FAECAL MICROBIOME AND THE ASSOCIATED ANTIMICROBIAL RESISTANCE GENES IN AUSTRALIAN COMPANION AND FOOD ANIMALS

by

Yuhong Liu

ORCID ID: https://orcid.org/0000-0003-0166-7467

This thesis is submitted in total fulfilment of the requirement of the

Doctor of Philosophy

February 2020

Faculty of Veterinary and Agricultural Sciences

The University of Melbourne

Abstract

Antimicrobial resistance (AMR) has become a global issue for human and animal health and welfare. It narrows down the choice of antimicrobials to treat infections and increases the costs of treatment. Effective antimicrobial stewardship is critical to ensure antimicrobials are used appropriately. Monitoring the antimicrobial resistance genes (ARGs) in bacteria is one of the important parts of antimicrobial stewardship.

Bacteria in animal faeces harbor various ARGs. The traditional culture-based approaches to detect ARGs in the faecal bacteria are limited to the culturable bacteria, which only represent a small fraction of the faecal microbiome. The culture-independent approaches, such as high-throughput qPCR (HT-qPCR) and the next generation sequencing, bypass the limitation of culture and allow us to characterise the ARG profiles of the total bacterial community. These approaches have been widely used to detect and quantify ARGs in the faecal microbiomes of animals in various countries, but few of such studies have been done in Australia.

In this project, faecal samples were collected from foals, calves, chickens and pigs, raised in commercial farms. The foals and chickens had no history of antimicrobial treatment. Calves were expected to have low chance being exposed to antimicrobials and only the pigs might be medicated. ARGs of the major antimicrobial classes, mobile genetic elements (MGEs) and metal resistance genes (MRGs) in the faecal samples were detected and quantified using a HT-qPCR array. The faecal microbiomes were determined by amplifying the V3-V4 region of the bacterial 16S rRNA gene and sequencing the amplicons on an Illumina platform. In addition, environmental swabs were collected from the chicken and pig farms and the microbiome and ARGs profiles of the swab samples were analysed to determine whether the environmental sampling could be used to study ARGs in animals raised intensively on a large scale.

Overall, tetracycline resistance genes (*tet*) encoding ribosomal protection proteins and macrolide resistance genes (*mef* and *erm*) were the dominant ARGs in the faecal samples regardless of the animal species. The high level of these genes could be explained by the predominance of the potential bacterial hosts, such as *Bacteroides* spp., in the faecal microbiomes. The ubiquitous presence of the *tet* and *mef*/*erm* genes

in animals suggested these genes are persistent and are not necessarily associated with the antimicrobial usage. On the other hand, the faecal samples had very low level of extended spectrum beta lactamase genes, bla_{CMY} and bla_{CTX-M}, carbapenems resistance gene *cph*A, fluoroquinolones resistance gene *qnr*B and virginiamycin resistance gene *vat*E, which suggested animals with low antimicrobial exposure had low risk of carrying ARGs that confer resistance to antimicrobials of high clinical concern.

Manure belt swabs collected in the cage chicken sheds were appropriate to study ARGs associated with caged poultry. However, the floor swabs in the chicken and pig farms did not reflect the ARG profiles of the animal faeces due to insufficient biomass for DNA extraction or great changes in the microbiome composition compared to the faeces.

In conclusion, this project showed that HT-PCR is an efficient method to screen a range of ARGs in complex samples. Analysis of ARG profile of animals without antimicrobial exposure set the baseline of presence and abundance of ARGs in animal faeces. Diverse ARGs detected in these animals suggests the persistence of ARGs in faecal microbiome. The results are useful for future studies investigating whether the current antimicrobial use practices can select and enrich pre-existing ARGs in farm animals.

Declaration

This is to declare that:

1. The thesis comprises only my original work towards the PhD degree except where indicated in the preface.
2. Due acknowledgement has been made in the text to all other material used.
3. The thesis is fewer than the 100,000 words in length, exclusive of tables, maps, bibliographies and appendices.

Signed

Yuhong Liu

5/2/2020

Preface

All the works performed in this thesis were performed by Yuhong Liu, except for the following:

1) Chapter 3: Foal faecal samples were collected by Dr. Kirsten Bailey, Department of Veterinary Biosciences, Faculty of Veterinary and Agricultural Sciences, The University of Melbourne.

2) Chapter 4: Calf faecal samples were collected by Mr. Monoar Pallab and Professor Peter Mansell, Department of Veterinary Biosciences, Faculty of Veterinary and Agricultural Sciences, The University of Melbourne.

3) Chapter 5 and 6: Floor and manure belt swabs in the layer and broiler chicken farms were collected by Dr. Helen Crabb, Department of Veterinary Biosciences, Faculty of Veterinary and Agricultural Sciences, The University of Melbourne.

All the works were performed under the supervision of A/Prof. Helen Billman-Jacobe, A/Prof. Michael Dyall-Smith and Dr. Marc Marenda. The project was funded by the National Health and Medical Research Council through the Centres of Research Excellence Programme, Grant no. 1079625. The PhD candidate received a Postgraduate Research Scholarship from The University of Melbourne.

Works towards the thesis that have been submitted for publication are listed below:

1) Chapter 3 was submitted for publication to Equine Veterinary Journal on 3/1/2020 and is under review.

Liu, Y., Bailey, KE., Dyall-Smith, M., Marenda, MM., Hardefeldt, LY., Browning, GF., Gilkerson, JR., & Billman-Jacobe, H. Faecal microbiota and antimicrobial resistance gene profiles of healthy foals.

Y. Liu, laboratory work, data analysis and interpretation, and manuscript preparation; K. E. Bailey, sample collection, data interpretation and manuscript review. M. Dyall-Smith, M. S. Marenda, L. Y. Hardefeldt and G. F. Browning, study design, data interpretation, manuscript review; J. R. Gilkerson, sampling study design, manuscript

review; H. Billman-Jacobe, study design, project supervision, data interpretation and manuscript review.

2) Chapter 5 and 6 were combined as one paper which was submitted for publication to Antibiotics on 30/1/2020 and published on 13/3/2020.

Liu, Y., Dyall-Smith, M., Marenda, M., Hu, H.W., Browning, G. and Billman-Jacobe, H. (2020) Antibiotic resistance genes in antibiotic-free chicken farms. *Antibiotics* 9, 120. Study design, experiments, data analysis and manuscript preparation were completed by Liu, Y. Hu, H-W. assisted with some HT-qPCR experiments. Billman-Jacobe, H. supervised the study and all authors assisted with manuscript review.

3) Other publications towards the thesis:

Dyall-Smith, M. L., Liu, Y. & Billman-Jacobe, H. 2017. Genome sequence of an Australian monophasic *Salmonella enterica* subsp. *enterica* Typhimurium Isolate (TW-Stm6) carrying a large plasmid with multiple antimicrobial resistance genes. *Genome Announc.*, 5, e00793-17.

Billman-Jacobe, H., Liu, Y., Haites, R., Weaver, T., Robinson, L., Marenda, M. & Dyall-Smith, M. 2018. pSTM6-275, a conjugative IncHI2 plasmid of *Salmonella* that confers antibiotic and heavy metal resistance under changing physiological conditions. *Antimicrob. Agents Chemother*., 62:e02357-17.

Billman-Jacobe, H., Dyall-Smith, M., Hawkey, J. & Liu, Y. 2019. Heavy metal and antibiotic resistance genes in bacteria from porcine monophasic *Salmonella* 1,4,[5],12:i:-. *Journal of Integrated OMICS*, 9, 37.

Acknowledgments

First of all, I would like to thank my principle supervisor Associate Professor Helen Billman-Jacobe for providing me this opportunity to do my PhD. I appreciate to your patience, valuable guidance and support from experimental design to the final writing. I am grateful to have this journey to learn how to do scientific research.

I would like to thank my supervisor Dr. Marc Marenda for teaching me how to do MinION sequencing and all your suggestions and inspirations for my research. I would like to thank my supervisor Associate Professor Michael Dyall-Smith for the meetings whenever you travelled to Melbourne. I appreciate to your valuable comments on my writing which helped me a lot improving my writing skill.

In addition, I would like to thank my committee members Professor Glenn Browning and Professor Frank Dunshea for your help and all the support to my work.

My gratitude goes to Dr. Kirsten Bailey, Mr. Monoar Pallab, Professor Peter Mansell and Dr. Helen Crabb for providing me valuable samples, which is one of the most important parts of my research. I would like to thank Dr. Hang-Wei Hu for your help with the HT-qPCR array. I also thank all the staff and students in the University animal house for your support for my sampling.

I would like to thank Professor Mark Stevenson, Dr. Simon Firestone, Dr. Andrew Woodward and students in the Epidemiology group for spending time helping me with the statistical analysis. I also thank Dr. Alistair Legione and Ms. Lina Martìnez Lopez for generously sharing the knowledge of bioinformatic analysis. Moreover, I would like to thank Kanishka, Fannana and all my wonderful colleagues in the Vet Micro group. I am grateful to your support and encouragement throughout my PhD life.

I would like to thank my parents for all your love and support throughout the years. You are always keen to know my life here, and I really enjoy telling you all the stories.

Finally, I would like to thank my husband, Tongda. I am lucky and grateful to have your companionship and support every single day.

Abbreviations

ASV	Amplicon sequence variant
ANCOM	Analysis of composition of microbiomes
ANOSIM	Analysis of similarity
ANOVA	Analysis of variance
AMR	Antimicrobial resistance
ARG	Antimicrobial resistance gene
APVMA	Australian Pesticides and Veterinary Medicines Authority
BLAST	Basic Local Alignment Search Tool
˚C	Celsius
CARD	Comprehensive Antibiotic Resistance Database
Ct	Cycle threshold
dNTP	Deoxynucleotide triphosphate
DNA	Deoxyribonucleic acid
ESBL	Extended spectrum beta lactamase
HT-qPCR	High throughput quantitative polymerase chain reaction
HGT	Horizontal gene transfer
MRG	Metal resistance gene
m/s	Meter per second
µl	Microlitre
mM	Millimolar
MIC	Minimum inhibitory concentration
min	Minute
MGE	Mobile genetic element
MDR	Multidrug resistance
ng	Nanogram
nM	Nanomolar
NCBI	National Center for Biotechnology Information
NTC	No template control
NMDS	Nonmetric multidimensional scaling
ORF	Open reading frame
ONT	Oxford Nanopore Technologies
PERMANOVA	Permutational multivariate analysis of variance
qPCR	quantitative polymerase chain reaction
rRNA	Ribosomal ribonucleic acid
s	Second
SNPs	Single nucleotide polymorphisms
×g	Unit of gravity

Table of Contents

List of Tables

List of Figures

Chapter 1 Literature Review

1.1 Use of antimicrobials in animals

Antimicrobial agents are substances that kill or inhibit the growth of microorganisms (Prescott and Dowling, 2013). Antimicrobials include substances that are naturally produced by microorganisms, semisynthetic substances that are primarily produced naturally and then chemically modified (e.g. methicillin and amoxicillin), and synthetic substances that are completely synthesised by chemical approaches (e.g. quinolones). The majority of antimicrobial agents used in animals are in the same classes as those used in human medicine (Schwarz et al., 2001). There are four ways that antimicrobials are used in animals: therapy, metaphylaxis, prophylaxis and growth promotion (Schwarz et al., 2001). Therapeutic use is to treat diseased animals to control an existing bacterial infection. The animals may be treated individually, especially for companion animals, or as a group. For food producing animals raised on a large scale, treating individual animals is not always practicable. Metaphylaxis is where antimicrobials are dispensed to an entire group of animals when individual animals present signs of infection in order to avoid the group being affected. Prophylactic treatment is usually used in key production stages of food animals, such as weaning, mixing animals from different groups or at the end of the lactation period of dairy cows to prevent disease and improve animal welfare (Schwarz et al., 2001, McEwen and Fedorka-Cray, 2002). However, prophylactic treatment is criticised for creating the basis to select antimicrobial resistant bacteria (Schwarz et al., 2001). Some antimicrobials are used as growth promoters to increase feed conversion efficiency and enhance growth rates. In these cases, antimicrobials are usually used in low concentrations for an extended period of time, which provides the environment to select for antimicrobial resistance (AMR) in bacteria through selection of antimicrobial resistance genes (ARGs) (Roy Chowdhury et al., 2014). For example, the discovery of glycopeptide-resistant enterococci in food animals in Great Britain, Germany and Denmark (Bates et al., 1994, Klare et al., 1995, Bager et al., 1997) was associated with the use of avoparcin (a glycopeptide antibiotic) as a growth promoter. To prevent further selection of resistant bacteria in food animal production systems, from 1995 to 1999, the European Union member countries banned avoparcin and other growth

promoters belonging to antimicrobial classes used in human medicine (tylosin, spiramycin, bacitracin and virginiamycin) or that were cytotoxic (olaquindox and carbadox) (Dibner and Richards, 2005).

In Australia, antimicrobials are assigned an importance rating of 'high', 'medium' and 'low' based on the threat they pose to human health if their misuse generated AMR (Office of Health, 2018). Veterinary antimicrobial drugs are prescription-only medicines, and the use of antimicrobials by veterinarians is regulated by state legislation and label restraints (Barton, 2007). Antimicrobials of high importance used in food animal production in some countries include fluoroquinolones, extended-spectrum cephalosporins (third and fourth generation cephalosporins) and streptogramins (Jordan et al., 2009). Unlike other countries, use of fluoroquinolone and gentamicin is prohibited in food animals in Australia (JETACAR, 1999). Ceftiofur, a third-generation cephalosporin, is registered to treat respiratory infections in cattle and can be prescribed in pigs as 'off-label use' (Jordan et al., 2009). Virginiamycin is the only streptogramin registered for livestock in Australia. Antimicrobial drugs with medium importance to human health used in food animals include amoxycillin-clavulanate (beta-lactamase inhibitor combinations), first and second generation cephalosporins for mastitis treatment in dairy cattle; spectinomycin to treat gastrointestinal and respiratory infections in pigs and broiler chickens (Shaban et al., 2014). Veterinary antimicrobial drugs of low human importance include macrolides, tetracyclines, penicillins and florfenicol (Jordan et al., 2009, Shaban et al., 2014).

According to the most recent report by Australian Pesticides and Veterinary Medicines Authority (APVMA) in 2014 (APVMA, 2014), the total quantity of antimicrobial products sold (in tonnes of active constituents) for animal use from 2005 to 2010 was between 492 to 661.2 tonnes, with no significant difference in total quantity of sales between the years. Antimicrobials sold for administration to food animals comprised an average 98% of the total sales. In non-food animals, antimicrobials are sold for therapeutic purposes. In food animals, an average 43% of antimicrobials sold were for therapeutic purposes, 6% for growth promotion and 51% were coccidiostats for control of parasites. Antimicrobials used as coccidiostats are not in the same antimicrobial classes used in human medicines, thus are thought not contribute to

AMR risks in human health. Among the antimicrobials used for therapeutic purposes in food animals, approximately 30% of sales were contributed by bacitracin for treatment of necrotic enteritis caused by *Clostridium perfringens* in poultry. The next largest antimicrobial sales were macrolide-streptogramins and tetracycline, which contributed 24% and 23% of sales, respectively. The majority of macrolides were given to pigs and poultry through food or water, and the streptogramins are administered in feed for reducing the risk of acidosis in cattle and sheep and preventing necrotic enteritis in broiler chickens. Tetracyclines are administered to various species through different routes. Most tetracycline products are administered in feed and used in pigs, poultry and, to a limited extent, in calves. A limitation of the APVMA data is that the usage is reported according to which animal species the antimicrobial is registered for and does not account for off label use.

Moreover, in a survey of antimicrobial use in the Australian pig industry published in 2009 (Jordan et al., 2009), which covered approximately 51% of the commercial pig producers in Australia, producers reported the most used antimicrobials were penicillins (including amoxicillin and ampicillin), followed by tetracyclines, macrolide and sulfonamides. All these antimicrobials are classified as low importance to human health. For antimicrobials of high importance, about 25% of producers reported the use of ceftiofur and no producer reported use of virginiamycin.

1.2 Acquisition of AMR and horizontal gene transfer

Bacteria can have AMR through intrinsic and acquired mechanisms (Alekshun and Levy, 2007). Intrinsic resistance is attained by genes naturally present on the host chromosome, such as genes encoding AmpC β-lactamase in Gram-negative bacteria (Galán et al., 2013) and the AcrAB-tolC efflux pump (Li and Nikaido, 2004, Blair et al., 2015). The mechanisms of acquired resistance include the mutation of endogenous genes associated with AMR and the acquisition of ARGs on mobile genetic elements (MGEs) through horizontal gene transfer (HGT) (Wales and Davies, 2015, Alekshun and Levy, 2007). HGT may involve three mechanisms: conjugation, transformation or transduction (von Wintersdorff et al., 2016, Baquero et al., 2009, Levy and Marshall, 2004).

1.2.1 Conjugation

Conjugation is the process in which DNA is transferred from donor to recipient cells via direct cell contact through cell surface pili or adhesins (von Wintersdorff et al., 2016). This machinery is encoded by autonomously replicative plasmids or chromosome-borne integrative conjugative elements, such as conjugative transposons (Wozniak and Waldor, 2010). Those MGEs are often associated with ARGs and some MGEs can be exchanged between bacteria belonging to different genera, thus contributing to the wide spread of ARGs. Some MGEs possess a large locus encoding their entire transfer machinery, while others, for example, the IncQ-like plasmids, are small in size and can be mobilised by other self-transmissible plasmids. The IncQ-like plasmids have been found in a broad range of host species and from different environments (Rawlings and Tietze, 2001). The initial version of IncQ plasmids carried sulfonamide (*sul*2) and streptomycin resistance genes (*str*A and *str*B) (Scholz et al., 1989) while later examples were found to carry a variety of ARGs including $bla_{\text{GES-1}}$, bla_{OXA}, $bla_{\text{GES-5}}$ and *qnr*S2 (Loftie-Eaton and Rawlings, 2012). Another well-studied example is the Tn916 family of transposons, which have been found in both Gram-positive and Gram-negative bacteria (Salyers et al., 1995, Roberts and Mullany, 2009). Tn916-like transposons often carry the tetracycline resistance gene *tet*M and some also carry macrolide resistance genes, such as *erm*B (Roberts and Mullany, 2011).

1.2.2 Transformation

Transformation is the process in which bacteria take up and incorporate extracellular DNA from the environment (Barlow, 2009). Transformation requires several conditions and steps: the dispersed extracellular DNA, recipient cells that are in a competent state to take up DNA, and the ability of the transferred DNA to integrate into the host genome or replicate as plasmids (Thomas and Nielsen, 2005). Some bacteria become competent at a specific stage in growth, such as streptococci; and some require a specific sequence within the DNA to enable uptake, such as *Neisseria* (Van Hoek et al., 2011). Natural transformation has been observed in variety of bacterial genera, including *Acinetobacter*, *Haemophilus*, *Neisseria*, *Pseudomonas*, *Staphylococcus*, *Streptococcus* and *Campylobacter* (Lerminiaux and Cameron, 2019, Bae et al., 2014). While *Escherichia coli* is well known to take up DNA naturally via conjugation (previous

section) and transduction (next section), it is generally not naturally transformable, however, some factors, such as Ca^{2+} and Mg^{2+} ions that accumulate in the environment, might induce detectable competence in *E. coli* (Hasegawa et al., 2018).

Several *in vitro* studies have clearly demonstrated the role of transformation in the movement of ARGs between bacteria (Bowler et al., 1994, Ferrándiz et al., 2000, Hamilton and Dillard, 2006). In environmental samples, Mao et al. extracted extracellular and intracellular (microbial) DNA from water and sediment samples, and found that ARGs were more frequently detected in extracellular DNA than intracellular DNA (Mao et al., 2013). Furthermore, Naquin et al. demonstrated that the methicillin resistant gene, *mec*A, in the free DNA from sewage samples could be transformed to sensitive *Staphylococcus aureus* strains and confer the corresponding resistance (Naquin et al., 2015). These results suggested that free DNA in nature provides a gene pool for bacteria acquiring ARGs via transformation. Moreover, Domingues et al. found natural transformation would facilitate the transfer of transposons, integrons and gene cassettes between different bacterial species (Domingues et al., 2012). Keen et al. showed a subset of natural lytic phage population could release intact, transformable plasmid DNA which could assist the spreading of carried ARGs (Keen et al., 2017).

Additionally, it has been found that antimicrobials can induce competence in bacteria as stress responses under laboratory conditions (Charpentier et al., 2012). For example, Prudhomme et al. demonstrated the treatment of streptococcus cultures with streptomycin and norfloxacin induced the expression of *com* regulon, which facilitates the competent cell to take up extracellular DNA and integrate it into the host genome (Prudhomme et al., 2006). Similarly, a recent study by Mantilla-Calderon et al. showed the use of bromoacetic acid in *Acinetobacter baylyi* induced the expression of *rec*A gene, which was involved in DNA repair and integration during transformation (Mantilla-Calderon et al., 2019). However, it is challenging to detect the uptake of ARGs via transformation in natural bacterial populations. Nordgard et al. attempted to determine the natural transformation of ARGs in the aerobic microbiota of the rat gastrointestinal, but no transformants could be identified among more than 400 tested isolates (Nordgård et al., 2012).

1.2.3 Transduction

Gene transfer from one bacterial cell to another via bacteriophages is known as transduction (Perry and Wright, 2013). Bacteriophages can transfer genes by either specialised or generalised transduction. Specialised transduction only transfers a few specific genes from donor bacterial cell to a recipient cell, whereas, generalised transduction can mobilise any portion of the donor genome to the recipient cell (Balcazar, 2014). This includes normal chromosomal genes, chromosomally embedded MGEs such as transposons and genomic islands, and fragments of extrachromosomal elements, such as plasmids (Brown-Jaque et al., 2015).

Transfer of ARGs via transduction has been reported in a range of bacterial species, such as *Streptococcus*, *Escherichia*, *Salmonella* and methicillin-resistant *S. aureus* (von Wintersdorff et al., 2016). Recent studies investigating DNA from complex natural samples with metagenomic approaches indicate the important role of bacteriophages in carrying and spreading ARGs. Using quantitative PCR (qPCR), Ross and Topp found bacteriophages in agricultural soil harboured *str*A, *str*B, *aad*A and *sul*1 genes (Ross and Topp, 2015). They also reported that purified bacteriophage could confer AMR to soil bacteria in the presence of antimicrobials, providing evidence that selective pressure aids ARGs transfer by transduction. Similarly, Colomer-Lluch et al. found that the copy number of quinolone resistance genes *qnrA* and *qnrS* increased in the phage DNA fraction of water samples treated with EDTA or sodium citrate (Colomer-Lluch et al., 2014). In addition, bacteriophage-related ARGs have been also detected in faecal specimens from food animals and humans (Colomer-Lluch et al., 2011, Wang et al., 2018, Quirós et al., 2014, Fernández-Orth et al., 2019, Gómez-Gómez et al., 2019). Studies showed phages carrying ARGs isolated from chicken meat were infective (Gómez-Gómez et al., 2019) and could transfer resistance to *E. coli* (Shousha et al., 2015). These results suggested the role of phages in spreading ARGs to bacteria. Virome studies of murine and human faeces have demonstrated that antimicrobial treatment led enrichment of ARGs in the faecal phageome (Modi et al., 2013, Fernández-Orth et al., 2019). However, Enault et al. pointed out the lower threshold used to identify ARGs in the viromes based on amino-acid sequence similarity could

overestimate the ARG abundance in some studies, and much fewer ARGs were detected when a conservative threshold was applied (Enault et al., 2017).

1.2.4 Concern of MGEs with multiple ARGs

The natural clustering of ARGs on a single MGE is a particular concern. This allows multidrug resistance (MDR) to transfer in one event, and assists in maintaining the resistance phenotype in bacteria even if the use of specific antimicrobials is ceased (Barlow, 2009). ARGs clustered on a single plasmid have been reported in a range of bacteria (Guerra et al., 2001, Billman-Jacobe et al., 2018, Jiang et al., 2010, Palmer et al., 2010), and the spreading of the MDR plasmids is expected to continue. For example, a current issue of high clinical concern is the carriage by *Klebsiella pneumoniae* ST258 strains of plasmids with multiple ARGs, especially genes specifying carbapenem resistance (DeLeo et al., 2014). However, even in the absence of any direct antimicrobial selection to maintain the presence of ARGs carrying plasmids in bacterial cells, many plasmids also carry toxin-antitoxin gene systems that function independently to prevent plasmid loss (Carattoli, 2013).

Another important type of MGE that can carry multiple ARGs are integrons, which are characterised by an integrase gene (*int*I), a recombination site (*att*I) and a promoter (P_c) (Partridge et al., 2018). Exogenous genes are captured and inserted into gene cassettes by a combination of the integrase (encoded by *int*I) and *att*I sites, and expressed under the control of P_c (Gillings et al., 2015). These gene cassettes can contain different ARGs and multiple cassettes can insert into the same integron, which then confers resistance to multiple antimicrobials (Partridge et al., 2009, Partridge et al., 2018). Integrons can transfer between bacterial strains of the same or different species with the assistance of transposons (Cambray et al., 2010). Integrons have been grouped into different classes based on the homology of *int*I (Gillings et al., 2015). Class 1 integrons were the first discovered, and so far, have been found in both pathogenic and commensal bacteria, and with many diverse ARGs (Goldstein et al., 2001, Yang et al., 2010, Byrne-Bailey et al., 2011, Sultan et al., 2018). Frequently detected ARGs in these gene cassettes include *aad*A, encoding streptomycin-spectinomycin resistance, and the trimethoprim resistance determinants (Sultan et al.,

2018), and they are often embedded in plasmids and transposons, which have contributed to the extensive dispersal of this integron in the environment (Fluit and Schmitz, 1999). For example, the *int*I1 gene has been frequently detected in metagenomic DNA of samples from both natural and human-involved environments, such as sediment, livestock manure and the impacted wastewater and soil (Hardwick et al., 2008, Hsu et al., 2014, Binh et al., 2009).

1.3 The gut microbiome and the influence of antimicrobial usage

1.3.1 The general gut microbiome ecosystem

The gastrointestinal tracts of humans and animals harbour highly complex bacterial communities. Based on 16S rRNA gene diversity, it has been estimated that over 7000 different strains comprising of approximately 800 different bacterial species live in the human gastrointestinal tract (Bäckhed et al., 2005). The majority of bacteria inhabiting the gastrointestinal tract belong to the phyla *Firmicutes* and *Bacteroidetes*. Other common phyla include *Proteobacteria*, *Actinobacteria* and *Verrucomicrobia* but they generally comprise small proportions of the bacterial community (Lozupone et al., 2012). Nevertheless, large variations in the relative proportion of each bacterial phylum have been observed between individuals. Arumugan et al. proposed the concept of enterotype to catalogue the variation of gut microbiome composition. The enterotype is based on 16S rRNA sequence analysis of the gut microbiome, which clusters the samples into bins based on the taxonomic similarity for intra- and inter-individual microbiome comparisons (Arumugam et al., 2011). Three enterotypes were identified in the human gut microbiome using the genera *Bacteroides*, *Prevotella* and *Ruminococcus* as indicators. The enterotype may be related to host life-history factors such as genetics, age, diet and environment, and may be associated with complex diseases (Ding and Schloss, 2014). In addition to humans, the concept has been applied to different animal species, including chimpanzees, mice and horses (Moeller et al., 2012, Hildebrand et al., 2013, Pesesky et al., 2016, Mach et al., 2017).

The initial gut microbiome of human infants and new born animals has relatively low richness and diversity which gradually increases with age (Arrieta et al., 2014, Costa et al., 2016, Oikonomou et al., 2013). Facultative anaerobes, such as *Enterobacteriaceae*,

are considered pioneer colonisers in the intestinal tract as they exhaust oxygen in the environment and enable colonisation by obligate anaerobic bacteria, such as *Bacteroides* spp. (Favier et al., 2002, Arrieta et al., 2014). The infant gut is colonised with bacteria from the mother and the external environment immediately after birth (Dominguez-Bello et al., 2011), then the maturation of microbiome is affected by factors such as diet and antimicrobial exposure (Arrieta et al., 2014).

The gut microbiome is critical to the health and physiological development of humans and animals, and microbial dysbiosis has been found associated with diseases in both groups. For example, studies in humans and mice suggest that changes in the abundance of *Firmicutes* and *Bacteroidetes* are associated with inflammatory bowel disease (Frank et al., 2007, Jeffery et al., 2012, Alam et al., 2020), autoimmune disease (De Luca and Shoenfeld, 2019), obesity and energy harvest (Turnbaugh et al., 2006, Ley et al., 2005, Koliada et al., 2017). In horses, a reduction in the proportion of *Firmicutes* was associated with colitis and diarrhoea (Costa et al., 2012, Rodriguez et al., 2015), while a reduction in the proportion of members of the genus *Fibrobacter* was associated with equine metabolic syndrome (Elzinga et al., 2016). In young calves, the proportion of *Faecalibacterium prausnitzii* was found associated with body weight gain and the incidence of diarrhoea (Oikonomou et al., 2013).

1.3.2 The impact of antimicrobial exposure on the gut microbiome

Antimicrobial usage is an important factor in disturbance of the gut microbiome. Antimicrobial treatment changes the proportion of bacterial species in the gut community by eradicating sensitive strains and allowing overgrowth and establishment of resistant strains (Sommer and Dantas, 2011). In some cases, antimicrobials can induce serious gut microbiome imbalance and lead to infections (Khanna and Pardi, 2016). Indeed, members of a microbiome form a complex co-dependent network in which many bacteria are reliant on other species for nutrients or secondary metabolites (Belenguer et al., 2006, Belzer et al., 2017). Thus, in addition to the target bacteria, the use of antimicrobials may indirectly affect other bacterial members associated with the co-dependent network.

Generally, antimicrobial treatment in humans and animals causes a decrease in species richness and evenness of the gut microbiome (Costa et al., 2015, Dethlefsen and Relman, 2011, Ghanbari et al., 2019, Janczyk et al., 2007), but exceptions have also been observed (Bessegatto et al., 2017). The gut microbiome responds to different antimicrobials in different ways. The major factors that determine the impact of antimicrobials on gut flora include the spectrum of the antimicrobial agents, the administration route, the dosage and duration of treatment and the pharmacokinetic and pharmacodynamic properties of the antimicrobial compound (Jernberg et al., 2010). Interestingly, studies in mice and pigs demonstrated transient increases of *Proteobacteria*, specifically *E. coli,* in the gut microbiome after administration of different antimicrobials through feed (including amoxicillin, tetracycline, sulfamethazine, penicillin) (Antonopoulos et al., 2009, Looft et al., 2012, Ghanbari et al., 2019). Although it was not confirmed that the increased proportion of *Proteobacteria* or *E. coli* was due to AMR in all cases, the common trends in response to various antimicrobial agents was intriguing given the concern for spread of AMR in proteobacterial pathogens, such as extended spectrum beta lactamase (ESBL)-producing species of *Enterobacteriaceae* (Nakai et al., 2016).

The gut bacterial community is resilient, in that the majority of microbiota can return to their pre-antimicrobial treatment state, but the duration over which this occurs can vary from days to months (Dethlefsen et al., 2008, Jakobsson et al., 2010). In some cases, taxa can be removed from the community indefinitely. For example, Jernberg et al, examined the impact of a 7-day clindamycin treatment on the faecal microbiome of four healthy humans over two years. The clonal diversity of *Bacteroides* decreased dramatically and the *Bacteroides* community did not return to the pre-treatment state during the study period (Jernberg et al., 2007). Similarly, in a later study, Dethlefsen and Relman monitored the gut microbiome composition of three individuals over 10 months during which they received two 5-day ciprofloxacin treatments with 6 months apart. Richness and diversity of their microbiome decreased as soon as 3 to 4 days after treatment. Although the disturbed microbiome began to recover to the initial state one week after treatment, taxa accounting for 25-50% of the community were eliminated. The microbiome of each subjects became stable by the end of the study,

but the composition was different from that prior to the antibiotic treatment (Dethlefsen and Relman, 2011).

In animals, Holman and Chenier assessed the effect of tylosin and chlortetracycline at subtherapeutic level on the pig gut microbiome over 19 weeks (Holman and Chénier, 2014). Researchers found both antibiotics did not affect the diversity and richness of the microbiome, however, pigs fed with tylosin had a shift in the proportion of specific phylum and genus, as well as 26 operational taxonomic units. Nevertheless, changes in the taxa proportion were not persistent, suggesting the microbiome was resilient to long-term changes of antibiotic usage (Holman and Chénier, 2014). Moreover, Looft et al. characterised the gut microbiome of pigs fed with carbodox for three weeks followed by six weeks withdrawal period (Looft et al., 2014). In the first four days of treatment, the medicated pigs had significant changes in the proportion of a few taxa with a notable increase in the proportion of *Prevotella* spp*.* The microbiome diversity and richness of medicated pig was significantly lower than the non-medicated pigs in the first week of antibiotic exposure, but the difference was no longer observed thereafter. Results of both studies showed the resilience of pig gut microbiome towards antibiotic perturbations, but the responses differed depends on antibiotics, duration and dosage (Looft et al., 2014).

The disrupted gut microbiome could be brought into normality by introducing gut flora from healthy donors. The use of broad-spectrum antimicrobials, such as the third generation cephalosporins, was found associated with *Clostridium difficile* infection diarrhoea which could greatly disrupt the balance of commensal gut microbiota (Khoruts et al., 2010, Poutanen and Simor, 2004). Previous studies have demonstrated that patients with *C. difficile* infection, who received faecal transplantation from healthy donors, had their gut microbiome reset from a low-diversity state to one resembling that of the donors, which was dominated by *Bacteroidetes* and butyrate-producing bacteria (Fuentes et al., 2014, Khoruts et al., 2010).

In addition, antimicrobials induce changes in the functional capacity of the bacterial community, which can affect the health of the host (Ferrer et al., 2017). Antimicrobial treatment has been shown to alter the functional gene abundance in the faecal

microbiome. A study in pigs showed that feeding with antimicrobial growth promoters (chlortetracycline, sulfamethazine and penicillin) produced increases in the abundance of gut microbial functional genes relating to energy production and conversion, which might reflect the growth promoting properties of the antimicrobials (Looft et al., 2012). Moreover, antimicrobial treatment has been shown to affect specific enzymatic activities of the gut microbiome. For example, a 14-day treatment with cefazolin, ampicillin and sulbactam in humans was associated with increases in the glycosidase activity of the lower gastrointestinal tract microbiome (Hernández et al., 2013).

Antimicrobial treatment impacts microbial gene expression and alters the metabolite fluxes of the microbiota. Pérez-Cobas et al. showed a combination treatment using ampicillin/sulbactam and cefazolin in a patient led to a great shift in the microbial gene expression level, 6 days after the treatment (Pérez-Cobas et al., 2013). Moreover, the authors found the antimicrobial treatment altered the level by about 4.4% of the total detected intestinal metabolites. However, an earlier study by Antunes et al. showed that streptomycin treatment in mice affected the levels of over 87% of total faecal metabolites (Antunes et al., 2011). In a recent study by Gadde et al., researchers compared the impact of virginiamycin and bacitracin on chicken intestinal metabolites (Gadde et al., 2018). The level of 31% of total metabolites were altered by virginiamycin and 17% by bacitracin. Different antimicrobials lead to varied alterations in microbiome composition which can result in differences in the extent of metabolite flux changes. Also, gut microbiome of different hosts responses to the antibiotic exposure to a different extent. This highlights the importance of better understanding the functions of bacterial members in the intestinal community.

1.4 The resistomes of human and animal gut microbiomes

Current molecular technologies have advanced to the point that they can overcome previous limitations of analysing only cultivable bacteria and can extend the study of AMR to encompass the entire bacterial community level, or the resistome. The resistome is defined as the set of resistance determinants within a particular biological or ecological context, which ranges from a genome to a microbial community, an ecosystem or the whole biosphere (Forslund et al., 2014).

1.4.1 The various approaches used to study AMR

Two different types of approaches have been used to study AMR: culture-based and culture-independent. Traditionally, culture-based approaches focus on specific bacterial isolates and examine them by antimicrobial susceptibility testing. The commonly used testing methods include broth dilution tests, antimicrobial gradient methods using commercial test strips (e.g. Etest®) and disk diffusion tests (Reller et al., 2009). Some of those testing methods have been developed into automated instrument systems to increase the accuracy and work efficiency, for example, the Sensititre™ ARIS™ system developed by Thermo Fisher Scientific. Nevertheless, culture-based methods take time, and can only target a fraction of the bacterial community because the majority of bacteria are unculturable (or difficult to culture), especially those of the gut microbiome. To overcome these difficulties and limitations, culture-independent approaches are often applied to study ARGs from the microbiome perspective. The commonly used techniques include quantitative polymerase chain reaction (qPCR), sequence-based metagenomics and functional metagenomics.

1.4.1.1 High-throughput qPCR to study resistomes

The comprehensive characterisation of ARG profiles in environmental samples requires detecting and quantifying many gene targets. Conventional qPCR is time consuming because relatively few targets can be tested in a single experiment. High-throughput qPCR (HT-qPCR) is a relatively efficient solution for screening hundreds of ARGs simultaneously, and has other advantages compared to competing technologies. First, HT-qPCR has better detection limits compared to sequence-based metagenomics. HT-qPCR was reported to detect ARGs as low as 10^{-5} to 10^{-4} relative to the 16S rRNA gene (Muziasari et al., 2016, Guo et al., 2018). To have similar detection limits for one ARG using a metagenomic approach would require at least 10^{8} reads (Waseem et al., 2019). For samples with high bacteria load, such as faeces, this requires a large amount sequencing data, which would increase experimental cost and the difficulties in bioinformatic analysis to process and analyse the reads. In addition, HT-qPCR usually requires less DNA quantity than metagenomic sequencing. For example, the WaferGen platform performs each qPCR reaction in 100nl, thus consuming only a small amount of DNA (Lin et al., 2016). Where researchers need to increase the DNA quantity by

whole genome amplification to meet the sequencing requirement, this may cause bias in the samples (Pinard et al., 2006).

Like other PCR-based techniques, the detection of ARGs by HT-qPCR is limited by the choice of primers used. Moreover, since assays performed in the same experiment are subjected to the same qPCR conditions, this makes it challenging for primer design as all primers need to bind to specific targets at the same annealing temperature (Waseem et al., 2019). So far, 307 primer sets have been published that target ARGs from the main antimicrobial classes, as wells as 21 primer sets that target MGEs, including plasmids, transposons and insertion sequences (Looft et al., 2012, Zhu et al., 2013).

1.4.1.2 Sequence-based metagenomics

The development of next generation sequencing techniques has greatly increased the throughput and reduced the cost of sequencing, which makes large scale sequencing projects possible, such as metagenomic studies. Sequence-based metagenomics is achieved by randomly sequencing DNA that has been directly extracted from samples. Then, the raw metagenomic reads are trimmed and filtered to remove poor quality sequences, and the cleaned reads or assembled contigs are used for various types of analysis, such as mapping them against reference ARG sequences to detect their presence, abundance and diversity (Schmieder and Edwards, 2012, Lim et al., 2018). Reference gene sequences are available from public databases, such as the Antibiotic Resistance Genes Database (Liu and Pop, 2008), the Comprehensive Antibiotic Resistance Database (CARD) (McArthur et al., 2013, Jia et al., 2017) and ResFinder (Zankari et al., 2012).

Currently, metagenomics sequencing used in most studies were performed on platforms producing short reads (e.g. 300bp on Illumina MiSeq). While they are high-throughput and have a low sequence error rates, the short reads cannot cover a whole ARG which can make it difficult to assemble whole-gene contigs or determine the co-localisation of ARGs (Martínez et al., 2017). In contrast, long-read sequencing techniques, such as Pacific Biosciences (PacBio) or Oxford Nanopore, can generate sequences longer than 10kb (Rhoads and Au, 2015, Deamer et al., 2016), which make

it possible to discover genetic linkage of ARGs and determine whether the genes are on a chromosome or plasmid. However, long-read sequencing techniques have higher sequencing errors rates and require greater amounts of high-quality DNA. In a recent study, a hybrid metagenomic assembly pipeline was described which combined Nanopore (long read) and Illumina (short read) data in order to achieve good coverage and larger, high quality contigs of the human gut metagenome (Bertrand et al., 2019). These hybrid assemblies were able to capture closed plasmids and phage genome sequences and could differentiate bacteria at the subspecies level.

Compared to qPCR-based techniques, sequence-based metagenomics has the advantage of being able to detect sequences that are homologous (but not identical) to reference ARGs, although the threshold of sequence similarity used to classify sequences as ARGs is somewhat arbitrary (Lim et al., 2018). In general, reads mapping to reference ARGs with amino acid similarity values of 80% to 95% are considered reasonable (Bengtsson-Palme et al., 2017), and such values have been used in some metagenomic studies (Hu et al., 2013, Ma et al., 2016, Li et al., 2015). Nevertheless, the detection ability of sequence-based metagenomics is still limited to the previously known ARGs, and novel genes that are not present in the sequence databases would not be identified.

As the DNA fragments are sequenced randomly, another limitation for sequence-based metagenomic is the possibility of overlooking the ARGs associated with less-abundant bacteria in the microbiome. Therefore, combining culture-based techniques with sequencing is useful to detect ARGs associated with less-abundant but clinically important bacteria. Some studies have tried to improve the chances of detecting ARGs from diverse bacteria by cultivation with various media and under aerobic and anaerobic conditions (Raymond et al., 2019, Lau et al., 2016).

In addition to identifying ARGs, predicting the antimicrobial susceptibility of bacteria is another aim of metagenomic analysis. Genotype-based AMR prediction is based on genotype-to-phenotype correspondence. Bradley et al. developed a method which assembles a *de Bruijn* graph to represent alleles with or without ARGs on different genetic backgrounds (Bradley et al., 2015). The graph of a sample can be compared to

reference graphs to predict antimicrobial susceptibility. Pesesky et al. combined rules-based and machine learning methods to generate a genotype-based-antibiotic susceptibility prediction algorithm, which showed potential to provide early treatment suggestions before the results of phenotypic susceptibility testing methods (Pesesky et al., 2016).

1.4.1.3 Functional metagenomics

For functional metagenomics, genomic DNA is sheared and cloned into plasmid vectors and transformed into heterologous hosts (e.g. *E. coli*) to create metagenomic libraries (Lim et al., 2018). The cloned metagenomic library is plated on antimicrobial selective media to isolate resistant strains, and the vector containing resistance genes are sequenced (Monier et al., 2011, Forslund et al., 2014, Schmieder and Edwards, 2012). The major advantage of functional metagenomics is the ability to discover novel resistance genes. Sommer et al. built functional metagenomic libraries of DNA from saliva and faecal samples from two healthy humans (Sommer et al., 2009). The vectors were transformed in *E. coli* and selected for genes conferring resistance to 13 antimicrobials. They identified 95 functional ARGs which, on average, showed 69.5% nucleotide sequence similarity and 65.3% amino acid sequence similarity to reference ARGs in GenBank. Wichmann et al. built metagenomic libraries of DNA from cow manure and selected for ARGs to beta-lactams, phenicols, aminoglycosides and tetracycline (Wichmann et al., 2014). Researchers identified 80 ARGs whose deduced protein sequences had on average 50 to 60% similarity to the sequences in GenBank. Zhou et al. selected functional ARGs from the chicken gut microbiome and discovered two novel ARGs conferring resistance to ampicillin and spectinomycin (Zhou et al., 2012). The genes were further transformed into *Campylobacter* and retained function in the new host, indicating that HGT of such genes in nature, even between phylogenetically divergent species, is likely to result in cells with increased AMR.

The functional metagenomics approach is not without technical challenges, as gene expression in heterologous hosts may be hampered by the host's gene expression machinery and environmental conditions (Schmieder and Edwards, 2012). Diaz-Torre et al. pointed out the difficulties when using *E. coli* as host to express certain tetracycline resistance genes from the human oral microbiome (Diaz-Torres et al.,

2006). It is possible that many genes cannot be expressed in *E. coli*, therefore, a wider range of both Gram-positive and Gram-negative candidate hosts is needed.

1.4.2 ARGs associated with the human faecal microbiome

The normal flora of human and animal gut microbiomes are often associated with diverse ARGs (Fitzpatrick and Walsh, 2016, Asante and Sekyere, 2019). In a large-scale metagenomic study of humans, Hu et al. examined 162 faecal metagenomes; 85 from Denmark, 39 from Spain and 38 from China (Hu et al., 2013). A total of 1,093 ARGs were identified. Among them, 9 types of resistance genes, *ant(6')*-Ia, *bac*A, *van*RA, *van*RG, *vat*32, *vat*40, *tet*O, *tet*Q and *tet*W, were found in all 162 samples, while the *erm*B gene was found in 161 samples. The Chinese cohort had the highest ARG abundance, followed by the Spanish. The *tet*Q gene was the most abundant gene type in all three countries, although gene abundances varied from country to country. In the Chinese and Spanish populations, the *erm*B ranked the second and the fourth most abundant gene, respectively. The cephalosporin resistance gene *bl2e_cfxA* was one of the top ten most abundant ARGs in the three countries. Moreover, ARGs for tetracycline, macrolide-lincosamide-streptogramin and beta lactams counted for more than 75% of the total relative abundance in each country, while the tetracycline resistance gene accounted for nearly 50% of the abundance.

Forslund et al. screened 252 faecal metagenomes from Danish, Spanish and American volunteers to detect ARGs in the human gut microbiome (Forslund et al., 2013). ARGs for 50 antimicrobial classes and subclasses were found in the samples, with an average 21 ARGs per sample. Tetracycline, bacitracin and cephalosporins were the top three classes whose ARGs were detected with high abundance across the samples. In addition, the antimicrobial exposure was positively correlated with the resistant potential at county-level, which suggested the national antimicrobial use policy may impact the relative abundance of ARGs in the human gut flora. Similar results were reported in a later metagenomic study that analysed the data from 1267 human gut microbiomes (139 American, 368 Chinese, 401 Danish and 359 Spanish) accessed from the Human Microbiome Project, NCBI database, Metagenomics of the Human Intestinal Tract project, EBI and Gene Expression Omnibus database (Yang et al., 2016).

1.4.3 ARGs associated with the animal faecal microbiome

Animal gut microbiomes are associated with various ARGs regardless of antimicrobial exposure (Table 1-1). Irrespective of animal species and background, tetracycline and macrolide resistance genes, particularly *tet*Q, *tet*W, *erm*B and *mef*A, were found to be ubiquitous in the animal gut microbiome. The high prevalence of these ARGs might be explained by the high level of species in the normal gut flora that naturally carry these genes, such as *Bacteroides* (Roberts et al., 2012, Shoemaker et al., 2001).

In a recent large-scale metagenome study, the resistomes of 181 pig and 178 poultry farms from nine European countries were analysed (Munk et al., 2018). Pigs generally had higher ARG loads, whereas poultry had a more diverse resistome. Tetracycline and macrolide resistance genes contributed most to ARG levels in the majority of pig and poultry samples. Sulfonamide and trimethoprim resistance genes were more abundant in poultry than pig samples across countries. Nevertheless, the pig and poultry resistomes appeared to show country-dependent patterns, particularly for pigs. It was found that the total country level veterinary antimicrobial usage was positively associated with the overall AMR in both pig and poultry samples, but exceptions were observed for particular genes. For example, the plasmid-borne colistin resistance gene *mcr*-1 had relatively high abundance in poultry samples from Bulgaria, whereas that country had a low reported usage of veterinary colistin.

Consistent findings were reported in another large-scale metagenomic study of pigs, which involved 287 individual pig samples from France, Denmark and China (Xiao et al., 2016). Chinese pigs which received diets with low doses of antimicrobials had a higher level of ARGs and different resistome profile compared to French and Danish pigs which had not received antimicrobials for prophylactical proposes. However, vancomycin and teicoplanin ARGs were found with higher level in French and Danish pigs than Chinese pigs.

Table 1-1: ARGs carried in animal faeces characterised by HT-qPCR or sequence-based metagenomic methods

Number of samples	Age	Origin of samples	Method	Antimicrobial treatment	Dominance/presence of ARG type	Reference
6 faecal samples from 6 individual, healthy beef heifers	6-8 months	USA	Sequence-based metagenomic	No	Multidrug resistance efflux pumps, followed by fluoroquinolones, beta lactamase, tetracycline resistance (ribosome protection type)	(Durso et al., 2011)
14 composite faeces from 2 dairies, 2 feedlots and a ranch	Not mentioned	USA (n=10) Canada (n=4)	Sequence-based metagenomic	No treatment for organic dairy; Macrolides and ionophores in feed in feedlots	Tetracycline resistance (*tet*Q, *tet*W); macrolide efflux pumps, erm methyltransferases, lincosamide nucleotidyltransferases	(Noyes et al., 2016)
Digests from rumen, colon and cecum of 5 treated and 5 non-treated beef steers	An average 13 month for non-treated steers, 14 months for treated steers.	USA	Sequence-based metagenomic	Tylosin in feed for treated steers. Treatment last for 74 days.	*tet*O, *tet*Q, *tet*W, *tet*40, *cfx*A, *lnu*C and *mef*A were frequently detected; No correlations between the presence of ARGs and the administration of antibiotic feed additives.	(Thomas et al., 2017)
Faeces from 3 antimicrobial treated and 3 non-treated cows	Not mentioned	USA	Sequence-based metagenomic	Ceftiofur through injection for treated cows	Tetracycline resistance genes were abundant in both groups; beta-lactam and multidrug resistance gene were more abundant in treated cows; No measurable effect of ceftiofur treatment on total ARGs abundance	(Chambers et al., 2015)

Number of samples	Age	Origin of samples	Method	Antimicrobial treatment	Dominance/presence of ARG type	Reference
Faeces from 176 cattle	Not mentioned	USA	Sequence-based metagenomic	Ceftiofur, tetracycline, or ceftiofur plus tetracycline	Tetracycline resistance genes (*tet*Q followed by *tet*40); macrolide resistance efflux pump genes. Tetracycline treatment increased the level of *tet* genes, while ceftiofur treatment did not change the beta-lactamase resistance genes significantly.	(Weinroth et al., 2018)
24 composite faeces from 6 grassland-based cattle farms	Not mentioned	Colombia	PCR targeting tetracycline resistance genes	Might be exposed to oxytetracycline dihydrate	*tet*O was the most frequently detected genes followed by *tet*W, *tet*Q and *tet*B(P)	(Santamaría et al., 2011)
17 pooled faecal samples from 17 dairy farms	Not mentioned	China	Sequence-based metagenomic	Not mentioned	The top three abundant ARGs were *cfx*A, *tet*Q and *tet*W.	(Zhou et al., 2016)
2 caecum contents from 2 chickens. One bird was challenged with *Campylobacter jejuni*	28 days old	USA	Sequence-based metagenomic	No	Tetracycline resistance (ribosome protection type), followed by fluoroquinolones resistance	(Qu et al., 2008)
32 fresh faecal droppings from 10 chicken households raising birds in production or household type	Not mentioned	Ecuador	HT-qPCR with 248 primer sets targeting ARGs from the major antimicrobial classes	Antimicrobials were added in feed for production birds, not used in household birds.	The most abundant genes were *str*B, *sul*2 and *tet*W. Production birds generally had higher ARG richness and abundance than household chickens.	(Guo et al., 2018)
142 fresh faecal droppings from egg	Not mentioned	Czech Republic (n=67) Slovenia (n=15)	qPCR targeting *str*A, *sul*1, *sul*2, *tet*A, *tet*B, *cat*	No	*str*A	(Videnska et al., 2014)

Number of samples	Age	Origin of samples	Method	Antimicrobial treatment	Dominance/presence of ARG type	Reference
laying hens and broiler farms		Croatia (n=30) Hungary (n=30)				
Chicken manure from 6 free range broiler farms and 5 indoor broiler farms	20-120 days old	China	qPCR targeting 18 ARGs from sulfonamide, tetracycline and chloramphenicol class	Not mentioned	*fex*A, *fex*B, *cfr*, *sul*1 and *tet*W	(He et al., 2014)
Fresh faeces from 8 treated and 8 non-treated pigs	~28 days old	Austria	Sequence-based metagenomic	Treated pigs received diet added with oxytetracycline for 7 days	Tetracycline resistance gene (ribosome protection type), beta-lactam resistance (Class A beta-lactamases), multidrug efflux pumps, macrolide efflux pumps, lincosamide nucleotidyltransferases; treated pigs were enriched with ARGs mainly from the tetracycline, beta-lactam and multidrug resistance classes	(Ghanbari et al., 2019)
Fresh faeces from 16 healthy pigs from the same farm	Not mentioned	Ireland	Sequence-based metagenomic	No	*tet*W, *tet*Q, *tet*44, *tet*37, *tet*40, *mef*A, *aad*E, *ant*(9)-1, *erm*B and *cfx*A2	(Joyce et al., 2019)
12 pool manure from 3 swine farms	Not mentioned	China	HT-qPCR targeting 244 ARGs from the major antimicrobial classes	No data for Beijing farm; chlortetracycline in feed for Jiaxing farm; florfenicol, doxycycline, oxycycline, polymyxin E,	*tet*Q, *tet*W, *mef*A, *aph*A3. Manures from swine farms generally had higher ARGs abundance than manure from pigs not exposed to antimicrobials.	(Zhu et al., 2013)

Number of samples	Age	Origin of samples	Method	Antimicrobial treatment	Dominance/presence of ARG type	Reference
				kitasamycin tartrate in feed for Putian farm		
Fresh faeces from 3 antimicrobial treated and 3 non-treated pigs	After weaning	USA	Sequence-based metagenomic and qPCR	Treated pigs received feed added with chlortetracycline, sulfamethazine and penicillin.	*erm*A, *erm*B, *mef*A, *tet*(32) and *aad*A were frequently detected in treated and non-treated pigs. Antimicrobial treatment caused increase in the corresponding ARGs abundances and co-selected ARGs belonging to aminoglycoside class.	(Looft et al., 2012)
Faeces from 287 individual pigs	Varied	France (n=100) Denmark(n=100) China (n=87)	Sequence-based metagenomic	Not mentioned	Tetracycline, macrolide, streptogramin B, bacitracin and cephalosporin resistance genes. Chinese pigs generally had higher ARGs abundance except for vancomycin and teicoplanin, than French and Danish pigs.	(Xiao et al., 2016)
Fresh faecal droppings from 181 pig herds and 178 broiler flocks	Varied between farms	Belgium Bulgaria Germany Denmark Spain France Italy Netherlands Poland	Sequence-based metagenomic	Varied between countries.	Tetracycline, macrolide AMR made up the majority in both pigs and poultry. Pigs generally had higher AMR level than poultry. Resistome varied between pig and poultry reservoirs and countries within each animal species.	(Munk et al., 2018)

1.4.4 Co-selection and persistence of ARGs in the gut microbiome

Exposure of gut microbial flora to antimicrobials is generally expected to be associated with the increase of corresponding ARGs and, sometimes co-selection of ARGs of non-administered antimicrobials. Looft et al. analysed the changes in ARG profiles of the gut microbiomes of pigs given antimicrobial treatments (chlortetracycline, sulfamethazine and penicillin) via their diet for two weeks (Looft et al., 2012). The medicated pigs showed an increase in abundance and diversity of ARGs, potentially conferring resistance to the given antimicrobials. In addition, genes encoding aminoglycoside O-phosphotransferases were found enriched in the medicated pigs, which suggested the co-selection of resistance to one antimicrobial by the use of other antimicrobials. A similar example in humans was reported by Gibson et al. (Gibson et al., 2016), which described the results of a large-scale gut resistome study of 84 preterm infants. They examined the changes in the ARGs of the gut microbiomes of infants treated with different antimicrobials, and found not only enrichment of ARGs to the corresponding antimicrobials, but also enrichment of ARGs to a wide range of non-cognate antimicrobials. For example, treatment with meropenem was associated with increased levels of ARGs potentially conferring resistance to fluoroquinolones, macrolides, tetracycline and trimethoprim. Metagenomic data revealed the MDR gene clusters carried by the gut microbiome could be both plasmid-mediated and genome-encoded.

Whether the selective effects of antimicrobials can be detected or not is influenced by various factors of the experimental design. For example, Chambers et al. compared the resistome of the bovine gut microbiome three days after ceftiofur injection in non-treated cows (Chambers et al., 2015). They found that ceftiofur treatment did not cause a measurable effect on the total ARG-like sequence abundance in the faeces, but this could have been masked by the dominance of sequences of tetracycline resistance genes in the faecal microbiome of both treated and non-treat cows. Nevertheless, the proportion of sequences carrying ARGs belonging to the beta-lactamase class, particularly *cfx*A2 and *cfx*A3, and the multidrug class were significantly increased in the treated cows. In contrast, Weinroth et al. analysed the effect of parenterally administered ceftiofur treatment in cattle and found no significant changes in beta-

lactamase genes of CMY, CTX and CFX groups 26 days after treatment (Weinroth et al., 2018). Therefore, the selective pressure for AMR in the gut microbiome by antimicrobial treatment should be interpreted with caution.

AMR is thought to bring a fitness cost to bacteria, and reduction in the use of antimicrobials might help reduce the proportion of the resistant strains by natural selection (Andersson and Hughes, 2010). However, in some cases, AMR in the gut microbiome can be maintained even when treatment with antimicrobial agents is ceased. Culture-based studies have shown that ARGs are maintained in various bacteria species isolated from healthy human subjects who had no recent antimicrobial usage (Shoemaker et al., 2001, Bailey et al., 2010, Sjölund et al., 2005, Sjölund et al., 2003). In a culture-independent study, Jernberg et al. analysed the impact of a 7-day clindamycin treatment on the abundance of macrolide resistance genes in the human faecal microbiome (Jernberg et al., 2007). The results showed the level of *erm*F could be still over 1000-fold higher than the pre-treatment levels even two years after the antimicrobial treatment ceased.

Factors that contribute to persistence of ARGs in bacteria are complex. In addition to direct selection by antimicrobial treatments, other factors include low antimicrobial concentration selective pressure, co-selection due to genetic linkages of ARGs on MGEs, cost-free resistances and reduction of fitness costs by compensatory evolution (Andersson and Hughes, 2011). Natural environments can have antimicrobial residues at concentrations much lower than their minimum inhibitory concentrations (MICs) (Li et al., 2008), and there is evidence that low antimicrobial concentration selection is an important force to maintain ARGs. Gullberg et al. tested the effects of sublethal levels of antimicrobials (tetracycline, trimethoprim, erythromycin and kanamycin) on the maintenance of an ESBL plasmid in both *K. pneumoniae* and *E. coli* (Gullberg et al., 2014). They found that the minimum concentrations of antimicrobials that maintained the plasmid in the bacteria were 1/17 to 2/3 of the MICs for the susceptible plasmid-free strains, which suggested that low antimicrobial levels might be sufficient to maintain carriage of ARGs by bacteria in the environment, as well as the intestinal bacteria of animals and humans.

Co-selection of ARGs due to genetic linkage on MGEs can potentially maintain resistance to the antimicrobial not currently in use (Andersson and Hughes, 2011). Co-selection of ARGs at the gut microbiome level has been reported in human and animal studies as discussed above. Moreover, clusters or networks of ARGs have been found in the faecal microbiomes of various animal species and humans (Johnson et al., 2016, Li et al., 2015, Qian et al., 2018, Guo et al., 2018), which indicate the co-localisation of those genes in the bacterial community. Johnson et al. analysed the ARG profiles and bacterial phylogeny of the microbiomes from three Chinese swine farms. Two major clusters of ARGs and MGEs were identified (Johnson et al., 2016). One comprised ARGs from six antimicrobial classes with a class 1 integrase, and the other consisted of IS6100-type transposons with tetracycline ribosomal protection resistance genes. The clustered ARGs could confer resistance to antimicrobials not used on the farms. Thus, selection for these clusters might be attributed to the use of one antimicrobial to which the cluster confers resistance. Moreover, Li et al. analysed the co-occurrence pattern of ARGs in the metagenomes of various environmental and faecal samples (Li et al., 2015). The *tet*M and aminoglycoside resistance genes had dense connections to other ARGs, thus, were proposed as indicators to estimate the level of associated ARGs in microbiomes.

Co-selection of genes might partially explain why the frequency of resistance to a particular antimicrobial changes little after its consumption has been reduced. For example, Sundqvist et al. analysed the effect of a 24-month restriction in the use of trimethoprim on the frequency of trimethoprim resistance in *E. coli* among residents of Kronoberg County, Sweden (Sundqvist et al., 2009). Over the study period, trimethoprim consumption reduced by 85% but no clear decrease in the rate of resistance was observed. In fact, a marginal but statistically significant increase in trimethoprim resistance was detected. The small fitness cost of trimethoprim resistance observed *in vitro* and co-selection of the other used antimicrobials (e.g. pivmecillinam, nitrofurantoin and ciprofloxacin) during the experiment were thought to be the possible reasons. Consistent findings were reported in an earlier study investigating the effect of restriction of sulfonamide use in UK on the frequency of corresponding resistance in *E. coli* (Enne et al., 2001). Moreover, an Australian study by

Ingram et al. reported the presence of ciprofloxacin resistant *E. coli* in poultry meat despite fluoroquinolone was not allowed to be used in food animals (Ingram et al., 2013). As the isolates were also resistant to amoxicillin, gentamicin, tetracycline and trimethoprim/sulfamethoxazole, which could be used in meat chicken production, the emergence of fluoroquinolone resistance might attribute to the co-selection by other antimicrobials.

1.4.5 Selection of AMR by heavy metals

In addition to antimicrobials, AMR can be selected by other compounds, such as heavy metals. Co-resistance and cross-resistance are the two main mechanisms that select AMR by heavy metals (Wales and Davies, 2015). Co-resistance is due to the genes responsible for resistance to antimicrobial and heavy metals being co-located on the same MGE, such as plasmids. Cross-resistance results from certain antimicrobials sharing a common pathway to cell death or a common route to access their own target, which stimulates a physiological adaptation against multiple agents (Baker-Austin et al., 2006). Copper and zinc are commonly added to feed for food animals to meet their basic nutritional needs and to increase feed efficiency (Wales and Davies, 2015). The concentrations of copper and zinc in pig manure were associated with resistance to beta-lactams in the *E. coli* isolates (Hölzel et al., 2012). Vahjen et al. reported when pigs received a diet supplemented with high doses of zinc (2425mg/kg, ZnO), the levels of *tet*A and *sul*1 significantly increased in the flora of their gastrointestinal tracts (Vahjen et al., 2015).

Gene operons encoding efflux systems that confer resistance to metals were found genetically linked to ARGs, such as, the *pco* and *sil* operon. The *pco* operon is comprised of gene *pco*A, B, C, D, R, S and E, which confers resistance to copper; and the *sil* operon is comprised of gene *sil*P,A, B, D, C, R, S and E, which confers resistance to silver (Hobman and Crossman, 2015). These operons are frequently present in different genera of the *Enterobacteriaceae* and are particularly well-studies in *Escherichia* sp. and *Salmonella* sp. (Hao et al., 2015). They are often associated with Tn7-like transposons, either on plasmids or integrated into chromosome, and

sometimes co-localised with ARGs (Fang et al., 2016, Chalmers et al., 2018, Billman-Jacobe et al., 2018, Tassinari et al., 2019).

Moreover, Agga et al. quantified the levels of *tet*A, *tet*B, bla_{CMY-2} and *pco*D in faeces from pigs that had received chlortetracycline, copper or both compounds added in their diet for three weeks (Agga et al., 2015). Copper tended to decrease the level of *tet*A but increase the level of *tet*B and bla_{CMY-2} in the faeces of treated pigs compared to controls. The levels of *pco*D in treated pigs varied over time in all treatment groups but treated pigs had lower levels of *pco*D than control pigs in the first two weeks of treatment, after which the gene level in treated pigs increased so that it was above that of control pigs by the end of the treatment period.

1.4.6 Transfer of ARGs within and between bacterial communities

The gut microbiome has a high density of bacteria that are in close proximity to each other, which provides an ideal environment for gene transfer (Van Hoek et al., 2011). Shoemaker et al. analysed ARGs carried by *Bacteroides* spp. isolated from the human gut over a period of three-decades (Shoemaker et al., 2001). The carriage of *tet*Q was observed to increase from about 30% to 80% of strains, and the alleles of *tet*Q in different *Bacteroides* species were commonly 96 to 100% identical in sequence, suggesting that this spread was attributed to HGT. Notably, gene *tet*Q is commonly associated with the conjugative transposon CTnDOT in *Bacteroides* (Chopra and Roberts, 2001, Shoemaker et al., 2001), and the presence of tetracycline was found to stimulate the excision and conjugative transfer of this transposon (Whittle et al., 2002). Additionally, bacteria of the *Proteobacteria* phylum present in the gut microbiome of humans and animals represent another reservoir of mobile ARGs. Hu et al. examined 23,425 bacterial genomes in GenBank and found the most mobile ARGs were carried by bacteria of the phylum *Proteobacteria*, followed by *Firmicutes, Bacteroidetes and Actinobacteria* (Hu et al., 2016). Recent exchanges of mobile ARGs (alleles with more than 99% nucleotide identity) were also found overrepresented in *Proteobacteria.*

Transient colonisation of animal-origin bacteria carrying ARGs may spread the genes into human gut flora. One study demonstrated that the *in vivo* transfer of *van*A from a

chicken-origin *Enterococcus faecium* isolate to a human-origin *E. faecium* isolate could occur in the intestines of healthy human subjects (Lester et al., 2006). Moreover, Smet et al. demonstrated the HGT of a beta-lactamase gene between *E. coli* in a fermentation system simulating the human gastrointestinal tract (Smet et al., 2011). In the study, a poultry isolate of *E. coli* with a plasmid carrying the bla_{TEM} gene was inoculated into the system, and human-origin *E. coli* transconjugants could be detected 24h after inoculation even without antimicrobial selective pressure. Those findings raised concerns about the spread of AMR between animals and humans through transient colonisation of bacterial strains.

From a microbiome perspective, Pal et al. analysed 864 metagenomes from humans, animals and natural environments (Pal et al., 2016). They found that the microbiomes of humans and animals had relatively high levels of ARGs but a low abundance and diversity of MGEs, whereas the natural environment metagenomes, particularly sediments, water, soil and mines, displayed the opposite trend. Human gut, oral and urogenital microbiomes and animal-associated microbiomes carried a high level of tetracycline resistance genes; however, the environmental microbiomes were more abundant with beta-lactam resistance genes. Human gut microbiomes commonly shared similar ARGs with animals, and the overall ARG profiles of those two sample types overlapped in principal component analysis, which suggested the potential of ARGs transmission through dissemination of resistant bacteria or HGT.

Due to the important role of HGT in spreading ARGs, the drivers governing HGT have been explored. The barriers for gene acquisition in nature include phylogenetic, ecological and functional barriers (Popa and Dagan, 2011). Hu et al. examined mobile ARGs from various bacterial taxa and found the HGT frequency of the gene within taxon was significantly higher than the HGT frequency between taxon, indicating there are phylogenetic barriers to gene transfer (Hu et al., 2016). Supporting evidence was found by Forsberg et al., who applied functional metagenomics to 18 soil samples to detect ARGs against 18 antimicrobials (Forsberg et al., 2014). Their results showed the soil resistome composition was associated with microbial phylogenetic and taxonomic structure regardless of soil type. At the ecological niche level, Smillie et al. examined 10,770 unique, recently transferred genes identified in 2,235 bacterial genomes

(Smillie et al., 2011). They found that most gene exchange occurred between isolates that were ecologically similar but from geographically separate environments. For example, human-associated bacteria were 25-fold more likely to share transferred genes than non-human bacteria from diverse environments, indicating the key role of ecology in shaping the resistome. Moreover, Gibson et al. developed an annotation method combining the Resfams database of ARG families and associated profile hidden Markov models to identify and annotate AMR functions in bacterial communities and genomes (Gibson et al., 2014). Researchers applied the method to compare the functional metagenomic data sets from soils and human gut microbiomes and found the two types of microbiomes had distinct resistome profiles. The method was further applied to predict antimicrobial functions in more than 6000 bacteria from diverse phylogenies and habitats. AMR mechanisms were found to be significantly enriched in certain bacterial phyla and habitats. For example, beta-lactam resistance was significantly enriched in *Actinobacteria* compared to other phyla, and soil bacteria were significantly enriched for tetracycline major facilitator superfamily efflux pumps compared to two other habitats (water and human associated bacteria).

1.5 AMR in Australian food animals

Studies of AMR in Australian food animals focused on specific bacterial species. Although the proportion of resistant bacteria varied between studies, the occurrence of bacteria resistant to high clinical important antimicrobials is rare. So far, carbapenem resistant *Enterobacteriaceae* has not been found in food animals. Resistance to fluoroquinolone and third generation cephalosporins has been reported in a few *E. coli* isolates from pigs, chicken and beef cattle (Kidsley et al., 2018, Smith et al., 2016, Abraham et al., 2015, Abraham et al., 2019, Ingram et al., 2013). Notably, study by McLellan et al. reported *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Serratia fonticola* isolates from chicken meat and pork were detected with resistance to 3rd generation cephalosporins at a high frequency, suggesting the possibility of underestimating the level of third generation cephalosporins resistant bacteria if only focusing on *E. coli* (McLellan et al., 2018). Moreover, virginiamycin resistance was reported in some *E. faecium* and *E. faecalis* isolates from pig, and no

*van*A and *van*B genes were detected in those isolates which was in line with the withdraw of avoparcin from market in 2000 (Fard et al., 2011).

Resistance to tetracycline, ampicillin, florfenicol/chloramphenicol, trimethoprim, sulfonamide, gentamicin was relatively frequently detected in *E. coli* and *Salmonella* spp. isolates mostly from pigs and chicken (Abraham et al., 2019, Abraham et al., 2015, Obeng et al., 2012b, Kidsley et al., 2018, Smith et al., 2016). AMR in *E. coli* and *Salmonella* spp. isolates from dairy cattle was detected at low rate (Izzo et al., 2011, Barlow et al., 2015). Enterococci isolates were commonly detected with resistance to tetracycline and tylosin in pigs (Fard et al., 2011), bacitracin, tylosin and lincomycin in chicken (Obeng et al., 2013), and flavomycin and lincomycin in cattle (Barlow et al., 2017). Moreover, *Campylobacter* spp. with tetracycline, ampicillin and lincomycin resistance was reported in chicken (Obeng et al., 2012a, Miflin et al., 2007).

1.6 Project aims and hypothesis

Culture-independent techniques have been widely applied to study the gut resistome of animals in various countries, but there have been few such studies on animals in Australia. This project analysed ARGs and faecal microbiomes of Australian animals with no or low chance of antimicrobial exposure in order to provide base line information regarding the background levels and diversity of ARGs in animal faeces. The specific aims of this project were to:

1) Establish a pipeline from sampling to the detection and quantification of ARGs in animal faeces using HT-qPCR and sequence-based metagenomic techniques.

2) Explore the ARGs and microbiome profiles of faecal samples from foals, calves, chickens and pigs.

3) Identify the co-occurrence patterns of ARGs and MGEs in the animal faecal microbiomes.

The hypothesis is that animals with low or no antimicrobial exposure have a low abundance of high clinical important ARGs in their faecal microbiome.

Chapter 2 General Materials and Methods

2.1 DNA extraction from faecal and swab samples

DNA was extracted from 0.2g of faeces or from biomass stored in one 2mL centrifuge tube of each swab sample. DNA extraction was performed using the DNeasy PowerSoil kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, except that faeces or swabs were mixed with buffer C1 and processed with a FastPrep bead homogeniser at 5.5 m/s for 30 s. The quality and quantity of DNA were assessed using Nanodrop ND-1000 (Thermo Fisher Scientific, USA) and Qubit Fluorometer (Thermo Fisher Scientific, USA). DNA was stored at -20˚C until analysis.

2.2 Bacterial 16S rRNA gene sequencing and bioinformatic analysis

Bacterial communities were characterised by sequencing the V3-V4 region of the 16S rRNA genes on an Illumina MiSeq platform using primers 341F'(CCTAYGGGRBGCASCAG) and 806R'(GGACTACNNGGGTATCTAAT) (Yu et al., 2005, Thijs et al., 2017). Library preparation and sequencing was performed at Australian Genome Research Facility Ltd.

Sequences were analysed through a QIIME2 pipeline (Version qiime2-2018.11) (Bolyen et al., 2019). De-multiplexed reads were trimmed to 250 bp and de-noised, paired and grouped into amplicon sequence variants (ASVs) by DADA2 (Callahan et al., 2016). Taxonomy was assigned using a pre-trained Naïve Bayes classifier (Bokulich et al., 2018) with the Greengenes database (2013 August release) (DeSantis et al., 2006). ASVs that were only present in a single sample or classified as non-bacterial were removed. For fair comparison, sequence depth was equalised by randomly subsampling the same number of reads from each sample from the original dataset. The minimum number of reads that would not affect coverage or result in discarding any samples were used. The adequacy of reads was tested by Good's coverage index. Chao1, Shannon and Simpson indices based on ASV were calculated to estimate the alpha diversity of the faecal microbiota of each sample.

Sequence data have been deposited to NCBI database. The accession numbers are: PRJNA638757 for Chapter 3; PRJNA649671 for Chapter 4; PRJNA659336 for Chapter 5; PRJNA656586 for Chapter 7; PRJNA657439 for Chapter 8.

2.3 HT-qPCR array for gene detection and quantification

ARGs were detected using the SmartChip Real-time PCR system (Takara Bio USA, USA). The HT-qPCR assay was performed at University of Melbourne (Chapter 5) or Michigan State University's Genomic Core (Chapter 3, 4, 6,7). The qPCR array used in Chapter 5 contained 296 primer pairs, including 285 ARG-specific primer pairs to amplifying genes encoding resistance to the following antimicrobial classes: aminoglycoside, beta lactamase, fluoroquinolone/florfenicol (FC), macrolide-lincosamide-streptogramin B (MLSB), sulfonamide/trimethoprim, tetracycline, vancomycin, streptothricin, bacitracin, fosfomycin, nitroimidazole and pyrazinamide; 10 MGE primer pairs, including two class I integron genes and eight transposase genes; one primer pair to amplify the bacterial 16S rRNA gene (Zhu et al., 2013, Wang et al., 2014, Muziasari et al., 2016) (Appendix II). Results of Chapter 5 showed many qPCRs were negative in animals without antimicrobial exposure. This informed the primer choice for studies described in other chapters. Moreover, intrinsic genes encoding efflux pumps were excluded. Therefore, a reduced set of 49 primers was selected to improve the cost-efficiency and still cover the most frequent ARGs and MGEs. Moreover, three primers targeting MRG: *sil*R, *pco*A and *pco*R were designed in this study and included in the assay (Appendix I).

The qPCR conditions were previously described by Wang et al. (Wang et al., 2014). Each 100nL reaction contained 1X LightCycler 480 SYBR Green I Master Mix (Roche Applied Science, USA), 500nM of each primer, and 5ng/µl to 12.5ng/µl DNA template. The qPCR cycle was 95˚C for 10min, then 40 cycles of; 95˚C for 30s, 60˚C for 45s. The cycle threshold (Ct) cut-off was 27 (Zhu et al., 2013). Nuclease-free water replacing the DNA was used as no template control. Amplicons with multiple melting peaks were excluded from analysis.

The abundance of each ARG was normalised to the 16S rRNA gene in each sample using the equation: relative abundance = $\frac{\mathrm{E(16S)}^{\mathrm{Ct(16S)}}}{\mathrm{E(ARG)}^{\mathrm{Ct(ARG)}}}$, where E(16S) is the efficiency of amplification of the 16S rRNA gene, E(ARG) is the efficiency of amplification of each ARG, and Ct is the threshold cycle (Muller et al., 2002a, Muller et al., 2002b).

2.4 Co-occurrence of resistance genes and MGEs

The patterns of co-occurrence of resistance genes and MGEs were analysed using the Spearman rank correlation using the 'rcorr' function in the Hmisc R package. Correlations were represented as networks with only strongly co-occurring ARGs (Spearman's rho ≥ 0.75, $P < 0.01$) shown. To avoid false positives, ARGs detected in less than 30% of samples were excluded (Liu et al., 2018). *P*-values were adjusted using a Benjamini-Hochberg correction method (Benjamini and Hochberg, 1995). The network visualisation was performed using Gephi (Bastian et al., 2009).

Chapter 3 Faecal microbiota and antimicrobial resistance gene profiles of healthy foals

This chapter was submitted for publication to Equine Veterinary Journal on 3/1/2020. The content in this chapter is the same to the submitted manuscript, except the format was adjusted to match the rest of the thesis.

Yuhong LIU†, Kirsten E. BAILEY†‡, Michael DYALL-SMITH†, Marc S. MARENDA†, Laura Y. HARDEFELDT†‡, Glenn F. BROWNING†‡, James R. GILKERSON† and Helen BILLMAN-JACOBE†‡*

†Asia-Pacific Centre for Animal Health, Melbourne Veterinary School, Faculty of Veterinary and Agricultural Sciences, University of Melbourne, Melbourne, Australia

‡National Centre for Antimicrobial Stewardship, Peter Doherty Institute, Melbourne, Australia

*Correspondence email: hbj@unimelb.edu.au

Authorship: Y. Liu, laboratory work, data analysis and interpretation, and manuscript preparation; K. E. Bailey, sample collection, data interpretation and manuscript review. M. Dyall-Smith, M. S. Marenda, L. Y. Hardefeldt and G. F. Browning, study design, data interpretation, manuscript review; J. R. Gilkerson, sampling study design, manuscript review; H. Billman-Jacobe, study design, project supervision, data interpretation and manuscript review.

Source of funding: This study was funded by the National Health and Medical Research Council through a Centres of Research Excellence grant to the National Centre for Antimicrobial Stewardship. Y. Liu received a Melbourne Research Scholarship from the University of Melbourne.

Competing Interests: The authors declared no competing interests.

Ethical animal research: Approval for inclusion of the foals in the study was obtained from the stud farm managers prior to commencing the study.

Acknowledgements: We would like to thank the managers and staff of the participating stud farms for their assistance in conducting this study.

3.1 Summary

Background: The human and domestic animal faecal microbiota can carry various antimicrobial resistance genes (ARGs), especially if they have been exposed to antimicrobials. However, little is known about the ARG profile of the faecal microbiota of healthy foals. A high-throughput qPCR array was used to detect ARGs in the faecal microbiota of healthy foals.

Objectives: To characterise the faecal microbiota and ARG profiles in healthy Australian foals aged less than one month.

Study design: Observational study

Methods: The faecal microbiota and ARG profiles of 37 Thoroughbred foals with no known gastrointestinal disease or antimicrobial treatment were determined using 16S rRNA gene sequencing and a high-throughput ARG qPCR array. Each foal was sampled on one occasion.

Results: *Firmicutes* and *Bacteroidetes* were dominant in the faecal microbiota. Foals aged 1-2 weeks had significantly lower microbiota richness than older foals. Tetracycline resistance genes were the most common ARGs in the majority of foals, regardless of age. ARGs of high clinical concern were rarely detected in the faeces. The presence of ARGs was associated with the presence of class I integron genes.

Main Limitations: Samples were collected for a case-control study so foals were not sampled longitudinally, and thus the development of the microbiota as individual foals aged could not be proven. The history of antimicrobial treatment of the dams was not collected and may have affected the microbiota of the foals.

Conclusion: The ARGs in foal faeces varied concomitantly with age related microbiota shifts. The high abundance of tetracycline resistance genes was likely due to the dominance of *Bacteroides* spp.

Keywords: faecal microbiota; antimicrobial resistance gene; farm environment

3.2 Introduction

The gastrointestinal tract of neonatal foals is colonised in the first few days of life by bacteria from the mare's microbiota (vaginal, perineal and milk) and the foal's environment, and later by core members from the adult faecal microbiota through coprophagy (Quercia et al., 2018, Husso et al., 2020). Progressive, age-dependent changes in the foal faecal microbiota have been identified (Costa et al., 2016, Quercia et al., 2018, De La Torre et al., 2019, Husso et al., 2020) and, after 2 months, the foal faecal microbiota becomes relatively stable and closely resembles the adult faecal microbiota (De La Torre et al., 2019, Costa et al., 2016). Bacteria associated with fibre digestion (e.g. *Lachnospiraceae*, *Ruminococcaceae* and *Fibrobacteres*) increase proportionally with age in the faecal microbiota of the foal to become dominant (De La Torre et al., 2019, Costa et al., 2016). Changes in the faecal microbiota have been associated with age, diet (Dougal et al., 2014), temperature (Salem et al., 2018), diarrhoea (Schoster et al., 2017) and antimicrobial exposure (Costa et al., 2015). Use of trimethoprim-sulfadiazine in healthy horses has been found to result in a decrease in faecal microbiota diversity and a reduction in *Verrucomicrobia* for at least 9 days after treatment (Costa et al., 2015).

Bacteria within the microbiota of humans and other animals can harbour antimicrobial resistance genes (ARGs) (Zhu et al., 2013, Li et al., 2015). Many studies of ARGs associated with horse intestinal bacteria have been limited to the readily culturable bacteria, such as *Escherichia coli* and *Enterococcus* species (Dolejska et al., 2011, Moura et al., 2010, Ahmed et al., 2010, Moura et al., 2013). Although these bacteria are useful indicators for monitoring ARGs in faeces, they only represent a small fraction of the bacteria in the equine microbiota. The abundance of ARGs in the total faecal microbiota from horses remains largely unexplored. Culture independent approaches, such as deep sequencing and high-throughput qPCR (HT-qPCR), bypass the limitations of culture and enable a holistic evaluation of ARGs in the microbiota. High-throughput qPCR has been used to investigate a broad range of ARGs in faecal samples of various animal species (Zhu et al., 2013, Qian et al., 2018, Muziasari et al., 2016).

The aim of this study was to characterise the faecal microbiota and ARG profiles of farm-raised foals less than 30 days of age that had had no direct exposure to antimicrobial agents. Factors with a potential to affect the microbiota and the ARG profile were also analysed.

3.3 Materials and methods

3.3.1 Sample collection and DNA extraction

Foal faecal samples and medical records were collected as part of a previous age-matched case-control study of diarrhoea (Bailey, 2017). Only samples from control foals aged less than 30 days were included in the current study. A single faecal sample was obtained from each of 37 Thoroughbred foals on four stud farms in the Hunter Valley, New South Wales, Australia, between September and December 2010 (Supplementary item 1). The foals had no known history of diarrhoea in the week preceding sampling and there was no record of prior antimicrobial treatment.

Freshly voided faeces was collected from foals while their dams were being examined for breeding purposes. Faecal samples were collected into sterile containers and kept overnight at -20˚C, then stored at -70˚C until processing. DNA was extracted from 0.2 g of faeces using the DNeasy PowerSoil kit[a] following the manufacturer's instructions. A FastPrep bead homogeniser[b] was used at 5.5 m/s for 30 s to break the bacterial cells open. The quality and quantity of DNA were determined using a Nanodrop ND-1000[c]. DNA was stored at -20°C until analysis.

3.3.2 Bacterial 16S rRNA gene sequencing and bioinformatic analysis

The V3-V4 region of the bacterial 16S rRNA genes was amplified using primers 341F'(CCTAYGGGRBGCASCAG) and 806R'(GGACTACNNGGGTATCTAAT) (Yu et al., 2005, Thijs et al., 2017). Amplicons were sequenced on an Illumina Miseq platform. Sequences were analysed through a QIIME2 pipeline (Version qiime2-2018.11) (Bolyen et al., 2019). DADA2 was used to trim the de-multiplexed reads to 250 bp and they were de-noised, paired and grouped into amplicon sequence variants (ASV) (Callahan et al., 2016). A pre-trained Naïve Bayes classifier and the Greengenes database (2013 August release) were used for taxonomic assignment (Bokulich et al., 2018, DeSantis et

al., 2006). ASVs only present in a single sample or classified as non-bacterial were removed. Sequences from each foal were randomly subsampled to the same depth for fair comparison. The minimum number of reads that would not affect coverage or result in the discard of any samples were used. Good's coverage was calculated for each sample to assess the adequacy of the reads. Sequence data are available in the NCBI database under accession number PRJNA638757.

3.3.3 Detection and quantification of ARGs in foals

ARGs were detected using the SmartChip Real-time PCR system[d]. Forty-four primer pairs were used to amplify ARGs encoding resistance to the following groups of antimicrobials: aminoglycosides, beta lactams, quinolones and phenicols (FC), macrolide-lincosamide-streptogramin B (MLSB), sulphonamides/trimethoprim, tetracyclines, virginiamycin and streptothricin. Primer pair "blaCTX-M-04" amplified the group 2 type $bla_{CTX\text{-}M}$ genes, such as $bla_{CTX\text{-}M\text{-}2}$. Two primer pairs were used to amplify the class I integron markers *int*I1 and *qac*EΔ, and one primer pair was specific for the bacterial 16S rRNA gene (Supplementary item 2) (Zhu et al., 2013, Wang et al., 2014). The primers used were a subset of 295 primers validated in previous studies (Zhu et al., 2013, Wang et al., 2014, Looft et al., 2012). Primers for intrinsic resistance genes were excluded and genes detected in livestock in previous studies were included (Zhu et al., 2013, Li et al., 2015, Liu et al., 2020).

The qPCR conditions were previously described by Wang *et al*. (Wang et al., 2014). A cycle threshold (Ct) of 27 was used as the limit of detection (Zhu et al., 2013). The abundance of each ARG was normalised to the 16S rRNA gene abundance in each sample using the equation: relative abundance = $\frac{\mathrm{E(16S)}^{\mathrm{Ct(16S)}}}{\mathrm{E(ARG)}^{\mathrm{Ct(ARG)}}}$, where E(16S) was the efficiency of amplification of the 16S rRNA gene, E(ARG) was the efficiency of amplification of each ARG, and Ct was the threshold cycle (Muller et al., 2002b, Muller et al., 2002a).

3.3.4 Statistical analysis

Statistical analyses were completed using QIIME2 (version qiime2-2018.11) (Bolyen et al., 2019) and R (version 3.6.3) (R Core Team, 2020). Foals were grouped by sex, farm

or age. The impact of these factors on the faecal microbiota and ARG profiles was tested using non-metric multidimensional scaling (NMDS) based on a Bray-Curtis distance matrix calculated from genus-level ASVs and ARG relative abundance, respectively. The similarity of the faecal microbiota and ARG profiles between groups was tested by permutational multivariate analysis of variance (PERMANOVA) using the 'adonis' function in the R package vegan (Oksanen et al., 2019). The P value of pairwise PERMANOVA was adjusted using the Benjamini-Hochberg method using the R package RVAideMemoire (Hervé, 2019).

Differences in the abundance of bacterial taxa, at family and genus level, between the age groups were tested using the analysis of composition of microbiomes method (ANCOM) (Mandal et al., 2015). Alpha diversity of the faecal microbiota of each foal was measured based on ASVs. Richness indices Chao1 and ACE were used to estimate the number of ASVs in the sample, and the Shannon and Simpson diversity indices were used to estimate the richness and evenness of the microbiota of each sample. Differences in Chao1, ACE and Shannon indices between age groups were tested using one-way ANOVA with *post-hoc* analysis by Tukey's Honest Significant Difference (HSD) test. Differences in the Simpson index between age groups were tested using the pairwise Wilcoxon rank sum test with P values adjusted using the Benjamini-Hochberg correction method (Benjamini and Hochberg, 1995). A P value of less than 0.01 was considered significant.

Differences in the relative abundance of ARGs between foal age groups were tested with the pairwise Wilcoxon rank sum test with the P value adjusted using the Benjamini-Hochberg correction method (Benjamini and Hochberg, 1995). A P value of less than 0.01 was considered significant. The patterns of co-occurrence of ARGs were analysed using the Spearman rank correlation using the 'rcorr' function in the R package Hmisc (Harrell Jr, 2019). Correlations were represented as networks with only strongly co-occurring ARGs (Spearman's rho≥0.75, P<0.01) shown. To avoid false positives, ARGs detected in fewer than 30% of samples were excluded. P values were adjusted using a Benjamini-Hochberg correction method (Benjamini and Hochberg, 1995). The network visualization was performed using Gephi (Bastian et al., 2009).

3.4 Results

3.4.1 Impact of sex, farm and age on the faecal microbiota profile

A total of 1,855,588 reads from 37 foals passed all quality filters and were partitioned into ASVs. The original read depth of all samples ranged from 22,443 to 454,745. A subsample of 22,443 reads was randomly selected for each sample to enable fair comparisons. The rarefied reads were partitioned into 2,085 ASVs. The Good's coverage index based on ASV ranged from 0.99 to 1, indicating a sample depth of 22,443 reads was sufficient for accurate estimates of the microbiota richness and evenness.

The foals were grouped based on their sex, farm of origin or age: 23 foals were female and 14 were male; 13 were from farm A, 10 from farm C, 4 from farm D and 10 from farm E. They were categorised into three age groups: 1-2 weeks (n = 8, aged 8-14 days), 2-3 weeks (n = 14, aged 15-21 days) and > 3 weeks old (n = 15, aged 23-29 days). The differences in the microbiota profiles between the three age groups are shown in Figure 3-1a-c. No significant separation was seen between the sex or farm groups (Fig 3-1a, 3-1b, Supplementary item 3). Significant separation was seen between foals aged 1-2 weeks and >3 weeks (P=0.003), with foals aged 2-3 weeks intermediate, with no significant separation from the other two groups (Fig 3-1c, Supplementary item 3).

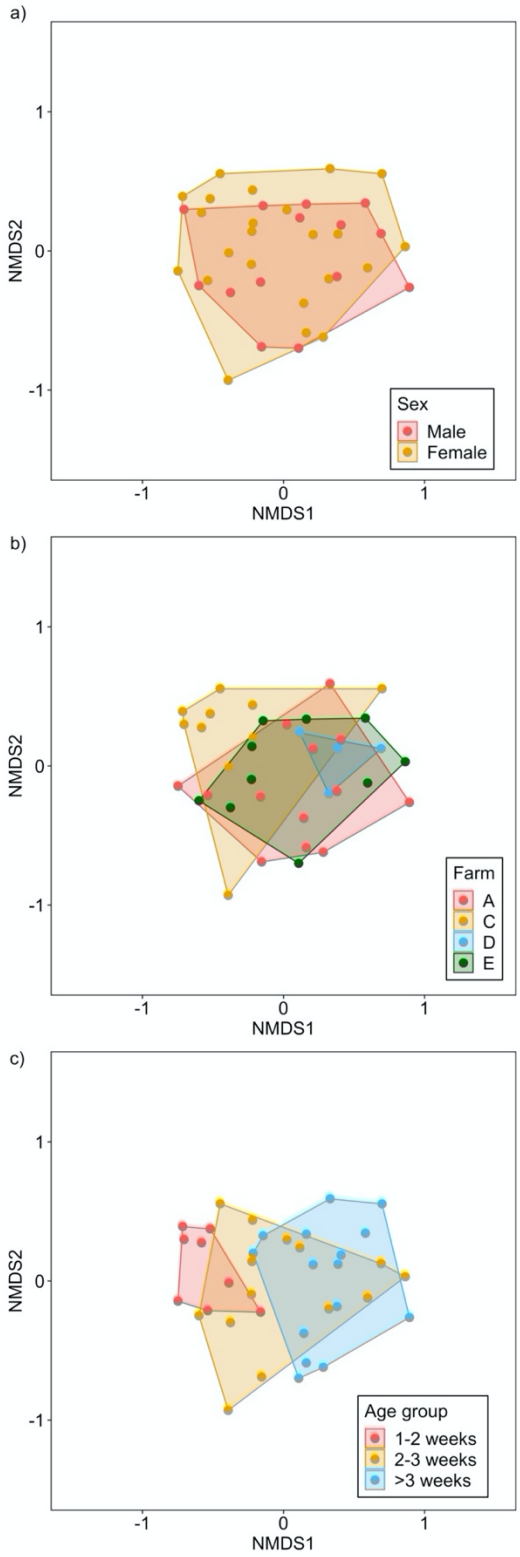

Figure 3-1: Similarity of the faecal microbiota of foals grouped by sex (a), farm (b) and age (c). NMDS analysis with Bray-Curtis distances was based on genus-level ASVs (NMDS stress = 0.18).

3.4.2 Faecal bacterial composition of foals in different age groups

Bacterial taxa with relative abundances above 0.5% within a sample, and detected in at least 33% of samples, were considered to represent the main taxa (Fig 3-2). *Firmicutes* and *Bacteroidetes* were dominant in the faecal microbiota and comprised up to 80% of the reads from each age group (Fig. 3-2a). *Verrucomicrobia* was the third most abundant phylum, and comprised 8%, 14% and 12% of the total reads from foals aged 1-2 weeks, 2-3 weeks and >3 weeks, respectively.

At the family level (Fig 3-2b.), *Ruminococcaceae*, *Bacteroidaceae, Lachnospiraceae* and *Verrucomicrobiaceae* were abundant in all age groups, and comprised more than 50% of the reads from each age group. At the genus level (Fig. 3-2c), *Bacteroides* spp., *Akkermansia* spp., *Lactobacillus* spp., *Blautia* spp., *Dorea* spp., *Parabacteroides* spp. and *Oscillospira* spp. were abundant in all age groups, with each genus comprising at least 1% of the reads from each age group.

Differences in the abundances of bacterial taxa at family and genus level between age groups are shown in Table 3-1. Foals aged >3 weeks had a significantly higher abundance of genus *Treponema* than the foals aged 1-2 weeks. Moreover, the family C*hristensenellaceae* was significantly more abundant in foals aged >3 weeks than in the younger foals. On the other hand, the abundance of the genus *Butyricimonas*, as well as its family, *Odoribacteraceae*, was significantly lower in foals aged >3 weeks than in foals in the other two age groups. No family or genus had a significantly different abundance between foals aged 1-2 weeks and 2-3 weeks.

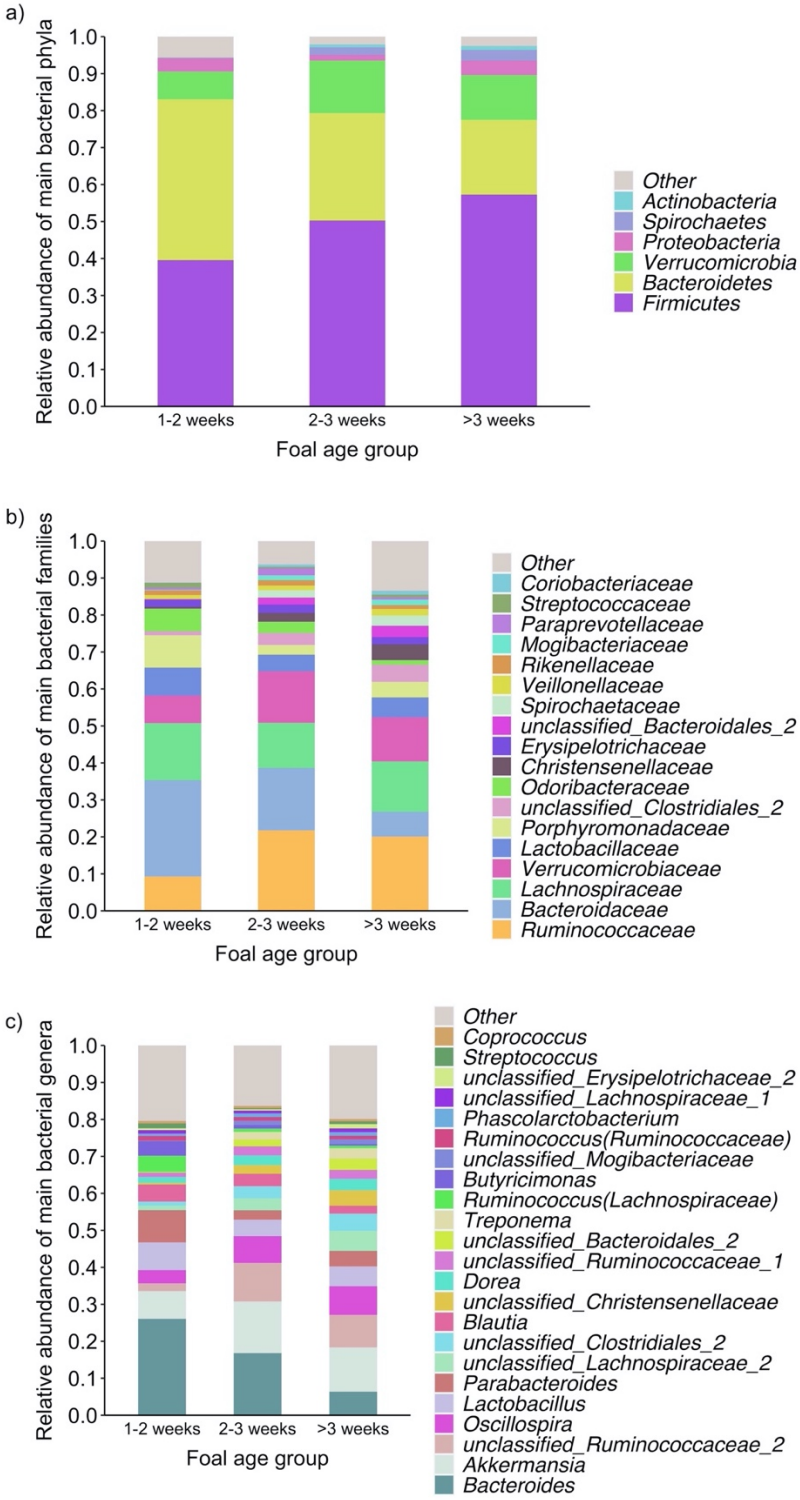

Figure 3-2: Relative abundance of dominant bacterial phyla (a), families (b) and genera (c) in the faecal microbiota of foals in each age group. Only taxa with a relative abundance above 0.5% in more than 33% of samples are shown. Phyla, families and genera below the cutoff were assigned to “Other”.

3.4.3 Comparison of alpha diversity of faecal microbiota between age groups

Four alpha diversity indices were calculated for the faecal microbiota in each group of foals (Fig 3-3). Foals aged 2-3 weeks and >3 weeks had significantly higher faecal microbiota richness than foals aged 1-2 weeks, as indicated by the Chao1 richness estimator ($P<0.01$) and the ACE index ($P<0.01$) (Fig 3-3a, 3-3b). No significant difference was found between the age groups in any of the diversity indices (Fig 3-3c, d).

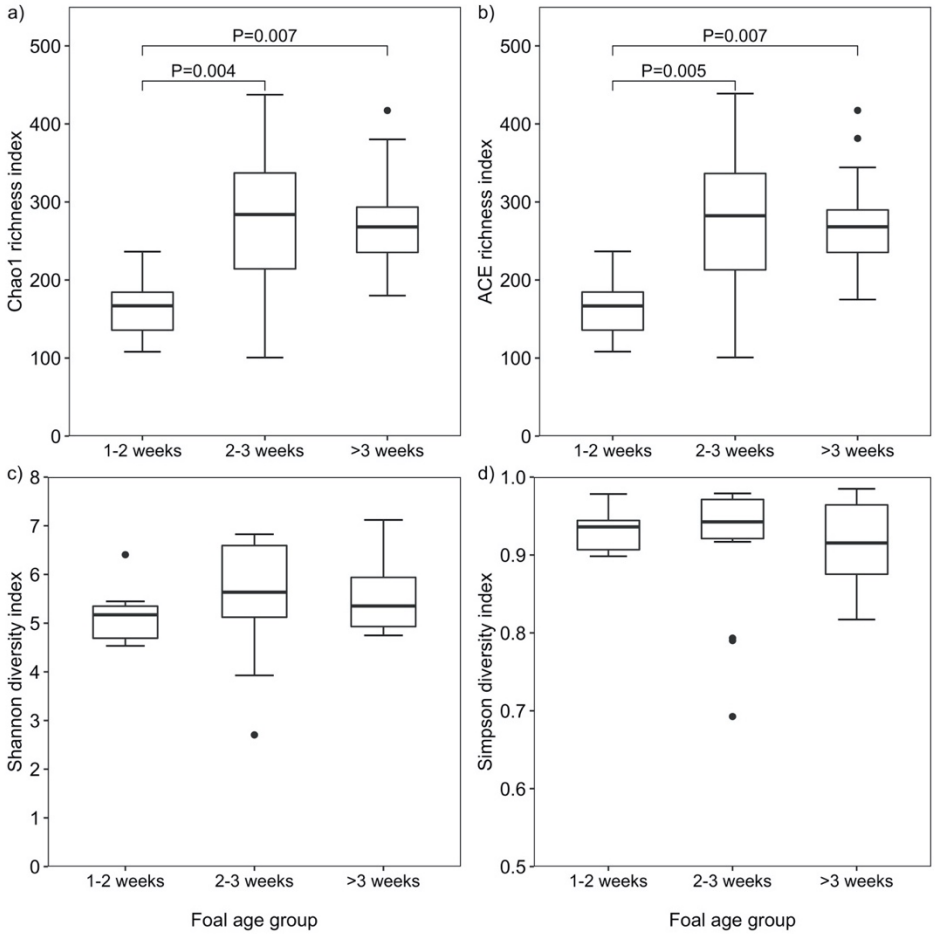

Figure 3-3: Alpha diversity at ASV level of the faecal microbiota of foals in each age group. The richness indices Chao1 (a) and ACE (b), and the Shannon (c) and Simpson (d) diversity indices are shown as box plots, where the upper and lower ends of the boxes indicate the interquartile range, and the whiskers indicate 1.5 times the interquartile ranges. Individual dots indicate outliers. P values were calculated by one-way ANOVA with post-hoc analysis by Tukey's Honest Significant Difference (HSD) test.

3.4.4 Detection of ARGs in foal faeces

Foal samples were screened for a total of 44 ARGs using qPCR. Most ARGs were detected in at least one foal, with the exception of *sul*1, *lnu*B, *mph*C, *msr*A, *qnr*A, *tet*K, *vat*E and *vgb*B. The relative abundances of ARGs in individual foals are presented in Figure 3-4 as proportions relative to the 16S rRNA gene. A total of 10 ARGs were detected in 30/37 of samples (81%) (Table 3-2). The tetracycline resistance genes *tet*Q, *tet*O and *tet*W contributed most to the total ARG abundance in the faecal microbiota, with median relative abundances of 2.3×10^{-2}, 2×10^{-2} and 7.9×10^{-3}, respectively. Other abundant ARGs were *aph*A3, *sat*4 and *mef*A, which had median relative abundances above 3.9×10^{-3}.

The similarities of the ARG profiles were compared between foals grouped by sex, farm and age. There was no significant separation of foals grouped by sex or farm (Fig 3-5a, 3-5b, Supplementary item 3), but foals aged 1-2 weeks had significantly different ARG profiles from those of the older foals ($P<0.01$) (Fig 3-5c, Supplementary item 3).

The relative abundance of each ARG was compared between the age groups. Foals aged 1-2 weeks had a significantly greater abundance of *aph*A3, *sat*4, *tet*M and *tet*X than the older foals ($P<0.01$). ARG abundances did not differ significantly between foals aged 2-3 weeks and those >3 weeks. This is demonstrated in Figure 3-4, with *aph*A3 representative of the aminoglycoside resistance genes, *sat*4 representative of the streptothricin resistance genes, and the *tet* genes representative of the tetracycline resistance genes.

The bla_{SHV} and group 2 type $bla_{CTX\text{-}M}$ extended spectrum beta lactamase (ESBL) genes, as well as the fluoroquinolone resistance gene *qnr*B, were detected in fewer than 10 foals. The relative abundances of these genes were low, from ~10^{-6} to 10^{-4}.

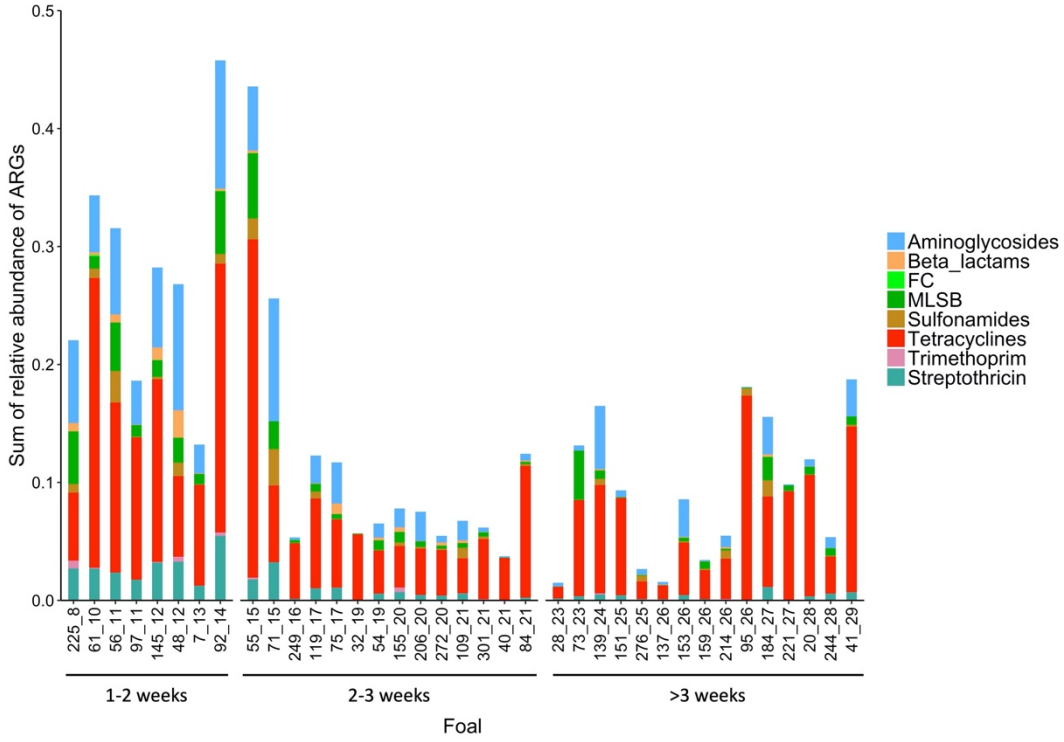

Figure 3-4: Sum of the relative abundances of ARGs (in proportion to the 16S rRNA gene) in each sample. The number after the underscore is the age in days. FC, quinolone/phenicol; MLSB, macrolide-lincosamide-streptogramin B.

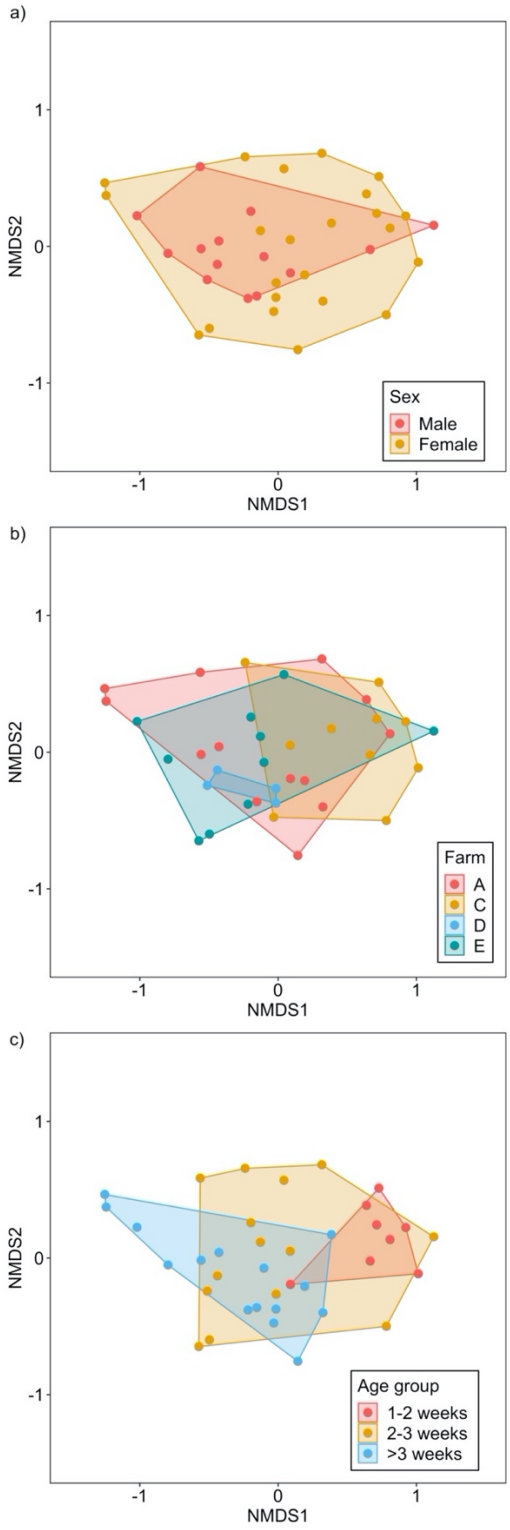

Figure 3-5: Similarity of the ARG profile between foals grouped by sex (a), farm (b) and age (c). NMDS analysis was based on Bray-Curtis distances derived from the relative abundance of ARGs (NMDS stress = 0.15).

3.4.5 Co-occurrence of ARGs in foal faeces

The patterns of co-occurrence of ARGs were analysed using Spearman's rank correlation and examined as networks (Fig 3-6, Supplementary item 4). A cluster comprised of ARGs encoding resistance to the aminoglycosides, beta lactams and trimethoprim, and the class I integron markers *int*I1 and *qac*EΔ, was detected. Other clusters contained *aph*A3, *aad*E, *sat*4 and *tet*M, *str*B and *sul*2, and *erm*F and *tet*X.

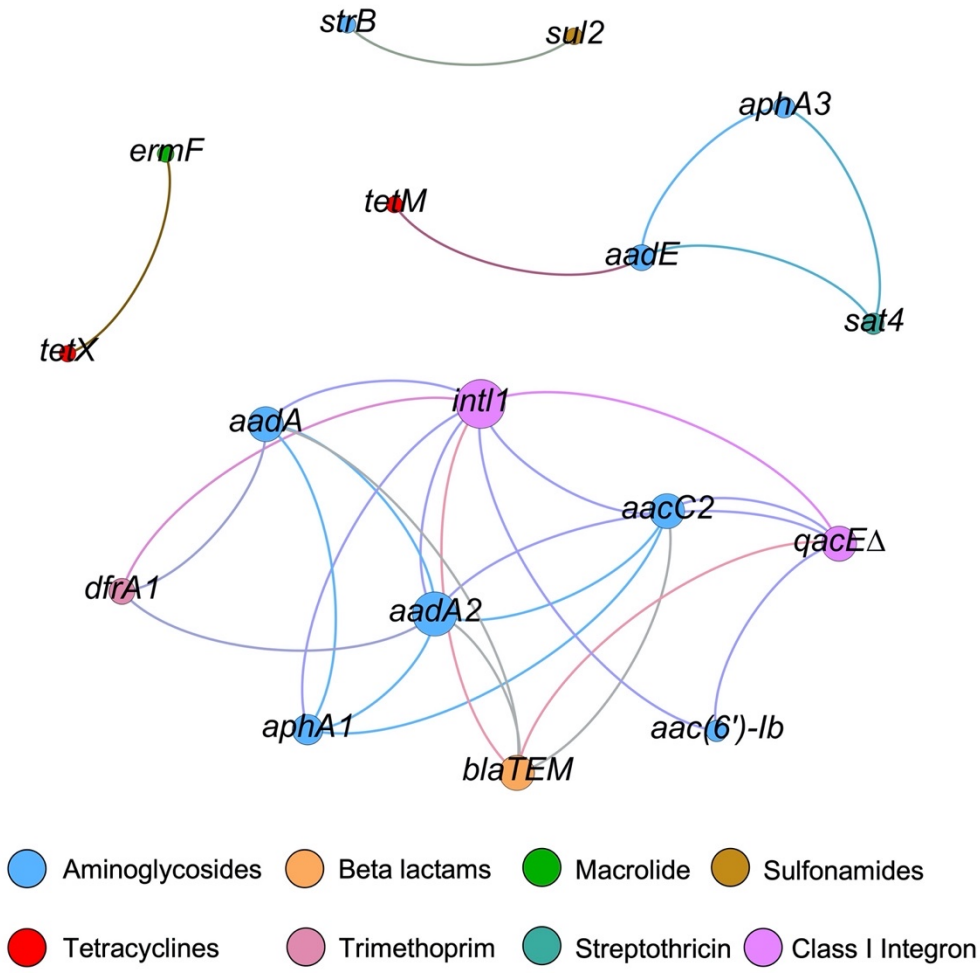

Figure 3-6: Network of co-occurrence patterns of ARGs detected in foals (Spearman's rho$\geqslant$0.75, P<0.01). The size of each node is proportional to the number of connections.

3.5 Discussion

This study described the faecal microbiota and ARG profiles of foals younger than one month of age that were being raised on commercial stud farms. The influence of sex, farm and age on the microbiota and the ARG profiles were examined, and only age was found to distinguish between the groups.

Foals were not sampled longitudinally in this study, so the data do not conclusively prove that the changes in the faecal microbiota were a result of aging, but the features detected in the microbiota of the foals in different age groups were consistent with previous longitudinal studies (Costa et al., 2016, De La Torre et al., 2019). Foals aged 1-2 weeks were found to have significantly lower microbiota richness than the older foals, suggesting that colonisation of the gastrointestinal tract with new bacterial species increased with age. Previous studies have shown that the number of operational taxonomic units (OTUs) increases in the faecal microbiota of foals as they age from 7 days to 28 days and then 60 days (De La Torre et al., 2019), and that foals in the first month of life had fewer OTUs than older foals (Costa et al., 2016).

Our results confirm previous studies that have shown that *Treponema* species are abundant in the adult horse faecal microbiota (O'Donnell et al., 2013, Metcalf et al., 2017) and are likely to interact with cellulolytic bacteria to promote the digestion of fibre (Kudo et al., 1987, Stanton and Canale-Parola, 1980). Members of the phylum *Spirochaetes* have been shown to increase in abundance in the horse faecal microbiota when haylage is introduced into the diet (Salem et al., 2018). Thus, the increase in abundance of *Treponema* species in foals >3 weeks old may be a result of the increasing proportion of forage in their diet. The family *Christensenellaceae* (phylum *Firmicutes*) was also more abundant in foals >3 weeks old. The role of members of the *Christensenellaceae* is less well understood, but their abundance has been found to differ between lean and obese horses (Biddle et al., 2018).

The phyla *Firmicutes* and *Bacteroides* were the two most abundant phyla in the majority of foals, consistent with previous observations in foals older than one week of age (De La Torre et al., 2019, Husso et al., 2020). Other studies have found *Verrucomicrobia* or *Proteobacteria* to be among the most abundant phyla (Costa et al.,

2016, Schoster et al., 2017). Although these earlier reports used the same Illumina MiSeq platform, only the V4 region of the bacterial 16S rRNA gene was sequenced, while our study targeted the V3-V4 region, perhaps contributing to these differences (Bukin et al., 2019). Differences in horse breed, diet and environmental factors could also have affected the faecal microbiota profiles.

Forty-four ARGs encoding resistance to the major antimicrobial classes were screened for using a HT-qPCR array. The tetracycline resistance genes *tet*Q*, tet*O and *tet*W contributed greatly to the total abundance of ARGs. These genes encode cytoplasmic proteins that protect ribosomes from tetracycline, and have been found in a broad range of bacteria, including genera dominant in the foal faecal microbiota (e.g. *Bacteroides, Lactobacillus*, *Ruminococcus* and *Streptococcus*) (Roberts et al., 2012). In addition, these *tet* genes are often associated with mobile genetic elements that can transfer between bacterial species (Chopra and Roberts, 2001). Metagenomic studies have shown that the ribosomal protection *tet* genes are commonly detected at a high abundance in the faecal microbiota of humans and several livestock species (Li et al., 2015, Hu et al., 2013).

Analyses of gene co-occurrence revealed that the abundances of some ARGs could be correlated with class I integron genes. Although analyses of co-occurrence are not sufficient to prove a physical linkage in the genome, they do indicate the potential for horizontal spread of ARGs within the bacterial microbiota.

The co-occurence of *str*B and *sul*2 may be a result of carriage of the *sul*2-*str*A-*str*B cluster on plasmids. RSF1010-like plasmids carrying this gene cluster are widespread in the environment (Yau et al., 2010, Partridge, 2011). The formation and dissemination of this ARG cluster can occur without the selective pressure of antimicrobial agents (Okubo et al., 2019), suggesting a natural prevalence of these genes in the foal faeces. The co-occurence of *aph*A3, *sat*4 and *aad*E could be a result of carriage of the gene cluster *aph*A3-*sat*4-*aad*E on Tn5404-like elements and members of the Tn1545/Tn6003 family, which have been found in Gram-positive bacteria (Werner et al., 2003, Palmieri et al., 2012).

The *bla*$_{SHV}$ and group 2 type *bla*$_{CTX-M}$ ESBL genes, and the fluoroquinolone resistance gene *qnr*B, were rarely detected in the foals, suggesting that young foals without direct antimicrobial exposure were unlikely to carry these genes in their faecal microbiota. A limitation of this study is that the group 1 type *bla*$_{CTX-M}$ genes were not included in the HT-qPCR assay. These genes have often been detected in ESBL-producing *E. coli* from horses and could be added in future studies (Dolejska et al., 2011, Walther et al., 2018). If comprehensive coverage of ARGs is required, then alternative sequencing strategies could be used, for example, metagenomic sequencing (Li et al., 2015).

In our study, the overall faecal microbiota of foals did not differ between farms. Differences in farm management, such as the amount of access to pasture and the housing conditions, have been identified as factors influencing the faecal microbiota in other studies (De La Torre et al., 2019, Kaiser-Thom et al., 2020), but an evaluation of the impact of management practices on the microbiota was not a primary aim of this study. While the majority of mares and foals were kept in grass paddocks, detailed information on this variable was not collected. In addition, the small number of foals from each farm evaluated in this study restrained the statistical power of any investigations of potential differences in the microbiota attributable to differences in farm management. This should be a fruitful area for future research. Another limitation was the lack of any history about antimicrobial treatment of the dams of the foals. A study in humans demonstrated that antimicrobial treatment of mothers during delivery may have affected the composition of the microbiota and the level of ARGs and mobile genetic elements in the infant gut (Pärnänen et al., 2018). This, in addition to the coprophagic behaviour of foals, may have influenced our results. Therefore, future studies that aim to describe the influences of age and farm management on the development of the faecal microbiota of healthy foals should have a larger sample size and record farm management practices in greater detail. This will allow investigation of any variation in the foal faecal microbiota and ARG profiles associated with differences in farm management.

In conclusion, this study analysed faecal microbiota and ARG profiles in Thoroughbred foals aged from 8 to 29 days raised on commercial stud farms in Australia. Age was found to influence the richness and composition of the faecal microbiota. The

tetracycline resistance genes *tet*Q, *tet*O and *tet*W were the largest contributors to the total abundance of ARGs in the faeces, probably because of the high concentrations of potential host genera for these genes in the normal foal faecal microbiota. The observation of correlations between specific ARGs and class I integron genes suggested indirect exposure to antimicrobials on the farms.

Manufacturers' addresses:

[a]Qiagen, Hilden, Germany.

[b]MP Biomedicals, Irvine, California, USA.

[c]NanoDrop Technologies, Wilmington, Delaware, USA.

[d]Takara Bio USA, Mountain View, California, USA.

Table 3-1: Taxa with significant differences in abundance between different age groups of foals

		ANCOM W†	Relative abundance (%)		Abundance ratio (>3 w/1-2 w)
			1-2 w	>3 w	
1-2 weeks vs >3 weeks	**Family**				
	Christensenellaceae	40	0.48	4.27	8.9
	Odoribacteraceae	37	6.13	1.24	0.2
	Genus				
	Unclassified Christensenellaceae	78	0.48	4.27	8.9
	Butyricimonas	68	3.92	0.41	0.1
	Unclassified Clostridiales 1	64	0.02	0.46	23
	Eubacterium	62	0.43	0.04	0.1
	Treponema	61	0.02	2.78	139
		ANCOM W†	**Relative abundance (%)**		**Abundance ratio (>3 w/2-3 w)**
			2-3 w	**>3 w**	
2-3 weeks vs >3 weeks	**Family**				
	Odoribacteraceae	34	3.05	1.24	0.4
	Christensenellaceae	33	2.38	4.27	1.8
	Genus				
	Butyricimonas	65	1	0.41	0.4
	Unclassified Christensenellaceae	64	2.38	4.27	1.8

† Differing abundances of taxa between age groups were assessed using ANCOM, adjusting for farm. For each of the n taxa, the mean abundance between two age groups was compared to the data for the other n-1 taxa. W is the number of null hypotheses rejected for the taxa (which can range from 0 to n-1). Taxa with a proportion of rejected hypotheses greater than 0.7 were considered to have a significantly different abundance between age groups. The ANCOM comparison was performed using 86 genera and 47 families. ANCOM does not assign a P value for the final result, but P values with a Benjamini-Hochberg correction were used in the pipeline to calculate W.

w, weeks of age

Table 3-2: Relative abundances of ARGs detected in more than 80% samples

Gene	Relative abundance to 16S rRNA gene		
	Min	Max	Median
*aad*E	4.3×10^{-5}	2.9×10^{-2}	1.5×10^{-3}
*aph*A3	1.8×10^{-4}	7.7×10^{-2}	9.6×10^{-3}
*str*B	1.4×10^{-5}	4.3×10^{-2}	2×10^{-3}
*mef*A	8.9×10^{-5}	5×10^{-2}	3.9×10^{-3}
*sul*2	9.6×10^{-6}	3.1×10^{-2}	1.4×10^{-3}
*tet*M	2.3×10^{-5}	2.5×10^{-2}	8.1×10^{-4}
*tet*O	1.2×10^{-3}	3.1×10^{-2}	7.9×10^{-3}
*tet*Q	1.7×10^{-3}	2.4×10^{-1}	2.3×10^{-2}
*tet*W	4.6×10^{-3}	1.2×10^{-1}	2×10^{-2}
*sat*4	2.3×10^{-4}	5.5×10^{-2}	5.1×10^{-3}

3.6 Supplementary items

Supplementary item 1: Information on sampled foals.

SampleID	Farm	Age	Sex
F_225	C	8	female
F_61	C	10	female
F_56	A	11	female
F_97	A	11	female
F_145	C	12	female
F_92	C	14	female
F_71	C	15	female
F_75	C	17	female
F_119	D	17	female
F_54	C	19	female
F_32	E	19	female
F_155	E	20	female
F_206	E	20	female
F_84	A	21	female
F_301	E	21	female
F_28	A	23	female
F_73	C	23	female
F_139	A	24	female
F_137	A	26	female
F_95	A	26	female
F_184	C	27	female
F_20	D	28	female
F_41	A	29	female
F_48	C	12	male
F_7	A	13	male
F_55	E	15	male
F_249	D	16	male
F_272	D	20	male
F_40	A	21	male
F_109	E	21	male
F_276	E	25	male
F_151	E	25	male
F_214	A	26	male
F_153	E	26	male
F_159	E	26	male
F_221	A	27	male
F_244	A	28	male

Supplementary item 2: List of primer pairs for each of the target genes.

Primers in supplementary item 2 are showed in Appendix I.

Supplementary item 3: Adjusted P-values of PERMANOVA analysis of pairwise comparisons of microbiota and ARGs data between sex, farm and age groups.

Microbiota data:

Sex			
	Male		
Female	0.74		
Farm			
	A	C	D
C	0.021	-	-
D	0.131	0.021	-
E	0.319	0.086	0.163
Age			
	1-2 weeks	2-3 weeks	
2-3 weeks	0.022	-	
>3 weeks	**0.003**	0.223	

ARGs data:

Sex			
	Male		
Female	0.055		
Farm			
	A	C	D
C	0.034	-	-
D	0.544	0.018	-
E	0.568	0.018	0.884
Age			
	1-2 weeks	2-3 weeks	
2-3 weeks	**0.003**	-	
>3 weeks	**0.003**	0.924	

Supplementary item 4: Spearman ‘s rho of co-occurring ARGs and MGEs.

Gene	Gene	Spearman’s rho
aac(6')-Ib	*qacEΔ*	0.84
aac(6')-Ib	*intl1*	0.77
aacC2	*intl1*	0.93
aacC2	*aphA1*	0.86
aacC2	*blaTEM*	0.82
aacC2	*aadA2*	0.82
aacC2	*qacEΔ*	0.82
aadA	*aadA2*	0.81
aadA	*aphA1*	0.79
aadA	*dfrA1*	0.78
aadA	*blaTEM*	0.77
aadA2	*intl1*	0.85
aadA2	*qacEΔ*	0.83
aadA2	*blaTEM*	0.83
aadA2	*aphA1*	0.79
aadA2	*dfrA1*	0.75
aadE	*sat4*	0.84
aadE	*aphA3*	0.81
aadE	*tetM*	0.78
aphA1	*intl1*	0.81
aphA3	*sat4*	0.92
blaTEM	*intl1*	0.85
blaTEM	*qacEΔ*	0.8
dfrA1	*intl1*	0.79
sul2	*strB*	0.82
ermF	*tetX*	0.87
intl1	*qacEΔ*	0.9

Chapter 4 Analysis of faecal microbiome and ARG profile of bobby calves from a wide range of farms in Victoria

4.1 Introduction

Calves undergo a serial of physiological changes in their gastrointestinal tracts from born to weaning (Guilloteau et al., 2009). Calves younger than two to three weeks are almost entirely fed with milk (Drackley, 2008). Due to the reflex closure of the reticular groove, milk will not pass through the rumen but directly into the abomasum and small intestine for digestion (Drackley, 2008). Functionally, calves in this stage are considered as non-ruminant. The pre-weaned calves have a dynamic gut microbiome, whose species richness and diversity have been shown to increase with calf age (Klein-Jöbstl et al., 2014, Mayer et al., 2012, Oikonomou et al., 2013). In addition, diet (Deng et al., 2017) and feed additives (Malmuthuge et al., 2015, Foditsch et al., 2015) can influence microbiome composition. Dysbiosis in the gut microbiome was found associated with diarrhoea in calves in the first month of life (Gomez et al., 2017, Oikonomou et al., 2013). In addition, Arumugam et al. proposed the concept of enterotype which is based on the taxonomic similarity to cluster samples into bins to investigate the microbiome variation (Arumugam et al., 2011). Human and animal studies have shown the enterotype might be associated with the host age, living environment, diet, health state, and metabolism, which suggests the role of enterotype in reflecting some host characteristics (Wu et al., 2011, Costea et al., 2018, Ramayo-Caldas et al., 2016, Le Sciellour et al., 2019, Wang et al., 2020, Yuan et al., 2020).

Antimicrobial exposure is one of the important factors that impacts the cattle gut microbiome composition. Thomas et al. showed that feeding steers with monensin and tylosin additives reduced both richness and diversity of microbiome in the rumen (Thomas et al., 2017). The treated steers had a reduced level of Gram-positive bacteria, such as *Ruminococcus* spp., *Erysipelotrichaceae* and *Lachnospiraceae*, in their gastrointestinal tract, however, the level of Gram-negative *Veillonellaceae* family increased. Moreover, Chambers et al. demonstrated that the 3-day ceftiofur injection

treatment in dairy cattle lead to increase in class *Bacteroidia* and a decrease in class *Actinobacteria* in their faeces (Chambers et al., 2015).

Recent studies using metagenomic sequencing and qPCR techniques have investigated ARG profiles of the faecal microbiome in dairy and beef cattle (Santamaría et al., 2011, Noyes et al., 2016, Zhou et al., 2016, Thomas et al., 2017, Weinroth et al., 2018). Although variations in ARG abundance were observed between individual animals, tetracycline resistance genes encoding ribosome protection proteins, particularly *tet*Q, *tet*O and *tet*W, were generally the most abundant genes in the cattle microbiome. Different antimicrobial treatments have been found to affect ARG levels in various ways. Weinroth et al. examined the effect of ceftiofur or ceftiofur combined with chlortetracycline treatment on the gut resistome of steers (Weinroth et al., 2018). Ceftiofur treatment was not associated with changes in the level of beta-lactamase genes whereas chlortetracycline treatment led to a significantly increase in level of tetracycline resistance gene 26 days after treatment. Nevertheless, few studies used culture-independent approaches to examine ARGs in pre-weaned calves (Noyes et al., 2016, Thames et al., 2012). Noyes et al. showed the faecal resistome was higher in diversity and richness in calves than in adult cattle, which might reflect the differences in the antimicrobial use practice between these two groups (Noyes et al., 2016). Thames et al. showed adding tetracycline and neomycin in milk replacer for calves had little effect on the level of tetracycline, macrolide and sulfonamide resistance genes (Thames et al., 2012). Tetracycline resistance genes increased along with age regardless of antimicrobial exposure.

In Australia, a bobby calf is a calf not accompanied by its dam and under the age of 4 weeks (Agriculture Victoria, 2017). Bobby calves are usually male and most are slaughtered. Handling and transporting calves to the abattoir can incur complex stimuli (Jongman and Butler, 2013), which could affected the calves psychologically, physically and physiologically (Trunkfield and Broom, 1990). Studies have assessed the effect of transportation on calf mortality (Cave et al., 2005), physiological responses (Kent and Ewbank, 1986, Fell and Shutt, 1986) and immunological responses (Kent and Ewbank, 1986, Mackenzie et al., 1997). However, little is known about the gut microbiome profile of young calves after transportation. In addition, as food animals, the shedding

of ARGs in the calf faecal microbiome is a concern for food safety but there has been lack of study analysing the ARGs from a microbiome perspective in calves raised in commercial farms.

The aims of this study were to 1) characterise the faecal microbiome of bobby calves after being transported; 2) quantify ARGs of the major antimicrobial classes in the calve faeces. Bobby calves involved in this study were originally from 76 farms in Victoria. The faecal microbiome and ARG profile were analysed by 16S rRNA sequencing and high throughput qPCR, respectively.

4.2 Methods

4.2.1 Animals and sample collection

Seventy-six calves were sampled in a commercial abattoir in Victoria during spring in 2015 and autumn in 2016. They came from 76 farms and one calf per farm was sampled. These farms were distributed across the north and south-east parts of Victoria. The transportation and lairage duration were from 15 to 23h based on 45 calves with time records but the duration should not go over 30h for any calves.

Faeces was collected from the rectum content of each calf soon after slaughter, stored in a sterile tube at -4˚C and transported to laboratory within approximately 2 hours. Then, samples were aliquoted into sterile microcentrifuge tubes and stored at -80˚C until DNA extraction.

4.2.2 DNA extraction

Faecal DNA extraction was according to “Section 2.1 DNA extraction from faecal and swab samples”.

4.2.3 Bacterial 16S rRNA gene sequencing and bioinformatics analysis

Bacterial communities were characterised according to “Section 2.2 Bacterial 16S rRNA gene sequencing and bioinformatic analysis”.

4.2.4 Detection and quantification of ARGs in calf faeces

ARGs were detected and quantified according to “Section 2.3 HT-qPCR array for detection and quantification of ARGs”.

4.2.5 Statistical analysis

All statistical analyses were completed using QIIME2 (Bolyen et al., 2019) and RStudio. The enterotypes of calf faecal microbiomes were determined following the method of (Arumugam et al., 2011), except the weighted UniFrac matrix was used in this study. Differences in the relative abundance of bacterial taxa at phylum, family and genus level between calves grouped by enterotypes were tested by ANCOM (Mandal et al., 2015).

Alpha diversity of the faecal microbiome of each calf was measured based on ASV using the Chao1 index for richness and Shannon and Simpson diversity indices for both richness and evenness. Data were tested for normality based on the Shapiro-Wilk method. Differences in alpha diversity between calves grouped by enterotypes were tested by one-way ANOVA or the Kruskal-Wallis rank sum test. A *p* value less than 0.05 was considered as a significant difference.

The similarity of faecal microbiomes between calves grouped by farm location (northern vs southern Victoria) and enterotype was analysed using NMDS with weighted UniFrac matrix derived from ASVs. The similarity was tested by PERMANOVA using the 'adonis' function in the Vegan R package.

The similarity of ARG profiles between calves with different enterotypes was analysed by NMDS using a Bray-Curtis matrix. The similarity was validated as shown above.

The co-occurrence patterns of ARGs were analysed according to "Section 2.4 Co-occurrence of ARGs and MGEs".

4.3 Results

4.3.1 Diversity of the calf faecal microbiome

A total of 5,027,518 high quality reads were obtained from the 76 calf samples. The number of sequences per sample ranged from 20,369 to 988,983. Sequence depth in each sample was rarefied to 20,369 for fair comparison and all samples had sufficient depth for accurate estimate of richness and diversity. The rarefied reads were partitioned into 622 ASVs which were further assigned with taxonomic information.

Good's coverage index of all samples ranged from 0.99 to 1, indicating sufficient sequence depth for accurate estimation of microbiota richness and diversity.

A total of six phyla were identified from all samples (Fig 4-1a). *Firmicutes* and *Bacteroidetes* were the most dominant phyla which accounted for 39% and 35% of the total reads, respectively. *Proteobacteria* and *Fusobacteria* accounted for 12%, respectively. *Actinobacteria* and *Verrucomicrobia* together accounted for only 2% of the total reads.

The six phyla comprised sequences that could be classified into 35 families and 92 genera (Fig4-1b, 4-2c). Eight families and 13 genera had a relative abundance greater than 0.5% in at least 33% samples. The most abundant family was *Bacteroidaceae* (32.6%), followed by *Ruminococcaceae* (19.2%), *Fusobacteriaceae* (11.8%), *Enterobacteriaceae* (9.1%), *Lactobacillaceae* (7.2%), *Lachnospiraceae* (6.3%), *Alcaligenaceae* (1.7%) and *Veillonellaceae* (1.6%). The most abundant genus was *Bacteroides* spp. (31.7%), followed by *Faecalibacterium* spp. (13.3%), unclassified *Enterobacteriaceae* (7.7%), *Lactobacillus* spp. (7.2%), *Fusobacterium* spp. (6%) and *Butyricicoccus* spp. (3.9%).

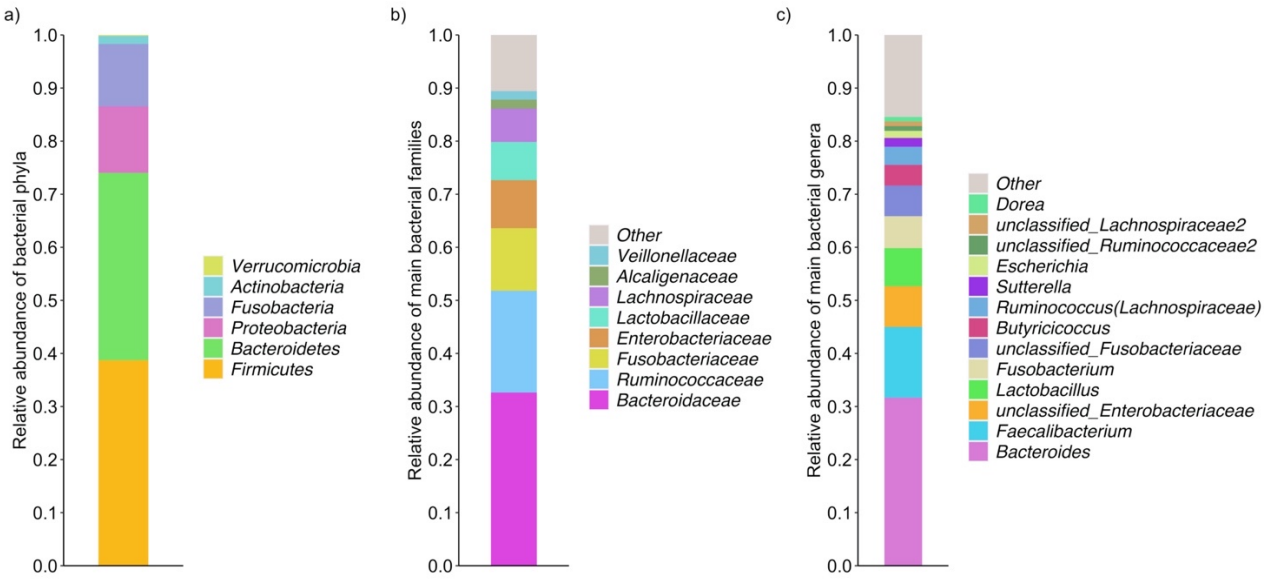

Figure 4-1: The overall relative abundance of bacterial phyla (a), dominant family (b) and genus (c) in the calf faecal microbiome. Only families and genera with a relative abundance above 0.5% in more than 33% samples are listed. Families and genera below the cutoff were assigned as "Other".

Faecal microbiome richness of each calf faecal sample was measured by the Chao1 index, which ranged from 23 to 118.1, and the microbiome diversity was measured by Shannon and Simpson diversity indices, which ranged from 1.5 to 5.1 and 0.4 to 0.95, respectively. Correlations of alpha diversity indices with transportation and lairage duration were tested by Spearman rank correlation. None of the diversity indices were found to statistically correlate to the transportation or lairage duration.

Similarity of the faecal microbiome at genus level between calves from north (n = 25) and south (n = 51) Victorian farms were compared by NMDS using a weighted UniFrac matrix (Fig 4-2). PERMANOVA analysis indicated no significant differences in calf faecal microbiome based on the farm locations ($R^2 < 0.003$, $p = 0.98$).

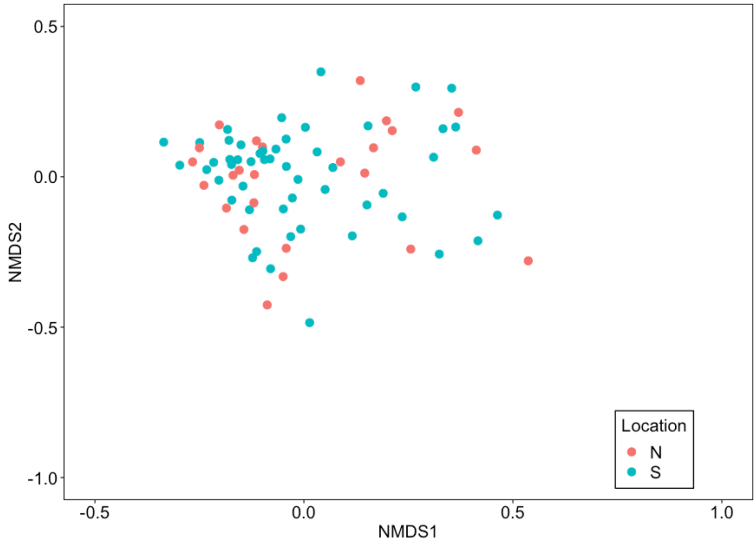

Figure 4-2: Similarity of faecal microbiome at genus level between calves grouped by farm location. N: Northern Victoria; S: Southern Victoria.

4.3.2 Detection of enterotype

The enterotype of the faecal microbiomes of the calves was detected following the clustering method of Arumugam et al (Arumugam et al., 2011), except the weighted UniFrac matrix was used in this study. The analysis showed that calf faecal microbiomes at genus level were grouped into two clusters, or enterotypes. Thirty-six calves had the faecal microbiome of enterotype 1 and 40 calves had enterotype 2.

PERMANOVA analysis showed 23% of the variations in the microbiome composition could be explained by the enterotypes (Fig 4-3a) ($R^2 = 0.23$, $P < 0.01$).

The relative abundance of bacterial taxa between the two enterotypes was compared by ANCOM analysis. Statistical differences at the genus level between the two enterotypes is summarised in Figure 4-3b. Calves with enterotype 1 had a statistically higher abundance of *Faecalibacterium* spp. than calves with enterotype 2, whereas calves with microbiomes of enterotype 2 showed significantly higher abundances of *Escherichia* spp., an unclassified *Enterobacteriaceae* and *Clostridium* spp. (Table 4-1). At the family level, enterotype 2 microbiomes displayed higher abundances of *Clostridiaceae* and *Enterobacteriaceae* than those of enterotype 1. In addition, phylum *Proteobacteria* and *Fusobacteria* were generally at higher levels in calves of enterotype 2 than enterotype 1.

Table 4-1: Taxa with significant difference in abundance between calves of each enterotype

Genus	ANCOM W[a]	Relative abundance (%)		Abundance ratio (Enterptype1/Enterotype2)
		Enterotype1	Enterotype2	
Faecalibacterium	36	23.4	4.3	5.44
Escherichia	33	0.4	2.1	0.19
unclassified_Enterobacteriaceae	32	2.4	12.4	0.19
Clostridium (*Clostridiaceae* family)	32	0.1	4.7	0.03
Family				
Clostridiaceae	21	0.2	5.3	0.04
Enterobacteriaceae	21	2.8	14.7	0.19

[a] In ANCOM, for each of the n taxa, the mean abundance between two age groups is compared to the data on the other n-1 taxa. The W is denoted as the number of null hypotheses rejected for the taxa (which can range from 0 to n-1). ANCOM comparison was done within 39 genera or 24 families in total

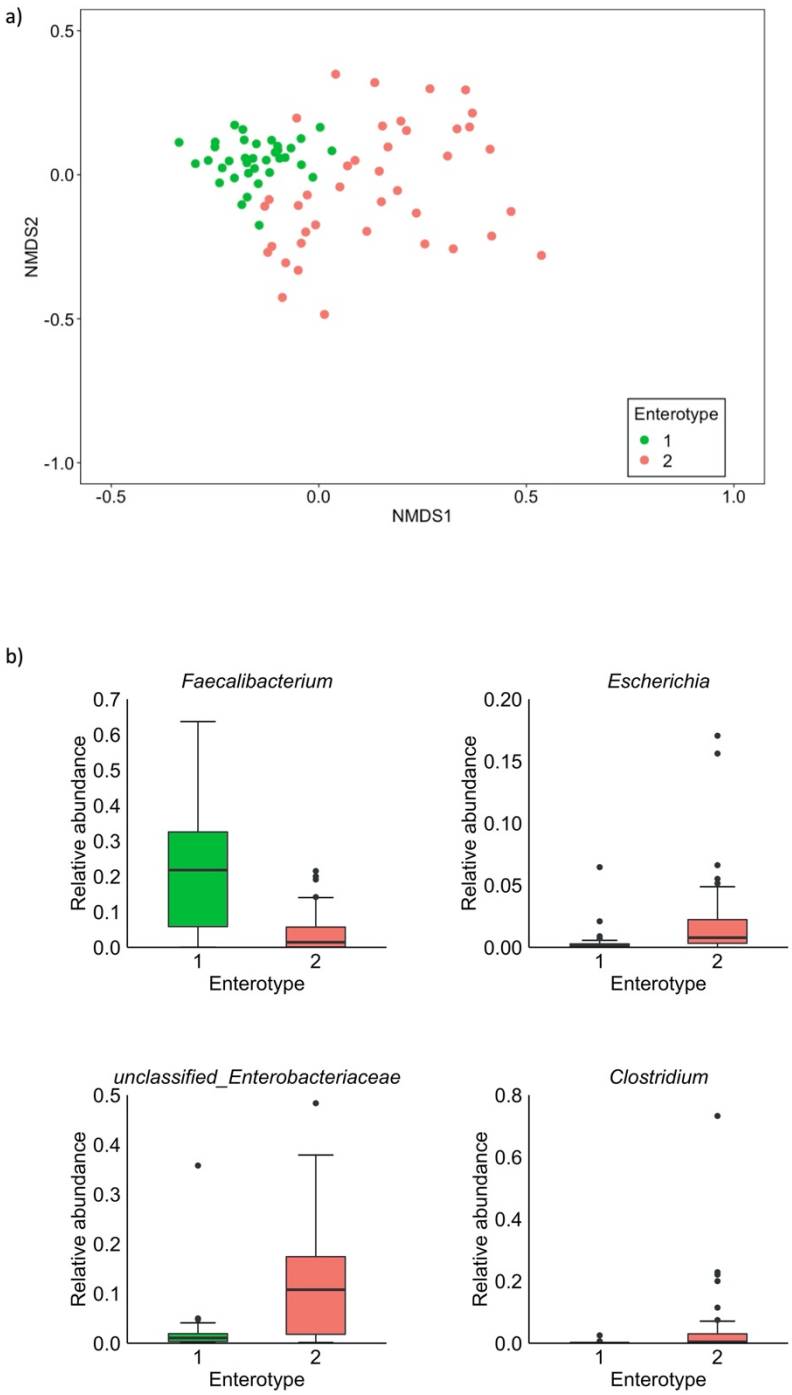

Figure 4-3: Presence of two enterotypes in the calf faecal microbiome at genus level (a) and the genera with statistically different abundance between calves grouped by enterotypes (b). The relative abundance of each genus in each group is shown as a box plot, where the lower and upper end of the box represent the interquartile range, and whiskers represent 1.5 times the interquartile range. The individual dots represent outliers.

4.3.3 Comparison of alpha diversity between enterotypes

Microbiome richness measured by Chao1 showed enterotype 1 had a significantly higher richness than enterotype 2 (ANOVA, $P < 0.01$) (Fig 4-4a). Microbiome diversity measured by Shannon and Simpson indices showed no statistical differences between the two enterotypes (Fig 4-4b, 4-4c).

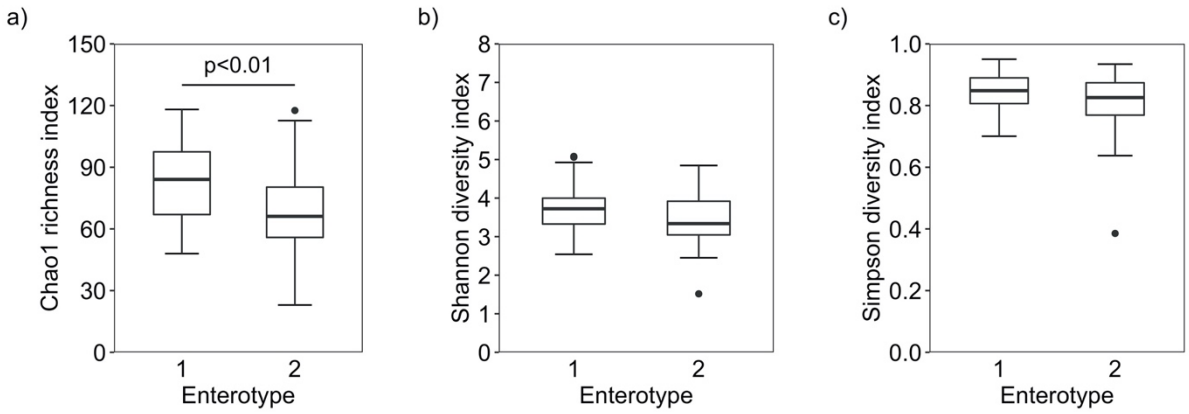

Figure 4-4: Alpha diversity based on ASV of faecal microbiomes of calves grouped by enterotype. The Chao1 index (a), Shannon index (b) and Simpson index (c) for each group are shown as box plots, where the lower and upper end of the box represents the interquartile range, and the whiskers represent 1.5 times interquartile range. Individual dots represent outliers. Enterotype 1 had a significantly higher Chao1 richness index than enterotype 2 (ANOVA*, $P < 0.01$*). There were no significant differences of Shannon and Simpson diversity index between the two enterotypes.

4.3.4 Detection of ARGs

From the total 48 primers targeting 44 ARGs and three MRGs, 41 ARGs and all three MRGs were detected in at least one sample. The number of detected genes in each sample ranged from 4 to 27, with the median of 18 (Fig 4-5a). The relative abundance of ARGs and MRGs are presented as proportions to 16S rRNA (Fig 4-5b), and varied greatly between individuals, ranging from 7.7×10^{-3} to 5.3, with the median of 3.4×10^{-1}.

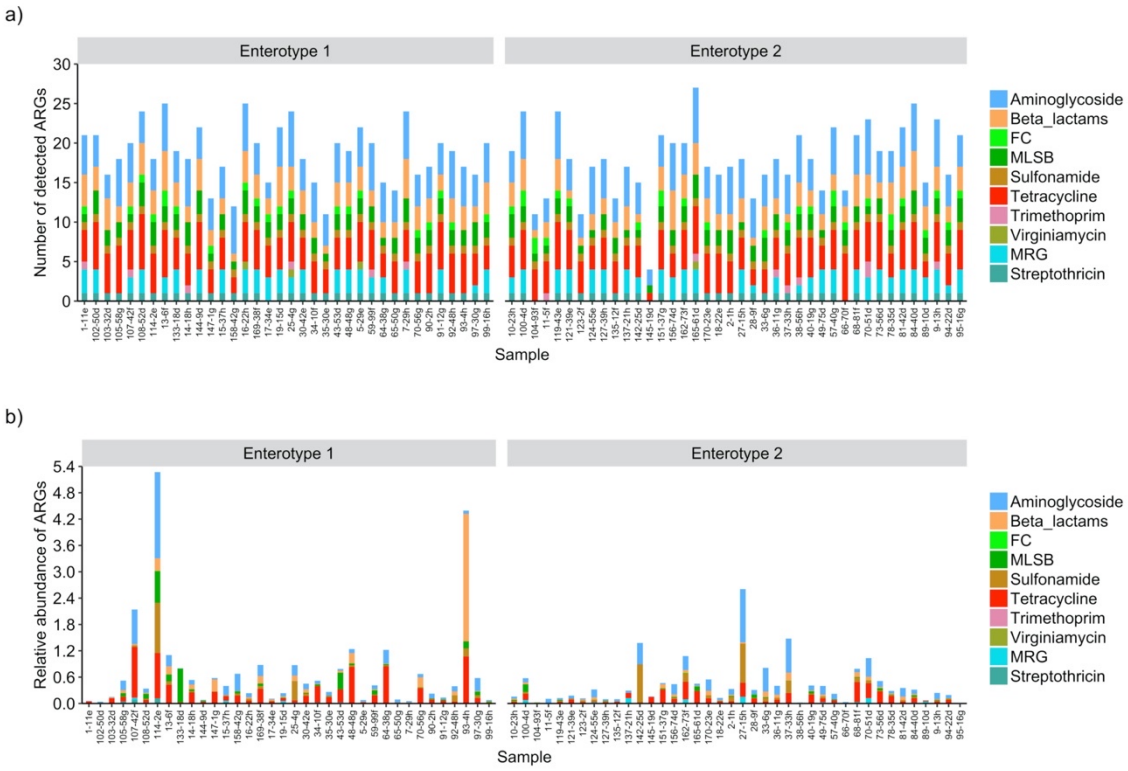

Figure 4-5: Number of detected ARGs (a) and the cumulative relative abundance (in proportion to the 16S rRNA gene) of detected ARGs (b). Each bar represents an individual calf. FC, fluoroquinolone/florfenicol; MLSB, macrolide-lincosamide-streptogramin B; MRG, metal resistance gene.

Among the detected resistance genes, 14 ARGs were detected in more than 80% of samples (60 out of 76) (Fig 4-6a). These ARGs contributed most to the total ARG abundance of all calves (Fig 6b), which potentially conferred resistance to aminoglycoside, beta-lactamase, macrolide, sulfonamide, tetracycline and streptothricin. Among these ARGs, *tet*Q, *tet*W, *mef*A, *str*B, *sul*2 and *cfx*A were generally at higher abundance (~10^{-2}) than the other ARGs.

As virginiamycin is permitted to be used in dairy cattle in Australia, two virginiamycin resistance genes *vatE* and *vgbB* were tested in the samples. No calves were positive for *vgbB* while *vatE* was only detected in four calves, and their low relative abundances (10^{-5} to 10^{-4}) approached the limits of detection (~10^{-5}).

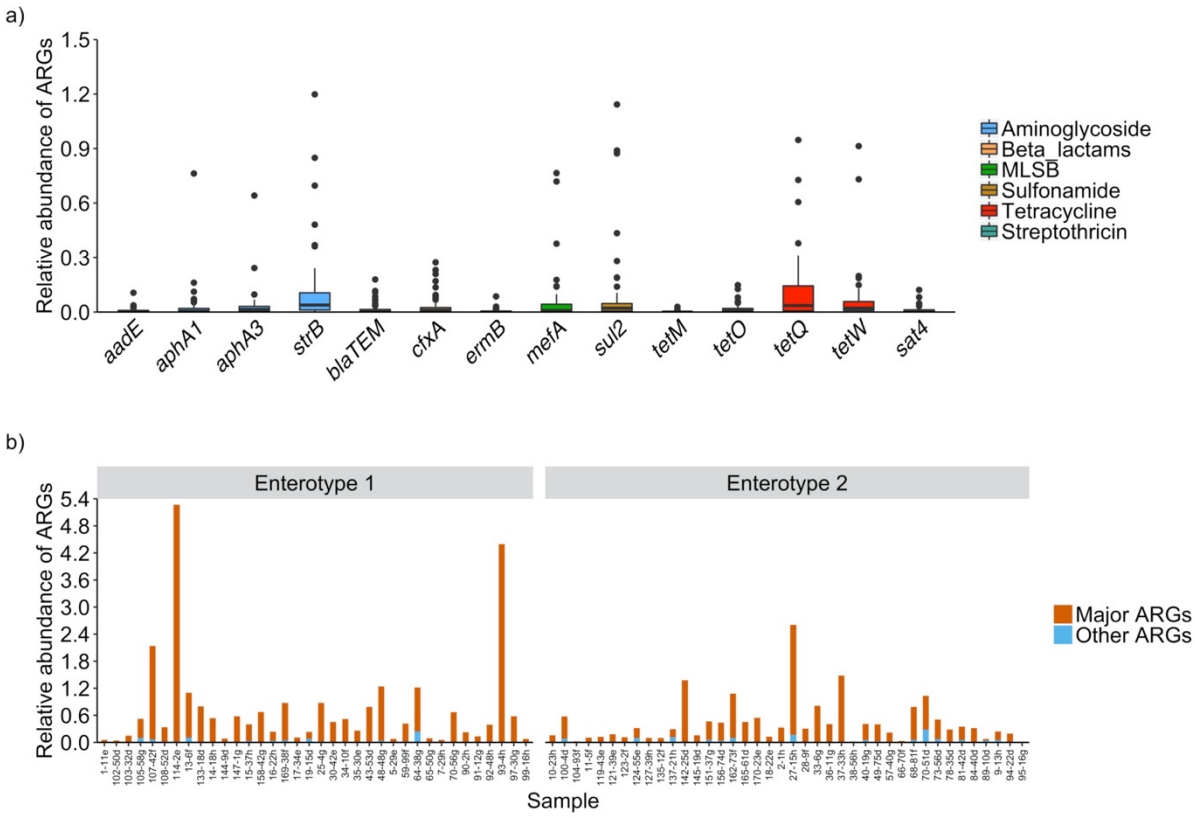

Figure 4-6: Relative abundance of the 14 ARGs detected in more than 80% of samples (a) and the total relative abundance of the 14 ARGs (major ARGs) in each sample (b).

4.3.5 Similarity of ARG profiles between calves

The ARG composition was compared between calves based on farm locations (north vs south Victoria) and based on enterotypes. PERMANOVA analysis indicated no distinct separation of ARG profile between calves grouped by farm locations (R^2 = 0.02, p = 0.2) or by enterotype (R^2 = 0.05, p = 0.001) (Fig 4-7a, 4-7b).

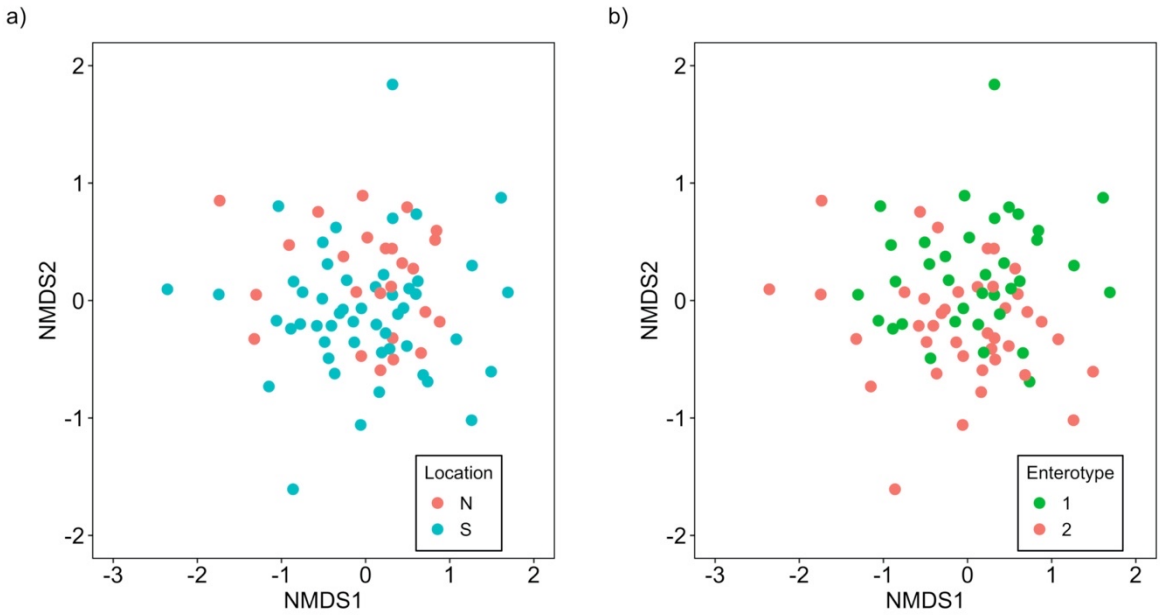

Figure 4-7: Similarity of faecal resistance gene profiles based on the gene relative abundance of resistance genes and MGEs between calves grouped by farm location (a) and enterotype (b). N: Northern Victoria; S: Southern Victoria. Samples did not cluster based on grouping.

4.3.6 Co-occurrence of ARGs in calf faeces

To identify potential ARG associations or genetic linkage, the co-occurred ARGs were analysed by Spearman rank correlation and displayed as networks (Spearman's rho ≥ 0 .75, $P < 0.01$) (Fig 4-8). The network was parsed into six clusters made of 13 genes in total with each cluster comprising two or three genes. The aminoglycoside resistance gene *aad*A connected to *aad*A2; *aph*A1 connected to beta-lactamase resistance gene bla_{TEM}; *str*B connected to sulfonamide resistance gene *sul*2, and *aph*A3 connected to streptothricin resistance gene *sat*4. The macrolide resistance gene *mef*A connected to mobile element *IS614*. The MRGs, *sil*R, *pco*A and *pco*R, formed one cluster without connecting to any ARGs.

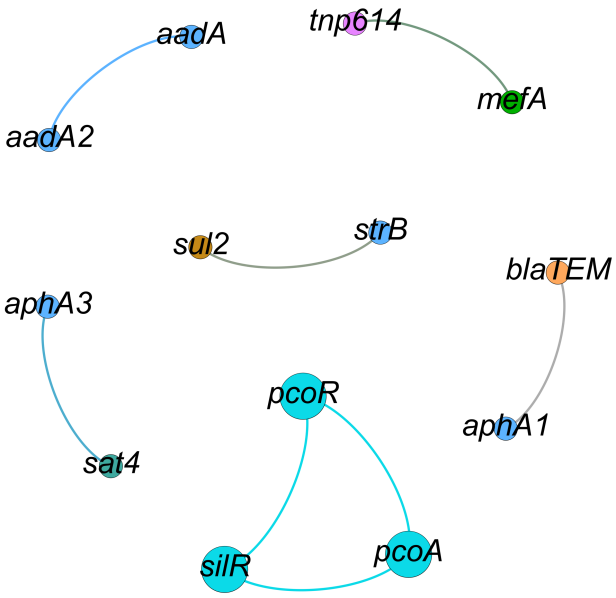

Figure 4-8: Network of co-occurred ARGs in calf faeces (Spearman's rho ≥ 0.75, $P < 0.01$). The size of each node is proportional to the number of connections.

4.4 Discussion

This study analysed faecal microbiome and ARG profiles in 76 bobby calves collected from a commercial abattoir in Victoria. Calves were originally from a wide range of farms in north and south parts of Victoria. Calves were transported to the abattoir and rested overnight before slaughter. The transportation and lairage duration ranged between 15 to 23h based on calves with time records. The results showed the faecal microbiome composition of young calves that had come from wide geographic locations in Victoria and after being transported. ARG analysis was undertaken in order to better understand the background carriage of ARGs in the faecal bacterial community of calves, and its potential significance in food safety.

In this study, *Firmicutes* and *Bacteroidetes* dominated the faecal bacterial community in most samples, which was also reported in prior calf faecal microbiome studies (Uyeno et al., 2010, Oikonomou et al., 2013, Klein-Jöbstl et al., 2014). Nevertheless, the relative abundance of each phylum varied between studies. Our study found the

overall *Firmicutes/Bacteroidetes* ratio was nearly 1:1. Klein-Jobstl et al found the ratio varied between 0.1 to 0.4 with calves aged from 12h to 11 weeks (Klein-Jöbstl et al., 2014) , whereas, Oikonomou et al. reported higher ratios (6.2 to 46.1) in calves aged from one to seven weeks (Oikonomou et al., 2013). In calves aged from one to four weeks fed with pasteurised or unpasteurised waste milk, the ratio varied between 0.7 to 1.3 and 1.5 to 2.5, respectively (Edrington et al., 2012). The divergence may due to differences in animals, geographical locations and methods, such as the bacterial 16S rRNA hypervariable regions targeted in the various studies (Bukin et al., 2019). Human studies suggested the ratio was correlated with obesity (Ley et al., 2005), however, conflicting results were reported about how the ratio differs between obesity and lean humans, and whether the difference was statistically significant (Graessler et al., 2013, Walters et al., 2014).

The *Firmicutes* phylum was largely represented by bacteria of *Ruminococcaceae*, *Lachnospiraceae* and *Lactobacillaceae* families. *Ruminococcaceae* and *Lachnospiraceae* have been found to be abundant in the mammalian gut environment, and their ability to assist in fibre digestion is particularly important for herbivores extracting energy from their normal diet (Biddle et al., 2013, Dougal et al., 2013, Li, 2017). The relatively high abundance of *Lactobacillus* spp., found in the present study was similar to results of prior studies on pre-weaned calves (Klein-Jöbstl et al., 2014, Song et al., 2017). Schwaiger et al. demonstrated that the counts of *Lactobacillus* spp. isolates from calf faeces increased in the first week of life and *Lactobacillus reuteri* was the most common species (Schwaiger et al., 2020). Moreover, the number of *Lactobacillus* was associated with milk feeding. Alimirzaei et al., found calves fed with more milk had increased number of *Lactobacillus* spp., in their faeces (Alimirzaei et al., 2020). Ma et al., demonstrated the proportion of colon mucosa-associated *Lactobacillus* spp., decreased in neonatal calves when the first colostrum feeding was delayed for 12h (Ma et al., 2019). In addition, the faeces of post-weaned calves have been shown to have reduced levels of *Lactobacillus* spp*.* (Uyeno et al., 2010, Meale et al., 2016).

Moreover, the relatively high level of *Bacteroidetes* in calf faeces was mainly attributed to members of the genus *Bacteroides,* which was consistent with previous microbiome studies of the calf rumen (Jami et al., 2013), hindgut mucosa (Song et al., 2017), and

faeces (Klein-Jöbstl et al., 2014). The relatively high level of *Bacteroides* spp. in the young calves might be acquired from the birth canal of cows (Klein-Jöbstl et al., 2014). Studies showed, as the calf grew, the level of *Bacteroides* spp. decreased in rumen and faeces, while the level of *Prevotella* spp. increased, which were assumed to be associated with the elevated proportion of fibre in the diet (Jami et al., 2013, Meale et al., 2016).

A wide range in both richness and evenness of the faecal microbiome was found among individual calves. However, no distinct differences in the bacterial composition were found between calves grouped by farm location. The results suggested other factors were driving these variations in diversity and composition, such as age. Unfortunately, this information was not available, however there are recommended procedures and regulations regarding the transport and slaughter of calves. In Australia, calves should not be transported until 5 days old, but if the date of birth was not identified on farms, the calves could be transported earlier and most calves are transported within 3 to 10 days old (Jongman and Butler, 2013). The age of calves in the current study was estimated to range from 5 to 30 days. In addition, variations in animal handling, fluctuating temperatures and deprivation of food and water during transportation could also influence microbiome diversity and composition.

In order to analyse the inter-individual variations in the faecal microbiome, the enterotype concept was used (Arumugam et al., 2011). Two enterotypes were identified among the animals sampled in this study. Calves with enterotype 1 had microbiomes with significantly higher levels of *Faecalibacterium* spp. compared to calves with enterotype 2. A study by Oikonomou et al. showed that the higher prevalence of *Faecalibacterium prausnitzii* in calves younger than one week was associated with higher body weight gain and lower incidence of diarrhoea (Oikonomou et al., 2013). Foditsch et al. suggested that *F. prausnitzii* was a potential probiotic for intestinal health and growth of pre-weaned calves (Foditsch et al., 2015). These benefits of *F. prausnitzii* were considered to relate to its butyrate production ability and anti-inflammatory properties. Butyrate is a major energy source to epithelial cell metabolism and has multiple roles in regulation of cell life cycles, thus plays an important role in maintaining gut health (Plöger et al., 2012).

One the other hand, calves with microbiomes of enterotype 2 had a higher abundance of *Proteobacteria*, especially *Enterobacteriaceae* bacteria compared to calves with enterotype 1. High levels of *Enterobacteriaceae* were found in the hindgut mucosa of calves in the first week of life (Song et al., 2017). These facultative anaerobes are considered to be pioneer gut colonisers in human infants, as they utilise oxygen in the gut environment that enable the colonisation of obligate anaerobic species (Favier et al., 2002, Arrieta et al., 2014). While several commensal bacterial species of the *Proteobacteria* are considered natural flora in a healthy mammalian gut, recent studies have reported that blooms of *Proteobacteria* are associated with dysbiosis and inflammation in the gut (reviewed by Shin et al, (Shin et al., 2015)). In calves, high levels of *Proteobacteria* have been found to be associated with diarrhoea in one month old calves (Gomez et al., 2017). However, other studies have found that healthy dairy and beef calves can have relatively high levels of *Proteobacteria* in particular farms, suggesting a farm-associated effect on the bacterial prevalence (Gomez et al., 2017, Weese and Jelinski, 2017). In addition, calves with enterotype 2 microbiomes were found to have higher abundances of species belonging to the genus *Clostridium* and the phylum *Fusobacteria*. These bacteria are commonly detected in calf faeces and considered part of the natural gut flora (Uyeno et al., 2010, Oikonomou et al., 2013, Klein-Jöbstl et al., 2014). Although changes in relative abundance were observed in calves of different age, the changes did not follow a consistent trend, suggesting the influence of other factors. Moreover, some members of *Clostridium* spp. and *Fusobacteria* spp. are opportunistic pathogens and associated with human gut inflammatory diseases (Britton and Young, 2014, Shen, 2012, Allen-Vercoe et al., 2011). Nevertheless, little is known about the correlation between *Clostridium* spp. or *Fusobacteria* spp. abundances in the calf faecal microbiome and diseases in the animal.

Moreover, calves with enterotype 1 had a statistically higher faecal microbiome richness than calves with enterotype 2. Species richness in faecal microbiome has been reported to increase with calf age (Klein-Jöbstl et al., 2014, Edrington et al., 2012, Oikonomou et al., 2013) and was associated with diarrhoea in calves younger than one month old (Oikonomou et al., 2013, Gomez et al., 2017). Given this information, along

with the differences observed in the genus abundance between the two enterotypes, we suggest that calves with enterotype 1 might be generally older than calves with enterotype 2 or have a more stable gut environment. However, the age of calves in the current study was not available.

Another objective of this study was to detect and quantify ARGs covering the major antimicrobial classes in the calf faeces. Variations were observed in number and relative abundances of detected ARGs among individual calves, however, the total ARGs abundances of each calf were mostly contributed by a few ARGs, especially the genes *tet*Q, *tet*W*, mef*A*, cfx*A*, str*B and *sul*2*.* Genes *tet*Q and *tet*W encode cytoplasmic proteins that protect ribosomes from tetracycline (Chopra and Roberts, 2001). These genes have been detected in a range of Gram-positive and Gram-negative bacteria that are part of the common gut flora, such as *Bacteroides* spp., *Ruminococcus* spp., *Lactobacillus* spp. and *Fusobacterium* spp. (Roberts et al., 2012). Due to the short period to withdraw antimicrobials prior to them being slaughtered for meat, bobby calves were unlikely to be treated with antimicrobials or fed with waste milk from treated cows. Therefore, the relatively high level of *tet*Q and *tet*W in the calf faeces is most likely due to the high level of bacterial hosts naturally present in the faeces. Similarly, genes *tet*O, *tet*M and *mef*A were detected in bacteria belonging to normal gut flora, such as *Bacteroides* (Roberts et al., 2012). In addition, these genes were commonly detected with a relatively high abundance in cattle faeces regardless of antimicrobial treatment (Alexander et al., 2011, Zhou et al., 2016, Noyes et al., 2016, Santamaría et al., 2011).

Moreover, gene *cfx*A was the most dominant beta-lactamase ARGs in the present study, which agrees with previous studies in cattle (Zhou et al., 2016, Thomas et al., 2017). Gene *cfx*A encodes a class A cephalosporinase that hydrolyzes cephaloridine and cephalothin. This gene is frequently associated with a variety of conjugative transposons in *Bacteroides* spp. and *Prevotella* spp. which could potentially assist HGT among the species (Garcia et al., 2008). A metagenomic study of cows treated with ceftiofur showed an increased relative abundance of class *Bacteroidia* and a decreased abundance of class *Actinobacteria* (Chambers et al., 2015) which, like the *tet* genes discussed above, indicates that the presence of relatively high levels of *cfx*A in the calf

faeces was more likely due to the presence of normal gut bacteria that naturally carry this gene.

The *str*B and *sul*2 genes were not only abundant in most calves but also co-occurred with each other. Indeed, previous studies reported the *sul*2-*str*A-*str*B cluster on RSF1010-like plasmids have been widely dispersed in the environment for decades, and occur in a range of hosts (Yau et al., 2010, Scholz et al., 1989, Tietze et al., 1989). A recent study showed this gene cluster was present in ice core samples from Antarctica and with highly sequence identity to the cluster carried by recent bacteria, suggesting the this cluster could form and disseminate with no selective pressure from human interventions involving antimicrobial use (Okubo et al., 2019). Similar to co-occurred *aph*A3 and *sat*4, the gene cluster *aph*A3-*sat4*-*aad*E was found on Tn5404-like elements in staphylococci (Derbise et al., 1997b), enterococci (Werner et al., 2003) and Tn1545/Tn6003 in streptococci (Palmieri et al., 2012), suggesting a wide dispersal of the cluster in the Gram-positive bacteria.

An interesting result from this study was the co-occurrence of the copper resistance genes *pco*A, *pco*R and *sil*R in the calf faeces, although the average abundances of these genes were low, around 10^{-3} relative to the 16S rRNA genes. The *pco* cluster consists of gene *pco*A, B, C, D, R, S and E; and *sil* cluster consists gene *sil*P, A, B, D, C, R, S and E (Hobman and Crossman, 2015). The *pco/sil* gene clusters have previously been detected on mobile genetic elements, particularly Tn7-like transposons and IncHI2 plasmids, and on the chromosomes of *E. coli* (Chalmers et al., 2018, Fang et al., 2016) and *Salmonella* spp. isolates (Billman-Jacobe et al., 2018, Tassinari et al., 2019) from food-producing animals. Plasmids carrying this gene cluster are known to be transferrable between genera and often carry ARGs (Billman-Jacobe et al., 2018, Chalmers et al., 2018). However, no ARGs showed a strong correlation to the *pco* and *sil* genes in the present study. To validate the genetic linkage of the *pco* and *sil* genes, as well as *pco*/*sil* with ARGs, one method is to isolate bacteria with copper resistance and analyse their genome to reveal the genetic linkage. Another method is to sequence the faecal metagenome using the long-read technique. Reads with the target genes are selected to explore the gene arrangement.

In conclusion, this study explored the faecal microbiome and the ARG profile of bobby calves after being transported. Overall, the calf faecal microbiome structure was consistent with previous studies of healthy dairy calves. Farm location, transportation and lairage duration did not appear to affect the faecal bacterial community. Faecal microbiomes grouped into two distinct enterotypes, which might reflect differences in calf age or the stability of the gut environment. ARGs with high clinical concern were rarely detected in the calves. The major ARGs that contributed most to the overall ARG background in calf faeces were likely due to the high level of bacterial hosts naturally present in the gut microbiome rather selected by antimicrobials.

Chapter 5 A longitudinal study of ARGs in an Australian antimicrobial-free layer chicken farm

Parts of chapter 5 and 6 have been combined as a manuscript that has been submitted to Antibiotics for publication on 31/1/2020 and accepted on 10/3/2020. A copy of the manuscript is provided at the end of the References section.

5.1 Introduction

Antimicrobial usage in food animal raises concerns over the potential emergence and spread of resistant bacteria (Marshall and Levy, 2011, Landers et al., 2012). To limit the negative impacts, many countries have restricted or banned the use of specific antimicrobials in food animals, especially those antimicrobials used for nontherapeutic purposes (Marshall and Levy, 2011). In Australia, gentamicin and fluoroquinolones are not used in any food animals, and the use of third generation cephalosporins is restricted (APVMA, 2017). In poultry, chlortetracycline is registered for treatment of egg-producing chickens, and several antimicrobials, including amoxycillin, neomycin, lincomycin, spectinomycin and oxytetracycline, are registered for use in meat-producing chickens (Obeng et al., 2012a). Virginiamycin and zinc-bacitracin are allowed for prevention and treatment of necrotic enteritis (Obeng et al., 2012a). All veterinary antimicrobial usage requires a prescription.

Previous studies of AMR in Australian poultry have revealed AMR in specific bacterial species. *E.* coli isolates have been found with resistance to tetracycline, ampicillin, trimethoprim-sufamethoxazole, streptomycin, spectinomycin, neomycin and florfenicol (Obeng et al., 2012b). *Enterococcus* spp. isolates have been reported with resistance to lincomycin, bacitracin, tetracycline and tylosin but no resistance to vancomycin or virginiamycin was reported in isolates collected between 2008 and 2009 (Obeng et al., 2013). *Campylobacter* spp. isolates were found resistant to ampicillin, tetracycline and lincomycin, but no resistance to ciprofloxacin, gentamicin, erythromycin and tylosin (Obeng et al., 2012a, Miflin et al., 2007).

Studies of the specific resistant strains provided valuable information to assess the risk of antimicrobial resistant bacteria in chicken, and to assist in the development of culture-independent methods to explore AMR in a broader bacterial population. HT-

qPCR and deep sequencing approaches have been used in a variety of human, animal and environmental samples to characterise the diversity of ARGs (Looft et al., 2012, Zhu et al., 2013, Li et al., 2015, Lin et al., 2016, Johnson et al., 2016). These methods have been found efficient and effective for the broad-spectrum detection and quantification of ARGs in complex samples.

In Chapters 3 and 4, we demonstrated how to analyse the faecal microbiome and ARG profile of individual animals. However, sampling individual animal is not always practical, especially for animals raised on a large scale, such as caged chickens in commercial farms. In this case, environmental sampling is a cost-effective and efficient method to collect biomass covering a large population, and this has been used for monitoring the prevalence of pathogens in chicken flocks (Arnold et al., 2010, Crabb et al., 2019).

The aims of this study were to detect and quantify ARGs in an antimicrobial-free layer farm using environmental samples. The studied farm had both cage and free-range production systems. Swabs of the floor of chicken sheds and manure belts underneath cages were collected. Sample enrichment was performed when the initial biomass was not sufficient for DNA extraction. DNA extracted from swabs was analysed by HT-qPCR and 16S rRNA sequencing to characterise the ARG profile and microbiome structure, respectively. Differences in ARGs and microbiome profiles between enriched and unenriched swabs, floor and manure belt swabs, and swabs from different types of sheds were compared. The results provided useful information for sampling design for monitoring ARGs in chicken farms for future studies.

5.2 Methods

5.2.1 Study farm and chickens

The study was carried out in an antimicrobial-free layer farm which used both caged and free-range production systems. Pullets were raised in a cage shed until they were 13 weeks old and then transferred into either a cage layer shed or free-range layer sheds. Birds in the caged shed were kept in tiered cages with six birds per cage. Manure was collected on a conveyor belt beneath each tier of cages and was removed weekly. Birds in the two free range sheds had access to the outdoors during daylight

hours and were confined to the shed at other times. Manure in the free range shed dropped through the slatted floor and was collected under the shed. The faeces were removed at the end of production when the shed was depopulated.

5.2.2 Sampling

The pullet shed was sampled when the birds were 13 weeks old. The two free-range sheds (A and B) were sampled immediately after cleaning with high pressure hoses before the study bird cohort was introduced to the site. Then, the layer cage and free-range sheds were each sampled three times with at least a two-week interval between samplings when the birds were 18 to 33 weeks old.

Two types of swabs were collected in the study sheds according to a method described in previous study (Crabb et al., 2019). Floor swabs were collected by a person wearing sterile covers outside clean dry boots and walking within the shed. Manure belt swabs were collected by wiping the edge of one end of the manure belt, using sterile 10×10cm cotton gauze swabs (ZebraVet, Australia) premoistened with sterile buffered peptone water. On each sampling timepoint, one manure belt per frame was sampled and there were five frames in total. Each manure belt swab sampling about 0.3m^2 area. Floor swabs were taken in all cage and free-range sheds and manure belt swabs were only taken in the layer cage shed. The sampling floor area in the cage shed was about 33m^2 and in the free-range sheds was about 18.5m^2. The sampling routes in the cage and free sheds are shown in Figure 5-1 and Figure 5-2. Boot 1 and 2 covered one half of the route and boot 3 and 4 covered the other half, except the sampling in the cleaned free range shed A and the second sampling in both free range sheds, boot 1 and 2 covered the whole sampling route.

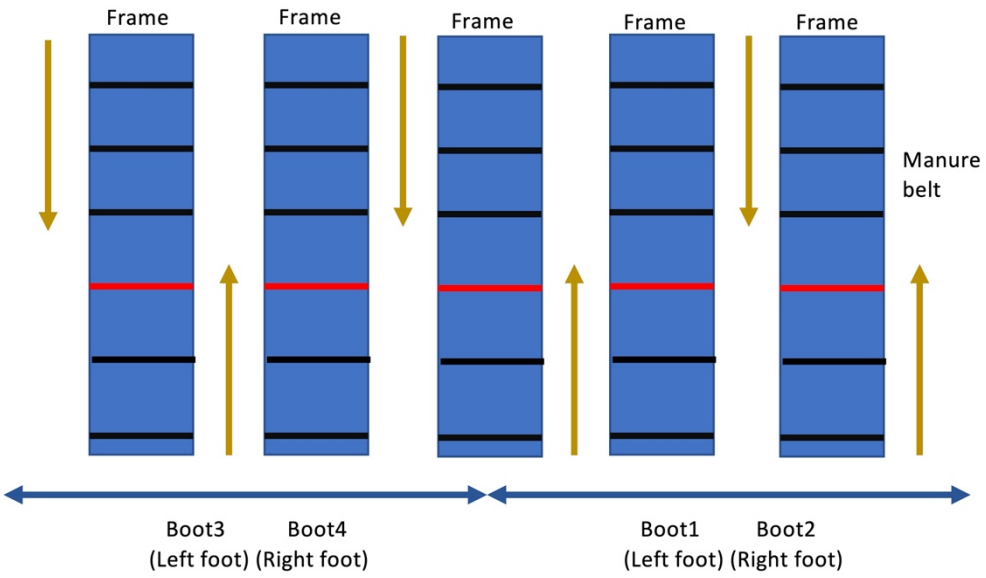

Figure 5-1: Sampling routes in cage sheds. The floor swab route is shown by the brown arrows and the sampled manure belt is highlighted in red.

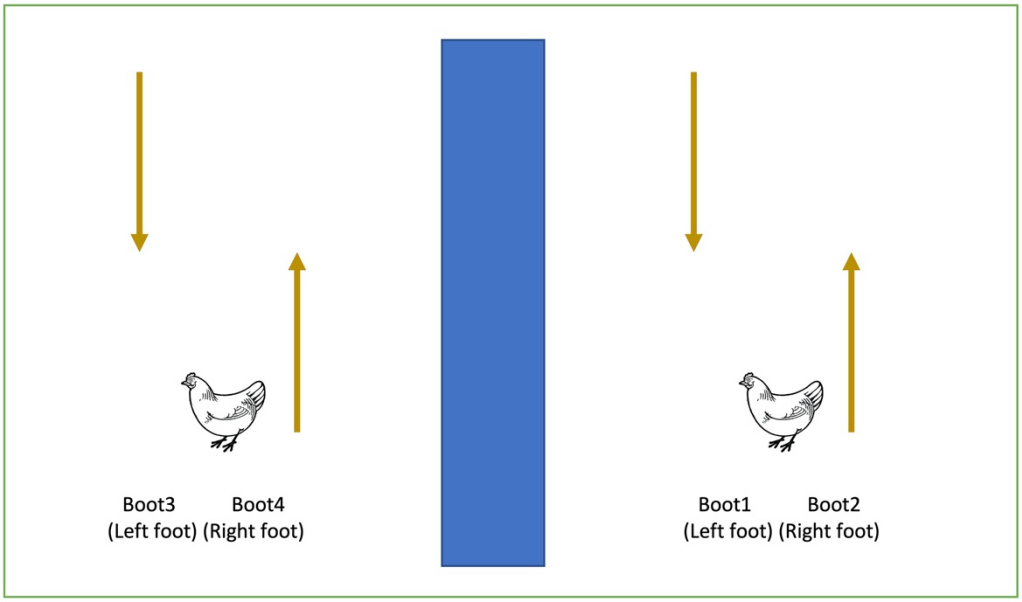

Figure 5-2: Floor swab sampling area in the free range sheds. The floor swab route is shown by the brown arrows.

Swabs were stored in sterile plastic bags and transported to the laboratory at ambient temperature. The swabs were then rinsed in 200mL peptone water and 50mL was processed directly as an unenriched sample for DNA extraction. The remaining volume (150mL) was incubated overnight at 37°C, and 50mL was then processed as an enriched sample for DNA extraction.

The biomass in each 50mL peptone water was collected by centrifugation at 2885×g for 30min at 4°C (Allegra X-12R centrifuge, Beckman Coulter, USA). For the unenriched sample, the pellet was stored in one 2mL microcentrifuge tube. For the enriched sample, pellet was suspended in 4mL fresh peptone water and then 1mL was aliquoted into a microcentrifuge tube. Bacterial material in each microcentrifuge tube was centrifuged at 10,000×g for 2min (Eppendorf, USA) to collect the bacterial pellet. Samples were stored at -80°C until DNA extraction.

5.2.3 DNA extraction

DNA was extracted using biomass in one microcentrifuge tube per sample. DNA extraction was according to "Section 2.1 DNA extraction from faecal and swab samples".

5.2.4 HT-qPCR array for detection and quantification of ARGs

ARGs were detected and quantified according to "Section 2.3 HT-qPCR array for gene detection and quantification", except the array included 296 validated primers targeting 285 ARGs, 10 MGEs, and one 16S rRNA gene (Appendix II) (Zhu et al., 2013, Wang et al., 2014, Muziasari et al., 2016). The assay includes genes from all major classes of ARGs, including aminoglycoside, beta lactamase, fluoroquinolone/florfenicol (FC), macrolide-lincosamide-streptogramin B (MLSB), sulfonamide, tetracycline, vancomycin, multidrug efflux pump, streptothricin and bacitracin resistance genes.

5.2.5 Nanopore long-read sequencing of DNA from pooled enriched swabs

Nanopore long-read sequencing was used to identify the potential bacterial host of detected ARGs. However, DNA extracts used for HT-qPCR and 16S rRNA sequencing were not sufficient for library preparation of Nanopore sequencing, therefore, DNA was re-extracted from the enriched floor and manure belt swabs from the second sampling in the layer caged and free-range sheds. DNA extracts of the same swab type and from the same shed were pooled at equal volume to meet the library preparation requirement for DNA quantity. The Nanopore sequencing libraries of the four pooled samples were prepared using the 1D genomic DNA sequencing kit SQK-LSK108 kit (Oxford Nanopore Technologies (ONT), UK) and barcoded using the EXP-NBD103 kit (ONT, UK) following the manufacture's instruction. Sequencing was performed on the

MinION device with a R9.5 flow cell (ONT, UK). Basecalling and demultiplexing of raw reads (in fastq format) were done with the program Albacore version 2.1.7 (ONT, UK). Reads below 750bp and above 50,000bp were excluded and the reads past filtering were converted into fasta format using the Fastaq program (https://github.com/sanger-pathogens/Fastaq).

5.2.6 ARG identification and taxonomic classification using long read sequences

ARGs were identified from the filtered Nanopore long reads using ABRicate program (https://github.com/tseemann/abricate) with the CARD database (McArthur et al., 2013) and the ARG-ANNOT database (Gupta et al., 2014). ARGs having BLAST hits with ≥ 70% coverage and ≥ 80% identity with the gene sequences from the database was accepted into the analysis (Altschul et al., 1990, Kamathewatta et al., 2019). Taxonomic classification of each long read was done with the Kraken taxonomic classifier version 1 (Wood and Salzberg, 2014) using the pre-built database (MiniKraken DB_4GB, https://ccb.jhu.edu/software/kraken/). ARGs and taxonomy information of each read were joined based on the sequence ID in R.

5.2.7 Bacterial 16S rRNA sequencing and bioinformatic analyses

Bacterial communities in the floor and manure belt swabs were characterised according to "Section 2.2 Bacterial 16S rRNA gene sequencing and bioinformatic analysis". For each sample, same DNA extract used for HT-qPCR array was used for the 16s rRNA sequencing.

5.2.8 Statistical analysis

Alpha diversity of each swab sample was calculated based on ASVs using the Chao1 index for richness and Shannon and Simpson diversity indices for richness and evenness.

ARG profiles were compared between swab samples grouped by processing method (enriched and unenriched), and the enriched samples were further grouped by sample type (floor and manure belt swab) and shed (pullet cage shed, layer cage shed, and free range shed). Similarity of ARG profiles and bacterial communities at genus level between swab sample groups was tested using the NMDS method with Bray-Curtis

distance matrix. Similarity among groups was tested by analysis of similarity (ANOSIM) using the 'anosim' function in the vegan package of R. NMDS analysis results were plotted using the ggplot2 R package with ellipses indicating the 95% confidence region of each cluster. Heatmaps of ARG abundance in each sample were done using the ComplexHeatmap package within R (Gu et al., 2016).

5.3 Results

5.3.1 Number of ARGs in caged and free-range chickens

Unenriched floor swabs did not have enough biomass for analysis, and sufficient quantities of DNA could only be extracted from shed floor swabs that had been culture-enriched. The overall number of ARGs and MGEs detected in the enriched floor swabs from each shed at each sampling visit ranged from 62 to 129 (Fig 5-3a). The highest number of ARGs and MGEs was detected in the pullet cage shed and the lowest number was in the cleaned layer free range cage A before the birds were introduced. Floor swabs collected from the free-range sheds were generally found to have less ARGs than those collected from the cage sheds. Variations in the number of detected genes were observed between sampling visit but did not follow a steady increasing or decreasing trend.

DNA extraction was done on each enriched and unenriched manure belt swab from the layer cage shed. Two unenriched swabs from the second sampling timepoint were pooled in order to have enough biomass for DNA extraction. The overall number of ARGs and MGEs detected in the enriched and unenriched swabs at each sampling timepoint ranged from 99 to 125 (Fig 5-3b). Enriched swabs generally showed more ARGs than the unenriched swabs.

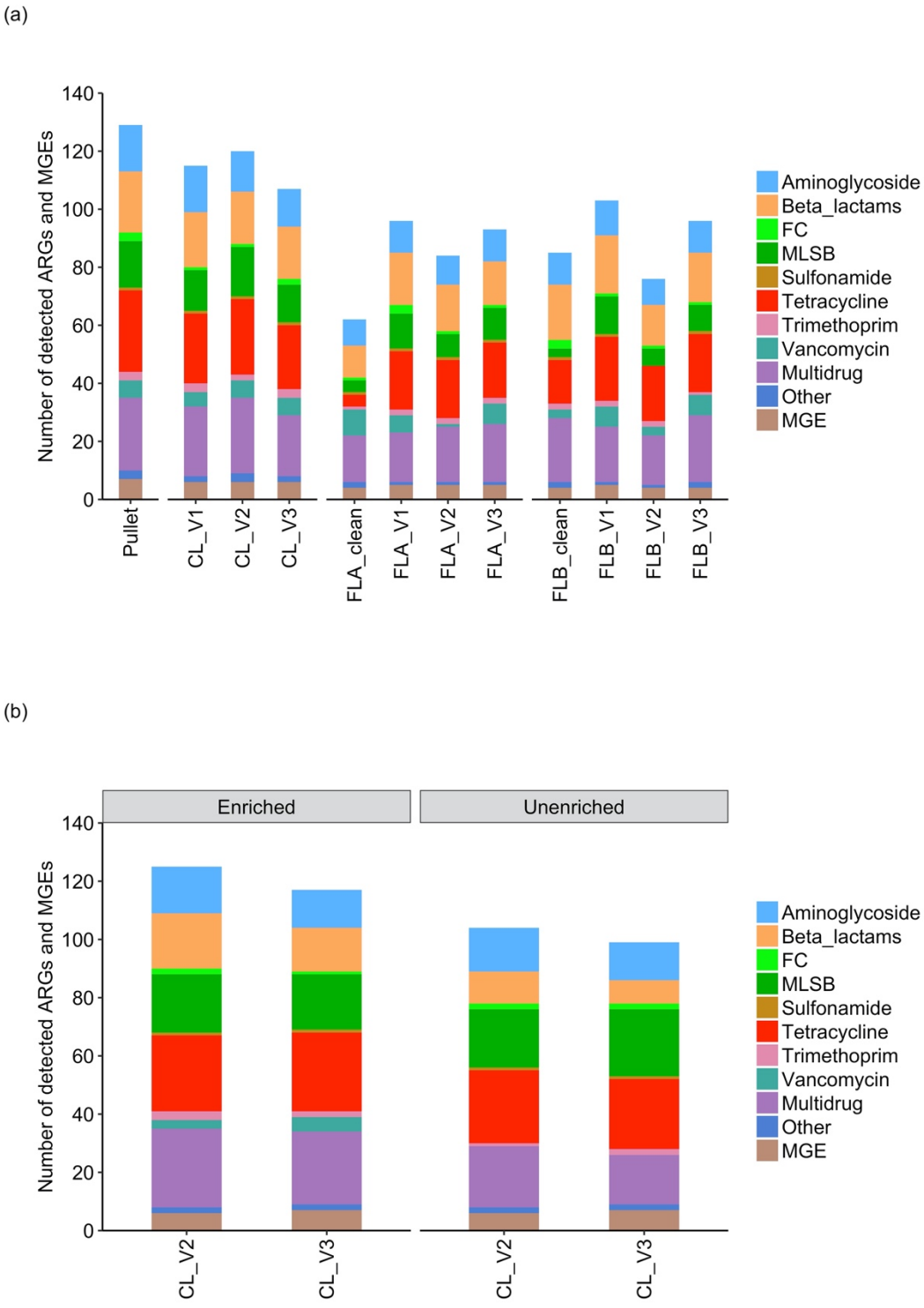

Figure 5-3: Number of detected ARGs in the enriched floor swabs of each shed and each sampling visit (a).Number of detected ARGs in the manure belt swabs from the layer cage shed of each sampling visit (b). FC, fluoroquinolone/florfenicol; MLSB, macrolide-lincosamide-streptogramin B; MGE, mobile genetic element. Pullet, cage pullet shed; CL, cage layer shed; FLA, free-range layer shed A; FLB, free range layer shed B. Numbers indicate the sampling visit and “clean” indicates sampling occurred in the cleaned shed before bird introduction.

5.3.2 Abundance of detected ARGs

The relative abundance of ARGs was represented by their proportion relative to the 16S rRNA gene for each individual sample (Fig 5-4). The enriched samples showed a consistent pattern regardless of the swab type and shed. The beta lactamase gene *amp*C, bacitracin resistance gene *bac*A and MDR genes acrA, *acr*B, *acr*F, *acr*R, *tol*C, *rar*D, *mdt*E/*yhi*U, *yce*E/*mdt*G, *yce*L/*mdt*H and *yid*Y/*mdt*L were detected with relative abundances above 10^{-3} to 16S rRNA gene in at least 45 enriched samples (52 enriched samples in total) (Table 5-1). These genes were highlighted orange in the heatmap (Fig 5-4). In contrast, the level of these genes decreased in the unenriched samples (Fig 5-4). ARGs with relative abundances above 10^{-3} to 16S rRNA gene in all the nine unenriched manure belt swabs were aminoglycoside resistance genes *aad*A, *aad*A1, *aad*A2 and *str*B, sulfonamide resistance gene *sul*2 and tetracycline resistance genes *tet*M, *tet*K and *tet*X (Table 5-2). Notably, the *cph*A gene was detected at high abundance (~ 10^{-1} to 16S rRNA gene) in one manure belt swab from the second sampling in the layer cage shed but the gene was below the detection limit in the next sampling.

Table 5-1: Most abundant ARGs detected in the enriched floor and manure belt swabs

Gene	Class	Relative abundance to 16S rRNA gene		
		Min	Max	Median
*acr*A	Multidrug efflux pump	2.6×10^{-3}	$2.7\times10\text{-}^{1}$	6.8×10^{-2}
*acr*B		5.5×10^{-5}	1.9×10^{-1}	4.6×10^{-2}
*acr*F		2.8×10^{-3}	2.2×10^{-1}	6.4×10^{-2}
*acr*R		1.9×10^{-3}	2.9×10^{-1}	5.0×10^{-2}
*mdt*E/*yhi*U		1.6×10^{-3}	1.9×10^{-1}	5.5×10^{-2}
*tol*C		2.2×10^{-3}	2.8×10^{-1}	5.2×10^{-2}
*rar*D		1.3×10^{-3}	1.8×10^{-1}	4.7×10^{-2}
*yce*E/*mdt*G		4.6×10^{-4}	1.5×10^{-1}	3.2×10^{-2}
*yce*L/*mdt*H		1.6×10^{-3}	2.0×10^{-1}	5.4×10^{-2}
*yid*Y/*mdt*L		1.1×10^{-3}	1.4×10^{-1}	3.9×10^{-2}
*amp*C	Beta lactamase	1.5×10^{-3}	1.6×10^{-1}	4.5×10^{-2}
*bac*A	Bacitracin	1.5×10^{-3}	5.7×10^{-1}	4.5×10^{-2}

Table 5-2: Most abundant ARGs detected in the unenriched manure belt swabs

Gene	Class	Relative abundance to 16S rRNA gene		
		Min	Max	Median
*aad*A	Aminoglycoside	6.5×10^{-3}	2.9×10^{-2}	1.2×10^{-2}
*aad*A1		3.4×10^{-3}	1.9×10^{-2}	6.0×10^{-3}
*aad*A2		1.0×10^{-3}	3.7×10^{-3}	2.1×10^{-3}
*str*B		1.2×10^{-2}	4.0×10^{-2}	2.3×10^{-2}
*sul*2	Sulfonamide	1.3×10^{-2}	4.5×10^{-2}	2.5×10^{-2}
*tet*K	Tetracycline	1.9×10^{-3}	1.3×10^{-2}	7.5×10^{-3}
*tet*M		1.1×10^{-2}	3.2×10^{-2}	2.3×10^{-2}
*tet*W		2.4×10^{-3}	4.6×10^{-2}	4.9×10^{-3}
*tet*X		6.7×10^{-3}	3.7×10^{-2}	1.1×10^{-2}

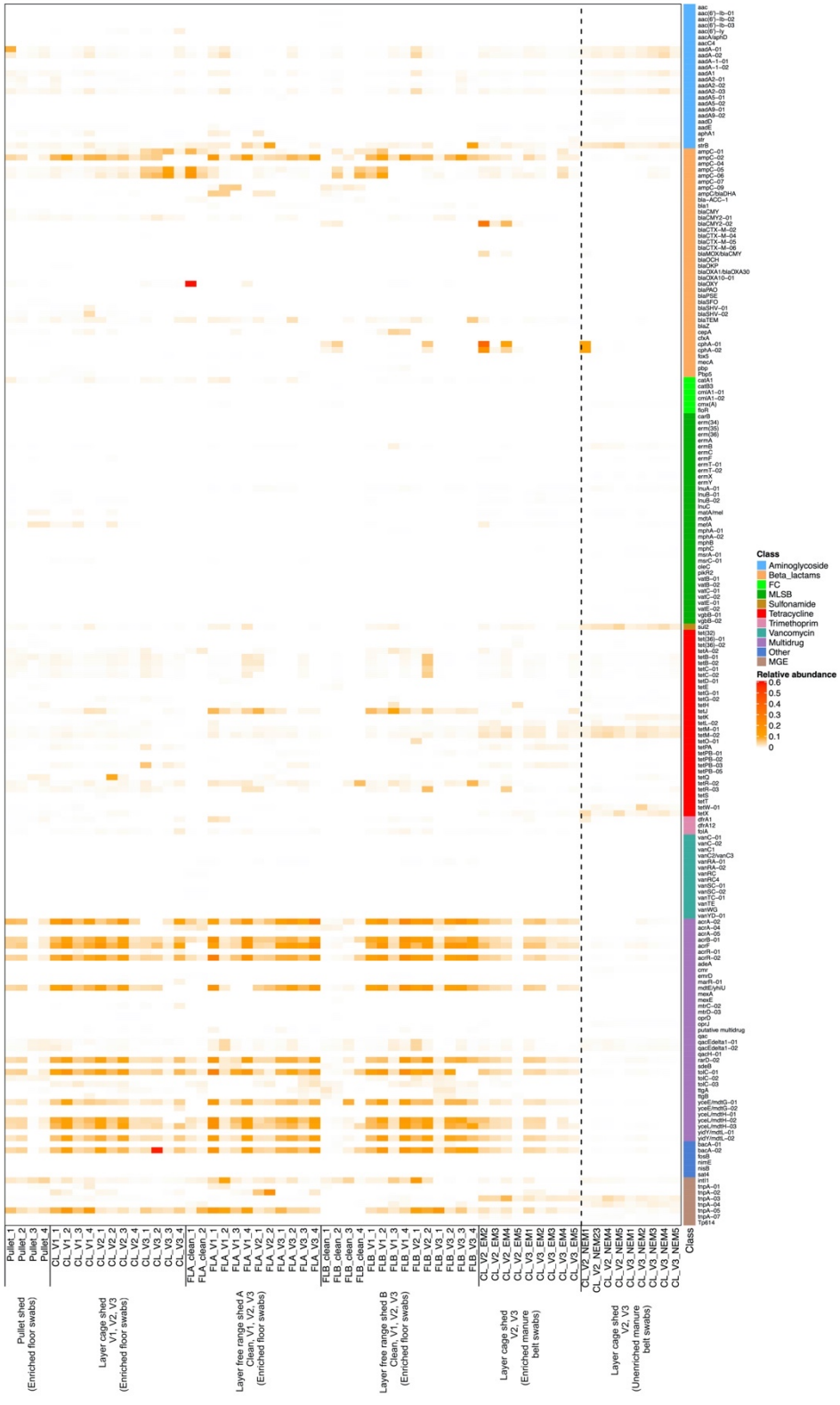

Figure 5-4: Heatmap of the relative abundance of ARGs and MGEs in individual samples. FC, fluoroquinolone/florfenicol; MLSB, macrolide-lincosamide-streptogramin B; MGE, mobile genetic element. Number after letter V indicate the sampling visit and "clean" indicates sampling occurred in the cleaned shed before bird introduction.

5.3.3 Similarity of ARG profiles in different sample groups

ARG profiles were compared between swab samples grouped by processing method (enriched and unenriched); and the enriched samples were further grouped by sample type (floor and manure belt swab) and shed (pullet cage shed, layer cage and free range shed). Enriched and unenriched manure belt swabs had a clear separation based on their ARG profiles (ANOSIM, R = 0.7, P = 0.001) (Fig 5-5a) and the enriched floor and manure belt swabs from the cage shed showed a mild separation (ANOSIM, R = 0.28, P = 0.003) (Fig 5-5b). The enriched floor swabs from the different sheds were not separated in this analysis (ANOSIM, R = 0.12, P = 0.02) (Fig 5-5c).

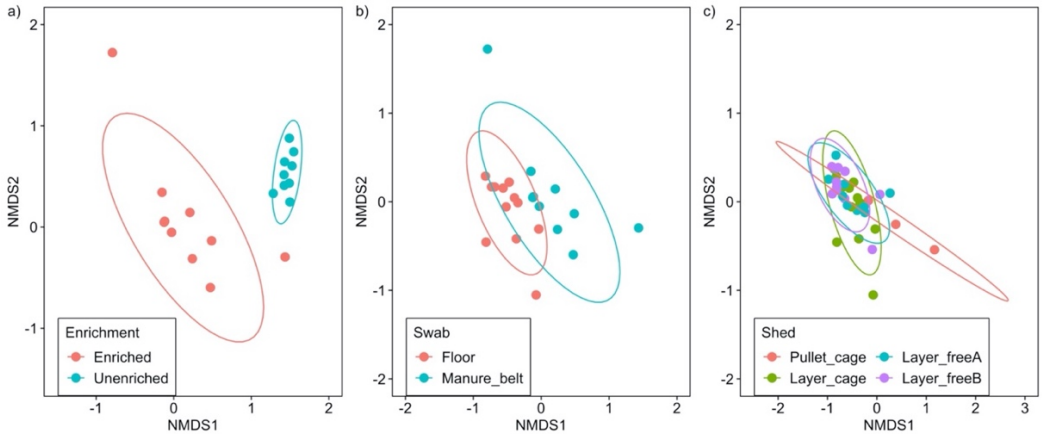

Figure 5-5: NMDS analysis of the ARG profiles of swab samples grouped by (a) processing method (enriched and unenriched); (b) sample type (floor and manure belt swab); (c) sheds (pullet and layer sheds).

5.3.4 Taxonomic classification of long reads carrying ARGs

To determine the potential bacterial hosts of the detected ARGs, DNA extracts from the enriched floor and manure belt swabs were sequenced using the MinION platform. The swabs from the second sampling timepoint in the layer cage and free-range sheds were pooled according to swab type and shed. The results showed that the *amp*C, *bac*A and MDR genes that had high abundance in the enriched samples were detected on reads classified as belonging to the genera *Escherichia* spp. and *Enterobacter* spp. In addition, some ARGs with relatively low abundance in the enriched samples were also detected by MinION sequencing. For example, *erm*F and *tet*Q were found on reads classified as *Bacteroides* spp., *mef*A and *mel* were on reads classified as *Streptococcus* spp., and *cph*A2 were on reads classified as *Aeromonas* spp. (Table 5-3).

Table 5-3: Taxonomy assignment of MinION long reads of enriched samples carrying ARGs

Gene	Class	Genus	Swab type	Shed
aadA1	Aminoglycoside	*Escherichia*	Floor	Free range B
aadD	Aminoglycoside	*Staphylococcus*	Floor	Free range A
aphA1	Aminoglycoside	*Escherichia*	Floor	Free range A
strA	Aminoglycosed	*Escherichia*	Floor	Free range A
strB	Aminoglycoside	*Salmonella, Escherichia*	Floor	Layer cage, free range A
bacA	Bacitracin	*Escherichia*	Floor, Manure belt	Layer cage, free range A and B
ampC	Beta lactamase	*Escherichia*	Floor, Manure belt	Layer cage, free range A and B
bla_{ACT}	Beta lactamase	*Enterobacter*	Floor	Free range A
bla_{CMY}	Beta lactamase	*Salmonella*	Floor	Layer cage
bla_{DHA}	Beta lactamase	*Morganella*	Floor	Layer cage, free range A
bla_{OXA}	Beta lactamase	*Pseudomonas*	Floor	Free range B
cblA	Beta lactamase	*Bacteroides*	Floor	Layer cage, free range A
cphA2	Beta Lactamase	*Aeromonas*	Manure belt	Layer cage
cepH	Beta Lactamase	*Aeromonas*	Manure belt	Layer cage
cepS	Beta Lactamase	*Aeromonas*	Floor	Free range B
fosA	Fosfomycin	*Enterobacter*	Floor	Free range A
acrA	MDR	*Escherichia, Enterobacter*	Floor	Layer cage, free range A and B
acrB	MDR	*Enterobacter*	Floor	Layer cage, free range A and B
acrD	MDR	*Escherichia*	Floor, Manure belt	Layer cage, free range A and B
acrE	MDR	*Escherichia*	Floor, Manure belt	Layer cage, free range A and B
acrF	MDR	*Escherichia*	Floor	Layer cage, free range A and B
mdtE	MDR	*Escherichia*	Floor, Manure belt	Layer cage, free range A and B
mdtG	MDR	*Escherichia*	Floor	Layer cage, free range A and B

mdtH	MDR	*Escherichia*	Floor	Layer cage, free range A and B
mdtL	MDR	*Escherichia*	Floor	Layer cage, free range A and B
ermB	MLSB	*Lactobacillus*	Floor	Free range A
ermF	MLSB	*Bacteroides*	Floor	Layer cage
lnuA	MLSB	*Lactobacillus*	Floor	Free range A
mefA	MLSB	*Enterococcus, Streptococcus*	Floor, Manure belt	Layer cage
mel	MLSB	*Streptococcus*	Manure belt	Layer cage
msrD	MLSB	*Streptococcus*	Manure belt	Layer cage
cat	Phenicol	*Proteus*	Floor	Layer cage, free range A
catA2	Phenicol	*Morganella*	Floor	Free range A
catII	Phenicol	*Proteus*	Floor	Free range A
sul1	Sulfonamide	*Klebsiella*	Floor	Layer cage
tetA	Tetracycline	*Escherichia, Klebsiella*	Floor	Free range A and B
tetA(P)	Tetracycline	*Clostridium*	Floor	Layer cage
tetB	Tetracycline	*Escherichia*	Floor	Free range A
tetB(P)	Tetracycline	*Clostridium*	Floor, Manure belt	Layer cage
tetD	Tetracycline	*Morganella*	Floor	Layer cage
tetG	Tetracycline	*Pseudomonas*	Floor	Layer cage
tetH	Tetracycline	*Pasteurellaceae* (Family)	Manure belt	Layer cage
tetJ	Tetracycline	*Proteus*	Floor	Layer cage, free range A and B
tetL	Tetracycline	*Lactobacillales* (Order)	Floor, Manure belt	Layer cage
tetQ	Tetracycline	*Bacteroides*	Floor	Layer cage
tetR	Tetracycline	*Escherichia, Klebsiella*	Floor	Free range B
tetX	Tetracycline	*Riemerella*	Floor, Manure belt	Layer cage
Tet39	Tetracycline	*Acinetobacter*	Floor	Layer cage
dfrA1	Trimethoprim	*Escherichia*	Floor	Free range B

5.3.5 Bacterial composition in different sample types

A total of 2,639,530 reads from 35 enriched and 4 unenriched samples passed all quality filters. The original read depth of all samples ranged between 22,092 to 122,790. Reads of each sample were rarefied to 22,092 which was sufficient for accurate analysis of microbiome richness and evenness. The rarefied reads were partitioned into 882 ASVs.

Comparison of microbiomes between enriched and unenriched manure belt swabs showed *Proteobacteria* increased from 46.3% to 80% after enrichment while *Actinobacteria* and *Firmicutes* decreased from 19.3% to 0.4% and 27.2% to 13.3%, respectively (Fig 5-6a). For the enriched sample, *Proteobacteria* represented more than half of the total bacterial community regardless of the swab type or shed (Fig 5-6b, 5-6c).

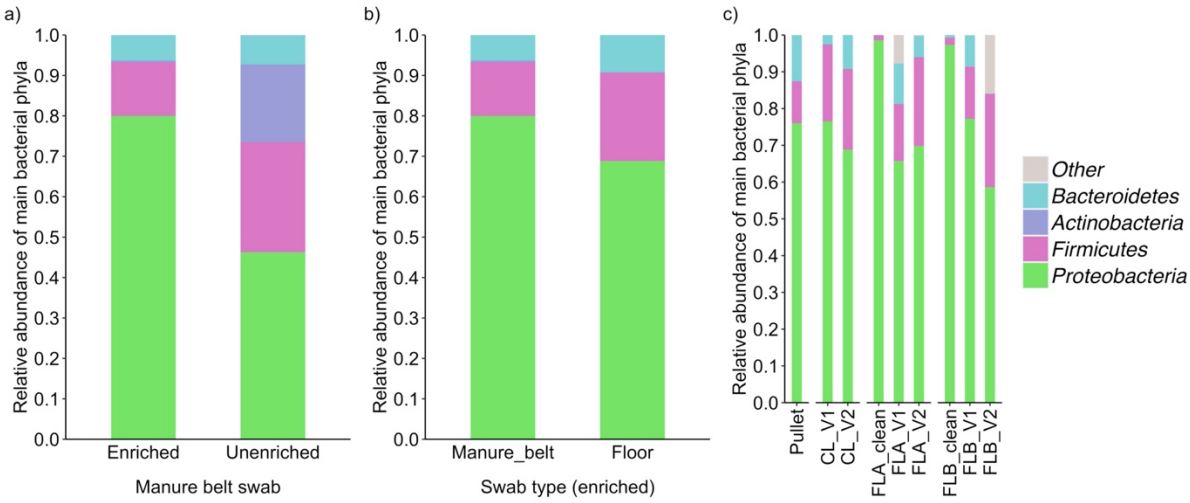

Figure 5-6: Relative abundance of dominant bacterial phyla in samples grouped by (a) processing method (enriched and unenriched); (b) sample type (floor and manure belt swab of the same sampling visit in layer cage shed); (c) sheds. Only phyla with a relative abundance above 0.1% in more than 20% of all samples are shown. Phyla below the cutoff were assigned as “Other”. Pullet, cage pullet shed; CL, cage layer shed; FLA, free range layer shed A; FLB, free range layer shed B. Numbers indicated the sampling visit and clean indicated the sampling in the cleaned shed before bird introduction.

At the family level, *Moraxellaceae* increased by 27% after manure belt swab enrichment, followed by *Aeromonadaceae*, *Enterobacteriaceae* and *Campylobacteraceae* which increased by 9.9%, 4.7% and 3.3%, respectively (Fig 5-7a, Fig 5-8). On the other hand, the most reduced family in the enriched manure belt swabs was *Staphylococcaceae* which decreased by 8.2%, followed by *Corynebacteriaceae*, *Carnobacteriaceae Dermabacteraceae* and *Pseudomonadaceae*, which decreased by 6.7%, 5.8%, 4.7% and 4.6%, respectively (Fig 5-7a, Fig 5-8). Comparison of the enriched samples collected from the layer cage shed at the same sampling visit showed floor swabs had a higher proportion of *Enterobacteriaceae* than the manure belt swabs, whereas, the manure belt swabs were more abundant with *Moraxellaceae* and *Aeromonadaceae* than the floor swabs (Fig 5-7b). In enriched floor swabs across all sheds and sampling visit, *Enterobacteriaceae* dominated the bacterial community, and *Moraxellaceae* were more abundant in swabs from the cage sheds than the free-range sheds (Fig 5-7c).

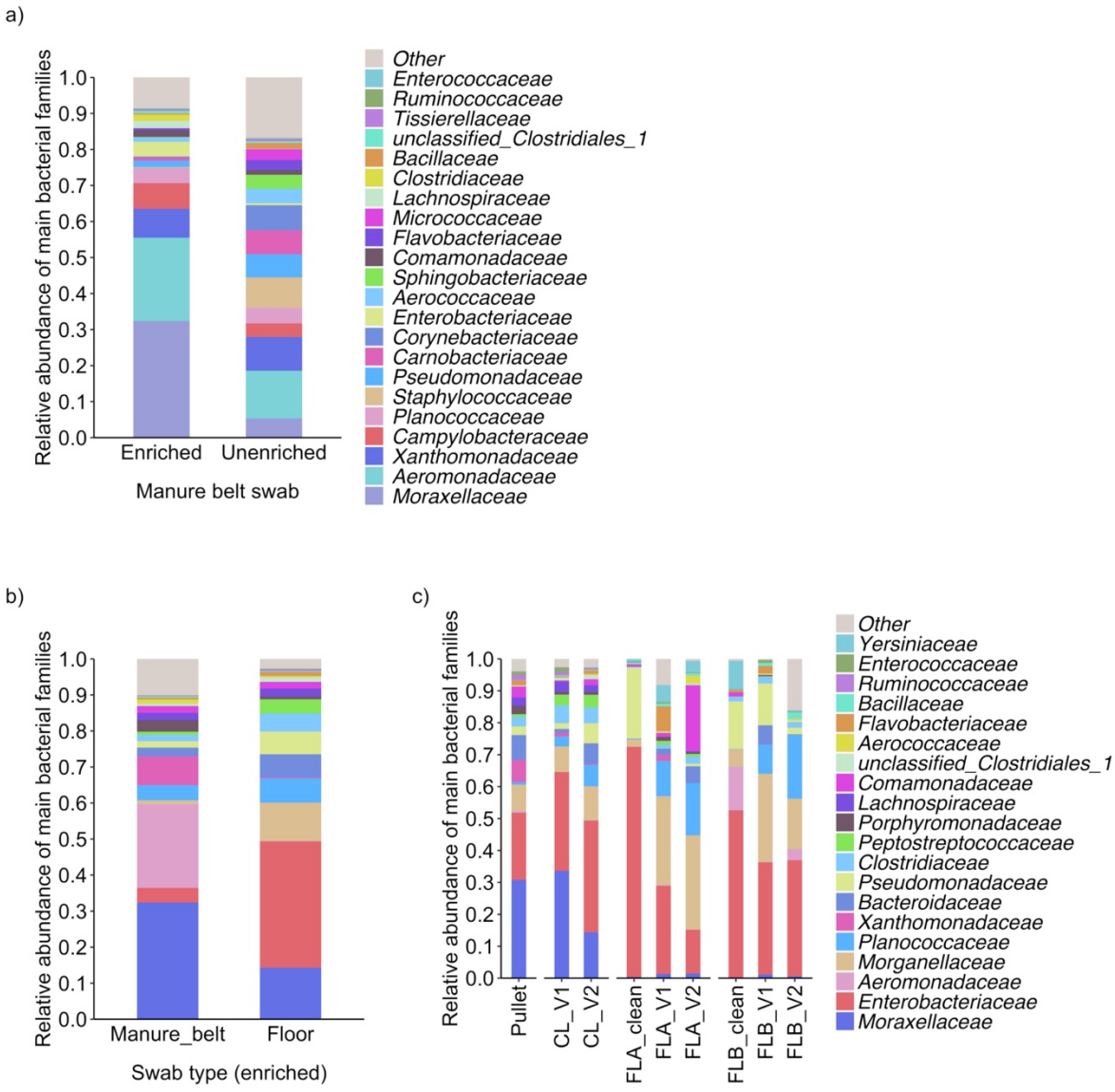

Figure 5-7: Relative abundance of dominant bacterial families in samples grouped by (a) processing method (enriched and unenriched); (b) sample type (floor and manure belt swab of the same sampling visit in layer cage shed); (c) sheds. Only families with a relative abundance above 0.1% in more than 5 unenriched samples or above 0.5% in more than 20% enriched samples are shown. Families below the cutoff were assigned as “Other”. Pullet, cage pullet shed; CL, cage layer shed; FLA, free range layer shed A; FLB, free range layer shed B. Numbers indicate the sampling visit and “clean” indicates the sampling occurred in the cleaned shed before bird introduction.

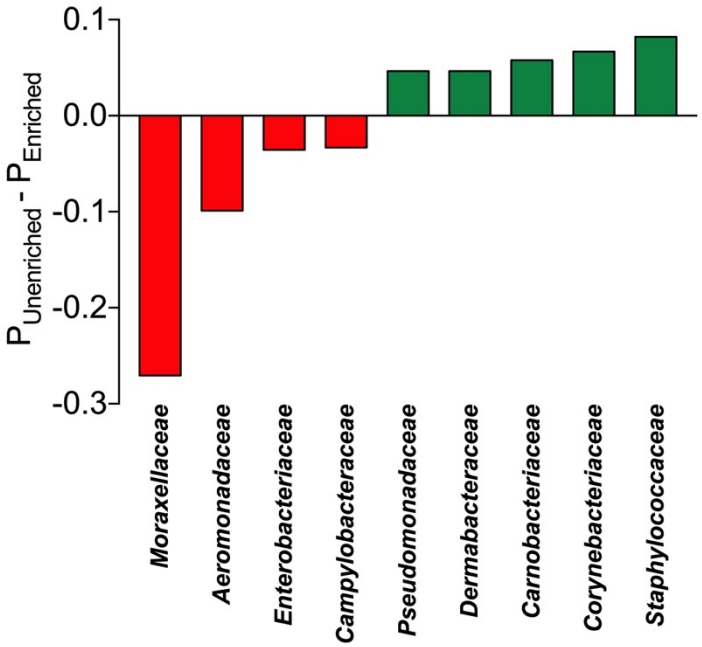

Figure 5-8: The top nine families that differed in relative abundance between unenriched and enriched manure belt swabs. $P_{unenriched}$, relative abundance of a family in unenriched samples; $P_{enriched}$, relative abundance of a family in enriched samples. Families coloured in red increased after enrichment and families coloured in green decreased after enrichment.

5.3.6 Comparison of microbiome diversity in different swab sample groups

Distinct clusters based on the microbiome at genus level were found between the enriched and unenriched manure belt swabs (ANOSIM, R = 0.69, P = 0.008) and the enriched floor and manure belt swabs from the layer cage shed (ANOSIM, R = 0.62, P = 0.002) (Fig 5-9a, 5-9b). The R value in the ANOSIM analysis indicated the swab microbiome differed more between enriched and unenriched samples than between the enriched floor and manure belt swabs. Moreover, the analysis of alpha diversity between the enriched and unenriched manure belt samples showed both the richness and evenness were greatly decreased after enrichment (Fig 5-10). For the enriched floor swabs of all sheds, the pullet and layer cage shed were separated from the layer free range sheds, and no separation was found between the two free range sheds (ANOSIM, R = 0.48, P = 0.001) (Fig 5-9c).

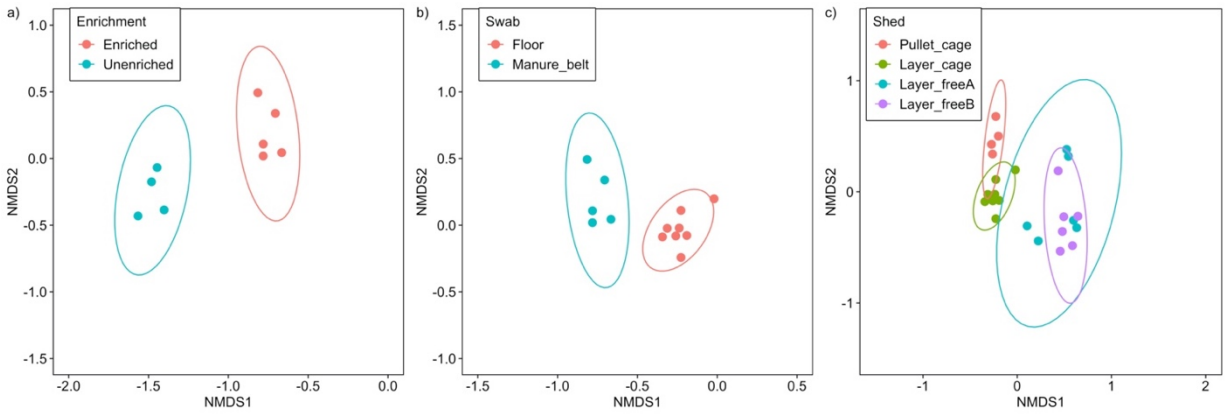

Figure 5-9: NMDS analysis of the microbiome at genus level of swab samples grouped by (a) processing method (enriched and unenriched); (b) sample type (floor and manure belt swab); (c) sheds (pullet and layer sheds).

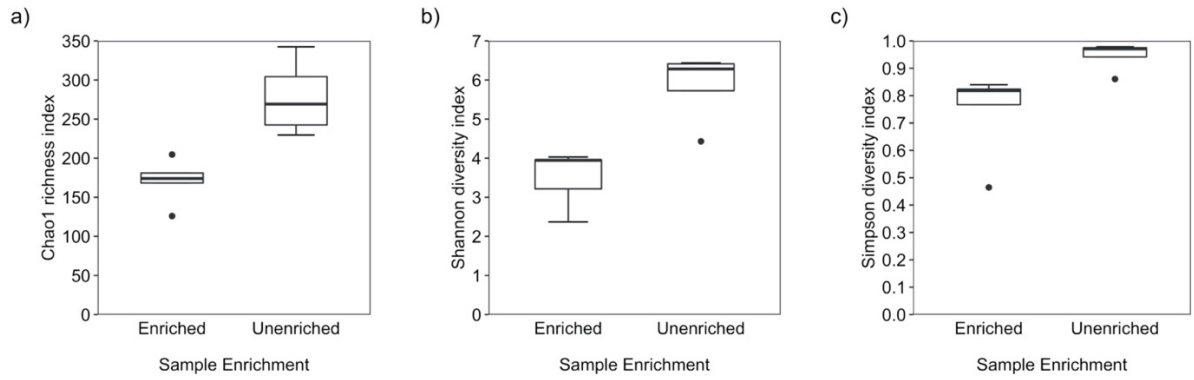

Figure 5-10: Alpha diversity of microbiome based on ASV of enriched and unenriched manure belt swabs collected in the layer cage shed at the same sampling timepoint. The richness index Chao1 (a), and diversity index Shannon index (b) and Simpson index (c) were showed as box plot, where the lower and upper end of the box represents the interquartile range. The whiskers represent 1.5 times interquartile range. The individual dots represent the outliers.

5.4 Discussion

This work explored the ARG profile in an antimicrobial-free layer farm that used both cage and free-range production systems. Floor and manure belt swabs were collected, and sample enrichment was performed as the initial biomass attached on the swabs, particularly the floor swabs, was not sufficient for DNA extraction.

Results showed the sample enrichment dramatically promoted the growth of aerobes and facultative anaerobes in the swabs, especially bacteria from *Moraxellaceae*, *Aeromonadaceae* and *Enterobacteriaceae* families. Therefore, it was not surprising to find increased numbers of ARGs from the *Escherichia* and *Enterobacter* genera in the enriched swabs. The abundant multidrug efflux pump genes are reported to be carried mainly on the bacterial chromosome, encoding efflux pumps that contribute to intrinsic resistance to antimicrobials in Gram-negative bacteria (Li and Nikaido, 2004). A well-known example is the AcrAB-tolC efflux pump in *E. coli*, which can extrude a broad range of antimicrobials and other toxicants (Li and Nikaido, 2004, Blair et al., 2015). Although the overexpression of these efflux pumps is associated with increased MICs of some antimicrobials, efflux-mediated resistance does not necessarily affect clinical antimicrobial treatment of bacterial infections (Piddock, 2006).

For the *bac*A and *amp*C genes that were abundant in the enriched sample, *bacA* is a chromosomal gene in *E. coli* which encodes undecaprenyl pyrophosphate phosphatase that confers resistance to bacitracin (Cain et al., 1993, El Ghachi et al., 2004). *Amp*C is usually found on the chromosomes of *Enterobacteriaceae* bacteria (Alekshun and Levy, 2007), and is normally expressed at low-levels in *E. coli*, although some clinical isolates with stronger *ampC* promoters or mutations that destabilise the normal *ampC* attenuator show high levels of expression (Jacoby, 2009).

The *cph*A gene was detected with high abundance in one unenriched manure belt sample from one sampling timepoint in the layer cage shed. Although no MinION sequencing was performed on the unenriched manure belt swabs due to insufficient DNA, analysis of long reads from the enriched manure belt swab suggested the gene might be carried by *Aeromonas* spp. The *cph*A gene encodes a class B metallo-beta-lactamase that hydrolyses carbapenems (Segatore et al., 1993), and CphA is the most common metallo-beta-lactamase produced by *Aeromonas* spp. (Janda and Abbott, 2010). As *cph*A was not continuously detected in the shed, the gene was more likely distributed in the environment transiently. Moreover, the plasmid-mediated ESBL genes $bla_{\text{CTX-M}}$, $bla_{\text{CMY-2}}$ and bla_{SHV} were detected with low abundance or below the detection limits in most swabs across all sampling visits, which suggests a low prevalence of these genes in the study layer farm.

The unenriched manure belt swabs showed a high abundance of *tet*M, *sul*2 and *str*B, which agreed with the previous observation of the chicken resistome (Guo et al., 2018, He et al., 2014). Indeed, tetracycline resistance genes, particularly, *tet*Q, *tet*W and *tet*M, have been found to be ubiquitous in faecal specimens from human and animals (Seville et al., 2009, Zhu et al., 2013, Li et al., 2015). The *tet*M gene encodes a ribosomal protection protein that reduces the sensitivity of ribosomes to the action of tetracyclines (Chopra and Roberts, 2001). *tet*M has been found in a wide range of bacterial species (Roberts et al., 2012), including those forming the normal gut flora in humans and animals (e.g. *Bacteroides* spp., *Lactobacillus* spp. and *Clostridium* spp.) (Lozupone et al., 2012, Wei et al., 2013). Therefore, the abundance of *tet*M could be due to the abundance of its bacterial hosts in the unenriched manure belts swabs. Moreover, *tet*M was often associated with conjugative transposons, especially the those of the Tn916-Tn1545 family (Roberts, 2005, Agersø et al., 2006). Tn916 family transposons can be transferred to a range of different bacterial genera and species (Clewell et al., 1995), which could contribute to the distribution of *tet*M.

The *sul2* gene is plasmid-borne, and has been found in plasmids from different incompatibility groups and often linked to streptomycin resistance genes *strA* and *strB* (Bean et al., 2009, Yau et al., 2010, Vinué et al., 2010). A recent study reported the *sul*2-*str*A-*str*B cluster could be found in ice core samples from Antarctica that were over 1,000 years old, suggesting the formation and dissemination of this ARG cluster can happen without human intervention involving antimicrobial use (Okubo et al., 2019). In the current study, no antimicrobial had been used on this farm for more than five years, so the prevalence of these ARGs in this layer farm was not likely driven by antimicrobial selective pressure and is more likely a reflection of their baseline abundances in the farming environment.

This study compared the ARG profiles and microbiomes between enriched and unenriched manure belt swabs, floor and manure belt swabs in the cage shed, and floor swabs from the cage and free-range sheds. Swab enrichment significantly biased the original microbiome and the estimates of ARG abundances. However, cultivation enrichment also increased the detection sensitivity of ARGs from bacteria able to grow aerobically, such as the *Enterobacteriaceae* bacteria. Some *Enterobacteriaceae* species

are considered to constitute a reservoir of ARGs (Boucher et al., 2009). In this case, sample enrichment could be used to screen ARGs in aerobic bacteria in complex samples.

Although the floor and manure belt swabs were enriched under the same conditions, the floor swabs exhibited higher levels of *Enterobacteriaceae* than manure belt swabs, while the manure belt swabs were more abundant with *Moraxellaceae* and *Aeromonadaceae.* The results suggested there were differences in the original microbiome compositions of the two swab types. Manure belt swabs mainly collected bird faeces, whereas the floor swabs tended to collect the dust present around the shed. Additionally, manure belt swabs usually collected more biomass than the floor swabs, so that sample enrichment was not necessary. Therefore, manure belt swabs are more suitable samples for detecting ARGs associated with birds than floor swabs. Moreover, the comparison of microbiome composition in the enriched floor swabs between different shed types showed the two free-range sheds had a similar microbiome which was different from the one in the cage shed. Variations, such as the shed constructions and bird movement, could affect the biomass collected by the floor swabs. These factors should be considered when using floor swabs to compare ARG abundances between different shed types.

In conclusion, consistently low levels of ARGs were detected in the layer farm to antimicrobials used for treatment of infections in humans, which is in line with the absence of antimicrobial usage on this farm. Sample enrichment could be used to increase the sensitivity of detecting ARGs in aerobes but should be avoided to characterise ARG profiles of the original bacterial community as enrichment greatly altered the community composition. Manure belt swabs are more suitable than floor swab for detecting ARGs associated with chickens. Variations between cage and free-range sheds should be taken into consideration when comparing ARG abundances using swab samples.

Chapter 6 Detection and quantification of ARGs in an Australian antimicrobial free broiler breeding farm

Parts of chapter 5 and 6 have been combined as a manuscript that has been submitted to Antibiotics for publication on 31/1/2020 and accepted on 10/3/2020. A copy of the manuscript is provided at the end of the References section.

6.1 Introduction

The bacteria that are shed in chicken faeces could serve as reservoirs of ARGs (Miranda et al., 2008, Van den Bogaard et al., 2001). Antimicrobial resistant bacteria present in faeces represent a potential hazard to health, as they may transfer ARGs to pathogens that increases the difficulties in treating infections in human and animals (Hammerum and Heuer, 2009). Furthermore, the use of chicken manure for fertilisation can promote spreading of ARGs into the environment (Heuer et al., 2011, He et al., 2014). In Australia, antimicrobials registered for use in meat-producing chickens include amoxycillin, neomycin, lincomycin, spectinomycin and oxytetracycline (Obeng et al., 2012a). Virginiamycin and zinc-bacitracin are prescription approved drugs for the prevention and treatment of necrotic enteritis but only for a restricted time period (Obeng et al., 2012a). Previous Australian studies have investigated ARGs in specific bacterial species in chicken faeces, and have reported various ARGs of the major antimicrobial classes carried in *Escherichia coli*, Enterococci and *Campylobacter* spp. strains (Obeng et al., 2012b, Obeng et al., 2013, Obeng et al., 2012a, Pratt and Korolik, 2005).

In addition to the culturable bacteria, the major proportion of the faecal microbiome in chickens is unculturable (Qu et al., 2008, Wei et al., 2013). Culture independent techniques bypassed this limitation and allowed detecting ARGs in the broader bacterial population. Previous studies conducted in chicken feedlots and impacted environments generally reported frequent detection of tetracycline (e.g. *tet*Q, *tet*M, *tet*W) and sulfonamide (e.g. *sul*1 and *sul*2) resistance genes (Cheng et al., 2013, He et al., 2014, Ji et al., 2012). Moreover, several studies showed a correlation between ARGs and MGEs, such as class 1 integron marker genes (Xie et al., 2016, Guo et al., 2018), suggesting the potential of ARGs to disseminate through HGT in the faecal

bacterial community. Nevertheless, little research has been done using culture-independent methods to characterise the ARG profiles in chickens in Australia.

Based on the results of Chapter 5, manure belt swabs were suitable to collect chicken faeces in a shed which provided enough biomass for DNA extraction for ARG detection. Moreover, the number of detected genes in the unenriched manure belts of each sampling visit was 99 and 104 out of the 295 ARGs tested, respectively. The results indicated that many qPCR assays were negative, and it was not cost effective to include all of the 295 ARGs and MGEs in further testing. In addition, the number and relative abundance of detected genes were consistent between visits (Figure 1, 2, Table 1 in (Liu et al., 2020), sampling multiple times is not necessary.

In this study, three cage sheds were sampled in an antimicrobial-free broiler breeding farm. No antimicrobials had been used on this farm for five years prior to this study. Manure belt swabs were used to sample the cage sheds and the HT-qPCR assay was reduced to 44 ARGs, four MGEs and three MRGs (Appendix I). The results were expected to provide useful baseline information regarding ARG prevalence in the chicken production system without direct selective pressure.

6.2 Methods

6.2.1 Study farm and sampling

The study farm was a broiler breeding farm which used a cage production system. The birds were housed in multitiered frames with one cage per frame width. Each shed contained eight frames which housed around 24,000 birds in total. Three sheds on the farm were sampled. Birds in shed B and C were originally separated from the same cohort of chicks and birds in shed A were from a different cohort.

The bird manure was collected by the conveyor belt beneath each tier of the cages and removed weekly. Biomass from the manure belt was obtained by swabbing the edge of one end of the belt using sterile cotton swabs premoistened with buffered peptone water. The middle three manure belts of each frame were sampled, and 24 swabs were collected from each shed (A, B and C). Each manure belt swab sampling about $0.2m^2$ area.

Swabs were stored in Whirl-Pak® sterile sample bags (Nasco, USA) and transported without delay to the laboratory at ambient temperature. The swabs were then rinsed in 50mL peptone water and the contents were homogenised by gently squeezing the bag. Then, all the liquid was transferred into a 50mL plastic tube and centrifuged at 2885×g for 30min at 4°C (Beckman Coulter, USA). The sediment was resuspended in 4mL buffered peptone water and transferred into two 2mL microcentrifuge tubes and centrifuged at 10,000×g for 2min (Eppendorf, USA). The supernatants were removed and all the biomass of one swab was transferred into a 2mL microcentrifuge tube and stored at -80°C until DNA extraction.

6.2.2 DNA extraction

DNA extraction was according to "Section 2.1 DNA extraction from faecal and swab samples".

6.2.3 HT-qPCR array for gene detection and quantification

ARGs were detected and quantified according to "Section 2.3 HT-qPCR array for gene detection and quantification".

6.2.4 Statistical analysis

A heatmap of the relative abundance of each gene in each sample was generated using the ComplexHeatmap R package (Gu et al., 2016). Differences in relative abundance of ARGs between sheds were tested by pairwise Wilcoxon rank sum test with *p* value adjusted by the Benjamini-Hochberg correction method (Benjamini and Hochberg, 1995) using the basic stats R package. A *p* value less than 0.05 was considered as a significant difference. Similarity of ARG profiles of the manure belt swabs grouped according to shed was tested. Moreover, the overall ARG profile of the manure belt swabs from the broiler breeding farm was compared to that from the layer farm described in Chapter 5. The comparison was done using the NMDS method with Bray-Curtis distance matrix. Similarity between groups was tested by ANOSIM using the 'anosim' function in the vegan R package. The NMDS result was plotted using the ggplot2 R package with ellipses indicating the 95% confidence region of each cluster.

The patterns of co-occurrence of ARGs and MGEs were analysed according to “Section 2.4 Co-occurrence of ARGs and MGEs”.

6.3 Results

6.3.1 Detection and quantification of resistance genes and MGEs

Samples that failed to amplify the 16S rRNA gene were excluded in the analysis, leaving 22 swabs that were validated in shed A and B, respectively, and 16 swabs in shed C. Out of the 53 primer pairs, the HT-qPCR assays detected 38 ARGs, three MRGs and four MGEs from all manure belt swabs. The number of genes detected in each sample ranged from 27 to 41 (Fig 6-1). Forty-four genes were detected in each shed in total, and the three sheds shared 42 genes with no genes detected in only one shed (Fig 6-2).

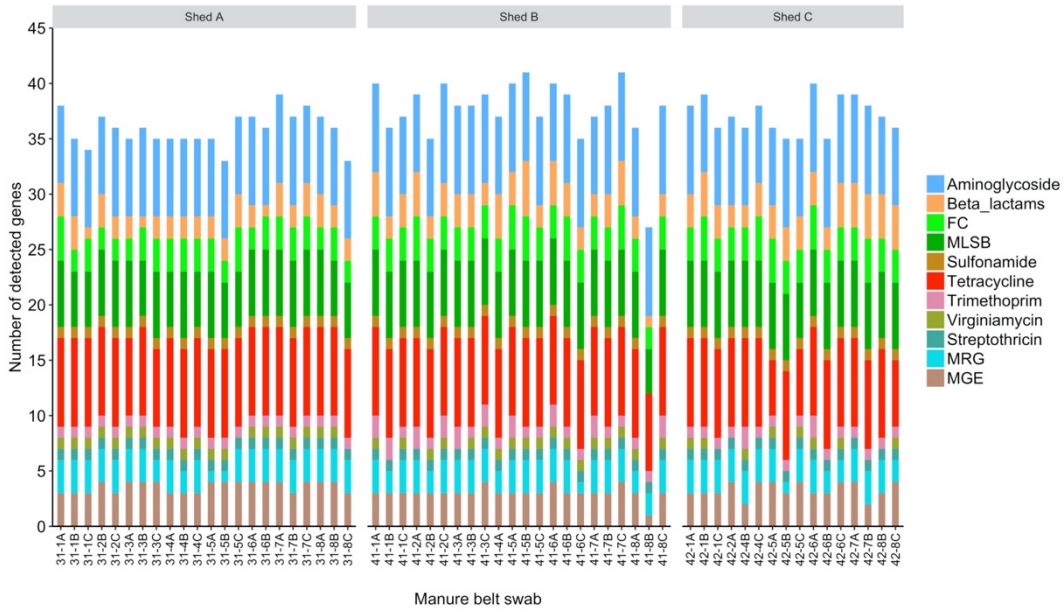

Figure 6-1: Number of detected ARGs, MRGs and MGEs in sheds A, B and C. FC, fluoroquinolone/florfenicol; MLSB, macrolide-lincosamide-streptogramin B; MRG, metal resistance gene; MGE, mobile genetic element.

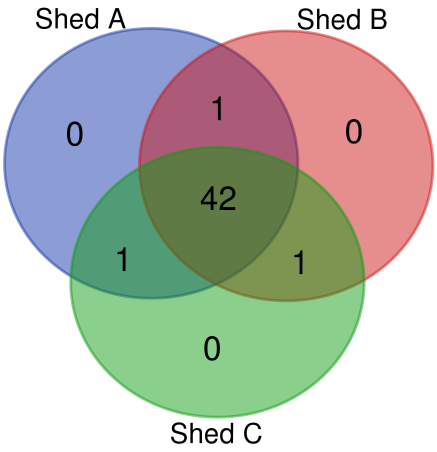

Figure 6-2: Number of detected resistant genes and MGEs shared among sheds A, B and C.

The relative abundance of the detected genes was represented by their proportion relative to the 16S rRNA gene (Fig 6-3). A total of 34 genes were detected in more than 80% samples (48 out of 60). The top 10 abundant genes based on median relative abundance across the sheds are listed in Table 6-1. The most abundant ARG was the aminoglycoside resistance gene *str*B, with the median relative abundance of 1.3×10^{-2}. The next most abundant ARG was tetracycline resistance genes *tet*L, with the median relative abundance of 1.2×10^{-2}, and the other two abundant *tet* genes were *tet*M and *tet*X. Other abundant genes were *sul*2, *erm*B, *aad*A and *aad*A2. In addition, the class I integron markers *int*I1 and *qac*EΔ were detected at high levels in the sheds, with median relative abundances of 1.8×10^{-2} and 1.1×10^{-2}, respectively. Relative abundances of these genes were further compared between the sheds using a pairwise Wilcoxon rank sum test. While three genes (*tet*X, *aad*A and *aad*A2) showed no significant differences in abundance between sheds, the other eight genes had significantly different abundances in shed A between shed B and/or shed C. Shed B and C had significantly different abundances in *tet*L, *erm*B, *int*I and *qac*EΔ (Fig 6-4).

ARGs with high clinical importance, such as the beta lactamase resistance genes bla_{SHV}, $bla_{CTX\text{-}M}$, *cph*A, the fluoroquinolone resistance gene *qnr*B and the virginiamycin resistance gene *vat*E, were detected at very low abundance (~10^{-6} to 10^{-5}) in the samples.

Table 6-1: The top 10 abundant ARGs and MGEs detected in the manure swab samples taken across all sheds

		Relative abundance to 16S rRNA gene		
Gene	**Class**	**Min**	**Max**	**Median**
*aad*A	Aminoglycosides	6.4×10^{-4}	6.7×10^{-2}	7.2×10^{-3}
*aad*A2		5.0×10^{-4}	3.8×10^{-2}	5.6×10^{-3}
*str*B		7.0×10^{-4}	2.1×10^{-1}	1.3×10^{-2}
*erm*B	MLSB	1.4×10^{-3}	3.0×10^{-2}	6.3×10^{-3}
*sul*2	Sulfonamides	1.5×10^{-4}	3.4×10^{-1}	8.0×10^{-3}
*intI*1	MGE	6.8×10^{-4}	7.9×10^{-2}	1.8×10^{-2}
*qac*EΔ		1.3×10^{-3}	5.4×10^{-2}	1.1×10^{-2}
*tet*L	Tetracyclines	1.0×10^{-3}	5.5×10^{-2}	1.2×10^{-2}
*tet*M		1.8×10^{-3}	3.0×10^{-2}	9.2×10^{-3}
*tet*X		2.2×10^{-4}	3.8×10^{-2}	7.7×10^{-3}

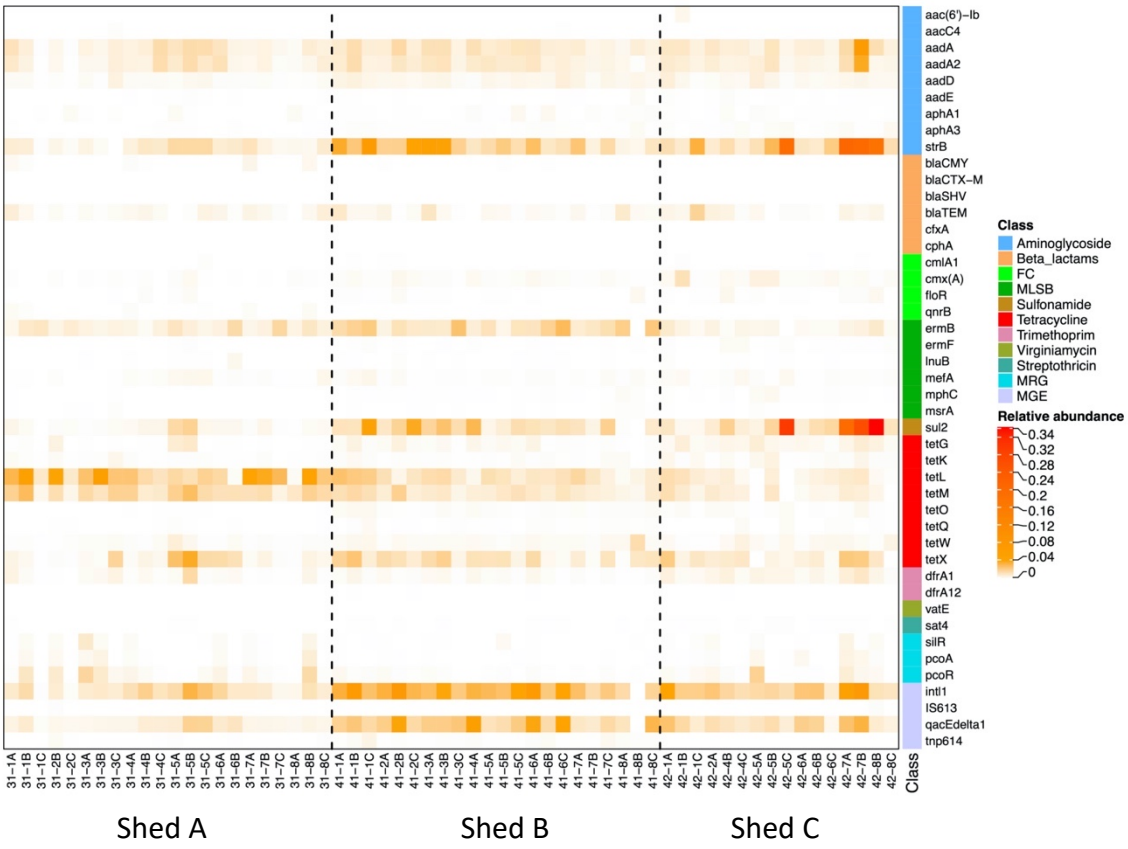

Figure 6-3: Heatmap of the relative abundance of resistance genes and MGEs in individual samples. FC, fluoroquinolone/florfenicol; MLSB, macrolide-lincosamide-streptogramin B; MRG, metal resistance gene; MGE, mobile genetic element.

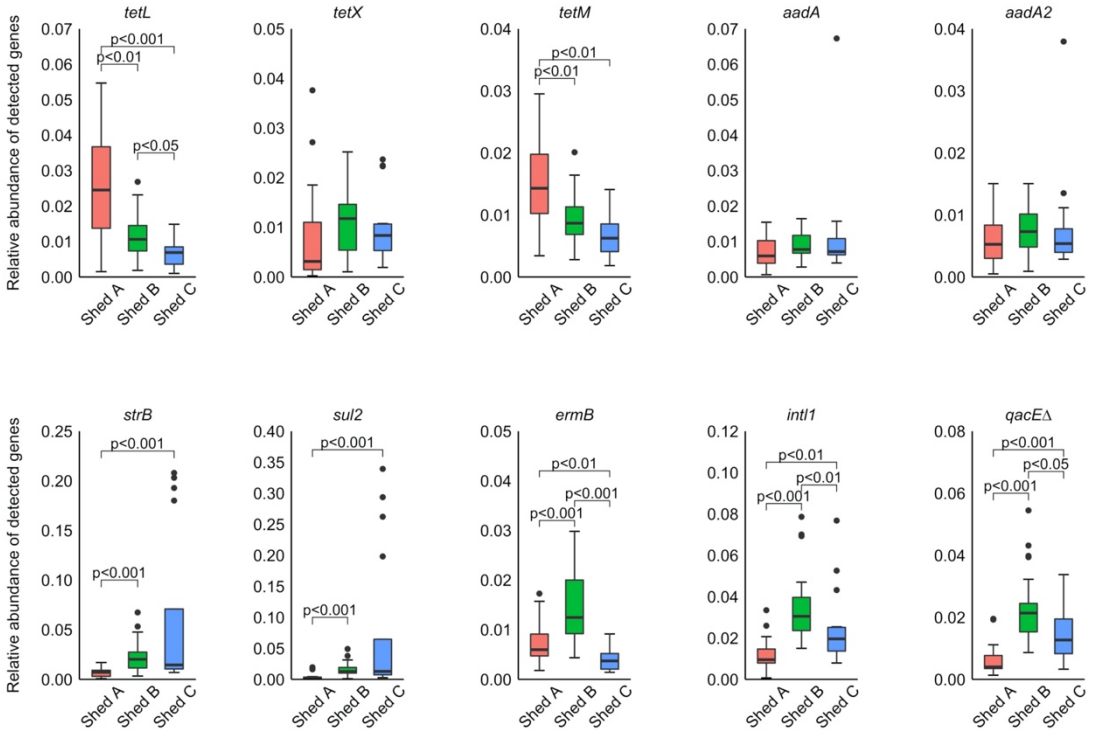

Figure 6-4: Relative abundances of the top 10 abundant genes in sheds A, B and C. The relative abundance of each gene in each shed is shown as a box plot, where the lower and upper ends of each box represents the interquartile range. The whiskers represent the 1.5 times interquartile range. The individual dots represent outliers. Different letters above the boxes indicate a significant difference in the relative abundance.

6.3.2 Similarity of resistance genes and MGE profiles between the sheds and farms

NMDS analysis based on the Bray-Curtis distance metric derived from the relative abundance of resistance genes and MGEs was used to explore sample clustering. The results are shown in Figure 6-5 and reveal a moderate clustering of samples grouped by shed (ANOSIM, R = 0.38, P = 0.001) in the study broiler breeding farm.

The overall ARG profile of manure belt swabs from the broiler breeding farm were compared to that from the layer farm described in Chapter 5. NMDS analysis was based on 33 ARGs detected in both farms, including ARGs with high levels in both farms (e.g. *tet*M, *tet*X, *str*B and *sul*2). Results showed little separation between samples of the two farms (ANOSIM, R = 0.21, P = 0.05) (Fig 6-6).

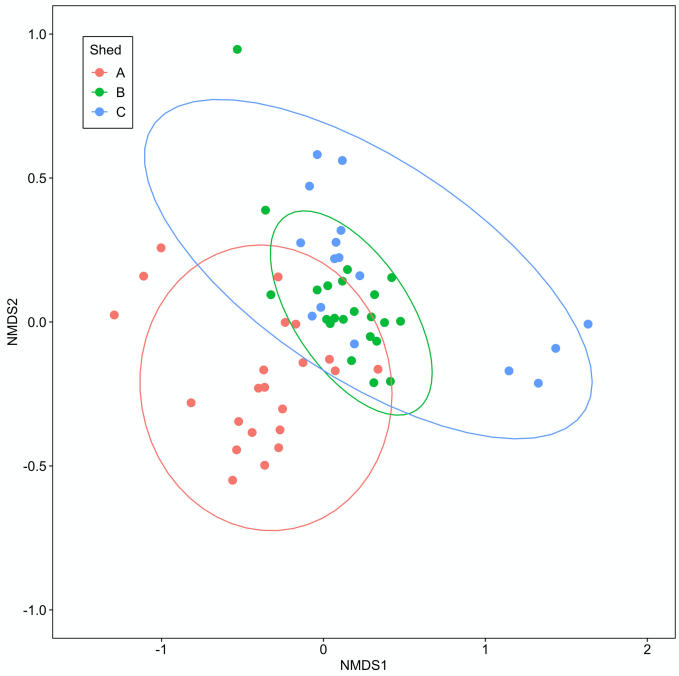

Figure 6-5: NMDS using Bray-Curtis distances to compare the similarity of resistance genes and MGE profiles among sheds A, B and C. The ellipses indicated 95% confidence region of each cluster.

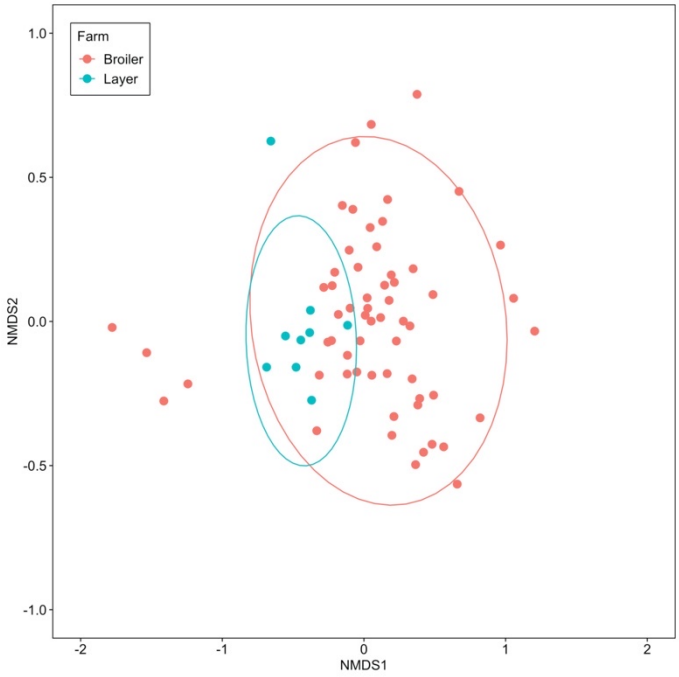

Figure 6-6: NMDS using Bray-Curtis distances to compare the similarity of ARGs profile based on the relative abundance of 33 ARGs between the broiler breeding farm and layer farm. The ellipses indicated 95% confidence region of each cluster.

6.3.3 Co-occurrence of resistance genes and MGEs in manure belt swabs

The co-occurrence pattern of ARGs, MRGs and MGEs were analysed by Spearman rank correlation and displayed in a network (Fig 6-7). The network consists of 9 ARGs, all three MRGs and the class I integron markers. Gene *cml*A1 co-occurred with class I integron genes, although *cml*A1 was generally detected with a low abundance (10^{-6} to 10^{-4}). Gene *aph*A3 co-occurred with *erm*F, *sat*4 and *tet*W, while gene *str*B was found to co-occur with *sul*2. The three MRGs were corelated with each other but not with any ARGs.

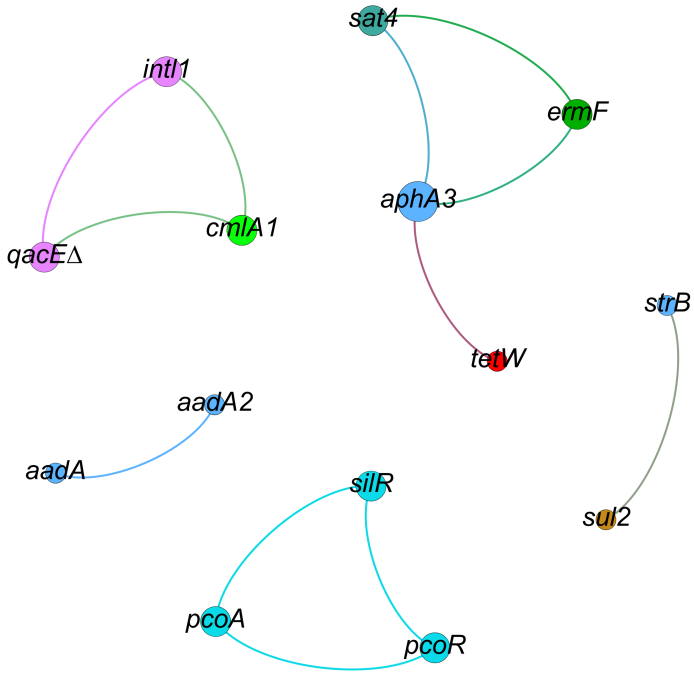

Figure 6-7: Network of co-occurred ARGs, MRGs and MGEs in samples across all three sheds (Spearman's rho ≥ 0.75, $P < 0.01$). The size of each node is proportional to the number of connections.

6.4 Discussion

This work explored the ARG profiles in an antimicrobial-free broiler breeding farm that used a cage production system and had not used antimicrobials for at least five years. Based on the results of Chapter 5, manure belt swabs were selected as the best method for sampling the sheds. The aim of the current study was to determine the

faecal ARG profiles of chickens in a commercial intensive farm where there was no direct antimicrobial selective pressure, and so provide baseline information for future studies exploring faecal ARG profiles in chickens, such as comparisons with other management practices.

The tetracycline resistance genes *tet*L, *tet*M and *tet*X were abundant in most samples. Consistent findings were reported in previous studies quantifying ARGs in faecal samples from poultry (Ji et al., 2012, Cheng et al., 2013), swine (Wang et al., 2017, Zhu et al., 2013) and humans (Li et al., 2015, Seville et al., 2009). The *tet*L gene encodes a tetracycline efflux pump, while *tet*X encodes an enzyme that alters tetracycline, and *tet*M encodes a ribosomal protection protein that reduces the sensitivity of ribosomes to the action of tetracycline (Chopra and Roberts, 2001). These *tet* genes are known to be present in a wide range of bacterial species (Roberts et al., 2012), some of which are members of the dominant flora of the normal chicken gastrointestinal tract, such as *Clostridium* spp., *Lactobacillus* spp. and *Bacteroides* spp. (Wei et al., 2013). Metagenomic studies have shown that the ribosome protection type *tet* genes contribute about 30% to the total ARG abundance in the chicken cecum (Qu et al., 2008), and many *tet* genes are often associated with conjugative elements. One of the most characterised examples is *tet*M, carried on the conjugative transposons of the Tn916-Tn1545 family (Chopra and Roberts, 2001). Transposon-borne *tet* genes have been found in many different bacterial species, including those of the enterococci (Agersø et al., 2006, De Leener et al., 2004), streptococci (De Vries et al., 2009) and *Bacillus* (Agersø et al., 2002). The prevalence of these transposon-linked resistance genes indicates the success with which these transposons can move between diverse species.

The macrolide resistance gene *erm*B was also found to be abundant in samples across all sheds. Similar to the *tet* genes, *erm*B was also detected in a range of bacterial species (Roberts et al., 2012) and associated with conjugative transposons (Cochetti et al., 2007, Okitsu et al., 2005, Gupta et al., 2003). Consistent with these results, a human faecal metagenomic study showed that *erm*B was the most common and abundant MLSB gene type in populations from different countries (Hu et al., 2013). Moreover, *erm*B was often found associated with *tet*M on the conjugative transposons

of the Tn916-Tn1545 family (Cochetti et al., 2007, De Leener et al., 2004), which may explain the wide distribution of the gene in the environment.

The overall ARG profile of the manure belt swabs from the broiler breeding farm was similar to that from the layer farm. The comparison included ARGs that were detected with high level in both farms, such as *tet*M, *tet*X, *str*B and *sul*2. As no antimicrobial has been applied to both farms for at least five year prior to sampling, the result indicated those ARGs were ubiquitous in poultry manure and their presence was not necessarily related to antimicrobial use.

Analysis of co-occurring genes revealed correlations among ARGs, and between ARGs and MGEs. Correlations of ARGs could indicate they are carried together on mobile elements. For example, the co-occurrence of *str*B and *sul*2 gene might be due to plasmids carrying the *sul*2-*str*A-*str*B cluster (Yau et al., 2010, Bean et al., 2009). This gene cluster occurs on RSF1010-like plasmids, which are found in a broad range of bacteria that occur widely in the environment and have been reported in various studies over decades (Scholz et al., 1989, Daly et al., 2005, Yau et al., 2010). Similarly, co-occurrence of *aph*A3 and *sat*4 might reflect a gene cluster such as *aph*A3-*sat*4-*aad*E, which is known to be carried by transposons that have been commonly detected in Gram-positive bacteria (Derbise et al., 1997b, Werner et al., 2001, Palmieri et al., 2012).

Interestingly, the high level of class I integron genes *int*I1 and *qac*EΔ and their correlation to the chloramphenicol resistance gene *cml*A1 indicated the potential of gene transfer in the sheds, and previous studies have shown that these genes can be found as integron cassettes (Partridge et al., 2018, Moura et al., 2009). Moreover, *intI*1 is widely distributed in pathogenic and commensal bacteria and has been proposed as a gene marker to assess anthropogenic pollution (Gillings et al., 2015). As chloramphenicol has been banned from use in farm animals in Australia since 1985, the results of the current study suggest that class I integrons might assist in the maintenance and dissemination of the *cml*A1 gene. Nevertheless, the findings in this study are based on statistical analysis and would need to be validated. One approach would be to isolate chloramphenicol resistant bacteria from the faecal samples and

examine the presence of *cml*A gene and its genetic linkage with class I integron. Another approach would be to sequence the faecal metagenome using long-read technique, such as Nanopore, to capture reads showing genetic linkage of *cml*A and the class I integron genes.

In conclusion, this study showed that ARGs with high abundances in chicken faeces, particularly tetracycline and macrolide resistance genes, were likely due to their carriage by bacterial species naturally present in the chicken faecal microbiome, or because these genes have been already widely distributed in the environment, such as *str*B and *sul*2. The clustering of ARGs and class I integron genes suggests a potential for HGT among bacteria found in sheds. Nevertheless, the genetic linkage of ARGs and class I integron needs further investigation.

Chapter 7 ARGs carried by faecal microbiome of commercially raised pigs

7.1 Introduction

Antimicrobials play an important role in livestock production in terms of disease prevention and animal welfare, however extensive antimicrobial use is often associated with selection of bacteria with AMR (Marshall and Levy, 2011). AMR of zoonotic pathogens and commensal bacteria in food animals is a potential risk to human health due to HGT of ARGs between bacteria (von Wintersdorff et al., 2016, Goldstein et al., 2001). Studies based on key indicator bacteria, such as *E. coli*, have found a significant correlation between antimicrobial usage and the occurrence of AMR in bacteria (Chantziaras et al., 2013, Asai et al., 2005). In addition, recent pig metagenome studies have revealed that the level of ARGs in pig faecal microbiomes appear to reflect the overall country-specific antimicrobial usage (Munk et al., 2018, Xiao et al., 2016).

Australia has a comparatively conservative use of antimicrobials in food animals (Cheng et al., 2012). Antimicrobial use in agriculture in Australia was the fifth lowest compared to the USA and European countries (O'Neill, 2015), and some antimicrobials, such as gentamicin and fluoroquinolones are not used in food animals (APVMA, 2017). Antimicrobials approved for use in pigs include sulfonamide-trimethoprim combinations, tetracycline, beta-lactams, and aminoglycosides (neomycin, apramycin and spectinomycin) (Smith et al., 2016). The third generation cephalosporin, ceftiofur, is registered in Australia for the treatment of cattle respiratory disease but can be prescribed for 'off-label use' in pigs (Jordan et al., 2009). A national survey covering half of the large pig herds (n=196) in Australia showed the most used antimicrobials in pig herds were penicillins, tetracyclines and sulphonamides (Jordan et al., 2009), which are considered to be of low importance in human medicine (Office of Health, 2018). Ceftiofur was reportedly used in only 25% of herds and virginiamycin was not used (Jordan et al., 2009).

Studies of *E. coli* and *Salmonella* isolated from pig faeces of different Australian pig farms showed that the overall resistance of the isolates to ceftiofur and fluoroquinolones was low, and the corresponding resistance genes bla_{CMY}, $bla_{CTX\text{-}M}$ and *qnr* were not detected (Smith et al., 2016, Kidsley et al., 2018). On the other hand, resistance to ampicillin, florfenicol/chloramphenicol, gentamicin and trimethoprim-sulfonamide was relatively frequent and the associated ARGs were common (Smith et al., 2016, Kidsley et al., 2018). Nevertheless, the indicator bacteria only make up a small proportion of the gut microbiome and may not represent the true diversity of ARGs present. Metagenomic approaches bypass the limitation of culturing and provide a more comprehensive understanding of ARGs associated with the animals.

To study the presence and diversity of ARGs in pigs, we collected faeces of gilts from multiple cohorts of one herd in a commercial swine farm. Total faecal DNA was used to detect and quantify ARGs and MGEs using HT-qPCR. In addition, floor swabs from herd housing areas were collected opportunistically, and their ARG profiles were compared to faecal samples. Finally, the microbiomes of faeces and swabs were analysed in order to understand the differences between ARG profiles of different sample types.

7.2 Methods

7.2.1 Swine farm and faecal material sampling

Sampled gilts in this study were from one commercial swine farm. A recent survey of Australian swine farms (Little, unpublished 2019) indicated that antimicrobial use on this farm was moderate compared to that of similar enterprises. Broad-spectrum antimicrobials were administered for control of respiratory and enteric diseases through the pigs' feed and drinking water. Vaccination was also a component of the farm's animal health management strategy. Fresh faeces were collected from four cohorts of gilts. Each cohort consisted of eight to ten gilts and sampling between each cohort was one to two weeks apart. The animals were collected from the farm and transported for less than 2 hours to the animal house at The University of Melbourne. The animals were unloaded and housed in individual pens. The pen was hosed thoroughly to remove any faeces before housing the new cohort. The animals were sampled two to four hours after transportation and before they were fed. Fresh faeces

of each animal were collected post-defecation into a 5mL sterile plastic container and transported to laboratory on ice within 30 minutes. Samples were stored at -80˚C until DNA extraction.

7.2.2 Herd floor swabs

The gilt herds of the swine farm were sampled opportunistically one year before the faecal sampling. Although these samples did not match the study pig cohorts, they were included in the analysis for comparison of sample type. Three swabs were collected from the floors just outside the pens in the gilt herd by persons wearing sterile covers outside the clean dry boots and walking through the herds. Each swab was stored in a Whirl-Pak® sterile sample bag (Nasco, USA) and transported to the laboratory at ambient temperature. The swabs were rinsed in 50mL peptone water and homogenised by gently squeezing the bag. Then, all the liquid was transferred into a 50mL plastic tube and centrifuged at 2885×g for 30min at 4°C (Beckman Coulter, USA). The precipitate was resuspended in 4mL buffered peptone water and transferred into 2mL microcentrifuge tubes and centrifuged at 10,000×g for 2min (Eppendorf, USA). Then, all the biomass of one swab was transferred into a 2mL microcentrifuge tube and stored at -80°C until DNA extraction.

7.2.3 DNA extraction of faecal and swab samples

DNA extraction of faecal and swab samples was according to “Section 2.1 DNA extraction from faecal and swab samples”.

7.2.4 HT-qPCR array for gene detection and quantification

ARGs, MRGs and MGEs were detected and quantified according to ““Section 2.3 HT-qPCR array for gene detection and quantification”.

7.2.5 Bacterial 16S rRNA gene sequencing and bioinformatics analysis

DNA extracts of each faecal sample from each cohort and all three swabs were used to analyse the bacterial community composition according to methods in “Section 2.2 Bacterial 16S rRNA gene sequencing and bioinformatic analysis”.

7.2.6 Statistical analysis

All statistical analyses were completed using QIIME2 (Bolyen et al., 2019) and RStudio. A heatmap of the relative abundance of each gene in each sample was built using the ComplexHeatmap R package (Gu et al., 2016). The similarity of ARG profiles of faecal samples, grouped by cohort, was analysed using the NMDS method with Bray-Curtis distance matrix. Similarity among groups was tested by ANOSIM using the 'anosim' function in the vegan R package. The NMDS result was plotted using the ggplot2 R package with ellipses indicating 95% confidence region of each cluster.

The co-occurrence patterns of ARGs were analysed according to "Section 2.4 Co-occurrence of ARGs and MGEs".

7.3 Results

7.3.1 Detection and quantification of resistance genes and MGEs

After excluding samples which failed to amplify the 16S rRNA gene there remained nine, nine, eight and 10 faecal samples from cohorts 1 to 4, respectively, and all three herd floor swabs were validated for the following analysis. From qPCR of the faecal DNA samples, the 53 primer pairs detected 37 ARGs, three MRGs and four MGEs in at least one sample. The number of detected genes in each sample ranged from 23 to 34, with the median of 29. For the frequently detected genes, 22 ARGs and two MGEs were detected in more than 80% samples (29 out of 36). These ARGs were from the following antimicrobial classes; aminoglycoside, beta-lactamase, florfenicol, macrolide/lincosamide, sulfonamide and tetracycline.

The relative abundance of the detected genes was represented by their proportion relative to the 16S rRNA gene (Fig 7-1). For the 22 frequently detected ARGs, 10 had median relative abundances above 10^{-2} across detected samples (Table 7-1). The most abundant ARG was the macrolide resistance gene *mef*A and the tetracycline resistance gene *tet*Q, with median relative abundances of 1.3 and 6.2×10^{-1}, respectively. The next most abundant ARGs were the macrolide resistance genes *erm*F and *erm*B and the tetracycline resistance genes *tet*O, *tet*W and *tet*L. Other abundant ARGs were the aminoglycoside resistance gene *aph*A3, the beta lactamase resistance gene *cfx*A and the streptothricin resistance gene *sat*4. For the two frequently detected MGEs, *tnp614*

and *IS613* were found with high levels in pig faeces, with the median relative abundance of 2.9 and 9.8×10^{-1}, respectively.

The ARG and MGE profiles in the three herd floor swabs were notably different from that observed in pig faeces. The ARGs that were notably more abundant in faecal samples than in floor swabs were *mef*A, *erm*B, *tet*Q, *tet*W, *cfx*A and MGE genes *IS613* and *tnp614*, which can be seen as highlighted orange/red in the heatmap (Fig 7-1). All ARGs or MGEs found in faeces were detected in the floor swabs. In contrast, several genes that were detected in the floor swabs were either in low relative abundance (*aph*A1, *str*B, *sul*2, *tet*X, *int*I1) or undetectable (*aac(6')-Ib*, bla_{CMY}, $bla_{OXA1/OXA30}$) in the faecal samples (Fig 7-1). The range of the relative abundance values of genes with high levels in faecal and swab samples are shown in Table 7-1 and Table 7-2, respectively.

Neither the ceftiofur resistance gene bla_{CMY} or the fluoroquinolone resistance gene *qnr*B were detected in any sample. The other ceftiofur resistance gene bla_{CTX-M} and virginiamycin resistance gene *vat*E were detected in 16 and 4 samples, respectively, with low abundances (10^{-6} to 10^{-4}). These genes were also found with low levels in the herd swabs.

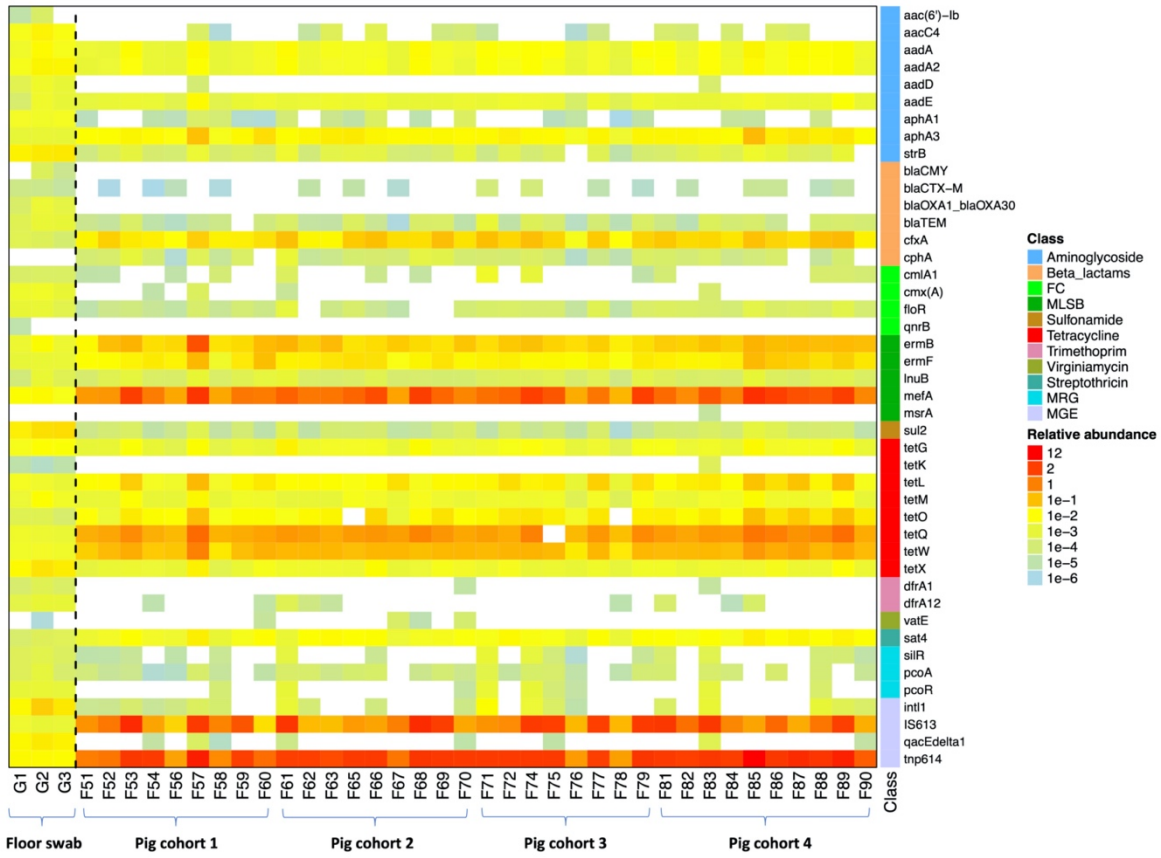

Figure 7-1: Heatmap of the relative abundance of ARGs, MRGs and MGEs in individual faecal and floor swab samples. FC, fluoroquinolone/florfenicol; MLSB, macrolide-lincosamide-streptogramin B; MRG, metal resistance gene.

Table 7-1: Genes detected at high levels in pig faeces

		Relative abundance to 16S rRNA genes		
Gene	**Class**	**Min**	**Max**	**Median**
*aph*A3	Aminoglycoside	3.9×10^{-3}	1.6×10^{-1}	2.1×10^{-2}
*cfx*A	Beta lactamase	7.7×10^{-3}	1.7×10^{-1}	6.3×10^{-2}
*erm*B	MLSB	2×10^{-2}	1.8	8.1×10^{-2}
*erm*F		4.7×10^{-3}	1.8×10^{-1}	3.1×10^{-2}
*mef*A		2.3×10^{-1}	6.2	1.3
*tet*L	Tetracycline	1.8×10^{-3}	1.7×10^{-1}	2×10^{-2}
*tet*O		2.2×10^{-3}	1.2×10^{-1}	2.8×10^{-2}
*tet*Q		1×10^{-1}	1.4	6.2×10^{-1}
*tet*W		4×10^{-2}	9.9×10^{-1}	2×10^{-1}
*sat*4	Streptothricin	1.6×10^{-3}	3.5×10^{-2}	1×10^{-2}
IS613	Mobile genetic element	5.5×10^{-2}	7.6	9.8×10^{-1}
tnp614		5.3×10^{-1}	12	2.9

Table 7-2: Genes detected at high levels in herd floor swabs

Gene	Class	Relative abundance to 16S rRNA genes		
		Min	Max	Median
*aad*A2	Aminoglycoside	7.7×10^{-3}	2.6×10^{-2}	2.5×10^{-2}
*aph*A1		5.2×10^{-3}	6.4×10^{-3}	6.1×10^{-3}
*str*B		2.7×10^{-2}	4.2×10^{-2}	4×10^{-2}
*sul*2	Sulfonamide	3.3×10^{-2}	5.9×10^{-2}	5.3×10^{-2}
*tet*X	Tetracycline	1.7×10^{-2}	5×10^{-2}	3.6×10^{-2}
intI1	MGE	1.9×10^{-2}	8.2×10^{-2}	4.2×10^{-2}
tnp614		1.5×10^{-2}	2×10^{-2}	1.7×10^{-2}

7.3.2 Similarity of ARGs and MGEs profiles of faeces between cohorts

The composition of ARGs and MGEs in faecal samples was compared between pig cohorts. For these comparisons, NMDS analysis was performed using Bray-Curtis distance metrics derived from the relative abundance of resistance genes and MGEs. No distinct clustering of samples was showed based on cohorts (ANOSIM, R = 0.05, P = 0.11), which indicated a consistent resistance gene and MGE profile of pigs from the same herd (Fig 7-2).

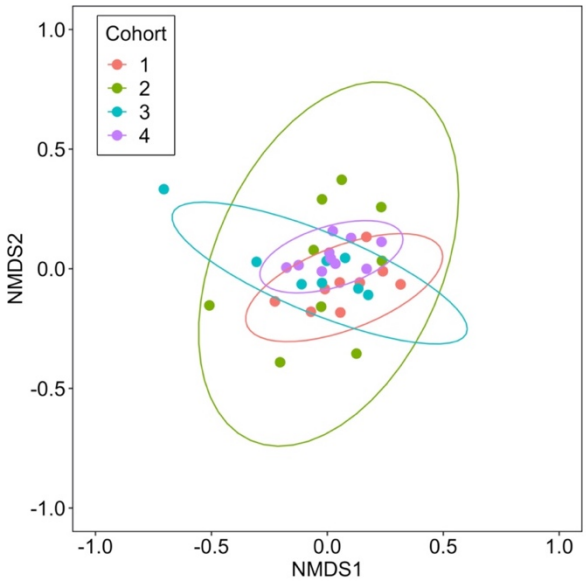

Figure 7-2: NMDS with Bray-Curtis distance comparing relative abundance of resistance genes and MEGs among the pig cohorts. The ellipses indicated 95% confidence region of each cluster. No significant separations were detected between cohorts.

7.3.3 Co-occurrence of resistance genes and MGEs in pig faeces

To examine potential associations between ARGs and MGEs, their co-occurrence patterns were analysed by Spearman rank correlation and the results displayed as networks (Fig 7-3). A total of 18 ARGs were found to form three separate networks. The lower section of the Figure 7-3 shows a large and complex network that includes 14 genes, while the upper section shows two small networks, each with two genes. In total, there are ARGs to aminoglycoside, beta lactamase, macrolide, sulfonamide, tetracycline and streptothricin classes, as well as MGEs belonging to two transposons, *IS*613 and *tnp*614. The major gene cluster showed the correlation of *tet*Q, *tet*O, *mef*A with *tnp*614. These genes were detected with high abundance in pig faeces. Another cluster showed the correlation between *aph*A3, *aad*E and *sat*4. In addition, the beta lactamase resistance gene *cph*A co-occurred with *IS613*, although *cph*A was only detected at low levels, with a median relative abundance of 1.7×10^{-4}.

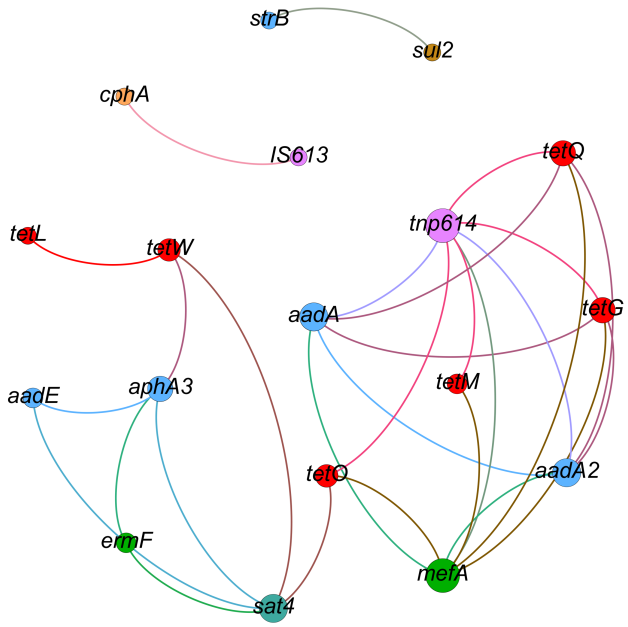

Figure 7-3: Network summary of co-occurring ARGs and MGEs in pig faecal samples (Spearman's rho ≥ 0.75, $P < 0.01$). The size of each node is proportional to the number of connections.

7.3.4 Bacterial community composition of pig faeces and herd floor swabs

After PCR of 16S rRNA genes from the four pig faecal samples and three herd floor swabs, a total of 576,464 high quality reads were obtained. The number of sequences per sample ranged from 62,683 to 101,837. For fair comparison among samples, the sequence depth in each sample was rarefied to 62,683 and all samples had sufficient depth for accurate estimation of richness and diversity. The rarefied reads were partitioned into 1,382 ASVs.

The bacterial composition of pig faeces was very different from that of the floor swabs. At phylum level, the most dominant phyla in the pig faecal microbiome were *Firmicutes* and *Bacteroidetes*. *Firmicutes* accounted for 48% to 61% of the total bacterial community and *Bacteroidetes* accounted for another 34% to 45% (Fig 7-4a). However, the swab samples showed a different composition, with the most abundant phylum being *Proteobacteria* which accounted for more than 70% of the total microbiome. In faeces, the proportion of *Proteobacteria* was less than 4%.

A total of 74 families and 227 genera were identified in the faecal and swab samples. In faeces, the five major families were *Prevotellaceae*, *Ruminococcaceae*, *Lachnospiraceae, Paraprevotellaceae and Streptococcaceae*, which together accounted for 54% to 75% of the bacterial community (Fig 7-4b). In contrast, the most dominant family in the swabs was *Moraxellaceae,* with abundances of 32% to 38%. At genus level, the most dominant bacteria in the faecal microbiome was *Prevotella* (21% to 35%), followed by *Streptococcus* (3% to 14%), *unclassified Ruminococcaceae* (2% to 9%) and *Lactobacillus* (2 to 21%). In the swab microbiome, the most dominant genus was *Acinetobacter*, which ranged from 26% to 34% (Fig 7-4c).

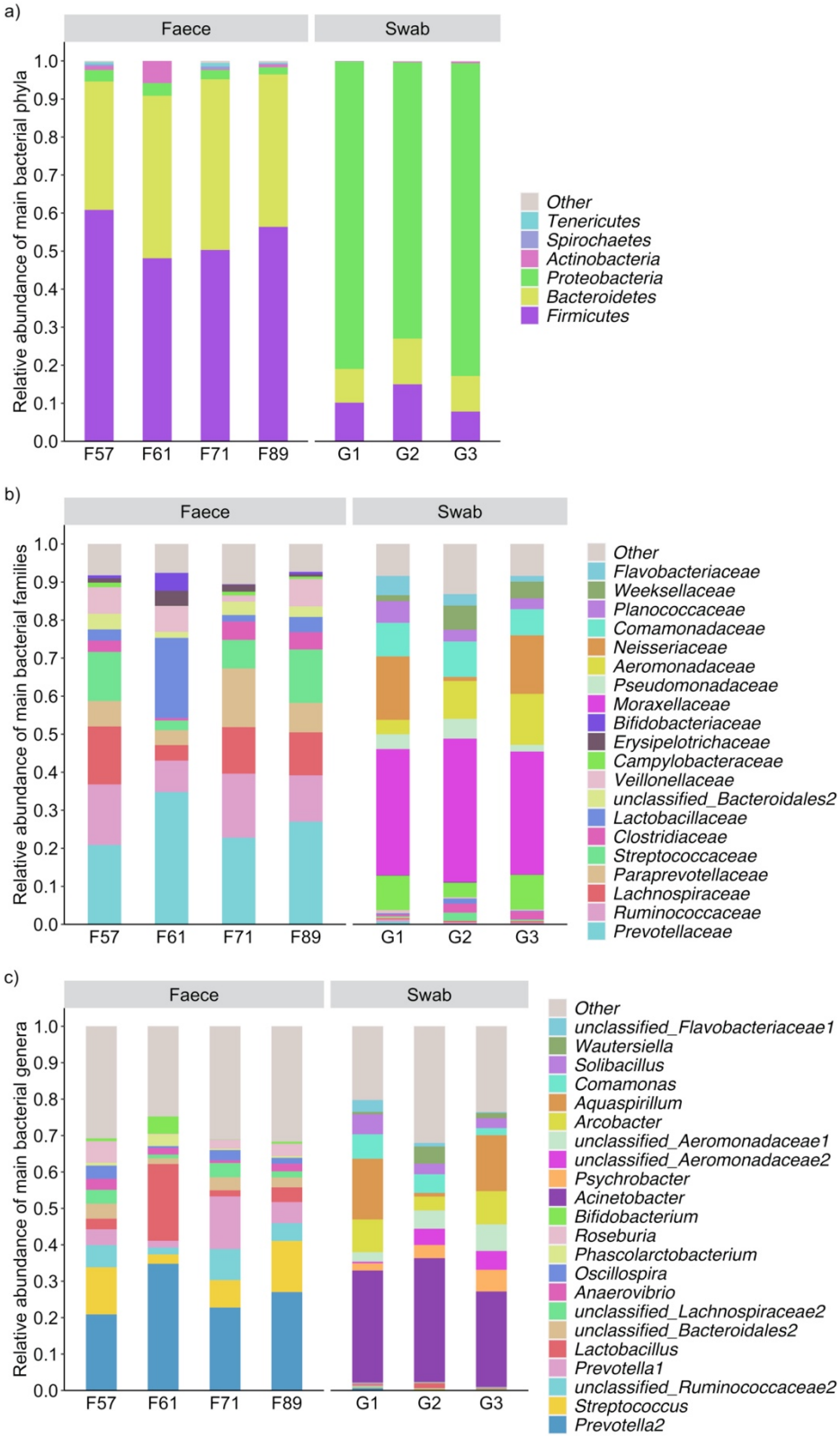

Figure 7-4: Relative abundance of dominant bacterial phyla (a), families (b) and genera (c) in faecal and swab samples. Only phyla with a relative abundance above 0.5%, families and genera with a relative abundance above 3% in at least one sample are listed. Taxa below the cutoff were assigned as “Other”.

7.4 Discussion

In this study, we collected faecal samples from four gilt cohorts after transport from a commercial swine farm. The sampled animals were raised in the same herd on the farm and sampling between cohorts was one to two weeks apart. In addition, swabs of floors just outside the pens in the gilt herd were collected opportunistically one year before the collection of faecal samples. ARGs covering the majority of antimicrobial classes and MGEs were quantified in the faecal and swab samples. The results showed that the faecal samples across all cohorts had a consistent ARG profile, and that this profile was greatly different to that of the swab samples. Nevertheless, ceftiofur and fluoroquinolone resistance genes were detected with very low levels in both sample types, which was consistent with the food animal antimicrobial use regulations in Australia and agreed with a previous culture-based study using *E. coli* (Smith et al., 2016). Microbiome analysis of each sample type suggested the differences in the ARG profile were due to the differences in the bacterial composition.

Faecal samples frequently displayed high levels of tetracycline resistance genes; *tet*Q being the most abundant followed by *tet*W, *tet*O and *tet*L. These results agreed with previous studies which reported a ubiquitous prevalence of these *tet* resistance genes in faecal samples from pigs (Munk et al., 2018, Wang et al., 2017, Xiao et al., 2016, Zhu et al., 2013, Patterson et al., 2007) and humans (Hu et al., 2013, Seville et al., 2009). These genes had a broad range of bacterial hosts, including the genera dominant in the pig gut microbiome, such as *Prevotella* spp*., Bacteroides* spp*., Streptococcus* spp., and *Lactobacillus* spp*.* (Roberts et al., 2012). Therefore, the high level of the *tet* genes may be due to the predominance of potential hosts in the pig gastrointestinal tract. In addition, the majority of *tet* genes were often associated with mobile elements that could facilitate the gene spreading among bacterial species (Chopra and Roberts, 2001). *tet*Q was found associated with CTnDOT type conjugative transposons in *Bacteroides* which could facilitate gene transfer within the species (Shoemaker et al., 2001). Moreover, studies reported the transfer of *tet*Q between *Bacteroides* and *Prevotella* (Nikolich et al., 1994) and transfer of *tet*Q from *Bacteroidetes* species into *Enterococcus faecalis* (Chung et al., 1999, Leng et al., 1997). It was hypothesised that the ribosomal protection genes, such as *tet*Q, *tet*W and *tet*O, originated from Gram-

positive bacteria and have been exchanged throughout the bacterial population regardless of species or genus and ended up in a wide range of bacterial host backgrounds (Chopra and Roberts, 2001).

The faecal samples also showed high levels of macrolide resistance genes, the highest being *mef*A followed by *erm*B and *erm*F. The *mef* genes encode an efflux-mediated macrolide resistance while the *erm* genes encode rRNA methylases that add one or two methyl groups to a single adenine in 23S rRNA, thereby protecting ribosomes from inhibition by macrolides (Roberts, 2011). Similar to the *tet* genes, *mef*A, *erm*B and *erm*F genes have been found to be carried by a variety of bacterial genera present in the pig gut microbiome, including *Prevotella* spp*., Bacteroides* spp*., Streptococcus* spp*.*, and *Lactobacillus* spp.(Roberts et al., 2012, Roberts, 2011). Also, the *erm* genes have been commonly reported to be abundant in human and animal faecal metagenomes (Hu et al., 2013, Munk et al., 2018, Patterson et al., 2007, Sui et al., 2016). In addition, the three genes have been found associated with conjugative elements which could allow transfer across species and genus barriers (Roberts et al., 1999, Soge et al., 2009, Santagati et al., 2003, Del Grosso et al., 2002, Roberts and Mullany, 2011). These genes are often associated with the *tet* genes. For example, the linkage of *mef*A-*tet*O and *erm*B-*tet*M were found on conjugative transposons of Tn1545/Tn916 family (Giovanetti et al., 2003, Seral et al., 2001, Cochetti et al., 2007, De Leener et al., 2004, Roberts, 2005). And *erm*F is linked with *tet*Q on transposons of the Tc^rEm^r DOT type (Chung et al., 1999). The wide dispersal of these transposons may explain the prevalence of the *erm* and *tet* genes.

Two mobile genetic markers, *IS613* and *tnp614,* were found to be abundant in the pig microbiome, and were correlated with ARGs. Primer pairs tnp614 amplify the transposase on IS614, which is wide spreading in *Bacteroides* spp. (Sóki, 2013). Therefore, the cluster of *tet*Q, *mef*A and *tnp*614 could be explained by the co-existence of these genes in the common bacterial hosts. *IS613* was found co-related to *cph*A gene, which encodes a class B metallo-beta-lactamase that could hydrolyse carbapenems (Segatore et al., 1993). *cph*A was detected with low abundance (~10^{-6} to 10^{-3}) in this study, although it was found in more than 90% (33 out of 36) samples. *cph*A was commonly reported on the chromosome of *Aeromonas* spp (Janda and

Abbott, 2010, Walsh et al., 2005), which, however, was below detection in the faecal microbiome in this study. The result may reflect the unknown bacterial hosts of *cph*A which co-exist with hosts of the *IS613*. Future studies could use long read metagenomic sequencing to reveal the identities of the bacterial hosts and precise relationship of these genes.

Moreover, *str*B and *sul*2 were both found co-related in the faecal samples but both genes were detected with a relatively low abundance (~10^{-6} to 10^{-3}). The RSF1010-like plasmids carrying *sul*2-*str*A-*str*B cluster have been widely disseminated in a broad range of hosts in the environment for decades (Yau et al., 2010, Hochhut et al., 2001, Scholz et al., 1989, Partridge, 2011). This result suggests spread of gene clusters in pig intestinal tracts, however, low gene abundances indicate that only a small proportion of bacteria harbor these genes.

Compared to the faecal samples, the herd floor swabs had a notably different ARG profile. The greatest change is the reduction in the relative abundance of *tet*Q and *mef*A, most likely due to differences in the microbiome composition of the sample types. Swabs had a much lower level of *Firmicutes* and *Bacteroidetes* than the faecal samples, and the bacterial community was mainly comprised of *Proteobacteria*, especially the *Acinetobacter* spp, which are Gram-negative, strict aerobes and ubiquitous in human, animal and environment (Al Atrouni et al., 2016). Biomass collected by the swab was mainly dust and small proportion of animal faeces. As material picked up by floor swabs had been exposed to the air for some time, the proportion of anaerobes is expected to reduce, and the proportion of aerobes and facultative anaerobes increase. Although the floor swabs were collected one year prior to the sampling of faeces and did not match the cohorts, the large difference in the ratio of aerobes to anaerobes in microbiome between floor swab and pig faeces was expected to be observed one year later. This divergency in microbiome composition led to changes in ARG abundance, therefore, swabs are not suitable to study ARG profile associated with pig gut microbiomes.

In conclusion, pigs from the studied herd had a consistent ARG profile during the one-month sampling period. The sample size could provide guidance for further studies of

ARGs in other swine farms. Tetracycline and macrolide resistance genes, especially *tet*Q and *mef*A genes, were detected at high levels in faecal samples, and this was most likely due to the dominance of *Firmicutes* and *Bacteroidetes* in the faecal microbiome. On the other hand, the relatively low level of these ARGs in herd floor swabs may be explained by very different microbial community composition of these samples, and the dominance of *Proteobacteria*.

Chapter 8 Other Findings

8.1 Background

The major work in this thesis describes monitoring the ARG profiles of various livestock from the microbiome perspective, using culture-independent methods. Some culture-based experiments were also conducted as an adjunct to the culture-independent methods. This section entitled, "Other findings" describes some of those experiments which led to publications:

- Dyall-Smith, M. L., Liu, Y. & Billman-Jacobe, H. 2017. Genome sequence of an Australian monophasic *Salmonella enterica* subsp. *enterica* Typhimurium Isolate (TW-Stm6) carrying a large plasmid with multiple antimicrobial resistance genes. *Genome Announc.*, 5, e00793-17.
- Billman-Jacobe, H., Liu, Y., Haites, R., Weaver, T., Robinson, L., Marenda, M. & Dyall-Smith, M. 2018. pSTM6-275, a conjugative IncHI2 plasmid of *Salmonella* that confers antibiotic and heavy metal resistance under changing physiological conditions. *Antimicrob. Agents Chemother*., 62:e02357-17.
- Billman-Jacobe, H., Dyall-Smith, M., Hawkey, J. & Liu, Y. 2019. Heavy metal and antibiotic resistance genes in bacteria from porcine monophasic *Salmonella* 1,4,[5],12:i:-. *Journal of Integrated OMICS*, 9, 37.

In a previous study by Tom Weaver (Weaver et al., 2017), *Salmonella enterica* serotype 1,4,[5],12:i:- strains were isolated from fresh pen-floor pig faecal samples from six pig farms and an abattoir between 2011 to 2015. Selected isolates were sequenced on an Illumina NextSeq® platform. During my candidature, one isolate, TW-Stm6, was further sequenced by Pacific Biosciences (PacBio) RS II platform and used as a reference strain (Genbank accession numbers: CP019647, CP019648, and CP019649). I contributed to the annotation of the TW-Stm6 genome, including its plasmids, and in writing two manuscripts reporting this work (Dyall-Smith et al., 2017, Billman-Jacobe et al., 2018).

The TW-Stm6 genome contains a 4,999,862bp chromosome, a 275.8kb IncHI2 plasmid (pSTM6-275) and a 4 kb MOB_Q plasmid (pSTM6-4). The annotated open reading frames (ORFs) showed the chromosome had a 15,725bp deletion of the *fliAB* region, which

was replaced by a 28,209bp insertion carrying ARGs *bla*$_{TEM}$, *str*B, *str*A, *sul*2, *tet*B and *tet*R and the mercury resistance operon (Dyall-Smith et al., 2017). In addition, the chromosome carried a *sil-pco* locus on the genomic island SGI-4. ARGs and MRGs were also detected on the plasmid pSTM6-275. ARGs (*bla*$_{TEM}$, *str*A, *str*B, *sul*3, *aad*A1, *aad*A2, *cml*A, *aph*A2 and *tet*A) on pSTM-275 were associated with IS elements, transposons and integrons; and a *sil-pco* locus with the same gene arrangement on the chromosome was carried by a Tn7-family transposon on the plasmid. Conjugal transfer of pSTM6-275 was demonstrated from TW-Stm6 to *E. coli* DH5α at 27˚C. The transconjugants showed resistance to ampicillin, sulfonamides, streptomycin, spectinomycin, kanamycin, tetracycline, trimethoprim and chloramphenicol corresponding to the ARGs carried by the plasmid. Sensitivity to $CuSO_4$ of transconjugants were decreased under anaerobic conditions. The genome announcement of TW-Stm6 was published in 2017 (Dyall-Smith et al., 2017). Details of characterisation of pSTM6-275 sequence, plasmid conjugal transfer and testing of sensitivity to antimicrobials and metals were published in 2018 (Billman-Jacobe et al., 2018).

Phylogenetic analysis was done using 55 isolates based on their core genome sequences using short read libraries and the assembled contigs, respectively. Isolates had little diversity based on the core genome (less than 81 single nucleotide polymorphisms (SNPs)) and isolates were clustered based on the source (herds and abattoir), indicating the persistence of *S*. 1,4,[5],12:i:- in the sampled properties (Billman-Jacobe et al., 2019).

I performed CRISPR analysis on the genomes and showed there were 33 spacers in CRISPR1 loci and 25 in CRISPR2 loci. The overall spacer patterns of CRISPR1 loci were similar across the isolates. Whereas, for CRISPR2 loci, isolates from 2011 had one pattern and the rest of the isolates had a distinct pattern with three spacers deleted. Furthermore, phylogenetic analysis showed the Australian *S*. 1,4,[5],12:i:- isolates had a close relationship with the isolates from the current European epidemic clade described in a study by Petrovska et al. (Petrovska et al., 2016). The details of this work were published in 2019 (Billman-Jacobe et al., 2019).

This work was extended in this thesis. The purpose of doing some culture-based examination of pig feces was principally to demonstrate there were copper tolerant bacteria in some of the faeces and that those specimens were suitable to test newly designed qPCR primers for *pco* and *sil* loci. Copper tolerant isolates were purified, and then phenotypically and genoptypically characterised.

The approach was to screen faecal samples for the presence of copper tolerant bacteria by culturing the bacteria on $CuSO_4$ selective agar and analysing the isolates. These isolates could then be used as positive controls in qPCRs to detect *pco* and *sil* genes in DNA extracted from manure. In addition, 16S rRNA gene sequences were also analysed in order to determine the abundance of bacterial species carrying those genes.

8.2 Methods

8.2.1 Primers targeting copper and silver resistance genes

The TW-Stm6 genome sequence was used to design PCR primers targeting *sil*R, *pco*A and *pco*R genes. These primers would then be used for screening MRGs present in faecal samples described in the previous chapters of this thesis (Table 8-1). The primers were first tested *in silico* against the NCBI Genbank database, and then in PCRs using DNA from TW-Stm6.

Table 8-1: Primer sequences of target genes

Assay	Forward Primer	Reverse Primer	Length (bp)
*sil*R	CTGATGTGAACGGCTGGGAT	ACCCTGTGTTCRATCGTGC	100
*pco*A	ATGCCGGGTATGCAAAGTCA	TTTCGGAGAGACGCTCATCG	81
*pco*R	CGTGCGGGACAAAGTGAAAG	CGCAGTAGGGTTCTTACACG	102

8.2.2 Isolation of copper resistant *Escherichia* spp. from pig faeces

8.2.2.1 Screening of MRGs in pig faecal DNA

Fresh faeces were collected from 13 gilts purchased for a separate project and kept in the animal house at The University of Melbourne. The faeces were collected less than 3 hours after the animals arrived from the farm. Faeces from each animal were

collected post defecation into a 5mL sterile plastic container and stored at -80˚C until DNA extraction.

Faecal DNA extraction was carried out as described in Chapter 2. *sil*R, *pco*A and *pco*R genes in the faecal DNA were screened by qPCR using the primer pairs shown in Table 8-1. The qPCR was conducted using the Rotor-Gene® Q system (QIAGEN, Hilden, German). Each qPCR run was as follows: 95˚C for 10 min, followed by 35 cycles of 95˚C for 30 s, and annealing at 60 ˚C for 30 s. A 20µl reaction mixture contained 400nM of each primer (IDT, USA), 0.2mM of each dNTPs (Bioline), 1.5mM of $MgCl_2$ (Promega), 8µl syto9 (Thermo Fisher Scientific), 0.05U/µl of GoTaq® Flexi DNA Polymerase (Promega), 4 µl of 5X Flexi Reaction Buffers (Promega), 7.2 µl of nuclease-free water and 1 µl of DNA template. Standard curves were generated using genomic DNA of strain TW-Stm6, which was also used as a positive control. Genomic DNA of *E. coli* DH5α and nuclease-free water were included in each qPCR run as negative control and no template control, respectively. Specific amplification of each amplicon is validated by high resolution melting curve.

8.2.2.2 Isolation of copper-tolerant bacterial strains from pig faeces

One gram of fresh faeces from each pig was suspended in 9mL phosphate buffered saline and homogenised. Then, 100 µl of the mixture was spread on a Luria broth plate containing 6.25mM $CuSO_4$ and the plates were incubated aerobically at 37 ˚C overnight (Billman-Jacobe et al., 2018).

Bacterial colonies were recovered from the faeces of 10 pigs. One isolate per pig was selected randomly for further analysis. MICs of $CuSO_4$ of the 10 isolates were determined by agar dilution assays under aerobic and anaerobic conditions as described in a previous study (Billman-Jacobe et al., 2018).

8.2.2.3 Analysis of the genome sequences of faecal isolates

The genomes of the 10 copper-tolerant isolates were sequenced using a combination of Illumina MiSeq and Nanopore technologies. Hybrid assembly of each bacterial genome using paired-end Illumina reads and Nanopore long reads was conducted using Unicycler (Version 0.4.7) (Wick et al., 2017, Walker et al., 2014). Taxonomic classification was done using the whole genome sequence with the Kraken taxonomic

classifier version 2 (Wood and Salzberg, 2014) using a custom made database containing all completed bacterial reference genomes in NCBI. Plasmid typing was performed using the ABRicate program (https://github.com/tseemann/abricate) with the PlasmidFinder database (Carattoli et al., 2014).

ARGs in the isolate genomes were identified using the ABRicate program and the CARD database (McArthur et al., 2013). MRGs were identified using a custom-made metal resistance database containing 64 MRGs.

8.2.2.4 Analysis of the pig faecal microbiome

The bacterial community present in the faecal samples from which copper tolerant strains were isolated, was characterised by PCR amplifying the V3-V4 region of 16S rRNA gene, and sequencing the amplimers on an Illumina MiSeq platform, as described in "Section 2.2 Bacterial 16S rRNA gene sequencing and bioinformatic analysis".

8.3 Results

8.3.1 Detection of *sil*R, *pco*A and *pco*R genes in pig faeces

PCR amplification using primers specific for *sil*R, *pco*A and *pco*R genes produced similar high-resolution melting curves for both the positive control (strain TW-Stm6 genomic DNA) and faecal DNA preparations, which indicated that these genes were present the pig faecal microbiome (Fig 8-1). The slight shift of the peak of faecal amplicons of *sil*R and *pco*R compared to the amplicon of the TW-Stm6 genomic DNA might due to the presence of SNPs in the amplicons.

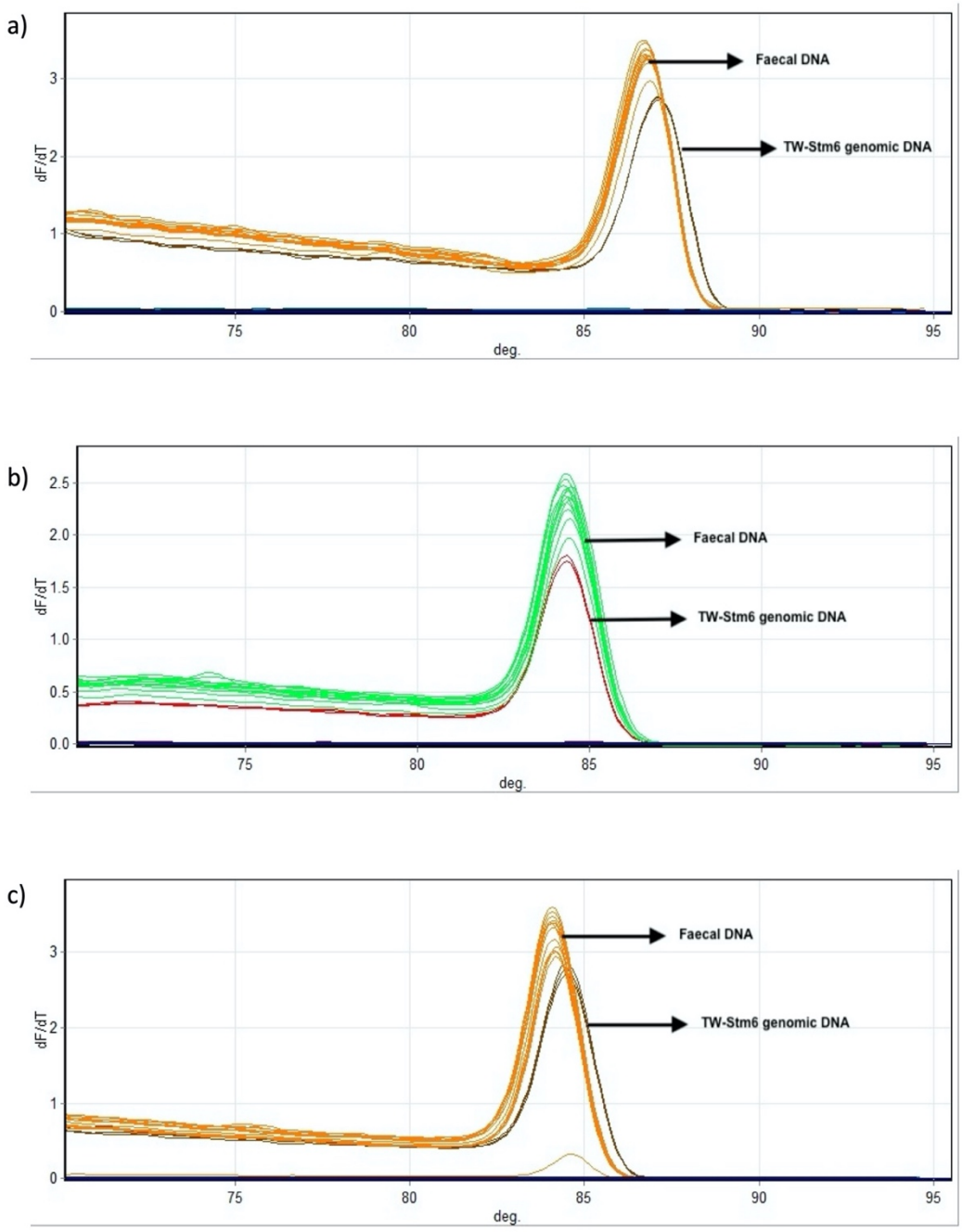

Figure 8-1: High resolution melting curves of (a) *sil*R, (b) *pco*A, and (c) *pco*R amplicons derived from faecal and strain TW-Stm6 genomic DNA. The y axis represents the derivative fluorescent signal and x axis represents temperature (˚C).

8.3.2 Copper sulphate tolerant bacterial isolates

Numerous but still countable single bacterial colonies derived from 1/10 diluted pig faeces, grew on the $CuSO_4$ agar plates. This indicated that the copper tolerant species able to grow on the media represented a very small fraction of the faecal microflora. One copper tolerant bacterial colony was randomly picked from each pig sample for further analysis. The $CuSO_4$ MICs of selected isolates showed they were more copper-tolerant than controls (*E. coli* DH5α) (Table 8-2).

Table 8-2: MICs of $CuSO_4$ (mM)

Strain	MIC ($CuSO_4$, mM)	
	Aerobic	**Anaerobic**
DH5α	6.25	3.13
YL-EF21	12.5	6.25
YL-EF22	12.5	6.25
YL-EF23	12.5	6.25
YL-EF24	12.5	6.25
YL-EF25	12.5	6.25
YL-EF32	12.5	6.25
YL-EF35A	12.5	6.25
YL-EF42	12.5	6.25
YL-EC43	12.5	6.25
YL-EF44	12.5	6.25

8.3.3 Analysis of the genome sequences of copper sulphate tolerant isolates

Genomic sequencing and taxonomic classification showed isolate 43 was *E. coli* and the other nine isolates were *Escherichia fergusonii* (Table 8-3). Three plasmids with the sizes around 250kb, 143kb and 93kb were found in the *E. fergusonii* isolates. The *E. coli* isolate had a very similar (> 99% nucleotide identity) 143kb plasmid and another two plasmids, of approximately 95kb and 48kb. The results of plasmid typing and the presence of plasmids in each isolate are shown in Table 8-3.

Table 8-3: Plasmid type and presence in the isolates

Strain	Species	Plasmid Type				
		p-250 IncHI1	p-143 IncI1	p-93 p0111_1 like	p-95 IncY	p-48 IncX
YL-EF21	*E. fergusonii*	+	+	+	–	–
YL-EF22	*E. fergusonii*	+	+	+	–	–
YL-EF23	*E. fergusonii*	–	+	+	–	–
YL-EF24	*E. fergusonii*	+	+	+	–	–
YL-EF25	*E. fergusonii*	+	+	+	–	–
YL-EF32	*E. fergusonii*	+	+	+	–	–
YL-EF35A	*E. fergusonii*	+	+	+	–	–
YL-EF42	*E. fergusonii*	+	+	+	–	–
YL-EF44	*E. fergusonii*	+	+	+	–	–
YL-EC43	*E. coli*	–	+	–	+	+

No ARGs were found on the chromosomes of the *E. fergusonii* isolates. On the chromosome of *E. coli* strain YL-EC43, only *aad*A was found.

The plasmids carried multiple ARGs and MRGs, and those present on the 250kb and 143kb plasmids are described in Table 8-4.

Table 8-4: ARGs and MRGs detected on the plasmids

Plasmid	ARGs	MRGs
pEF21-250	*aad*A, *aad*A2, *bla*$_{TEM-33}$, *cml*A6, *dfr*A12, *flo*R, *qnr*S1, *sul*2, *sul*3, *tet*A, *tet*M	*sil*E, *sil*S, *sil*R, *sil*C, *sil*F, *sil*B, *sil*A, *sil*P; *pco*E1, *pco*C, *pco*B, *pco*A, *pco*E2
pEF-143	*aad*A2, *cml*A6, *aad*A, *sul*3	*sil*E, *sil*S, *sil*R, *sil*C, *sil*F, *sil*B, *sil*A, *sil*P; *pco*E1, *pco*S, *pco*R, *pco*D, *pco*C, *pco*B, *pco*A, *pco*E2; *mer*R, *mer*T, *mer*P, *mer*C, *mer*A, *mer*D, *mer*E

Interestingly, both the 250kb and 143kb plasmids carried the *sil* and *pco* genes. To explore the gene arrangement, YL-EF21 was used as the reference strain and its genome was annotated using the RAST server (Aziz et al., 2008, Overbeek et al., 2014).

Annotation of the MRG loci were further refined using the loci described in the *Salmonella* strain TW-Stm6 (Billman-Jacobe et al., 2019). The comparison showed that both strains had the complete *sil* locus, whereas the *pco* locus on the 250kb plasmid in YL-EF21 (pEF21-250) had a deletion of *pco*S, *pco*R and *pco*D genes (Fig 8-2).

Moreover, the comparison of sequences that were amplified by the *sil*R, *pco*A and *pco*R primers from plasmids in strain YL-EF21, the TW-STM6 chromosome and plasmid pSTM5-275, showed SNPs in the amplicons of *silR* and *pcoR* (Fig 8-3). These results supported the slight peak shifts observed in the high-resolution melting curves of these genes shown in Figure 8-1.

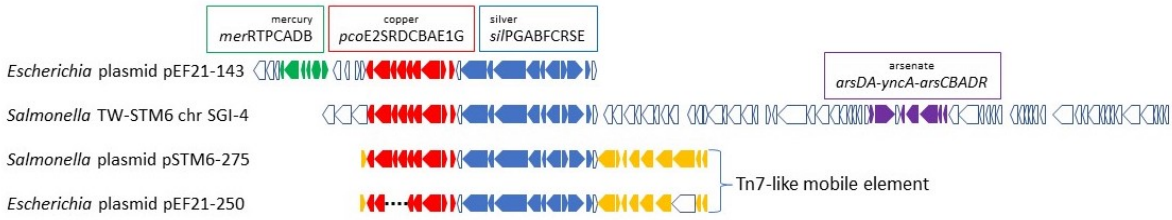

Figure 8-2: Gene arrangement of the MRG loci on TW-Stm6 chromosome (SGI-4) and plasmids found in TW-Stm6 (pSTM-275) and YL-EF21 (pEF21-143, pEF-250).

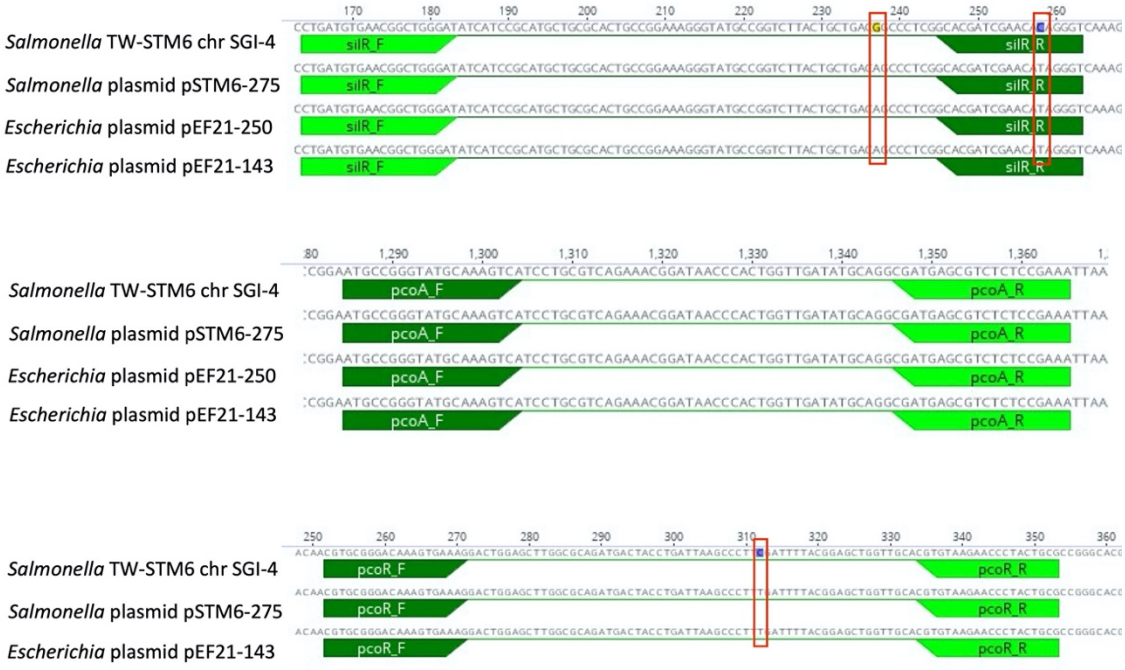

Figure 8-3: Sequence alignments of amplicons of *sil*R, *pco*A and *pco*R on TW-Stm6 chromosome (SGI-4) and plasmids found in TW-Stm6 (pSTM-275) and YL-EF21 (pEF21-250, pEF-143).

Plasmid pEF21-143 is a novel plasmid. It carries a complete Tn7-like element that includes the *sil* and *pco* operons, while the *aad*A2, *cml*A6, *aad*A, *sul*3 genes are part of an integron.

Plasmid pEF21-250 shares the same backbone as pSa-CIP, a plasmid found in a *Salmonella* strain isolated from a Chinese meat sample (Genbank accession number: MG874042). Both have a *sil* locus and an incomplete *pco* locus in which *pco*D and *pco*S are pseudogenes and *pco*R is absent. pEF21-250 may have been derived from pSa-CIP through several rearrangements, especially in the MDR region (Fig 8-3a, orange, outer circle) which has many mobile element genes. Preliminary analysis of the plasmids suggested that two sections of the sequence are inverted (Fig 3a. pale green, middle circle) and there are two sections in pEF21-250 that are missing from pSa-CIP (Fig 8-3a, black, middle circle).

The MDR region of pEF21-250 is 49,846bp long, flanked by inverted copies of IS26, and contains a complex mosaic of mobile elements and ARGs (Fig 8-3c). The tetracycline resistance genes, *tet*R, *tet*A and *tet*M occur within a IS26-flanked sequence, that is not present pSa-CIP, and may have been acquired as a unit via IS26-mediated insertion. The *dfr*A12, *aad*A1, *aad*A2, *cml*A genes occur on an integron. A *qnr*S1 gene occurs at the 5' end of the MDR region on an IS26-flanked segment that also contains *bla* and *sul*3. This section is inverted in pSa-CIP but the sequences upstream of *qnr*S1, and required for induction of fluoroquinolone resistance are conserved (Monárrez et al., 2018).

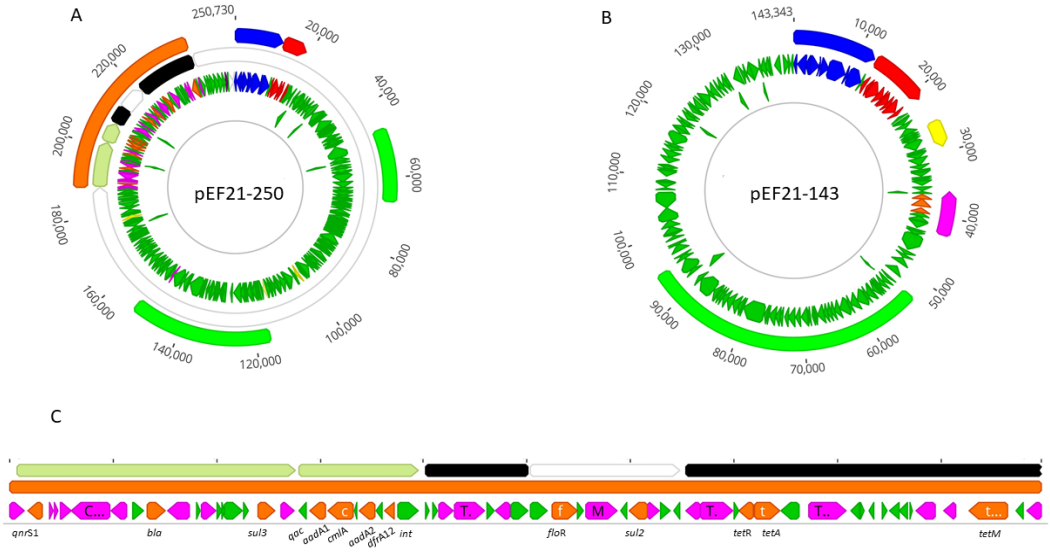

Figure 8-4: Plasmid maps showing ARGs (orange), mobile elements (pink), *sil* genes (blue), *pco* gene (red), *mer* gene (yellow). (a) pEF21-250, outer ring shows MDR region (orange), *sil* locus (blue), *pco* locus (red) and plasmid transfer region (green); middle ring show arrangement of segments relative to pSa-CIP, light green (inverted), black (absent from pSa-CIP) and white (present in same orientation); centre ring shows ORFs. (b) pEF21-143, outer ring shows MDR region (orange), *sil* locus (blue), *pco* locus (red) and plasmid transfer region (green) and centre ring shows ORFs. (c) MDR region of pEF2-250. ARGs (orange), other ORFs (green). Top line refers to the presence and orientation of sections with respect to pSa-CIP.

8.3.4 Low proportion of *Escherichia* spp. in the faecal microbiome

Analysis of the faecal microbiomes in the 10 faecal samples showed the proportion of bacteria belonging to the *Proteobacteria* phylum ranged between 0.8% and 6.1%. The proportion of *Enterobacteriaceae* family members ranged between 0.1% and 4.8%. *Escherichia* spp. had a level between 0.1% and 1.2% and was below the limit of detection in three samples (Fig 8-4). The results showed that *Escherichia* spp. carrying ARGs and MRGs only represented a minor component of the faecal bacterial community.

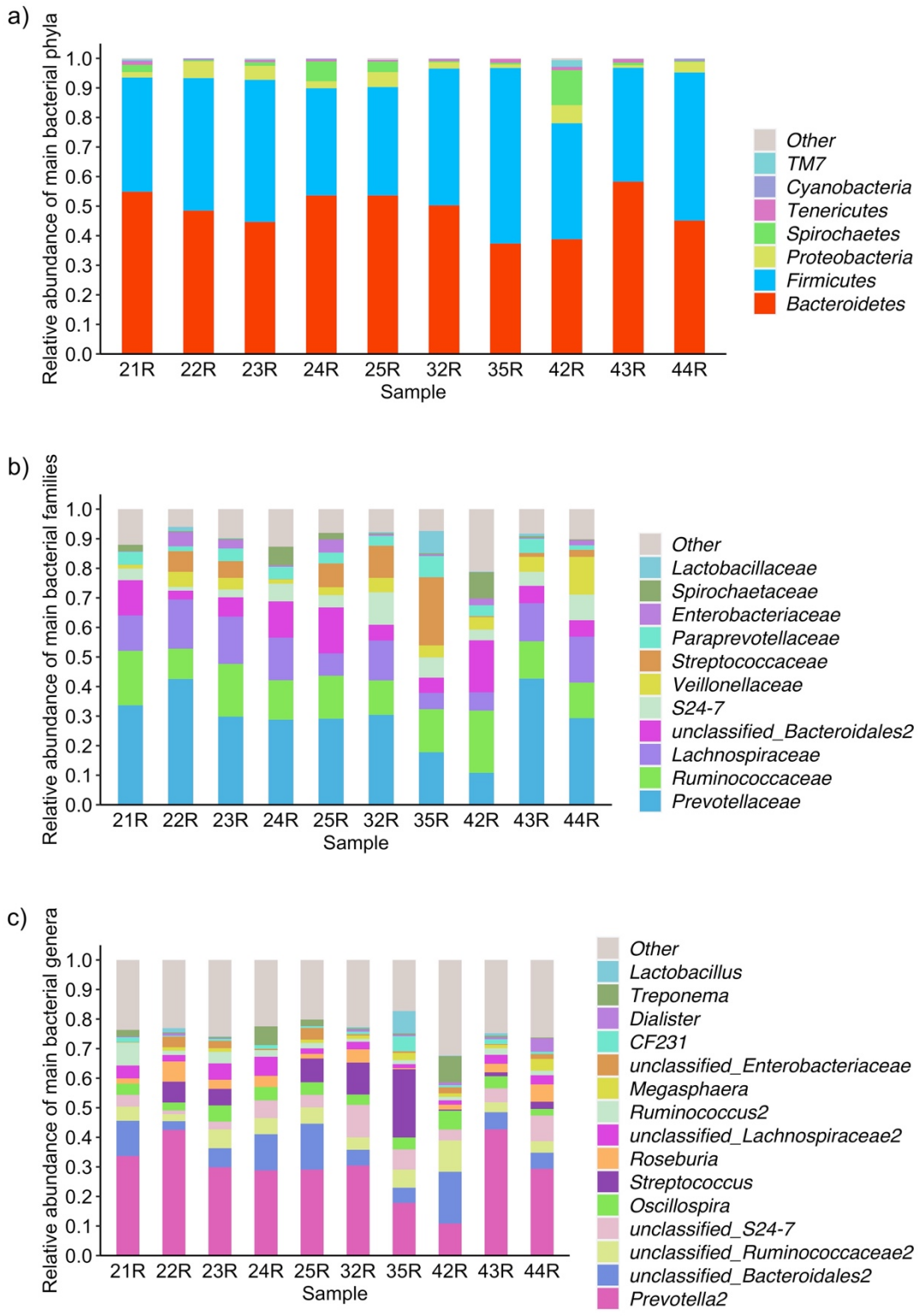

Figure 8-5: Relative abundance of dominant bacterial phyla (a), families (b) and genera (c) in faecal samples. Only phyla with relative abundances above 0.5%, and families and genera with relative abundances above 3% in at least one sample are listed. Taxa below the cutoff were assigned as “Other”.

8.4 Summary

The current analysis showed that plasmids carrying *sil*, *pco* genes and ARGs were persistent in *Salmonella*, *E. coli* and *E. fergusonii* in the pig faecal flora. Use of antimicrobials or metals in animals might contribute to the maintenance of plasmids in these bacterial populations. However, these bacteria only represent a small fraction of the faecal flora, thus their ARG and MRG profiles cannot be generalised to the overall resistome. The culture-based and culture-independent methods have different strengths and weaknesses in studying antimicrobial and metal resistance in bacteria. Culture-based methods allow targeting of specific bacterial species to test their resistant genotype and phenotype in depth, whereas, the culture-independent methods have the potential to explore the resistance genes carried by a broad range of bacteria, and to evaluate the risk of a complex bacterial community.

General Discussion

The major objective of this project was to characterise the faecal ARG profiles of Australian animals with no or low direct antimicrobial exposure. Although ARGs carried by specific faecal bacterial species from Australian animals have been tested in previous studies (Obeng et al., 2012b, Obeng et al., 2012a, Obeng et al., 2013, Smith et al., 2016, Kidsley et al., 2018), this is the first study to detect and quantify ARGs in animal faeces from the microbiome perspective using culture independent approaches.

Tetracycline resistance genes encoding the ribosomal protection proteins and macrolide resistance genes, *mef*A, *erm*B and *erm*F, were always found at high levels in the faecal microbiomes regardless of the animal species. Consistent results were reported in previous human and animal faecal microbiome studies (Literature review, Table 1-1). These genes have a wide range of bacterial hosts, including the genera dominant in the animal gut microbiome, such as *Bacteroides*, *Prevotella*, *Ruminococcus* and *Lactobacillus* (Roberts et al., 2012). Therefore, the predominance of the potential bacterial hosts, naturally present in the gastrointestinal tract, would explain the high level of ARGs detected in these analyses. Moreover, the *tet*, *mef* and *erm* genes are usually associated with conjugative elements, such as conjugative transposons of the Tn916-Tn1545 family (Chopra and Roberts, 2001, Cochetti et al., 2007, De Leener et al., 2004). The transmission of MGEs between different bacterial species and genera would contribute to the widespread occurrence of these genes. The foals, calves and chickens sampled in this project had a low chance of being directly exposed to antimicrobials. Despite the low to no-antimicrobial exposure, tetracycline and macrolide resistance genes were abundant in the animal faecal flora.

The persistence of these ARGs might reflect the long-term effect of past antimicrobial usage. ARGs do not always cause a fitness cost to the hosts in the absence of antimicrobial selection (Andersson and Hughes, 2011), therefore, once ARGs are acquired they could persist. In addition, antimicrobials at low concentrations could be sufficient to maintain carriage of ARGs by bacteria (Gullberg et al., 2014). In nature, tetracyclines are produced by *Streptomyces aureofaciens* and *S. rimosus* (Chopra and

Roberts, 2001), and erythromycin, a member of the macrolide group, can be produced by the soil bacterium, *Saccharopolyspora erythraea* (Bryskier, 2010). The natural production even at a low level might create a selective pressure sufficient to maintain the corresponding ARGs in the animal faecal microbiome.

Indeed, AMR is an ancient, naturally occurring phenomenon which have appeared far before the modern antimicrobial use (D'Costa et al., 2011). Studies have shown the presence of ARGs in soil, ice and cave samples over 1000 years old (D'Costa et al., 2011, Okubo et al., 2019, Bhullar et al., 2012). Moreover, Clemente et al. analysed the faecal microbiome of a group of Yanomami Amerindians, who have been isolated from modern lifestyle for more than 11,000 years and had no history of antimicrobial exposure (Clemente et al., 2015). Researchers detected functional ARGs in the subjects' faeces, suggesting that carrying ARGs might be a feature of human microbiome (Clemente et al., 2015). The preexisting of ARGs in various ecological environments emphasizes the importance of prudent antimicrobial usage to reduce the driving force of ARG expansion.

The co-occurrence of ARGs was explored using statistical analyses, and showed a consistent correlation of *str*B-*sul*2 and *aad*E-*aph*A3-*sat*4 in the animal faeces. Previous culture-based studies have shown the widespread occurrence of RSF1010-like plasmids carrying the *sul*2-*str*A-*str*B cluster in a broad range of hosts in the environment (Scholz et al., 1989, Yau et al., 2010), and the spread of transposons carrying the *aph*A3-*sat*4-*aad*E cluster in Gram-positive bacteria (Derbise et al., 1997a, Werner et al., 2003, Palmieri et al., 2012). Our results suggest that animal faecal microbiome could be a reservoir of those MGEs carrying these gene clusters.

Other approaches to analyse the faecal resistome and microbiome include sequencing the entire extracted DNA. A comprehensive faecal metagenome could be determined using next generation sequencing but currently this is usually performed on platforms producing short reads (e.g. Illumina). These are high throughput systems with low sequencing error rates, however, the short reads are difficult to assemble into contigs that cover whole genes. Hi-C is one of the new powerful tools that improves bacterial genome assembly using metagenomics sequencing data. This method cross-links DNA

molecules that exist in close physical proximity within intact cells, thus, the paired reads originating from the same cell can be identified and clustered (Burton et al., 2014, Beitel et al., 2014, Stewart et al., 2018). In this way, individual bacterial genomes could be deconvoluted from the metagenome reads which allows exploration of the physical linkage of genes. A recent study showed the Hi-C method could cluster plasmid sequences with their core genomes (Stewart et al., 2018), which suggests the potential in identifying bacterial hosts for plasmids as well as ARGs.

In addition, metagenomics using long-read sequencing techniques are able to yield reads that capture multiple genes without assembly. This approach has been demonstrated in finding co-localised ARGs and MGEs in environmental samples from a veterinary hospital (Kamathewatta et al., 2019). However, the low sequence quality and the need for large amounts of high quality DNA could be challenging for gene detection using the long-reads metagenomics (Bengtsson-Palme et al., 2017). Moreover, detecting ARGs in faecal metagenomes requires significant sequencing depth. Due to the cost of sequencing and need for computational resources, there is a trade-off between having sufficient sequencing data to study the gene context in individual samples and the number of samples to generalize the discovery (Bengtsson-Palme et al., 2017).

The animal faeces studied in this project had very low levels of ARGs that confer resistance to antimicrobials of high clinical concern. The ESBL genes *bla*$_{CMY}$ and *bla*$_{CTX-M}$, carbapenems resistance gene *cph*A, fluoroquinolone resistance gene (*qnr*B) and virginiamycin resistance gene (*vat*E) were detected in a small proportion of samples and close to the detection limit. This result is in line with the limited use of the corresponding antimicrobials in food animals in Australia. Animals with no or low chance of antimicrobial exposure have low risk in maintaining those ARGs.

This project also explored the sampling strategy when animals were raised on a large scale and where sampling individual animals was not practical. The layer and broiler chicken studies suggest the manure belt swabs were ideal to study ARGs associated with the poultry. Manure belt swabs were easy to collect and provided enough biomass for extraction of sufficient amounts of high quality DNA. Moreover, the faecal

material collected on the swabs represented a large number of birds, which reduced the bias of a particular bird to the analysis.

In the pig study, swabs of floors just outside the pens in a gilt herd were compared to the animal faeces to determine whether the floor swabs could be a suitable sample type to accurately reflect the pig faecal ARGs profile. The results showed the floor swabs had a notably different ARG profile compared to the faecal samples. Floor swabs had a large reduction in the relative abundance of *tet*Q and *mef*A genes, which was most likely due to the decreased proportion of the anaerobes, *Firmicutes* and *Bacteroidetes* in the bacterial community. *Proteobacteria* were the most dominant bacteria in the swab microbiome, which suggested although the floor swabs could collect some pig faeces, the biomass has been exposed to air for some time which led to reduction of anaerobes and promoted the growth of aerobes and facultative anaerobes. Therefore, a floor swab is not an ideal sample type to accurately represent the pig faecal ARG profile. Schmidt et al. demonstrated that swabs of floors inside the pens and slurry tank were suitable to quantify ARGs in herds (Schmidt et al., 2015). Those methods are worthwhile trying in future studies.

On the other hand, pigs from the study herd had consistent faecal ARG profiles regardless of the sampling cohorts. These results suggest reducing the number of sampled animals or pooling faeces of multiple animals from the same herd could be appropriate to increase the experimental efficiency. A previous study showed increasing the number of faecal samples in a pool could reduce the variation in quantifying ARGs between pooled samples (Clasen et al., 2016). However, the optimal number for pooling needs to be determined for the particular herd.

In future studies, characterisation of ARGs in animal faeces and surrounding environment at microbiome level should be done on more farms to explore the factors that impact the ARG profile. Specifically, the risk of antimicrobial usage in real farm practice in selecting ARGs in animals need more investigation. Furthermore, genetic linkages of ARGs and MGEs in animal faecal microbiome should be confirmed using sequencing based metagenomic approaches. Also, the potential and frequency of ARGs transfer between bacteria assisted by these MGEs need to measure. In addition

to ARG profile, the resistant phenotype is another important aspect to assess the AMR risk. Functional metagenomics as well as genotype-based prediction models would be useful tools for determining AMR and identifying novel ARGs.

Appendices

Appendix I: qPCR primers used in Chapter 3,4,6 and 7.

Assay	Forward Primer (5'-3')	Reverse Primer (5'-3')	Class	Chapter
16S rRNA	GGGTTGCGCTCGTTGC	ATGGYTGTCGTCAGCTCGTG	16S rRNA	3,4,6 and 7
aac(6')-Ib-01	GTTTGAGAGGCAAGGTACCGTAA	GAATGCCTGGCGTGTTTGA	Aminoglycoside	3,4,6 and 7
aacC2	ACGGCATTCTCGATTGCTTT	CCGAGCTTCACGTAAGCATTT	Aminoglycoside	3,4,6 and 7
aacC4	CGGCGTGGGACACGAT	AGGGAACCTTTGCCATCAACT	Aminoglycoside	3,4,6 and 7
aadA-01	GTTGTGCACGACGACATCATT	GGCTCGAAGATACCTGCAAGAA	Aminoglycoside	3,4,6 and 7
aadA2-03	CAATGACATTCTTGCGGGTATC	GACCTACCAAGGCAACGCTATG	Aminoglycoside	3,4,6 and 7
aadD	CCGACAACATTTCTACCATCCTT	ACCGAAGCGCTCGTCGTATA	Aminoglycoside	3,4,6 and 7
aphA1	TGAACAAGTCTGGAAAGAAATGCA	CCTATTAATTTCCCCTCGTCAAAAA	Aminoglycoside	3,4,6 and 7
aphA3-01	AAAAGCCCGAAGAGGAACTTG	CATCTTTCACAAAGATGTTGCTGTCT	Aminoglycoside	3,4,6 and 7
aphA3-02	CGGAATTGAAAAAACTGATCGAA	ATACCGGCTGTCCGTCATTT	Aminoglycoside	3,4,6 and 7
strB	GCTCGGTCGTGAGAACAATCT	CAATTTCGGTCGCCTGGTAGT	Aminoglycoside	3,4,6 and 7
aadE	TACCTTATTGCCCTTGGAAGAGTTA	GGAACTATGTCCCTTTTAATTCTACAATCT	Aminoglycoside	3,4,6 and 7
blaOXA1_blaOXA30	CGGATGGTTTGAAGGGTTTATTAT	TCTTGGCTTTTATGCTTGATGTTAA	Beta_lactamase	3,4,6 and 7
blaSHV-01	TCCCATGATGAGCACCTTTAAA	TTCGTCACCGGCATCCA	Beta_lactamase	3,4,6 and 7
blaTEM	AGCATCTTACGGATGGCATGA	TCCTCCGATCGTTGTCAGAAGT	Beta_lactamase	3,4,6 and 7
cfxA	TCATTCCTCGTTCAAGTTTTCAGA	TGCAGCACCAAGAGGAGATGT	Beta_lactamase	3,4,6 and 7
cphA	GCGAGCTGCACAAGCTGAT	CGGCCCAGTCGCTCTTC	Beta_lactamase	3,4,6 and 7
blaCMY	CCGCGGCGAAATTAAGC	GCCACTGTTTGCCTGTCAGTT	Beta_lactamase	3,4,6 and 7
blaCTX-M-04	CTTGGCGTTGCGCTGAT	CGTTCATCGGCACGGTAGA	Beta_lactamase	3,4,6 and 7
cfiA	GCAGCGTTGCTGGACACA	GTTCGGGATAAACGTGGTGACT	Beta_lactamase	3,4,6 and 7
silR	CTGATGTGAACGGCTGGGAT	ACCCTGTGTTCRATCGTGC	Copper	4,6 and 7
pcoA	ATGCCGGGTATGCAAAGTCA	TTTCGGAGAGACGCTCATCG	Copper	4,6 and 7
pcoR	CGTGCGGGACAAAGTGAAAG	CGCAGTAGGGTTCTTACACG	Copper	4,6 and 7
cmlA1-01	TAGGAAGCATCGGAACGTTGAT	CAGACCGAGCACGACTGTTG	FC	3,4,6 and 7
cmx(A)	GCGATCGCCATCCTCTGT	TCGACACGGAGCCTTGGT	FC	3,4,6 and 7
floR-01	ATTGTCTTCACGGTGTCCGTTA	CCGCGATGTCGTCGAACT	FC	3,4,6 and 7
qnrA	AGGATTTCTCACGCCAGGATT	CCGCTTTCAATGAAACTGCAA	FC	3,4,6 and 7
qnrB	GCGACGTTCAGTGGTTCAGA	GCTGCTCGCCAGTCGAA	FC	3,4,6 and 7
intl1	GCCTTGATGTTACCCGAGAG	GATCGGTCGAATGCGTGT	MGE	3,4,6 and 7
IS613	AGGTTCGGACTCAATGCAACA	TTCAGCACATACCGCCTTGAT	MGE	4,6 and 7
tnp614	GGAAATCAACGGCATCCAGTT	CATCCATGCGCTTTTGTCTCT	MGE	4,6 and 7
qacEdelta1-01	TCGCAACATCCGCATTAAAA	ATGGATTTCAGAACCAGAGAAAGAAA	MGE	3,4,6 and 7
ermB	TAAAGGGCATTTAACGACGAAACT	TTTATACCTCTGTTTGTTAGGGAATTGAA	MLSB	3,4,6 and 7
mefA	CCGTAGCATTGGAACAGCTTTT	AAACGGAGTATAAGAGTGCTGCAA	MLSB	3,4,6 and 7
msrA	CTGCTAACACAAGTACGATTCCAAAT	TCAAGTAAAGTTGTCTTACCTACACCATT	MLSB	3,4,6 and 7
ermF	CAGCTTTGGTTGAACATTTACGAA	AAATTCCTAAAATCACAACCGACAA	MLSB	3,4,6 and 7
lnuB-01	TGAACATAATCCCCTCGTTTAAAGAT	TAATTGCCCTGTTTCATCGTAAATAA	MLSB	3,4,6 and 7
mphC	CGTTTGAAGTACCGAATTGGAAA	GCTGCGGGTTTGCCTGTA	MLSB	3,4,6 and 7

Assay	Forward Primer (5'-3')	Reverse Primer (5'-3')	Class	Chapter
sat4	GAATGGGCAAAGCATAAAAACTTG	CCGATTTTGAAACCACAATTATGATA	Streptothricin	3,4,6 and 7
disul	TCATCTGCCAAACTCGTCGTTA	GTCAAAGAACGCCGCAATGT	Sulfonamide	3,4,6 and 7
sul1-01	CAGCGCTATGCGCTCAAG	ATCCCGCTGCGCTGAGT	Sulfonamide	3,4,6 and 7
tetG-02	CATCAGCGCCGGTCTTATG	CCCCATGTAGCCGAACCA	Tetracycline	3,4,6 and 7
tetL-02	ATGGTTGTAGTTGCGCGCTATAT	ATCGCTGGACCGACTCCTT	Tetracycline	3,4,6 and 7
tetM-01	CATCATAGACACGCCAGGACATAT	CGCCATCTTTTGCAGAAATCA	Tetracycline	3,4,6 and 7
tetO-01	ATGTGGATACTACAACGCATGAGATT	TGCCTCCACATGATATTTTTCCT	Tetracycline	3,4,6 and 7
tetQ	CGCCTCAGAAGTAAGTTCATACACTAAG	TCGTTCATGCGGATATTATCAGAAT	Tetracycline	3,4,6 and 7
tetW	ATGAACATTCCCACCGTTATCTTT	ATATCGGCGGAGAGCTTATCC	Tetracycline	3,4,6 and 7
tetX	AAATTTGTTACCGACACGGAAGTT	CATAGCTGAAAAAATCCAGGACAGTT	Tetracycline	3,4,6 and 7
tetK	CAGCAGTCATTGGAAAATTATCTGATTATA	CCTTGTACTAACCTACCAAAAATCAAAATA	Tetracycline	3,4,6 and 7
dfrA1-01	GGAATGGCCCTGATATTCCA	AGTCTTGCGTCCAACCAACAG	Trimethoprim	3,4,6 and 7
dfrA12	CCTCTACCGAACCGTCACACA	GCGACAGCGTTGAAACAACTAC	Trimethoprim	3,4,6 and 7
vatE	GACCGTCCTACCAGGCGTAA	TTGGATTGCCACCGACAATT	Virginiamycin	3,4,6 and 7
vgbB-01	CAGCCGGATTCTGGTCCTT	TACGATCTCCATTCAATTGGGTAAA	Virginiamycin	3,4,6 and 7

Appendix II: qPCR primers used in Chapter 5.

Assay	Forward Primer (5'-3')	Reverse Primer (5'-3')	Class
16S rRNA	GGGTTGCGCTCGTTGC	ATGGYTGTCGTCAGCTCGTG	16S rRNA
aac	CCCTGCGTTGTGGCTATGT	TTGGCCACGCCAATCC	Aminoglycoside
aacC1	GGTCGTGAGTTCGGAGACGTA	GCAAGTTCCCGAGGTAATCG	Aminoglycoside
aacC2	ACGGCATTCTCGATTGCTTT	CCGAGCTTCACGTAAGCATTT	Aminoglycoside
aacC4	CGGCGTGGGACACGAT	AGGGAACCTTTGCCATCAACT	Aminoglycoside
aac(6')I1	GACCGGATTAAGGCCGATG	CTTGCCTTGATATTCAGTTTTTATAACCA	Aminoglycoside
aacA/aphD	AGAGCCTTGGGAAGATGAAGTTT	TTGATCCATACCATAGACTATCTCATCA	Aminoglycoside
aac(6')-Iy	GCTTTGCGGATGCCTCAAT	GGAGAACAAAAATACCTTCAAGGAAA	Aminoglycoside
aac(6')-II	CGACCCGACTCCGAACAA	GCACGAATCCTGCCTTCTCA	Aminoglycoside
aacC	CGTCACTTATTCGATGCCCTTAC	GTCGGGCGCGGCATA	Aminoglycoside
aac(6')-Ib-01	GTTTGAGAGGCAAGGTACCGTAA	GAATGCCTGGCGTGTTTGA	Aminoglycoside
aac(6')-Ib-02	CGTCGCCGAGCAACTTG	CGGTACCTTGCCTCTCAAACC	Aminoglycoside
aadA5-01	ATCACGATCTTGCGATTTTGCT	CTGCGGATGGGCCTAGAAG	Aminoglycoside
aadA5-02	GTTCTTGCTCTTGCTCGCATT	GATGCTCGGCAGGCAAAC	Aminoglycoside
aac(6')-Ib-03	AGAAGCACGCCCGACACTT	GCTCTCCATTCAGCATTGCA	Aminoglycoside
aadA1	AGCTAAGCGCGAACTGCAAT	TGGCTCGAAGATACCTGCAA	Aminoglycoside
aadA2-01	ACGGCTCCGCAGTGGAT	GGCCACAGTAACCAACAAATCA	Aminoglycoside
aadA-01	GTTGTGCACGACGACATCATT	GGCTCGAAGATACCTGCAAGAA	Aminoglycoside
aadA2-02	CTTGTCGTGCATGACGACATC	TCGAAGATACCCGCAAGAATG	Aminoglycoside
aadD	CCGACAACATTTCTACCATCCTT	ACCGAAGCGCTCGTCGTATA	Aminoglycoside
aadA2-03	CAATGACATTCTTGCGGGTATC	GACCTACCAAGGCAACGCTATG	Aminoglycoside
aadA9-01	CGCGGCAAGCCTATCTTG	CAAATCAGCGACCGCAGACT	Aminoglycoside
aadA-02	CGAGATTCTCCGCGCTGTA	GCTGCCATTCTCCAAATTGC	Aminoglycoside
aadA9-02	GGATGCACGCTTGGATGAA	CCTCTAGCGGCCGGAGTATT	Aminoglycoside
aadE	TACCTTATTGCCCTTGGAAGAGTTA	GGAACTATGTCCCTTTTAATTCTACAATCT	Aminoglycoside
spcN-01	AAAAGTTCGATGAAACACGCCTAT	TCCAGTGGTAGTCCCCGAATC	Aminoglycoside
spcN-02	CAGAATCTTCCTGAAAAGTTTGATGAA	CGCAGACACGCCGAATC	Aminoglycoside
aadA-1-01	AAAAGCCCGAAGAGGAACTTG	CATCTTTCACAAAGATGTTGCTGTCT	Aminoglycoside
aph6ia	CCCATCCCATGTGTAAGGAAA	GCCACCGCTTCTGCTGTAC	Aminoglycoside
aph(2')-Id-02	TGAGCAGTATCATAAGTTGAGTGAAAAG	GACAGAACAATCAATCTCTATGGAATG	Aminoglycoside
aph(2')-Id-01	TAAGGATATACCGACAGTTTTGGAAA	TTTAATCCCTCTTCATACCAATCCATA	Aminoglycoside
aph	TTTCAGCAAGTGGATCATGTTAAAAT	CCAAGCTGTTTCCACTGTTTTTC	Aminoglycoside
aphA1	TGAACAAGTCTGGAAAGAAATGCA	CCTATTAATTTCCCCTCGTCAAAAA	Aminoglycoside
aadA-1-02	CGGAATTGAAAAAACTGATCGAA	ATACCGGCTGTCCGTCATTT	Aminoglycoside
str	AATGAGTTTTGGAGTGTCTCAACGTA	AATCAAAACCCCTATTAAAGCCAAT	Aminoglycoside
strA	CCGGTGGCATTTGAGAAAAA	GTGGCTCAACCTGCGAAAAG	Aminoglycoside
strB	GCTCGGTCGTGAGAACAATCT	CAATTTCGGTCGCCTGGTAGT	Aminoglycoside
ampC-01	TGGCGTATCGGGTCAATGT	CTCCACGGGCCAGTTGAG	Beta_lactamase
ampC-02	GCAGCACGCCCCGTAA	TGTACCCATGATGCGCGTACT	Beta_lactamase
ampC-04	TCCGGTGACGCGACAGA	CAGCACGCCGGTGAAAGT	Beta_lactamase

Assay	Forward Primer (5'-3')	Reverse Primer (5'-3')	Class
ampC-05	CTGTTCGAGCTGGGTTCTATAAGTAAA	CAGTATCTGGTCACCGGATCGT	Beta_lactamase
ampC-06	CCGCTCAAGCTGGACCATAC	CCATATCCTGCACGTTGGTTT	Beta_lactamase
ampC-07	CCGCCCAGAGCAAGGACTA	GCTCGACTTCACGCCGTAAG	Beta_lactamase
ampC-09	CAGCCGCTGATGAAAAAATATG	CAGCGAGCCCACTTCGA	Beta_lactamase
ampC/blaDHA	TGGCCGCAGCAGAAAGA	CCGTTTTATGCACCCAGGAA	Beta_lactamase
bla1	GCAAGTTGAAGCGAAAGAAAAGA	TACCAGTATCAATCGCATATACACCTAA	Beta_lactamase
bla-ACC-1	CACACAGCTGATGGCTTATCTAAAA	AATAAACGCGATGGGTTCCA	Beta_lactamase
blaCMY	CCGCGGCGAAATTAAGC	GCCACTGTTTGCCTGTCAGTT	Beta_lactamase
blaCMY2-01	AAAGCCTCAT GGGTGCATAAA	ATAGCTTTTGTTTGCCAGCATCA	Beta_lactamase
blaCMY2-02	GCGAGCAGCCTGAAGCA	CGGATGGGCTTGTCCTCTT	Beta_lactamase
blaCTX-M-01	GGAGGCGTGACGGCTTTT	TTCAGTGCGATCCAGACGAA	Beta_lactamase
blaCTX-M-02	GCCGCGGTGCTGAAGA	ATCGGATTATAGTTAACCAGGTCAGATTT	Beta_lactamase
blaCTX-M-03	CGATACCACCACGCCGTTA	GCATTGCCCAACGTCAGATT	Beta_lactamase
blaCTX-M-04	CTTGGCGTTGCGCTGAT	CGTTCATCGGCACGGTAGA	Beta_lactamase
blaCTX-M-05	GCGATAACGTGGCGATGAAT	GTCGAGACGGAACGTTTCGT	Beta_lactamase
blaCTX-M-06	CACAGTTGGTGACGTGGCTTAA	CTCCGCTGCCGGTTTTATC	Beta_lactamase
blaGES	GCAATGTGCTCAACGTTCAAG	GTGCCTGAGTCAATTCTTTCAAAG	Beta_lactamase
blaIMP-01	AACACGGTTTGGTGGTTCTTGTA	GCGCTCCACAAACCAATTG	Beta_lactamase
blaIMP-02	AAGGCAGCATTTCCTCTCATTTT	GGATAGATCGAGAATTAAGCCACTCT	Beta_lactamase
bla-L1	CACCGGGTTACCAGCTGAAG	GCGAAGCTGCGCTTGTAGTC	Beta_lactamase
blaMOX/blaCMY	CTATGTCAATGTGCCGAAGCA	GGCTTGTCCTCTTTCGAATAGC	Beta_lactamase
blaOCH	GGCGACTTGCGCCGTAT	TTTTCTGCTCGGCCATGAG	Beta_lactamase
blaOKP	GCCGCCATCACCATGAG	GGTGACGTTGTCACCGATCTG	Beta_lactamase
blaOXA1/blaOXA30	CGGATGGTTTGAAGGGTTTATTAT	TCTTGGCTTTTATGCTTGATGTTAA	Beta_lactamase
blaOXA10-01	CGCAATTATCGGCCTAGAAACT	TTGGCTTTCCGTCCCATTT	Beta_lactamase
blaOXA10-02	CGCAATTATCGGCCTAGAAACT	TTGGCTTTCCGTCCCATTT	Beta_lactamase
blaOXY	CGTTCAGGCGGCAGGTT	GCCGCGATATAAGATTTGAGAATT	Beta_lactamase
blaPAO	CGCCGTACAACCGGTGAT	GAAGTAATGCGGTTCTCCTTTCA	Beta_lactamase
blaPER	TGCTGGTTGCTGTTTTTGTGA	CCTGCGCAATGATAGCTTCAT	Beta_lactamase
blaPSE	TTGTGACCTATTCCCCTGTAATAGAA	TGCGAAGCACGCATCATC	Beta_lactamase
blaROB	GCAAAGGCATGACGATTGC	CGCGCTGTTGTCGCTAAA	Beta_lactamase
blaSFO	CCGCCGCCATCCAGTA	GGGCCGCCAAGATGCT	Beta_lactamase
blaSHV-01	TCCCATGATGAGCACCTTTAAA	TTCGTCACCGGCATCCA	Beta_lactamase
blaSHV-02	CTTTCCCATGATGAGCACCTTT	TCCTGCTGGCGATAGTGGAT	Beta_lactamase
blaTEM	AGCATCTTACGGATGGCATGA	TCCTCCGATCGTTGTCAGAAGT	Beta_lactamase
blaTLA	ACACTTTGCCATTGCTGTTTATGT	TGCAAATTTCGGCAATAATCTTT	Beta_lactamase
blaVEB	CCCGATGCAAAGCGTTATG	GAAAGATTCCCTTTATCTATCTCAGACAA	Beta_lactamase
blaVIM	GCACTTCTCGCGGAGATTG	CGACGGTGATGCGTACGTT	Beta_lactamase
blaZ	GGAGATAAAGTAACAAATCCAGTTAGATATGA	TGCTTAATTTTCCATTTGCGATAAG	Beta_lactamase
cepA	AGTTGCGCAGAACAGTCCTCTT	TCGTATCTTGCCCGTCGATAAT	Beta_lactamase
cfiA	GCAGCGTTGCTGGACACA	GTTCGGGATAAACGTGGTGACT	Beta_lactamase
cfxA	TCATTCCTCGTTCAAGTTTTCAGA	TGCAGCACCAAGAGGAGATGT	Beta_lactamase

Assay	Forward Primer (5'-3')	Reverse Primer (5'-3')	Class
cphA-01	GCGAGCTGCACAAGCTGAT	CGGCCCAGTCGCTCTTC	Beta_lactamase
cphA-02	GTGCTGATGGCGAGTTTCTG	GGTGTGGTAGTTGGTGTTGATCAC	Beta_lactamase
fox5	GGTTTGCCGCTGCAGTTC	GCGGCCAGGTGACCAA	Beta_lactamase
mecA	GGTTACGGACAAGGTGAAATACTGAT	TGTCTTTTAATAAGTGAGGTGCGTTAATA	Beta_lactamase
NDM1	ATTAGCCGCTGCATTGAT	CATGTCGAGATAGGAAGTG	Beta_lactamase
pbp	CCGGTGCCATTGGTTTAGA	AAAATAGCCGCCCCAAGATT	Beta_lactamase
pbp2x	TTTCATAAGTATCTGGACATGGAAGAA	CCAAAGGAAACTTGCTTGAGATTAG	Beta_lactamase
Pbp5	GGCGAACTTCTAATTAATCCTATCCA	CGCCGATGACATTCTTCTTATCTT	Beta_lactamase
penA	AGACGGTAACGTATAACTTTTTGAAAGA	GCGTGTAGCCGGCAATG	Beta_lactamase
catB3	GCACTCGATGCCTTCCAAAA	AGAGCCGATCCAAACGTCAT	FC
catB8	CACTCGACGCCTTCCAAAG	CCGAGCCTATCCAGACATCATT	FC
cfr	GCAAAATTCAGAGCAAGTTACGAA	AAAATGACTCCCAACCTGCTTTAT	FC
floR	ATTGTCTTCACGGTGTCCGTTA	CCGCGATGTCGTCGAACT	FC
cmlA1-01	TAGGAAGCATCGGAACGTTGAT	CAGACCGAGCACGACTGTTG	FC
cmlA1-02	AGGAAGCATCGGAACGTTGA	ACAGACCGAGCACGACTGTTG	FC
cmx(A)	GCGATCGCCATCCTCTGT	TCGACACGGAGCCTTGGT	FC
catA1	GGGTGAGTTTCACCAGTTTTGATT	CACCTTGTCGCCTTGCGTATA	FC
qnrA	AGGATTTCTCACGCCAGGATT	CCGCTTTCAATGAAACTGCAA	FC
intI	GGCATCCAAGCAGCAAG	AAGCAGACTTGACCTGA	MGE
intI1	CGAACGAGTGGCGGAGGGTG	TACCCGAGAGCTTGGCACCCA	MGE
tnpA-03	AATTGATGCGGACGGCTTAA	TCACCAAACTGTTTATGGAGTCGTT	MGE
IS613	AGGTTCGGACTCAATGCAACA	TTCAGCACATACCGCCTTGAT	MGE
tnpA-01	CATCATCGGACGGACAGAATT	GTCGGAGATGTGGGTGTAGAAAGT	MGE
tnpA-04	CCGATCACGGAAAGCTCAAG	GGCTCGCATGACTTCGAATC	MGE
tnpA-07	GAAACCGATGCTACAATATCCAATTT	CAGCACCGTTTGCAGTGTAAG	MGE
tnpA-05	GCCGCACTGTCGATTTTTATC	GCGGGATCTGCCACTTCTT	MGE
Tp614	GGAAATCAACGGCATCCAGTT	CATCCATGCGCTTTTGTCTCT	MGE
tnpA-02	GGGCGGGTCGATTGAAA	GTGGGCGGGATCTGCTT	MGE
carB	GGAGTGAGGCTGACCGTAGAAG	ATCGGCGAAACGCACAAA	MLSB
ereA	CCTGTGGTACGGAGAATTCATGT	ACCGCATTCGCTTTGCTT	MLSB
ereB	GCTTTATTTCAGGAGGCGGAAT	TTTTAAATGCCACAGCACAGAATC	MLSB
erm(34)	GCGCGTTGACGACGATTT	TGGTCATACTCGACGGCTAGAAC	MLSB
erm(35)	TTGAAAACGATGTTGCATTAAGTCA	TCTATAATCACAACTAACCACTTGAACGT	MLSB
erm(36)	GGCGGACCGACTTGCAT	TCTGCGTTGACGACGGTTAC	MLSB
ermA	TTGAGAAGGGATTTGCGAAAAG	ATATCCATCTCCACCATTAATAGTAAACC	MLSB
ermA/ermTR	ACATTTTACCAAGGAACTTGTGGAA	GTGGCATGACATAAACCTTCATCA	MLSB
ermB	TAAAGGGCATTTAACGACGAAACT	TTTATACCTCTGTTTGTTAGGGAATTGAA	MLSB
ermC	TTTGAAATCGGCTCAGGAAAA	ATGGTCTATTTCAATGGCAGTTACG	MLSB
ermF	CAGCTTTGGTTGAACATTTACGAA	AAATTCCTAAAATCACAACCGACAA	MLSB
ermJ/ermD	GGACTCGGCAATGGTCAGAA	CCCCGAAACGCAATATAATGTT	MLSB
ermK-01	GTTTGATATTGGCATTGTCAGAGAAA	ACCATTGCCGAGTCCACTTT	MLSB
ermK-02	GAGCCGCAAGCCCCTTT	GTGTTTCATTTGACGCGGAGTAA	MLSB

Assay	Forward Primer (5'-3')	Reverse Primer (5'-3')	Class
ermT-01	GTTCACTAGCACTATTTTTAATGACAGAAGT	GAAGGGTGTCTTTTTAATACAATTAACGA	MLSB
ermT-02	GTAAAATCCCTAGAGAATACTTTCATCCA	TGAGTGATATTTTTGAAGGGTGTCTT	MLSB
ermX	GCTCAGTGGTCCCCATGGT	ATCCCCCCGTCAACGTTT	MLSB
ermY	TTGTCTTTGAAAGTGAAGCAACAGT	TAACGCTAGAGAACGATTTGTATTGAG	MLSB
lmrA-01	TCGACGTGACCGTAGTGAACA	CGTGACTACCCAGGTGAGTTGA	MLSB
lnuA-01	TGACGCTCAACACACTCAAAAA	TTCATGCTTAAGTTCCATACGTGAA	MLSB
lnuB-01	TGAACATAATCCCCTCGTTTAAAGAT	TAATTGCCCTGTTTCATCGTAAATAA	MLSB
lnuB-02	AAAGGAGAAGGTGACCAATACTCTGA	GGAGCTACGTCAAACAACCAGTT	MLSB
lnuC	TGGTCAATATAACAGATGTAAACCAGATTT	CACCCCAGCCACCATCAA	MLSB
matA/mel	TAGTAGGCAAGCTCGGTGTTGA	CCTGTGCTATTTTAAGCCTTGTTTCT	MLSB
mdtA	CCTAACGGGCGTGACTTCA	TTCACCTGTTTCAAGGGTCAAA	MLSB
mefA	CCGTAGCATTGGAACAGCTTTT	AAACGGAGTATAAGAGTGCTGCAA	MLSB
mphA-01	CTGACGCGCTCCGTGTT	GGTGGTGCATGGCGATCT	MLSB
mphA-02	TGATGACCCTGCCATCGA	TTCGCGAGCCCCTCTTC	MLSB
mphB	CGCAGCGCTTGATCTTGTAG	TTACTGCATCCATACGCTGCTT	MLSB
mphC	CGTTTGAAGTACCGAATTGGAAA	GCTGCGGGTTTGCCTGTA	MLSB
msrA-01	CTGCTAACACAAGTACGATTCCAAAT	TCAAGTAAAGTTGTCTTACCTACACCATT	MLSB
msrC-01	TCAGACCGGATCGGTTGTC	CCTATTTTTTGGAGTCTTCTCTCTAATGTT	MLSB
oleC	CCCGGAGTCGATGTTCGA	GCCGAAGACGTACACGAACAG	MLSB
pikR1	TCGACATGCGTGACGAGATT	CCGCGAATTAGGCCAGAA	MLSB
pikR2	TCGTGGGCCAGGTGAAGA	TTCCCCTTGCCGGTGAA	MLSB
vatB-01	GGAAAAAGCAACTCCATCTCTTGA	TCCTGGCATAACAGTAACATTCTGA	MLSB
vatB-02	TTGGGAAAAAGCAACTCCATCT	CAATCCACACATCATTTCCAACA	MLSB
vatC-01	CGGAAATTGGGAACGATGTT	GCAATAATAGCCCCGTTTCCTA	MLSB
vatC-02	CGATGTTTGGATTGGACGAGAT	GCTGCAATAATAGCCCCGTTT	MLSB
vatE-01	GGTGCCATTATCGGAGCAAAT	TTGGATTGCCACCGACAAT	MLSB
vatE-02	GACCGTCCTACCAGGCGTAA	TTGGATTGCCACCGACAATT	MLSB
vgaA-01	CGAGTATTGTGGAAAGCAGCTAGTT	CCCGTACCGTTAGAGCCGATA	MLSB
vgaA-02	GACGGGTATTGTGGAAAGCAA	TTTCCTGTACCATTAGATCCGATAATT	MLSB
vgb-01	AGGGAGGGTATCCATGCAGAT	ACCAAATGCGCCCGTTT	MLSB
vgbB-01	CAGCCGGATTCTGGTCCTT	TACGATCTCCATTCAATTGGGTAAA	MLSB
vgbB-02	ATACGAGCTGCCTAATAAAGGATCTT	TGTGAACCACAGGGCATTATCA	MLSB
acrA-01	CAACGATCGGACGGGTTTC	TGGCGATGCCACCGTACT	Multidrug efflux pump
acrA-02	GGTCTATCACCCTACGCGCTATC	GCGCGCACGAACATACC	Multidrug efflux pump
acrA-03	CAGACCCGCATCGCATATT	CGACAATTTCGCGCTCATG	Multidrug efflux pump
acrA-04	TACTTTGCGCGCCATCTTC	CGTGCGCGAACGAACAT	Multidrug efflux pump
acrB-01	AGTCGGTGTTCGCCGTTAAC	CAAGGAAACGAACGCAATACC	Multidrug efflux pump
acrR-01	GCGCTGGAGACACGACAAC	GCCTTGCTGCGAGAACAAA	Multidrug efflux pump
acrR-02	GATGATACCCCCTGCTGTGAGA	ACCAAACAAGAAGCGCAAGAA	Multidrug efflux pump
adeA	CAGTTCGAGCGCCTATTTCTG	CGCCCTGACCGACCAAT	Multidrug efflux pump
acrA-05	CGTGCGCGAACGAACA	ACTTTGCGCGCCATCTTC	Multidrug efflux pump
acrF	GCGGCCAGGCACAAAA	TACGCTCTTCCCACGGTTTC	Multidrug efflux pump

Assay	Forward Primer (5'-3')	Reverse Primer (5'-3')	Class
ceoA	ATCAACACGGACCAGGACAAG	GGAAAGTCCGCTCACGATGA	Multidrug efflux pump
cmeA	GCAGCAAAGAAGAAGCACCAA	AGCAGGGTAAGTAAAACTAAGTGGTAAATCT	Multidrug efflux pump
cmr	CGGCATCGTCAGTGGAATT	CGGTTCCGAAAAAGATGGAA	Multidrug efflux pump
emrD	CTCAGCAGTATGGTGGTAAGCATT	ACCAGGCGCCGAAGAAC	Multidrug efflux pump
marR-01	GCGGCGTACTGGTGAAGCTA	TGCCCTGGTCGTTGATGA	Multidrug efflux pump
mdetl1	ATACAGCAGTGGATATTGGTTTAATTGT	TGCATAAGGTGAATGTTCCATGA	Multidrug efflux pump
mdtE/yhiU	CGTCGGCGCACTCGTT	TCCAGACGTTGTACGGTAACCA	Multidrug efflux pump
mepA	ATCGGTCGCTCTTCGTTCAC	ATAAATAGGATCGAGCTGCTGGAT	Multidrug efflux pump
mexA	AGGACAACGCTATGCAACGAA	CCGGAAAGGGCCGAAAT	Multidrug efflux pump
mexD	TTGCCACTGGCTTTCATGAG	CACTGCGGAGAACTGTCTGTAGA	Multidrug efflux pump
mexE	GGTCAGCACCGACAAGGTCTAC	AGCTCGACGTACTTGAGGAACAC	Multidrug efflux pump
mexF	CCGCGAGAAGGCCAAGA	TTGAGTTCGGCGGTGATGA	Multidrug efflux pump
mtrC-01	GGACGGGAAGATGGTCCAA	CGTAGCGTTCCGGTTCGAT	Multidrug efflux pump
mtrC-02	CGGAGTCCATCGACCATTTG	ATCGTCGGCAAGGAGAATCA	Multidrug efflux pump
mtrD-02	GGTCGGCACGCTCTTGTC	TGAAGAATTTGCGCACCACTAC	Multidrug efflux pump
mtrD-03	CCGCCAAGCCGATATAGACA	GGCCGGGTTGCCAAA	Multidrug efflux pump
oprD	ATGAAGTGGAGCGCCATTG	GGCCACGGCGAACTGA	Multidrug efflux pump
oprJ	ACGAGAGTGGCGTCGACAA	AAGGCGATCTCGTTGAGGAA	Multidrug efflux pump
pmrA	TTTGCAGGTTTTGTTCCTAATGC	GCAGAGCCTGATTTCTCCTTTG	Multidrug efflux pump
putative Multidrug	AATTTTGCCGATTATTGCTGAAA	GATTGTCATCATTCGTTTATCACCAA	Multidrug efflux pump
qac	CAATAATAACCGAAATAATAGGGACAAGTT	AATAAGTGTTCCTAGTGTTGGCCATAG	Multidrug efflux pump
qacA	TGGCAATAGGAGCTATGGTGTTT	AAGGTAACACTATTTTCGGTCCAAATC	Multidrug efflux pump
qacA/qacB	TTTAGGCAGCCTCGCTTCA	CCGAATCCAAATAAAACCCAATAA	Multidrug efflux pump
qacEdelta1-01	TCGCAACATCCGCATTAAAA	ATGGATTTCAGAACCAGAGAAAGAAA	Multidrug efflux pump
qacEdelta1-02	CCCCTTCCGCCGTTGT	CGACCAGACTGCATAAGCAACA	Multidrug efflux pump
qacH-01	GTGGCAGCTATCGCTTGGAT	CCAACGAACGCCCACAA	Multidrug efflux pump
qacH-02	CATCGTGCTTGTGGCAGCTA	TGAACGCCCAGAAGTCTAGTTTT	Multidrug efflux pump
rarD-02	TGACGCATCGCGTGATCT	AAATTTTCTGTGGCGTCTGAATC	Multidrug efflux pump
sdeB	CACTACCGCTTCCGCACTTAA	TGAAAAAACGGGAAAAGTCCAT	Multidrug efflux pump
tolC-01	GGCCGAGAACCTGATGCA	AGACTTACGCAATTCCGGGTTA	Multidrug efflux pump
tolC-02	CAGGCAGAGAACCTGATGCA	CGCAATTCCGGGTTGCT	Multidrug efflux pump
tolC-03	GCCAGGCAGAGAACCTGATG	CGCAATTCCGGGTTGCT	Multidrug efflux pump
ttgA	ACGCCAATGCCAAACGATT	GTCACGGCGCAGCTTGA	Multidrug efflux pump
ttgB	TCGCCCTGGATGTACACCTT	ACCATTGCCGACATCAACAAC	Multidrug efflux pump
yceE/mdtG-01	TGGCACAAAATATCTGGCAGTT	TTGTGTGGCGATAAGAGCATTAG	Multidrug efflux pump
yceE/mdtG-02	TTATCTGTTTTCTGCTCACCTTCTTTT	GCGTGGTGACAAACAGGCTTA	Multidrug efflux pump
yceL/mdtH-01	TCGGGATGGTGGGCAAT	CGATAACCGAGCCGATGTAGA	Multidrug efflux pump
yceL/mdtH-02	CGCGTGAAACCTTAAGTGCTT	AGACGGCTAAACCCCATATAGCT	Multidrug efflux pump
yceL/mdtH-03	CTGCCGTTAAATGGATGTATGC	ACTCCAGCGGGCGATAGG	Multidrug efflux pump
yidY/mdtL-01	GCAGTTGCATATCGCCTTCTC	CTTCCCGGCAAACAGCAT	Multidrug efflux pump
yidY/mdtL-02	TGCTGATCGGGATTCTGATTG	CAGGCGCGACGAACATAAT	Multidrug efflux pump
fabK	TTTCAGCTCAGCACTTTGGTCAT	AAGGCATCTTTTTCAGCCAGTTC	Other

Assay	Forward Primer (5'-3')	Reverse Primer (5'-3')	Class
imiR	CCGGACTAGAGCTTCATGTAAGC	CCCACGCGGTACTCTTGTAAA	Other
nisB	GGGAGAGTTGCCGATGTTGTA	AGCCACTCGTTAAAGGGCAAT	other
speA	GCAAGAGGTATTTGCTCAACAAGA	CAGGGTCACCCTCATAAAGAAAA	other
bacA-01	CGGCTTCGTGACCTCGTT	ACAATGCGATACCAGGCAAAT	other/bacitracin
bacA-02	TTCCACGACACGATTAAGTCATTG	CGGCTCTTTCGGCTTCAG	other/bacitracin
fosB	TCACTGTAACTAATGAAGCATTAGACCAT	CCATCTGGATCTGTAAAGTAAAGAGATC	other/fosfomycin
fosX	GATTAAGCCATATCACTTTAATTGTGAAAG	TCTCCTTCCATAATGCAAATCCA	other/fosfomycin
nimE	TGCGCCAAGATAGGGCATA	GTCGTGAATTCGGCAGGTTTA	other/nitroimidazole
pncA	GCAATCGAGGCGGTGTTC	TTGCCGCAGCCAATTCA	other/Pyrazinamide
sat4	GAATGGGCAAAGCATAAAAACTTG	CCGATTTTGAAACCACAATTATGATA	other/streptothricin
sul2	TCATCTGCCAAACTCGTCGTTA	GTCAAAGAACGCCGCAATGT	Sulfonamide
sul1	CAGCGCTATGCGCTCAAG	ATCCCGCTGCGCTGAGT	Sulfonamide
sulA/folP-01	CAGGCTCGTAAATTGATAGCAGAAG	CTTTCCTTGCGAATCGCTTT	Sulfonamide
sulA/folP-03	CACGGCTTCGGCTCATGT	TGCCATCCTGTGACTAGCTACGT	Sulfonamide
dfrA1	GGAATGGCCCTGATATTCCA	AGTCTTGCGTCCAACCAACAG	Sulfonamide
dfrA12	CCTCTACCGAACCGTCACACA	GCGACAGCGTTGAAACAACTAC	Sulfonamide
folA	CGAGCAGTTCCTGCCAAAG	CCCAGTCATCCGGTTCATAATC	Sulfonamide
tet(32)	CCATTACTTCGGACAACGGTAGA	CAATCTCTGTGAGGGCATTTAACA	Tetracycline
tet(34)	CTTAGCGCAAACAGCAATCAGT	CGGTGATACAGCGCGTAAACT	Tetracycline
tet(35)	ACCCCATGACGTACCTGTAGAGA	CAACCCACACTGGCTACCAGTT	Tetracycline
tet(36)-01	AGAATACTCAGCAGAGGTCAGTTCCT	TGGTAGGTCGATAACCCGAAAAT	Tetracycline
tet(36)-02	TGCAGGAAAGACCTCCATTACAG	CTTTGTCCACACTTCCACGTACTATG	Tetracycline
tet(37)	GAGAACGTTGAAAAGGTGGTGAA	AACCAAGCCTGGATCAGTCTCA	Tetracycline
tetA-01	GCTGTTTGTTCTGCCGGAAA	GGTTAAGTTCCTTGAACGCAAACT	Tetracycline
tetA-02	CTCACCAGCCTGACCTCGAT	CACGTTGTTATAGAAGCCGCATAG	Tetracycline
tetB-01	AGTGCGCTTTGGATGCTGTA	AGCCCCAGTAGCTCCTGTGA	Tetracycline
tetB-02	GCCCAGTGCTGTTGTTGTCAT	TGAAAGCAAACGGCCTAAATACA	Tetracycline
tetC-01	CATATCGCAATACATGCGAAAAA	AAAGCCGCGGTAAATAGCAA	Tetracycline
tetC-02	ACTGGTAAGGTAAACGCCATTGTC	ATGCATAAACCAGCCATTGAGTAAG	Tetracycline
tetD-01	TGCCGCGTTTGATTACACA	CACCAGTGATCCCGGAGATAA	Tetracycline
tetD-02	TGTCATCGCGCTGGTGATT	CATCCGCTTCCGGGAGAT	Tetracycline
tetE	TTGGCGCTGTATGCAATGAT	CGACGACCTATGCGATCTGA	Tetracycline
tetG-01	TCAACCATTGCCGATTCGA	TGGCCCGGCAATCATG	Tetracycline
tetG-02	CATCAGCGCCGGTCTTATG	CCCCATGTAGCCGAACCA	Tetracycline
tetH	TTTGGGTCATCTTACCAGCATTAA	TTGCGCATTATCATCGACAGA	Tetracycline
tetJ	GGGTGCCGCATTAGATTACCT	TCGTCCAATGTAGAGCATCCATA	Tetracycline
tetK	CAGCAGTCATTGGAAAATTATCTGATTATA	CCTTGTACTAACCTACCAAAAATCAAAATA	Tetracycline
tetL-01	AGCCCGATTTATTCAAGGAATTG	CAAATGCTTTCCCCCTGTTCT	Tetracycline
tetL-02	ATGGTTGTAGTTGCGCGCTATAT	ATCGCTGGACCGACTCCTT	Tetracycline
tetM-01	CATCATAGACACGCCAGGACATAT	CGCCATCTTTTGCAGAAATCA	Tetracycline
tetM-02	TAATATTGGAGTTTTAGCTCATGTTGATG	CCTCTCTGACGTTCTAAAAGCGTATTAT	Tetracycline
tetO-01	ATGTGGATACTACAACGCATGAGATT	TGCCTCCACATGATATTTTTCCT	Tetracycline

Assay	Forward Primer (5′-3′)	Reverse Primer (5′-3′)	Class
tetPA	AGTTGCAGATGTGTATAGTCGTAAACTATCTATT	TGCTACAAGTACGAAAACAAAACTAGAA	Tetracycline
tetPB-01	ACACCTGGACACGCTGATTTT	ACCGTCTAGAACGCGGAATG	Tetracycline
tetPB-02	TGATACACCTGGACACGCTGAT	CGTCCAAAACGCGGAATG	Tetracycline
tetPB-03	TGGGCGACAGTAGGCTTAGAA	TGACCCTACTGAAACATTAGAAATATACCT	Tetracycline
tetPB-05	CTGAAGTGGAGCGATCATTCC	CCCTCAACGGCAGAAATAACTAA	Tetracycline
tetQ	CGCCTCAGAAGTAAGTTCATACACTAAG	TCGTTCATGCGGATATTATCAGAAT	Tetracycline
tetR-02	CGCGATAGACGCCTTCGA	TCCTGACAACGAGCCTCCTT	Tetracycline
tetR-03	CGCGATGGAGCAAAAGTACAT	AGTGAAAAACCTTGTTGGCATAAAA	Tetracycline
tetS	TTAAGGACAAACTTTCTGACGACATC	TGTCTCCCATTGTTCTGGTTCA	Tetracycline
tetT	CCATATAGAGGTTCCACCAAATCC	TGACCCTATTGGTAGTGGTTCTATTG	Tetracycline
tetU-01	GTGGCAAAGCAACGGATTG	TGCGGGCTTGCAAAACTATC	Tetracycline
tetV	GCGGGAACGACGATGTATATC	CCGCTATCTCACGACCATGAT	Tetracycline
tetW-01	ATGAACATTCCCACCGTTATCTTT	ATATCGGCGGAGAGCTTATCC	Tetracycline
tetX	AAATTTGTTACCGACACGGAAGTT	CATAGCTGAAAAAATCCAGGACAGTT	Tetracycline
vanA	AAAAGGCTCTGAAAACGCAGTTAT	CGGCCGTTATCTTGTAAAAACAT	Vancomycin
vanB-01	TTGTCGGCGAAGTGGATCA	AGCCTTTTTCCGGCTCGTT	Vancomycin
vanC-01	ACAGGGATTGGCTATGAACCAT	TGACTGGCGATGATTTGACTATG	Vancomycin
vanC-02	CCTGCCACAATCGATCGTT	CGGCTTCATTCGGCTTGATA	Vancomycin
vanC-03	AAATCAATACTATGCCGGGCTTT	CCGACCGCTGCCATCA	Vancomycin
vanC1	AGGCGATAGCGGGTATTGAA	CAATCGTCAATTGCTCATTTCC	Vancomycin
vanC2/vanC3	TTTGACTGTCGGTGCTTGTGA	TCAATCGTTTCAGGCAATGG	Vancomycin
vanG	ATTTGAATTGGCAGGTATACAGGTTA	TGATTTGTCTTTGTCCATACATAATGC	Vancomycin
vanHB	GAGGTTTCCGAGGCGACAA	CTCTCGGCGGCAGTCGTAT	Vancomycin
vanHD	GTGGCCGATTATACCGTCATG	CGCAGGTCATTCAGGCAAT	Vancomycin
vanRA-01	CCCTTACTCCCACCGAGTTTT	TTCGTCGCCCCATATCTCAT	Vancomycin
vanRA-02	CCACTCCGGCCTTGTCATT	GCTAACCACATTCCCCTTGTTTT	Vancomycin
vanRB	GCCCTGTCGGATGACGAA	TTACATAGTCGTCTGCCTCTGCAT	Vancomycin
vanRC	TGCGGGAAAAACTGAACGA	CCCCCCATACGGTTTTGATTA	Vancomycin
vanRC4	AGTGCTTTGGCTTATCTCGAAAA	TCCGGCAGCATCACATCTAA	Vancomycin
vanRD	TTATAATGGCAAGGATGCACTAAAGT	CGTCTACATCCGGAAGCATGA	Vancomycin
vanSA	CGCGTCATGCTTTCAAAATTC	TCCGCAGAAAGCTCAATTTGTT	Vancomycin
vanSB	GCGCGGCAAATGACAAC	TTTGCCATTTTATTCGCACTGT	Vancomycin
vanSC-01	ATCAACTGCGGGAGAAAAGTCT	TCCGCTGTTCCGCTTCTT	Vancomycin
vanSC-02	GCCATCAGCGAGTCTGATGA	CAGCTGGGATCGTTTTTCCTT	Vancomycin
vanTC-01	CACACGCATTTTTTCCCATCTAG	CAGCCAACAGATCATCAAAACAA	Vancomycin
vanTC-02	ACAGTTGCCGCTGGTGAAG	CGTGGCTGGTCGATCAAAA	Vancomycin
vanTE	GTGGTGCCAAGGAAGTTGCT	CGTAGCCACCGCAAAAAAAT	Vancomycin
vanTG	CGTGTAGCCGTTCCGTTCTT	CGGCATTACAGGTATATCTGGAAA	Vancomycin
vanWB	CGGACAAAGATACCCCCTATAAAG	AAATAGTAAATTGCTCATCTGGCACAT	Vancomycin
vanWG	ACATTTTCATTTTGGCAGCTTGTAC	CCGCCATAAGAGCCTACAATCT	Vancomycin
vanXA	CGCTAAATATGCCACTTGGGATA	TCAAAAGCGATTCAGCCAACT	Vancomycin
vanXB	AGGCACAAAATCGAAGATGCTT	GGGTATGGCTCATCAATCAACTT	Vancomycin

Assay	Forward Primer (5’-3’)	Reverse Primer (5’-3’)	Class
vanXD	TAAACCGTGTTATGGGAACGAA	GCGATAGCCGTCCCATAAGA	Vancomycin
vanYB	GGCTAAAGCGGAAGCAGAAA	GATATCCACAGCAAGACCAAGCT	Vancomycin
vanYD-01	AAGGCGATACCCTGACTGTCA	ATTGCCGGACGGAAGCA	Vancomycin
vanYD-02	CAAACGGAAGAGAGGTCACTTACA	CGGACGGTAATAGGGACTGTTC	Vancomycin

References

Abraham, S., Jordan, D., Wong, H. S., Johnson, J. R., Toleman, M. A., Wakeham, D. L., Gordon, D. M., Turnidge, J. D., Mollinger, J. L., Gibson, J. S. & Trott, D. J. 2015. First detection of extended-spectrum cephalosporin- and fluoroquinolone-resistant *Escherichia coli* in Australian food-producing animals. *J Glob Antimicrob Resist,* 3**,** 273-277.

Abraham, S., O'Dea, M., Sahibzada, S., Hewson, K., Pavic, A., Veltman, T., Abraham, R., Harris, T., Trott, D. J. & Jordan, D. 2019. *Escherichia coli* and *Salmonella* spp. isolated from Australian meat chickens remain susceptible to critically important antimicrobial agents. *PLoS One,* 14**,** e0224281.

Agersø, Y., Jensen, L. B., Givskov, M. & Roberts, M. C. 2002. The identification of a tetracycline resistance gene *tet*(M), on a Tn 916-like transposon, in the *Bacillus cereus* group. *FEMS Microbiol. Lett.,* 214**,** 251-256.

Agersø, Y., Pedersen, A. G. & Aarestrup, F. M. 2006. Identification of Tn5397-like and Tn916-like transposons and diversity of the tetracycline resistance gene *tet*(M) in enterococci from humans, pigs and poultry. *J. Antimicrob. Chemother.,* 57**,** 832-839.

Agga, G. E., Scott, H. M., Vinasco, J., Nagaraja, T., Amachawadi, R. G., Bai, J., Norby, B., Renter, D. G., Dritz, S. S. & Nelssen, J. L. 2015. Effects of chlortetracycline and copper supplementation on the prevalence, distribution, and quantity of antimicrobial resistance genes in the fecal metagenome of weaned pigs. *Prev. Vet. Med.,* 119**,** 179-189.

Agriculture Victoria. 2017. Code of accepted farming practice for the welfare of cattle [Online]. Available: http://agriculture.vic.gov.au/agriculture/animal-health-and-welfare/animal-welfare/animal-welfare-legislation/victorian-codes-of-practice-for-animal-welfare/code-of-accepted-farming-practice-for-the-welfare-of-cattle [Accessed 1/1/2019].

Ahmed, M. O., Clegg, P. D., Williams, N. J., Baptiste, K. E. & Bennett, M. 2010. Antimicrobial resistance in equine faecal *Escherichia coli* isolates from North West England. *Ann. Clin. Microbiol. Antimicrob.,* 9**,** 12.

Al Atrouni, A., Joly-Guillou, M.-L., Hamze, M. & Kempf, M. 2016. Reservoirs of Non-*baumannii Acinetobacter* Species. *Front. Microbiol.,* 7**,** 49.

Alam, M. T., Amos, G. C. A., Murphy, A. R. J., Murch, S., Wellington, E. M. H. & Arasaradnam, R. P. 2020. Microbial imbalance in inflammatory bowel disease patients at different taxonomic levels. *Gut Pathog.,* 12**,** 1.

Alekshun, M. N. & Levy, S. B. 2007. Molecular mechanisms of antibacterial multidrug resistance. *Cell,* 128**,** 1037-50.

Alexander, T. W., Yanke, J. L., Reuter, T., Topp, E., Read, R. R., Selinger, B. L. & McAllister, T. A. 2011. Longitudinal characterization of antimicrobial resistance genes in feces shed from cattle fed different subtherapeutic antibiotics. *BMC Microbiol.,* 11**,** 19.

Alimirzaei, M., Alijoo, Y. A., Dehghan-Banadaky, M. & Eslamizad, M. 2020. The effects of feeding high or low milk levels in early life on growth performance, fecal microbial count and metabolic and inflammatory status of Holstein female calves. *Animal,* 14**,** 303-311.

Allen-Vercoe, E., Strauss, J. & Chadee, K. J. G. m. 2011. *Fusobacterium nucleatum*: an emerging gut pathogen? *Gut Microbes,* 2**,** 294-298.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. 1990. Basic local alignment search tool. *J. Mol. Biol.,* 215**,** 403-410.

Andersson, D. I. & Hughes, D. 2010. Antibiotic resistance and its cost: is it possible to reverse resistance? *Nat. Rev. Microbiol.,* 8**,** 260-271.

Andersson, D. I. & Hughes, D. 2011. Persistence of antibiotic resistance in bacterial populations. *FEMS Microbiol. Rev.,* 35**,** 901-911.

Antonopoulos, D. A., Huse, S. M., Morrison, H. G., Schmidt, T. M., Sogin, M. L. & Young, V. B. 2009. Reproducible community dynamics of the gastrointestinal microbiota following antibiotic perturbation. *Infect. Immun.,* 77**,** 2367-2375.

Antunes, L. C. M., Han, J., Ferreira, R. B., Lolić, P., Borchers, C. H. & Finlay, B. B. 2011. Effect of antibiotic treatment on the intestinal metabolome. *Antimicrob. Agents Chemother.,* 55**,** 1494-1503.

APVMA. 2014. Quantity of antimicrobial products sold for veterinary use in Australia. *Australian Pesticides and Veterinary Medicines Authority (APVMA)*. Available: https://apvma.gov.au/sites/default/files/images/antimicrobial_sales_report_march-2014.pdf [Accessed 1/7/2018].

APVMA. 2017. Antibiotic resistance in animals. *Australian Pesticides and Veterinary Medicines Authority (APVMA)*. Available: https://apvma.gov.au/our-science/14596/publications/27326 [Accessed 27/6/2018].

Arnold, M., Carrique-Mas, J. & Davies, R. 2010. Sensitivity of environmental sampling methods for detecting *Salmonella Enteritidis* in commercial laying flocks relative to the within-flock prevalence. *Epidemiol. Infect.,* 138**,** 330-339.

Arrieta, M.-C., Stiemsma, L. T., Amenyogbe, N., Brown, E. M. & Finlay, B. 2014. The intestinal microbiome in early life: health and disease. *Front. Immunol.,* 5**,** 427.

Arumugam, M., Raes, J., Pelletier, E., Le Paslier, D., Yamada, T., Mende, D. R., Fernandes, G. R., Tap, J., Bruls, T. & Batto, J.-M. 2011. Enterotypes of the human gut microbiome. *Nature,* 473**,** 174-180.

Asai, T., Kojima, A., Harada, K., Ishihara, K., Takahashi, T. & Tamura, Y. 2005. Correlation between the usage volume of veterinary therapeutic antimicrobials and resistance in *Escherichia coli* isolated from the feces of food-producing animals in Japan. *Jpn. J. Infect. Dis.,* 58**,** 369-372.

Asante, J. & Sekyere, J. O. 2019. Understanding antimicrobial discovery and resistance from a metagenomic and metatranscriptomic perspective: advances and applications. *Environ. Microbiol. Rep.,* 11**,** 62-86.

Aziz, R. K., Bartels, D., Best, A. A., DeJongh, M., Disz, T., Edwards, R. A., Formsma, K., Gerdes, S., Glass, E. M., Kubal, M., Meyer, F., Olsen, G. J., Olson, R., Osterman, A. L., Overbeek, R. A., McNeil, L. K., Paarmann, D., Paczian, T., Parrello, B., Pusch, G. D., Reich, C., Stevens, R., Vassieva, O., Vonstein, V., Wilke, A. & Zagnitko, O. 2008. The RAST Server: rapid annotations using subsystems technology. *BMC Genomics,* 9**,** 75.

Bäckhed, F., Ley, R. E., Sonnenburg, J. L., Peterson, D. A. & Gordon, J. I. 2005. Host-bacterial mutualism in the human intestine. *Science,* 307**,** 1915-1920.

Bae, J., Oh, E. & Jeon, B. 2014. Enhanced transmission of antibiotic resistance in *Campylobacter jejuni* biofilms by natural transformation. *Antimicrob. Agents Chemother.,* 58**,** 7573-7575.

Bager, F., Madsen, M., Christensen, J. & Aarestrup, F. M. 1997. Avoparcin used as a growth promoter is associated with the occurrence of vancomycin-resistant *Enterococcus faecium* on Danish poultry and pig farms. *Prev. Vet. Med.,* 31**,** 95-112.

Bailey, J. K., Pinyon, J. L., Anantham, S. & Hall, R. M. 2010. Commensal *Escherichia coli* of healthy humans: a reservoir for antibiotic-resistance determinants. *J. Med. Microbiol.,* 59**,** 1331-1339.

Bailey, K. E. 2017. *Investigation of the Infectious Causes of Diarrhoea in Australian Thoroughbred Foals.* PhD Thesis PhD Thesis, The University of Melbourne.

Baker-Austin, C., Wright, M. S., Stepanauskas, R. & McArthur, J. 2006. Co-selection of antibiotic and metal resistance. *Trends Microbiol.,* 14**,** 176-182.

Balcazar, J. L. 2014. Bacteriophages as vehicles for antibiotic resistance genes in the environment. *PLoS pathogens,* 10**,** e1004219.

Baquero, F., Alvarez‐Ortega, C. & Martinez, J. 2009. Ecology and evolution of antibiotic resistance. *Environ. Microbiol. Rep.,* 1**,** 469-476.

Barlow, M. 2009. What antimicrobial resistance has taught us about horizontal gene transfer. *In: Horizontal Gene Transfer: Genomes in Flux.* Humana Press. Totowa, NJ, 397-411

Barlow, R. S., Mcmillan, K. E., Duffy, L. L., Fegan, N., Jordan, D. & Mellor, G. E. 2015. Prevalence and antimicrobial resistance of *Salmonella* and *Escherichia coli* from Australian cattle populations at slaughter. *J. Food Prot.,* 78**,** 912-920.

Barlow, R. S., McMillan, K. E., Duffy, L. L., Fegan, N., Jordan, D. & Mellor, G. E. 2017. Antimicrobial resistance status of *Enterococcus* from Australian cattle populations at slaughter. *PLoS One,* 12**,** e0177728.

Barton, M. D. 2007. Antimicrobial resistance in veterinary medicine? Issues and controversies from an Australian perspective. *Microbiol. Aust.,* 28**,** 180-181.

Bastian, M., Heymann, S. & Jacomy, M. 2009. Gephi: an open source software for exploring and manipulating networks. International AAAI Conference on Weblogs and Social Media, 2009.

Bates, J., Jordens, J. Z. & Griffiths, D. T. 1994. Farm animals as a putative reservoir for vancomycin-resistant enterococcal infection in man. *J. Antimicrob. Chemother.,* 34**,** 507-514.

Bean, D. C., Livermore, D. M. & Hall, L. M. 2009. Plasmids imparting sulfonamide resistance in *Escherichia coli*: implications for persistence. *Antimicrob. Agents Chemother.,* 53**,** 1088-1093.

Beitel, C. W., Froenicke, L., Lang, J. M., Korf, I. F., Michelmore, R. W., Eisen, J. A. & Darling, A. E. 2014. Strain-and plasmid-level deconvolution of a synthetic metagenome by sequencing proximity ligation products. *PeerJ,* 2**,** e415.

Belenguer, A., Duncan, S. H., Calder, A. G., Holtrop, G., Louis, P., Lobley, G. E. & Flint, H. J. 2006. Two routes of metabolic cross-feeding between *Bifidobacterium adolescentis* and butyrate-producing anaerobes from the human gut. *Appl. Environ. Microbiol.,* 72**,** 3593-3599.

Belzer, C., Chia, L. W., Aalvink, S., Chamlagain, B., Piironen, V., Knol, J. & de Vos, W. M. 2017. Microbial metabolic networks at the mucus layer lead to diet-independent butyrate and vitamin B12 production by intestinal symbionts. *mBio,* 8**,** e00770-17.

Bengtsson-Palme, J., Larsson, D. G. J. & Kristiansson, E. 2017. Using metagenomics to investigate human and environmental resistomes. *J. Antimicrob. Chemother.,* 72**,** 2690-2703.

Benjamini, Y. & Hochberg, Y. 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. Roy. Stat. Soc. Ser. B. (Stat. Method.),* 57**,** 289-300.

Bertrand, D., Shaw, J., Kalathiyappan, M., Ng, A. H. Q., Kumar, M. S., Li, C. H., Dvornicic, M., Soldo, J. P., Koh, J. Y., Tong, C. X., Ng, O. T., Barkham, T., Young, B., Marimuthu, K., Chng, K. R., Sikic, M. & Nagarajan, N. 2019. Hybrid metagenomic assembly enables high-resolution analysis of resistance

determinants and mobile elements in human microbiomes. *Nat. Biotechnol.,* 37**,** 937-944.

Bessegatto, J. A., Paulino, L. R., Lisbôa, J. A. N., Alfieri, A. A., Montemor, C. H., Medeiros, L. P., Kobayashi, R. K. T., Weese, J. S. & Costa, M. C. 2017. Changes in the fecal microbiota of beef cattle caused by change in management and the use of virginiamycin as a growth promoter. *Res. Vet. Sci.,* 114**,** 355-362.

Bhullar, K., Waglechner, N., Pawlowski, A., Koteva, K., Banks, E. D., Johnston, M. D., Barton, H. A. & Wright, G. D. 2012. Antibiotic resistance is prevalent in an isolated cave microbiome. *PLoS One,* 7**,** e34953.

Biddle, A., Stewart, L., Blanchard, J. & Leschine, S. 2013. Untangling the genetic basis of fibrolytic specialization by *Lachnospiraceae* and *Ruminococcaceae* in diverse gut communities. *Diversity,* 5**,** 627-640.

Biddle, A. S., Tomb, J.-F. & Fan, Z. 2018. Microbiome and blood analyte differences point to community and metabolic signatures in lean and obese horses. *Front. Vet. Sci.,* 5**,** 225.

Billman-Jacobe, H., Dyall-Smith, M., Hawkey, J. & Liu, Y. 2019. Heavy metal and antibiotic resistance genes in bacteria from porcine monophasic *Salmonella* 1,4,[5],12:i:-. *Journal of Integrated OMICS,* 9**,** 37.

Billman-Jacobe, H., Liu, Y., Haites, R., Weaver, T., Robinson, L., Marenda, M. & Dyall-Smith, M. 2018. pSTM6-275, a conjugative IncHI2 plasmid of *Salmonella* that confers antibiotic and heavy metal resistance under changing physiological conditions. *Antimicrob. Agents Chemother.***,** 62:e02357-17.

Binh, C. T., Heuer, H., Kaupenjohann, M. & Smalla, K. 2009. Diverse *aad*A gene cassettes on class 1 integrons introduced into soil via spread manure. *Res. Microbiol.,* 160**,** 427-433.

Blair, J. M., Webber, M. A., Baylay, A. J., Ogbolu, D. O. & Piddock, L. J. 2015. Molecular mechanisms of antibiotic resistance. *Nat. Rev. Microbiol.,* 13**,** 42-51.

Bokulich, N. A., Kaehler, B. D., Rideout, J. R., Dillon, M., Bolyen, E., Knight, R., Huttley, G. A. & Caporaso, J. G. 2018. Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. *Microbiome,* 6**,** 90.

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., Alexander, H., Alm, E. J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J. E., Bittinger, K., Brejnrod, A., Brislawn, C. J., Brown, C. T., Callahan, B. J., Caraballo-Rodríguez, A. M., Chase, J., Cope, E. K., Da Silva, R., Diener, C., Dorrestein, P. C., Douglas, G. M., Durall, D. M., Duvallet, C., Edwardson, C. F., Ernst, M., Estaki, M., Fouquier, J., Gauglitz, J. M., Gibbons, S. M., Gibson, D. L., Gonzalez, A., Gorlick, K., Guo, J., Hillmann, B., Holmes, S., Holste, H., Huttenhower, C., Huttley, G. A., Janssen, S., Jarmusch, A. K., Jiang, L., Kaehler, B. D., Kang, K. B.,

Keefe, C. R., Keim, P., Kelley, S. T., Knights, D., Koester, I., Kosciolek, T., Kreps, J., Langille, M. G. I., Lee, J., Ley, R., Liu, Y.-X., Loftfield, E., Lozupone, C., Maher, M., Marotz, C., Martin, B. D., McDonald, D., McIver, L. J., Melnik, A. V., Metcalf, J. L., Morgan, S. C., Morton, J. T., Naimey, A. T., Navas-Molina, J. A., Nothias, L. F., Orchanian, S. B., Pearson, T., Peoples, S. L., Petras, D., Preuss, M. L., Pruesse, E., Rasmussen, L. B., Rivers, A., Robeson, M. S., Rosenthal, P., Segata, N., Shaffer, M., Shiffer, A., Sinha, R., Song, S. J., Spear, J. R., Swafford, A. D., Thompson, L. R., Torres, P. J., Trinh, P., Tripathi, A., Turnbaugh, P. J., Ul-Hasan, S., van der Hooft, J. J. J., Vargas, F., Vázquez-Baeza, Y., Vogtmann, E., von Hippel, M., Walters, W., et al. 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.,* 37**,** 852-857.

Boucher, H. W., Talbot, G. H., Bradley, J. S., Edwards, J. E., Gilbert, D., Rice, L. B., Scheld, M., Spellberg, B. & Bartlett, J. 2009. Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. *Clin. Infect. Dis.,* 48**,** 1-12.

Bowler, L. D., Zhang, Q.-Y., Riou, J.-Y. & Spratt, B. G. 1994. Interspecies recombination between the *pen*A genes of *Neisseria meningitidis* and commensal *Neisseria* species during the emergence of penicillin resistance in *N. meningitidis*: natural events and laboratory simulation. *J. Bacteriol.,* 176**,** 333-337.

Bradley, P., Gordon, N. C., Walker, T. M., Dunn, L., Heys, S., Huang, B., Earle, S., Pankhurst, L. J., Anson, L. & De Cesare, M. 2015. Rapid antibiotic-resistance predictions from genome sequence data for *Staphylococcus aureus* and *Mycobacterium tuberculosis*. *Nat. Commun.,* 6**,** 10063.

Britton, R. A. & Young, V. B. 2014. Role of the intestinal microbiota in resistance to colonization by *Clostridium difficile*. *Gastroenterology,* 146**,** 1547-1553.

Brown-Jaque, M., Calero-Cáceres, W. & Muniesa, M. 2015. Transfer of antibiotic-resistance genes via phage-related mobile elements. *Plasmid,* 79**,** 1-7.

Bryskier, A. 2010. Macrolides. *In: Antibiotic and Chemotherapy (Ninth Edition).* W.B. Saunders. London, 276-289

Bukin, Y. S., Galachyants, Y. P., Morozov, I., Bukin, S., Zakharenko, A. & Zemskaya, T. 2019. The effect of 16S rRNA region choice on bacterial community metabarcoding results. *Sci. Data.,* 6**,** 190007.

Burton, J. N., Liachko, I., Dunham, M. J. & Shendure, J. 2014. Species-level deconvolution of metagenome assemblies with Hi-C–based contact probability maps. *G3: Genes, Genomes, Genetics,* 4**,** 1339-1346.

Byrne-Bailey, K., Gaze, W. H., Zhang, L., Kay, P., Boxall, A., Hawkey, P. & Wellington, E. 2011. Integron prevalence and diversity in manured soil. *Appl. Environ. Microbiol.,* 77**,** 684-687.

Cain, B. D., Norton, P. J., Eubanks, W., Nick, H. S. & Allen, C. M. 1993. Amplification of the *bacA* gene confers bacitracin resistance to *Escherichia coli*. *J. Bacteriol.,* 175**,** 3784-3789.

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A. & Holmes, S. P. 2016. DADA2: high-resolution sample inference from Illumina amplicon data. *Nat. Methods,* 13**,** 581-583.

Cambray, G., Guerout, A.-M. & Mazel, D. 2010. Integrons. *Annu. Rev. Genet.,* 44**,** 141-166.

Carattoli, A. 2013. Plasmids and the spread of resistance. *Int. J. Med. Microbiol.,* 303**,** 298-304.

Carattoli, A., Zankari, E., Garcia-Fernandez, A., Voldby Larsen, M., Lund, O., Villa, L., Moller Aarestrup, F. & Hasman, H. 2014. *In silico* detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. *Antimicrob. Agents Chemother.,* 58**,** 3895-903.

Cave, J., Callinan, A. & Woonton, W. 2005. Mortalities in bobby calves associated with long distance transport. *Aust. Vet. J.,* 83**,** 82-84.

Chalmers, G., Rozas, K., Amachawadi, R., Scott, H., Norman, K., Nagaraja, T., Tokach, M. & Boerlin, P. 2018. Distribution of the *pco* gene cluster and associated genetic determinants among swine *Escherichia coli* from a controlled feeding trial. *Genes,* 9**,** 504.

Chambers, L., Yang, Y., Littier, H., Ray, P., Zhang, T., Pruden, A., Strickland, M. & Knowlton, K. 2015. Metagenomic analysis of antibiotic resistance genes in dairy cow feces following therapeutic administration of third generation cephalosporin. *PLoS One,* 10**,** e0133764.

Chantziaras, I., Boyen, F., Callens, B. & Dewulf, J. 2013. Correlation between veterinary antimicrobial use and antimicrobial resistance in food-producing animals: a report on seven countries. *J. Antimicrob. Chemother.,* 69**,** 827-834.

Charpentier, X., Polard, P. & Claverys, J.-P. 2012. Induction of competence for genetic transformation by antibiotics: convergent evolution of stress responses in distant bacterial species lacking SOS? *Curr. Opin. Microbiol.,* 15**,** 570-576.

Cheng, A. C., Turnidge, J., Collignon, P., Looke, D., Barton, M. & Gottlieb, T. 2012. Control of fluoroquinolone resistance through successful regulation, Australia. *Emerg. Infect. Dis.,* 18**,** 1453-1460.

Cheng, W., Chen, H., Su, C. & Yan, S. 2013. Abundance and persistence of antibiotic resistance genes in livestock farms: a comprehensive investigation in eastern China. *Environ. Int.,* 61**,** 1-7.

Chopra, I. & Roberts, M. 2001. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol. Mol. Biol. Rev.,* 65**,** 232-260.

Chung, W. O., Young, K., Leng, Z. & Roberts, M. C. 1999. Mobile elements carrying *erm*F and *tet*Q genes in Gram-positive and Gram-negative bacteria. *J. Antimicrob. Chemother.,* 44**,** 329-335.

Clasen, J., Mellerup, A., Olsen, J. E., Angen, Ø., Folkesson, A., Halasa, T., Toft, N. & Birkegård, A. C. 2016. Determining the optimal number of individual samples to pool for quantification of average herd levels of antimicrobial resistance genes in Danish pig herds using high-throughput qPCR. *Vet. Microbiol.,* 189**,** 46-51.

Clemente, J. C., Pehrsson, E. C., Blaser, M. J., Sandhu, K., Gao, Z., Wang, B., Magris, M., Hidalgo, G., Contreras, M., Noya-Alarcón, Ó., Lander, O., McDonald, J., Cox, M., Walter, J., Oh, P. L., Ruiz, J. F., Rodriguez, S., Shen, N., Song, S. J., Metcalf, J., Knight, R., Dantas, G. & Dominguez-Bello, M. G. 2015. The microbiome of uncontacted Amerindians. *Sci Adv,* 1**,** e1500183.

Clewell, D. B., Flannagan, S. E. & Jaworski, D. D. 1995. Unconstrained bacterial promiscuity: the Tn916–Tn1545 family of conjugative transposons. *Trends Microbiol.,* 3**,** 229-236.

Cochetti, I., Tili, E., Vecchi, M., Manzin, A., Mingoia, M., Varaldo, P. E. & Montanari, M. P. 2007. New Tn 916-related elements causing *erm*(B)-mediated erythromycin resistance in tetracycline-susceptible pneumococci. *J. Antimicrob. Chemother.,* 60**,** 127-131.

Colomer-Lluch, M., Imamovic, L., Jofre, J. & Muniesa, M. 2011. Bacteriophages carrying antibiotic resistance genes in fecal waste from cattle, pigs, and poultry. *Antimicrob. Agents Chemother.,* 55**,** 4908-4911.

Colomer-Lluch, M., Jofre, J. & Muniesa, M. 2014. Quinolone resistance genes (*qnr*A and *qnr*S) in bacteriophage particles from wastewater samples and the effect of inducing agents on packaged antibiotic resistance genes. *J. Antimicrob. Chemother.,* 69**,** 1265-1274.

Costa, M. C., Arroyo, L. G., Allen-Vercoe, E., Stämpfli, H. R., Kim, P. T., Sturgeon, A. & Weese, J. S. 2012. Comparison of the fecal microbiota of healthy horses and horses with colitis by high throughput sequencing of the V3-V5 region of the 16S rRNA gene. *PLoS One,* 7**,** e41484.

Costa, M. C., Stämpfli, H. R., Allen‐Vercoe, E. & Weese, J. S. 2016. Development of the faecal microbiota in foals. *Equine Vet. J.,* 48**,** 681-688.

Costa, M. C., Stämpfli, H. R., Arroyo, L. G., Allen-Vercoe, E., Gomes, R. G. & Weese, J. S. 2015. Changes in the equine fecal microbiota associated with the use of systemic antimicrobial drugs. *BMC Vet. Res.,* 11**,** 19.

Costea, P. I., Hildebrand, F., Arumugam, M., Bäckhed, F., Blaser, M. J., Bushman, F. D., de Vos, W. M., Ehrlich, S. D., Fraser, C. M., Hattori, M., Huttenhower, C., Jeffery, I. B., Knights, D., Lewis, J. D., Ley, R. E., Ochman, H., O'Toole, P. W., Quince, C., Relman, D. A., Shanahan, F., Sunagawa, S., Wang, J., Weinstock, G. M., Wu, G. D., Zeller, G., Zhao, L., Raes, J., Knight, R. & Bork, P. 2018. Enterotypes in the landscape of gut microbial community composition. *Nat Microbiol,* 3**,** 8-16.

Crabb, H. K., Allen, J. L., Devlin, J. M., Wilks, C. R. & Gilkerson, J. R. 2019. Spatial distribution of *Salmonella enterica* in poultry shed environments observed by intensive longitudinal environmental sampling. *Appl. Environ. Microbiol.***,** AEM. 00333-19.

D'Costa, V. M., King, C. E., Kalan, L., Morar, M., Sung, W. W., Schwarz, C., Froese, D., Zazula, G., Calmels, F. & Debruyne, R. 2011. Antibiotic resistance is ancient. *Nature,* 477**,** 457.

Daly, M., Villa, L., Pezzella, C., Fanning, S. & Carattoli, A. 2005. Comparison of multidrug resistance gene regions between two geographically unrelated *Salmonella* serotypes. *J. Antimicrob. Chemother.,* 55**,** 558-561.

De La Torre, U., Henderson, J. D., Furtado, K. L., Pedroja, M., Mora, A., Pechanec, M. Y., Maga, E. A. & Mienaltowski, M. J. 2019. Utilizing the fecal microbiota to understand foal gut transitions from birth to weaning. *PLoS One,* 14**,** e0216211.

De Leener, E., Martel, A., Decostere, A. & Haesebrouck, F. 2004. Distribution of the *erm*(B) gene, tetracycline resistance genes, and Tn1545-like transposons in macrolide- and lincosamide-resistant enterococci from pigs and humans. *Microb. Drug Resist.,* 10**,** 341-345.

De Luca, F. & Shoenfeld, Y. 2019. The microbiome in autoimmune diseases. *Clin. Exp. Immunol.,* 195**,** 74-85.

De Vries, L. E., Christensen, H., Skov, R. L., Aarestrup, F. M. & Agersø, Y. 2009. Diversity of the tetracycline resistance gene *tet*(M) and identification of Tn 916-and Tn 5801-like (Tn 6014) transposons in *Staphylococcus aureus* from humans and animals. *J. Antimicrob. Chemother.,* 64**,** 490-500.

Deamer, D., Akeson, M. & Branton, D. 2016. Three decades of Nanopore sequencing. *Nat. Biotechnol.,* 34**,** 518-524.

Del Grosso, M., Iannelli, F., Messina, C., Santagati, M., Petrosillo, N., Stefani, S., Pozzi, G. & Pantosti, A. 2002. Macrolide efflux genes *mef*(A) and *mef*(E) are carried by different genetic elements in *Streptococcus pneumoniae*. *J. Clin. Microbiol.,* 40**,** 774-778.

DeLeo, F. R., Chen, L., Porcella, S. F., Martens, C. A., Kobayashi, S. D., Porter, A. R., Chavda, K. D., Jacobs, M. R., Mathema, B. & Olsen, R. J. 2014. Molecular dissection of the evolution of carbapenem-resistant multilocus sequence type 258 *Klebsiella pneumoniae*. *Proc. Natl. Acad. Sci. USA,* 111**,** 4988-4993.

Deng, Y., Wang, Y., Zou, Y., Azarfar, A., Wei, X., Ji, S., Zhang, J., Wu, Z., Wang, S. & Dong, S. 2017. Influence of dairy by-product waste milk on the microbiomes of different gastrointestinal tract components in pre-weaned dairy calves. *Sci. Rep.,* 7**,** 42689.

Derbise, A., Aubert, S. & El Solh, N. 1997a. Mapping the regions carrying the three contiguous antibiotic resistance genes *aad*E, *sat*4, and *aph*A-3 in the genomes of staphylococci. *Antimicrob. Agents Chemother.,* 41**,** 1024-1032.

Derbise, A., de Cespedes, G. & Solh, N. E. 1997b. Nucleotide sequence of the *Staphylococcus aureus* transposon, Tn5405, carrying aminoglycosides resistance genes. *J. Basic Microbiol.,* 37**,** 379-384.

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., Huber, T., Dalevi, D., Hu, P. & Andersen, G. L. 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl. Environ. Microbiol.,* 72**,** 5069-5072.

Dethlefsen, L., Huse, S., Sogin, M. L. & Relman, D. A. 2008. The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. *PLoS Biol.,* 6**,** e280.

Dethlefsen, L. & Relman, D. A. 2011. Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. *Proc. Natl. Acad. Sci. USA,* 108**,** 4554-4561.

Diaz-Torres, M. L., Villedieu, A., Hunt, N., McNab, R., Spratt, D. A., Allan, E., Mullany, P. & Wilson, M. 2006. Determining the antibiotic resistance potential of the indigenous oral microbiota of humans using a metagenomic approach. *FEMS Microbiol. Lett.,* 258**,** 257-262.

Dibner, J. & Richards, J. 2005. Antibiotic growth promoters in agriculture: history and mode of action. *Poult. Sci.,* 84**,** 634-643.

Dolejska, M., Duskova, E., Rybarikova, J., Janoszowska, D., Roubalova, E., Dibdakova, K., Maceckova, G., Kohoutova, L., Literak, I. & Smola, J. 2011. Plasmids carrying *bla*CTX-M-1 and *qnr* genes in *Escherichia coli* isolates from an equine clinic and a horseback riding centre. *J. Antimicrob. Chemother.,* 66**,** 757-764.

Domingues, S., Harms, K., Fricke, W. F., Johnsen, P. J., Da Silva, G. J. & Nielsen, K. M. 2012. Natural transformation facilitates transfer of transposons, integrons and gene cassettes between bacterial species. *PLoS pathogens,* 8**,** e1002837.

Dominguez-Bello, M. G., Blaser, M. J., Ley, R. E. & Knight, R. 2011. Development of the human gastrointestinal microbiota and insights from high-throughput sequencing. *Gastroenterology,* 140**,** 1713-1719.

Dougal, K., de la Fuente, G., Harris, P. A., Girdwood, S. E., Pinloche, E., Geor, R. J., Nielsen, B. D., Schott II, H. C., Elzinga, S. & Newbold, C. J. 2014. Characterisation

of the faecal bacterial community in adult and elderly horses fed a high fibre, high oil or high starch diet using 454 pyrosequencing. *PLoS One,* 9**,** e87424.

Dougal, K., de la Fuente, G., Harris, P. A., Girdwood, S. E., Pinloche, E. & Newbold, C. J. 2013. Identification of a core bacterial community within the large intestine of the horse. *PLoS One,* 8**,** e77660.

Drackley, J. K. 2008. Calf nutrition from birth to breeding. *Vet. Clin. North Am. Food Anim. Pract.,* 24**,** 55-86.

Durso, L. M., Harhay, G. P., Bono, J. L. & Smith, T. P. 2011. Virulence-associated and antibiotic resistance genes of microbial populations in cattle feces analyzed using a metagenomic approach. *J. Microbiol. Methods,* 84**,** 278-282.

Dyall-Smith, M. L., Liu, Y. & Billman-Jacobe, H. 2017. Genome sequence of an Australian monophasic *Salmonella enterica* subsp. *enterica* Typhimurium Isolate (TW-Stm6) carrying a large plasmid with multiple antimicrobial resistance genes. *Genome Announc.,* 5**,** e00793-17.

Edrington, T., Dowd, S., Farrow, R., Hagevoort, G., Callaway, T., Anderson, R. & Nisbet, D. 2012. Development of colonic microflora as assessed by pyrosequencing in dairy calves fed waste milk. *J. Dairy Sci.,* 95**,** 4519-4525.

El Ghachi, M., Bouhss, A., Blanot, D. & Mengin-Lecreulx, D. 2004. The *bac*A gene of *Escherichia coli* encodes an undecaprenyl pyrophosphate phosphatase activity. *J. Biol. Chem.,* 279**,** 30106-30113.

Elzinga, S. E., Weese, J. S. & Adams, A. A. 2016. Comparison of the fecal microbiota in horses with equine metabolic syndrome and metabolically normal controls fed a similar all-forage diet. *J. Equine Vet. Sci.,* 44**,** 9-16.

Enault, F., Briet, A., Bouteille, L., Roux, S., Sullivan, M. B. & Petit, M.-A. 2017. Phages rarely encode antibiotic resistance genes: a cautionary tale for virome analyses. *ISME J,* 11**,** 237-247.

Enne, V. I., Livermore, D. M., Stephens, P. & Hall, L. M. 2001. Persistence of sulphonamide resistance in *Escherichia coli* in the UK despite national prescribing restriction. *The Lancet,* 357**,** 1325-1328.

Fang, L., Li, X., Li, L., Li, S., Liao, X., Sun, J. & Liu, Y. 2016. Co-spread of metal and antibiotic resistance within ST3-IncHI2 plasmids from *E. coli* isolates of food-producing animals. *Sci. Rep.,* 6**,** 25312.

Fard, R. M. N., Heuzenroeder, M. W. & Barton, M. D. 2011. Antimicrobial and heavy metal resistance in commensal enterococci isolated from pigs. *Vet. Microbiol.,* 148**,** 276-282.

Favier, C. F., Vaughan, E. E., De Vos, W. M. & Akkermans, A. D. 2002. Molecular monitoring of succession of bacterial communities in human neonates. *Appl. Environ. Microbiol.,* 68**,** 219-226.

Fell, L. & Shutt, D. 1986. Adrenocortical response of calves to transport stress as measured by salivary cortisol. *Canadian Journal of Animal Science,* 66**,** 637-641.

Fernández-Orth, D., Miró, E., Brown-Jaque, M., Rodríguez-Rubio, L., Espinal, P., Rodriguez-Navarro, J., González-López, J. J., Muniesa, M. & Navarro, F. 2019. Faecal phageome of healthy individuals: presence of antibiotic resistance genes and variations caused by ciprofloxacin treatment. *J. Antimicrob. Chemother.,* 74**,** 854-864.

Ferrándiz, M. J., Fenoll, A., Liñares, J. & Adela, G. 2000. Horizontal transfer of *par*C and *gyr*A in fluoroquinolone-resistant clinical isolates of *Streptococcus pneumoniae*. *Antimicrob. Agents Chemother.,* 44**,** 840-847.

Ferrer, M., Mendez-Garcia, C., Rojo, D., Barbas, C. & Moya, A. 2017. Antibiotic use and microbiome function. *Biochem. Pharmacol.,* 134**,** 114-126.

Fitzpatrick, D. & Walsh, F. 2016. Antibiotic resistance genes across a wide variety of metagenomes. *FEMS Microbiol. Ecol.,* 92**,** fiv168.

Fluit, A. & Schmitz, F. 1999. Class 1 integrons, gene cassettes, mobility, and epidemiology. *Eur. J. Clin. Microbiol. Infect. Dis.,* 18**,** 761-770.

Foditsch, C., Pereira, R. V. V., Ganda, E. K., Gomez, M. S., Marques, E. C., Santin, T. & Bicalho, R. C. 2015. Oral administration of *Faecalibacterium prausnitzii* decreased the incidence of severe diarrhea and related mortality rate and increased weight gain in preweaned dairy heifers. *PLoS One,* 10**,** e0145485.

Forsberg, K. J., Patel, S., Gibson, M. K., Lauber, C. L., Knight, R., Fierer, N. & Dantas, G. 2014. Bacterial phylogeny structures soil resistomes across habitats. *Nature,* 509**,** 612-616.

Forslund, K., Sunagawa, S., Coelho, L. P. & Bork, P. 2014. Metagenomic insights into the human gut resistome and the forces that shape it. *Bioessays,* 36**,** 316-329.

Forslund, K., Sunagawa, S., Kultima, J. R., Mende, D. R., Arumugam, M., Typas, A. & Bork, P. 2013. Country-specific antibiotic use practices impact the human gut resistome. *Genome Res.,* 23**,** 1163-1169.

Frank, D. N., Amand, A. L. S., Feldman, R. A., Boedeker, E. C., Harpaz, N. & Pace, N. R. 2007. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proc. Natl. Acad. Sci. USA,* 104**,** 13780-13785.

Fuentes, S., Van Nood, E., Tims, S., Heikamp-de Jong, I., Ter Braak, C. J., Keller, J. J., Zoetendal, E. G. & De Vos, W. M. 2014. Reset of a critically disturbed microbial

ecosystem: faecal transplant in recurrent *Clostridium difficile* infection. *ISME J,* 8**,** 1621-1633.

Gadde, U. D., Oh, S., Lillehoj, H. S. & Lillehoj, E. P. 2018. Antibiotic growth promoters virginiamycin and bacitracin methylene disalicylate alter the chicken intestinal metabolome. *Sci. Rep.,* 8**,** 3592.

Galán, J.-C., González-Candelas, F., Rolain, J.-M. & Cantón, R. 2013. Antibiotics as selectors and accelerators of diversity in the mechanisms of resistance: from the resistome to genetic plasticity in the β-lactamases world. *Front. Microbiol.,* 4**,** 9.

Garcia, N., Gutierrez, G., Lorenzo, M., Garcia, J. E., Piriz, S. & Quesada, A. 2008. Genetic determinants for *cfx*A expression in Bacteroides strains isolated from human infections. *J. Antimicrob. Chemother.,* 62**,** 942-947.

Ghanbari, M., Klose, V., Crispie, F. & Cotter, P. D. 2019. The dynamics of the antibiotic resistome in the feces of freshly weaned pigs following therapeutic administration of oxytetracycline. *Sci. Rep.,* 9**,** 4062.

Gibson, M. K., Forsberg, K. J. & Dantas, G. 2014. Improved annotation of antibiotic resistance determinants reveals microbial resistomes cluster by ecology. *ISME J,* 9**,** 207-216.

Gibson, M. K., Wang, B., Ahmadi, S., Burnham, C.-A. D., Tarr, P. I., Warner, B. B. & Dantas, G. 2016. Developmental dynamics of the preterm infant gut microbiota and antibiotic resistome. *Nat Microbiol,* 1**,** 16024.

Gillings, M. R., Gaze, W. H., Pruden, A., Smalla, K., Tiedje, J. M. & Zhu, Y.-G. 2015. Using the class 1 integron-integrase gene as a proxy for anthropogenic pollution. *ISME J,* 9**,** 1269.

Giovanetti, E., Brenciani, A., Lupidi, R., Roberts, M. C. & Varaldo, P. E. 2003. Presence of the *tet*(O) gene in erythromycin-and tetracycline-resistant strains of *Streptococcus pyogenes* and linkage with either the *mef*(A) or the *erm*(A) gene. *Antimicrob. Agents Chemother.,* 47**,** 2844-2849.

Goldstein, C., Lee, M. D., Sanchez, S., Hudson, C., Phillips, B., Register, B., Grady, M., Liebert, C., Summers, A. O. & White, D. G. 2001. Incidence of class 1 and 2 integrases in clinical and commensal bacteria from livestock, companion animals, and exotics. *Antimicrob. Agents Chemother.,* 45**,** 723-726.

Gomez, D., Arroyo, L., Costa, M., Viel, L. & Weese, J. 2017. Characterization of the fecal bacterial microbiota of healthy and diarrheic dairy calves. *J. Vet. Intern. Med.,* 31**,** 928-939.

Gómez-Gómez, C., Blanco-Picazo, P., Brown-Jaque, M., Quirós, P., Rodríguez-Rubio, L., Cerdà-Cuellar, M. & Muniesa, M. 2019. Infectious phage particles packaging

antibiotic resistance genes found in meat products and chicken feces. *Sci. Rep.,* 9**,** 13281.

Graessler, J., Qin, Y., Zhong, H., Zhang, J., Licinio, J., Wong, M.-L., Xu, A., Chavakis, T., Bornstein, A. & Ehrhart-Bornstein, M. 2013. Metagenomic sequencing of the human gut microbiome before and after bariatric surgery in obese patients with type 2 diabetes: correlation with inflammatory and metabolic parameters. *Pharmacogenomics J,* 13**,** 514-522.

Gu, Z., Eils, R. & Schlesner, M. 2016. Complex heatmaps reveal patterns and correlations in multidimensional genomic data. *Bioinformatics,* 32**,** 2847-2849.

Guerra, B., Soto, S. M., Argüelles, J. M. & Mendoza, M. C. 2001. Multidrug resistance is mediated by large plasmids carrying a class 1 integron in the emergent *Salmonella enterica* serotype [4, 5, 12: i:−]. *Antimicrob. Agents Chemother.,* 45**,** 1305-1308.

Guilloteau, P., Zabielski, R. & Blum, J. W. 2009. Gastrointestinal tract and digestion in the young ruminant: ontogenesis, adaptations, consequences and manipulations. *J. Physiol. Pharmacol.,* 60 Suppl 3**,** 37-46.

Gullberg, E., Albrecht, L. M., Karlsson, C., Sandegren, L. & Andersson, D. I. 2014. Selection of a multidrug resistance plasmid by sublethal levels of antibiotics and heavy metals. *MBio,* 5**,** e01918-14.

Guo, X., Stedtfeld, R. D., Hedman, H., Eisenberg, J. N., Trueba, G., Yin, D., Tiedje, J. M. & Zhang, L. 2018. Antibiotic resistome associated with small-scale poultry production in rural Ecuador. *Environ. Sci. Technol.,* 52**,** 8165-8172.

Gupta, A., Vlamakis, H., Shoemaker, N. & Salyers, A. A. 2003. A new *Bacteroides* conjugative transposon that carries an *ermB* gene. *Appl. Environ. Microbiol.,* 69**,** 6455-6463.

Gupta, S. K., Padmanabhan, B. R., Diene, S. M., Lopez-Rojas, R., Kempf, M., Landraud, L. & Rolain, J. M. 2014. ARG-ANNOT, a new bioinformatic tool to discover antibiotic resistance genes in bacterial genomes. *Antimicrob. Agents Chemother.,* 58**,** 212-20.

Hamilton, H. L. & Dillard, J. P. 2006. Natural transformation of *Neisseria gonorrhoeae*: from DNA donation to homologous recombination. *Mol. Microbiol.,* 59**,** 376-385.

Hammerum, A. M. & Heuer, O. E. 2009. Human health hazards from antimicrobial-resistant *Escherichia coli* of animal origin. *Clin. Infect. Dis.,* 48**,** 916-21.

Hao, X., Lüthje, F. L., Qin, Y., McDevitt, S. F., Lutay, N., Hobman, J. L., Asiani, K., Soncini, F. C., German, N. & Zhang, S. 2015. Survival in amoeba—a major selection pressure on the presence of bacterial copper and zinc resistance determinants?

Identification of a “copper pathogenicity island”. *Appl. Microbiol. Biotechnol.,* 99**,** 5817-5824.

Hardwick, S. A., Stokes, H., Findlay, S., Taylor, M. & Gillings, M. R. 2008. Quantification of class 1 integron abundance in natural environments using real-time quantitative PCR. *FEMS Microbiol. Lett.,* 278**,** 207-212.

Harrell Jr, F. E. 2019. Hmisc: harrell miscellaneous [Online]. Available: URL https://CRAN.R-project.org/package=Hmisc [Accessed 1/2/2019].

Hasegawa, H., Suzuki, E. & Maeda, S. 2018. Horizontal plasmid transfer by transformation in *Escherichia coli*: environmental factors and possible mechanisms. *Front. Microbiol.,* 9**,** 2365.

He, L. Y., Liu, Y. S., Su, H. C., Zhao, J. L., Liu, S. S., Chen, J., Liu, W. R. & Ying, G. G. 2014. Dissemination of antibiotic resistance genes in representative broiler feedlots environments: identification of indicator ARGs and correlations with environmental variables. *Environ. Sci. Technol.,* 48**,** 13120-13129.

Hernández, E., Bargiela, R., Diez, M. S., Friedrichs, A., Pérez-Cobas, A. E., Gosalbes, M. J., Knecht, H., Martínez-Martínez, M., Seifert, J. & Von Bergen, M. 2013. Functional consequences of microbial shifts in the human gastrointestinal tract linked to antibiotic treatment and obesity. *Gut Microbes,* 4**,** 306-315.

Hervé, M. 2019. RVAideMemoire: testing and plotting procedures for biostatistics [Online]. Available: URL https://CRAN.R-project.org/package=RVAideMemoire [Accessed 1/2/2019].

Heuer, H., Schmitt, H. & Smalla, K. 2011. Antibiotic resistance gene spread due to manure application on agricultural fields. *Curr. Opin. Microbiol.,* 14**,** 236-243.

Hildebrand, F., Nguyen, T. L. A., Brinkman, B., Yunta, R. G., Cauwe, B., Vandenabeele, P., Liston, A. & Raes, J. 2013. Inflammation-associated enterotypes, host genotype, cage and inter-individual effects drive gut microbiota variation in common laboratory mice. *Genome Biol.,* 14**,** R4.

Hobman, J. L. & Crossman, L. C. 2015. Bacterial antimicrobial metal ion resistance. *J. Med. Microbiol.,* 64**,** 471-497.

Hochhut, B., Lotfi, Y., Mazel, D., Faruque, S. M., Woodgate, R. & Waldor, M. K. 2001. Molecular analysis of antibiotic resistance gene clusters in *Vibrio cholerae* O139 and O1 SXT constins. *Antimicrob. Agents Chemother.,* 45**,** 2991-3000.

Holman, D. B. & Chénier, M. R. 2014. Temporal changes and the effect of subtherapeutic concentrations of antibiotics in the gut microbiota of swine. *FEMS Microbiol. Ecol.,* 90**,** 599-608.

Hölzel, C. S., Müller, C., Harms, K. S., Mikolajewski, S., Schäfer, S., Schwaiger, K. & Bauer, J. 2012. Heavy metals in liquid pig manure in light of bacterial antimicrobial resistance. *Environ. Res.,* 113**,** 21-27.

Hsu, J.-T., Chen, C.-Y., Young, C.-W., Chao, W.-L., Li, M.-H., Liu, Y.-H., Lin, C.-M. & Ying, C. 2014. Prevalence of sulfonamide-resistant bacteria, resistance genes and integron-associated horizontal gene transfer in natural water bodies and soils adjacent to a swine feedlot in northern Taiwan. *J. Hazard. Mater.,* 277**,** 34-43.

Hu, Y., Yang, X., Li, J., Lv, N., Liu, F., Wu, J., Lin, I. Y., Wu, N., Weimer, B. C. & Gao, G. F. 2016. The bacterial mobile resistome transfer network connecting the animal and human microbiomes. *Appl. Environ. Microbiol.,* 82**,** 6672-6681.

Hu, Y., Yang, X., Qin, J., Lu, N., Cheng, G., Wu, N., Pan, Y., Li, J., Zhu, L., Wang, X., Meng, Z., Zhao, F., Liu, D., Ma, J., Qin, N., Xiang, C., Xiao, Y., Li, L., Yang, H., Wang, J., Yang, R., Gao, G. F., Wang, J. & Zhu, B. 2013. Metagenome-wide analysis of antibiotic resistance genes in a large cohort of human gut microbiota. *Nat. Commun.,* 4**,** 2151.

Husso, A., Jalanka, J., Alipour, M. J., Huhti, P., Kareskoski, M., Pessa-Morikawa, T., Iivanainen, A. & Niku, M. 2020. The composition of the perinatal intestinal microbiota in horse. *Sci. Rep.,* 10**,** 441.

Ingram, P. R., Rogers, B. A., Sidjabat, H. E., Gibson, J. S. & Inglis, T. J. J. 2013. Co-selection may explain high rates of ciprofloxacin non-susceptible *Escherichia coli* from retail poultry reared without prior fluoroquinolone exposure. *J. Med. Microbiol.,* 62**,** 1743-1746.

Izzo, M., Mohler, V. & House, J. 2011. Antimicrobial susceptibility of Salmonella isolates recovered from calves with diarrhoea in Australia. *Aust. Vet. J.,* 89**,** 402-8.

Jacoby, G. A. 2009. AmpC beta-lactamases. *Clin. Microbiol. Rev.,* 22**,** 161-182.

Jakobsson, H. E., Jernberg, C., Andersson, A. F., Sjölund-Karlsson, M., Jansson, J. K. & Engstrand, L. 2010. Short-term antibiotic treatment has differing long-term impacts on the human throat and gut microbiome. *PLoS One,* 5**,** e9836.

Jami, E., Israel, A., Kotser, A. & Mizrahi, I. 2013. Exploring the bovine rumen bacterial community from birth to adulthood. *ISME J,* 7**,** 1069-1079.

Janczyk, P., Pieper, R., Souffrant, W. B., Bimczok, D., Rothkötter, H.-J. & Smidt, H. 2007. Parenteral long-acting amoxicillin reduces intestinal bacterial community diversity in piglets even 5 weeks after the administration. *ISME J,* 1**,** 180-183.

Janda, J. M. & Abbott, S. L. 2010. The genus *Aeromonas*: taxonomy, pathogenicity, and infection. *Clin. Microbiol. Rev.,* 23**,** 35-73.

Jeffery, I. B., O'Toole, P. W., Öhman, L., Claesson, M. J., Deane, J., Quigley, E. M. M. & Simrén, M. 2012. An irritable bowel syndrome subtype defined by species-specific alterations in faecal microbiota. *Gut,* 61**,** 997-1006.

Jernberg, C., Löfmark, S., Edlund, C. & Jansson, J. K. 2007. Long-term ecological impacts of antibiotic administration on the human intestinal microbiota. *ISME J,* 1**,** 56-66.

Jernberg, C., Löfmark, S., Edlund, C. & Jansson, J. K. 2010. Long-term impacts of antibiotic exposure on the human intestinal microbiota. *Microbiology,* 156**,** 3216-3223.

JETACAR. 1999. The use of antibiotics in food-producing animals: antibiotic resistant bacteria in animals and humans. *The Joint Expert Technical Advisory Commettee on Antibiotic Resistance*. Available: https://www1.health.gov.au/internet/main/publishing.nsf/Content/health-pubs-jetacar-cnt.htm/$FILE/jetacar.pdf [Accessed 1/1/2017].

Ji, X., Shen, Q., Liu, F., Ma, J., Xu, G., Wang, Y. & Wu, M. 2012. Antibiotic resistance gene abundances associated with antibiotics and heavy metals in animal manures and agricultural soils adjacent to feedlots in Shanghai; China. *J. Hazard. Mater.,* 235–236**,** 178-185.

Jia, B., Raphenya, A. R., Alcock, B., Waglechner, N., Guo, P., Tsang, K. K., Lago, B. A., Dave, B. M., Pereira, S. & Sharma, A. N. 2017. CARD 2017: expansion and model-centric curation of the comprehensive antibiotic resistance database. *Nucleic Acids Res.,* 45**,** D566–D573.

Jiang, Y., Yu, D., Wei, Z., Shen, P., Zhou, Z. & Yu, Y. 2010. Complete nucleotide sequence of *Klebsiella pneumoniae* multidrug resistance plasmid pKP048, carrying *bla*KPC-2, *bla*DHA-1, *qnr*B4, and *arm*A. *Antimicrob. Agents Chemother.,* 54**,** 3967-3969.

Johnson, T. A., Stedtfeld, R. D., Wang, Q., Cole, J. R., Hashsham, S. A., Looft, T., Zhu, Y.-G. & Tiedje, J. M. 2016. Clusters of antibiotic resistance genes enriched together stay together in swine agriculture. *mBio,* 7**,** e02214-15.

Jongman, E. & Butler, K. 2013. Ease of moving young calves at different ages. *Aust. Vet. J.,* 91**,** 94-98.

Jordan, D., Chin, J. C., Fahy, V., Barton, M., Smith, M. & Trott, D. 2009. Antimicrobial use in the Australian pig industry: results of a national survey. *Aust. Vet. J.,* 87**,** 222-229.

Joyce, A., McCarthy, C. G. P., Murphy, S. & Walsh, F. 2019. Antibiotic resistomes of healthy pig faecal metagenomes. *Microbial Genomics,* 5**,** e000272.

Kaiser-Thom, S., Hilty, M. & Gerber, V. 2020. Effects of hypersensitivity disorders and environmental factors on the equine intestinal microbiota. *Vet. Q.,* 40**,** 97-107.

Kamathewatta, K. I., Bushell, R. N., Young, N. D., Stevenson, M. A., Billman-Jacobe, H., Browning, G. F. & Marenda, M. S. 2019. Exploration of antibiotic resistance risks in a veterinary teaching hospital with Oxford Nanopore long read sequencing. *PLoS One,* 14**,** e0217600.

Keen, E. C., Bliskovsky, V. V., Malagon, F., Baker, J. D., Prince, J. S., Klaus, J. S. & Adhya, S. L. 2017. Novel "Superspreader" Bacteriophages Promote Horizontal Gene Transfer by Transformation. *mBio,* 8**,** e02115-16.

Kent, J. & Ewbank, R. 1986. The effect of road transportation on the blood constituents and behaviour of calves. II. One to three weeks old. *Br. Vet. J.,* 142**,** 131-140.

Khanna, S. & Pardi, D. S. 2016. Clinical implications of antibiotic impact on gastrointestinal microbiota and *Clostridium difficile* infection. *Expert Rev. Gastroenterol. Hepatol.,* 10**,** 1145-1152.

Khoruts, A., Dicksved, J., Jansson, J. K. & Sadowsky, M. J. 2010. Changes in the composition of the human fecal microbiome after bacteriotherapy for recurrent *Clostridium difficile*-associated diarrhea. *J. Clin. Gastroenterol.,* 44**,** 354-360.

Kidsley, A. K., Abraham, S., Bell, J. M., O'Dea, M., Laird, T. J., Jordan, D., Mitchell, P., McDevitt, C. A. & Trott, D. J. 2018. Antimicrobial susceptibility of *Escherichia coli* and *Salmonella* spp. isolates from healthy pigs in Australia: results of a pilot national survey. *Front. Microbiol.,* 9**,** 1207.

Klare, I., Heier, H., Claus, H., Böhme, G., Marin, S., Seltmann, G., Hakenbeck, R., Antanassova, V. & Witte, W. 1995. *Enterococcus faecium* strains with *van*A-mediated high-level glycopeptide resistance isolated from animal foodstuffs and fecal samples of humans in the community. *Microb. Drug Resist.,* 1**,** 265-272.

Klein-Jöbstl, D., Schornsteiner, E., Mann, E., Wagner, M., Drillich, M. & Schmitz-Esser, S. 2014. Pyrosequencing reveals diverse fecal microbiota in Simmental calves during early development. *Front. Microbiol.,* 5**,** 622.

Koliada, A., Syzenko, G., Moseiko, V., Budovska, L., Puchkov, K., Perederiy, V., Gavalko, Y., Dorofeyev, A., Romanenko, M., Tkach, S., Sineok, L., Lushchak, O. & Vaiserman, A. 2017. Association between body mass index and *Firmicutes*/*Bacteroidetes* ratio in an adult Ukrainian population. *BMC Microbiol.,* 17**,** 120.

Kudo, H., Cheng, K.-J. & Costerton, J. 1987. Interactions between *Treponema bryantii* and cellulolytic bacteria in the *in vitro* degradation of straw cellulose. *Can. J. Microbiol.,* 33**,** 244-248.

Landers, T. F., Cohen, B., Wittum, T. E. & Larson, E. L. 2012. A review of antibiotic use in food animals: perspective, policy, and potential. *Public Health Rep.,* 127**,** 4-22.

Lau, J. T., Whelan, F. J., Herath, I., Lee, C. H., Collins, S. M., Bercik, P. & Surette, M. G. 2016. Capturing the diversity of the human gut microbiota through culture-enriched molecular profiling. *Genome Med.,* 8**,** 72.

Le Sciellour, M., Zemb, O., Hochu, I., Riquet, J., Gilbert, H., Giorgi, M., Billon, Y., Gourdine, J. L. & Renaudeau, D. 2019. Effect of chronic and acute heat challenges on fecal microbiota composition, production, and thermoregulation traits in growing pigs. *J. Anim. Sci.,* 97**,** 3845-3858.

Leng, Z., Riley, D., Berger, R., Krieger, J. & Roberts, M. 1997. Distribution and mobility of the tetracycline resistance determinant *tet*Q. *J. Antimicrob. Chemother.,* 40**,** 551-559.

Lerminiaux, N. A. & Cameron, A. D. S. 2019. Horizontal transfer of antibiotic resistance genes in clinical environments. *Can. J. Microbiol.,* 65**,** 34-44.

Lester, C. H., Frimodt-Møller, N., Sørensen, T. L., Monnet, D. L. & Hammerum, A. M. 2006. *In Vivo* transfer of the *van*A resistance gene from an *Enterococcus faecium* isolate of animal origin to an *E. faecium* isolate of human origin in the intestines of human volunteers. *Antimicrob. Agents Chemother.,* 50**,** 596-599.

Levy, S. B. & Marshall, B. 2004. Antibacterial resistance worldwide: causes, challenges and responses. *Nat. Med.,* 10**,** S122-S129.

Ley, R. E., Bäckhed, F., Turnbaugh, P., Lozupone, C. A., Knight, R. D. & Gordon, J. I. 2005. Obesity alters gut microbial ecology. *Proceedings of the National Academy of Sciences,* 102**,** 11070-11075.

Li, B., Yang, Y., Ma, L., Ju, F., Guo, F., Tiedje, J. M. & Zhang, T. 2015. Metagenomic and network analysis reveal wide distribution and co-occurrence of environmental antibiotic resistance genes. *ISME J,* 9**,** 2490-2502.

Li, D., Yang, M., Hu, J., Ren, L., Zhang, Y. & Li, K. 2008. Determination and fate of oxytetracycline and related compounds in oxytetracycline production wastewater and the receiving river. *Environmental Toxicology and Chemistry,* 27**,** 80-86.

Li, F. 2017. Metatranscriptomic profiling reveals linkages between the active rumen microbiome and feed efficiency in beef cattle. *Appl. Environ. Microbiol.,* 83**,** e00061-17.

Li, X.-Z. & Nikaido, H. 2004. Efflux-mediated drug resistance in bacteria. *Drugs,* 64**,** 159-204.

Lim, M. Y., Cho, Y. & Rho, M. 2018. Diverse distribution of resistomes in the human and environmental microbiomes. *Curr. Genomics,* 19**,** 701-711.

Lin, W., Zhang, M., Zhang, S. & Yu, X. 2016. Can chlorination co-select antibiotic-resistance genes? *Chemosphere,* 156**,** 412-419.

Liu, B. & Pop, M. 2008. ARDB—Antibiotic Resistance Genes Database. *Nucleic Acids Res.,* 37**,** D443-D447.

Liu, L., Su, J.-Q., Guo, Y., Wilkinson, D. M., Liu, Z., Zhu, Y.-G. & Yang, J. 2018. Large-scale biogeographical patterns of bacterial antibiotic resistome in the waterbodies of China. *Environ. Int.,* 117**,** 292-299.

Liu, Y., Dyall-Smith, M., Marenda, M., Hu, H. W., Browning, G. & Billman-Jacobe, H. 2020. Antibiotic resistance genes in antibiotic-free chicken farms. *Antibiotics,* 9**,** 120.

Loftie-Eaton, W. & Rawlings, D. E. 2012. Diversity, biology and evolution of IncQ-family plasmids. *Plasmid,* 67**,** 15-34.

Looft, T., Allen, H. K., Casey, T. A., Alt, D. P. & Stanton, T. B. 2014. Carbadox has both temporary and lasting effects on the swine gut microbiota. *Front. Microbiol.,* 5.

Looft, T., Johnson, T. A., Allen, H. K., Bayles, D. O., Alt, D. P., Stedtfeld, R. D., Sul, W. J., Stedtfeld, T. M., Chai, B. & Cole, J. R. 2012. In-feed antibiotic effects on the swine intestinal microbiome. *Proc. Natl. Acad. Sci. USA,* 109**,** 1691-1696.

Lozupone, C. A., Stombaugh, J. I., Gordon, J. I., Jansson, J. K. & Knight, R. 2012. Diversity, stability and resilience of the human gut microbiota. *Nature,* 489**,** 220.

Ma, L., Xia, Y., Li, B., Yang, Y., Li, L. G., Tiedje, J. M. & Zhang, T. 2016. Metagenomic assembly reveals hosts of antibiotic resistance genes and the shared resistome in pig, chicken, and human feces. *Environ. Sci. Technol.,* 50**,** 420-427.

Ma, T., O'Hara, E., Song, Y., Fischer, A. J., He, Z., Steele, M. A. & Guan, L. L. 2019. Altered mucosa-associated microbiota in the ileum and colon of neonatal calves in response to delayed first colostrum feeding. *J. Dairy Sci.,* 102**,** 7073-7086.

Mach, N., Foury, A., Kittelmann, S., Reigner, F., Moroldo, M., Ballester, M., Esquerré, D., Rivière, J., Sallé, G., Gérard, P., Moisan, M.-P. & Lansade, L. 2017. The effects of weaning methods on gut microbiota composition and horse physiology. *Front. Physiol.,* 8**,** 535.

Mackenzie, A., Drennan, M., Rowan, T., Dixon, J. & Carter, S. 1997. Effect of transportation and weaning on humoral immune responses of calves. *Res. Vet. Sci.,* 63**,** 227-230.

Malmuthuge, N., Griebel, P. J. & Guan, L. L. 2015. The gut microbiome and its potential role in the development and function of newborn calf gastrointestinal tract. *Front. Vet. Sci.,* 2**,** 36.

Mandal, S., Van Treuren, W., White, R. A., Eggesbø, M., Knight, R. & Peddada, S. D. 2015. Analysis of composition of microbiomes: a novel method for studying microbial composition. *Microb. Ecol. Health Dis.,* 26**,** 27663.

Mantilla-Calderon, D., Plewa, M. J., Michoud, G., Fodelianakis, S., Daffonchio, D. & Hong, P.-Y. 2019. Water disinfection byproducts increase natural transformation rates of environmental DNA in *Acinetobacter baylyi* ADP1. *Environ. Sci. Technol.,* 53**,** 6520-6528.

Mao, D., Luo, Y., Mathieu, J., Wang, Q., Feng, L., Mu, Q., Feng, C. & Alvarez, P. 2013. Persistence of extracellular DNA in river sediment facilitates antibiotic resistance gene propagation. *Environ. Sci. Technol.,* 48**,** 71-78.

Marshall, B. M. & Levy, S. B. 2011. Food animals and antimicrobials: impacts on human health. *Clin. Microbiol. Rev.,* 24**,** 718-733.

Martínez, J. L., Coque, T. M., Lanza, V. F., de la Cruz, F. & Baquero, F. 2017. Genomic and metagenomic technologies to explore the antibiotic resistance mobilome. *Ann. N. Y. Acad. Sci.,* 1388**,** 26-41.

Mayer, M., Abenthum, A., Matthes, J., Kleeberger, D., Ege, M., Hölzel, C., Bauer, J. & Schwaiger, K. 2012. Development and genetic influence of the rectal bacterial flora of newborn calves. *Vet. Microbiol.,* 161**,** 179-185.

McArthur, A. G., Waglechner, N., Nizam, F., Yan, A., Azad, M. A., Baylay, A. J., Bhullar, K., Canova, M. J., De Pascale, G. & Ejim, L. 2013. The comprehensive antibiotic resistance database. *Antimicrob. Agents Chemother.,* 57**,** 3348-3357.

McEwen, S. A. & Fedorka-Cray, P. J. 2002. Antimicrobial use and resistance in animals. *Clin. Infect. Dis.,* 34**,** S93-S106.

McLellan, J. E., Pitcher, J. I., Ballard, S. A., Grabsch, E. A., Bell, J. M., Barton, M. & Grayson, M. L. 2018. Superbugs in the supermarket? Assessing the rate of contamination with third-generation cephalosporin-resistant gram-negative bacteria in fresh Australian pork and chicken. *Antimicrob Resist Infect Control,* 7**,** 30.

Meale, S. J., Li, S., Azevedo, P., Derakhshani, H., Plaizier, J. C., Khafipour, E. & Steele, M. A. 2016. Development of ruminal and fecal microbiomes are affected by weaning but not weaning strategy in dairy calves. *Front. Microbiol.,* 7**,** 582.

Metcalf, J. L., Song, S. J., Morton, J. T., Weiss, S., Seguin-Orlando, A., Joly, F., Feh, C., Taberlet, P., Coissac, E., Amir, A., Willerslev, E., Knight, R., McKenzie, V. & Orlando, L. 2017. Evaluating the impact of domestication and captivity on the horse gut microbiome. *Sci. Rep.,* 7**,** 15497.

Miflin, J. K., Templeton, J. M. & Blackall, P. 2007. Antibiotic resistance in *Campylobacter jejuni* and *Campylobacter coli* isolated from poultry in the South-East Queensland region. *J. Antimicrob. Chemother.,* 59**,** 775-778.

Miranda, J., Vázquez, B., Fente, C., Barros-Velázquez, J., Cepeda, A. & Franco, C. 2008. Evolution of resistance in poultry intestinal Escherichia coli during three commonly used antimicrobial therapeutic treatments in poultry. *Poult. Sci.,* 87**,** 1643-1648.

Modi, S. R., Lee, H. H., Spina, C. S. & Collins, J. J. 2013. Antibiotic treatment expands the resistance reservoir and ecological network of the phage metagenome. *Nature,* 499**,** 219-222.

Moeller, A. H., Degnan, P. H., Pusey, A. E., Wilson, M. L., Hahn, B. H. & Ochman, H. 2012. Chimpanzees and humans harbour compositionally similar gut enterotypes. *Nat. Commun.,* 3**,** 1179.

Monárrez, R., Wang, Y., Fu, Y., Liao, C.-H., Okumura, R., Braun, M. R., Jacoby, G. A. & Hooper, D. C. 2018. Genes and proteins involved in *qnr*S1 induction. *Antimicrob. Agents Chemother.,* 62**,** e00806-18.

Monier, J.-M., Demanèche, S., Delmont, T. O., Mathieu, A., Vogel, T. M. & Simonet, P. 2011. Metagenomic exploration of antibiotic resistance in soil. *Curr. Opin. Microbiol.,* 14**,** 229-235.

Moura, A., Soares, M., Pereira, C., Leitão, N., Henriques, I. & Correia, A. 2009. INTEGRALL: a database and search engine for integrons, integrases and gene cassettes. *Bioinformatics,* 25**,** 1096-1098.

Moura, I., Radhouani, H., Torres, C., Poeta, P. & Igrejas, G. 2010. Detection and genetic characterisation of *van*A ‐ containing *Enterococcus* strains in healthy Lusitano horses. *Equine Vet. J.,* 42**,** 181-183.

Moura, I., Torres, C., Silva, N., Somalo, S., Igrejas, G. & Poeta, P. 2013. Genomic description of antibiotic resistance in *Escherichia coli* and *Enterococci* isolates from healthy Lusitano horses. *J. Equine Vet. Sci.,* 33**,** 1057-1063.

Muller, P. Y., Janovjak, H., Miserez, A. R. & Dobbie, Z. 2002a. Erratum. *BioTechniques,* 33**,** 514.

Muller, P. Y., Janovjak, H., Miserez, A. R. & Dobbie, Z. 2002b. Processing of gene expression data generated by quantitative real-time RT-PCR. *BioTechniques,* 32**,** 1372-1379.

Munk, P., Knudsen, B. E., Lukjancenko, O., Duarte, A. S. R., Van Gompel, L., Luiken, R. E., Smit, L., Schmitt, H., Garcia, A. D. & Hansen, R. B. 2018. Abundance and diversity of the faecal resistome in slaughter pigs and broilers in nine European countries. *Nat Microbiol,* 3**,** 898-908.

Muziasari, W. I., Pitkanen, L. K., Sorum, H., Stedtfeld, R. D., Tiedje, J. M. & Virta, M. 2016. The resistome of farmed fish feces contributes to the enrichment of antibiotic resistance genes in sediments below Baltic sea fish farms. *Front. Microbiol.,* 7**,** 2137.

Nakai, H., Hagihara, M., Kato, H., Hirai, J., Nishiyama, N., Koizumi, Y., Sakanashi, D., Suematsu, H., Yamagishi, Y. & Mikamo, H. 2016. Prevalence and risk factors of infections caused by extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae*. *J. Infect. Chemother.,* 22**,** 319-326.

Naquin, A., Shrestha, A., Sherpa, M., Nathaniel, R. & Boopathy, R. 2015. Presence of antibiotic resistance genes in a sewage treatment plant in Thibodaux, Louisiana, USA. *Bioresour. Technol.,* 188**,** 79-83.

Nikolich, M. P., Hong, G., Shoemaker, N. B. & Salyers, A. A. 1994. Evidence for natural horizontal transfer of *tet*Q between bacteria that normally colonize humans and bacteria that normally colonize livestock. *Appl. Environ. Microbiol.,* 60**,** 3255-3260.

Nordgård, L., Brusetti, L., Raddadi, N., Traavik, T., Averhoff, B. & Nielsen, K. M. 2012. An investigation of horizontal transfer of feed introduced DNA to the aerobic microbiota of the gastrointestinal tract of rats. *BMC Res. Notes,* 5**,** 170.

Noyes, N. R., Yang, X., Linke, L. M., Magnuson, R. J., Cook, S. R., Zaheer, R., Yang, H., Woerner, D. R., Geornaras, I. & McArt, J. A. 2016. Characterization of the resistome in manure, soil and wastewater from dairy and beef production systems. *Sci. Rep.,* 6**,** 24645.

O'Donnell, M., Harris, H., Jeffery, I., Claesson, M., Younge, B., O'Toole, P. & Ross, R. 2013. The core faecal bacterial microbiome of Irish Thoroughbred racehorses. *Lett. Appl. Microbiol.,* 57**,** 492-501.

O'Neill, J. 2015. Antimicrobials in agriculture and the environment: reducing unnecessary use and waste. Review on antimicrobial resistance. London, England: Wellcome Trust.

Obeng, A., Rickard, H., Sexton, M., Pang, Y., Peng, H. & Barton, M. 2012a. Antimicrobial susceptibilities and resistance genes in *Campylobacter* strains isolated from poultry and pigs in Australia. *J. Appl. Microbiol.,* 113**,** 294-307.

Obeng, A. S., Rickard, H., Ndi, O., Sexton, M. & Barton, M. 2012b. Antibiotic resistance, phylogenetic grouping and virulence potential of *Escherichia coli* isolated from the faeces of intensively farmed and free range poultry. *Vet. Microbiol.,* 154**,** 305-315.

Obeng, A. S., Rickard, H., Ndi, O., Sexton, M. & Barton, M. 2013. Comparison of antimicrobial resistance patterns in enterococci from intensive and free range chickens in Australia. *Avian Pathol.,* 42**,** 45-54.

Office of Health. 2018. Importance ratings and summary of antibacterial uses in human and animal health in Australia, version 1.0. *Australian Government*. Available: https://www.amr.gov.au/resources/importance-ratings-and-summary-antibacterial-uses-human-and-animal-health-australia [Accessed 1/1/2019].

Oikonomou, G., Teixeira, A. G. V., Foditsch, C., Bicalho, M. L., Machado, V. S. & Bicalho, R. C. 2013. Fecal microbial diversity in pre-weaned dairy calves as described by pyrosequencing of metagenomic 16S rDNA. Associations of *Faecalibacterium* species with health and growth. *PLoS One,* 8**,** e63157.

Okitsu, N., Kaieda, S., Yano, H., Nakano, R., Hosaka, Y., Okamoto, R., Kobayashi, T. & Inoue, M. 2005. Characterization of *erm*B gene transposition by Tn1545 and Tn917 in macrolide-resistant *Streptococcus pneumoniae* isolates. *J. Clin. Microbiol.,* 43**,** 168-173.

Oksanen, J., Blanchet, F., Friendly, M., Kindt, R., Legendre, P. & McGlinn, D. 2019. vegan: community ecology package [Online]. Available: URL https://CRAN.R-project.org/package=vegan [Accessed 1/2/2019].

Okubo, T., Ae, R., Noda, J., Iizuka, Y., Usui, M. & Tamura, Y. 2019. Detection of the *sul*2–*str*A–*str*B gene cluster in an ice core from Dome Fuji Station, East Antarctica. *J Glob Antimicrob Resist,* 17**,** 72-78.

Overbeek, R., Olson, R., Pusch, G. D., Olsen, G. J., Davis, J. J., Disz, T., Edwards, R. A., Gerdes, S., Parrello, B., Shukla, M., Vonstein, V., Wattam, A. R., Xia, F. & Stevens, R. 2014. The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). *Nucleic Acids Res.,* 42**,** D206-14.

Pal, C., Bengtsson-Palme, J., Kristiansson, E. & Larsson, D. J. 2016. The structure and diversity of human, animal and environmental resistomes. *Microbiome,* 4**,** 54.

Palmer, K. L., Kos, V. N. & Gilmore, M. S. 2010. Horizontal gene transfer and the genomics of enterococcal antibiotic resistance. *Curr. Opin. Microbiol.,* 13**,** 632-639.

Palmieri, C., Mingoia, M., Massidda, O., Giovanetti, E. & Varaldo, P. E. 2012. *Streptococcus pneumoniae* transposon Tn1545/Tn6003 changes to Tn6002 due to spontaneous excision in circular form of the *erm*(B)-and *aph*A3-containing macrolide-aminoglycoside-streptothricin (MAS) element. *Antimicrob. Agents Chemother.,* 56**,** 5994-5997.

Pärnänen, K., Karkman, A., Hultman, J., Lyra, C., Bengtsson-Palme, J., Larsson, D. G. J., Rautava, S., Isolauri, E., Salminen, S., Kumar, H., Satokari, R. & Virta, M. 2018. Maternal gut and breast milk microbiota affect infant gut antibiotic resistome and mobile genetic elements. *Nat. Commun.,* 9**,** 3891.

Partridge, S. R. 2011. Analysis of antibiotic resistance regions in Gram-negative bacteria. *FEMS Microbiol. Rev.,* 35**,** 820-855.

Partridge, S. R., Kwong, S. M., Firth, N. & Jensen, S. O. 2018. Mobile genetic elements associated with antimicrobial resistance. *Clin. Microbiol. Rev.,* 31**,** e00088-17.

Partridge, S. R., Tsafnat, G., Coiera, E. & Iredell, J. R. 2009. Gene cassettes and cassette arrays in mobile resistance integrons. *FEMS Microbiol. Rev.,* 33**,** 757-784.

Patterson, A. J., Colangeli, R., Spigaglia, P. & Scott, K. P. 2007. Distribution of specific tetracycline and erythromycin resistance genes in environmental samples assessed by macroarray detection. *Environ. Microbiol.,* 9**,** 703-715.

Pérez-Cobas, A. E., Gosalbes, M. J., Friedrichs, A., Knecht, H., Artacho, A., Eismann, K., Otto, W., Rojo, D., Bargiela, R. & von Bergen, M. 2013. Gut microbiota disturbance during antibiotic therapy: a multi-omic approach. *Gut,* 62**,** 1591-1601.

Perry, J. & Wright, G. 2013. The antibiotic resistance "mobilome": searching for the link between environment and clinic. *Front. Microbiol.,* 4**,** 138.

Pesesky, M. W., Hussain, T., Wallace, M., Patel, S., Andleeb, S., Burnham, C.-A. D. & Dantas, G. 2016. Evaluation of machine learning and rules-based approaches for predicting antimicrobial resistance profiles in Gram-negative bacilli from whole genome sequence data. *Front. Microbiol.,* 7**,** 1887.

Petrovska, L., Mather, A. E., AbuOun, M., Branchu, P., Harris, S. R., Connor, T., Hopkins, K., Underwood, A., Lettini, A. A. & Page, A. 2016. Microevolution of monophasic *Salmonella Typhimurium* during epidemic, United Kingdom, 2005–2010. *Emerg. Infect. Dis.,* 22**,** 617-624.

Piddock, L. J. 2006. Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. *Clin. Microbiol. Rev.,* 19**,** 382-402.

Pinard, R., de Winter, A., Sarkis, G. J., Gerstein, M. B., Tartaro, K. R., Plant, R. N., Egholm, M., Rothberg, J. M. & Leamon, J. H. 2006. Assessment of whole genome amplification-induced bias through high-throughput, massively parallel whole genome sequencing. *BMC Genomics,* 7**,** 216.

Plöger, S., Stumpff, F., Penner, G. B., Schulzke, J.-D., Gäbel, G., Martens, H., Shen, Z., Günzel, D. & Aschenbach, J. R. 2012. Microbial butyrate and its role for barrier function in the gastrointestinal tract. *Ann. N. Y. Acad. Sci.,* 1258**,** 52-59.

Popa, O. & Dagan, T. 2011. Trends and barriers to lateral gene transfer in prokaryotes. *Curr. Opin. Microbiol.,* 14**,** 615-623.

Poutanen, S. M. & Simor, A. E. 2004. *Clostridium difficile*-associated diarrhea in adults. *CMAJ,* 171**,** 51-58.

Pratt, A. & Korolik, V. 2005. Tetracycline resistance of Australian *Campylobacter jejuni* and *Campylobacter coli* isolates. *J. Antimicrob. Chemother.,* 55**,** 452-460.

Prescott, J. F. & Dowling, P. M. 2013. *Antimicrobial Therapy in Veterinary Medicine,* Oxford, UK, John Wiley & Sons.

Prudhomme, M., Attaiech, L., Sanchez, G., Martin, B. & Claverys, J.-P. 2006. Antibiotic stress induces genetic transformability in the human pathogen *Streptococcus pneumoniae*. *Science,* 313**,** 89-92.

Qian, X., Gu, J., Sun, W., Wang, X.-J., Su, J.-Q. & Stedfeld, R. 2018. Diversity, abundance, and persistence of antibiotic resistance genes in various types of animal manure following industrial composting. *J. Hazard. Mater.,* 344**,** 716-722.

Qu, A., Brulc, J. M., Wilson, M. K., Law, B. F., Theoret, J. R., Joens, L. A., Konkel, M. E., Angly, F., Dinsdale, E. A. & Edwards, R. A. 2008. Comparative metagenomics reveals host specific metavirulomes and horizontal gene transfer elements in the chicken cecum microbiome. *PLoS One,* 3**,** e2945.

Quercia, S., Freccero, F., Castagnetti, C., Soverini, M., Turroni, S., Biagi, E., Rampelli, S., Lanci, A., Mariella, J. & Chinellato, E. 2018. Early colonisation and temporal dynamics of the gut microbial ecosystem in Standardbred foals. *Equine Vet. J.,* 51**,** 231-237.

Quirós, P., Colomer-Lluch, M., Martínez-Castillo, A., Miró, E., Argente, M., Jofre, J., Navarro, F. & Muniesa, M. 2014. Antibiotic resistance genes in the bacteriophage DNA fraction of human fecal samples. *Antimicrob. Agents Chemother.,* 58**,** 606-609.

R Core Team. 2020. R: A language and environment for statistical computing [Online]. R Foundation for Statistical Computing, Vienna, Austria. Available: URL https://www.R-project.org/ [Accessed 1/2/2020].

Ramayo-Caldas, Y., Mach, N., Lepage, P., Levenez, F., Denis, C., Lemonnier, G., Leplat, J. J., Billon, Y., Berri, M., Dore, J., Rogel-Gaillard, C. & Estelle, J. 2016. Phylogenetic network analysis applied to pig gut microbiota identifies an ecosystem structure linked with growth traits. *Isme J.,* 10**,** 2973-2977.

Rawlings, D. E. & Tietze, E. 2001. Comparative biology of IncQ and IncQ-like plasmids. *Microbiol. Mol. Biol. Rev.,* 65**,** 481-496.

Raymond, F., Boissinot, M., Ouameur, A. A., Deraspe, M., Plante, P. L., Kpanou, S. R., Berube, E., Huletsky, A., Roy, P. H., Ouellette, M., Bergeron, M. G. & Corbeil, J. 2019. Culture-enriched human gut microbiomes reveal core and accessory resistance genes. *Microbiome,* 7**,** 56.

Reller, L. B., Weinstein, M., Jorgensen, J. H. & Ferraro, M. J. 2009. Antimicrobial susceptibility testing: a review of general principles and contemporary practices. *Clin. Infect. Dis.,* 49**,** 1749-1755.

Rhoads, A. & Au, K. F. 2015. PacBio sequencing and its applications. *Genomics Proteomics Bioinformatics,* 13**,** 278-289.

Roberts, A. P. & Mullany, P. 2009. A modular master on the move: the Tn916 family of mobile genetic elements. *Trends Microbiol.,* 17**,** 251-258.

Roberts, A. P. & Mullany, P. 2011. Tn 916-like genetic elements: a diverse group of modular mobile elements conferring antibiotic resistance. *FEMS Microbiol. Rev.,* 35**,** 856-871.

Roberts, M. C. 2005. Update on acquired tetracycline resistance genes. *FEMS Microbiol. Lett.,* 245**,** 195-203.

Roberts, M. C. 2011. Environmental macrolide–lincosamide–streptogramin and tetracycline resistant bacteria. *Front. Microbiol.,* 2**,** 40.

Roberts, M. C., Schwarz, S. & Aarts, H. J. 2012. Erratum: Acquired antibiotic resistance genes: an overview. *Front. Microbiol.,* 3**,** 384.

Roberts, M. C., Sutcliffe, J., Courvalin, P., Jensen, L. B., Rood, J. & Seppala, H. 1999. Nomenclature for macrolide and macrolide-lincosamide-streptogramin B resistance determinants. *Antimicrob. Agents Chemother.,* 43**,** 2823-2830.

Rodriguez, C., Taminiau, B., Brévers, B., Avesani, V., Van Broeck, J., Leroux, A., Gallot, M., Bruwier, A., Amory, H. & Delmée, M. 2015. Faecal microbiota characterisation of horses using 16 rdna barcoded pyrosequencing, and carriage rate of *Clostridium difficile* at hospital admission. *BMC Microbiol.,* 15**,** 181.

Ross, J. & Topp, E. 2015. Abundance of antibiotic resistance genes in bacteriophage following soil fertilization with dairy manure or municipal biosolids, and evidence for potential transduction. *Appl. Environ. Microbiol.,* 81**,** 7905-7913.

Roy Chowdhury, P., McKinnon, J., Wyrsch, E., Hammond, J. M., Charles, I. G. & Djordjevic, S. P. 2014. Genomic interplay in bacterial communities: implications for growth promoting practices in animal husbandry. *Front. Microbiol.,* 5**,** 394.

Salem, S. E., Maddox, T. W., Berg, A., Antczak, P., Ketley, J. M., Williams, N. J. & Archer, D. C. 2018. Variation in faecal microbiota in a group of horses managed at pasture over a 12-month period. *Sci. Rep.,* 8**,** 8510.

Salyers, A. A., Shoemaker, N. B., Stevens, A. M. & Li, L.-Y. 1995. Conjugative transposons: an unusual and diverse set of integrated gene transfer elements. *Microbiol. Mol. Biol. Rev.,* 59**,** 579-590.

Santagati, M., Iannelli, F., Cascone, C., Campanile, F., Oggioni, M. R., Stefani, S. & Pozzi, G. 2003. The novel conjugative transposon Tn1207.3 carries the macrolide efflux gene *mef*(A) in *Streptococcus pyogenes*. *Microb. Drug Resist.,* 9**,** 243-247.

Santamaría, J., López, L. & Soto, C. 2011. Detection and diversity evaluation of tetracycline resistance genes in grassland-based production systems in Colombia, South America. *Front. Microbiol.,* 2**,** 252.

Schmidt, G. V., Mellerup, A., Christiansen, L. E., Ståhl, M., Olsen, J. E. & Angen, Ø. 2015. Sampling and pooling methods for capturing herd level antibiotic resistance in swine feces using qPCR and CFU approaches. *PLoS One,* 10**,** e0131672.

Schmieder, R. & Edwards, R. 2012. Insights into antibiotic resistance through metagenomic approaches. *Future Microbiol.,* 7**,** 73-89.

Scholz, P., Haring, V., Wittmann-Liebold, B., Ashman, K., Bagdasarian, M. & Scherzinger, E. 1989. Complete nucleotide sequence and gene organization of the broad-host-range plasmid RSF1010. *Gene,* 75**,** 271-288.

Schoster, A., Staempfli, H., Guardabassi, L., Jalali, M. & Weese, J. 2017. Comparison of the fecal bacterial microbiota of healthy and diarrheic foals at two and four weeks of life. *BMC Vet. Res.,* 13**,** 144.

Schwaiger, K., Storch, J., Bauer, C. & Bauer, J. 2020. Development of selected bacterial groups of the rectal microbiota of healthy calves during the first week postpartum. *J. Appl. Microbiol.,* 128**,** 366-375.

Schwarz, S., Kehrenberg, C. & Walsh, T. 2001. Use of antimicrobial agents in veterinary medicine and food animal production. *Int. J. Antimicrob. Agents,* 17**,** 431-437.

Segatore, B., Massidda, O., Satta, G., Setacci, D. & Amicosante, G. 1993. High specificity of *cph*A-encoded metallo-beta-lactamase from *Aeromonas hydrophila* AE036 for carbapenems and its contribution to beta-lactam resistance. *Antimicrob. Agents Chemother.,* 37**,** 1324-1328.

Seral, C., Castillo, F. J., Rubio-Calvo, M. C., Fenoll, A., García, C. & Gómez-Lus, R. 2001. Distribution of resistance genes *tet*(M), *aph*3′-III, *cat*pC194 and the integrase gene of Tn1545 in clinical *Streptococcus pneumoniae* harbouring *erm*(B) and *mef*(A) genes in Spain. *J. Antimicrob. Chemother.,* 47**,** 863-866.

Seville, L. A., Patterson, A. J., Scott, K. P., Mullany, P., Quail, M. A., Parkhill, J., Ready, D., Wilson, M., Spratt, D. & Roberts, A. P. 2009. Distribution of tetracycline and erythromycin resistance genes among human oral and fecal metagenomic DNA. *Microb. Drug Resist.,* 15**,** 159-166.

Shaban, R. Z., Simon, G. I., Trott, D. J., Turnidge, J. & Jordan, D. 2014. Surveillance and reporting of antimicrobial resistance and antibiotic usage in animals and agriculture in Australia, report to the Department of Agriculture. *Griffith University and The University of Adelaide*. Available: https://www.agriculture.gov.au/animal/health/amr/surveillance-antimicrobial-resistance [Accessed 1/7/2017].

Shen, A. 2012. *Clostridium difficile* toxins: mediators of inflammation. *J. Innate Immun.,* 4**,** 149-158.

Shin, N.-R., Whon, T. W. & Bae, J.-W. 2015. Proteobacteria: microbial signature of dysbiosis in gut microbiota. *Trends Biotechnol.,* 33**,** 496-503.

Shoemaker, N., Vlamakis, H., Hayes, K. & Salyers, A. 2001. Evidence for extensive resistance gene transfer among *Bacteroides* spp. and among *Bacteroides* and other genera in the human colon. *Appl. Environ. Microbiol.,* 67**,** 561-568.

Shousha, A., Awaiwanont, N., Sofka, D., Smulders, F. J., Paulsen, P., Szostak, M. P., Humphrey, T. & Hilbert, F. 2015. Bacteriophages isolated from chicken meat and the horizontal transfer of antimicrobial resistance genes. *Appl. Environ. Microbiol.,* 81**,** 4600-4606.

Sjölund, M., Tano, E., Blaser, M. J., Andersson, D. I. & Engstrand, L. 2005. Persistence of resistant *Staphylococcus epidermidis* after single course of clarithromycin. *Emerg. Infect. Dis.,* 11**,** 1389-1393.

Sjölund, M., Wreiber, K., Andersson, D. I., Blaser, M. J. & Engstrand, L. 2003. Long-term persistence of resistant *Enterococcus* species after antibiotics to eradicate *Helicobacter pylori*. *Ann. Intern. Med.,* 139**,** 483-487.

Smet, A., Rasschaert, G., Martel, A., Persoons, D., Dewulf, J., Butaye, P., Catry, B., Haesebrouck, F., Herman, L. & Heyndrickx, M. 2011. *In situ* ESBL conjugation from avian to human *Escherichia coli* during cefotaxime administration. *J. Appl. Microbiol.,* 110**,** 541-549.

Smillie, C. S., Smith, M. B., Friedman, J., Cordero, O. X., David, L. A. & Alm, E. J. 2011. Ecology drives a global network of gene exchange connecting the human microbiome. *Nature,* 480**,** 241-244.

Smith, M., Jordan, D., Gibson, J., Cobbold, R., Chapman, T., Abraham, S. & Trott, D. 2016. Phenotypic and genotypic profiling of antimicrobial resistance in enteric *Escherichia coli* communities isolated from finisher pigs in Australia. *Aust. Vet. J.,* 94**,** 371-376.

Soge, O., Tivoli, L., Meschke, J. & Roberts, M. 2009. A conjugative macrolide resistance gene, *mef*(A), in environmental *Clostridium perfringens* carrying multiple macrolide and/or tetracycline resistance genes. *J. Appl. Microbiol.,* 106**,** 34-40.

Sóki, J. 2013. Extended role for insertion sequence elements in the antibiotic resistance of *Bacteroides*. *World J Clin Infect Dis,* 3**,** 1-12.

Sommer, M. O., Dantas, G. & Church, G. M. 2009. Functional characterization of the antibiotic resistance reservoir in the human microflora. *Science,* 325**,** 1128-1131.

Sommer, M. O. A. & Dantas, G. 2011. Antibiotics and the resistant microbiome. *Curr. Opin. Microbiol.,* 14**,** 556-563.

Song, Y., Malmuthuge, N., Steele, M. A. & Guan, L. L. 2017. Shift of hindgut microbiota and microbial short chain fatty acids profiles in dairy calves from birth to pre-weaning. *FEMS Microbiol. Ecol.,* 94**,** fix179.

Stanton, T. & Canale-Parola, E. 1980. *Treponema bryantii* sp. nov., a rumen spirochete that interacts with cellulolytic bacteria. *Arch. Microbiol.,* 127**,** 145-156.

Stewart, R. D., Auffret, M. D., Warr, A., Wiser, A. H., Press, M. O., Langford, K. W., Liachko, I., Snelling, T. J., Dewhurst, R. J., Walker, A. W., Roehe, R. & Watson, M. 2018. Assembly of 913 microbial genomes from metagenomic sequencing of the cow rumen. *Nat. Commun.,* 9**,** 870.

Sui, Q., Zhang, J., Chen, M., Tong, J., Wang, R. & Wei, Y. 2016. Distribution of antibiotic resistance genes (ARGs) in anaerobic digestion and land application of swine wastewater. *Environ. Pollut.,* 213**,** 751-759.

Sultan, I., Rahman, S., Jan, A. T., Siddiqui, M. T., Mondal, A. H. & Haq, Q. M. R. 2018. Antibiotics, resistome and resistance mechanisms: A bacterial perspective. *Front. Microbiol.,* 9**,** 2066.

Sundqvist, M., Geli, P., Andersson, D. I., Sjölund-Karlsson, M., Runehagen, A., Cars, H., Abelson-Storby, K., Cars, O. & Kahlmeter, G. 2009. Little evidence for reversibility of trimethoprim resistance after a drastic reduction in trimethoprim use. *J. Antimicrob. Chemother.,* 65**,** 350-360.

Tassinari, E., Duffy, G., Bawn, M., Burgess, C. M., McCabe, E. M., Lawlor, P. G., Gardiner, G. & Kingsley, R. A. 2019. Microevolution of antimicrobial resistance and biofilm formation of *Salmonella Typhimurium* during persistence on pig farms. *Sci. Rep.,* 9**,** 8832.

Thames, C. H., Pruden, A., James, R. E., Ray, P. P. & Knowlton, K. F. 2012. Excretion of antibiotic resistance genes by dairy calves fed milk replacers with varying doses of antibiotics. *Front. Microbiol.,* 3**,** 139.

Thijs, S., Op De Beeck, M., Beckers, B., Truyens, S., Stevens, V., Van Hamme, J. D., Weyens, N. & Vangronsveld, J. 2017. Comparative evaluation of four bacteria-specific primer pairs for 16S rRNA gene surveys. *Front. Microbiol.,* 8**,** 494.

Thomas, C. M. & Nielsen, K. M. 2005. Mechanisms of, and barriers to, horizontal gene transfer between bacteria. *Nat. Rev. Microbiol.,* 3**,** 711-721.

Thomas, M., Webb, M., Ghimire, S., Blair, A., Olson, K., Fenske, G. J., Fonder, A. T., Christopher-Hennings, J., Brake, D. & Scaria, J. 2017. Metagenomic characterization of the effect of feed additives on the gut microbiome and antibiotic resistome of feedlot cattle. *Sci. Rep.,* 7**,** 12257.

Tietze, E., Tschäpe, H. & Voigt, W. 1989. Characterization of new resistance plasmids belonging to incompatibility group IncQ. *J. Basic Microbiol.,* 29**,** 695-706.

Trunkfield, H. & Broom, D. 1990. The welfare of calves during handling and transport. *Appl. Anim. Behav. Sci.,* 28**,** 135-152.

Turnbaugh, P. J., Ley, R. E., Mahowald, M. A., Magrini, V., Mardis, E. R. & Gordon, J. I. 2006. An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature,* 444**,** 1027-1031.

Uyeno, Y., Sekiguchi, Y. & Kamagata, Y. 2010. rRNA‐based analysis to monitor succession of faecal bacterial communities in Holstein calves. *Lett. Appl. Microbiol.,* 51**,** 570-577.

Vahjen, W., Pietruszyńska, D., Starke, I. C. & Zentek, J. 2015. High dietary zinc supplementation increases the occurrence of tetracycline and sulfonamide resistance genes in the intestine of weaned pigs. *Gut Pathog.,* 7**,** 23.

Van den Bogaard, A., London, N., Driessen, C. & Stobberingh, E. 2001. Antibiotic resistance of faecal *Escherichia coli* in poultry, poultry farmers and poultry slaughterers. *J. Antimicrob. Chemother.,* 47**,** 763-771.

Van Hoek, A. H., Mevius, D., Guerra, B., Mullany, P., Roberts, A. P. & Aarts, H. J. 2011. Acquired antibiotic resistance genes: an overview. *Front. Microbiol.,* 2**,** 203.

Videnska, P., Rahman, M. M., Faldynova, M., Babak, V., Matulova, M. E., Prukner-Radovcic, E., Krizek, I., Smole-Mozina, S., Kovac, J. & Szmolka, A. 2014. Characterization of egg laying hen and broiler fecal microbiota in poultry farms in Croatia, Czech Republic, Hungary and Slovenia. *PLoS One,* 9**,** e110076.

Vinué, L., Sáenz, Y., Rojo-Bezares, B., Olarte, I., Undabeitia, E., Somalo, S., Zarazaga, M. & Torres, C. 2010. Genetic environment of sul genes and characterisation of integrons in *Escherichia coli* isolates of blood origin in a Spanish hospital. *Int. J. Antimicrob. Agents,* 35**,** 492-496.

von Wintersdorff, C. J. H., Penders, J., van Niekerk, J. M., Mills, N. D., Majumder, S., van Alphen, L. B., Savelkoul, P. H. M. & Wolffs, P. F. G. 2016. Dissemination of antimicrobial resistance in microbial ecosystems through horizontal gene transfer. *Front. Microbiol.,* 7**,** 173.

Wales, A. D. & Davies, R. H. 2015. Co-selection of resistance to antibiotics, biocides and heavy metals, and its relevance to foodborne pathogens. *Antibiotics,* 4**,** 567-604.

Walker, B. J., Abeel, T., Shea, T., Priest, M., Abouelliel, A., Sakthikumar, S., Cuomo, C. A., Zeng, Q., Wortman, J. & Young, S. K. 2014. Pilon: an integrated tool for comprehensive microbial variant detection and genome assembly improvement. *PLoS One,* 9**,** e112963.

Walsh, T. R., Toleman, M. A., Poirel, L. & Nordmann, P. 2005. Metallo-β-lactamases: the quiet before the storm? *Clin. Microbiol. Rev.,* 18**,** 306-325.

Walters, W. A., Xu, Z. & Knight, R. 2014. Meta‐analyses of human gut microbes associated with obesity and IBD. *FEBS Lett.,* 588**,** 4223-4233.

Walther, B., Klein, K.-S., Barton, A.-K., Semmler, T., Huber, C., Wolf, S. A., Tedin, K., Merle, R., Mitrach, F. & Guenther, S. J. P. o. 2018. Extended-spectrum beta-lactamase (ESBL)-producing Escherichia coli and Acinetobacter baumannii among horses entering a veterinary teaching hospital: The contemporary" Trojan Horse". 13**,** e0191873.

Wang, F. H., Qiao, M., Su, J. Q., Chen, Z., Zhou, X. & Zhu, Y. G. 2014. High throughput profiling of antibiotic resistance genes in urban park soils with reclaimed water irrigation. *Environ. Sci. Technol.,* 48**,** 9079-9085.

Wang, J. J., Li, W. J., Wang, C., Wang, L. S., He, T. Y., Hu, H. Q., Song, J., Cui, C., Qiao, J. T., Qing, L., Li, L. L., Zang, N., Wang, K. W., Wu, C. L., Qi, L., Ma, A. X., Zheng, H. Z., Hou, X. G., Liu, F. Q. & Chen, L. 2020. Enterotype *Bacteroides* is associated with a high risk in patients with diabetes: a pilot study. *J. Diabetes Res.,* 2020**,** 11.

Wang, M., Liu, P., Zhou, Q., Tao, W., Sun, Y. & Zeng, Z. 2018. Estimating the contribution of bacteriophage to the dissemination of antibiotic resistance genes in pig feces. *Environ. Pollut.,* 238**,** 291-298.

Wang, R., Chen, M., Feng, F., Zhang, J., Sui, Q., Tong, J., Wei, Y. & Wei, D. 2017. Effects of chlortetracycline and copper on tetracyclines and copper resistance genes and microbial community during swine manure anaerobic digestion. *Bioresour. Technol.,* 238**,** 57-69.

Waseem, H., Jameel, S., Ali, J., Rehman, H. S. U., Tauseef, I., Farooq, U., Jamal, A. & Ali, M. I. 2019. Contributions and challenges of high throughput qPCR for determining antimicrobial resistance in the environment: a critical review. *Molecules,* 24**,** 163.

Weaver, T., Valcanis, M., Mercoulia, K., Sait, M., Tuke, J., Kiermeier, A., Hogg, G., Pointon, A., Hamilton, D. & Billman-Jacobe, H. 2017. Longitudinal study of *Salmonella* 1, 4,[5], 12: i:-shedding in five Australian pig herds. *Prev. Vet. Med.,* 136**,** 19-28.

Weese, J. & Jelinski, M. 2017. Assessment of the fecal microbiota in beef calves. *J. Vet. Intern. Med.,* 31**,** 176-185.

Wei, S., Morrison, M. & Yu, Z. 2013. Bacterial census of poultry intestinal microbiome. *Poult. Sci.,* 92**,** 671-683.

Weinroth, M. D., Scott, H. M., Norby, B., Loneragan, G. H., Noyes, N. R., Rovira, P., Doster, E., Yang, X., Woerner, D. R., Morley, P. S. & Belk, K. E. 2018. Effects of ceftiofur and chlortetracycline on the resistomes of feedlot cattle. *Appl. Environ. Microbiol.,* 84**,** e00610-18.

Werner, G., Hildebrandt, B. & Witte, W. 2001. Aminoglycoside-Streptothricin resistance gene cluster *aad*E–*sat*4–*aph*A-3 disseminated among multiresistant

isolates of *Enterococcus faecium*. *Antimicrob. Agents Chemother.,* 45**,** 3267-3269.

Werner, G., Hildebrandt, B. & Witte, W. 2003. Linkage of *erm*(B) and *aad*E-*sat*4-*aph*A-3 in multiple-resistant *Enterococcus faecium* isolates of different ecological origins. *Microb. Drug Resist.,* 9**,** 9-16.

Whittle, G., Shoemaker, N. B. & Salyers, A. A. 2002. Characterization of genes involved in modulation of conjugal transfer of the *Bacteroides* conjugative transposon CTnDOT. *J. Bacteriol.,* 184**,** 3839-3847.

Wichmann, F., Udikovic-Kolic, N., Andrew, S. & Handelsman, J. 2014. Diverse antibiotic resistance genes in dairy cow manure. *MBio,* 5**,** e01017-13.

Wick, R. R., Judd, L. M., Gorrie, C. L. & Holt, K. E. 2017. Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads. *PLoS Comput. Biol.,* 13**,** e1005595.

Wood, D. E. & Salzberg, S. L. 2014. Kraken: ultrafast metagenomic sequence classification using exact alignments. *Genome Biol.,* 15**,** R46.

Wozniak, R. A. & Waldor, M. K. 2010. Integrative and conjugative elements: mosaic mobile genetic elements enabling dynamic lateral gene flow. *Nat. Rev. Microbiol.,* 8**,** 552-563.

Wu, G. D., Chen, J., Hoffmann, C., Bittinger, K., Chen, Y.-Y., Keilbaugh, S. A., Bewtra, M., Knights, D., Walters, W. A., Knight, R., Sinha, R., Gilroy, E., Gupta, K., Baldassano, R., Nessel, L., Li, H., Bushman, F. D. & Lewis, J. D. 2011. Linking long-term dietary patterns with gut microbial enterotypes. *Science,* 334**,** 105-108.

Xiao, L., Estellé, J., Kiilerich, P., Ramayo-Caldas, Y., Xia, Z., Feng, Q., Liang, S., Pedersen, A. Ø., Kjeldsen, N. J., Liu, C., Maguin, E., Doré, J., Pons, N., Le Chatelier, E., Prifti, E., Li, J., Jia, H., Liu, X., Xu, X., Ehrlich, S. D., Madsen, L., Kristiansen, K., Rogel-Gaillard, C. & Wang, J. 2016. A reference gene catalogue of the pig gut microbiome. *Nat Microbiol,* 1**,** 16161.

Xie, W.-Y., Yang, X.-P., Li, Q., Wu, L.-H., Shen, Q.-R. & Zhao, F.-J. 2016. Changes in antibiotic concentrations and antibiotic resistome during commercial composting of animal manures. *Environ. Pollut.,* 219**,** 182-190.

Yang, H., Byelashov, O. A., Geornaras, I., Goodridge, L. D., Nightingale, K. K., Belk, K. E., Smith, G. C. & Sofos, J. N. 2010. Characterization and transferability of class 1 integrons in commensal bacteria isolated from farm and nonfarm environments. *Foodborne Pathog. Dis.,* 7**,** 1441-1451.

Yang, Z., Guo, Z., Qiu, C., Li, Y., Feng, X., Liu, Y., Zhang, Y., Pang, P., Wang, P. & Zhou, Q. 2016. Preliminary analysis showed country-specific gut resistome based on 1267 feces samples. *Gene,* 581**,** 178-182.

Yau, S., Liu, X., Djordjevic, S. P. & Hall, R. M. 2010. RSF1010-like plasmids in Australian *Salmonella enterica* serovar Typhimurium and origin of their *sul*2-*str*A-*str*B antibiotic resistance gene cluster. *Microb. Drug Resist.,* 16**,** 249-252.

Yu, Y., Lee, C., Kim, J. & Hwang, S. 2005. Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction. *Biotechnol Bioeng,* 89**,** 670-679.

Yuan, Z., Yan, W., Wen, C., Zheng, J., Yang, N. & Sun, C. 2020. Enterotype identification and its influence on regulating the duodenum metabolism in chickens. *Poult. Sci.,* 99**,** 1515-1527.

Zankari, E., Hasman, H., Cosentino, S., Vestergaard, M., Rasmussen, S., Lund, O., Aarestrup, F. M. & Larsen, M. V. 2012. Identification of acquired antimicrobial resistance genes. *J. Antimicrob. Chemother.,* 67**,** 2640-2644.

Zhou, B., Wang, C., Zhao, Q., Wang, Y., Huo, M., Wang, J. & Wang, S. 2016. Prevalence and dissemination of antibiotic resistance genes and coselection of heavy metals in Chinese dairy farms. *J. Hazard. Mater.,* 320**,** 10-17.

Zhou, W., Wang, Y. & Lin, J. 2012. Functional cloning and characterization of antibiotic resistance genes from the chicken gut microbiome. *Appl. Environ. Microbiol.,* 78**,** 3028-3032.

Zhu, Y.-G., Johnson, T. A., Su, J.-Q., Qiao, M., Guo, G.-X., Stedtfeld, R. D., Hashsham, S. A. & Tiedje, J. M. 2013. Diverse and abundant antibiotic resistance genes in Chinese swine farms. *Proc. Natl. Acad. Sci. USA,* 110**,** 3435-3440.

antibiotics

MDPI

Article

Antibiotic Resistance Genes in Antibiotic-Free Chicken Farms

Yuhong Liu [1,2], Michael Dyall-Smith [1], Marc Marenda [1], Hang-Wei Hu [3], Glenn Browning [1,2] and Helen Billman-Jacobe [1,2,*]

1 Asia-Pacific Centre for Animal Health, Department of Veterinary Biosciences, Faculty of Veterinary and Agricultural Sciences, Melbourne Veterinary School, The University of Melbourne, Parkville, VIC 3010, Australia; yuhongl@student.unimelb.edu.au (Y.L.); mike.dyallsmith@gmail.com (M.D.-S.); mmarenda@unimelb.edu.au (M.M.); glenfb@unimelb.edu.au (G.B.)

2 National Centre for Antimicrobial Stewardship, The Peter Doherty Institute, Elizabeth St Melbourne, VIC 3000, Australia

3 School of Agriculture and Food, Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Parkville, VIC 3010, Australia; hang-wei.hu@unimelb.edu.au

* Correspondence: hbj@unimelb.edu.au

Received: 30 January 2020; Accepted: 10 March 2020; Published: 13 March 2020

Abstract: Rising concern about the use of antibiotics in food production has resulted in many studies on the occurrence of antibiotic resistance genes (ARGs) in animal-associated bacterial communities. There are few baseline data on the abundance of ARGs on farms where chickens are intensively raised with little or no use of antibiotics. This study used a high-throughput quantitative PCR array to survey two antibiotic-free chicken farms for the occurrence of ARGs and mobile genetic elements known to enhance the spread of ARGs. No antibiotics had been used on the study farms for five years prior to this study. The results provide a baseline for the occurrence of resistance genes in the chicken production system without direct selective pressure.

Keywords: poultry; chicken; manure; microbiota; resistome

1. Introduction

Antibiotic usage in food animals raises concerns over the potential emergence and spread of resistant bacteria [1,2]. To limit the negative impacts, many countries have restricted or banned the use of specific antibiotics in food animals, especially those antibiotics used for nontherapeutic purposes or that are of high importance for human use [2]. In Australia, fluoroquinolones have never been approved for use in food animals, and the use of third generation cephalosporins is restricted [3]. In poultry, chlortetracycline is registered for the treatment of egg-producing chickens (layers), and several antibiotics, including amoxycillin, neomycin, lincomycin, spectinomycin and oxytetracycline, are registered for use in meat-producing chickens (broilers) [4]. Virginiamycin and zinc-bacitracin are allowed for the prevention and treatment of necrotic enteritis [4].

Previous studies of specific bacterial species isolated from Australian poultry have revealed antibiotic resistance. Some *Escherichia coli* isolates from chickens were resistant to tetracycline, ampicillin, trimethoprim-sufamethoxazole, streptomycin, spectinomycin, neomycin and florfenicol [5]. *Enterococcus* isolates have been reported with resistance to lincomycin, bacitracin, tetracycline and tylosin but no resistance to vancomycin or virginiamycin was reported [6]. *Campylobacter* isolates were found to be resistant to ampicillin, tetracycline and lincomycin, but no resistance to ciprofloxacin, gentamicin, erythromycin and tylosin was reported [4,7].

Sampling of individual animals is not always practical, especially when animals are raised on a large scale, such as caged chickens in commercial farms. In this case, environmental sampling is a

cost-effective and efficient method to collect biomass representing a large population, and this has been used for monitoring the prevalence of pathogens in chicken flocks [8,9]. High-throughput quantitative PCR (HT-qPCR) and deep sequencing approaches have been used in a variety of human, animal and environmental samples to characterize the diversity of antibiotic resistance genes (ARGs) [10–14]. These methods are efficient and effective for the broad-spectrum detection and quantification of ARGs in complex samples.

The aim of this study was to assess the presence and diversity of ARGs and mobile genetic elements (MGEs) in poultry farms that had not used antibiotics for 5 years. The WaferGen HT-qPCR system was used to detect and quantify ARGs and MGEs in environmental samples from two chicken farms. The PCR primers targeted ARGs of the major antibiotic classes, and integrases and transposase genes. Caged chicken sheds from two types of farms were selected for investigation; the first was an egg production enterprise which housed caged layer chickens (Farm L), while the second was a broiler breeder farm (Farm B). The results should provide useful baseline information regarding ARG prevalence in the chicken production system without direct selective pressure.

2. Results

2.1. ARGs and MGEs in the Caged Layer Shed in Farm L

2.1.1. Number of ARGs and MGEs

The caged layer shed on farm L was sampled twice when the chickens were 23 and 33 weeks old. Five manure belt swabs were collected from the shed each time. The biomass from two manure belt swabs in the first sampling were combined (sample W23_2 and W23_ 3) in order to have sufficient biomass for DNA extraction. The ARGs and MGEs in the DNA extracts were quantified using the WaferGen HT-qPCR system and the detection limit was set at Ct 27. Most of the ARGs were below the limits of detection. The combined number of ARGs and MGEs detected in each sample were between 80 and 89, out of the 285 ARGs tested, and either 6 or 7 out of 10 MGEs tested (Figure 1). The proportions of the classes of ARGs did not change substantially between samples.

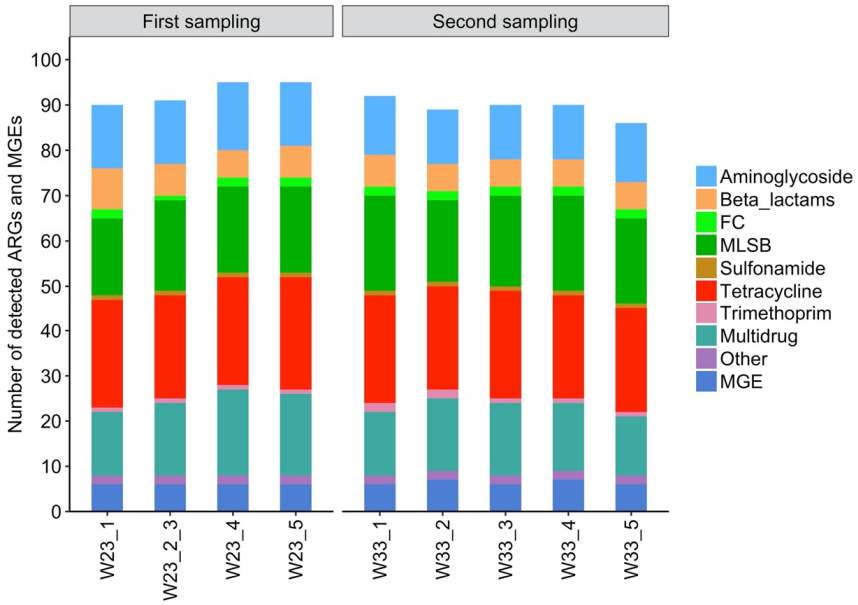

Figure 1. Number of detected antibiotic resistance genes (ARGs) and mobile genetic elements (MGEs) in each manure belt swab from the caged layer shed of each sampling visit on Farm L. The sampling was performed when the chickens were 23 weeks (W23) and 33 weeks old (W33). Sample 2 and 3 for W23 were combined to provide a sufficient biomass for processing. FC, fluoroquinolone/florfenicol/chloramphenicol; MLSB, macrolide-lincosamide-streptogramin B; MGE, mobile genetic element.

2.1.2. Abundance of ARGs and MGEs

The relative abundance of ARGs within their bacterial community was represented by their proportion relative to the 16S rRNA gene for each individual sample (Figure 2). The Ct for the 16S rRNA gene ranged between 8 and 12 and, after normalisation, the lowest relative abundance of ARG or MGE genes ranged between 10^{-6} and 10^{-5}. ARGs with relative abundances above 10^{-3} in all the nine manure belt swab samples were aminoglycoside resistance genes *aad*A, *aad*A1, *aad*A2 and *str*B, sulfonamide resistance gene *sul*2 and tetracycline resistance genes *tet*M, *tet*K and *tet*X (Table 1). The *cph*A gene was detected at high abundance (~ 10^{-1}) in one manure belt swab from the first sampling, but the gene was below the detection limit in the next sampling.

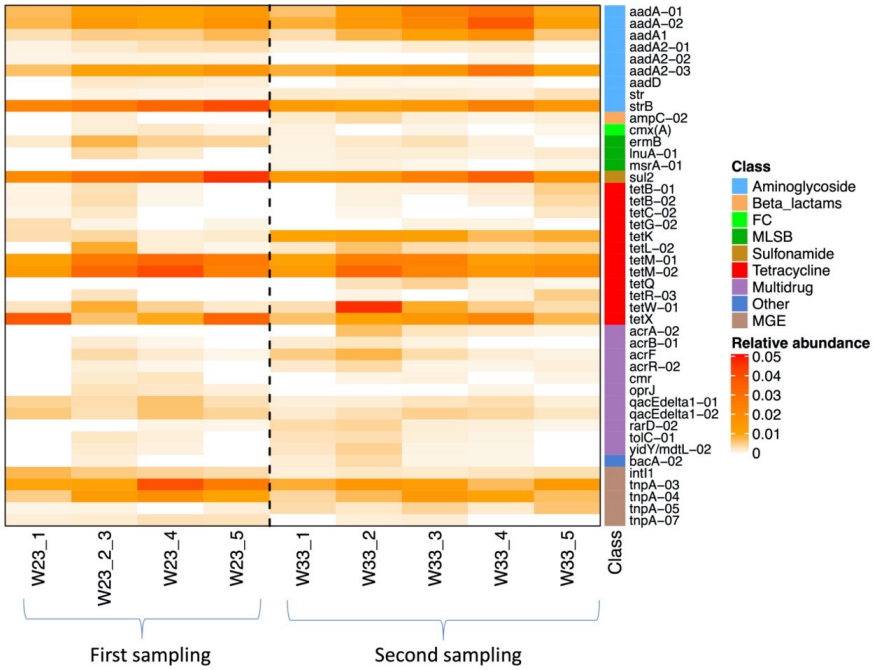

Figure 2. Heatmap of the relative abundance of ARGs and MGEs in each manure swab sample in Farm L. Only genes with a relative abundance above 10^{-3} in at least four samples are shown. FC, fluoroquinolone/florfenicol/chloramphenicol; MLSB, macrolide-lincosamide-streptogramin B; MGE, mobile genetic element.

Table 1. Most abundant ARGs in the layer Farm L.

Gene	Relative Abundance		
	Min	Max	Median
*aad*A	6.5×10^{-3}	2.9×10^{-2}	1.2×10^{-2}
*aad*A1	3.4×10^{-3}	1.9×10^{-2}	6.0×10^{-3}
*aad*A2	1.0×10^{-3}	3.7×10^{-3}	2.1×10^{-3}
*str*B	1.2×10^{-2}	4.0×10^{-2}	2.3×10^{-2}
*sul*2	1.3×10^{-2}	4.5×10^{-2}	2.5×10^{-2}
*tet*K	1.9×10^{-3}	1.3×10^{-2}	7.5×10^{-3}
*tet*M	1.1×10^{-2}	3.2×10^{-2}	2.3×10^{-2}
*tet*W	2.4×10^{-3}	4.6×10^{-2}	4.9×10^{-3}
*tet*X	6.7×10^{-3}	3.7×10^{-2}	1.1×10^{-2}

2.1.3. Microbiota Analysis of Manure Belt swabs of Farm L

A total of 316,232 reads of the four manure belt swabs from the first sampling passed the quality filter. The original read depth of all samples ranged between 68,312 and 86,951. Reads of each sample

were rarefied to 68,312, which was sufficient for the accurate analysis of microbiota richness and evenness. The rarefied reads were partitioned into 450 ASVs and assigned with taxonomic information.

Proteobacteria was the most dominant phylum in sample W23_1 and W23_5, which accounted for 80% and 49% of the total reads, respectively (Figure 3a). In sample W23_2_3 and W23_4, the most abundant phylum was *Firmicutes*, which accounted for 37% and 45% of the total reads, respectively (Figure 3a). *Actinobacteria* was the next most abundant phylum in sample W23_2_3, W23_4 and W23_5, which accounted for 25%, 30% and 19% of the total reads, respectively (Figure 3a).

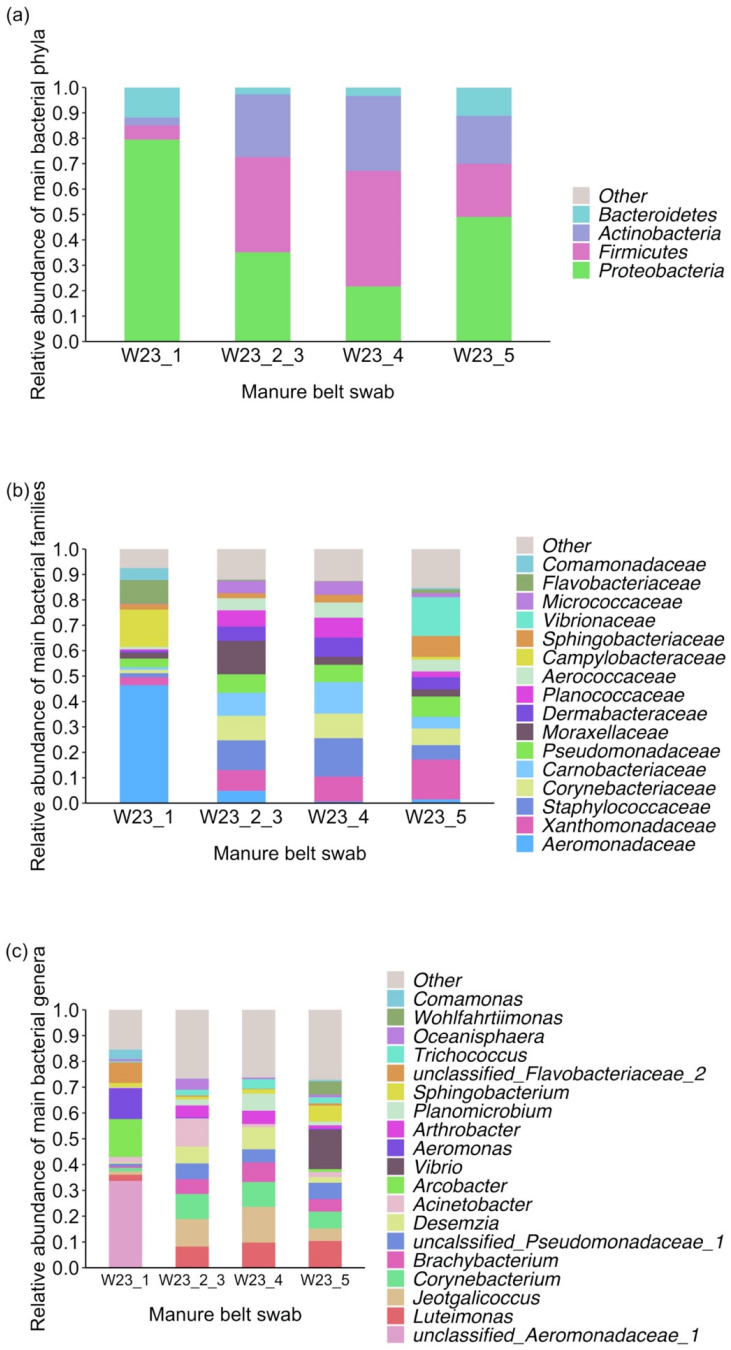

Figure 3. Relative abundance of dominant bacterial phyla (**a**), families (**b**) and genera (**c**) in the manure belt swabs of the first sampling in Farm L. Only taxa with relative abundance above 3% in at least one sample are shown. Taxa below the cut off were assigned as "other".

Proteobacteria in sample W23_1 was mainly represented by bacteria belonging to the *Aeromonadaceae* family and *Arcobacter* genus (*Campylobacteraceae* family), which accounted for 46% and 15% of the total reads, respectively (Figure 3c). In the other three samples, genus *Luteimonas*, *Jeotgalicoccus*, *Corynebacterium* and *Brachybacterium* were predominant in the microbiota, which together represented 27% to 41% of the total reads (Figure 3c). Moreover, sample W23_2_3 had a high level of *Acinetobacter* genus (11%) and sample W23_5 had a high level of *Vibrio* genus (15%) (Figure 3c).

2.2. ARGs and MGEs in the Caged Broiler Breeder Sheds in Farm B

2.2.1. Number and Abundance of ARGs and MGEs

The results of Farm L showed that the Ct of many of ARGs was above the cutoff of 27. It was not cost-effective to include all of the 295 ARGs and MGEs in further testing. Consequently, the HT-qPCR assay was reduced to 45 ARGs and four MGEs for samples from Farm B.

Three sheds of caged broiler breeder chickens were sampled on Farm B and 24 manure belt swabs were collected from each shed. Samples that failed to amplify the 16S rRNA gene were excluded in the analysis, leaving 22 swabs that were validated for sheds A and B, respectively, and 16 swabs in shed C.

Each shed was detected with 44 genes and the three sheds shared 42 genes. The relative abundance of genes detected in Farm B is shown in the heatmap (Figure 4). A total of 34 genes were detected in more than 80% samples (48 out of 60). The most abundant ARG was the aminoglycoside resistance gene *str*B, with a median relative abundance of 1.3×10^{-2}. The next most abundant ARG was tetracycline resistance gene *tet*L, with the median relative abundance of 1.2×10^{-2}, and the other two abundant *tet* genes were *tet*M and *tet*X. Other abundant genes were *sul*2, *erm*B, *aad*A and *aad*A2. In addition, the class I integron markers *intI*1 and *qac*EΔ were detected at high levels in the sheds, with median relative abundances of 1.8×10^{-2} and 1.1×10^{-2}, respectively (Table 2). Relative abundances of these genes were further compared among the sheds using a pairwise Wilcoxon rank sum test. Three genes (*tet*X, *aad*A and *aad*A2) showed no significant differences in abundance between sheds. Shed A had significantly different abundances of gene *tet*L, *tet*M, *str*B, *sul*2, *erm*B, *int*I1, *qac*EΔ compared to shed B and/or shed C ($p < 0.05$). Sheds B and C had significantly different abundances of gene *tet*L, *erm*B, i*nt*I1 and *qac*EΔ ($p < 0.05$). The ARGs with high human clinical importance, such as the beta lactamase resistance genes *bla*SHV, *bla*CTX-M, *cph*A, the fluoroquinolone resistance gene *qnr*B and the virginiamycin resistance gene *vat*E, detected at very low abundances (~10^{-6} to 10^{-5}) in the samples.

Table 2. Most abundant ARGs and MGEs in the broiler breeder Farm B.

Gene	Relative Abundance		
	Min	**Max**	**Median**
*aad*A	6.4×10^{-4}	6.7×10^{-2}	7.2×10^{-3}
*aad*A2	5.0×10^{-4}	3.8×10^{-2}	5.6×10^{-3}
*str*B	7.0×10^{-4}	2.1×10^{-1}	1.3×10^{-2}
*erm*B	1.4×10^{-3}	3.0×10^{-2}	6.3×10^{-3}
*sul*2	1.5×10^{-4}	3.4×10^{-1}	8.0×10^{-3}
*intl*1	6.8×10^{-4}	7.9×10^{-2}	1.8×10^{-2}
*qac*EΔ	1.3×10^{-3}	5.4×10^{-2}	1.1×10^{-2}
*tet*L	1.0×10^{-3}	5.5×10^{-2}	1.2×10^{-2}
*tet*M	1.8×10^{-3}	3.0×10^{-2}	9.2×10^{-3}
*tet*X	2.2×10^{-4}	3.8×10^{-2}	7.7×10^{-3}

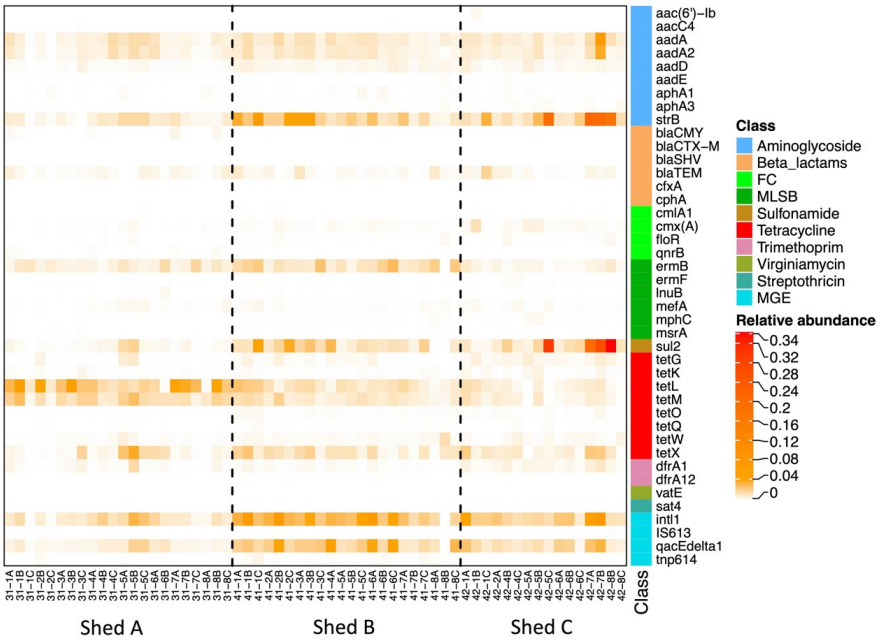

Figure 4. Heatmap of the relative abundance of ARGs and MGEs in all manure belt samples from three sheds in Farm B. FC, fluoroquinolone/florfenicol/chloramphenicol; MLSB, macrolide-lincosamide-streptogramin B; MGE, mobile genetic element.

2.2.2. Similarity of ARG and MGE Profiles between Sheds and Farms

NMDS analysis using Bray–Curtis distance derived from the relative abundance of ARGs and MGEs was used to explore sample clustering based on sheds in Farm B. The results are shown in Figure 5 and reveal a separation of shed A from sheds B and C (R = 0.39, p = 0.001, ANOSIM).

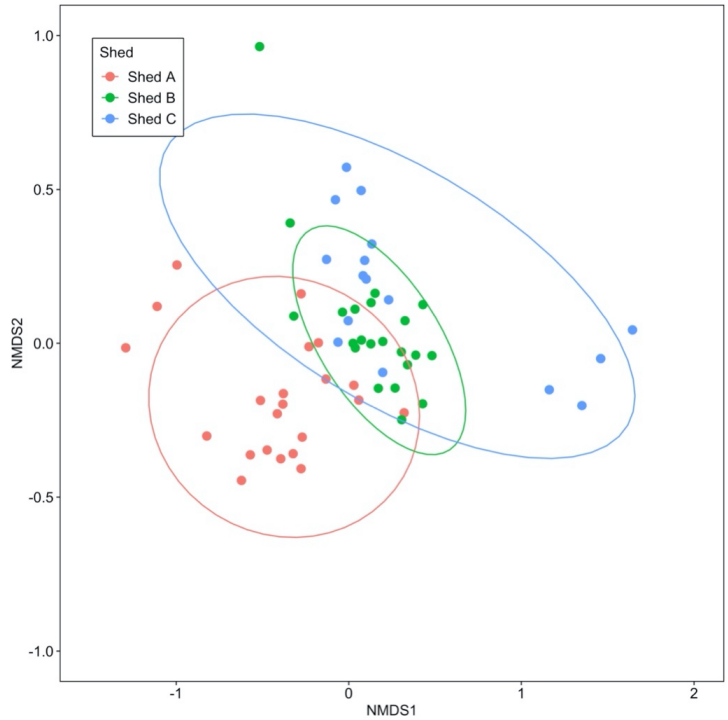

Figure 5. NMDS using Bray–Curtis distances to compare the similarity of ARG profiles of the manure belt swabs between the sheds in the broiler breeder Farm B. The ellipses indicate 95% confidence region of each cluster.

The ARG profile of manure belt swabs of Farm B was further compared to that of the layer Farm L. NMDS analysis was based on the relative abundance of 33 ARGs detected in both farms. No significant difference was found between the two farms (R = 0.17, p = 0.08, ANOSIM) (Figure 6).

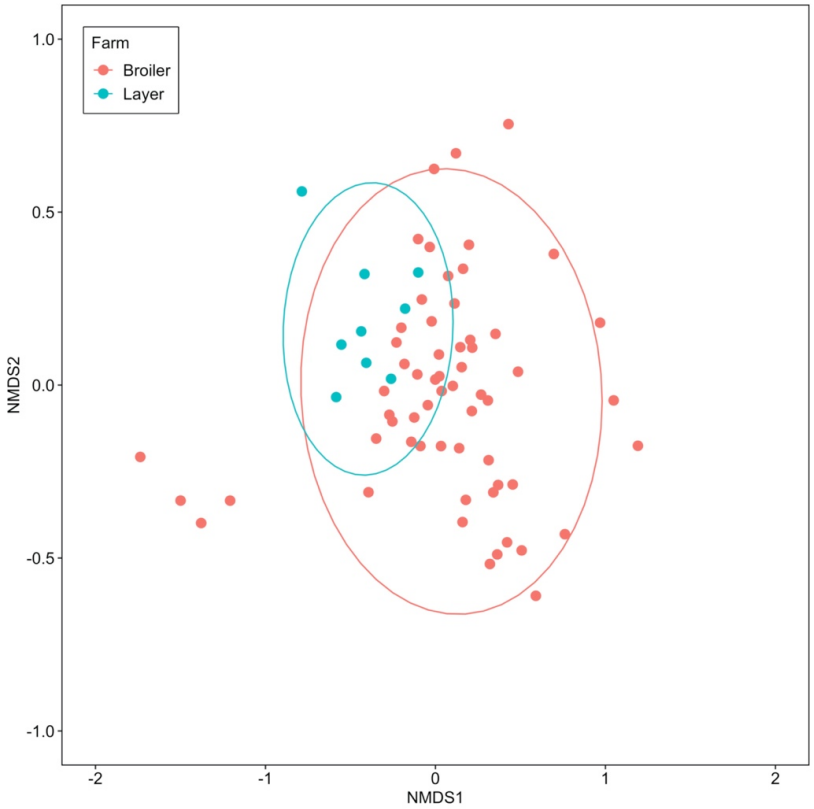

Figure 6. NMDS using Bray–Curtis distances to compare the similarity of the manure belt swab ARG profiles between layer Farm L and broiler breeder Farm B. The analysis was based on the relative abundance of 33 ARGs detected in both farms. The ellipses indicate 95% confidence region of each cluster.

3. Discussion

This work explored the ARG profile in two antibiotic-free farms, a layer farm and a broiler breeder farm. Manure belt swabs were used for sampling as this was easy to perform in a large chicken shed, and the biomass collected on the swabs represented a large number of birds. The manure belt swabs collected in layer Farm L showed a high abundance of *tet*M, *sul*2 and *str*B, which agreed with the results of previous studies that examined the resistome of intensively reared chickens [15,16]. Indeed, tetracycline resistance genes, particularly, *tet*Q, *tet*W and *tet*M, have been found to be ubiquitous in faecal specimens from human and animals [11,14,17].

The *sul*2 gene is plasmid borne, and has been found in plasmids from different incompatibility groups and often linked to streptomycin resistance genes *str*A and *str*B [18–20]. A recent study reported that the *sul*2-*str*A-*str*B cluster could be found in ice core samples from Antarctica that were over 1000 years old, suggesting that the formation and dissemination of this ARG cluster can happen without human interventions involving antibiotic use [21]. In the current study, no antibiotics had been used on this farm for more than 5 years, so the prevalence of these ARGs in this layer farm was not likely driven by antibiotic selective pressure and is more likely a reflection of their baseline abundances in the farming environment.

Analysis of the microbiota profile of the manure belt swabs showed a dominance of family *Aeromonadaceae* and genus *Luteimonas*, *Jeotgalicoccus*, *Corynebacterium* and *Brachybacterium* in the samples.

The *Corynebacterium* genus was found abundant in chicken duodenum, jejunum, ileum, and colon [22]. However, other genera, such as *Lactobacillus*, *Enterococcus*, *Bacteriodes* and *Ruminococcus,* reported as abundant in the chicken gastrointestinal tract [22,23], were rarely detected in our results. The divergency might be explained by the differences between samples. Previous studies usually collected fresh chicken faeces or gut contents, while, in our study, faecal material collected from the manure belt would have been exposed and the faecal microbiota could be impacted by environmental factors, such as oxygen, temperature and moisture. Indeed, *Luteimonas, Corynebacterium* and *Brachybacterium* were usually detected in chicken litter and *Corynebacterium* was one of the major genera in the litter microbiota [24,25].

The results of the Farm L study showed that many of the qPCRs were negative, which was consistent with the antibiotic-free policy of this farm. The results from the study of Farm L informed the experimental design of the study of Farm B, which also did not use antibiotics. A reduced set of HT-qPCR primers was selected for use in the Farm B study, which allowed more samples to be analysed while still maintaining broad coverage of the most frequent ARGs and MGEs present.

Farm B samples had abundant tetracycline resistance genes in most samples. This is consistent with findings on Farm L and other studies quantifying ARGs in faecal samples from poultry [26,27], swine [14,28] and humans [11,17]. These *tet* genes are known to be present in a wide range of bacterial species [29], some of which are members of the dominant flora of the normal chicken gastrointestinal tract and chicken litter, such as *Clostridium*, *Lactobacillus, Bacteroides* and *Corynebacterium* [23]. Metagenomic studies have shown that the ribosome protection type *tet* genes contribute about 30% to the total ARG abundance in the chicken cecum [30], and many *tet* genes are often associated with mobile elements in diverse species [31–35]. The macrolide resistance gene *erm*B was also found to be abundant in samples across all broiler sheds. Similar to the *tet* genes, *erm*B was also detected in a wide range of bacterial species [29] and associated with conjugative transposons [36–38], which may explain the wide dissemination of the gene in the environment.

Moreover, the ARG profile of the manure belt swabs from Farm B was similar to that from the Farm L. The comparison included ARGs that were detected at high levels in both farms, such as *tet*M, *tet*X, *str*B and *sul*2. As both farms were antibiotic-free for at least 5 years prior to sampling, the result indicates that those ARGs were ubiquitous in poultry manure and their presence was not necessarily related to human antibiotic usage.

In conclusion, this study showed that HT-qPCR can be used for surveying chicken farm environments, and that manure belt sampling is both convenient and useful as long as the physical design of the housing permits access to the belt. The ARG profiles for the two farms were similar. The ARGs with higher abundances, particularly *tet*M, *str*B and *sul*2, were likely due to their carriage by bacterial species naturally present in the chicken faecal microbiota, or because these genes have been already spread widely in the environment and were not the result of selection due to antibiotic use.

4. Materials and Methods

4.1. Study Farms and Chickens

In the layer Farm L, birds in the caged shed were kept in cages with six birds per cage. The study shed housed around 65,000 birds in five frames six tiers high. Manure accumulated on a conveyor belt beneath each tier of cages and was removed weekly.

In the broiler breeder Farm B, the birds were housed in frames three to four tiers high. Each shed contained eight frames which housed around 24,000 birds in total. Three sheds on the farm were sampled. Birds in shed B and C were originally from the same cohort of chicks and birds in shed A were from a different cohort.

4.2. Sampling

The caged layer shed in Farm L was sampled twice when the birds were 23 and 33 weeks old, respectively. One manure belt per frame was sampled and there were five frames in total. In Farm B, three manure belts of each frame were sampled, and 24 swabs were collected from each shed.

The manure belts were sampled according to the method described in [9]. Manure belt swabs were collected by wiping the edge of one end of the manure belt, using sterile 10 × 10cm cotton gauze swabs (ZebraVet, Australia) premoistened with buffered peptone water. Swabs were stored in sterile plastic bags and transported to the laboratory at ambient temperature. Samples from Farm L were transported to the laboratory on the same day of collection, and samples from Farm B were transported to the laboratory the next morning within 24h of collection. Upon receipt, the biomass was collected from the swabs and frozen at -80°C until DNA extraction.

4.3. DNA Extraction

The swabs were rinsed in 50ml peptone and the biomass was collected by centrifugation at 2885× *g* for 30min at 4 °C (Allegra X-12R centrifuge, Beckman Coulter, USA). The pellet was stored in one 2ml microcentrifuge tube and stored at −80 °C until DNA extraction.

DNA was extracted using the DNeasy PowerSoil Kit (Qiagen, Carlsbad, CA, USA) according to the manufacturer's instructions, except that the biomass mixed with buffer C1 was processed with a FastPrep homogeniser (Bio101, USA) at 5.5m/s for 30s. DNA quality and quantity were checked by Nanodrop (Thermo Fisher Scientific, USA) and Qubit (Thermo Fisher Scientific, USA), respectively. DNA was store at −20 °C until analysis.

4.4. HT-qPCR Array for Detection and Quantification of ARGs and MGEs

ARGs and MGEs were detected by the WaferGen SmartChip Real-time PCR system (WaferGen Inc. USA).

For Farm L, the array contained 296 validated primers targeting 285 ARGs, 10 mobile genetic elements (MGEs), and one 16S rRNA gene (Table S1) [13,14]. The assay includes genes from all major classes of ARGs, including aminoglycoside, beta lactamase, fluoroquinolone/florfenicol/chloramphenicol (FC), macrolide-lincosamide-streptogramin B (MLSB), sulfonamide, trimethoprim, tetracycline, vancomycin, multidrug resistance and other resistance genes. For Farm B, 45 primers were used to amplify ARGs, four primer pairs were used to amplify MGEs and one primer pair was specific to the bacterial 16S rRNA gene (Table S2). The qPCR conditions have been described previously [39]. The cycle threshold (Ct) cut-off was 27 [14]. The abundance of each ARG was normalised to the 16S rRNA gene in each sample using the equation Relative abundance = $\frac{E(16S)^{Ct(16S)}}{E(ARG)^{Ct(ARG)}}$, where E(16S) is the efficiency of the 16S rRNA gene, E(ARG) is the efficiency of each ARG or MGE, and Ct is the threshold cycle [40,41].

4.5. Bacterial 16S rRNA Sequencing and Bioinformatic Analyses

Bacterial communities in the manure belt swabs (Farm L) of the first sampling were characterised by sequencing the V3-V4 region of the 16S rRNA gene using the Illumina MiSeq platform. Primers were as follows: forward primer (342F): 5′-CCTAYGGGRBGCASCAG-3′, reverse primer (806R): 5′-GGACTACNNGGGTATCTAA-3′ [42,43]. Sequences were processed through the QIIME2 pipeline (Version qiime2-2018.11) [44]. De-multiplexed reads were trimmed to 250bp, de-noised, paired and grouped into amplicon sequence variants (ASVs) by DADA2 [45]. ASVs only present in a single sample or classified as non-bacteria were removed. Taxonomy was assigned using a pre-trained Naïve Bayes classifier with the Greengenes database (2013 August release) [46,47]. For fair comparison, sequence depth was equalised by randomly subsampling the same number of reads of each sample from the original dataset. The minimum number of reads that would not affect coverage or discard any samples was used.

4.6. Statistical Analysis

A heatmap of the relative abundance of each gene in each sample of Farm L and Farm B was generated using the ComplexHeatmap R package [48].

Differences in the relative abundance of ARGs between sheds in Farm B were tested by pairwise Wilcoxon rank sum test with P value adjusted by the Benjamini–Hochberg correction method [49] using the basic stats R package. A P value less than 0.05 was considered as a significant difference.

The similarity of ARG profiles of the manure belt swabs between sheds in Farm B was compared using the NMDS method with Bray–Curtis distance matrix based on the relative abundance of ARGs and MGEs. Similarity between sheds was tested by analysis of similarity (ANOSIM) using the 'anosim' function in the vegan package of R. NMDS analysis results were plotted using the ggplot2 R package with ellipses indicating the 95% confidence region of each cluster.

The similarity of ARG profiles of manure belt swabs between Farm L and Farm B was tested based on the relative abundance of ARGs tested in both farms, using the NMDS and ANOSIM methods described above.

Supplementary Materials: The following are available online at http://www.mdpi.com/2079-6382/9/3/120/s1, Table S1: qPCR primers for ARGs and MGEs in Farm L, Table S2: qPCR primers for ARGs and MGEs in Farm B.

Author Contributions: Conceptualization, Y.L., G.B., M.D.-S., M.M., H.B.-J.; methodology, Y.L., H.-W.H. and H.B.-J.; writing—original draft preparation, Y.L.; writing—review and editing, H.B.-J., M.D.-S., M.M.; G.B., supervision, H.B.-J., M.D.-S., M.M.; funding acquisition, H.B.-J. and G.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Health and Medical Research Council through the Centres of Research Excellence Programme, Grant no. 1079625.

Acknowledgments: YL received a Melbourne Research Scholarship from the University of Melbourne. The authors wish to thank Helen Crabb for assistance with sample collection.

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

References

1. Landers, T.F.; Cohen, B.; Wittum, T.E.; Larson, E.L. A review of antibiotic use in food animals: Perspective, policy, and potential. *Public Health Rep.* **2012**, *127*, 4–22. [CrossRef] [PubMed]
2. Marshall, B.M.; Levy, S.B. Food animals and antimicrobials: Impacts on human health. *Clin. Microbiol. Rev.* **2011**, *24*, 718–733. [CrossRef]
3. APVMA. Antibiotic Resistance in Animals. Available online: https://apvma.gov.au/our-science/14596/publications/27326 (accessed on 27 June 2018).
4. Obeng, A.; Rickard, H.; Sexton, M.; Pang, Y.; Peng, H.; Barton, M. Antimicrobial susceptibilities and resistance genes in *Campylobacter* strains isolated from poultry and pigs in Australia. *J. Appl. Microbiol.* **2012**, *113*, 294–307. [CrossRef] [PubMed]
5. Obeng, A.S.; Rickard, H.; Ndi, O.; Sexton, M.; Barton, M. Antibiotic resistance, phylogenetic grouping and virulence potential of *Escherichia coli* isolated from the faeces of intensively farmed and free range poultry. *Vet. Microbiol.* **2012**, *154*, 305–315. [CrossRef] [PubMed]
6. Obeng, A.S.; Rickard, H.; Ndi, O.; Sexton, M.; Barton, M. Comparison of antimicrobial resistance patterns in enterococci from intensive and free range chickens in Australia. *Avian Pathol.* **2013**, *42*, 45–54. [CrossRef]
7. Miflin, J.K.; Templeton, J.M.; Blackall, P. Antibiotic resistance in *Campylobacter jejuni* and *Campylobacter coli* isolated from poultry in the South-East Queensland region. *J. Antimicrob. Chemother.* **2007**, *59*, 775–778. [CrossRef]
8. Arnold, M.; Carrique-Mas, J.; Davies, R. Sensitivity of environmental sampling methods for detecting *Salmonella Enteritidis* in commercial laying flocks relative to the within-flock prevalence. *Epidemiol. Infect.* **2010**, *138*, 330–339. [CrossRef]
9. Crabb, H.K.; Allen, J.L.; Devlin, J.M.; Wilks, C.R.; Gilkerson, J.R. Spatial distribution of *Salmonella enterica* in poultry shed environments observed by intensive longitudinal environmental sampling. *Appl. Environ. Microbiol.* **2019**, *19*, e00333-19. [CrossRef]

10. Johnson, T.A.; Stedtfeld, R.D.; Wang, Q.; Cole, J.R.; Hashsham, S.A.; Looft, T.; Zhu, Y.G.; Tiedje, J.M. Clusters of antibiotic resistance genes enriched together stay together in swine agriculture. *mBio* **2016**, *7*, e02214–e02215. [CrossRef]
11. Li, B.; Yang, Y.; Ma, L.; Ju, F.; Guo, F.; Tiedje, J.M.; Zhang, T. Metagenomic and network analysis reveal wide distribution and co-occurrence of environmental antibiotic resistance genes. *ISME J.* **2015**, *9*, 2490–2502. [CrossRef]
12. Lin, W.; Zhang, M.; Zhang, S.; Yu, X. Can chlorination co-select antibiotic-resistance genes? *Chemosphere* **2016**, *156*, 412–419. [CrossRef] [PubMed]
13. Looft, T.; Johnson, T.A.; Allen, H.K.; Bayles, D.O.; Alt, D.P.; Stedtfeld, R.D.; Sul, W.J.; Stedtfeld, T.M.; Chai, B.; Cole, J.R. In-feed antibiotic effects on the swine intestinal microbiome. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 1691–1696. [CrossRef] [PubMed]
14. Zhu, Y.G.; Johnson, T.A.; Su, J.Q.; Qiao, M.; Guo, G.X.; Stedtfeld, R.D.; Hashsham, S.A.; Tiedje, J.M. Diverse and abundant antibiotic resistance genes in Chinese swine farms. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 3435–3440. [CrossRef] [PubMed]
15. Guo, X.; Stedtfeld, R.D.; Hedman, H.; Eisenberg, J.N.; Trueba, G.; Yin, D.; Tiedje, J.M.; Zhang, L. Antibiotic resistome associated with small-scale poultry production in rural Ecuador. *Environ. Sci. Technol.* **2018**, *52*, 8165–8172. [CrossRef]
16. He, L.Y.; Liu, Y.S.; Su, H.C.; Zhao, J.L.; Liu, S.S.; Chen, J.; Liu, W.R.; Ying, G.G. Dissemination of antibiotic resistance genes in representative broiler feedlots environments: Identification of indicator ARGs and correlations with environmental variables. *Environ. Sci. Technol.* **2014**, *48*, 13120–13129. [CrossRef]
17. Seville, L.A.; Patterson, A.J.; Scott, K.P.; Mullany, P.; Quail, M.A.; Parkhill, J.; Ready, D.; Wilson, M.; Spratt, D.; Roberts, A.P. Distribution of tetracycline and erythromycin resistance genes among human oral and fecal metagenomic DNA. *Microb. Drug Resist.* **2009**, *15*, 159–166. [CrossRef]
18. Bean, D.C.; Livermore, D.M.; Hall, L.M. Plasmids imparting sulfonamide resistance in *Escherichia coli*: Implications for persistence. *Antimicrob. Agents Chemother.* **2009**, *53*, 1088–1093. [CrossRef]
19. Vinué, L.; Sáenz, Y.; Rojo-Bezares, B.; Olarte, I.; Undabeitia, E.; Somalo, S.; Zarazaga, M.; Torres, C. Genetic environment of sul genes and characterisation of integrons in *Escherichia coli* isolates of blood origin in a Spanish hospital. *Int. J. Antimicrob. Agents* **2010**, *35*, 492–496. [CrossRef]
20. Yau, S.; Liu, X.; Djordjevic, S.P.; Hall, R.M. RSF1010-like plasmids in Australian *Salmonella enterica* serovar Typhimurium and origin of their *sul*2-*str*A-*str*B antibiotic resistance gene cluster. *Microb. Drug Resist.* **2010**, *16*, 249–252. [CrossRef]
21. Okubo, T.; Ae, R.; Noda, J.; Iizuka, Y.; Usui, M.; Tamura, Y. Detection of the *sul*2–*str*A–*str*B gene cluster in an ice core from Dome Fuji Station, East Antarctica. *J. Glob. Antimicrob Resist.* **2019**, *17*, 72–78. [CrossRef]
22. Xiao, Y.; Xiang, Y.; Zhou, W.; Chen, J.; Li, K.; Yang, H. Microbial community mapping in intestinal tract of broiler chicken. *Poult. Sci.* **2017**, *96*, 1387–1393. [CrossRef] [PubMed]
23. Wei, S.; Morrison, M.; Yu, Z. Bacterial census of poultry intestinal microbiome. *Poult. Sci.* **2013**, *92*, 671–683. [CrossRef] [PubMed]
24. Lu, J.; Sanchez, S.; Hofacre, C.; Maurer, J.J.; Harmon, B.G.; Lee, M.D. Evaluation of broiler litter with reference to the microbial composition as assessed by using 16S rRNA and functional gene markers. *Appl. Environ. Microbiol.* **2003**, *69*, 901–908. [CrossRef] [PubMed]
25. Wang, L.; Lilburn, M.; Yu, Z. Intestinal microbiota of broiler chickens as affected by litter management regimens. *Front. Microbiol.* **2016**, *7*, 1–12. [CrossRef] [PubMed]
26. Cheng, W.; Chen, H.; Su, C.; Yan, S. Abundance and persistence of antibiotic resistance genes in livestock farms: A comprehensive investigation in eastern China. *Environ. Int.* **2013**, *61*, 1–7. [CrossRef] [PubMed]
27. Ji, X.; Shen, Q.; Liu, F.; Ma, J.; Xu, G.; Wang, Y.; Wu, M. Antibiotic resistance gene abundances associated with antibiotics and heavy metals in animal manures and agricultural soils adjacent to feedlots in Shanghai; China. *J. Hazard. Mater.* **2012**, *235–236*, 178–185. [CrossRef] [PubMed]
28. Wang, R.; Chen, M.; Feng, F.; Zhang, J.; Sui, Q.; Tong, J.; Wei, Y.; Wei, D. Effects of chlortetracycline and copper on tetracyclines and copper resistance genes and microbial community during swine manure anaerobic digestion. *Bioresour. Technol.* **2017**, *238*, 57–69. [CrossRef]
29. Roberts, M.C.; Schwarz, S.; Aarts, H.J. Erratum: Acquired antibiotic resistance genes: An overview. *Front. Microbiol.* **2012**, *3*, 1–17. [CrossRef]

30. Qu, A.; Brulc, J.M.; Wilson, M.K.; Law, B.F.; Theoret, J.R.; Joens, L.A.; Konkel, M.E.; Angly, F.; Dinsdale, E.A.; Edwards, R.A. Comparative metagenomics reveals host specific metavirulomes and horizontal gene transfer elements in the chicken cecum microbiome. *PLoS ONE* **2008**, *3*, e2945. [CrossRef]
31. Agersø, Y.; Jensen, L.B.; Givskov, M.; Roberts, M.C. The identification of a tetracycline resistance gene *tet*(M), on a Tn 916-like transposon, in the *Bacillus cereus* group. *FEMS Microbiol. Lett.* **2002**, *214*, 251–256. [CrossRef]
32. Agersø, Y.; Pedersen, A.G.; Aarestrup, F.M. Identification of Tn5397-like and Tn916-like transposons and diversity of the tetracycline resistance gene *tet*(M) in enterococci from humans, pigs and poultry. *J. Antimicrob. Chemother.* **2006**, *57*, 832–839. [CrossRef] [PubMed]
33. Chopra, I.; Roberts, M. Tetracycline antibiotics: Mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol. Mol. Biol. Rev.* **2001**, *65*, 232–260. [CrossRef]
34. De Leener, E.; Martel, A.; Decostere, A.; Haesebrouck, F. Distribution of the *erm*(B) gene, tetracycline resistance genes, and Tn1545-like transposons in macrolide-and lincosamide-resistant enterococci from pigs and humans. *Microb. Drug Resist.* **2004**, *10*, 341–345. [CrossRef]
35. De Vries, L.E.; Christensen, H.; Skov, R.L.; Aarestrup, F.M.; Agersø, Y. Diversity of the tetracycline resistance gene *tet*(M) and identification of Tn 916-and Tn 5801-like (Tn 6014) transposons in *Staphylococcus aureus* from humans and animals. *J. Antimicrob. Chemother.* **2009**, *64*, 490–500. [CrossRef] [PubMed]
36. Cochetti, I.; Tili, E.; Vecchi, M.; Manzin, A.; Mingoia, M.; Varaldo, P.E.; Montanari, M.P. New Tn 916-related elements causing *erm*(B)-mediated erythromycin resistance in tetracycline-susceptible pneumococci. *J. Antimicrob. Chemother.* **2007**, *60*, 127–131. [CrossRef] [PubMed]
37. Gupta, A.; Vlamakis, H.; Shoemaker, N.; Salyers, A.A. A new *Bacteroides* conjugative transposon that carries an *ermB* gene. *Appl. Environ. Microbiol.* **2003**, *69*, 6455–6463. [CrossRef]
38. Okitsu, N.; Kaieda, S.; Yano, H.; Nakano, R.; Hosaka, Y.; Okamoto, R.; Kobayashi, T.; Inoue, M. Characterization of *erm*B gene transposition by Tn1545 and Tn917 in macrolide-resistant *Streptococcus pneumoniae* isolates. *J. Clin. Microbiol.* **2005**, *43*, 168–173. [CrossRef]
39. Wang, F.H.; Qiao, M.; Su, J.Q.; Chen, Z.; Zhou, X.; Zhu, Y.G. High throughput profiling of antibiotic resistance genes in urban park soils with reclaimed water irrigation. *Environ. Sci. Technol.* **2014**, *48*, 9079–9085. [CrossRef]
40. Muller, P.Y.; Janovjak, H.; Miserez, A.R.; Dobbie, Z. Erratum. *BioTechniques* **2002**, *33*, 514.
41. Muller, P.Y.; Janovjak, H.; Miserez, A.R.; Dobbie, Z. Processing of gene expression data generated by quantitative real-time RT-PCR. *BioTechniques* **2002**, *32*, 1372–1379.
42. Thijs, S.; Op De Beeck, M.; Beckers, B.; Truyens, S.; Stevens, V.; Van Hamme, J.D.; Weyens, N.; Vangronsveld, J. Comparative evaluation of four bacteria-specific primer pairs for 16S rRNA gene surveys. *Front. Microbiol.* **2017**, *8*, 1–15. [CrossRef] [PubMed]
43. Yu, Y.; Lee, C.; Kim, J.; Hwang, S. Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction. *Biotechnol Bioeng* **2005**, *89*, 670–679. [CrossRef] [PubMed]
44. Bolyen, E.; Rideout, J.R.; Dillon, M.R.; Bokulich, N.A.; Abnet, C.C.; Al-Ghalith, G.A.; Alexander, H.; Alm, E.J.; Arumugam, M.; Asnicar, F.; et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* **2019**, *37*, 852–857. [CrossRef] [PubMed]
45. Callahan, B.J.; McMurdie, P.J.; Rosen, M.J.; Han, A.W.; Johnson, A.J.A.; Holmes, S.P. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods* **2016**, *13*, 581–583. [CrossRef] [PubMed]
46. Bokulich, N.A.; Kaehler, B.D.; Rideout, J.R.; Dillon, M.; Bolyen, E.; Knight, R.; Huttley, G.A.; Caporaso, J.G. Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. *Microbiome* **2018**, *6*, 1–17. [CrossRef] [PubMed]
47. DeSantis, T.Z.; Hugenholtz, P.; Larsen, N.; Rojas, M.; Brodie, E.L.; Keller, K.; Huber, T.; Dalevi, D.; Hu, P.; Andersen, G.L. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl. Environ. Microbiol.* **2006**, *72*, 5069–5072. [CrossRef] [PubMed]
48. Gu, Z.; Eils, R.; Schlesner, M. Complex heatmaps reveal patterns and correlations in multidimensional genomic data. *Bioinformatics* **2016**, *32*, 2847–2849. [CrossRef]
49. Benjamini, Y.; Hochberg, Y. Controlling the false discovery rate: A practical and powerful approach to multiple testing. *J. R. Stat. Soc. Ser. B.* **1995**, *57*, 289–300. [CrossRef]