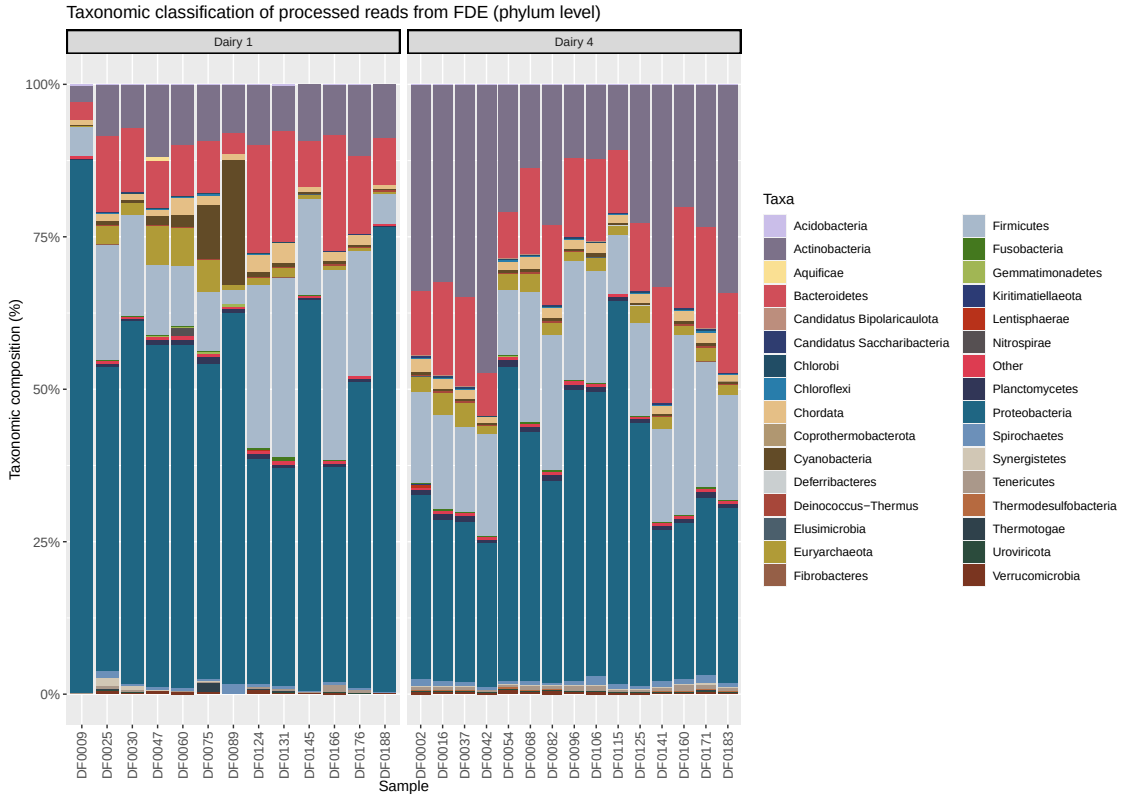
Isolate	**Virulence factors**
DG079c	*astA, cia, f17A, f17G, iss, terC, traT*
DG079h	*astA, cia, f17A, f17G, gad, iss, terC, traT*
DG082f	*astA, cia, f17A, f17G, iss, terC, traT*

Appendix T

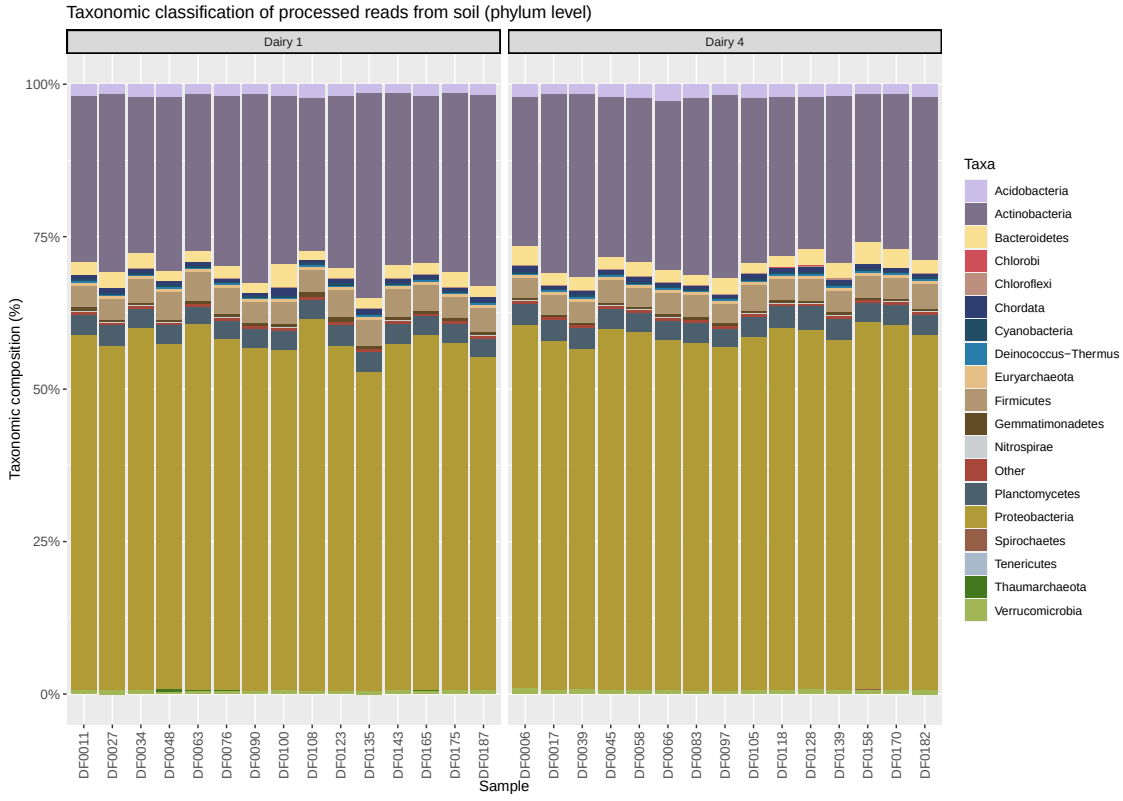
Taxonomic classification at the phylum level of processed reads from faeces, FDE, soil and milk



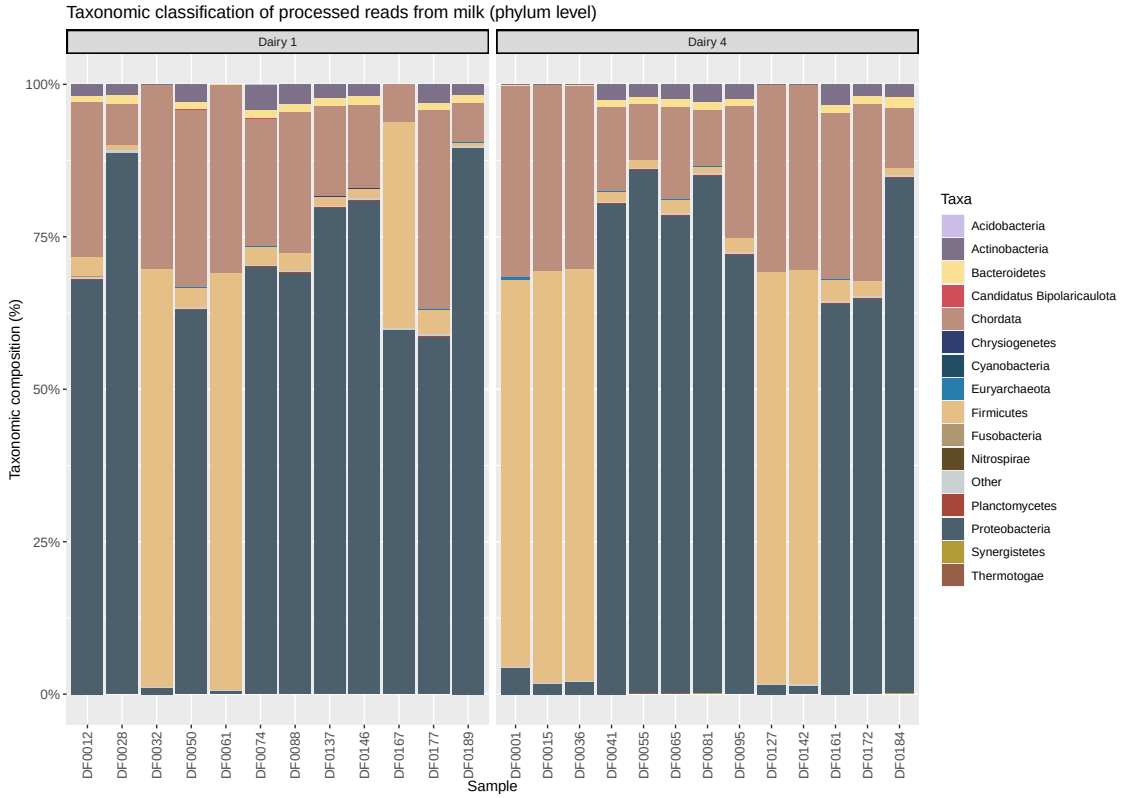
Taxonomic classification at the phylum level of processed reads from faeces.



Taxonomic classification at the phylum level of processed reads from farm dairy effluent (FDE)



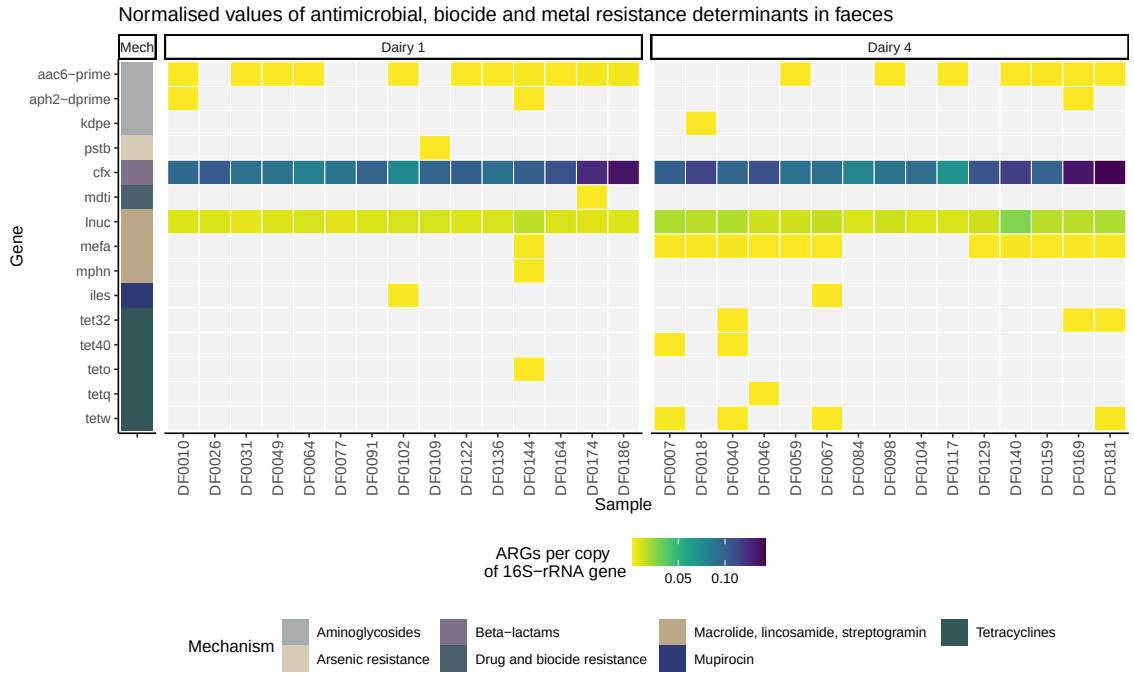
Taxonomic classification at the phylum level of processed reads from soil.



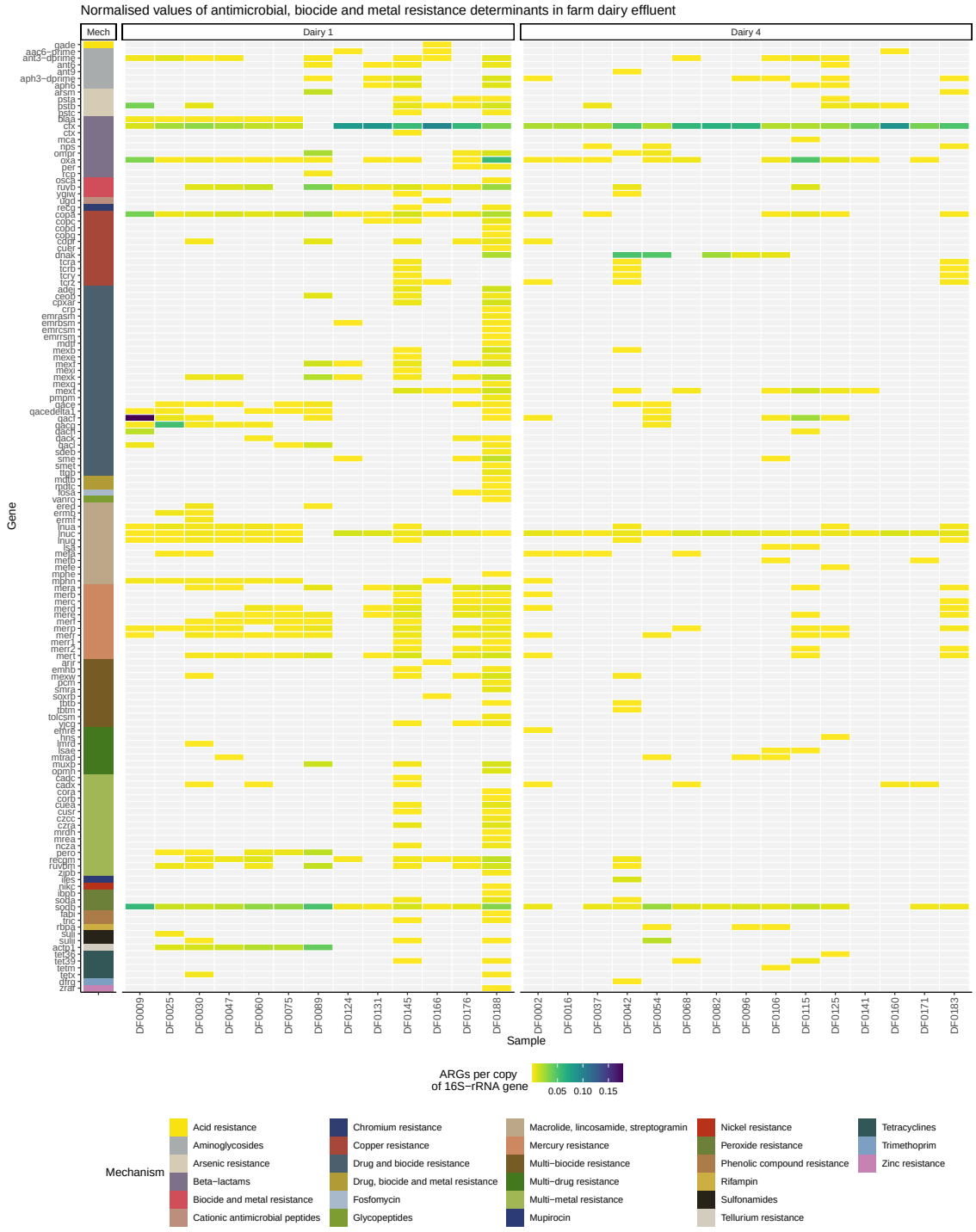
Taxonomic classification at the phylum level of processed reads from milk.

Appendix U

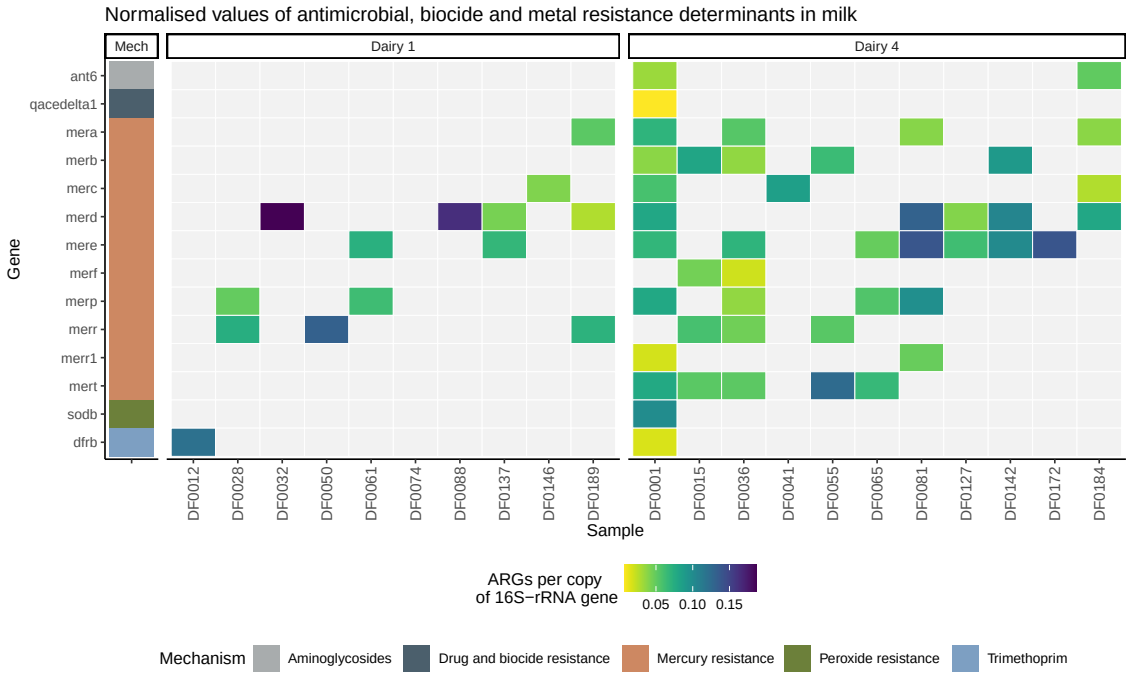
Heat map showing normalised values of antimicrobial drug, biocide and metal resistance determinants at the gene level



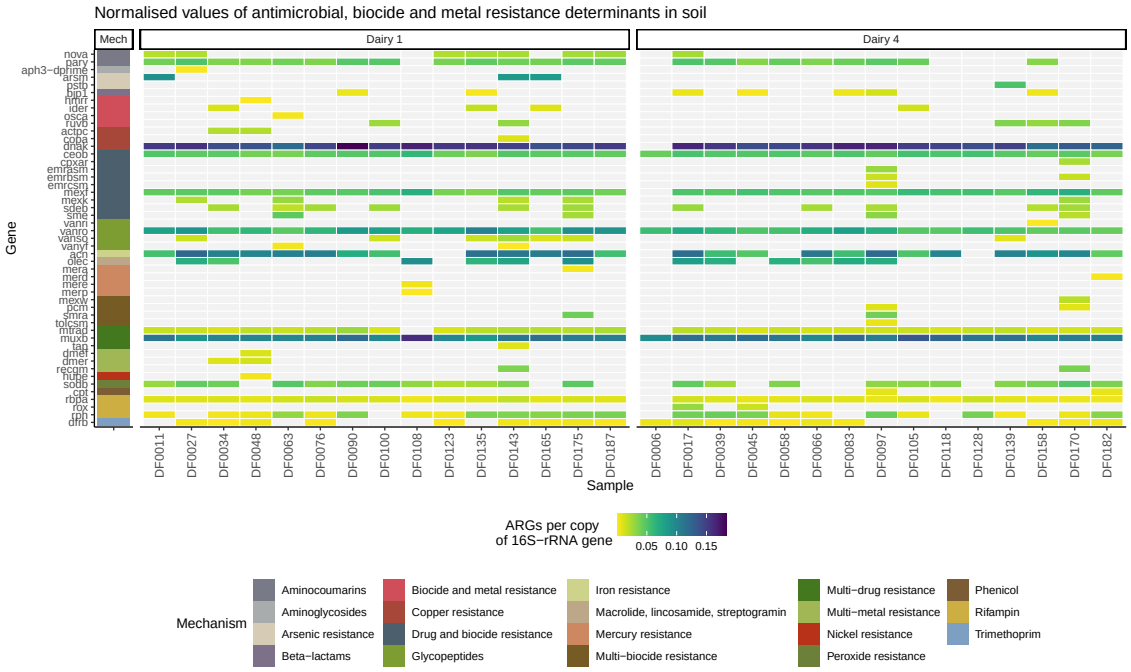
Heat map showing normalised values of antimicrobial drug, biocide and heavy metal resistance determinants at the gene level identified in faeces samples on Dairy 1 and Dairy 4 from October 2018 and December 2019. ARG abundance was expressed as copy of ARG per copy of 16S rRNA gene and individual gene variants were combined by group. The class of ARGs is indicated by colour in the figure legend.



Heat map showing normalised values of antimicrobial drug, biocide and metal resistance determinants at the gene level identified in farm dairy effluent samples on Dairy 1 and Dairy 4 from October 2018 and December 2019. ARG abundance was expressed as copy of ARG per copy of 16S rRNA gene and individual gene variants were combined by group. The class of ARGs is indicated by colour in the figure legend.



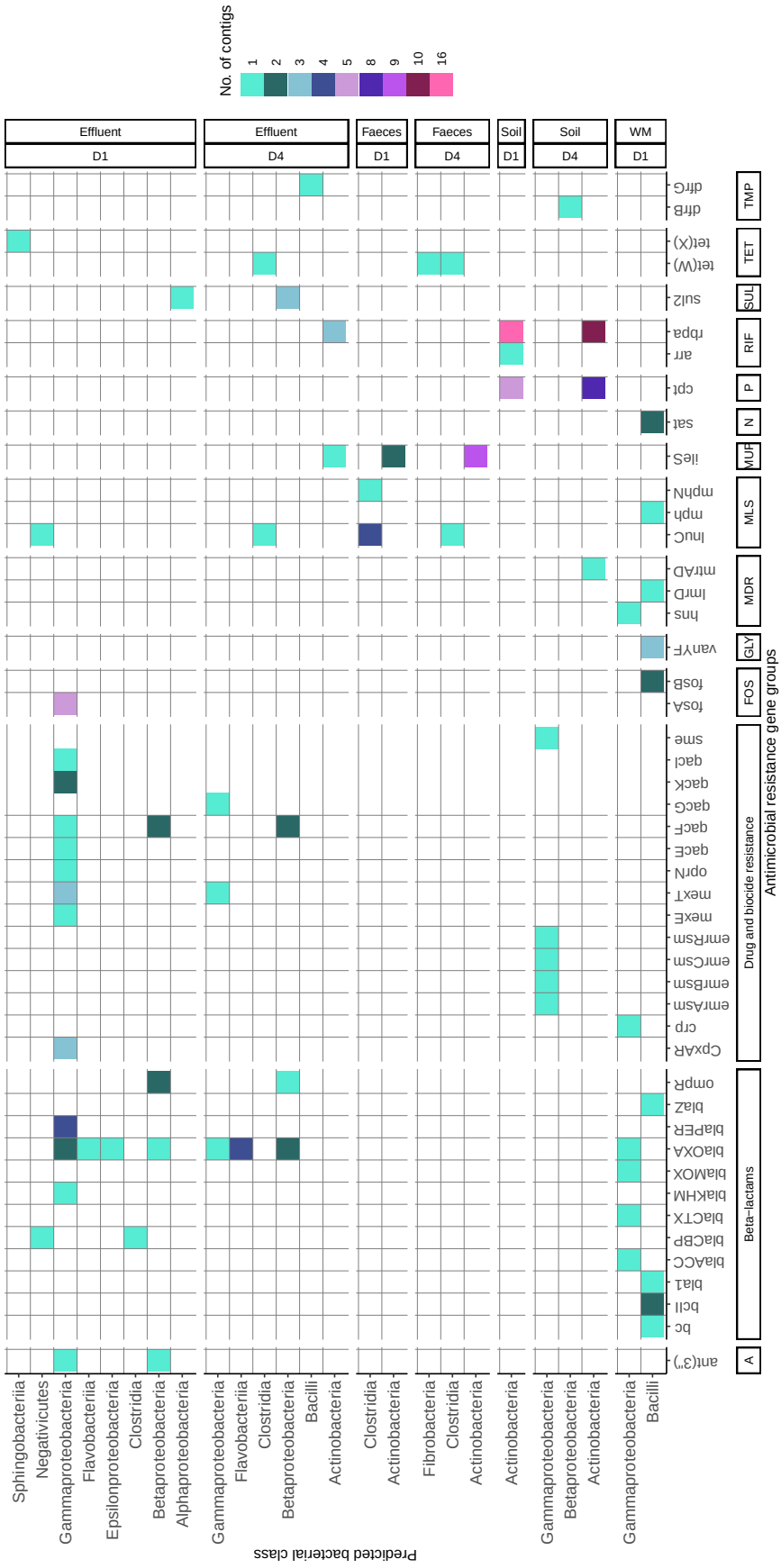
Heat map showing normalised values of antimicrobial drug, biocide and metal resistance determinants at the gene level identified in milk samples on Dairy 1 and Dairy 4 from October 2018 and December 2019. ARG abundance was expressed as copy of ARG per copy of 16S rRNA gene and individual gene variants were combined by group. The class of ARGs is indicated by colour in the figure legend. The waste milk sample (DF0167) was excluded from this analysis.



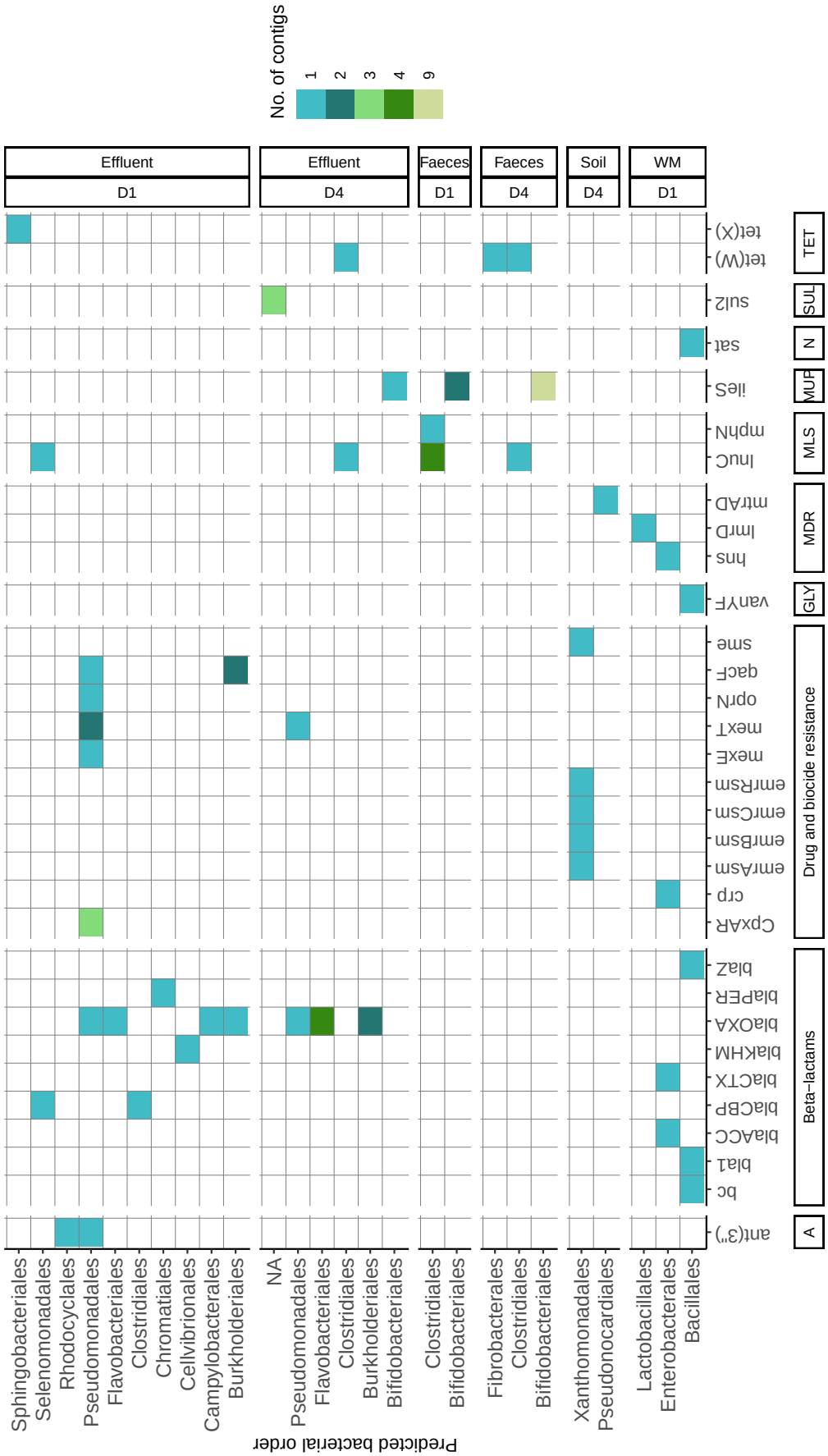
Heat map showing normalised values of antimicrobial drug, biocide and metal resistance determinants at the gene level identified in soil samples on Dairy 1 and Dairy 4 from October 2018 and December 2019. ARG abundance was expressed as copy of ARG per copy of 16S rRNA gene and individual gene variants were combined by group. The class of ARGs is indicated by colour in the figure legend.

Appendix V

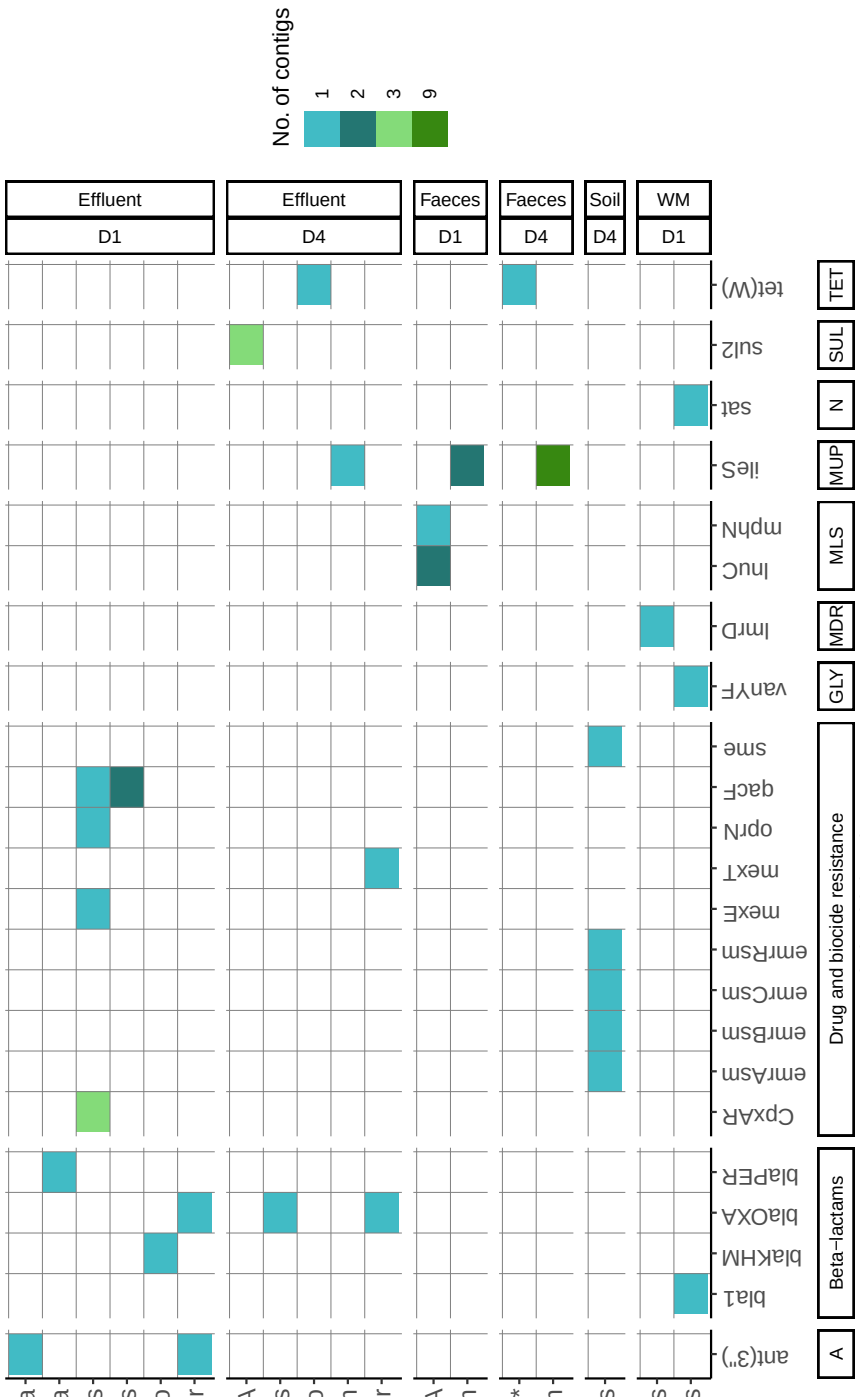
The predicted taxonomic ranks of contigs harbouring acquired antimicrobial resistance genes



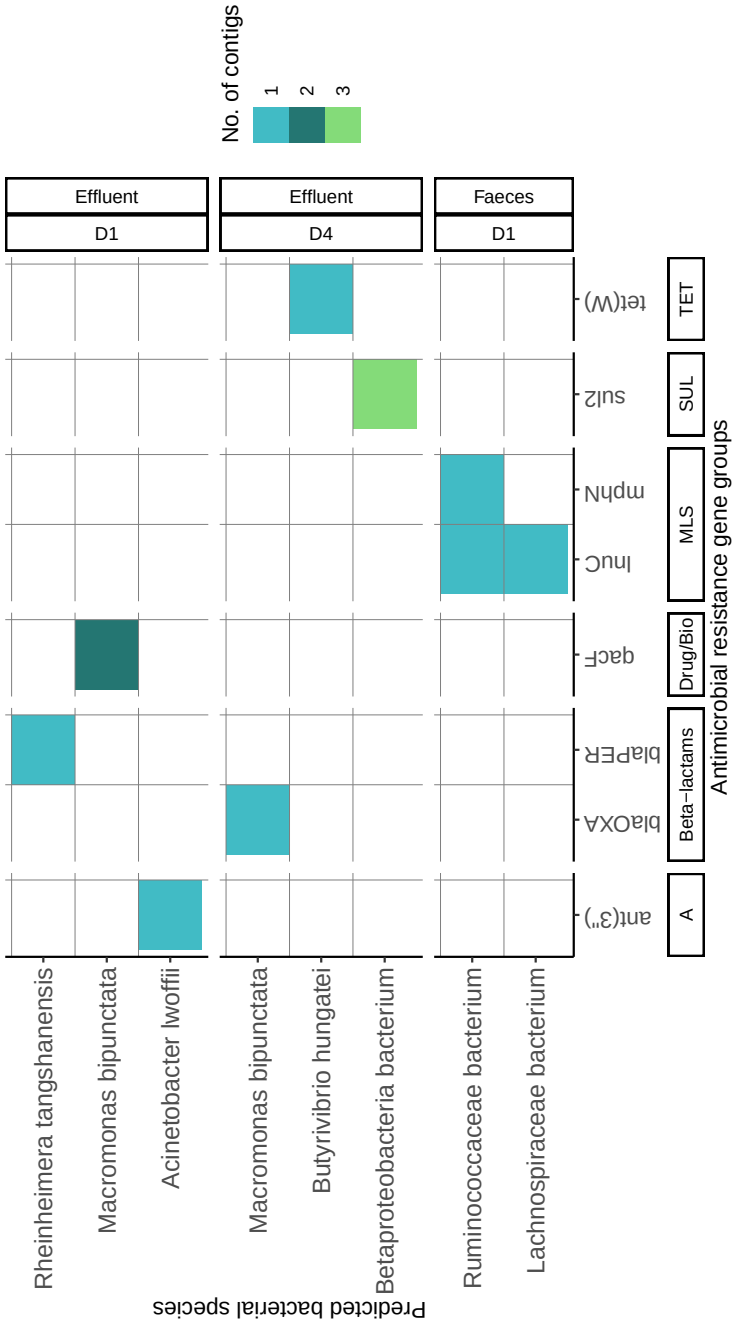
The predicted bacterial class of 140 contigs harbouring acquired antimicrobial resistance genes (ARGs). ARG gene groups were grouped per class of antibiotics and the contigs grouped by sample type and farm. The number of contigs with the unique class and ARG combination are indicated by the colour key on the Figure legend. A, Aminoglycoside; FOS, Fosfomycin; GLY, Glycopeptides; MDR, Multi-drug resistance; MLS, Macrolide, lincosamide and streptogramin; MUP, Mupirocin; N, Nucleosides; P, Phenicol; RIF, Rifampin; SUL, Sulfonamides; TET, Tetracycline; TMP, Trimethoprim; WM, Waste milk.



The predicted bacterial order of 67 contigs harbouring acquired antimicrobial resistance genes (ARGs). ARG gene groups were grouped per class of antibiotics and the contigs grouped by sample type and farm. The number of contigs with the unique order and ARG combination are indicated by the colour key on the Figure legend. A, Aminoglycoside; GLY, Glycopeptides; MDR, Multi-drug resistance; MLS, Macrolide, lincosamide and streptogramin; MUP, Mupirocin; N, Nucleosides; SUL, Sulfonamides; TET, Tetracycline; WM, Waste milk. NA, Bacterial family not identified by contig classified as *Betaproteobacteria* bacterium.



The predicted bacterial genus of 42 contigs harbouring acquired antimicrobial resistance genes (ARGs). ARG gene groups were grouped per class of antibiotics and the contigs grouped by sample type and farm. The number of contigs with the unique genus and ARG combination are indicated by the colour key on the Figure legend. A, Aminoglycoside; GLY, Glycopeptides; MDR, Multi-drug resistance; MLS, Macrolide, lincosamide and streptogramin; MUP, Mupirocin; N, Nucleosides; SUL, Sulfonamides; TET, Tetracycline; WM, Waste milk. The *Fibrobacteraceae* family is marked with an asterisk as no other sequences from the Order *Fibrobacterales* are present in the database. NA, Bacterial family not identified by contig classified as *Betaproteobacteria* bacterium.



The predicted bacterial species of 12 contigs harbouring acquired antimicrobial resistance genes (ARGs). ARG gene groups were grouped per class of antibiotics and the contigs grouped by sample type and farm. The number of contigs with the unique species and ARG combination are indicated by the colour key on the Figure legend. A, Aminoglycoside; Drug/Bio, Drug and Biocide resistance; MLS, Macrolide, lincosamide and streptogramin; SUL, Sulfonamides; TET, Tetracycline; WM, Waste milk.

Appendix W

Supplementary feed on Dairy 1 and Dairy 4 during the study period

Collection date	**Dairy 1**	**Dairy 4[a]**
October 2018	No extra feed	Dairy pellets, baleage, maize silage
November 2018	No extra feed	Maize silage, baleage, DDG, dairy pellets
December 2018	No extra feed	Maize silage, peas
January 2019	Crop (turnips)	Maize silage, dairy pellets
February 2019	Baleage, crop (turnips)	Grass silage
March 2019	Grass silage	Grass and maize silage
April 2019	Grass and maize silage, DDG, tapioca, lucerne, hay	Grass and maize silage, straw, soya hull, baleage
May 2019	Maize silage, DDG, tapioca, lucerne, hay	Maize silage
June 2019	Maize silage, kale	Straw and baleage
July 2019	Maize silage, kale	Maize silage
August 2019	Maize silage	DDG, soya meal, lucerne, maize silage
September 2019	Maize silage	Hay, DDG, lucerne (*ad libitum*), baleage (*ad libitum*)
October 2019	No extra feed	Maize silage, soya, DDG, molasses
November 2019	No extra feed	Maize silage
December 2019	No extra feed	Maize silage

[a] DDG, Distillers dried grains.

Appendix X

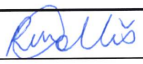
DRC16 form

DRC 16

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Rose Moyra Collis
Name/title of Primary Supervisor:	Prof. Patrick Biggs
Name of Research Output and full reference:	
s, P. J., Midwinter, A. C., French, N. P., Toombs-Ruane, L., & Cookson, A. L. (2019). Extended-spectrum beta-lactamase-producing Enterobacteriaceae in dairy farm environments: A New Zealand perspective. Foodborne Pathogens and Disease, 16(1),	
In which Chapter is the Manuscript /Published work:	Chapter 2 (Lit. review)
Please indicate:	
• The percentage of the manuscript/Published Work that was contributed by the candidate:	80
and	
• Describe the contribution that the candidate has made to the Manuscript/Published Work:	
This paper was the work of the candidate who researched the literature and wrote the review paper with the guidance and editing by the co-authors.	
For manuscripts intended for publication please indicate target journal:	
Candidate's Signature:	
Date:	28/01/2022
Primary Supervisor's Signature:	Prof Patrick Biggs Digitally signed by Prof Patrick Biggs DN: cn=Prof Patrick Biggs, c=NZ, o=Massey University, ou=School of Veterinary Science, email=p.biggs@massey.ac.nz Date: 2022.01.31 10:25:47 +13'00'
Date:	31-Jan-22

(This form should appear at the end of each thesis chapter/section/appendix submitted as a manuscript/ publication or collected as an appendix at the end of the thesis)

GRS Version 4– January 2019