Examining antibiotic resistance in the feedlot cattle industry using real-time, quantitative PCR (qPCR) and enterococci as an indicator bacterium

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Abstract

Antibiotics are administered to livestock at subtherapeutic levels to maintain animal health. Many of the antibiotics used are analogues or the same as those used in human medicine, raising the possibility that genes conferring resistance arise within agricultural production systems with implications for human health. In beef cattle, macrolides are administered for the control of bovine respiratory disease and liver abscesses and have been identified by the World Health Organization as critically important antibiotics for which management strategies are required to prevent resistance development. Enterococci are present in the gastrointestinal tract of humans and cattle and are also associated with nosocomial infections in humans. They are an indicator bacterium that can be used to monitor macrolide resistance. This thesis examined antibiotic resistance in the Canadian beef feedlot industry. Real-time, quantitative PCR was used to examine differences in the relative abundance of eighteen resistance genes across five antibiotic families including sulfonamides [*sul1* and *sul2*], tetracyclines [*tet*(A), *tet*(B), *tet*(M), *tet*(O), *tet*(Q) and *tet*(W)], macrolides [*erm*(A), *erm*(B), *erm*(C), *erm*(F) and *mef*(A)], fluoroquinolones [*qnrS* and *oqxB*] and β-lactams [bla_{SHV}, bla_{TEM1} and $bla_{\mathrm{CTX\text{-}M}}$] from feedlot cattle faeces and urban environments. The effect of in-feed administration and withdrawal of tylosin phosphate on macrolide resistance was examined using enterococci as an indicator bacterium. Resistant enterococci (n=21) were selected for whole-genome sequencing and comparative genomics. Results presented here show that the relative abundance of resistance genes differs between cattle feedlots and urban environments, likely a reflection of differences in antibiotic use. Sulfonamide, fluoroquinolone and β-lactam resistance genes predominated in urban wastewater, whilst tetracycline resistance genes were more prevalent in cattle faeces. The inclusion of tylosin in the diet of cattle at subtherapeutic levels increased the proportion of erythromycin- and

tylosin-resistant enterococci. However, withdrawal of tylosin from the diet appeared to contribute to a reduction in macrolide resistant enterococci. Comparative genomics revealed resistance to macrolides was present on mobile genetic elements, specifically the Tn*917* transposon harbouring *erm*(B). This transposon was identified in both *Enterococcus hirae* and *Enterococcus faecium*, suggesting inter-species transfer of resistance genes may occur in the bovine gastrointestinal tract. Furthermore, the integrative conjugative elements (ICEs) Tn*916* and Tn*5801*, both conferring tetracycline resistance, were identified in *E. faecium*. As the cost of genomic sequencing continues to decrease, further investigation of ICEs using whole genome sequencing will help determine if there are linkages between enterococci isolates from bovine environmental and human clinical sources and whether bovine enterococci represent a source of dissemination and spread of antibiotic resistance. Although macrolide resistance in enterococci decreased following the withdrawal of macrolides from cattle feed, this is not a reason to become complacent with the use of macrolides in cattle production. Investigating alternatives to macrolides for the control of bovine respiratory disease and liver abscesses, such as vaccines and plant bioactives, is becoming increasingly important. Furthermore, implementation of management practices by cattle producers that reduce the likelihood of disease spread is also essential to reduce the need to use antibiotics to control infectious diseases.

Declaration

The work described in this thesis was conducted under the supervision of Associate Professor Alexandre V. Chaves, Faculty of Veterinary Science, The University of Sydney, Australia and under the co-supervision of Professor Michael P. Ward, Faculty of Veterinary Science, The University of Sydney, Australia and Dr. Tim A. McAllister, Lethbridge Research Centre, Agriculture and Agri-Food Canada, Canada.

I declare that this thesis is the result of my own work, original and is not currently being submitted for any other degree or qualification. Full acknowledgement has been made where the work of others has been cited or used.

Alicia Grace Beukers

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AGISAR: Advisory Group on Integrated Surveillance of Antimicrobial Resistance

AMP: ampicillin

AMR: antimicrobial resistance

AMRPC: Australian Antimicrobial Resistance Prevention and Containment

ARGs: antibiotic resistance genes

AZ: azithromycin

β-lactam: beta-lactam

BEA: Bile-Esculin-Azide

BEA^{E}: Bile-Esculin-Azide amended with erythromycin (8µg/mL)

BEA$^{T:}$ Bile-Esculin-Azide amended with tylosin (32µg/mL)

BHI: brain heart infusion

Bp: base pair

BRD: bovine respiratory disease

°C: degrees centigrade

C: cattle

Ca: Cat

CARDs: Comprehensive Antibiotic Resistance Database

CC: clonal complex

CDC: Centers for Disease Control and Prevention

CFIA: Canadian Food Inspection Agency

CFU: coliform forming units

CH: clarithromycin

CI: chromosomal integrons

CI: collagen type I

CIPARS: Canadian Integrated Program for Antimicrobial Resistance Surveillance

CIV: collagen type IV

CL: chloramphenicol

Cls: cardiolipin synthase

CLSI: Clinical and Laboratory Standards Institute

CM: clindamycin

COGs: Clusters of Orthologous Groups

CRISPR: clustered, regularly interspaced short palindromic repeats

d: day

D: dog

D-Ala: D-alanine

D-Lac: D-lactate

D-Ser: D-serine

DAFF: Department of Agriculture, Fisheries and Forestry

DM: dry-matter

DNA: deoxyribonucleic acid

DoHA: Department of Health and Aging

DOX: doxycycline

DR: direct repeat

ECM: extracellular matrix

EDTA: ethylenediaminetetraacetic acid

EFSA: European Food Safety Authority

ERY: erythromycin

ery^R: erythromycin resistant

ESBLs: extend spectrum β-lactamase

Esp: enterococcal surface protein

EU: European Union

EUCAST: The European Committee on Antimicrobial Susceptibility Testing

FDA: Food and Drug Administration

Fi: fish

FWZID: Foodborne, Waterborne and Zoonotic Infections Division

***g*:** G-force

g: gram

GdpD: glycerophosphoryl diester phosphodiesterase

GEN: gentamicin

GI: gastrointestinal

H: horse

h: hour

HA: hospital acquired

HGT: horizontal gene transfer

HLRG: high level gentamicin resistance

I: intermediate resistance

ICEs: integrative conjugative elements

IMG: Integrated Microbial Genomes

ISs: insertion sequences

kb: kilobase pairs

kg: kilogram

KM: kanamycin

LAB: lactic acid bacteria

LFZ: Laboratory for Foodborne Zoonoses

LI: lincomycin

LN: lamina

ln: natural log

LVX: levofloxacin

LZD: linezolid

µg: microgram

µm: micrometre

µL: microlitre

M: molar

m: metre

mb: megabase pairs

mg: milligram

MGEs: mobile genetic elements

MI: mobile integrons

MIC: minimum inhibitory concentration

MIC_{90}: minimum inhibitory concentration for 90 percent of strains

min: minutes

mL: millilitre

MLS_B: macrolide-lincosamide-streptogramin B

MLST: multilocus sequence typing

mM: millimolar

MRSA: methicillin-resistant Staphylococcus aureus

MSCRAMMs: microbial surface components recognizing adhesive matrix molecules

NARMS: National Antimicrobial Resistance Monitoring System

NCBI: National Center for Biotechnology Information

NDM-1: New Delhi Metallo-beta-lactamase-1

NE: neomycin

NIT: nitrofurantoin

nM: nanomolar

NML: National Microbiology Laboratory

NU: nourseothricin sulphate

P: poultry

PBPs: penicillin binding proteins

PCR: polymerase chain reaction

PFGE: pulsed-field gel electrophoresis

ppm: parts per million

Q-D: quinupristin-dalfopristin

qPCR: real-time, quantitative polymerase chain reaction

R: complete resistance

RO: roxithromycin

rpm: revolutions per minute

rRNA: ribosomal ribonucleic acid

s: seconds

SAS: statistical analysis software

SDS: sodium lauryl sulfate

Sh: sheep

SNPs: single-nucleotide polymorphisms

ssDNA: single stranded DNA

ST: sequence type

STR: streptomycin

Sw: swine

TE: tris-EDTA

TGC: tigecycline

tmRNA: transfer-messenger RNA

TSA: Trypticase soy agar

TYL: tylosin

tyl^R: tylosin resistant

UPGMA: unweighted pair group method

USDA: US Department of Agriculture

VAN: vancomycin

VRE: vancomycin-resistant enterococci

WHO: World Health Organization

List of Publications and Presentations

Peer-reviewed articles

Beukers, A.G., Zaheer, R., Goji, N., Amoako, K.K., Chaves, A.V., Ward, M.P. and McAllister, T.A. Comparative genomic analysis of *Enterococcus* spp. isolated from bovine feces. *BMC Microbiol*. (Submitted).

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Beukers, A.G., Zaheer, R., Goji, N., Cook, S.R., Amoako, K.K., Chaves, A.V., Ward, M.P. and McAllister, T.A. (2016). Draft Genome Sequence of an *Enterococcus thailandicus* strain isolated from bovine feces. *Genome Announc*. 4(4):e00576-16.

Beukers, A.G., Zaheer, R., Cook, S.R., Stanford, K., Chaves, A.V., Ward, M.P. and McAllister, T.A. (2015). Effect of in-feed administration and withdrawal of tylosin phosphate on antibiotic resistance in enterococci isolated from feedlot steers. *Front. Microbiol.* 6:483

Conference presentations

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Beukers, A.G., Zaheer, R., Cook, S.R., Chaves, A.V., Ward, M.P., Tymensen, L., Morley, P.S., Hannon, S., Booker, C.W., Read, R.R. and McAllister, T.A. Comparison of antimicrobial resistant genes within feedlots to those in urban wastewater. 17th Annual Scientific Meeting – Antimicrobials 2016, February 25th-27th, 2016, Melbourne, Australia.

Chapter 1 – General Introduction

Antimicrobial resistance is a prominent issue in today's society. Multi-drug resistant pathogens such as carbapenem-resistant and extended spectrum β-lactamase (ESBLs) producing Enterobacteriaceae (e.g. New Delhi Metallo-beta-lactamase-1 [NDM-1] *Klebsiella pneumoniae* and ESBL *Escherichia coli*), vancomycin-resistant enterococci (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA) represent some of the current antibiotic resistant threats to public health (Centers for Disease Control and Prevention, 2013).

Antibiotics are frequently used for therapy and prophylaxis in both humans and animals, therefore exposing not only pathogenic and zoonotic bacteria, but also commensal bacteria to these compounds (Van den Bogaard and Stobberingh, 2000). A consequence of this is the emergence and spread of resistant bacteria. This has made it increasingly difficult to successfully treat infections that were in the past easily controlled by antibiotics. Resistant bacteria can be transferred among humans in health care settings or resistant bacteria of animal origin can be transferred to humans through direct contact with animals, or through the consumption of animal products contaminated with resistant bacteria (Centers for Disease Control and Prevention, 2013). Contamination of the environment with residual antibiotics entering the ecosystem through sewage, application of livestock and poultry manure to land, or from surface water run-off from farms also contributes to the spread of antibiotic resistance and can lead to selection of resistance in bacterial communities residing in these environments (Centers for Disease Control and Prevention, 2013). Some of the potential routes of transmission of antibiotic resistant bacteria to humans are summarised in Figure 1.1.

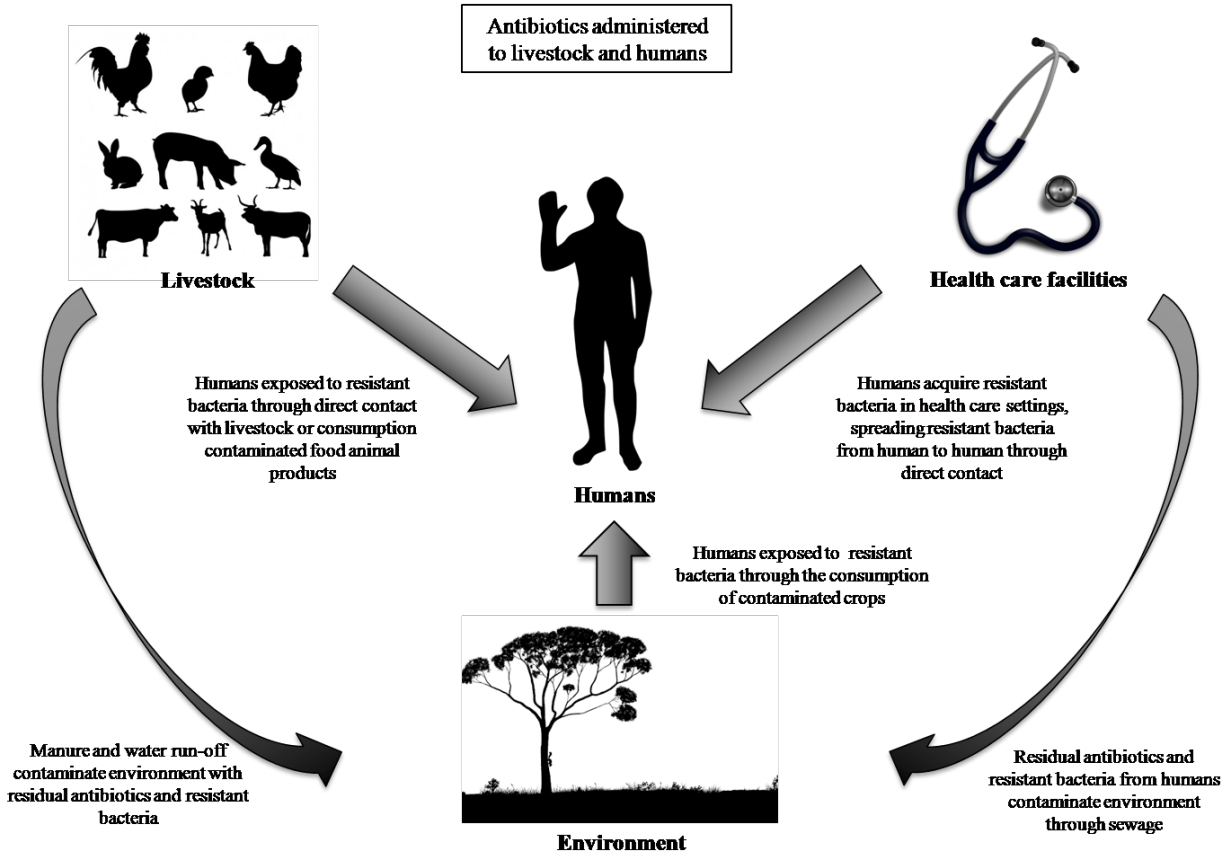

Figure 1.1. Potential routes of transmission of antibiotic resistant bacteria to humans. Figure adapted from Centers for Disease Control and Prevention (2013).

Compared to the pre-1970's, modern livestock and poultry production systems have intensified, with animals being housed at high densities (Silbergeld et al., 2008; Thornton, 2010). Consequently, infectious diseases are more easily spread (Otte et al., 2007) and antimicrobials are administered at sub-therapeutic levels in livestock and poultry feed to control and prevent disease (Marshall and Levy, 2011; Silbergeld et al., 2008), raising the possibility that genes conferring resistance arise within agricultural production systems. Many of the antimicrobials used are analogues or the same as antimicrobials used in human medicine.

Beef production is the third largest meat industry after swine and poultry production with >65 million tonnes of beef produced globally (Food and Agricultural Organization, 2015). Feedlots are used in the United States, Canada and Australia for intensive beef cattle production (Australian Lot Feeders Association, 2015; Galyean et al., 2011). They are generally used to 'finish' cattle before slaughter after these cattle have initially been raised on pasture. Most cattle are finished using a high-grain diet over a period of 100 to 120 days. Feeding cattle in this manner ensures that growth is maximised over the duration they are housed in the feedlot. However, the nature of this type of production means that disease can become a significant issue, in particular bovine respiratory disease (BRD) and liver abscesses.

BRD represents the primary disease of young calves and feedlot cattle in North America, accounting for >70% and >40% of feedlot morbidity and mortality, respectively (Hilton, 2014). The disease is multifactorial, involving a number of pathogens of both viral and bacterial origin, and arises when cattle are exposed to risk factors promoting stress, many of which are commonly experienced in the feedlot environment. The feeding of high-grain diets to cattle can cause acidosis, leading to ruminal lesions. This predisposes cattle to invasion by *Fusobacterium necrophorum*, the primary etiological agent involved in the formation of liver abscesses (Nagaraja and Chengappa, 1998). Cattle with liver abscesses experience reduced productivity caused by reduced feed intake and weight gain. In North America, macrolide antibiotics are frequently administered to cattle to prevent and treat these diseases (Pagel and Gautier, 2012). Tylosin phosphate is a common in-feed macrolide used to reduce the incidence of liver abscesses by inhibiting *F. necrophorum*, whilst tilmicosin, tulathromycin and gamithromycin are injectable macrolides used in the treatment of BRD (DeDonder and Apley, 2015; Nagaraja and Chengappa, 1998).

Commensal bacteria, such as *Escherichia coli* and enterococci, are considered suitable indicators to study selection pressure exerted on bacterial populations due to antibiotic use (Van den Bogaard and Stobberingh, 2000). They are also useful indicators of faecal contamination. In the case of macrolides, enterococci represent a more suitable candidate as *E. coli* is intrinsically resistant to this antibiotic class (Mao and Putterman, 1968). As enterococci are present in a number of environments, including the bovine and human gastrointestinal tract (Chenoweth and Schaberg, 1990; Devriese et al., 1992; Noble, 1978), they represent a potential source of resistance genes that could be transferred to other bacteria including pathogenic bacteria.

It is almost inevitable that bacteria that are exposed to antibiotics will develop resistance making it important that responsible stewardship be employed in their use. Surveillance and monitoring indicator bacteria such as enterococci for antibiotic resistance can provide information on the development of antibiotic resistance within the feedlot environment. As such, this thesis aims to provide insight on how antibiotic use in the Canadian beef feedlot industry contributes to resistance development by quantifying resistance genes using real-time, quantitative PCR (qPCR). Further, enterococci were selected as an indicator bacterium to investigate the effects of macrolide use, specifically tylosin phosphate, on the development of antibiotic resistance. Select isolates were further analysed using whole genome sequencing and comparative genomics to provide further insight into the genus *Enterococcus.*

Chapter 2 – Literature Review

2.1. Antimicrobials

Antimicrobials are defined by the World Organisation for Animal Health as a 'naturally occurring, semi-synthetic or synthetic substances that exhibit antimicrobial activity by killing or inhibiting the growth of micro-organisms' (World Organisation for Animal Health, 2013). This definition encompasses agents active against bacteria, viruses, protozoa and fungi. The term 'antibiotic' is used in this document to describe antimicrobial agents which are active against bacteria. Many classes of antibiotics are available for use in human and animal medicine with each class representing a group of structurally related antibiotics. The penicillin, cephalosporin, carbapenem and monobactam classes are grouped collectively as the beta-lactam (β-lactam) antibiotics and represent the largest group of antibiotics. Other classes of antibiotics include the aminoglycosides, tetracyclines, macrolides, quinolones, sulfonamides, chloramphenicals, oxazolidinones, ansamycins, streptogramins, lipopeptides and glycopeptides. Many of the antibiotics used in animal husbandry are from the same antibiotic class as those used in human medicine (Table 2.1; Marshall and Levy, 2011).

2.1.1. Ranking of antibiotics according to importance

In 2005, the World Health Organization (WHO) held 'the Canberra meeting' to develop a list ranking antimicrobial agents according to their importance in human medicine. In 2007, this list and rankings were reviewed at 'the Copenhagen meeting'. Since then, it has been revised twice, with the latest revision occurring in Oslo, Norway in 2011 (World Health Organization,

2012a). The WHO list represents the first international consensus to rank antimicrobials according to their importance in human medicine. In doing so, this list provides an important guideline with regard to which antimicrobials used in food animal production are most likely to compromise the treatment of infectious diseases in humans (Collignon et al., 2009).

In the WHO list, antimicrobials were placed into one of three categories based on two criteria. The first criteria addressed if the antimicrobial was the sole or one of a few alternative therapies available to treat serious infectious disease in humans. The second criteria addressed whether the antimicrobial was used to treat diseases caused by organisms that may be zoonotic or the extent to which they may acquire resistance genes from zoonotic sources (World Health Organization, 2012a). Based on these two criteria, antimicrobials meeting both were categorised as critically important, those meeting either were categorised as highly important and those satisfying neither criteria as important (Table 2.2; World Health Organistion, 2012a).

Antimicrobials within the critically important category were further prioritised to identify agents where management strategies were urgently needed to reduce the development of antimicrobial resistance. Selection was based on a number of guidelines, including if the antimicrobial was the sole or one of the few alternative therapies used to treat diseases affecting a significant portion of the human population, if the antimicrobial was frequently used and if it was used to treat diseases caused by organisms showing evidence of transmission from non-human sources to humans or able to acquire resistance genes from non-human sources. Following these guidelines, fluoroquinolones, third- and fourth-generation cephalosporins, macrolides and glycopeptides were given highest priority for risk management (World Health Organization, 2012a).

2.1.2. Antibiotic use in humans

Antibiotics have been used extensively in the treatment of infections in humans. Their use has revolutionised human medicine and can be credited with the control of many potentially fatal infections. Penicillin, discovered in 1928, was one of the first antibiotics used to treat clinical infections in humans and its use became widespread in 1941 (Shaban et al., 2014; Zaffiri et al., 2012). Since the discovery of penicillin, additional antibiotics have been discovered and developed (Figure 2.1). This development pipeline has in part been driven by the need to discover new antibiotics effective against resistant bacteria. Bacterial resistance to penicillin was documented shortly after its discovery. Finland et al. (1950) demonstrated a clear difference in penicillin sensitivity in staphylococci strains isolated from hospitalised patients before 1946 to those in later years, highlighting emerging resistance to penicillin following its widespread use (Finland et al., 1950). This pattern of emerging resistance following discovery and use is also apparent with other antibiotics (Figure 2.1; Centers for Disease Control and Prevention, 2013; Zaffiri et al., 2012; Zaffiri et al., 2013).

Inappropriate use of antibiotics in human medicine has contributed to the development of antibiotic resistant bacteria. In a bacterial population, both susceptible and resistant bacteria are present. Resistant bacteria may be intrinsically resistant to an antibiotic or may acquire resistance through mutation or horizontal gene transfer (HGT). Incorrect use of antibiotics such as inadequate treatment duration, too low of a dose, or selection of an antibiotic inappropriate for the target bacteria results in selective pressure enabling resistant bacteria to survive. Without competition, resistant bacteria proliferate and after a period of time replace susceptible bacteria, dominating the population (Figure 2.2; Rosenblatt-Farrell, 2009).

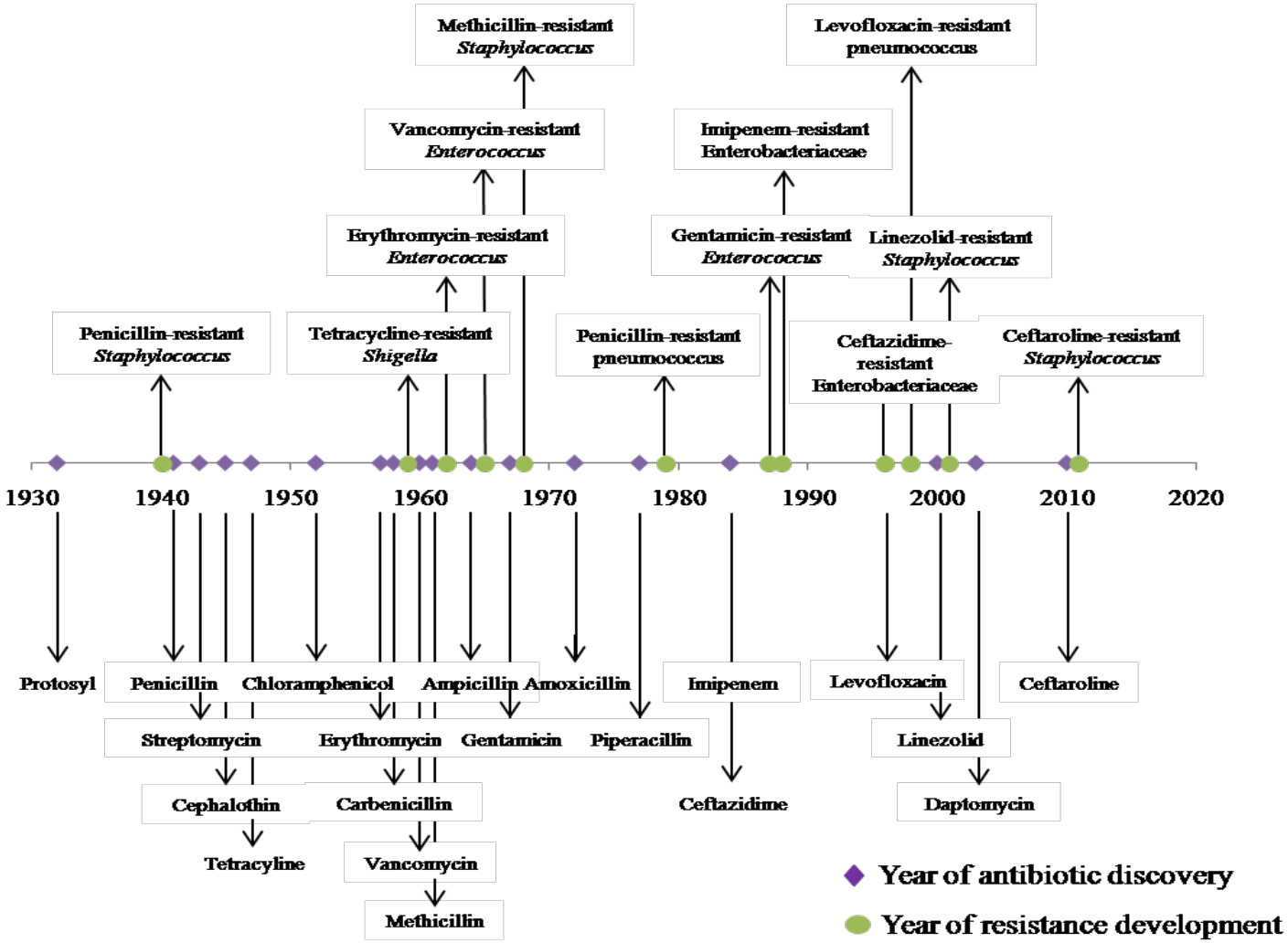

Figure 2.1. Timeline of antibiotic discovery and development of antibiotic resistance. Figure adapted from Centers for Disease Control and Prevention (2013); Zaffiri et al. (2012) and Zaffiri et al. (2013).

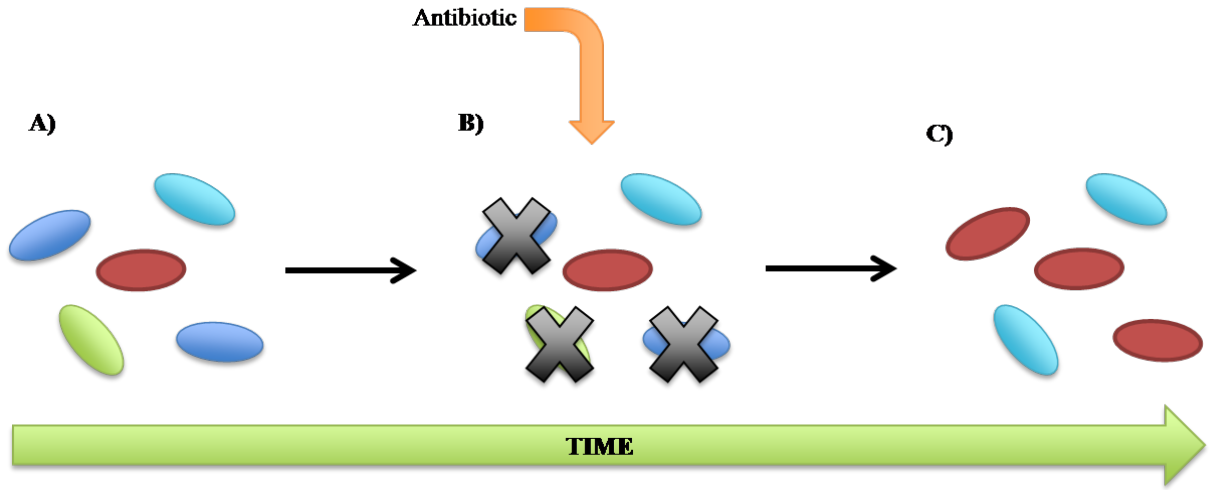

Figure 2.2. Selective pressure and antibiotic resistance development. (A) Bacterial population consists of a mixture of susceptible and resistant bacteria; (B) Antibiotics provide selective pressure, eliminating susceptible bacteria whilst resistant bacteria survive; (C) Resistant bacteria predominant the population. Figure adapted from Centers for Disease Control and Prevention (2013) and Rosenblatt-Farrell (2009).

2.1.3. Antibiotic use in food producing animals

Antibiotic use in food producing animals is also suggested to contribute to the emergence of resistant bacteria. There is growing concern resistant bacteria can be transmitted from animals to humans via the food chain. Antibiotic use in animal production can be separated into four different categories: therapeutic use in the treatment of disease, prophylactic use to prevent the development of disease, metaphylactic use for the control of disease, and for growth promotion (Australian Commission on Safety and Quality in Health Care, 2013; European Platform for the Responsible Use of Medicines in Animals, 2013). The USA, Canada, Australia and Europe have different regulatory laws regarding the use of antibiotics in livestock and each has their own governing body responsible for the regulation of antibiotics. The antibiotics listed include those approved for use in growth promotion as well as those approved for therapeutic and prophylactic use (Table 2.1.).

Concerns surrounding the use of antibiotics in food producing animals were evident in 1969 following the release of the Swann report by the Joint Committee on the use of Antibiotics in Animal Husbandry and Veterinary Medicine. This report provided recommendations on the use of antibiotics in food animals and suggested only antibiotics which have little or no application as therapeutic drugs in either humans or animals should be allowed for use as growth promoters. Subsequently, it was recommended that the antibiotics chlortetracycline, oxytetracycline, penicillin, tylosin and the sulphonamides no longer be used for growth promotion (House of Lords, 1998).

The Swann Report was one of the first reports to promote changes in the use of antibiotics in food producing animals. Some of these changes included the removal of antibiotics (such as penicillin) from animal feeds in the UK, Australia and several other countries, but this policy was not implemented in the USA (Barton, 2010).

Concern over the use of antibiotics for growth promotion continued, and in the 1990s it was demonstrated the use of the glycopeptide growth promotant, avoparcin, was selecting for vancomycin-resistant *Enterococcus faecium* in livestock and poultry (Bager et al., 1997). Vancomycin is used as an alternative to ampicillin in patients allergic to β-lactam antibiotics or to treat infections caused by penicillin resistant pathogens, in particular methicillin-resistant *Staphylococcus aureus* (MRSA) and penicillin-resistant *Streptococcus pneumoniae* (Arias and Murray, 2012; Levine, 2006). Widespread vancomycin-resistance is of concern as it would reduce the effectiveness of this last resort antibiotic, particularly with regard to vancomycin-resistant enterococci, increasing treatment failure. Consequently, this prompted the European Union (EU) to ban the use of antibiotics for growth promotion of livestock in 2006 (European Food Safety Authority, 2015a).

2.1.4. Monitoring and surveillance schemes for antimicrobial resistance

It was reported by the WHO Advisory Group on Integrated Surveillance of Antimicrobial Resistance (AGISAR) that 'the rate of antimicrobial resistance in bacteria causing serious and life-threatening infections is rising' (World Health Organization, 2012b). Human medicine has played a big part in this increase, but the use of antibiotics in agriculture is also a contributor. It has therefore become increasingly important for surveillance schemes to be in place to assess the impact of antibiotic use on the development of antibiotic resistance. The USA, Canada and Europe have implemented monitoring and surveillance schemes with Australia in the process of establishing one (Table 2.3.; Shaban et al., 2014).

In the USA, the Centers for Disease Control and Prevention (CDC) established the National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS) in 1996. Collaboration between the US CDC, the US Food and Drug Administration (FDA), the US Department of Agriculture (USDA) and state and local health departments established a system to monitor antimicrobial resistance within enteric bacteria. Three main sources of antimicrobial resistant bacteria are monitored; humans (CDC), retail meat (FDA) and food animals (USDA), with each department responsible for its specific source. Enteric bacteria collected from these sources undergo antimicrobial susceptibility testing and genetic analysis to determine the extent of resistance development. Information obtained from these isolates identifies emerging trends of resistance and links enteric illnesses to specific sources and possible risk factors. The molecular portion of the study provides information on the underlying genetic mechanisms of resistance and their possible spread amongst enteric bacteria. The program characterises enteric disease outbreaks, aides in the development of recommendations for the judicious use of antimicrobial agents and educates consumers on food safety and about foodborne antimicrobial resistance

threats. Information from NARMS is provided in an annual report, published on their website and in scientific articles (Centers for Disease Control and Prevention, 2015).

In Canada, the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) was established in 2002. It is a coordinated approach involving the Laboratory for Foodborne Zoonoses (LFZ), the Foodborne, Waterborne and Zoonotic Infections Division (FWZID), the National Microbiology Laboratory (NML), the Canadian Food Inspection Agency (CFIA), and provincial health and agricultural ministries. Together they monitor trends in resistance development in selected bacterial organisms collected from human, animal and food sources throughout Canada. Information obtained allows decisions to be made about policies to control antimicrobial use in hospital, community and agricultural settings and to identify measures to manage the emergence and spread of resistant bacteria (Public Health Agency of Canada, 2007).

Europe has extensive surveillance systems in place for monitoring antimicrobial resistance. National surveillance systems exist in many European countries including Denmark (DANMAP), Norway (NORM/NORMVET), France (ONERBA), Finland (FINRES-VET), the Netherlands (NETHMAP/MARAN), Sweden (SWEDRES/SVARM) and Italy (ITAVARM) (World Health Organization, 2014). These programs collect isolates from both animal and human sources. In addition to these programs, the European Food Safety Authority (EFSA), an independent European agency funded by the EU, reports on isolates collected from food-producing animals and products across 26 EU member states (European Food Safety Authority, 2012; European Food Safety Authority, 2015b). More recently, reports have included a section entitled 'Farm-to-Fork Analysis' including data on human isolates alongside the data of animal isolates, encompassing a 'one health' perspective (European Food Safety Authority, 2012).

Presently, Australia has no nationally coordinated veterinary or agricultural antimicrobial resistance monitoring and surveillance program in place. A number of pilot studies have been conducted and in 2013 the Australian Antimicrobial Resistance Prevention and Containment (AMRPC) Steering Group was established. The Steering Group is chaired by the Department of Health and Aging (DoHA) and the Department of Agriculture and Water Resources (previously the Department of Agriculture, Fisheries and Forestry; DAFF) with aims of establishing a comprehensive National Antimicrobial Resistance Prevention and Containment Strategy for Australia (Australian Commission on Safety and Quality in Health Care, 2013). In 2015, the Australian Government announced Australia's first National antimicrobial resistance strategy to be implemented (Australian Government, 2015). Presently, a strategy implementation plan is being developed in consultation with stakeholders (Department of Health, 2016).

2.2. Antibiotic Resistance

2.2.1. Development of antibiotic resistant bacteria

Antibiotics have various mechanisms of inhibiting and killing bacteria as summarised in Table 2.4. Bacteria have evolved five main mechanisms to counteract the activity of antibiotics through resistance. Resistance genes encode for enzymes which degrade or modify the target antibiotic, rendering it inactive. Alternatively, antibiotic-efflux pumps can pump the antibiotic out of the bacterial cell before it is able to cause damage. Other adaptations include modification of the bacterial cell surface to reduce uptake of the antibiotic through a reduction in cell wall or cell membrane permeability, the production of an alternative metabolic pathway bypassing the

action of the antibiotic, and alteration of the intracellular target of the antibiotic so it no longer has an effect on its target (Tenover, 2006).

Antibiotic resistance in bacteria can be intrinsic or acquired. Intrinsic (or innate) resistance is a term used to describe resistance to antibiotics whereby the general physiology or anatomy of the microorganism confers resistance (Rosenblatt-Farrell, 2009). Examples of this include the target for the antimicrobial agent being absent in the microorganism, the cell envelope being impermeable to the antimicrobial, or the natural presence of an enzyme or enzymes that degrade the antibiotic or remove it from the cell (Rosenblatt-Farrell, 2009).

Acquired resistance arises through spontaneous mutations or acquisition of new genetic material through gene transfer (Federation of Veterinarians of Europe, 2002). Spontaneous mutations in the bacterial chromosomal DNA can alter the target of an antibiotic leading to resistance. Such an event is rare, happening at a frequency of about 1 per 10^7–10^{10} bacteria (Mulvey and Simor, 2009). However, these types of changes are vertically transmissible and the exponential growth rate of bacteria can lead to a substantial increase in the number of resistant bacteria in a population within a short period of time.

2.2.2. Spread of resistance

In addition to treating the pathogen of interest, administering antibiotics to humans or animals also exposes commensal bacteria, leading to the elimination of susceptible organisms and selection for resistant strains. Bacteria carrying resistance genes can then disseminate into the environment or be acquired by other hosts where they may serve as a reservoir of resistance genes. Once disseminated, these bacteria can potentially transfer their resistance genes to

pathogenic bacteria or to other commensals facilitating the spread of resistance genes within the bacterial community (Shaban et al., 2014).

Metagenomic analysis of 30,000 year old DNA identified resistance genes to β-lactam, tetracycline and glycopeptide antibiotics (D'Costa et al., 2011). This phenomenon is not surprising considering the majority of antimicrobial classes originated from naturally occurring substances produced by fungi and bacteria within the environment (Shaban et al., 2014). Antibiotic use in any setting, whether in humans, animals or agriculture can select for antibiotic resistant bacteria. Selection of resistant strains occurs at both lethal and sub-lethal concentrations of the antibiotic (Figure 2.3.). At lethal concentrations, bacteria conferring high resistance are usually selected whilst at sub-lethal concentrations bacteria with low resistance are selected. Sub-lethal concentrations are likely to select for bacteria conferring high resistance with a low fitness cost. However, this scenario is rare because high-level resistance usually is accompanied by a high fitness cost. Consequently, sub-lethal concentrations of antibiotics are more likely to select for highly fit resistant bacteria (Andersson and Hughes, 2012). This scenario is particularly concerning in cases where residual antibiotics enter and contaminate the environment providing selection pressure at sub-lethal concentrations. It highlights the importance of appropriate dosing and length of exposure when using antibiotics to treat individuals, whether animal or human.

2.2.3. Horizontal gene transfer

Horizontal gene transfer (HGT) involves the transfer of antibiotic resistance genes on mobile segments of DNA such as plasmids, transposons or integrons (Mulvey and Simor, 2009). It can greatly accelerate the spread of antibiotic resistance because it can occur amongst strains

of the same species or between genera occupying the same ecological niche (De Niederhausern et al., 2004; Sparo et al., 2011; Vignaroli et al., 2011). Most concerning from a public health perspective, is the transfer of resistance genes from non-pathogenic to pathogenic bacteria, especially if they infect humans. Horizontal gene transfer occurs through three main mechanisms: conjugation, transformation and transduction.

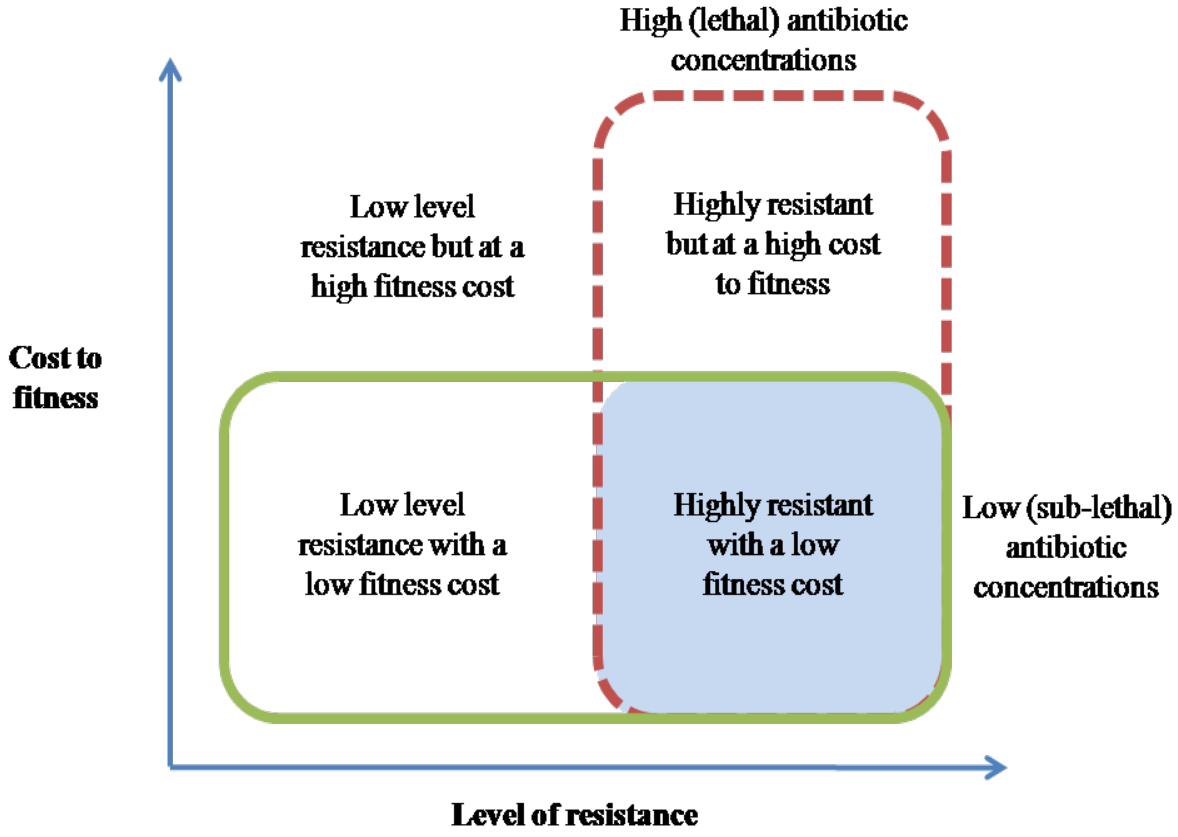

Figure 2.3. Schematic demonstrating how different concentrations of antibiotics influence the characteristics of resistant mutants in terms of their fitness and level of resistance. — — — At high (lethal) antibiotic concentrations, highly resistant mutants are selected, with either a high or low fitness cost. ——— At low (sub-lethal) antibiotic concentrations, mutants with a low fitness cost are selected that are either highly resistant or low level resistant. At both high or low antibiotic concentrations, highly resistant mutants with a low fitness cost can be selected, indicated by the blue shaded box. Figure adapted from Andersson and Hughes (2012).

Conjugation

Conjugation is the most common mechanism of HGT. It involves the transmission of plasmids (extrachromosomal circular fragments of DNA which can replicate semi-autonomously) between bacteria (Thomas, 2004). For plasmids to be transmissible, they require two key genes; the *tra* genes which encode the membrane proteins allowing the bacterium to form a mating pair, and the origin of transfer (*oriT*) genes, which initiate replication of the plasmid and its transfer. A conjugative plasmid contains both of these genes and is thus, self-transmissible. Mobilisable plasmids lack the *tra* genes, but can still be transferred provided the bacterium also contains a conjugative plasmid containing the *tra* genes (Kaiser and Suchman, 2014).

In Gram-negative bacteria, transmission of plasmids occurs through a pilus that extends from the donor to the recipient bacterium. The recipient bacterium has a receptor for the pilus, with bridges or pores being formed between the donor and recipient cell. A copy of the plasmid then passes through the bridge from the donor to the recipient cell. In contrast, Gram-positive bacteria do not form pili during conjugation and instead rely on chemical signalling to promote plasmid transfer. Little is understood about this mechanism of plasmid transfer. However, it is believed it involves a variety of cell surface components and the formation of mating aggregates (Andrup, 1998; Kaiser and Suchman, 2014).

Pheromone-responsive plasmids in enterococci are the most studied conjugal transfer system in Gram-positive bacteria. They are a unique type of plasmid transfer system first described in enterococci by Dunny et al. (1978). Short peptide pheromones are secreted by potential recipient cells, signalling donors carrying the respective plasmids to synthesise an

adhesion that aides in the formation of mating aggregates among recipients. Each pheromone produced corresponds to a particular plasmid and once the recipient has acquired this plasmid, the production of the corresponding pheromone ceases whilst the production of pheromones specific for other plasmids continues (Clewell et al., 2000). The production of pheromones mediates high-frequency plasmid transfer.

Transformation

Transformation involves the uptake of free DNA ("naked DNA") from the environment (Alanis, 2005). Transformation can be a natural or an artificial process with natural transformation only described in a limited number of bacterial species (reviewed in Chen and Dubnau, 2004; and Lorenz and Wackernagel, 1994). Artificial transformation involves the uptake of DNA by physical, chemical or enzymatic treatment and has been exploited by scientists for many years for use in molecular biology.

Transduction

Transduction is a form of gene transfer which involves the use of viral vectors known as bacteriophages to transfer genetic material amongst bacteria (Alanis, 2005). When bacteriophages undergo their replicative cycle inside bacterial cells, sometimes they incorporate the host's cell DNA into their capsids. When the bacteriophage infects a new host, this DNA can then be integrated into the new host's DNA. If the DNA carried by the bacteriophage happens to

contain antibiotic resistance genes then the new host has the potential to become antibiotic resistant (Griffiths et al., 2000).

2.2.4. Mobile genetic elements

Mobile genetic elements (MGEs) are segments of DNA containing the 'machinery' (enzymes and other proteins) required to facilitate their movement within genomes (intracellular movement) or between bacterial cells (intercellular movement) (Frost et al., 2005). Many resistance genes are located on MGEs, therefore, they play a significant role in HGT. A number of MGEs have been identified including transposons, integrons, plasmids and bacteriophages. Of these, transposons and plasmids are the most extensively studied, whilst the role of bacteriophages in the transfer of resistance genes is still under investigation.

Transposons

Transposons are mobile fragments of DNA with the ability to carry multiple resistance genes. They are not self-replicating, but have the ability to move within the genome, for example from chromosome to plasmid (Capita and Alonso-Calleja, 2013). Three different types of transposons have been identified. These are composite transposons, Tn*3* family of transposons and integrative conjugative elements (ICEs) (Weaver et al., 2002).

Composite transposons are composed of a segment of DNA flanked by two insertion sequences (ISs) of the same family, which encode enzymes to promote transposition (Werner et al., 2013). Several families of ISs exist, grouped based on their genetic organisation (Siguier et

al., 2006). Not only do these IS elements allow for mobility of resistance genes, they are also responsible for co-integration of plasmids with other plasmids and with the bacterial chromosome (Hollenbeck and Rice, 2012).

The Tn*3* family of transposons are identified by the presence of a transposase (TnpA) and the replicative mechanism, resolvase (TnpR), which allows them to transpose intracellularly within or between different replicons (Hegstad et al., 2010).

Integrative conjugative elements (ICEs), also known as conjugative transposons, are self-transmissible elements that typically contain three modules ensuring maintenance, dissemination and regulation. Maintenance modules are responsible for integration and excision of ICEs. ICEs integrate into a replicon of their host ensuring vertical inheritance. Dissemination modules contain an array of genes encoding 'mating machinery' which enables the transfer of ICEs via conjugation. Finally, regulation modules encode the genes and the mechanisms responsible for the regulation of ICE transfer (Burrus and Waldor, 2004; Werner et al., 2013). ICEs have been identified in both Gram-positive and Gram-negative bacteria, including Proteobacteria, Actinobacteria and Firmicutes (Roberts and Mullany, 2009; Wozniak and Waldor, 2010). As more information about ICEs is generated, it has become apparent they play a greater evolutionary role than just conferring resistance to antibiotics. ICEs often carry genes that code for other beneficial properties, including resistance to heavy-metals, virulence factors, biofilm formation, nitrogen fixation and metabolic adaptation (Bi et al., 2012; Wozniak and Waldor, 2010).

Integrons

Integrons are genetic units that capture small mobile elements known as gene cassettes (Hall, 2012). Integrons are not in themselves mobile, but are often found within transposons or plasmids (Rice, 2002; Werner et al., 2013). The defining features of an integron include an integrase gene (*intI*), a recombination site (*attI* site) where gene cassettes are inserted, and a promoter (P_c) that directs expression of the genes encoded by the cassette (Hall et al., 1999). Gene cassettes usually include only one gene or open reading frame and an *attC* recombination site. This recombination site is recognised by the integrase gene, enabling splicing of the cassettes into the *attI* site of the integron. This process can occur repeatedly, resulting in a string of gene cassettes. Thus, integrons are capable of containing a few to hundreds of cassettes (Hall, 2012). Although gene cassettes carry only one gene, a pool of more than 130 different cassettes within integrons has been identified with many of these genes coding for antibiotic resistance (Partridge et al., 2009).

Two types of integrons have been identified, mobile and chromosomal (reviewed by Cambray et al., 2010; and Mazel, 2006). Mobile integrons (MI) mostly carry gene cassettes that code for antibiotic resistance genes. These types of integrons are associated with MGEs, enabling their dissemination between bacteria of the same or different species. Within this group, five different classes have been identified and it is likely new classes will be discovered in the future. The different classes are grouped based on the sequence of the encoded integrase. The first three classes are typically involved in the spread of multi-resistance phenotypes, with class 1 integrons being the most ubiquitous. However, all five have been associated with antibiotic resistance determinants. In contrast, chromosomal integrons (CI) are non-mobile with a subset of

integrons within this group being termed superintegrons, as they contain large cassette arrays that contain more than 20 genes.

As gene cassettes are typically promoterless, they rely on the promoter within integrons to regulate their expression (Cambray et al., 2010). Gene cassettes in close proximity to the promoter are highly expressed, with this expression declining as the distance of gene cassettes from the promoter increases. Recombination events, such as excision and integration of cassettes, can displace cassettes to distal positions from the promoter, ultimately silencing them (Guerin et al., 2009).

It has been demonstrated that induction of the SOS response increases integrase expression 4.5-fold in *E. coli* and 37-fold in *Vibrio cholerae* (Guerin et al., 2009). The SOS response is an inducible, widespread regulatory network, allowing bacteria to survive sudden increases in DNA damage. The SOS response is regulated by two main proteins, LexA, a repressor that binds to the "SOS box" and prevents the expression of SOS genes, and RecA, an inducer which binds to single stranded DNA (ssDNA) forming a multimeric nucleoprotein filament that induces the self-cleavage of LexA. When bacteria undergo DNA damage, the presence of ssDNA increases in the cell, activating the RecA protein and subsequent cleavage of LexA. This leads to the expression of the SOS genes and subsequent DNA repair (Michel, 2005; Sutton, 2000). Under normal conditions, SOS repression inhibits the expression of the integrase gene, thus maintaining cassette arrays in their designated order. Certain antibiotics, such as fluoroquinolones, trimethoprim and β-lactams, can induce the SOS response and increase the expression of integrase (Erill et al., 2007; Kelley, 2006). This promotes recombination events which reorder gene cassette positioning, reactivating silenced cassettes or incorporating new cassettes from the surrounding bacterial communities (Guerin et al., 2009).

Resistance mechanisms are usually costly to bacterial fitness, so in the absence of selection by antibiotic exposure they are usually lost. However, the ability to silence these mechanisms when incorporated into cassette arrays ensures they impose no biological cost until they are required. Thus, in this sense, the SOS response ensures the persistence of resistance genes in bacteria whilst also influencing their regulation and expression (Guerin et al., 2009). Induction of the SOS response has also been shown to promote mobilisation of some ICE (Beaber et al., 2004) and transposons (Aleshkin et al., 1998). Induction of an SOS response therefore plays an important role in antibiotic resistance spread by promoting horizontal gene transfer.

Plasmids

Plasmids are extrachromosomal genetic elements which can replicate semi-autonomously (Thomas, 2004). They play a key role in bacterial evolution and horizontal gene transfer (Norman, 2009). Plasmids are classified based on a number of criteria such as mode of replication (rolling-circle, theta or strand displacement replication) and on incompatibility (Inc) which is based on groups of plasmids that fail to co-reside in the same cell (Del Solar, 1998; Novick, 1987). Pheromone responsive plasmids are a unique group of plasmids associated with enterococci which are transferred in response to the excretion of short peptide pheromones (Clewell et al., 2000; Dunny et al., 1978).

Bacteriophages

The role of bacteriophage in the transfer of antimicrobial resistance genes has been investigated with examples of bacteriophage mobilising resistance genes present in the literature. Lytic bacteriophages from the family *Siphoviridae* have been studied in enterococci and transfer of resistance genes by transduction has been demonstrated (Mazaheri Nezhad Fard et al., 2011; Yasmin et al., 2010). Yasmin et al. (2010) investigated transduction in *Enterococcus faecalis*. The genomes of eight representative phages were pyrosequenced with four distinct groups of phages identified. Transduction experiments were performed with generalised transduction occurring in each of the eight phages analysed (Yasmin et al., 2010). Mazaheri Nezhad Fard et al. (2011) was able to demonstrate the transfer of genes coding for resistance to tetracycline and gentamicin through transduction using bacteriophages obtained from strains of *Enterococcus gallinarum* and *E. faecalis* isolated from swine. Not only did this study demonstrate transduction in enterococci, it also demonstrated interspecies transduction: from *E. faecalis* to *E. faecium, Enterococcus hirae/durans* to *Enterococcus casseliflavus;* and from *E. gallinarum* to *E. faecalis* (Mazaheri Nezhad Fard et al., 2011). Despite these findings, further research is still required to determine the role of bacteriophages in transferring genes conferring antibiotic resistance.

2.3. Enterococci

2.3.1. Taxonomy

The genus *Enterococcus* includes more than 33 species and belongs to the phylum of bacteria known as the *Firmicutes* (Garrity et al., 2007). They are part of the lactic acid bacteria (LAB) group, identified by a low G+C (guanine plus cytosine) content of <50 mol% (Holzapfel

and Wood, 1995). This group consists of several other genera of bacteria including *Lactobacillus, Lactococcus, Weissella, Tetragenococcus, Streptococcus, Pediococcus, Leuconostoc* and *Carnobacterium* (Klein et al., 1998). Lactic acid bacteria share a number of similar characteristics including being Gram-positive, catalase negative, non-spore forming with an ability to grow in microaerobic/anaerobic conditions (Klein, 2003).

2.3.2. Physiology

Enterococci are Gram-positive bacteria that occur as cocci, both singly and as chains. They are facultative anaerobes, with the ability to grow in both aerobic and anaerobic conditions. Enterococci can grow over a broad range of temperatures (10 – 45°C) and pH (4.6 to 9.9) as well as in the presence of 40% (w/v) bile salts, a trait used in the formulation of selective media (reviewed in Fisher and Phillips, 2009; and Vu and Carvalho, 2011).

Enterococci are difficult to distinguish from *Streptococcus* spp. and were originally classified as Group D streptococci because both groups possess the Group D cell wall antigen. In 1984, enterococci were reclassified into the single genus, *Enterococcus* (Murray, 1990). Enterococci can be distinguished from streptococci by their ability to survive and grow at high salt concentrations (6.5% NaCl) and under highly alkaline conditions (Schleifer and Kilpper-Balz, 1984).

2.3.3. Distribution

Enterococci can be found in a range of habitats including soil, on plants, in fresh and salt water, sewage and in the gastrointestinal tract of animals (including mammals, birds, fish, reptiles and insects) and humans (Franz et al., 2011). They are often isolated from foods of animal origin due to their presence within the gastrointestinal tract. Enterococci have been isolated from meat, cheese, fish, sausages and ground meat, with *E. faecalis* and *E. faecium* being the predominant species identified (Aslam et al., 2012; Devriese et al., 1995; Peters et al., 2003). This differs from enterococci isolated from plants, where *Enterococcus mundtii* and *E. casseliflavus* are the most common species isolated (Klein, 2003; Micallef et al., 2013).

Enterococci make up an essential part of the gastrointestinal flora of both humans and animals. In humans, *E. faecalis* is the predominant species of enterococci isolated, but *E. faecium* also occurs in high numbers. Counts of *E. faecalis* and *E. faecium* in human faeces range from 10^5–10^7 CFU/g and 10^4–10^5 CFU/g, respectively (Chenoweth and Schaberg, 1990; Noble, 1978). The species of enterococci within the gastrointestinal tract tends to be host specific. In poultry, *E. faecium, E. faecalis* and *Enterococcus cecorum* are regularly isolated. The species of enterococci in the gastrointestinal tract of poultry also varies with the age of the host. Devriese et al. (1991) reported *E. faecium* and *E. faecalis* were dominant enterococci species in day old chicks whereas *E. faecium* was more common in the gastrointestinal tract of 3–4 week old broilers. *E. cecorum* was the dominate species isolated from mature poultry (Devriese et al., 1991). The species distribution of enterococci also varies with maturity in cattle. *Enterococcus avium, E. cecorum, E. durans, E. faecalis, E. faecium* and *E. hirae* have been isolated from suckling calves with *E. faecalis* making up the greatest proportion. In mature dairy cows, the enterococci population is less diverse with *E. faecalis, E. hirae* and *E. casseliflavus* being the

principal species isolated (Devriese et al., 1992). Finally, in pigs it has been reported *E. faecalis, E. faecium, E. cecorum* and *E. hirae* are the most common species isolated (Devriese et al., 1994). It is likely that the species distribution varies with age in pigs, as it does with poultry and cattle, a possibility that has yet to be investigated.

2.3.4. Role of enterococci in food, silage and health

Enterococci play an important role in the fermentation and spoilage of food. They are desirable components of the microflora of many traditional European cheeses where they play a role in the ripening and development of desirable aromas. Enterococci are also associated with the fermentation of sausages and vegetables, including table olives, sauerkraut, kimichi, tomato juice, fruit beans, caper berries and cereal-based products (Foulquie Moreno et al., 2006; M`hir et al., 2012), and the production of silage (Acosta Aragón et al., 2012; Weinberg and Muck, 1996). Not only do enterococci play a role in fermentation, they have also been shown to produce bacteriocins which protect against spoilage or pathogenic bacteria, such as *Listeria monocytogenes.* Known as enterocins in enterococci, they are ribosomally synthesised antimicrobial peptides with activity against closely related Gram-positive bacteria (Khan et al., 2010).

In addition to their role in food and silage production, certain strains of enterococci have been utilised as probiotics to improve human and animal health. They have been used to treat diseases such as irritable bowel syndrome (Enck et al. 2008; Gade and Thorn, 1989), diarrhoea or antibiotic associated diarrhoea (Wunderlich et al., 1989), or improve health through lowering

cholesterol levels (Agerholm-Larsen et al., 2000) and stimulating the immune system (Habermann et al., 2002; Stockert et al., 2007).

2.3.5. Pathogenesis

Although part of the normal microflora of humans, enterococci are often responsible for nosocomial and community-acquired infections, particularly targeting individuals that are immunocompromised or elderly. There are number of virulence factors that contribute to their pathogenicity including aggregation surface adhesin proteins, enterococcal surface protein (Esp), cytolysin, gelatinase and microbial surface components recognising adhesive matrix molecules (MSCRAMMs).

Aggregation substance is a surface adhesion protein, encoded by pheromone responsive plasmids and expression is stimulated by short peptide pheromones secreted by plasmid-free recipient cells (Olmsted et al., 1991; Yagi et al., 1983). Studies have demonstrated aggregation substance increases binding to cultured renal tubular cells (Kreft et al., 1992), promotes adherence and intercellular survival in human macrophages (Sußmuth et al., 2000) and affects the pathogenesis of experimental endocarditis (Schlievert et al., 1998). It is therefore believed to play an important role in enterococcal virulence by facilitating adherence and infection of host cells.

Enterococcal surface protein (Esp) is a cell wall associated protein identified in both *E. faecalis* (Tendolkar et al., 2004) and *E. faecium* (Heikens et al., 2007). It is believed to promote the adhesion, colonisation and evasion of the immune system and increased innate resistance to antibiotics through the formation of biofilms. The ability to form biofilms can also facilitate the

attachment to abiotic surfaces such as intrauterine devices and catheters, aiding in transmission and spread of hospital acquired infections (Donlan, 2002).

Cytolysin is a two-peptide lytic toxin that exhibits both haemolytic and bacteriocin activity and is usually encoded by pheromone-responsive plasmids (Clewell, 2007; Ike et al., 1990) or pathogenicity islands (Shankar et al., 2002) in strains of *E. faecalis* (Cox et al., 2005). Its bacteriocin activity is believed to assist in its growth and persistence by inhibiting the growth of other Gram-positive bacteria (Brock et al., 1963; Jett and Gilmore, 1990), whilst its haemolytic properties can lyse macrophages and neutrophils enabling it to circumvent immune responses (Miyazaki et al., 1993).

Gelatinase is a bacterial protease produced by *E. faecalis*. This enzyme hydrolyses gelatin, collagen, casein and haemoglobin (Su et al., 1991). Secretion of gelatinase is controlled by the two-component *fsr* system comprised of the genes *fsrA, fsrB, fsrC* and *fsrD*. This system plays a role in the expression of the protease genes, *gelE* and *spreE,* which encode for gelatinase and serine protease, respectively (Nakayama et al., 2006; Qin et al., 2000). The production of gelatinase is also suggested to play a role in biofilm formation in *E. faecalis* (Hancock and Perego, 2004).

Microbial surface components recognising adhesive matrix molecules (MSCRAMMs) are important in the establishment of infections. They facilitate adherence to the host's extracellular matrix (ECM). Two well-studied MSCRAMMs in enterococci are Ace in *E. faecalis* and Acm in *E. faecium*. Ace is conditionally expressed in the presence of collagen or serum, binding to the ECM components collagen type I (CI), collagen type IV (CIV) and lamina (LN) (Nallapareddy et al., 2000; Nallapareddy and Murray, 2006), whilst Acm binds to CI

(Nallapareddy et al., 2003). Under normal conditions, epithelial or endothelial cells cover ECMs and prevent binding. However, following trauma or damage to the host tissues it can result in ECMs becoming exposed, allowing enterococci to colonise and cause infection (Nallapareddy et al., 2000).

2.3.6. Clinical infections, epidemiology and VRE

Despite their usual commensal nature, enterococci are becoming increasingly important as pathogens. Their increased involvement in the development of clinical infections is in part due to their intrinsic resistance to certain antibiotics including clindamycin, cephalosporins and aminoglycosides, but also their ability to acquire resistance to antibiotics such as vancomycin. Resistance to vancomycin is of particular concern as it is a last resort antibiotic in the treatment of penicillin resistant pathogens such as MRSA, and an important alternative to ampicillin for patients allergic to β-lactam antibiotics (Arias and Murray, 2012; Levine, 2006).

In humans, enterococci are associated with urinary tract infections, hepatobiliary sepsis, endocarditis, surgical wound infections, bacteraemia and neonatal sepsis (Agudelo Higuita and Huycke, 2014; Poh et al., 2006). Healthcare-associated enterococcal infections are predominantly caused by *E. faecalis* and *E. faecium* (Sivert et al., 2013). *E. avium, E. casseliflavus, E. durans, E. gallinarum, E. hirae, Enterococcus raffinosus* and *E. mundtii* have also been known to cause clinical infections, but far less frequently than *E. faecalis* and *E. faecium* (De Perio et al., 2006; Gordon et al., 1992).

Increased use of vancomycin and broad-spectrum antibiotics has contributed to emerging resistance in enterococci and has changed the epidemiology of enterococcal infections. In the

past, *E. faecalis* was the predominant species isolated from clinical infections, but more recently *E. faecium* has been more frequently isolated (Deshpande et al., 2007; Mutnick et al., 2003). This trend follows the increase in VRE, as *E. faecium* is ten times more likely to be resistant to vancomycin than *E. faecalis* (Iwen et al., 1997). In the United States, the incidence of hospitalisations with VRE infections more than doubled between 2000 and 2006 (Ramsey and Zilberberg, 2009). VRE are now widely distributed having been isolated from patients in the United Kingdom, France, Belgium, Denmark, Germany, Italy, The Netherlands, Spain, Sweden, USA, Canada, Malaysia and Australia (Cetinkaya et al., 2000).

2.3.7. Antibiotic resistance in enterococci

Intrinsic resistance

<u>β-lactams</u>

The cell wall of Gram-positive bacteria consists of an outer thick peptidoglycan layer, with attached accessory molecules including teichoic acids, teichuronic acids, polyphosphates or carbohydrates. Assembly of the cell wall is catalysed by penicillin binding proteins (PBPs), such as transpeptidases and carboxypeptidases, which are the target of β-lactam antibiotics (Navarre and Schneewind, 1999). β-lactams bind covalently to PBPs and thereby inhibit cell wall synthesis (Zapun et al., 2008). Penicillin binding proteins produced by enterococci have a low-affinity for β-lactam antibiotics resulting in an inherent low-level of resistance (Fontana et al., 1983; Fontana et al., 1985). Overproduction of PBPs has also been attributed to increased resistance (Fontana et al., 1994). The minimum inhibitory concentration for 90 percent of strains (MIC_{90}) to penicillin for *E. faecalis* and *E. faecium* is 4 μg/mL and >64 μg/mL, respectively,

much higher than reported for streptococci and other related Gram-positive organisms (Murray, 1990; Weinstein, 2001).

Aminoglycosides

Enterococci have intrinsic resistance to low to moderate levels of aminoglycosides, such as streptomycin and gentamicin. This low to moderate level of resistance is attributed to a decreased uptake of these antibiotics (Kristich et al., 2014). This is generally overcome with the synergistic use of cell-wall active antibiotics such as β-lactams and glycopeptides, which increase the uptake of these molecules (Moellering and Weinberg, 1971). This therapeutic approach can be negated by the acquisition of high-level aminoglycoside resistance (Kristich et al., 2014).

Lincosamides and streptogramins

E. faecalis is intrinsically resistant to clindamycin (a lincosamide), quinupristin (a streptogramin B class) and dalpfopristin (a streptogramin A class). Resistance is conferred by the expression of the resistance gene *lsa*, believed to be responsible for encoding an ATP-binding cassette (ABC)-efflux pump targeted at these antibiotics (Singh et al., 2002).

Trimethoprim-sulfamethoxazole

Trimethoprim-sulfamethoxazole inhibits folate synthesis by targeting steps in the tetrahydrofolate synthesis pathway responsible for folate synthesis. Many bacteria rely on this pathway for the production of folate, as they lack the ability to acquire it from the environment. Without folate, bacteria cannot produce nucleic acids and therefore are killed by the activity of these antibiotics (Hollenbeck and Rice, 2012). Enterococci are intrinsically resistant to this combination of antibiotics as they have the ability to absorb folate from the environment, rendering trimethoprim-sulfamethoxazole ineffective (Zervos and Schaberg, 1985).

Acquired resistance

Glycopeptides

Glycopeptide resistance is well documented in enterococci (Clark et al., 1993; Liassine et al., 1998; Mascini and Bonten, 2005). High-level resistance to vancomycin, a critically important glycopeptide, has been increasingly reported in nosocomial infections (Ramsey and Zilberberg, 2009). This is important because of the ability of enterococci to transfer resistance not only to antibiotic-susceptible enterococci, but also potentially to other pathogens. Vancomycin is an essential antibiotic used in the treatment of infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) (Mascini and Bonten, 2005). Transfer of vancomycin resistance from VRE to MRSA has been documented (Centers for Disease Control and Prevention, 2002; Centers for Disease Control and Prevention, 2004; Chang et al., 2003).

Nine distinct gene clusters have been associated with glycopeptide resistance and described in enterococci. The most common among clinical isolates are the VanA and VanB types which have been studied in the greatest detail (Kristich et al., 2014). These two gene clusters are acquired and confer moderate to high-level glycopeptide resistance. Intrinsic resistance, conferred by the VanC operon, provides low levels of vancomycin resistance. It is chromosomally located and non-transferrable. Three species of enterococci have been shown to harbour the VanC operon, namely *E. gallinarum, E. casseliflavus* and *Enterococcus flavescens*. Each species has a unique set of genes contained in this operon which encode the ligase-related proteins; *vanC*-1 for *E. gallinarum, vanC*-2 for *E. casseliflavus* and *vanC*-3 for *E. flavescens* (Leclercq et al., 1992; Navarro and Courvalin, 1994).

In peptidoglycan synthesis, glycan chains composed of a repeating disaccharide, *N*-acetylmuramic acid-(β1-4)-*N*-acetyleglycosamine (MurNAc-GlcNAc), are linked by cross bridge peptides that connect short wall peptides (consisting of three to five amino acids) that branch off the MurNAc segment of the glycan chain (Navarre and Schneewind, 1999). These peptidoglycan precursors (glycan chain with branching chain of peptides) typically end with a D-alanine-D-alanine (D-Ala-D-Ala) dipeptide. Glycopeptides act to inhibit cell wall synthesis by binding to the D-Ala-D-Ala terminus of the peptidoglycan precursor thus preventing peptidoglycan synthesis (Kristich et al., 2014).

Glycopeptide resistance is achieved through the synergistic action of two pathways. The first pathway involves replacement of the terminal D-Ala in a peptidoglycan precursor with D-lactate (D-Lac) or D-serine (D-Ser) and the second is prevention of the synthesis or destruction of peptidoglycan precursors which end in D-Ala-D-Ala by action of specific D,D-carboxypeptidases. The production of modified peptidoglycan precursors and destruction of

those ending with D-Ala-D-Ala is achieved by the production of enzymes encoded by the glycopeptide gene clusters (Kristich et al., 2014). Replacement of D-Ala with D-Lac or D-Ser reduces the binding affinity of glycopeptides to peptidoglycan precursors, effectively reducing their ability to inhibit cell wall synthesis. In the case of D-Lac, the binding affinity is reduced 1,000 fold conferring high-level glycopeptide resistance while with D-Ser the reduction in affinity is less pronounced (approximately 7 fold), thus conferring low-level glycopeptide resistance (Billot-Klein et al., 1994; Bugg et al., 1991). The intrinsic VanC operon leads to the replacement of D-Ala with D-Ser, whilst VanA and VanB operons replace D-Ala with D-Lac (Arthur et al., 1996).

The VanA and VanB operons are acquired by enterococci through the transfer of transposons or plasmids, specifically, the Tn*1546* transposon for VanA and Tn*1549* and/or Tn*5382* for VanB (Arthur et al., 1993; Carias et al., 1998; Garnier et al., 2000). The VanA operon contains seven genes (*vanR, vanS, vanH, vanA, vanX, vanY* and *vanZ*) and confers inducible resistance to high levels of vancomycin and teicoplanin (Arthur et al., 1996). The VanB operon is organised and functions in a similar manner to the VanA operon. However, unlike VanA, resistance is induced by vancomycin but not teicoplanin (Ribeiro et al., 2011). The genes of the VanB operon consist of $vanR_B$, $vanS_B$, $vanH_B$, *vanB*, $vanX_B$, $vanY_B$, *vanW* and v*anV* (Evers and Courvalin, 1996; Ribeiro et al., 2011). The *vanR*/$vanR_B$ and *vanS*/$vanS_B$ genes encode a two-component regulatory system that regulates the expression of glycopeptide resistance genes. The *vanH*/vanH_B and *vanA/vanB* genes are involved in the synthesis of depsipeptide D-alanyl-D-lactate and *vanX*/$vanX_B$ and *vanY*/$vanY_B$ are responsible for the hydrolysis of peptidoglycan precursors containing the D-Ala-D-Ala dipeptide (Arthur et al., 1996). The *vanZ* gene confers low-level teicoplanin resistance through an unknown mechanism (Evers and

Courvalin, 1996). The function of the *vanW* and *vanV* genes of the VanB operon is also unknown, with *vanV* gene not found in all VanB operons (Ribeiro et al., 2011).

Aminoglycosides

As described above, enterococci have inherent resistance to low to moderate levels of aminoglycosides, and can acquire high-level resistance to all aminoglycosides, including gentamicin and streptomycin. High-level resistance to gentamicin and streptomycin is of particular concern because these antibiotics are used synergistically in the treatment of serious enterococcal infections (Chow, 2000).

Aminoglycosides bind to prokaryotic ribosomes thus disrupting protein synthesis. Genes conferring high-level aminoglycoside resistance are usually encoded on plasmids, but are also associated with transposons (Hodel-Christian and Murray, 1992; Simjee et al., 2000). High-level resistance to all aminoglycosides, except for streptomycin, is encoded by the bi-functional aminoglycoside-modifying enzyme AAC(6')-Ie-APH(2'')-Ia. High-level resistance to streptomycin arises from ribosomal mutations altering the S12 ribosomal protein or by the acquisition of a gene coding for a nucleotidyltransferases, ANT(3'')-Ia or ANT(6')-Ia, which inactivates this aminoglycoside (Chow, 2000). The binding affinity of aminoglycoside antibiotics to the bacterial ribosome is reduced by the action of these aminoglycoside-modifying enzymes which catalyse the covalent modification of amino and hydroxyl groups within the aminoglycoside molecule (Mingeot-Leclercq et al., 1999).

Oxazolidinones

Linezolid is an oxazolidinone used in the treatment of infections caused by VRE. Resistance is most often due to point mutations of the 23S ribosomal RNA ribosome-binding site or through acquisition of the *cfr* gene (Long et al, 2006; Prystowsky et al., 2001). Resistance to linezolid is still rare in enterococci, but has been documented in enterococci isolated from humans (Patel et al., 2013). The first report of the *cfr* gene in an *E. faecalis* strain isolated from cattle was from China in 2011 (Liu et al., 2012). This gene encodes for resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins and streptogramin A (Long et al., 2006). Liu et al. (2012) reported *cfr* to be located on a plasmid (pEF-01) in *E. faecalis* EF-01. The transferability of pEF-01 from *E. faecalis* EF-01 was assessed through conjugation and transformation assays. Transfer of pEF-01 from *E. faecalis* EF-01 to *E. faecalis* JH2-2 through conjugation was unsuccessful. However, successful transformation of pEF-01 to *E. faecalis* JH2-2 and *S. aureus* RN4220 by electrotransformation was demonstrated (Liu et al., 2012). The pEF-01 plasmid was functional in both *E. faecalis* JH2-2 and *S. aureus* RN4220 following transformation, suggesting dissemination of the *cfr* gene through plasmid transfer may occur. The *cfr* gene has also been recently identified in a human clinical isolate of *E. faecium* (Patel et al., 2013). This is a significant finding because even though linezolid resistance is rare, dissemination of the *cfr* gene may increase the prevalence of resistant enterococci.

Lipopeptides

Daptomycin is a lipopeptide that has bactericidal activity against enterococci (Akins and Rybak, 2001; Jorgensen et al., 2003). The mechanism of daptomycin resistance in enterococci is

not fully understood, but recent comparative whole-genome sequencing of a daptomycin-resistant *E. faecalis* suggested mutations that alter the ultrastructure of the cell membrane and cell wall may contribute to resistance (Arias et al., 2011). This study identified three genes with in-frame deletions in the daptomycin-resistant strain of *E. faecalis* that were absent in susceptible strains. Two genes encoded for the putative enzymes, glycerophosphoryl diester phosphodiesterase (GdpD) and cardiolipin synthase (Cls), which are believed to be involved in phospholipid metabolism. The third gene encoded for a putative membrane protein, lipid II cycle-interfering antibiotic protein (LiaF) believed to be a member of a three-component regulatory system (LiaFSR). This system is involved in the stress-sensing response to antibiotics by the cell envelope (Arias et al., 2011). It was determined mutations in LiaF and GdpD were necessary for enterococci to be resistant to daptomycin (Arias et al., 2011). In another study, genomic data obtained from the multidrug-resistant *E. faecalis* strain V583 identified seven proteins with mutations associated with daptomycin resistance, including Cls. They further confirmed the role of the *cls* mutant allele in daptomycin resistance through transfer studies. However, additional daptomycin-resistant mutants lacking the *cls* mutation were also observed suggesting alternative pathways to daptomycin resistance may also exist (Palmer et al., 2011).

Macrolide, lincosamide and streptogramins

The macrolide-lincosamide-streptogramin B (MLS_B) superfamily is a group of structurally unrelated antibiotics which act to bind the 50S ribosomal subunit in bacteria. Binding blocks peptide bond formation and translation thus inhibiting protein synthesis (Roberts et al, 1999; Roberts, 2008).

A number of resistance genes have been identified in enterococci which confer MLS_B resistance (Table 2.5.). Ribosomal methylation is a major mechanism of resistance, encoded by *erm* genes. The product of these genes (rRNA methylases) alter the binding site for antibiotics of the MLS_B superfamily resulting in resistance (Weisblum, 1995). Other mechanisms of resistance also exist, however these confer resistance to only one or two antibiotic classes of the MLS_B superfamily. These include efflux proteins and inactivating enzymes including esterases, lysases, transferases and phosphorylases (Roberts et al., 1999; Roberts, 2008).

Macrolides used in animal production are not the same as those used in human medicine. However, the ability of *erm* genes to confer resistance to multiple antibiotics in the MLS_B superfamily, including those used in human medicine such as erythromycin, is concerning. Macrolide resistance genes are often found linked with resistance genes conferring resistance to other antimicrobials, such as glycopeptides and tetracyclines. They are also often found located on MGEs such as plasmids and transposons suggesting the use of macrolides in animal production could also be co-selecting for resistance to antibiotics other than macrolides.

Examples of MGEs conferring MLS_B resistance include pheromone-responsive conjugative plasmids such as the one found in *E. faecalis* isolated from a chicken, described carrying five drug resistance determinants including *vanA*, *erm*(B), *aph(3')*, *aph(6')* and *aac(6')/aph(2')*, encoding for resistance to vancomycin, erythromycin, kanamycin, streptomycin and gentamicin/kanamycin, respectively (Lim et al., 2006). Plasmid co-localisation of *tet*(O) with *erm*(B) has also been described in *E. faecalis* isolates from poultry, and *tet*(M) with *erm*(B) in *E. faecalis* isolates from pigs (Tremblay et al., 2011; Tremblay et al., 2012). Transposons identified in enterococci carrying resistance to MLS_B antibiotics include the composite transposons Tn*5384* (Bonafede et al., 1997) and Tn*5385* (Rice and Carias, 1998; Rice, 2002)

both linked to the transfer of *erm*(B), Tn*3* family of transposons including Tn*917* and Tn*3871* (Banai and LeBlanc, 1984; Shaw and Clewell, 1985) and ICEs including Tn*1545* and variants (De Leener et al., 2004).

2.4. Implications of Horizontal Gene Transfer

One of the biggest concerns surrounding resistance development in enterococci is the horizontal transfer of resistance genes from non-pathogenic to pathogenic bacteria that cause infections in humans. It is hypothesised that human intestinal bacteria may serve as a reservoir of resistance genes, with transfer occurring among naturally residing intestinal bacteria or to ingested bacteria, including pathogenic bacteria that may contaminate food. Commensal bacteria, many of which are opportunistic pathogens, have the potential to cause post-surgical infections with acquisition of resistance genes increasing the difficulty of successful therapy (Salyers et al., 2004). This phenomenon is difficult to study in humans. However, a number of *in vitro* and *in vivo* studies have been conducted investigating the transfer of resistance genes in enterococci (Tables 2.6. and 2.7.). These studies demonstrated intra- and inter-species transfer of resistance genes in enterococci, transfer between enterococci and other bacterial genera and transfer between enterococci strains isolated from humans and livestock.

Despite the difficulty of studying horizontal gene transfer in humans, transient transfer of resistance genes has been demonstrated. Human volunteers were used to assess if a strain of *E. faecium* from chickens that contained *van*A, *erm*(B) and *vat*(E) could transfer resistance to *E. faecium* colonising the gut of the participants in the study. Transfer of *van*A was demonstrated in three out of the six humans participating in the study. Even though colonisation was transient,

this study demonstrated transfer of resistance genes between bacteria originating from chickens to bacteria from humans could occur within the human intestinal tract (Lester et al., 2006). If colonisation of the human gastrointestinal tract with antibiotic resistant bacteria was to occur, this could lead to further dissemination of resistance genes or hinder the effectiveness of antibiotics in the treatment of opportunistic infections. Thus, transmission of antibiotic resistant enterococci from animals to humans through direct contact, the environment or food represents a public health risk.

2.5. Comparative Genomics of Enterococci

The advancement of next-generation technologies has reduced the time and cost associated with sequencing bacterial genomes (Loman et al., 2012; Stahl and Lundeberg, 2012). Consequently, more and more genomes have been sequenced revolutionising the way we study bacteria. Comparative genomics is a technique used to compare the genomes of multiple bacteria, allowing identification of similarities and differences among organisms.

The first enterococcal genome sequenced was *E. faecalis* V583, published in 2003 (Paulsen et al., 2003). Since then, hundreds of enterococci have been sequenced with complete and draft genome sequences available (http://www.ncbi.nlm.nih.gov/genome). *E. faecium* and *E. faecalis* make up the bulk of genome sequences available, due to their association and importance as nosocomial and community-acquired infections in humans. Examination of enterococcal genomes has expanded our knowledge of their population structure, evolutionary history and basic biology.

Multilocus sequence typing (MLST) has been used to describe the genetic relatedness between strains of *E. faecium* and *E. faecalis* in order to define their evolutionary history (Ruiz-Garbajosa et al., 2006; Willems et al., 2005). MLST involves the sequencing and analysis of housekeeping genes present in different locations on a chromosome. A limitation of this technique is the limited number of alleles assessed, only seven for *E. faecium* and *E. faecalis* (Homan et al., 2002; Ruiz-Garbajosa et al., 2006). Despite this limitation, MLST analysis has assisted in the understanding of population structure and evolution of *E. faecium* and *E. faecalis* and has assisted in the selection of isolates for whole genome sequencing (Ruiz-Garbajosa et al., 2006; Willems et al., 2005; Willems et al., 2012).

Comparison of whole genome sequences can be used to overcome the limitations associated with MLST analysis and has been used to study the population structure and evolution of *E. faecium* and *E. faecalis*. Initial investigation of *E. faecium* population structure using MLST analysis revealed a major split in the *E. faecium* population (Willems et al., 2012). This split was confirmed following analysis of 6 *E. faecium* genomes which identified two clades designated as clade A and clade B (Palmer et al., 2012). A more recent study examined 51 newly sequenced *E. faecium* genomes and found evidence of a second split within clade A, designated clade A1 and A2 (Lebreton et al., 2013). Clade A and B separate hospital-associated and human commensal isolates, whilst clade A1 distinguishes clinical isolates from most animal-derived strains in A2. Mutation rates were also used to estimate the time of divergence between clades, with the split between clade A and B estimated to have occurred 3,000 years ago and the split between clade A1 and A2 occurring only 75 years ago (Lebreton et al., 2013). In this study, a commensal strain was found to cluster in clade A and an infecting hospital strain was found to

cluster in clade B, suggesting the ecological distinction between clades is not absolute. In contrast to *E. faecium*, *E. faecalis* shows little phylogenetic divergence (Palmer et al., 2012).

Comparative genomic analysis has been used to study the basic biology of enterococci and has identified important structures contributing to virulence, including pathogenicity islands in *E. faecalis* (Shankar et al., 2002) and *E. faecium* (Lam et al, 2012; van Schaik et al., 2010). It has been useful in the identification of plasmids and MGEs associated with antibiotic resistance (Hegstad et al, 2010; Palmer et al., 2010) and has provided insight into genome plasticity. Clustered, regularly interspaced short palindromic repeats (CRISPR) with CRISPR-associated (*cas*) genes are a system used by prokaryotes as a type of immune defence against the invasion of viruses and plasmids (Wiedenheft et al., 2012). Examination of *E. faecium* and *E. faecalis* genomes has revealed an inverse relationship between CRISPR-*cas* and antibiotic resistance, suggesting antibiotic use selects for enterococci with a compromised genome defence system, making them susceptible to the acquisition of antibiotic resistance genes (Palmer and Gilmore, 2010). Pan-genome analysis is used to estimate the total size of the gene pool accessible to a single species and investigate genomic diversity. The *E. faecium* pan-genome is considered open, meaning *E. faecium* can acquire and incorporate novel DNA into its gene pool contributing to the high genomic diversity between strains and enabling this species to adapt to different environments through the acquisition of new genes (Van Schaik et al., 2010). Investigation of the pan-genomes of other enterococci species has yet to be conducted.

There has been an increase in the number of genomes of other enterococci species that have been characterised. Investigation of these genomes is important in understanding the diversity of the genus *Enterococcus*. Already studies have provided insight into the genetic basis

for motility and pigmentation as seen in *E. casseliflavus* and *E. gallinarum*, and differences in metabolism that discriminate different enterococcal species (Palmer et al., 2012).

Comparative genomic analysis of enterococci is still in its infancy. A number of areas still need to be addressed for further advancements in this field. Firstly, available *E. faecium* and *E. faecalis* genomes are mostly isolates originating from human infection or from hospitalized patients colonized by antibiotic resistant strains. There is a poor representation of strains isolated from healthy humans and non-human sources as well as an overrepresentation of strains from Europe and North America (Palmer et al., 2014). Furthermore, there is a lack of sequence data available for species other than *E. faecium* and *E. faecalis*. As more sequences become available, comparative genomics offers a new way to search for traits unique to each species.

2.6. References

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Tables

Table 2.1. Antimicrobials registered for use in animals

Antibiotic class	Antibiotic	USA	Canada	Australia
Aminoglycosides	Amikacin	D, H	H	-
	Apramycin	Sw	Sw	C
	Gentamicin	D, Ca, H, P, Sw, C	C, Sw, P, H, Ca, D	H
	Neomycin	Ca, D, H, G, Sh, Sw, P	C, D, Ca, Sw, H, Sh, P	C, Sw, Sh, P
	Spectinomycin	P, Sw, D	Sw, P, Ca, D	Sw
	Streptomycin	P, C, D, H, Sw	C, Sw, P	C, Sw, Sh
Cephalosporins	Cefadroxil	Ca, D	Ca, D	-
	Ceftiofur	C, H, Sw, P, H, G, Sh, D	C, Sw, H, Sh, D	C, H
	Cephapirin	C	C	-
Chloramphenicol and Congeners	Chloramphenicol	D, Ca	Ca, D	-
	Florfenicol	C, Sw, Fi	Fi, C, Sw, P	-
Fluoroquinolones	Enrofloxacin	Ca, D, C, Sw	Ca, D, C, Sw	-
	Marbofloxacin	D, Ca	D, Ca	-
	Orbifloxacin	Ca, D	Ca, D	-
Glycopeptides	Avoparcin	-	-	P, Sw, C
Lincosamides	Clindamycin	D, Ca	D, Ca	-
	Lincomycin hydrochloride	Ca, D, Sw, P, Bees	Sw, P, Ca, D	C, Sw, P
	Pirlimycin	C	C	-
Macrolides	Erythromycin	C, P, Ca, D, Sw	C, Sw, Sh, P	C, Sw, P, Sh
	Tilimicosin	C, Sh, Sw	C, Sh	C, Sw
	Tildipirosin	C	C	-
	Tulthromycin	C, Sw	C, Sw	-

Table 2.1. Continued

Antibiotic class	Antibiotic	USA	Canada	Australia
	Tylosin	C, P, Sw, Ca, D, Bees	C, Sw, P	C, Sw, P, Sh
Nitrofurans	Nitrofurantoin	-	H, Ca, D	-
	Nitrofurazone	Ca, D, H	H, D, C, Sw, G, Sh, Ca	-
	Amoxicillin	D, Sw, Ca, C	Ca, D	C, Sw, Sh, P
Penicillins	Amoxicillin, Clavulanic acid	-	D, Ca	-
	Ampicillin	C, D, Ca, Sw, H	C, Sw, Ca, D	C, Sw
	Cloxacillin	D, C	C	C
	Penicillin G benzathine	C, D, H	C, Sw, H, Ca, Sh, D	Sh, C
	Penicillin G potassium	P, Ca, D	Ca, Sw, P	-
	Penicillin G procaine	Sw, P, C, H, Sh, D, Ca	Sw, C, H, Ca, Sh, D, P	C, Sw, Sh
	Polymixin B	Ca, C, D, H, Sh	C	-
	Virginiamycin	P, Sw	Sw, P	C, Sh, Sw
Polymixin	Chlortetracycline	Sw, C, P, Sh	C, Sw, P, Sh,	C, Sw, Sh, P
Streptogramins	Oxytetracycline	Ca, D, C, Bees, P, Fi, Sh, Sw, H	C, Sw, Sh, P, Fi, Bees	C, Sw, Sh, P, H, Bees
Tetracyclines	Tetracycline hydrochloride	D, C, Ca, P	C, Sw, P, Sh	-
	Doxycycline	D	D	-
	Tiamulin	Sw	Sw	-
	Sulfadiazine	D, H	D, Ca, H, Fi	-
Pleuromutilins	Sulfadimethoxine	Ca, D, C, P, H, Fi	Fi, Ca, D	-

Table 2.1. Continued

Antibiotic class	Antibiotic	USA	Canada	Australia
Sulfonamides	Sulfaguanidine	-	C, Sw, H, Sh, D, Ca	-
	Sulfamethazine	C, P, Sw, H	C, Sw, Sh, H, Ca, D, G, P	-
	Trimethoprim	D, H	C, Sw, Ca, D, Fi, H	H
	Ormetoprim	P, D, Fi	Fi	-
Diaminopyrimidines	Lasolocid sodium	C, P, Sh	C, P	C
	Maduramicin	P	P	-
Ionophores	Monensin	P, C, G	C, P	C
	Narasin	P, Sw	P, Sw	C
	Salinomycin sodium	P	P, C, Sw	Sw, C
	Arsanilic acid	P, Sw	P, Sw	-
	Bacitracin	P, C, Sw	Sw, P, Ca, D	P
Miscellaneous drugs	Bambermycins	P, Sw, C	P	Sw, P, C
Bacitracins	Olaquinodox	-	-	Sw
Bambermycins	Carbadox	Sw	-	-

Abbreviations: P, poultry; C, cattle; Ca, cat; D, dog; Fi, fish; Sw, swine; Sh, sheep; H, horse.

Table 2.2. Ranking of antimicrobials by the World Health Organization (WHO)

Classification	Antimicrobial class	Antimicrobial(s)	Criteria 1[a]	Criteria 2[b]
Critically important	Aminoglycosides	Amikacin, arbekacin, bekanamycin, dibekacin, dihydrostreptomycin, gentamicin, isepamicin, kanamycin, neomycin, netilmicin, ribostamycin, sisomicin, streptoduocin, streptomycin, tobramycin (*Veterinary use only:* apramycin, framycetin)	Yes	Yes
	Carbapenems and other penems	Biapenem, doripenem, ertapenem, faropenem, imipenem, meropenem, panipenem	Yes	Yes
	Cephalosporins, third and fourth generation	Cefcapene, cefdinir, cefditoren, cefepime, cefetamet, cefixime, cefmenoxime, cefodizime, cefoperazone, cefoselis, cefotaxime, cefozopran, cefpiramide, cefpirome, cefpodoxime, cefsulodin, ceftraoline, ceftazidime, ceftizoxime, ceftobiprole, ceftibuten, ceftriazone, latamoxef (*Veterinary use only*: cefovecin, cefquinome, ceftiofur)	Yes	Yes
	Cyclic esters	Fosfomycin	Yes	Yes
	Fluoro- and other quinolones	Cinoxacin, ciprofloxacin, enoxacin, fleroxacin, flumequine, garenoxacin, gatifloxacin, gemifloxacin, grepafloxacin, levofloxacin, lomefloxacin, moxifloxacin, nalidixic acid, norfloxacin, ofloxacin, oxolinic acid, pazufloxacin, pefloxacin, pipemidic acid, piromidic acid, prulifloxacin, rosoxacin, rufloxacin, sitafloxacin, sparfloxacin, temafloxacin, trovafloxacin (*Veterinary use only*: danofloxacin, difloxacin, enrofloxacin, ibafloxacin, marbofloxacin, orbifloxacin)	Yes	Yes

Table 2.2. Continued

Classification	Antimicrobial class	Antimicrobial(s)	Criteria 1[a]	Criteria 2[b]
	Glycopeptides	Dalbavancin, oritavancin, teicoplanin, telavancin, vancomycine (*Veterinary use only*: Avoparcin)	Yes	Yes
	Glycylcyclines	Tigecycline	Yes	Yes
	Lipopeptides	Daptomycin	Yes	Yes
	Macrolides and ketolides	Azithromycin, clarithromycin, erythromycin, dirithromycin, flurithromycin, josamycin, midecamycin, miocamycin, oleandomycin, rokitamycin, roxithromycin, spiramycin, telithromycin, trolandomycin (*Veterinary use only*: gamithromycin, kitasamycin, tildipirosin, tilmicosin, tulathromycin, tylosin, tylvalosin)	Yes	Yes
	Monobactams	Aztreonam, carumonam	Yes	Yes
	Oxazolidinones	Linezolid	Yes	Yes
	Penicillins, including natural penicillins, aminopenicillins, and antipseudomonals	Amoxicillin, ampicillin, azidocillin, azlocillin, bacampicillin, carbenicillin, carindacillin, clometocillin, epicillin, hetacillin, metampicillin, meticillin, mexlocillin, penamecillin, penicillin G, penicillin V, pheneticillin, piperacillin, pivampicillin, propicillin, sulbenicillin, sultamicillin, talampicillin, temocillin, ticarcillin (*Veterinary use only*: penethamate hydroiodide)	Yes	Yes
	Polymyxins	Colistin and polymyxin B	Yes	Yes

Table 2.2. Continued

Classification	Antimicrobial class	Antimicrobial(s)	Criteria 1[a]	Criteria 2[b]
	Rifamycins	Rifabutin, rifampicin, rifaximin, rifapentine, rifamycin	Yes	Yes
	Drugs solely to treat tuberculosis or other mycobacterial diseases	Calcium aminosalicylate, capreomycin, cycloserine, ethambutol, ethionamide, isoniazid, morinamide, para-aminosalicyclic acid, protionamide, pyrazinamide, sodium aminosalicylate, terizidone, tiocarlide	Yes	Yes
Highly important	Amdinopenicillins	Mecillinam, pivmecillinam	No	Yes
	Amphenicols	Chloramphenicol, thiamphenicol (*Veterinary use only*: florfenicol)	No	Yes
	Cephalosporins (first and second generations) and cephamycins	Cefaclor, cefacetrile, cefadroxil, cefaloridine, cefalexin, cefalotin, cefamandole, cefapirin, cefatrizine, cefazedone, cefazolin, cefbuperazone, cefmetazole, cefminox, cefonicid, ceforanide, cefotetan, cefotiam, cefoxitin, cefprozil, cefradine, cefroxadine, ceftezole, cefuroxime, flomoxef, loracarbef (*Veterinary use only*: cefalonium)	No	Yes
	Lincosamides	Clindamycin, lincomycin (*Veterinary use only*: pirlimycin)	No	Yes
	Penicillins (Antistaphylococcal)	Cloxacillin, dicloxacillin, flucloxacillin, oxacillin, nafcillin	No	Yes
	Pleuromutilins	Retapamulin	No	Yes
	Pseudomonic acids	Mupirocin	No	Yes

Table 2.2. Continued

Classification	Antimicrobial class	Antimicrobial(s)	Criteria 1[a]	Criteria 2[b]
	Riminofenazines	Clofazimine	Yes	No
	Steroid antibacterials	Fusidic acid	No	Yes
	Streptogramins	Quinupristin-dalfopristin, pristinamycin (*Veterinary use only*: virginiamycin)	No	Yes
	Sulfonamides, dihydrofolate reductase inhibitors, and combinations	Brodimoprim, iclaprim, pyrimethamine, sulfadiazine, sulfadimethoxine, sulfadimidine, sulfafurazole, sulfaisodimidine, sulfalene, sulfamazone, sulfamerazine, sulfamethizole, sulfamethoxazole, sulfamthoxypyridazine, sulfametomidine, sulfametoxydiazine, sulfametrole, sulfamoxole, subtherapeutic, sulfaperin, sulfaphenazole, sulfapyridine, sulfathiazole, sulfathiourea, tetroxoprim, trimethoprim (*Veterinary use only*: ormosulfathiazole, phthalylsulfathiazole)	No	Yes
	Sulfones	Dapsone, aldesulfone	Yes	No
	Tetracyclines	Chlortetracyline, clomocycline, demeclocycline, doxycycline, lymecycline, metacycline, minocycline, penimepicycline, rolitetracycline, oxytetracycline, tetracycline	Yes	No
Important	Aminocyclitols	Spectinomycin	No	No
	Cyclic polypeptides	Bacitracin	No	No

Table 2.2. Continued

Classification	Antimicrobial class	Antimicrobial(s)	Criteria 1[a]	Criteria 2[b]
	Nitrofurantoins	Furazolidone, nitrofurantoin, nifurtoinol, nitrofural (*Veterinary use only*: furaltadone)	No	No
	Nitroimidazoles	Metronidazole, tinidazole, ornidazole	No	No

Adapted from World Health Organization (2012a)
[a]Criteria 1: antimicrobial sole therapy or one of few alternatives available to treat serious human disease
[b]Criteria 2: antimicrobial used to treat diseases caused by organisms that may be transmitted via nonhuman sources or the diseases caused by organisms may acquire resistance genes from nonhuman sources

Table 2.3. Summary of surveillance programs and indicator bacteria in the USA, Canada and Europe

Surveillance program	Country	Indicator bacteria	Sample source	Participants	References
NARMS	USA	*Salmonella, Campylobacter, Shigella, Escherichia coli* O157 and *Vibrio*	Human clinical isolates	Centers for Disease Control and Prevention (CDC)	Centers for Disease Control and Prevention, (2015).
		Salmonella, Campylobacter, Enterococcus and *Escherichia coli*	Retail meat samples including chicken, ground turkey, ground beef and pork chops	US Food and Drug Administration (FDA)	
		Salmonella, Campylobacter, Enterococcus and *Escherichia coli*	Food-producing animal specimens	US Department of Agriculture (USDA)	
CIPARS	Canada	*Salmonella*	Human isolates	National Microbiology Laboratory (NML)	Government of Canada, (2013).
		Salmonella, Campylobacter and *Escherichia coli*	Animal and food samples	Laboratory for Foodborne Zoonoses (LFZ)	
EFSA	Europe	*Salmonella* and *Campylobacter* spp. (mandatory), *Escherichia coli* and *Enterococcus* (voluntary)	Isolates from humans, food of animal origin and food-producing animals	26 European Union (EU) Member States	European Food Safety Authority & European Centre for Disease Prevention and Control, (2013).

Abbreviations: NARMS, National Antimicrobial Resistance Monitoring System for Enteric Bacteria; CIPARS, Canadian Integrated Program for Antimicrobial Resistance Surveillance; EFSA, European Food Safety Authority

Table 2.4. Mode of action of major antibiotic classes

Mode of action	Antibiotic class
Inhibition of cell wall synthesis	β-lactams (penicillins, cephaplosporins, carbapenems, monobactams); glycocpeptides; cyclic lipopeptides (daptomycin)
Inhibition of protein synthesis	Tetracyclines; aminoglycosides; oxazolidonones (linezolid); streptogramins (quinupristin-dalfopristin); ketolides; macrolides; lincosamides
Inhibition of DNA synthesis	Fluroquinolones
Inhibition of RNA synthesis	Rifampin
Competitive inhibition of folic acid synthesis inhibition	Sulfonamides; trimethoprim
Membrane disorganising agents	Polymyxins (Polymixin-B, Colistin)
Other	Metronidazole

Adapted from Alanis et al. (2005) and Levy and Marshall (2004)

Table 2.5. MLS_B resistance genes identified in *Enterococcus* spp.

Resistance mechanism	Resistance genes
rRNA methylases	*erm*(A)
	erm(B)
	*erm*I
	erm(F)
	erm(T)
Efflux genes	*msr*(A)
	*msr*I
	msr(D)
	lsa(A)
	lsa(E)
	vga(B)
	vga(D)
	mef(A)
Inactivating genes	
Lysases	*vgb*(A)
Transferases	*lnu*(B)
	vat(B)
	vat(D)
	vat(E)
	vat(H)

Adapted from Roberts (2008); Werner et al. (2013);
http://faculty.washington.edu/marilynr/ermweb4.pdf

Table 2.6. *In vitro* transfer of resistance genes in *Enterococcus* spp.

Donor	Recipient	Transferred genes	Reference
E. faecalis (food of animal origin)	*E. faecalis*	HLGR	Sparo et al., 2011[a]
E. faecalis	*S. aureus*	*vanA*	De Niederhausern et al., 2011
E. faecium	*L. monocytogenes*	*vanA*	De Niederhausern et al., 2011
E. hirae	*E. faecalis*	*vanA*	Robredo et al., 1999
E. faecalis	*E. faecalis*	*tet*(M) and *tet*(L)	Hummel et al., 2007
E. faecalis	*E. faecalis, Lactoccus lactis* and *Listeria innocua*	pRE25 – KM, NE, STR, CM, LI, AZ, CH, EM, RO, TYL, CL, NU	Schwarz et al., 2001
E. faecalis	*E. faecalis* and *E. faecium*	70-kb plasmid – *vat*(E) – streptogramin	Simjee et al., 2002
E. faecium and *E. durans* (pig)	*E. faecium*	*vanA* and *erm*(B)	Vignaroli et al., 2011[a]
Lactobacillus spp.	*E. faecalis*	*tet*(M)	Gevers et al., 2003

Abbreviations: HLRG, high-level gentamicin resistance; KM, kanamycin; NE, neomycin; STR, streptomycin; CM, clindamycin; LI, lincomycin; AZ, azithromycin; CH, clarithromycin; RO, roxithromycin; TYL, tylosin; CL, chloramphenicol; NU, nourseothricin sulphate

[a]transfer of resistance genes between animal and human strains

Table 2.7. Transfer of resistance genes in *Enterococcus* spp. using *in vivo* models

Donor	Recipient	Model	Transferred genes	Reference
E. faecium (pig)	*E. faecium*	Germfree C3H mice	*tet*(M), *vanA*, *erm*(B)	Moubareck et al., 2003[a]
E. faecalis	*E. faecalis*	Streptomycin-treated mini-pigs	pCF10 [*tet*(M)]	Licht et al., 2002
L. plantarum	*E. faecalis*	Gnotobiotic rats	*tet*(M), *erm*(B)	Jacobsen et al., 2007
E. faecalis	*E. faecalis*	Ceftriazxone-treated BALB/c mice	HLGR	Sparo et al., 2011
E. faecium (chicken)	*E. faecium* (HA)	Cefuroxime-treated NMRI mice	*vanA*	Lester and Hammerum, 2010[a]
E. faecium (poultry or pig)	*E. faecium*	Germ-free NMRI mice	*vanA*, *vanB*	Dahl et al., 2007[a]
E. faecium	*L. monocytogenes*	Germ-free C3H mice	Tn*1545*	Doucet-Populaire et al., 1991
E. faecium (poultry)	*E. faecalis*	Germ-free C3H mice	*vanA*	Bourgeois-Nicolaos et al., 2006[a]

Abbreviations: HLGR, high-level gentamicin resistance; HA, hospital acquired
[a]transfer of resistance genes between animal and human strains

Chapter 3 – Objectives

This thesis aims to provide insight on the contribution of the beef feedlot industry to antibiotic resistance, with a focus on macrolide resistance and using enterococci as an indicator bacterium.

The objective of the first study was to use real-time, quantitative PCR to determine the resistance gene profile of Canadian beef feedlots by quantifying resistance genes across five antibiotic classes (sulfonamides, tetracyclines, macrolides, fluoroquinolones and β-lactams) and comparing this to resistance genes found in catch basins, a surrounding waterway and urban wastewater treatment plants.

The objective of study two was to examine the effect of in-feed administration and withdrawal of tylosin phosphate on the prevalence of macrolide resistant enterococci isolated from feedlot steers, and to characterise the enterococci recovered through species identification, antimicrobial susceptibility testing, identification of resistance determinants and pulsed-field gel electrophoresis (PFGE) profiling.

The objective of study three was to announce the submission of the first draft genome sequence of *Enterococcus thailandicus* isolated from the faeces of feedlot cattle in Southern Alberta. A summary of the genome was provided to highlight key findings of this newly sequenced genome.

The objective of the fourth and final study was to perform whole-genome sequencing on twenty-one isolates of *Enterococcus* spp. isolated from bovine faeces and to perform a comparative genomic analysis.

Chapter 4

Antimicrobial resistance genes within feedlots and urban wastewater[1]

[1]This chapter has been submitted and is under review: Beukers, A.G., Zaheer, R., Cook, S.R., Chaves, A.V., Ward, M.P., Tymensen, L., Morley, P.S., Hannon, S., Booker, C.W., Read, R.R., and McAllister, T.A. Antimicrobial resistance genes within feedlots and urban wastewater. *PLoS One*. (Submitted).

4.1. Abstract

The use of antibiotics in livestock production in North America and possible association with elevated abundance of detectable antimicrobial resistance genes (ARGs) is a growing concern. Real-time, quantitative PCR (qPCR) was used to determine the relative abundance and diversity of ARGs in faecal and catch basin samples from four beef feedlots in Alberta. Samples from a surrounding waterway and municipal wastewater treatment plants were also included to compare the ARG profile of urban environments and fresh water with that of feedlots. The relative abundance of eighteen resistance genes across five antibiotic families including sulfonamides, tetracyclines, macrolides, fluoroquinolones and β-lactams were examined. Sulfonamide, fluoroquinolone and β-lactam resistance genes predominated in human samples, while tetracycline resistance genes predominated in cattle faecal samples. These differences appear to reflect differences in antibiotic use in cattle versus humans however other factors such as co-selection of ARGs and differences in bacterial community diversity and distribution may also play a role. Antibiotic resistance is a complex issue with multiple factors influencing the selection and persistence of ARGs.

Key words: antibiotic resistance, cattle, wastewater, quantitative real-time PCR, Alberta

4.2. Introduction

The acquisition of antimicrobial resistance genes (ARGs) by bacterial pathogens is a serious concern that can impede the successful treatment of infectious diseases (Centers for

Disease Control and Prevention, 2013). Antibiotics used in livestock production are often analogues or the same as those used in human medicine, raising the possibility that genes conferring resistance arise within agricultural production systems. Consequently, ARGs entering the environment through runoff or via the food chain could be transferred to pathogenic bacteria reducing the effectiveness of antibiotics currently used for human medicine.

Canada is one of the largest beef-exporting nations in the world, with the industry contributing more than $20 billion each year to the Canadian economy (Canada Beef, 2012). A number of antimicrobials are approved for administration to cattle as feed additives or in drinking water, including aminoglycosides, macrolides, tetracyclines and sulfonamides (Silbergel et al., 2008). Commensal bacteria residing in the bovine gastrointestinal tract may become resistant to these antibiotics and once disseminated into the environment, transfer these genes to pathogenic bacteria (Andremont 2003; Harrison et al., 2013; Marshall et al., 2009). Furthermore, residual antibiotics may enter the environment through runoff or application of manure to land, exposing bacteria in these environments to antibiotics and possibly applying selective pressure for resistance development (Campagnolo et al., 2002; Heuer et al., 2011).

Real-time, quantitative PCR (qPCR) has been used to study the levels of ARGs in livestock and poultry systems (He et al., 2014; Mu et al., 2014; Zhu et al., 2013) and in wastewater from urban environments (Marti et al., 2013; Negreanu et al., 2012). It is a useful tool that can provide an approximation of the abundance of ARGs in the environment (Berendonk et al., 2015). The objective of this study was to use qPCR to compare the types and relative abundance of ARGs present in feedlot cattle faeces to those in feedlot catch basins, a surrounding waterway and municipal wastewater treatment plants in Alberta.

4.3. Materials and Methods

4.3.1. Study area and sample collection

Sample collection occurred from April to October 2014. Four beef feedlots (designated A to D) and two municipal (human) wastewater treatment plants located in Alberta were selected for this study (Appendix 1 Table S4.1.). Antibiotic usage in all feedlots was recorded (Appendix 1 Table S4.2.). In feedlots A, B and C, conventional production pens associated with the catch basins of interest at each feedlot were identified and 20 pens in each feedlot were randomly selected. At Feedlot D, pens were stratified by production type with 15 Conventional pens (D_c) and 5 Natural pens (D_n) randomly selected. Conventional pens contained cattle routinely administered antibiotics while natural pens contained cattle that were not receiving any antibiotics. Twenty fresh faecal pats were sampled from each pen and composited to provide one faecal sample per pen per feedlot. Three composite samples were then arbitrarily chosen from each feedlot (or within each production strata for feedlot D) for real-time qPCR. After collection, faecal samples were transported to the lab on ice, flash-frozen in liquid nitrogen within 24 h and stored at -80°C for DNA extraction. The research study was reviewed and approved by the Lethbridge Research Centre Animal Care Committee, an evaluation body that is accredited by the Canadian Council of Animal Care.

Catch basins, which received runoff from the cattle pens, were also sampled once at each feedlot. Sewage influent and effluent samples were collected from wastewater treatment plants located at two different municipal centres. Surface water was collected from an ephemeral creek that was adjacent to feedlot C, which drains land that receives regular manure application. Based on turbidity, catch basin, sewage treatment and surface water samples were processed by

centrifugation (30 mL for catch basin and 80 mL for sewage influent; 15,500 × *g*) or filtration (sewage effluent and surface water) through a 0.45 µm nitrocellulose filter membrane (until the filter was saturated) within 24 h of collection. The filter membrane or pellet from centrifugation was stored at -80°C for later DNA extraction.

4.3.2. DNA extraction

Total DNA was extracted from individual water samples (pellet or filter) and faecal composite samples (approximately 350 mg). Each 100 mg of sample was resuspended in 300 µL of resuspension buffer [600 mM NaCl, 120 mM Tris-HCl, 60 mM EDTA, 200 mM Guanidine isothyocynate] or 800 µL for filter samples. Aliquots (1 mL) of the resuspended faecal sample or pellet were transferred to 2 mL microfuge tubes containing 0.4 g of sterile zirconia beads (0.3 g of 0.1 mm and 0.1 g of 0.5 mm). For filtered samples, beads were added directly to the vial containing the filter paper. β-Mercaptoethanol (5 µL) and 200 µL pre-heated (70°C) 10% sodium lauryl sulfate (SDS) were sequentially added to the tubes and gently mixed. Cell lysis was carried out for 3 min at maximum speed (setting=30) using a Qiagen TissueLyser (Germantown, MD, USA) or for filter samples using an Omni Bead Ruptor (3.25 m/s for 5 min; Omni International, Kennesaw, GA, USA). Samples were then incubated at 70°C for 15 min, with gentle shaking. The filter paper was removed and all samples were centrifuged at 4°C for 5 min at 16,000 × *g*, with the supernatant transferred to a new 2 mL microfuge tube. The pellet was resuspended in 800 µL of resuspension buffer and the bead-beating process repeated. Duplicate lysates were subject to isopropanol precipitation of nucleic acid and the pellet was resuspended in 100 µL Tris-EDTA, pH 7.4 (TE). Nucleic acids in TE were pretreated with 2 µL of Dnase-free

Rnase (10 mg/mL) per 200 µL of sample and incubated at 37°C for 15 min. The resulting DNA was further purified using a QIAamp DNA Stool Mini Kit (Qiagen) with inclusion of proteinase K (Kit handbook), and the final elution accomplished using nuclease-free water. Extracted DNA was assessed for PCR inhibitors using 16S primers (Appendix 1 Table S4.3.) and where inhibition was indicated by the absence or low yield of a PCR product, an additional sepharose purification step was undertaken as described by Miller et al. (2001) using sepharose 2B resin. Purity of the DNA was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) to ensure that the 260/280 absorbance ratio was approximately 1.8 and the DNA was quantified using the Quant-iT PicoGreen dsDNA Assay Kit with a Nanodrop 3300 fluorospectrometer (Thermo Scientific).

4.3.3. Quantification of antimicrobial resistance genes

Real-time, quantitative PCR (qPCR) was used to estimate the copy numbers of 18 resistance genes across five antibiotic families including sulfonamides [*sul1* and *sul2*], tetracyclines [*tet*(A), *tet*(B), *tet*(M), *tet*(O), *tet*(Q) and *tet*(W)], macrolides [*erm*(A), *erm*(B), *erm*(C), *erm*(F) and *mef*(A)], fluoroquinolones [*qnrS* and *oqxB*] and β-lactams [bla_{SHV}, bla_{TEM1} and $bla_{\mathrm{CTX\text{-}M}}$]. Primers for the 16S-ribosomal RNA (rRNA) gene were also included to estimate the total amount of bacteria associated with each sample and to normalise the abundance of ARGs in collected samples. All qPCR assays were performed on an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using primers and conditions as described in Appendix 1 Table S4.3. For primers that were not from published information, available sequences encoding each respective antibiotic resistance gene were

downloaded from the GenBank Database (http://www.ncbi.nlm.nih.gov/) and aligned in Geneious (version 8.1.) to determine a consensus sequence that could be used for primer design. Using the primer design tool, forward and reverse primers that would anneal to regions of the consensus sequence were identified and the specificity of each primer pair verified using the BLAST alignment tool (http://www.ncbi.nlm.nih.gov/blast/).

Each reaction was carried out in a total volume of 25 µL, containing 2 µL of template, 0.2 µM of each primer and 1 × iQ SYBR Green Supermix (Bio-Rad, Saint-Laurent, QC, Canada). All qPCR reactions included an initial step of 95°C for 3 min, followed by the respective number of cycles, with denaturation at 95°C for 15 s, annealing at the respective temperature for 30 s, and an extension at 72°C for 30 s, except bla_{TEM1} which was extended for 40 s. Melt curve (55 to 95°C) analysis was performed to verify the uniformity of the amplicons.

Standard curves generated using known quantities of cloned or synthesised target genes were used to quantify gene copy numbers. Standards for *tet*(A), *qnrS*, *oqxB* and bla_{SHV} were synthesised by Eurofins Scientific (Lancaster, PA) whilst *tet*(W), bla_{TEM1} and $bla_{\mathrm{CTX\text{-}M}}$ were synthesised by Integrated DNA Technologies (San Diego, CA). The remaining standards were cloned in our laboratory and the presence of the target gene was verified by sequencing. Dilutions of cloned target genes at concentrations 10^8,10^7, 10^6, 10^5, 10^4, 10^3, 100 and 50 copies per reaction were amplified in duplicate to generate standard curves for each qPCR assay. All qPCR reactions were performed in triplicate for DNA samples with raw values averaged.

4.3.4. Statistical analysis

For each resistance gene, averaged raw values for each DNA sample were normalised by dividing each by 16S-rRNA values, providing the relative abundance (copies of ARGs/copies of 16S-rRNA). Using the Shapiro-Wilk test, it was determined that a natural log (ln) transformation on normalised data was required to achieve normal distribution. Statistical analyses were performed using SAS version 2.0.4 (SAS Institute Inc., Cary, NC). Natural log (ln) transformed data were analysed using the MIXED procedure of SAS with feedlot considered as a random effect. The model consisted of sample type (feedlot=A, B, C, D_C, D_N, catch basin=CB, sewage influent=Influent, sewage effluent=Effluent and creek=Ephemeral creek) as a fixed effect and the relative abundance of each ARG (ln transformed) as the dependant variable. The LSMEANS statement was used to separate means with statistical significance declared at a p-value ≤ 0.05. Samples that were unable to be detected/outside the standard curve range were arbitrarily assigned a value of zero for statistical analysis. The means and standard deviation of means of untransformed normalised data were used for figures.

4.4. Results

Antibiotics from the tetracycline, macrolide, phenicol, cephalosporin, fluoroquinolone and sulfonamide families as well as ionophores were used at feedlots A, B, C and D_C (Appendix 1 Table S4.2.). Antimicrobials administered to study animals were approved for use in cattle in Canada by the Veterinary Drugs Directorate, Health Canada and used under veterinary prescription issued by a licensed veterinarian with a valid veterinary-client-patient relationship. At the time of sampling, the majority of cattle from all feedlots were receiving chlortetracycline

for the control of liver abscesses and monensin for the control of bloat and coccidiosis. Sulfonamides were not being administered at the time of sampling, but parenteral or oral bolus sulfonamides had been administered to clinically ill cattle at all feedlots in the past. Tylosin, a commonly used macrolide, was only being administered in one pen of cattle from feedlot D_C for the control of liver abscesses.

Among the 18 target resistance genes, all genes with the exception of those associated with fluoroquinolone (*qnrS* and *oqxB*) and β-lactam resistance (bla_{SHV} and $bla_{CTX\text{-}M}$) were detected in faecal and catch basin water samples. Both the sewage influent and effluent samples possessed all genes except *erm*(A) and bla_{SHV}, which code for macrolide and β-lactam resistance, respectively. Only eight (*sul1*, *sul2*, *tet*(O), *tet*(Q), *tet*(W), *erm*(C), *mef*(A) and bla_{TEM1}) of the eighteen resistance genes were detected in water from the creek.

The relative abundance of *sul1* and *sul2* differed ($P < 0.0001$) among sample types, but were similar in faecal samples collected from the four feedlots (Figure 4.1.). The relative abundance of both *sul1* and *sul2* was greater ($P < 0.05$) in the catch basin and the sewage samples compared to the faecal and creek samples. There was no difference ($P > 0.05$) between the conventional and natural production systems for either *sul1* or *sul2*. Error bars for the catch basin sample for both *sul1* and *sul2* were large, indicating variability among individual samples.

The relative abundance of *tet* genes also differed among sample types ($P < 0.001$), except *tet*(A) and *tet*(B) ($P = 0.5$ and $P = 0.1$, respectively) (Figure 4.2.). Sewage influent and effluent samples were both lower ($P < 0.05$) in relative abundance for *tet*(M) than faecal and catch basin samples, but did not differ from each other ($P > 0.05$). For *tet*(O), *tet*(Q) and *tet*(W), the catch basin, sewage influent, sewage effluent and creek samples were all lower ($P < 0.05$) in relative

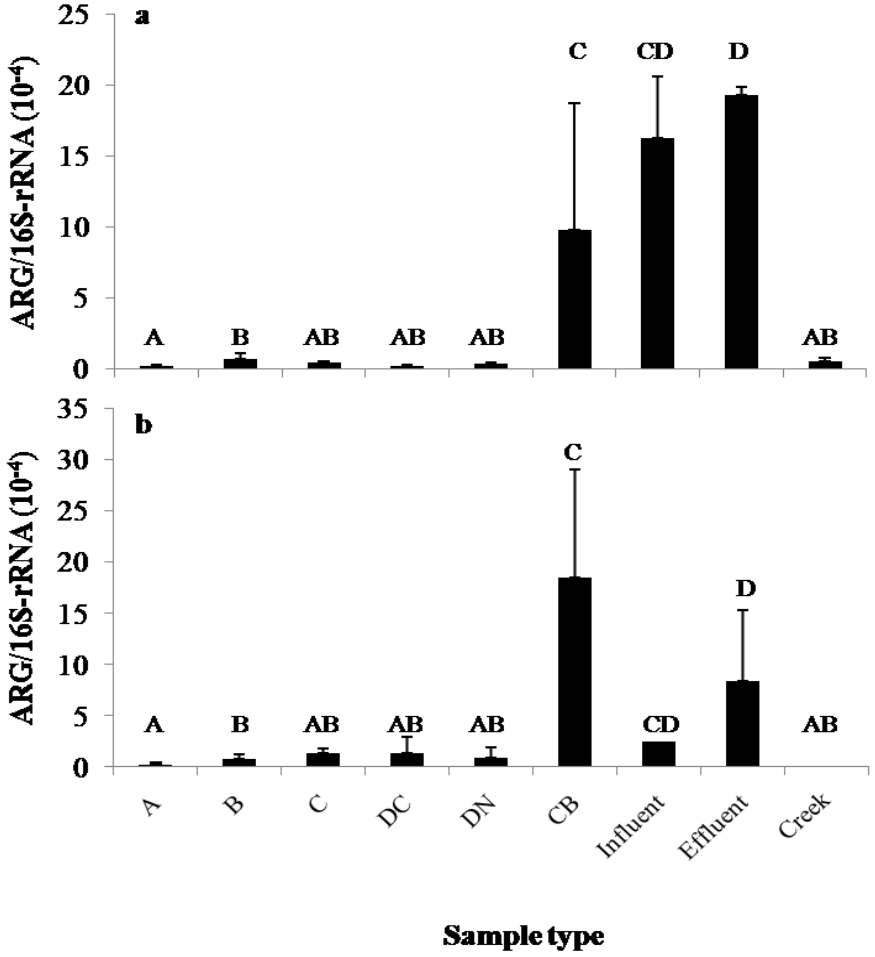

Figure 4.1. Relative abundance (copies of ARGs/copies of 16S-rRNA) of sulfonamide resistance genes. (a) *sul1* and (b) *sul2.* Error bars represent standard deviation of the means. A = feedlot A, B = feedlot B, C = feedlot C, DC = feedlot D (conventional production), DN = feedlot D (natural production), CB = catch basin, Influent = sewage influent, Effluent = sewage effluent, and Creek = Ephemeral creek. Means with different letters significantly differ ($P \leq 0.05$).

abundance than the faecal samples. The sewage influent did not differ ($P > 0.05$) in the relative abundance of *tet*(Q) and *tet*(W) from the catch basin sample, but were greater ($P < 0.05$) in relative abundance of *tet*(O). All three *tet* genes in sewage influent samples were greater ($P < 0.05$) in relative abundance than in sewage effluent and creek samples. The creek sample was

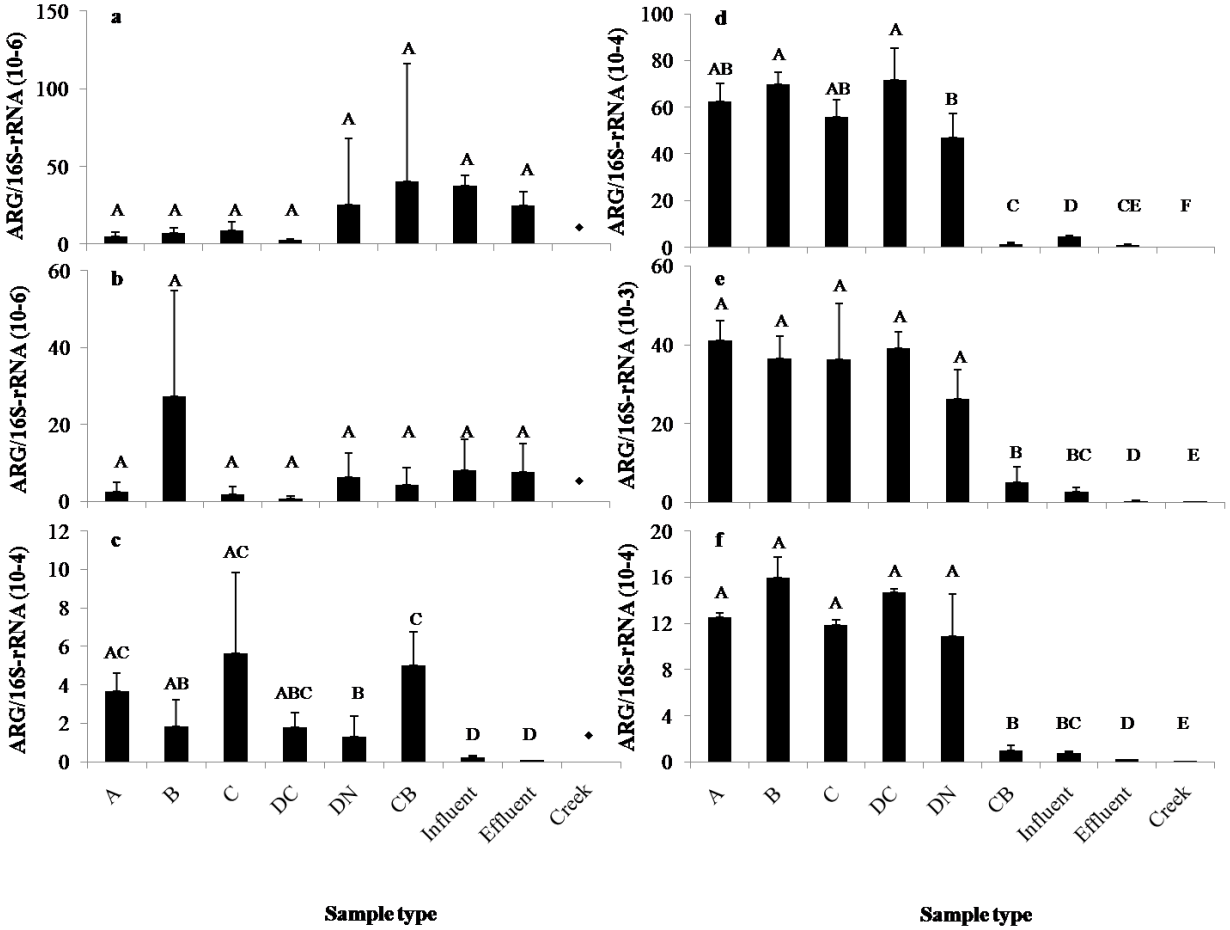

Figure 4.2. Relative abundance (copies of ARGs/copies of 16S-rRNA) of tetracycline resistance genes. (a) *tet*(A), (b) *tet*(B), (c) *tet*(M), (d) *tet*(O), (e) *tet*(Q) and (f) *tet*(W). Error bars represent standard deviation of the means. A = feedlot A, B = feedlot B, C = feedlot C, DC = feedlot D (conventional production), DN = feedlot D (natural production), CB = catch basin, Influent = sewage influent, Effluent = sewage effluent, and Creek = Ephemeral creek. Means with different letters significantly differ ($P \leq 0.05$). ◆ - unable to be detected/outside standard curve range.

lower ($P < 0.05$) in relative abundance of *tet*(O), *tet*(Q) and *tet*(W) than samples from all other environments. There was no difference ($P > 0.05$) between faecal samples collected from cattle raised in conventional versus natural production systems for *tet*(M), *tet*(Q) and *tet*(W). However, faecal samples from the conventional system had greater ($P < 0.05$) relative abundance of *tet*(O) than the natural system.

There was no difference ($P > 0.05$) in the relative abundance of macrolide resistance genes in faecal samples collected from conventional versus natural production systems (Figure 4.3.). The relative abundance of *erm*(B) was greater ($P < 0.05$) in catch basin samples than the faecal samples, whereas *mef*(A) was lower ($P < 0.05$). The relative abundance of *erm*(B) was greater ($P < 0.05$) in the sewage influent sample than in faecal, catch basin or sewage effluent samples. The relative abundance of *mef*(A) in the catch basin, sewage influent and effluent and creek samples were all lower ($P < 0.05$) than faecal samples.

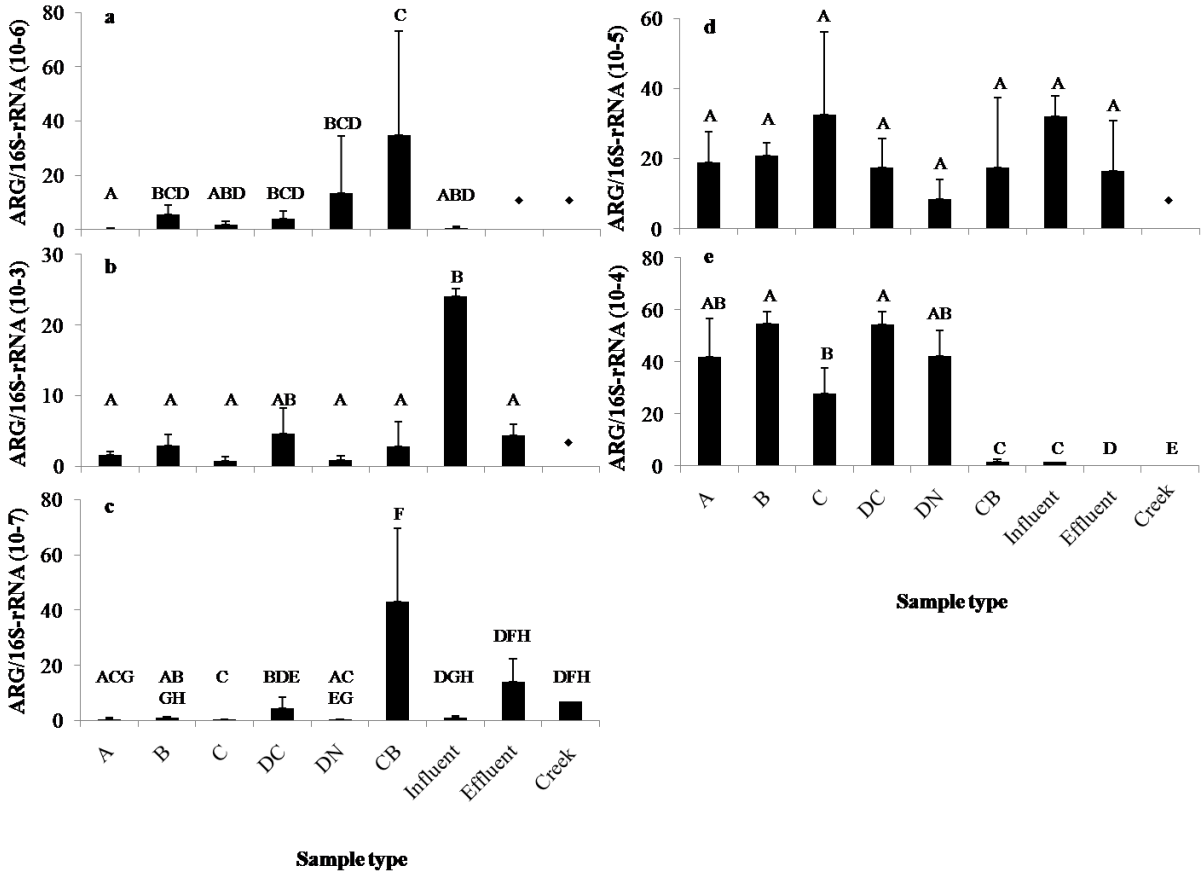

Figure 4.3. Relative abundance (copies of ARGs/copies of 16S-rRNA) of macrolide resistance genes. (a) *erm*(A), (b) *erm*(B), (c) *erm*(C), (d) *erm*(F) and (e) *mef*(A). Error bars represent standard deviation of the means. A = feedlot A, B = feedlot B, C = feedlot C, DC = feedlot D (conventional production), DN = feedlot D (natural production), CB = catch basin, Influent = sewage influent, Effluent = sewage effluent, and Creek = Ephemeral creek. Means with different letters significantly differ ($P \leq 0.05$). ◆ - unable to be detected/outside standard curve range.

The fluoroquinolone resistance genes (*qnrS* and *oqxB*) were only detected in the sewage samples (Figure 4.4.). Comparison of the relative gene abundances indicated that there was no difference ($P = 0.2$) for *qnrS* whilst *oqxB* was greater ($P < 0.05$) in relative abundance for influent than effluent sewage samples.

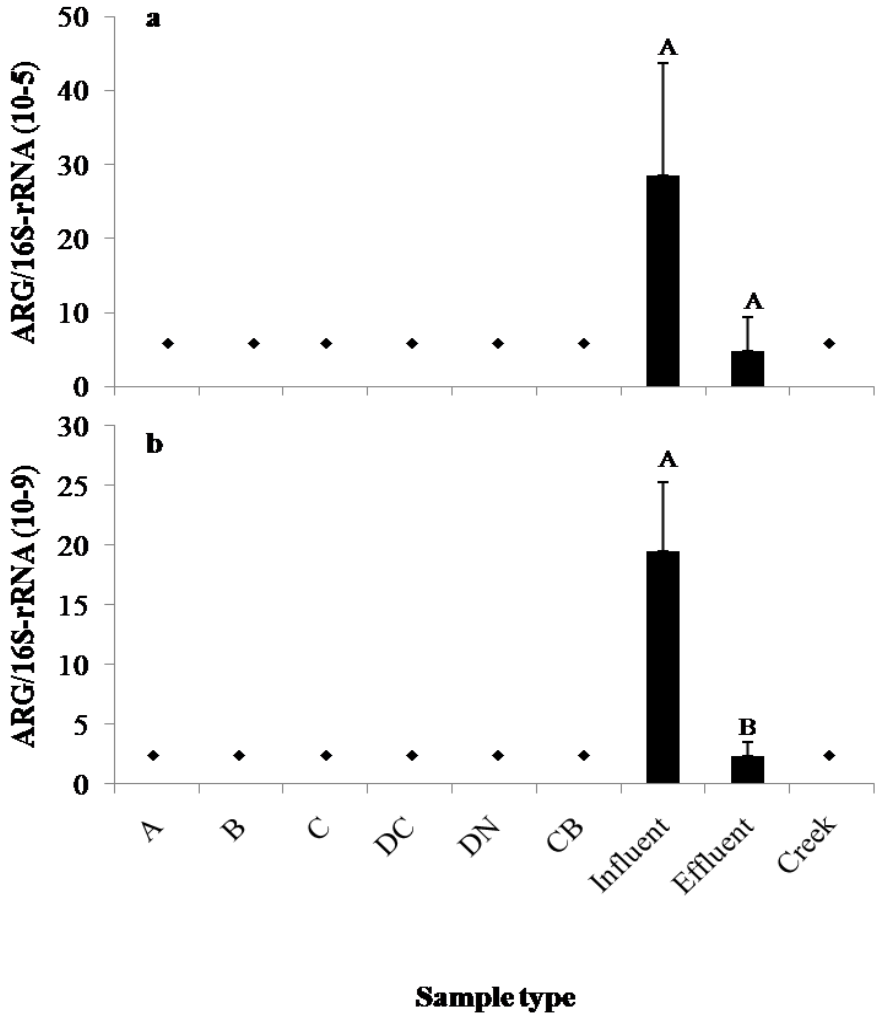

Figure 4.4. Relative abundance (copies of ARGs/copies of 16S-rRNA) of fluoroquinolone resistance genes. (a) *qnrS* and (b) *oqxB*. Error bars represent standard deviation of the means. A = feedlot A, B = feedlot B, C = feedlot C, DC = feedlot D (conventional production), DN = feedlot D (natural production), CB = catch basin, Influent = sewage influent, Effluent = sewage effluent, and Creek = Ephemeral creek. Means with different letters significantly differ ($P \leq 0.05$). ◆ - unable to be detected/outside standard curve range.

The β-lactam resistance gene bla_{SHV} was detected in both sewage influent and effluent samples, but the copy number was below the range of the standard curve for effluent samples and as a result not included in our analysis. Of the *bla* genes, $bla_{CTX\text{-}M}$ was the only one detected in the sewage treatment samples with no difference ($P = 0.1$) observed between influent and effluent samples, whereas bla_{TEM1} was detected in all samples (Figure 4.5.). Among sample types, the relative abundance of bla_{TEM1} was greater ($P < 0.05$) in sewage influent than in faecal, catch basin or creek samples, but did not differ ($P > 0.05$) from the sewage effluent sample.

4.5. Discussion

Real-time, quantitative PCR has been used to examine the abundance and distribution of ARGs in beef cattle faeces (Alexander et al., 2011; Yu et al., 2005), feedlot wastewater lagoons (McKinney et al. 2010; Peak et al. 2007; Zhang et al. 2009), urban wastewater (Gao et al., 2012; Lachmayr et al., 2009) and fresh water samples from a flowing river (Pei et al., 2006). Most of these studies have focused on one or two antibiotic families or on one type of environmental source. In contrast, this study aimed to examine the abundance and distribution of ARGs across five antibiotic families and over a range of environments including from beef cattle faeces, water catch basins at feedlots, municipal sewage samples and surface water from a creek, all collected within the same temporal period.

There were obvious differences in the relative abundance of ARGs among sample types, with some ARGs clearly predominant in certain environments. For example, the fluoroquinolone and β-lactam resistance genes were abundant in the human sewage treatment samples and the tetracycline resistance genes were abundant in the cattle faecal samples.

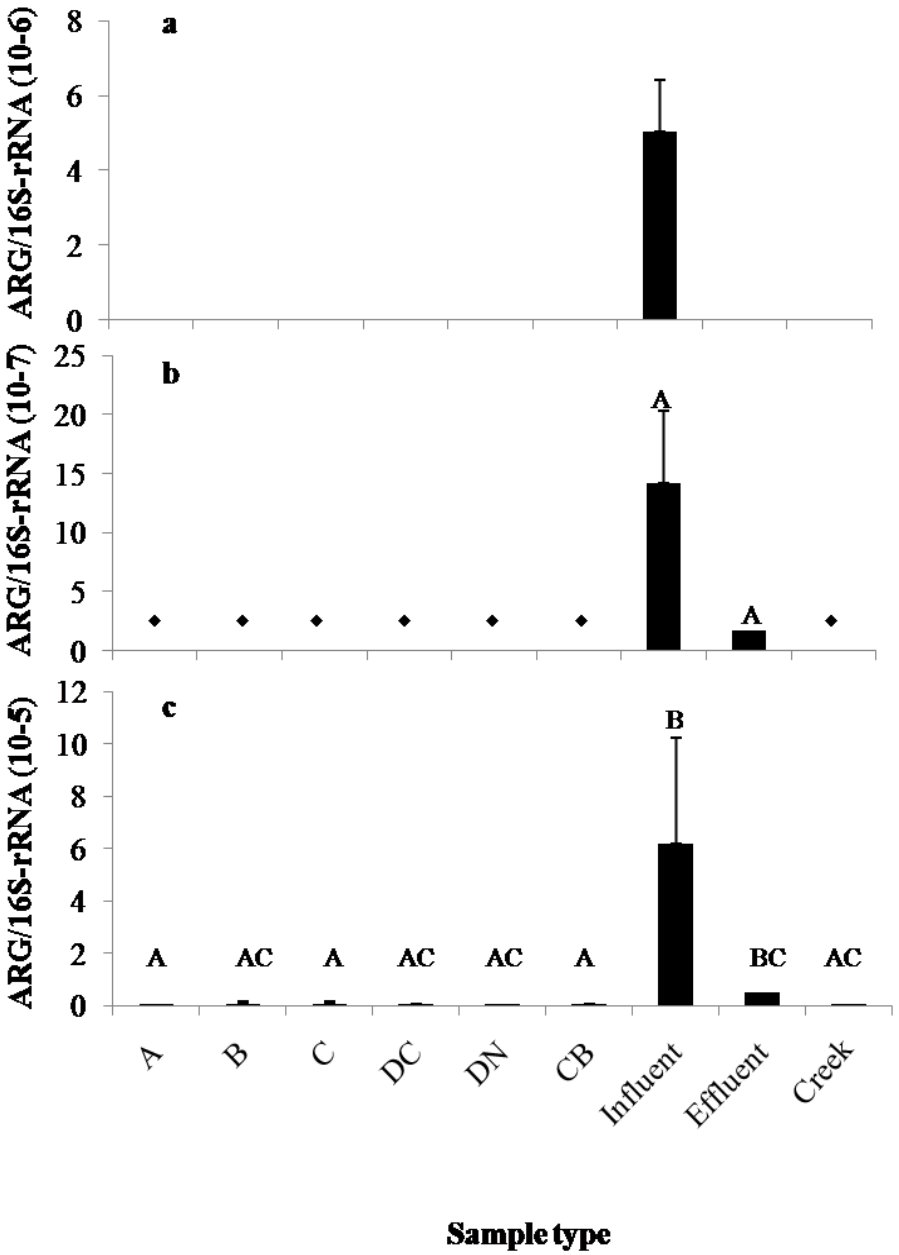

Figure 4.5. Relative abundance (copies of ARGs/copies of 16S-rRNA) of β-lactam resistance genes. (a) bla_{SHV}, (b) $bla_{CTX\text{-}M}$ and (c) bla_{TEM1}. Error bars represent standard deviation of the means. A = feedlot A, B = feedlot B, C = feedlot C, DC = feedlot D (conventional production), DN = feedlot D (natural production), CB = catch basin, Influent = sewage influent, Effluent = sewage effluent, and Creek = Ephemeral creek. Means with different letters significantly differ ($P \leq 0.05$). ◆ - unable to be detected/outside standard curve range.

Studies have demonstrated that administration of antibiotics can increase the abundance of ARGs, including in beef cattle faeces (Alexander et al., 2008; Alexander et al., 2011; Peak et

al., 2007). Consequently, antibiotic use in humans and in livestock production could play a role in the abundance and distribution of ARGs among environments. An aspect of this study was the collection of data related to antibiotic use from the feedlots sampled (Appendix 1 Table S4.2.). As such, inferences between the use of antibiotics in the feedlot environment and the distribution and abundance of ARGs could be made.

Sulfonamides were not being administered to cattle at feedlots A, B, C and D at the time of sampling, but they had been used to treat clinically ill cattle at all feedlots in the past (Appendix 1 Table S4.2.). Compared to other antibiotics used in feedlots, sulfonamides are more hydrophilic and this property combined with their low sorption to soil makes them among the most mobile of antibiotics (Chee-Sanford et al., 2009). Therefore, it is possible that sulfonamides flowed from the feedlot and accumulated within the catch basin. This would provide selective pressure for sulfonamide resistance and may explain the greater relative abundance of the *sul* genes in catch basin samples as compared to faecal samples, where limited use would have led to low selective pressure. The relative stability of sulfonamides in water may also explain the greater relative abundance of these genes in the sewage treatment samples as sulfonamides are excreted in the urine and faeces of humans and enter the environment through sewage (Yang et al., 2005). Testing samples for sulfonamide residues would help elucidate if this is the case. The relative abundance of *sul* genes was low in the creek sample suggesting that despite its close proximity to one of the feedlots, residual sulfonamides were contained within the catch basin and were not being transferred to the broader environment.

A large proportion of tetracycline resistance genes encode for efflux proteins which export tetracycline out of bacterial cells and are the most common *tet* genes found in Gram-negative bacteria (Roberts, 2005). In this study, the tetracycline resistance genes encoding for

efflux proteins (*tet*(A) and *tet*(B)) were present in all environments at similar levels, with the exception of the creek sample where *tet*(A) and *tet*(B) were not detected. In contrast, the genes encoding for ribosomal protection proteins (*tet*(M), *tet*(O), *tet*(Q) and *tet*(W)) were dominant in the faecal samples as compared to other sample types. In general, the relative abundance of ribosomal protection proteins was also much greater (3 orders of magnitude) compared to the genes encoding for efflux proteins. The ribosomal protection proteins are predominantly found in Gram-positive bacteria which account for the majority of bacteria found in bovine faeces (Roberts 2005; Shanks et al., 2011), possibly explaining the greater relative abundance of these genes.

Tetracyclines are usually fed at low concentrations to feedlot cattle for the control of liver abscesses and other bacterial diseases. All conventional feedlots sampled in this study used chlortetracycline in their production practices (Appendix 1 Table S4.2.) and at the time of sampling, most cattle were being administered chlortetracycline in their diet. This could account for the greater relative abundance of *tet* genes in faecal composite samples, as administration of tetracycline increases the abundance of *tet* genes in cattle faeces (Alexander et al., 2011). There was no difference between conventional and natural production systems for *tet*(M), *tet*(Q) and *tet*(W). However, *tet*(O) was more predominant in faeces collected from the conventional as compared to the natural production system, suggesting that in-feed chlortetracycline may preferentially select for certain *tet* genes. Tetracycline resistance genes in DNA isolated from the catch basin, sewage and creek samples were in low relative abundance compared to faecal samples. Tetracyclines have a high sorption to soil compared to other antibiotics making them less mobile (Chee-Sanford et al., 2009) and less likely to be transported in water runoff into the catch basin or nearby waterways. Their lower mobility in water could also account for the lower

presence of *tet* genes in urban wastewater. Consequently, selection pressure in the catch basin, sewage treatment and creek samples for tetracycline resistance would be lower and may explain the lower *tet* abundance in these environments.

Ribosomal methylation is the most widespread mechanism of macrolide resistance and is encoded for by the *erm* genes, *erm*(A), *erm*(B), *erm*(C) and *erm*(F). Drug efflux is another common resistance mechanism, encoded for by *mef*(A) (Leclercq, 2002). Of the macrolide resistance genes assessed, differences were observed among samples for all genes, with the exception of *erm*(F). The genes conferring resistance to macrolides are mostly associated with Gram-positive bacteria, with the host range varying among genes (Roberts, 2004). The nature of the bacterial microbiome within samples is likely to influence both the density and types of resistance determinants present, factors that may explain why the abundance of *erm*(A) and *erm*(C) is much lower than *erm*(B) and *mef*(A) even though all determinants code for macrolide resistance. As with tetracycline, administration of macrolides to cattle has also been demonstrated to increase the abundance of macrolide resistance genes in cattle faeces (Alexander et al., 2011). While macrolides (tylosin, tulathromycin and tilmicosin) were used at all conventional feedlots, only one out of the three conventional pens sampled from feedlot D were being administered macrolides at the time of sampling. This may explain why no difference was observed in the relative abundance of macrolide resistance genes in cattle faeces collected from conventional versus natural production systems for *erm*(A), *erm*(B) and *mef*(A).

The macrolide resistance gene *mef*(A) was the dominant gene within faecal samples. Its greater relative abundance in cattle faeces could be due to its common presence in enteric bacteria (Roberts, 2004) or a reflection of its co-selection along with other ARGs. Many tetracycline resistance genes can be linked with macrolide resistance genes on mobile genetic

elements, resulting in co-selection. For example, *erm*(F) is often linked with *tet*(Q) on a conjugative transposon described in *Bacteroides* spp. (Chung et al., 1999), e*rm*(B) with *tet*(M) on the Tn*1545* conjugative transposon described in *Enterococcus* spp. (De Leener et al., 2004) and *mef*(A) with *tet*(O) on a conjugative transposon described in *Streptococcus pyogenes* (Giovanetti et al., 2003). The *erm*(B) gene was more abundant in sewage influent than in other samples. This gene has been identified in a number of bacterial species, including *Enterococcus* and *Escherichia* (Roberts et al., 1999). Macrolides, such as erythromycin, are extensively used in human medicine (World Health Organization 2012) and may be influencing the relative abundance of *erm*(B) in the sewage influent sample. As observed with previous ARGs, the relative abundance of macrolide resistance genes was low in the creek sample.

The fluoroquinolone resistant genes *qnrS* and *oqxB* were only detected in the sewage influent and effluent treatment samples, which may reflect the use of fluoroquinolones in human medicine. There was a noticeable decrease in the relative abundance of *oqxB* when comparing sewage influent to effluent. The sewage treatment process has been shown to reduce the number of bacteria resistant to tetracycline and sulfonamides, although numbers of resistant bacteria in the effluent still remained high (Gao et al., 2012). In this study, it appears the sewage treatment process resulted in a decline in fluoroquinolone resistant bacteria, as indicated by a reduction of resistance genes detected. However, the fact that fluoroquinolone resistance genes were detected in the effluent sample even after sewage treatment indicates that these resistance genes still entered the environment. The fluoroquinolone genes assessed in this study are predominantly plasmid-mediated suggesting they could easily be transferred to other bacteria (Hata et al., 2005; Hansen et al., 2007; Norman et al., 2008). Similar to the fluoroquinolone resistance genes, the β-lactamase resistance genes were predominantly found in sewage samples. The bla_{TEM1} resistance

gene, which confers resistance to ampicillin, penicillin and first-generation cephalosporins (Rupp and Fey, 2003), was primarily detected in sewage samples, but low levels were also detected in the faecal and catch basin samples. Our results support those of Agga et al. (2015) describing a greater abundance of fluoroquinolone and β-lactamase resistance genes in sewage treatment samples compared to cattle faecal samples. The association between fluoroquinolone and β-lactamase resistance genes, in particular *qnrS* and bla_{TEM1} could possibly indicate co-selection of these ARGs in sewage samples (Hata et al., 2005).

Although the relative abundance of ARGs can be influenced by the use of antibiotics, there is a growing body of literature highlighting the relationship between antibiotic use and ARGs is complex and not necessarily linear. Jindal et al. (2006) demonstrated a high level of tylosin resistance persisted on swine farms years after antimicrobial use ceased. ARGs can also be detected in pristine environments not exposed to antibiotics and where the corresponding antibiotic residues are absent (D'Costa et al., 2011; Durso et al., 2012). Furthermore, the abundance of ARGs can be influenced by the bacterial community composition with ARGs more common in some bacterial species than in others. For example, the macrolide resistance genes *erm*(A) and *erm*(C) are typically associated with staphylococci whilst *erm*(B) is mostly found in streptococci and enterococci (Leclercq, 2002; Roberts et al., 1999). Other studies have also demonstrated links between the ARG profile and the bacterial taxonomic profile (Durso et al., 2012; Forsberg et al., 2014). Bacterial composition and diversity amongst sample types was not examined in this study but it is likely to have influenced the distribution and abundance of ARGs.

4.6. Conclusion

The results from this study demonstrate clear differences in the relative abundance of ARGs among feedlot and human related samples. Although samples were only collected at one point in time, it is clear that sulfonamide, fluoroquinolone and β-lactam resistance genes predominate in urban wastewater, whilst tetracycline resistance genes were more prevalent in cattle faeces. These differences appear to reflect differences in antibiotic use in cattle versus humans, however other factors such as co-selection of ARGs and differences in bacterial community diversity and distribution may also be playing a role. In conclusion, antibiotic resistance is a complex issue with multiple factors influencing the selection and persistence of ARGs.

4.6. References

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Chapter 5

Effect of in-feed administration and withdrawal of tylosin phosphate on antibiotic resistance in enterococci isolated from feedlot steers[2]

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5.1. Abstract

Tylosin phosphate is a macrolide commonly administered to cattle in North America for the control of liver abscesses. This study investigated the effect of in-feed administration of tylosin phosphate to cattle at subtherapeutic levels and its subsequent withdrawal on macrolide resistance using enterococci as an indicator bacterium. Faecal samples were collected from steers that received no antibiotics and steers administered tylosin phosphate (11 ppm) in-feed for 197 d and withdrawn 28 d before slaughter. *Enterococcus* species isolated from faecal samples were identified through sequencing the *groES-EL* intergenic spacer region and subject to antimicrobial susceptibility testing, identification of resistance determinants and pulsed-field gel electrophoresis profiling. Tylosin increased ($P < 0.05$) the proportion of ery^R and tyl^R enterococci within the population. Just prior to its removal, the proportion of ery^R and tyl^R resistant enterococci began decreasing and continued to decrease after tylosin was withdrawn from the diet until there was no difference ($P > 0.05$) between treatments on d 225. This suggests that antibiotic withdrawal prior to slaughter contributes to a reduction in the proportion of macrolide resistant enterococci entering the food chain. Among the 504 enterococci isolates characterised, *Enterococcus hirae* was found to predominate (n=431), followed by *Enterococcus villorum* (n=32), *Enterococcus faecium* (n=21), *Enterococcus durans* (n=7), *Enterococcus casseliflavus* (n=4), *Enterococcus mundtii* (n=4), *Enterococcus gallinarum* (n=3), *Enterococcus faecalis* (n=1), and *Enterococcus thailandicus* (n=1). The diversity of enterococci was greater in steers at arrival than at exit from the feedlot. Erythromycin resistant isolates harboured the *erm*(B) and/or *msrC* gene. Similar PFGE profiles of ery^R *E. hirae* pre- and post-antibiotic treatment suggest that increased abundance of ery^R enterococci after administration of tylosin

phosphate reflects selection for strains that were already present within the gastrointestinal tract of cattle at arrival.

Key words: enterococci, antimicrobial resistance, subtherapeutic macrolides, beef cattle, tylosin, erythromycin

5.2. Introduction

Subtherapeutic administration of antibiotics in livestock feed has come under increasing scrutiny due to concerns that such a practice increases the emergence of antibiotic resistant bacteria (Aarestrup, 1999). This concern is particularly relevant for bacteria that reside in livestock and are associated with clinical infections in humans.

Enterococci are commensal bacteria of the human and bovine gastrointestinal tract, but are also associated with nosocomial and community-acquired infections in humans (Franz et al., 2011; Poh et al., 2006). *Enterococcus faecalis* and *Enterococcus faecium* are the two species most frequently associated with enterococcal infections in humans, being responsible for as much as a third of the nosocomial infections worldwide (Werner et al., 2008). Whereas in cattle, *Enterococcus hirae*, a species not commonly associated with human infections is predominately isolated from bovine faeces (Anderson et al., 2008; Jackson et al., 2010; Zaheer et al., 2013).

In North America, tylosin phosphate is commonly included in cattle feed for the control of liver abscesses (Pagel and Gautier, 2012). Previous research has shown therapeutic and subtherapeutic administrations of macrolides to cattle increases the proportion of erythromycin

resistant enterococci in bovine faeces (Jacob et al., 2008; Zaheer et al., 2013). In 2005, the WHO identified macrolides as critically important antimicrobials for which management strategies are urgently required to reduce the prevalence of bacterial resistance (Collignon et al., 2009). Macrolides are part of the MLS_B (macrolide-lincosamide-streptogramin B) superfamily with each antibiotic having slight structural differences, but resistance to one member of the family can cross-select for resistance to other drugs in the family. Consequently, if the inclusion of tylosin in feed leads to tylosin resistant enterococci in cattle it may also select for enterococci that are resistance to other macrolides such as erythromycin, an antibiotic important for the treatment of bacterial infections in humans (Desmolaize et al., 2011; Roberts, 2008).

Enterococci resistant to macrolides commonly carry the resistance determinant *erm*(B), an rRNA methylase that confers cross-resistance to MLS_B antibiotics, or *msr*C, a macrolide efflux pump (Portillo et al., 2000). Very little is known about the nature and resistance characteristics of enterococci isolated from feedlot cattle. If *E. hirae* is consistently found as the predominant species in cattle faeces, administering macrolides to cattle may not pose as significant risk because this species is not commonly associated with human infections. Furthermore, antibiotics are often withdrawn prior to slaughter to reduce the risk of residues contaminating meat. In this study, we hypothesized that withdrawal of tylosin prior to slaughter would be an effective method of reducing the risk of resistant enterococci entering the food chain.

The objectives of this study were to determine the prevalence of macrolide resistant enterococci recovered from cattle continuously fed tylosin phosphate, and following its withdrawal. The recovered enterococci were characterised through species identification,

antimicrobial susceptibility testing, identification of resistance determinants and pulsed-field gel electrophoresis (PFGE) profiling.

5.3. Materials and Methods

5.3.1. Experimental design

The enterococci isolates investigated in this study were a subset of those archived during a larger study. Full methodological details have been described previously (Alexander et al., 2008; Sharma et al., 2008) and are summarised briefly below.

British crossbred steers (150±20 kg) were randomly assigned to 10 pens (10 steers per pen) at the Lethbridge Research Centre feedlot (Lethbridge, Alberta, Canada). Steers were obtained from a single ranch (Deseret Ranches, Raymond, Alberta, Canada) and received no antibiotics prior to the beginning of the experiment.

Five pens of cattle each were randomly assigned to one of two treatments: i) control, no antibiotics (denoted CON); ii) tylosin phosphate (Tylan®, Elanco Animal Health; treatment denoted T11) at 11 ppm in the diet. Tylosin was administered continuously for 197 d, starting on arrival at the feedlot and was withdrawn from the diet 28 d prior to slaughter (Figure 5.1.). To avoid cross contamination between diets, tylosin was mixed with 5 kg of supplement and manually spread over the surface of the feed during the morning feeding. Steers were fed once daily to ensure that all feed allotted to each pen was consumed. Steers in CON and T11 treatments were housed in opposite sides of the feed alley to ensure that steers in different treatments did not have direct contact with one another. The animals involved in this study were

cared for according to the guidelines set out by the Canadian Council on Animal Care (Canadian Council on Animal Care, 2003).

Steers were fed diets typical of the western Canadian feedlot industry during a growing and finishing period. For the growing period, a silage-based diet consisting of 70% barley silage, 25% barley grain, and 5% supplement on a dry-matter (DM) basis was fed for the first 80 days (Figure 5.1.). Cattle were transitioned from the silage-based growing diet to a grain-based finishing diet (85% barley grain, 10% barley silage, and 5% supplement on a DM basis) over 21 days and maintained on this diet for a further 124 days until slaughtered. A common watering bowl was shared between adjacent pens on the same treatment.

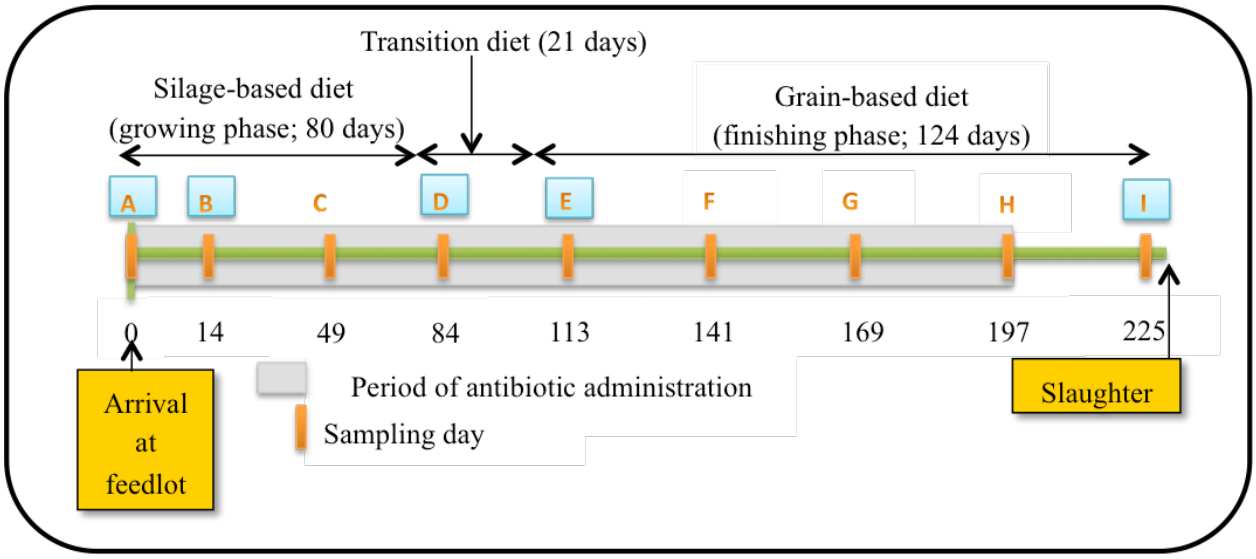


Figure 5.1. Schematic representation of experiment timeline (Figure reproduced from Sharma et al., 2008). Numbers indicate day of feeding period. Periodic orange rectangles indicate points where faecal samples were collected from steers. A, B, D, E and I represent points where isolates were selected for assessing antibiotic susceptibility, PFGE profiles and identifying resistance determinants. Grey shaded area represents the period that tylosin was administered in the diet.

5.3.2. Sample collection and processing

The study occurred from November 2004 to July 2005. Rectal faecal samples were collected from each steer upon arrival at the feedlot and monthly thereafter until slaughter (Figure 5.1.). Proportion of steers positive for macrolide resistant enterococci, CFU counts and the proportion of macrolide resistant enterococci in steers were estimated at all 9 sampling dates with enterococci isolates from 5 of these dates used for assessing antimicrobial susceptibility, identifying resistance determinants and PFGE profiles. The five sampling dates were selected to include isolates prior to administration of tylosin, during the growing and finishing feeding periods and post-withdrawal of tylosin from the diet.

On each sampling date, faecal grab samples were collected and immediately transported to the lab within 1 h after collection. At the lab, faecal slurries were created by mixing faeces (10 g) with 90 mL of 1 × phosphate-buffered saline in a stomacher bag (Fisher Scientific, Ottawa, Ontario, Canada) and using a Stomacher (2 min, 230 rpm, room temperature; Seward Ltd., Worthing, West Sussex, United Kingdom). Slurries were serially diluted 10-fold and 100 μL of the appropriate dilution plated in duplicate onto Bile-Esculin-Azide (BEA; BD, Franklin Lakes, New Jersey, USA) agar containing no antibiotics or onto BEA amended with erythromycin (8μg/mL; BEA^E), or tylosin (32μg/mL; BEA^T) to select for enterococci resistant to erythromycin or tylosin. The breakpoint for erythromycin was based on the Clinical and Laboratory Standards Institute (CLSI) guidelines whilst an arbitrary value, based on avoiding plate growth and the levels used by Davies and Roberts (1999), was selected for tylosin. Plates were incubated for 48 h at 37°C and colonies from BEA, BEA^E, and BEA^T were enumerated. Two isolates from control plates and four isolates from antibiotic selective plates were streaked onto Trypticase soy agar

(TSA; BD), incubated for 24 h, transferred to 20% glycerol in brain heart infusion broth (BD) and stored at -80°C until processed.

5.3.3. Characterisation of enterococci

A total of 1029 presumptive enterococci isolates representing one isolate from each steer faecal sample were revived on the same media from which they were initially isolated (BEA, BEA^E or BEA^T; BD). Cultures were grown over 36 h at 37°C and two colonies were selected and suspended in 75 µL of TE (10 mM Tris, 1 mM EDTA, pH 8.0). Samples were heat lysed for 5 min using a thermomixer set at 98°C with shaking at 1000 rpm, followed by centrifugation at 10,000 × *g* for 5 min. The supernatant containing the genomic DNA was used as a source of template for all PCR reactions. Simultaneously, a subset of presumptive enterococci consisting of ~50% isolates of each category including treatment type, media type and sampling day were randomly selected for species identification. In this manner, 519 presumptive enterococci isolates were selected (Table 5.1.). All of the 1029 isolates were screened by PCR with *Enterococcus* specific *groES-EL* primers Ent-ES-211-233-F and Ent-EL-74-95-R (Zaheer et al., 2012) for confirmation as *Enterococcus* spp. whereas the 519 selected isolates for species identification were further processed for sequencing of the *groES-EL* PCR product. Occasionally, the sequence results of the *groES-EL* PCR product varied from publically available databases. In order to characterise those *Enterococcus* spp. isolates correctly, multilocus sequencing including 16S rRNA, *atpA, pheS* and *rpoA* genes was used to identify species. Detailed methodology can be found in the Appendix 2 (Figure S5.1. and Table S5.1.). In cases where an isolate did not generate the *groES-EL* PCR product, i.e. was not an *Enterococcus*

spp., PCR amplification and sequencing of the 16S rRNA gene using primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') was conducted for taxonomic identification.

A subset of 171 isolates representing major species (~25% coverage) and all minor species were subject to antimicrobial susceptibility testing. These selected isolates were subject to PCR-based identification of resistance determinants and PFGE profiling.

5.3.4. Antimicrobial susceptibility testing

Disc susceptibility tests were conducted on 171 characterised enterococci isolates according to the CLSI documents M02-A11 and M100-S24 (Clinical and Laboratory Standards Institute, 2014a,b). The antimicrobials tested, suppliers and resistance breakpoints applied are listed in Table 5.2. Reference strains *Staphylococcus aureus* ATCC 25923® and *E. faecalis* ATCC 29212® were used as quality controls. Resulting zones of inhibition were read using the BioMic V3 imaging system (Giles Scientific, Inc., Santa Barbara, CA, USA) and classified as sensitive or resistant based on CLSI interpretive criteria (Clinical Laboratory Standards Institute, 2014b), except for tigecycline which used EUCAST interpretive criteria (The European Committee on Antimicrobial Susceptibility Testing, (EUCAST), 2014). Neither EUCAST nor CLSI defined breakpoints exist for enterococci with tylosin, however the quality control range of tylosin discs (30 μg) has recently been acknowledged for *S. aureus* ATCC 25923® (Buß et al., 2014). Tylosin minimum inhibitory concentration (MIC) were established for a sub-set of isolates containing *erm*(B) or *msr*C, both genes or neither gene according to CLSI documents M100-S24 and M07-A9, with results reported in the Appendix 2 (Figure S5.2.). Isolates

exhibiting a high MIC (≥128 µg/mL) to tylosin also contained the resistance determinant *erm*(B). Therefore, isolates harbouring the resistance determinant *erm*(B) were given the designation of resistant to tylosin.

5.3.5. Identification of resistance determinants

Of selected isolates, 125 isolates displaying intermediate or complete resistance to erythromycin were screened for the presence of macrolide resistance determinants. Isolates were first screened by PCR for the commonly found macrolide resistance determinants in enterococci, *erm*(B) and *msr*C (Portillo et al., 2000). For *erm*(B), PCR primers and reaction conditions were used as described by Chen et al. (2007). For *msr*C PCR, the forward and reverse primers, msrC_F1 (5'-TCGTTTTGTCATGAGACAAACAG-3') and msrC_R1 (5'-AAATTAGTCGGTTCATCTAACAG-3'), respectively were used. A 20 µL PCR reaction using 2 µL of template DNA was prepared with the following reaction conditions: initial denaturation for 5 min at 95°C, followed by 35 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 53°C, extension for 30 s at 72°C with a final extension for 10 min at 72°C. The PCR reaction product (5 µL) was resolved on a 2% agarose gel, and visualized for the presence of a 191 bp PCR product. An environmental sample, showing positive amplification for *msr*C and verified by DNA sequencing, was used as a positive control.

A subset of 40 isolates containing *erm*(B) or *msr*C or both genes and consisting of all identified species with a variety of PFGE profiles were further screened for the presence of other macrolide resistance determinants. These included *erm*(A), *erm*(C), *erm*(F), and *erm*(T) with primers and reaction conditions as described by Chen et al. (2007).

Isolates displaying intermediate or complete resistance to doxycycline were further screened for the tetracycline resistance determinants *tet*(B), *tet*(C), *tet*(L), and *tet*(M). A 20 µL PCR reaction using 2 µL of template DNA was prepared with products resolved on a 2% agarose gel. For *tet*(B), primers as described by Peak et al. (2007) were used with the following reaction conditions; initial denaturation for 5 min at 95°C, followed by 35 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 60°C, extension for 30 s at 72°C and a final extension for 10 min at 72°C. Primers and reaction conditions for *tet*(C), *tet*(L), and *tet*(M) were as described by Ng et al. (2001). The expected product size for *tet*(B), *tet*(C), *tet*(L), and *tet*(M) were 205, 418, 267, and 406 bp, respectively.

For all PCR reactions, the commercially available HotStarTaq Plus Master Mix Kit (Qiagen Canada, Inc., Mississauga, ON, Canada) was used according to manufacturer's instructions. Plasmids containing the corresponding gene fragments were used as positive controls (Alexander et al, 2009; Zaheer et al., 2013).

5.3.6. PFGE

One-hundred and seventy-one isolates were subject to PFGE profiling with *Sma*I restriction enzyme using a modified procedure of PulseNet USA (Centers for Disease Control and Prevention, 2012). Briefly, bacteria grown overnight on brain-heart infusion-agar (BHI-agar; BD) were harvested using sterile swabs and suspended in TE buffer to an OD of 1.85 at 610 nm. An aliquot (400 µL) of cell suspension was transferred to a 1.5 mL microfuge tube containing 20 µL of lysozyme (50 mg/mL; Sigma-Aldrich, Co., St Louis, Mo, USA), gently mixed and incubated at 55°C for 45 min. An equal volume of 1.2% molten SeaKem Gold agarose (Lornza,

Rockland, Maine, USA) in TE buffer was added and the mixture dispensed in duplicate into re-useable plug molds (Bio-Rad Laboratories, Hercules, CA, USA) and allowed to solidify at room temperature. Duplicate plugs were added to 2 mL microfuge tubes containing 1.8 mL cell lysis buffer [50mM Tris; 50mM EDTA; 1% sodium sarcosyl] and 9 μL of Proteinase K (20 mg/mL; Sigma-Aldrich, Co., St Louis, Mo, USA) and incubated for 2 h at 55°C with agitation (300 rpm). Plugs were washed twice in sterile, deionized H_2O (1.8 mL) and three times in TE (1.8 mL) for 10 min each using a thermomixer set at 50°C and 300 rpm. Restriction digestion and electrophoresis conditions were as described by Zaheer et al. (2013). Gels were photographed using an AlphaImager gel documentation system (Alpha Innotech Corp., St. Leandro, CA, USA) and banding patterns analysed with BioNumerics V6.6 software (Applied Maths Inc., Austin, TX, USA), using Dice coefficient and the unweighted pair group method (UPGMA). Optimisation and band tolerance were both set at 1%. *Salmonella* serotype Braenderup digested with XbaI was included in each gel as a control reference and for normalisation of band fragments.

5.3.7. Data and statistical analysis

Enumeration data were used to determine the proportion of steers positive for macrolide resistant enterococci and the proportion of macrolide resistant *Enterococcus* in the total population. For the purposes of enumeration, esculin hydrolysing colonies observed on BEA, BEA^E and BEA^T plates were assumed to be enterococci.

Data were analysed using commercially available statistical analysis software (SAS Systems for Windows, version 9.3, SAS Institute Inc, Cary, NC, USA). Prior to analysis,

enumeration data were normalised through a log transformation. When enumeration data for the antibiotic selective media exceeded that of the non-selective media for each sampling point, it was assumed that 100% of the population was resistant to the respective antibiotic. The MIXED procedure of SAS was used to assess CFU counts over time and the proportion of macrolide resistant *Enterococci* in the total population. The CFU counts over time were analysed with media type, day and media type × day in the model as fixed effects while for the proportion of macrolide resistant enterococci in the total population, day, treatment and day × treatment interaction were included in the model as fixed effects. For both analyses, day was included as a repeated measure. Results were considered significant when $P < 0.05$. For most sampling days, 50 samples were collected, but due to conflicts with other experiments in the feedlot facility, only 30 samples were collected on day 49, 141, 169, and 197.

5.4. Results

5.4.1. Prevalence of positive steers and CFU counts of macrolide resistant enterococci

Upon arrival at the feedlot, 28 and 24% (CON and T11, respectively) of the steers were positive for ery^R enterococci, whilst 44 and 38% (CON and T11, respectively) were positive for tyl^R enterococci, even though steers did not previously receive antibiotics (Figure 5.2.).

For the control group, the counts of tyl^R enterococci were higher ($P < 0.05$) than the counts of ery^R enterococci on d 0, 84, 113, 141, 169, 197, and 225 (Figure 5.2.A). Whilst for the tylosin treatment, the counts of tyl^R enterococci were higher ($P < 0.05$) than the counts of ery^R enterococci for d 84, 113, and 225 (Figure 5.2.B). In general, the counts of ery^R enterococci in the tylosin treatment group and counts of tyl^R enterococci in both treatment groups increased

over the sampling period as the cattle were transitioned from a silage-based growing diet to a grain-based finishing diet. The increased counts of macrolide resistant enterococci over the experiment were due to an increase in the proportion of macrolide resistant enterococci within the total population.

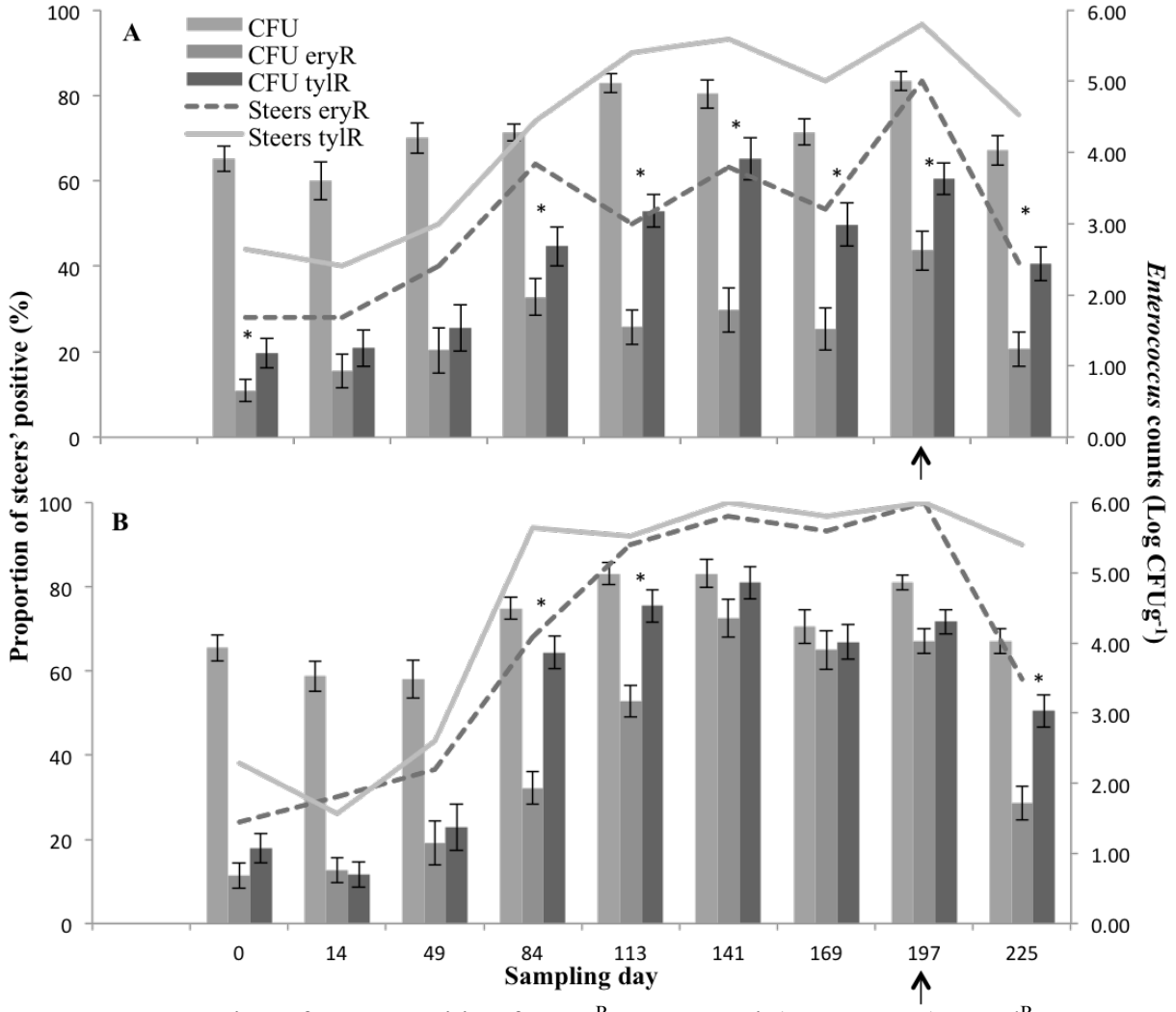


Figure 5.2. Proportion of steers positive for ery^R enterococci (Steers eryR) or tyl^R enterococci (Steers tylR) and *Enterococcus* counts (log CFUg^{-1}) of, total population (CFU), ery^R enterococci (CFU eryR) or tyl^R enterococci (CFU tylR) for CON (A) or T11 (B) treatments. Arrow indicates when antibiotics were withdrawn from the diet. An "*" indicates days for which there was a significant difference between treatments ($P < 0.05$). For each treatment (day 0, 14, 84, 113, and 225 n=50; day 49, 141, 169, and 197 n=30).

5.4.2. Proportion of macrolide resistant enterococci in the total enterococci population

No difference ($P > 0.05$) was observed between control and tylosin-fed steers on d 0, 14, 49, and 84 for the proportion of ery^R enterococci or d 0, 14, and 49 for the proportion of tyl^R enterococci (Figures 5.3.A,B, respectively). On d 113, 141, 169, and 197, the proportion of ery^R enterococci was higher ($P < 0.001$) for steers fed tylosin compared to controls. The proportion of tyl^R enterococci, resistance was higher ($P < 0.001$) for steers fed tylosin compared to controls on d 84, 113, 141, 169, and 197. After withdrawal of tylosin on d 197, the proportion of ery^R or tyl^R enterococci decreased until there was no difference ($P > 0.05$) between tylosin-fed and control steers on d 225 (Figures 5.3.A,B, respectively).

5.4.3. Characterisation of enterococci

Of the 1029 isolates analysed, 95.2% were confirmed as enterococci by PCR. Of the 519 isolates speciated, 504 were identified as *E. hirae* (*n*=431), *Enterococcus villorum* (*n*=32), *E. faecium* (*n*=21), *Enterococcus durans* (*n*=7), *Enterococcus casseliflavus* (*n*=4), *Enterococcus mundtii* (*n*=4), *Enterococcus gallinarum* (*n*=3), *E. faecalis* (*n*=1), and *Enterococcus thailandicus* (*n*=1). The remaining 15 non-enterococci were identified as *Lactobacillus* spp. (*n*=3), *Aerococcus* spp. (*n*=9), *Streptococcus* spp. (*n*=2), and *Staphylococcus epidermids* (*n*=1) as determined by 16S rRNA sequencing. All the species identified were represented by the 231 isolates originally recovered from BEA, whereas only six species (*E. hirae, E. villorum, E. faecium, E. durans, E. casseliflavus*, and *E. gallinarum*) were isolated from BEA^E and BEA^T (Figure 5.4.). Variants of the *groES-EL* sequence for two isolates of *E. faecium* and single

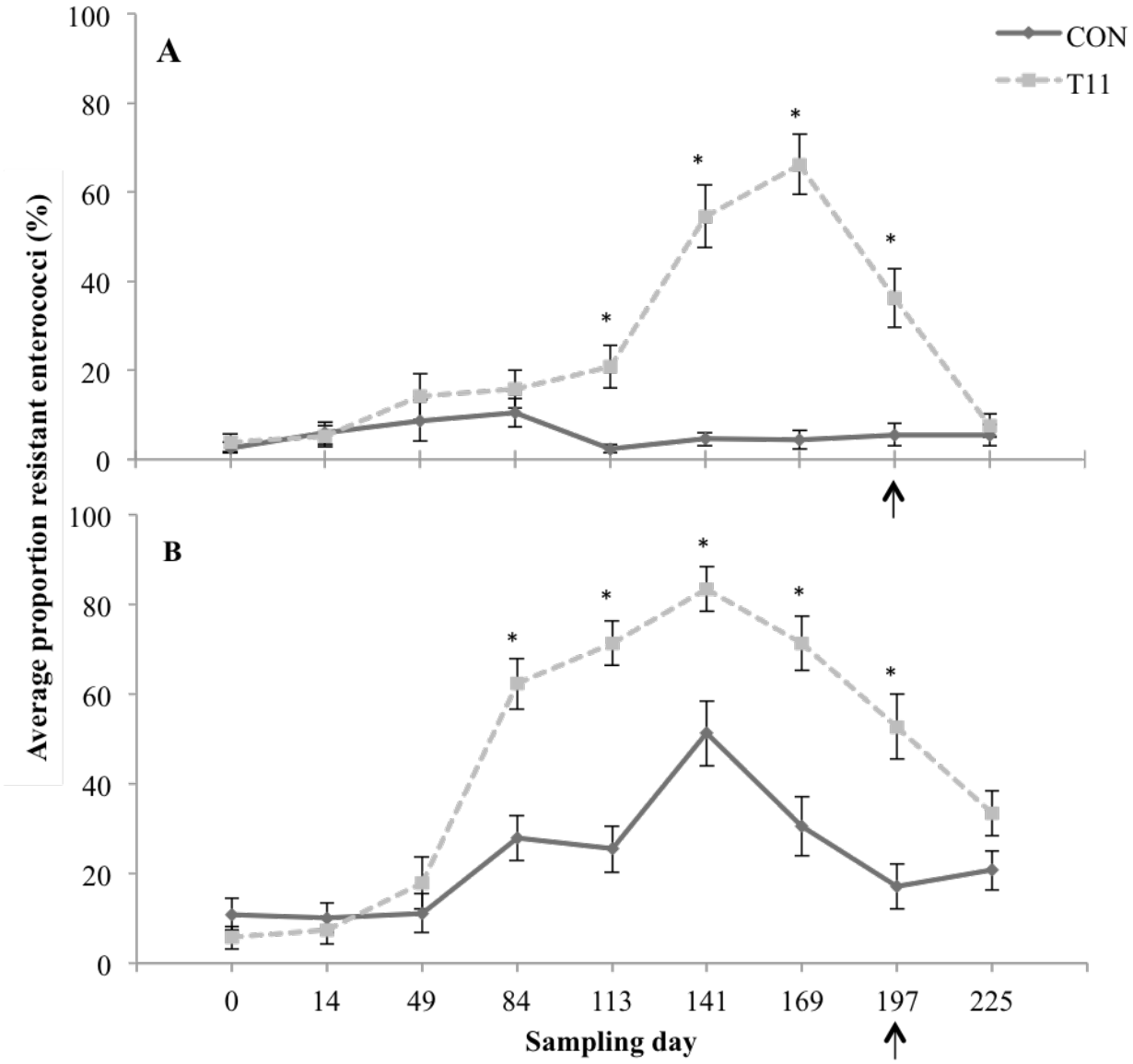


Figure 5.3. Proportion of erythromycin-resistant (A) or tylosin-resistant (B) faecal enterococci isolates for both treatments across all sampling days. Arrow indicates when antibiotics were withdrawn from the diet. Line styles distinguish the treatment. An "*" indicates days for which there was a significant difference between treatments ($P < 0.05$). For each treatment (day 0, 14, 84, 113, and 225 n=50; day 49, 141, 169, and 197 n=30).

isolates of *E. thailandicus* and *E. villorum* have been submitted to the NCBI database (Accession numbers KP993544, KP993545, KP993546, and KP993547, respectively). The diversity of enterococci tended to be greater in steers upon arrival than at exit from the feedlot. A greater

diversity of enterococci species were isolated from non-selective BEA compared with either BEA^E or BEA^T, with similar proportions of most species occurring in control and tylosin-fed steers. *E. hirae* was the predominant species isolated from both control and tylosin-fed steers across all sampling dates (Figure 5.4.).

5.4.4. Antibiotic susceptibility testing

A subset (*n*=171) of enterococci representing all of the isolated *Enterococcus* species were tested for antibiotic susceptibility (Table 5.3.). Resistance to ampicillin, gentamicin, linezolid, streptomycin or tigecycline was not detected in any of the isolates. Vancomycin resistance was also absent in all isolates except for one which displayed intermediate resistance. One isolate of *E. casseliflavus* exhibited ERY-TYL-Q-D-van resistance and one isolate of *E. durans* exhibited ERY-TYL-q-d (lower case denotes intermediate resistance and upper case complete resistance). One isolate of *E. faecium* was ERY-DOX-TYL-q-d resistant, with other single isolates exhibiting intermediate ery-nit, ery-lvx or dox-nit-lvx-q-d resistance. Two isolates of *E. gallinarum* showed ery-TYL resistance and a number of *E. hirae* isolates were resistant to ERY-TYL (*n*=27), ery-TYL (*n*=27), ERY-dox-TYL (*n*=8), or ERY-TYL-q-d (*n*=7). With one exception, all *E. villorum* isolates exhibited ERY-TYL (*n*=31) resistance.

In general, isolates grown on BEA^T also exhibited erythromycin resistance. An exception to this was three isolates of *E. durans* isolated on BEA^T, which remained susceptible to erythromycin.

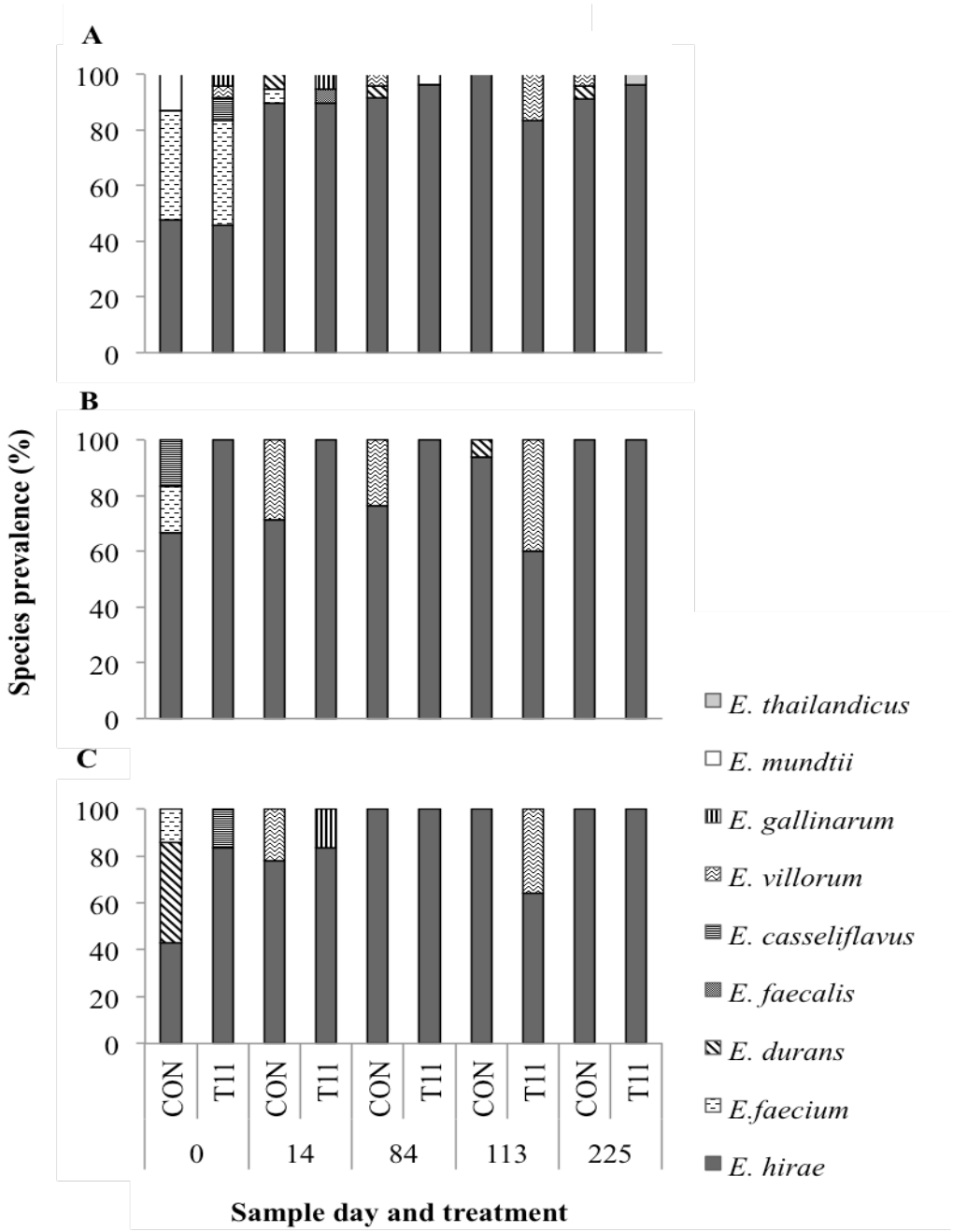


Figure 5.4. Species distribution of characterised isolates from (A) BEA (bile esculin azide agar), (B) BEA^E (bile esculin azide agar amended with erythromycin [8µg/mL]) and (C) BEA^T (bile esculin azide agar amended with tylosin [32µg/mL]). Prevalence was calculated by dividing the number of isolates for each species by the total number of isolates from each sample day and treatment.

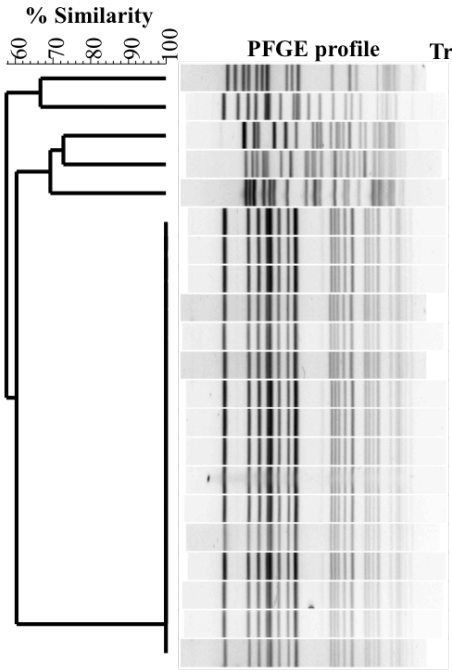
5.4.5. Identification of resistance determinants

Of the 125 enterococci isolates displaying intermediate or complete resistance to erythromycin, the *erm*(B) gene was detected in 106 isolates representing *E. hirae*, *E. durans, E. faecium, E. villorum, E. gallinarum*, and *E. casseliflavus.* Of the 19 erythromycin-resistant *E. faecium* isolates obtained all except one lacked *erm*(B), but all were positive for *msr*C. The isolate identified as *E. thailandicus* displayed intermediate resistance to erythromycin, but was negative for all of the macrolide resistance determinants tested. None of the isolates tested positive for the other macrolide resistance determinants.

A total of 10 isolates displayed intermediate or complete resistance to doxycycline. None of the isolates were positive for *tet*(B) or *tet*(C). All 10 isolates were positive for *tet(*M) and 9 were positive for *tet*(L).

5.4.6. PFGE

The PFGE profiles of *E. faecium, E. villorum* and erythromycin resistant *E. hirae* are displayed in Figures 5.5.-5.7., respectively. *E. faecium* had at least 16 isolates from different steers with the same PFGE profile, suggesting the presence of a clonal population. Isolates from this clonal population were isolated only on day 0 (Figure 5.5.). The similarity (>95%) of PFGE profiles of *E. villorum* also suggested clonality (Figure 5.6.). Unlike *E. faecium*, these profiles appeared on day 14 of the trial and persisted until the end of the experiment. PFGE profiles of erythromycin resistant *E. hirae* produced 8 clusters with >85% similarity (Figure 5.7.).



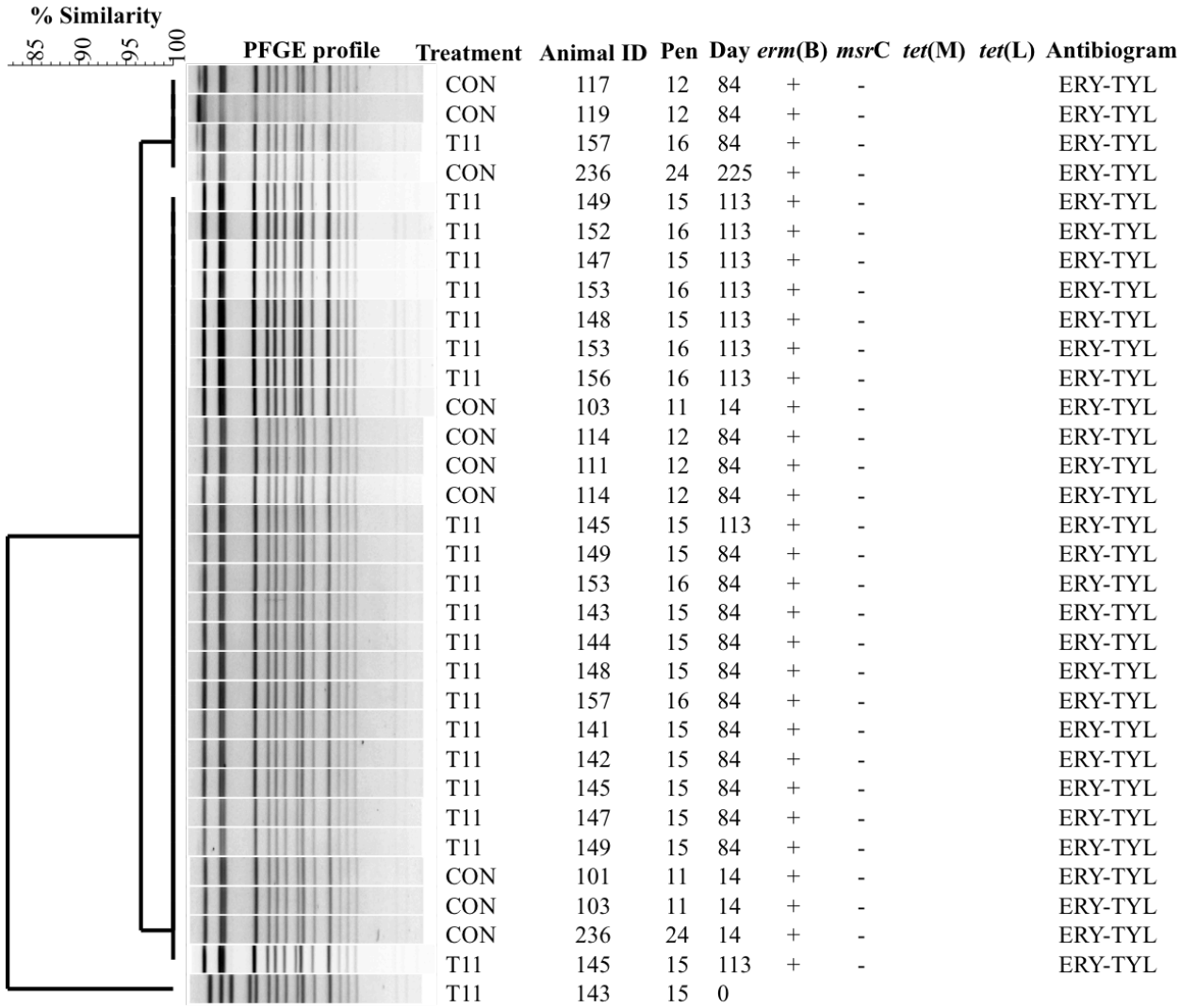
Treatment	Animal ID	Pen	Day	*erm*(B)	*msr*C	*tet*(M)	*tet*(L)	Antibiogram
T11	148	15	0			+	-	dox-nit-lvx-q-d
T11	229	23	0					
CON	114	12	0	-	+			ery
CON	09	1	0	+	+	+	+	ERY-TYL-DOX-q-d
CON	01	1	14	-	+			ery-lvx
CON	02	1	0	-	+			ERY
CON	101	11	0	-	+			ERY
CON	103	11	0	-	+			ery
CON	110	11	0	-	+			ery
CON	111	12	0	-	+			ERY
CON	120	12	0	-	+			ERY
T11	149	15	0	-	+			ERY
T11	153	16	0	-	+			ERY
T11	160	16	0	-	+			ERY
T11	226	23	0	-	+			ERY
CON	231	24	0	-	+			ERY
CON	240	24	0	-	+			ERY
T11	44	5	0	-	+			ERY
T11	46	5	0	-	+			ery-nit
T11	60	6	0	-	+			ERY
CON	20	2	0	-	+			ERY

Figure 5.5. Dendrogram of PFGE *Sma*I profiles from isolates identified as *Enterococcus faecium*. A "+" indicates PCR positive and "-" indicates PCR negative to the respective genes. A "blank" space indicates the gene was not screened for in the respective isolate. For the antibiogram, upper case denotes complete resistance and lower case denotes incomplete resistance.

5.5. Discussion

Enterococci are ubiquitous in nature and are frequently isolated from the gastrointestinal tract of mammals, including humans (Franz et al., 2011). Of the enterococci recovered from this study *E. hirae* was revealed to be the predominant species isolated, an observation consistent with previous studies (Anderson et al., 2008; Jackson et al., 2010; Zaheer et al., 2013).

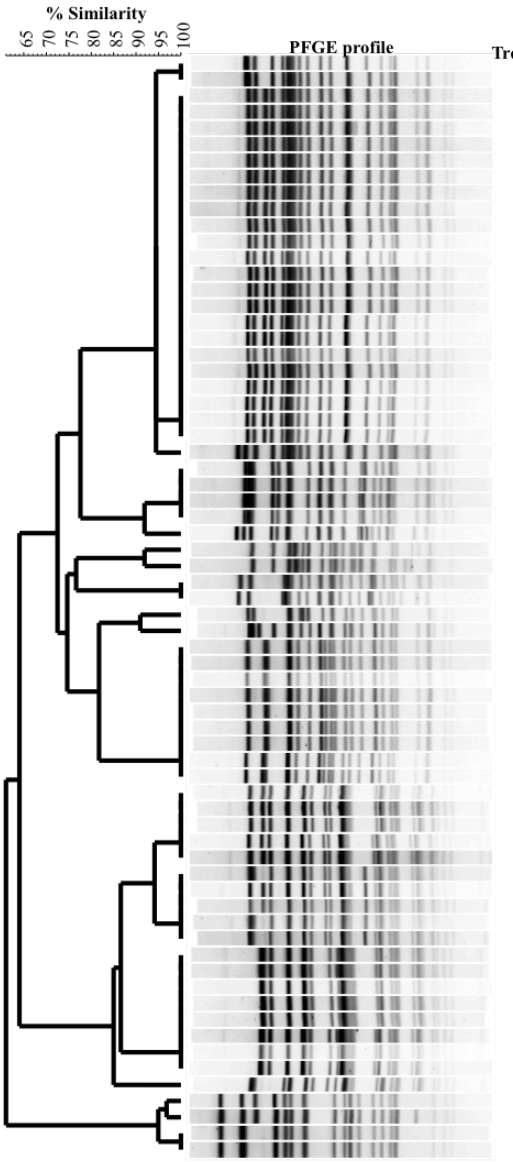
Enterococci have been described as a "drug resistance gene trafficker" due to the ease with which they can acquire and transfer resistance genes (Werner et al., 2013). They have emerged as a serious threat to human health, particularly due to the acquisition of vancomycin resistance, increasing the difficulty of successful treatment (Centers for Disease Control and Prevention, 2013). Of the 171 isolates examined for antibiotic resistance, only one isolate



Treatment	Animal ID	Pen	Day	*erm*(B)	*msr*C	*tet*(M)	*tet*(L)	Antibiogram
CON	117	12	84	+	-			ERY-TYL
CON	119	12	84	+	-			ERY-TYL
T11	157	16	84	+	-			ERY-TYL
CON	236	24	225	+	-			ERY-TYL
T11	149	15	113	+	-			ERY-TYL
T11	152	16	113	+	-			ERY-TYL
T11	147	15	113	+	-			ERY-TYL
T11	153	16	113	+	-			ERY-TYL
T11	148	15	113	+	-			ERY-TYL
T11	153	16	113	+	-			ERY-TYL
T11	156	16	113	+	-			ERY-TYL
CON	103	11	14	+	-			ERY-TYL
CON	114	12	84	+	-			ERY-TYL
CON	111	12	84	+	-			ERY-TYL
CON	114	12	84	+	-			ERY-TYL
T11	145	15	113	+	-			ERY-TYL
T11	149	15	84	+	-			ERY-TYL
T11	153	16	84	+	-			ERY-TYL
T11	143	15	84	+	-			ERY-TYL
T11	144	15	84	+	-			ERY-TYL
T11	148	15	84	+	-			ERY-TYL
T11	157	16	84	+	-			ERY-TYL
T11	141	15	84	+	-			ERY-TYL
T11	142	15	84	+	-			ERY-TYL
T11	145	15	84	+	-			ERY-TYL
T11	147	15	84	+	-			ERY-TYL
T11	149	15	84	+	-			ERY-TYL
CON	101	11	14	+	-			ERY-TYL
CON	103	11	14	+	-			ERY-TYL
CON	236	24	14	+	-			ERY-TYL
T11	145	15	113	+	-			ERY-TYL
T11	143	15	0					

Figure 5.6. Dendrogram of PFGE *Sma*I profiles from isolates identified as *Enterococcus villorum*. A "+" indicates PCR positive and "-" indicates PCR negative to the respective genes. A "blank" space indicates the gene was not screened for in the respective isolate. For the antibiogram, upper case denotes complete resistance and lower case denotes incomplete resistance.

displayed intermediate resistance to vancomycin. This isolate was identified as *E. casseliflavus*, an outcome that likely reflects the intrinsic resistance of *E. casseliflavus* and *E. gallinarum* to low levels of vancomcyin (Hollenbeck and Rice, 2012). This observation is encouraging, as the enterococci isolated from beef cattle do not appear to represent a significant source of vancomycin resistance.



Treatment	Animal ID	Pen	Day	*erm*(B)	*msr*C	*tet*(M)	*tet*(L)	Antibiogram
CON	101	11	113	+	-			ery-TYL
T11	160	16	113	+	-			ery-TYL
CON	115	12	84	+	-			ery-TYL
T11	141	15	84	+	-			ery-TYL
CON	235	24	84	+	-			ery-TYL
T11	49	5	84	+	-			ery-TYL
T11	55	6	84	+	-			ery-TYL
CON	102	11	113	+	-			ery-TYL
CON	108	11	113	+	-			ery-TYL
T11	154	16	113	+	-			ery-TYL
CON	08	1	225	+	-			ery-TYL
T11	226	23	225	+	-			ery-TYL
T11	59	6	225	+	-			ery-TYL
T11	147	15	84	+	-			ery-TYL
T11	224	23	84	+	-			ery-TYL
T11	227	23	84	+	-			ery-TYL
CON	106	11	113	+	-			ery-TYL
CON	119	12	113	+	-			ery-TYL
CON	235	24	113	+	-			ery-TYL
T11	51	6	113	+	-			ery-TYL
T11	55	6	113	+	-			ery-TYL
CON	103	11	225	+	-			ery-TYL
CON	112	12	225	+	-			ery-TYL
T11	225	23	225	+	-			ery-TYL
CON	12	2	113	+	-			ery-TYL
CON	118	12	225	+	-			ERY-TYL-q-d
CON	15	2	14	+	-			ERY-TYL-q-d
CON	13	2	84	+	-			ery-TYL
CON	240	24	113	+	-			ERY-TYL
T11	141	15	225	+	-			ERY-TYL-q-d
T11	53	6	14	+	-			ERY-TYL
T11	53	6	14	+	-			ERY-TYL
CON	14	2	113	+	-			ERY-TYL
T11	160	16	225	+	-			ERY-TYL
CON	10	1	225	+	-			ERY-TYL-q-d
T11	57	6	0	+	-			ERY-TYL
T11	46	5	84	+	-	+	+	ERY-TYL-dox
T11	55	6	84	+	-	+	+	ERY-TYL-dox
T11	42	5	225	+	-	+	+	ERY-TYL-dox
T11	56	6	113	+	-	+	+	ERY-TYL-dox
T11	45	5	84	+	-	+	+	ERY-TYL-dox
T11	46	5	84	+	-	+	+	ERY-TYL-dox
T11	60	6	84	+	-	+	+	ERY-TYL-dox
CON	10	1	225	+	-	+	+	ERY-TYL-dox
T11	57	6	225	+	-			ERY-TYL
CON	103	11	113	+	-			ERY-TYL
T11	55	6	225	+	-			ERY-TYL
T11	229	23	225	+	-			ERY-TYL
T11	226	23	225	+	-			ERY-TYL-q-d
CON	14	2	14	+	-			ERY-TYL
CON	19	2	84	+	-			ERY-TYL
T11	150	15	113	+	-			ERY-TYL
CON	113	12	0	+	-			ERY-TYL
CON	19	2	14	+	-			ERY-TYL
CON	19	2	0	+	-			ERY-TYL
CON	233	24	84	+	-			ERY-TYL
CON	237	24	84	+	-			ERY-TYL
CON	116	12	113	+	-			ERY-TYL
CON	16	2	84	+	-			ERY-TYL
CON	17	2	84	+	-			ERY-TYL
CON	237	24	84	+	-			ery-TYL
T11	156	16	225	+	-			ERY-TYL
CON	238	24	225	+	-			ERY-TYL
T11	227	23	0	+	-			ERY-TYL
CON	09	1	84	+	-			ERY-TYL
T11	144	15	113	+	-			ERY-TYL
T11	148	15	84	+	-			ERY-TYL
T11	143	15	14	+	-			ERY-TYL

Figure 5.7. Dendrogram of PFGE *Sma*I profiles from isolates identified as erythromycin resistant *Enterococcus hirae*. A "+" indicates PCR positive and "-" indicates PCR negative to the respective genes. A "blank" space indicates the gene was not screened for in the respective isolate. For the antibiogram, upper case denotes complete resistance and lower case denotes incomplete resistance.

E. faecium and *E. faecalis* are the two species most commonly associated with nosocomial human infections (Ruoff et al., 1990; Sievert et al., 2013; Werner et al., 2008). These species have been isolated from cattle (Anderson et al., 2008; Jackson et al., 2010; Kuhn et al., 2003), but they do not predominate, with our study suggesting that their prevalence declines after

cattle enter the feedlot. Although *E. hirae*, as well as other enterococcal species (i.e. *Enterococcus avium, E. durans, E. casseliflavus, E. gallinarum*, and *Enterococcus raffinosus*) can cause clinical infections in humans, they are rare and thought to be more opportunistic in nature than those caused by *E. faecium* and *E. faecalis* (Alfouzan et al., 2014; Ruoff et al., 1990). Presence of *E. hirae* predominantly in the bovine gastrointestinal tract suggests that cattle do not present a significant source of *Enterococcus* that could colonise and infect humans.

In the absence of selection, the predominant resistance phenotype observed in the enterococci recovered from cattle was to erythromycin or tylosin, including isolates recovered pre- and post- antibiotic treatment. Despite no prior treatment with antimicrobials, steers harboured ery^R (28 and 24%, CON and T11 respectively) and tyl^R (44 and 38%, CON and T11 respectively) enterococci upon arrival at the feedlot (Figure 5.2.). This suggests that naturally occurring resistance determinants coding for macrolide resistance are already present and circulating in bovine gut enterococci populations.

For some days, the counts of tyl^R enterococci were higher ($P < 0.05$) than ery^R enterococci for both treatment groups (Figure 5.2.). It would be expected that similar counts would be obtained for both ery^R and tyl^R enterococci as the same resistance mechanism confers resistance to both antibiotics (Desmolaize et al., 2011; Roberts, 2008). Enterococci with both intermediate and complete resistance to erythromycin were isolated from tylosin plates; whilst erythromycin plates only selected for enterococci with complete resistance to erythromycin, explaining some of the discrepancy seen between enumeration data for the two media. Isolates from tylosin media with intermediate resistance to erythromycin also carried the *erm*(B) gene. It appears that the MIC breakpoint for erythromycin may be too high, therefore missing enterococci with intermediate resistance which also carry a resistance determinant. Conversely,

the MIC breakpoint for tylosin may be too low thereby selecting for isolates that contain resistance determinants that may be compromised, resulting in an intermediate resistance phenotype. The fact that three isolates of *E. durans* from the tylosin media remained susceptible to erythromycin supports this theory. It is possible however, that these isolates carry a resistance determinant not screened for. It would be worthwhile to further explore the likely genetic differences between the resistance determinant(s) from complete and intermediate tylosin resistant isolates to identify the linkage between antimicrobial resistance (AMR) genotype and phenotype.

As the trial progressed, the number of steers positive for macrolide resistant enterococci increased in both treatment groups. This increase, even in the control group may be a reflection of increased transmission between steers due to close proximity in the feedlot environment. Likewise, the changing population dynamics of enterococci in the gastrointestinal tract of cattle may also contribute to increased transmission. Increased shedding of macrolide resistant enterococci would increase the likelihood of cattle being exposed to macrolide resistant enterococci and thus also increase the detection of positive cattle. Similarly, an increase in the proportion of the population that are macrolide resistant would increase the chances of isolating macrolide resistant enterococci. For a steer to be considered positive in this study, isolation of a single macrolide resistant enterococci colony was required. In order to make an assessment of resistance development it is important to look at resistance as a proportion of the total enterococci population.

The CFU counts of the overall enterococci population remained relatively constant over the experiment for both treatments (Figure 5.2.). This trend was also true for CFU counts of ery^R enterococci in the control group (Figure 5.2.A), whilst the CFU counts of ery^R enterococci in the

tylosin treatment group tended to increase during the period of tylosin administration before dropping off on d 197, presumably due to its withdrawal from the diet (Figure 5.2.B). This trend was also observed for the CFU counts of tyl^R enterococci for both treatments, with possible differences between ery^R and tyl^R CFU being attributed to the selection of intermediate resistant enterococci on the tylosin media (Figure 5.2.). A delay between the increase of CFU counts and tylosin administration can be seen, with increases coinciding with the transition from a silage-based diet to a grain-based diet.

High-grain diets tend to increase the amount of starch available in the lower intestinal tract, changing the nutrient availability for bacterial growth (Callaway et al., 2009). Previous researchers have reported a 1 (Scott et al., 2000) to 3 log (Diez-Gonzalez et al., 1998) increase in *Escherichia coli* when cattle were transitioned from a forage- to a grain-based diet. Changes that occur in the gastrointestinal environment of cattle as a result of increased starch in the diet alter the composition of the microbiome (Shanks et al., 2011). It is possible that the transition to a grain-based diet created conditions ideal for proliferation of macrolide resistant enterococci. Although not seen with the CFU of ery^R enterococci, the increase of tyl^R enterococci in both the control and tylosin treatment group suggest factors other than administration of tylosin may have been selecting for macrolide resistant enterococci.

Increases in ery^R enterococci in cattle as a result of the administration of tylosin has been previously documented (Jacob et al., 2008; Zaheer et al., 2013), but these authors did not study the effect of withdrawal of tylosin from the diet. As in previous studies, there was an increase in the proportion of ery^R and tyl^R resistant enterococci isolated from cattle administered tylosin. The proportion of ery^R and tyl^R resistant enterococci for the tylosin treatment began decreasing just prior to removal of tylosin from the diet and continued to decrease after its withdrawal, until

no difference ($P > 0.05$) was observed between treatments on d 225 (Figure 5.3.). It appears that withdrawal of tylosin phosphate prior to slaughter contributes to a reduction in the proportion of macrolide resistant enterococci entering the food chain. However, the possibility that other unknown factors such as stress, age and diet may also be influencing this decline cannot be eliminated. It would be interesting to investigate this phenomenon further to determine why this reduction is occurring prior to the withdrawal of tylosin from the diet.

A decrease in species diversity was observed as the experiment progressed, with *E. hirae* being the predominant species identified. Transitioning of the diet from a forage- to a grain-based diet alters the faecal microbiome of cattle (Shanks et al., 2011). Diet may be a contributing factor in the shift in species diversity seen in this study, but it is also possible that other factors, such as age, may also be influencing the faecal microbial community (Devriese et al., 1992).

In this study, *E. thailandicus* and *E. villorum* were identified using multilocus sequencing of 16S rRNA, *atpA, pheS*, and *rpoA* genes after the discovery of *groES-EL* PCR products that varied from publically available databases (Appendix 2 Figure S.5.1.). To our knowledge, these species have not been previously isolated from cattle. *E. thailandicus* was first isolated in 2008 from fermented sausage in Thailand (Tanasupawat et al., 2008) and has been found in swine faeces (Liu et al., 2013). *E. villorum* was first isolated in 2001 from piglets (Vancanneyt et. al., 2001). Traditional methods of identifying *Enterococcus* species rely on biochemical tests which are unreliable for atypical species or species that have not been previously isolated (Deasy et al., 2000; Jackson et al., 2004). Molecular techniques have the advantage of being able to differentiate between closely related enterococci species.

Erythromycin resistant enterococci possessed either *erm*(B) or *msr*C or both resistance genes. Isolates designated as tylosin resistant possessed *erm*(B). Other macrolide resistance determinants were absent in the subset of isolates screened and it is possible that isolates not screened may have contained macrolide resistance determinants other than *erm*(B) or *msr*C. Presence of at least one resistance determinant in these isolates however confirmed the association between resistance phenotype and genotype.

Eight isolates of *E. hirae* and one isolate of *E. faecium* displayed complete resistance to erythromycin and either complete or intermediate resistance to doxycycline. These isolates were all positive for *erm*(B), *tet*(L), and *tet*(M). The resistance genes *erm*(B) and *tet*(M) are often associated with the transposon Tn*1545* (Clewell et al., 1995; Rice, 1998). The transposon integrase gene (*int* gene) of Tn*916*/Tn*1545* family of transposons has been previously detected in enterococci (De Leener et al., 2004). The identification of *erm*(B) and *tet*(M) in the same isolate in this study could possibly suggest the presence of mobile genetic elements. It would be worthwhile to investigate this further as many *erm* genes are often linked with other antibiotic resistance genes, tetracycline in particular (Roberts et al., 1999). Linkage of macrolide and other resistance genes is potentially problematic as administrating tylosin to cattle may not only select for macrolide resistance, but also for resistance to antibiotics such as tetracycline. Co-selection of tetracycline resistance upon the administration of tylosin has been suggested to occur within the faecal microbial communities of beef cattle (Chen et. al., 2008). Linkage of these genes on mobile genetic elements increases the potential for the transfer of genes conferring resistance to multiple antibiotics (Hegstad et al., 2010; Tremblay et al., 2012).

Pulsed-field gel electrophoresis revealed a predominate cluster of *E. faecium* containing *msr*C and displaying a similar AMR profile of intermediate or complete resistance to

erythromycin. Sequencing of *msr*C revealed that all isolates within this cluster had identical sequences. However, there were sequence differences in the *msr*C gene among these isolates and isolates with unique PFGE profiles (Figure 5.5.). The four newly identified sequences have been submitted to the NCBI sequence database (Accession numbers KP775623, KP775624, KP775625, and KP775626).

Similar PFGE profiles were seen pre- and post-antibiotic treatment for erythromycin resistant *E. hirae*, highlighting that administration of tylosin selected for erythromycin resistant enterococci already present in the bovine gastrointestinal tract. These same profiles were still present after d 225; 28 days after tylosin had been removed from the diet. This suggests that although administration of tylosin increased the proportion of macrolide resistant enterococci in beef cattle it does not appear to be promoting the transfer of resistance between isolates. Once the selection pressure is removed (withdrawal of tylosin), the proportion of macrolide resistant enterococci returned to levels seen before antibiotic treatment.

5.6. Conclusion

Few studies have investigated the role that administration of tylosin in the feed of beef cattle has on the development of macrolide resistance in enterococci. This study demonstrated that administering tylosin to cattle increases the proportion of macrolide resistant enterococci. Withdrawal of tylosin from the diet appears to contribute to the decline in macrolide resistant enterococci but may not be the only factor influencing this decline. Furthermore, transitioning cattle to a grain-based diet appears to alter the species population of enterococci to one in favour of *E. hirae*, a species not commonly associated with infection in humans. PFGE profiling of

erythromycin resistant *E. hirae* suggest that antibiotic administration selects resistant strains already present in the intestinal microbial population.

5.7. References

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Tables

Table 5.1. Distribution of isolates characterised in this study

Treatment[a]	Media used for selection[b]	Sampling day[c]					Total
		0	**14**	**84**	**113**	**225**	
CON	BEA	24	20	25	25	23	119
	BEA^E	6	8	17	16	9	58
	BEA^T	9	9	19	22	19	79
T11	BEA	24	20	25	25	25	122
	BEA^E	6	8	15	20	14	65
	BEA^T	8	7	24	25	22	86
Total		77	72	125	133	112	519

[a]Steers fed no antibiotics (control, CON) or tylosin phosphate (11 ppm; T11); administered continuously and withdrawn on day 197.
[b]Isolates were streaked onto bile esculin azide agar (BEA) containing no antibiotics, or amended with erythromycin (8µg/mL; BEA^E) or with tylosin (32µg/mL; BEA^T).
[c]Sampling days began at day 0 (arrival at feedlot) prior to antibiotic administration and continued until the end of the feeding trial; sample day 0 and 14 were during the silage-based diet, day 84 during the transition diet and day 113 and 225 during the grain-based diet.

Table 5.2. Antibiotics, suppliers, disc content and breakpoints used for disc susceptibility testing

Antibiotic	Supplier	Disc content (µg)	Zone diameter (mm) breakpoints[d]		
			S	I	R
Ampicillin[a]	BD	10	≥17	-	≤16
Doxycycline[a]	BD	30	≥16	13-15	≤12
Erythromycin[a]	BD	15	≥23	14-22	≤13
Gentamicin[a]	BD	120	≥10	7-9	6
Levofloxacin[a]	BD	5	≥17	14-16	≤13
Linezolid[a]	BD	30	≥23	21-22	≤20
Nitrofurantoin[a]	BD	300	≥17	15-16	≤14
Quinupristin-dalfopristin[a]	BD	4.5/10.5	≥19	16-18	≤15
Streptomycin[a]	BD	300	≥10	7-9	6
Tigecycline	BD	15	≥18	-	<15
Tylosin[b]	Medox	30	n/a	n/a	n/a
Vancomycin[a,c]	BD	30	≥17	15-16	≤14
Ampicillin[a]	BD	10	≥17	-	≤16

[a]M100-S24: Performance standards for antimicrobial susceptibility testing; twenty-fourth informational supplement (CLSI, 2014b).
[b]Breakpoint tables for interpretation of MICs and zone diameters. Version 4.0. (EUCAST, 2014).
[c]Vancomycin requires 24 hours incubation while for all other antibiotics 16-18 hours incubation is sufficient.
[d]Zone diameter value used to indicate susceptible (S), intermediate (I), resistant I and not available (n/a).

Table 5.3. Number of enterococci isolates (percentage of total species[a]) showing intermediate or complete resistance to antibiotics pooled across treatments, isolation media and sample date

Enterococcus spp.		**Antibiotic[b] (No. isolates [%])**											
		AMP	**DOX**	**ERY**	**GEN**	**LVX**	**LZD**	**NIT**	**Q-D**	**STR**	**TGC**	**TYL[c]**	**VAN**
E. hirae (*n*=98)	I	n/a	8 (8.2)	27 (27.6)	0	0	0	0	7 (7.1)	0	n/a	n/a	0
	R	0	0	42 (42.9)	0	0	0	0	0	0	0	69 (70.4)	0
E. villorum (*n*=32)	I	n/a	0	0	0	0	0	0	0	0	n/a	n/a	0
	R	0	0	31 (96.9)	0	0	0	0	0	0	0	31 (96.9)	0
E. faecium (*n*=21)	I	n/a	1 (4.8)	5 (23.8)	0	2 (9.5)	0	2 (9.5)	2 (9.5)	0	n/a	n/a	0
	R	0	1 (4.8)	14 (66.7)	0	0	0	0	0	0	0	1 (4.8)	0
E. durans (*n*=7)	I	n/a	0	0	0	0	0	0	1 (14.3)	0	n/a	n/a	0
	R	0	0	1 (14.3)	0	0	0	0	0	0	0	1 (14.3)	0
E. casseliflavus (*n*=4)	I	n/a	0	0	0	0	0	0	0	0	n/a	n/a	1 (25.0)
	R	0	0	2 (50.0)	0	0	0	0	1 (25.0)	0	0	2 (50.0)	0
E. mundtii (n=4)	I	n/a	0	0	0	0	0	0	0	0	n/a	n/a	0
	R	0	0	0	0	0	0	0	0	0	0	0	0
E. gallinarum (n=3)	I	n/a	0	2 (66.7)	0	0	0	0	0	0	n/a	n/a	0
	R	0	0	0	0	0	0	0	0	0	0	2 (66.7)	0
E. faecalis (*n*=1)	I	n/a	0	0	0	0	0	0	0	0	n/a	n/a	0
	R	0	0	0	0	0	0	0	1 (100.0)	0	0	0	0
E. thailandicus (*n*=1)	I	n/a	0	1 (100.0)	0	0	0	0	0	0	n/a	n/a	0
	R	0	0	0	0	0	0	0	0	0	0	0	0

[a]Percentages were calculated by dividing resistant isolates with the total number of isolates for individual species and rounded to the first decimal place.

[b]AMP, ampicillin; DOX, doxycycline; ERY, erythromycin; GEN, gentamicin; LVX, levofloxacin; LZD, linezolid; NIT, nitrofurantoin; Q-D, quinupristin-dalfopristin; STR, streptomycin; TGC, tigecycline; TYL, tylosin; VAN, vancomycin.

[c]Resistance isolates were classified as those which carried the erm(B) resistance gene (see materials and methods for more information).

R, complete resistance; I, intermediate resistance; n/a, no interpretive criteria for intermediate resistance.

Chapter 6

Draft genome sequence of an *Enterococcus thailandicus* strain isolated from bovine faeces[3]

[3]This chapter was published in full: Beukers, A.G., Zaheer, R., Goji, N., Cook, S.R., Amoako, K.K., Chaves, A.V., Ward, M.P. and McAllister, T.A. (2016). Draft genome sequence of an *Enterococcus thailandicus* strain isolated from bovine feces. *Genome Announc*. 4(4):e00576-16.

6.1. Abstract

Here, we report the first draft genome sequence of *Enterococcus thailandicus* isolated from the faeces of feedlot cattle in Southern Alberta.

6.2. Introduction and Results

Enterococcus thailandicus was first isolated from fermented sausage in Thailand in 2008 (Tanasupawat et al., 2008) and has been identified in the faeces of swine (Liu et al., 2013). We isolated *E. thailandicus* with an intermediate resistance to erythromycin from bovine faeces in Alberta, Canada in 2005 (Beukers et al, 2015). This isolate was originally identified through a previously unobserved variance in the *groES-EL* spacer region (Zaheer et al., 2012). The nonexistence/unavailability of *E. thailandicus* genome sequence in the database provided motive for selecting this isolate for whole-genome sequencing. The present genome sequence will help provide further insight and understanding of *Enterococcus* genera.

Here, we report the first draft genome sequence of *E. thailandicus*. Genomic DNA was prepared as described by Klima et al. (2016). Indexed paired-end libraries were prepared using the Nextera XT DNA sample preparation kit (Illumina, Inc., CA) and paired-end (2 × 300 bp reads) sequenced on an Illumina MiSeq platform (Illumina) to yield a total of 1,169,142 reads. High quality reads were *de novo* assembled using SPAdes version 3.6.0 software (Bankevich et al., 2012).

The draft genome of *E. thailandicus* has a total size of 2,603,691 bp with a GC content of 36.7% and consists of 17 contigs ranging from 998 bp to 431,427 bp with an average coverage of

39× and an N_{50} length of 337,578 bp. Genome annotation was performed by use of the NCBI Prokaryotic Genome Annotation Pipeline (http://www.ncbi.nlm.nih.gov/genome/annotation_prok/), leading to the prediction of 2,397 protein-coding genes, 56 tRNAs, 1 transfer-messenger RNA (tmRNA), and 5 rRNA operons. At least four multidrug efflux pump proteins were annotated in the genome and may have contributed to the observed intermediate resistance to erythromycin (Beukers et al., 2015). A glycopeptide resistance protein with homology to VanZ was also identified in the genome. VanZ is known to confer low-level resistance to teicoplanin in *Enterococcus faecium* but not to vancomycin (Arthur et al., 1995). No resistance determinants were identified using the Comprehensive Antibiotic Resistance Database (CARDs) (McArthur et al., 2013) or the ResFinder version 2.1 server (Zankari et al., 2012). No virulence factors were identified using the VirulenceFinder version 1.5 server (Joensen et al., 2014). Limitations of databases for both antibiotic resistance and virulence genes could have resulted in unknown resistance or virulence genes remaining unidentified. It is possible that *E. thailandicus* contains further novel antibiotic resistance or virulence genes with further studies required to elucidate this.

The genome was ordered based on alignment against *E. faecium* T110 (Accession number CP006030.1) using progressive Mauve (Darling et al., 2010) and analysed for the presence of prophage using PHAST (Zhou et al., 2011). Three incomplete and one questionable prophage were predicted in the genome. Six confirmed clustered regularly interspaced short palindromic repeat (CRISPR) arrays were identified using CRISPRfinder (Grissa et al., 2007). Only one CRISPR array was linked to CRISPR-associated (*cas*) genes, consisting of *cas9*, *cas2*, *cas1* and *csn2* classifying this array as a type II-A system (Chylinski et al., 2014). Gene clusters

encoding for the production of a putative lantipeptide and a bacteriocin were predicted using the Antibiotics and Secondary Metabolite Analysis Shell (Medema et al., 2011).

6.3. Conclusion

The addition of the draft genome of *E. thailandicus* has expanded on the current *Enterococcus* genome database and will be a valuable addition in comparative genomic analysis studies to further understanding of the diversity of the genus *Enterococcus*.

Nucleotide sequence accession number. This Whole Genome Shotgun project has been deposited in DDBJ/ENA/GenBank under the accession LWMN00000000. The version described in this paper is the first version LWMN01000000.

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Chapter 7

Comparative genomic analysis of *Enterococcus* spp. isolated from bovine faeces[4]

[4] This chapter has been submitted and is under review: Beukers, A.G., Zaheer, R., Goji, N., Amoako, K.K., Chaves, A.V., Ward, M.P. and McAllister, T.A. Comparative genomic analysis of *Enterococcus* spp. isolated from bovine feces. *BMC Microbiol*. (Submitted).

7.1. Abstract

Enterococcus is ubiquitous in nature and is a commensal of both the bovine and human gastrointestinal (GI) tract. It is also associated with clinical infections in humans. Subtherapeutic administration of antibiotics to cattle selects for antibiotic resistant enterococci in the bovine GI tract. Antibiotic resistance genes (ARGs) may be present in enterococci following antibiotic use in cattle. If located on mobile genetic elements (MGEs) their dissemination between *Enterococcus* species and to pathogenic bacteria may be promoted, reducing the efficacy of antibiotics. We present a comparative genomic analysis of twenty-one *Enterococcus* spp. including *Enterococcus hirae* (*n*=10), *Enterococcus faecium* (*n*=3), *Enterococcus villorum* (*n*=2), *Enterococcus casseliflavus* (*n*=2), *Enterococcus faecalis* (*n*=1), *Enterococcus durans* (*n*=1), *Enterococcus gallinarum* (*n*=1) and *Enterococcus thailandicus* (*n*=1) isolated from bovine faeces. The analysis revealed *E. faecium* and *E. faecalis* from bovine faeces share features with human clinical isolates, including virulence factors. The Tn*917* transposon conferring macrolide-lincosamide-streptogramin B resistance was identified in both *E. faecium* and *E. hirae*, suggesting dissemination of ARGs on MGEs may occur in the bovine GI tract. An *E. faecium* isolate was also identified with two integrative conjugative elements (ICEs) belonging to the Tn*916* family of ICE, Tn*916* and Tn*5801*, both conferring tetracycline resistance. This study confirms the presence of enterococci in the bovine GI tract possessing ARGs on MGEs, but the predominant species in cattle, *E. hirae* is not commonly associated with infections in humans. As the cost of genomic sequencing continues to decrease, further investigation of ICE using whole genome sequencing will help determine if there are linkages between enterococci isolates from bovine environmental and human clinical sources and whether bovine enterococci represent a source of dissemination and spread of antibiotic resistance.

Key words: bovine faeces, *Enterococcus*, comparative genomics

7.2. Introduction

The genus *Enterococcus* is ubiquitous in nature and can be found in a range of habitats, being associated with soil, plants, fresh and salt water, sewage and the gastrointestinal (GI) tract of animals (including mammals, birds, fish, reptiles and insects) and humans (Franz et al., 2011). Although typically a commensal of the human GI tract, enterococci are often associated with a variety of clinical infections including urinary tract infections, hepatobiliary sepsis, endocarditis, surgical wound infections, bacteraemia and neonatal sepsis (Agudelo Higuita and Huycke, 2014; Poh et al., 2006). *Enterococcus faecalis* and *Enterococcus faecium* are the two species responsible for the majority of healthcare-associated enterococcal infections (Sivert et al., 2013). Difficulties in treating enterococcal infections have emerged due to their ability to readily acquire resistance to many antibiotics, most notably to vancomycin. As a result, the ability to successfully treat clinical infections has been reduced (Arias and Murray, 2008).

Antibiotic use in livestock production has been correlated with the emergence of antibiotic resistant bacteria. This was first recognised in the 1990s when use of the glycopeptide avoparcin as a subtherapeutic growth promotant led to the emergence of glycopeptide-resistant *E. faecium* in livestock and poultry (Bager et al., 1997). Consumption of meat products contaminated with resistant bacteria was suggested to lead to the transmission of glycopeptide-resistant *E. faecium* to healthy, non-hospitalised humans. This association demonstrated transmission of resistant bacteria from animals to humans through the food chain (Klare et al.,

1995; Schouten and Voss, 1997). Consequently, avoparacin was banned as a growth promotant in Europe in 1997 (European Commission, 1997). However, many antibiotics continue to be administered subtherapeutically to livestock in North America. For example, tylosin phosphate, a member of the macrolide family, is administered subtherapeutically to cattle to control liver abscesses. We recently demonstrated subtherapeutic administration of tylosin phosphate selected for macrolide resistant enterococci in the bovine GI tract (Beukers et al., 2015). Enterococci have the ability to transfer antibiotic resistance and virulence genes horizontally to other bacteria (Coburn et al., 2007). The creation of a reservoir of resistant enterococci in the bovine GI tract could promote the dissemination of antibiotic resistance genes (ARGs) to other bacteria, particularly if they are associated with mobile genetic elements (MGEs).

Comparative genomic analysis can be used to identify genes coding for virulence, antibiotic resistance and gene mobility as well as elucidate the evolutionary relationship among bacteria. The number of complete or draft genome sequences available for *E. faecalis* and *E. faecium* is 446 and 436, respectively, comprising the bulk of enterococcal genome sequences available (http://www.ncbi.nlm.nih.gov/genome), as several comparative genomic studies of these species has been conducted (Palmer et al., 2012; Qin et al., 2012; Van Schaik et al., 2010). There are comparatively few draft genome sequences available for other *Enterococcus* spp. with only 11, 10, 6, 5, 2 and 1 genomes are available for *Enterococcus casseliflavus*, *Enterococcus hirae*, *Enterococcus durans*, *Enterococcus gallinarum*, *Enterococcus villorum* and *Enterococcus thailandicus*, respectively (http://www.ncbi.nlm.nih.gov/genome). Furthermore, there is a poor representation of genomic sequences available for enterococci isolated from non-human sources because the majority of enterococcal genomic sequences available originate from human clinical

infections (Palmer et al., 2014). Therefore, there is a need to expand the currently available dataset of enterococcal genomic sequences.

Previously, we identified a number of enterococci from bovine faeces that carried at least one ARG, but only a few isolates carrying multiple ARGs (Beukers et al., 2015). We also identified *E. hirae* as the principle species of the bovine GI tract, with infrequent isolation of *E. faecium* and *E. faecalis*, the species associated with nosocomial infections in humans. In the current study, we selected twenty-one isolates of enterococci originating from bovine faeces for whole-genome sequencing and comparative genomic analysis. We hypothesised that *E. faecium* and *E. faecalis* would present more genes coding for virulence and antibiotic resistance than other *Enterococcus* spp. isolated from bovine faeces.

7.3. Methods

7.3.1. Isolate selection

Twenty-one *Enterococcus* spp. isolated from bovine faeces including *E. hirae* (*n*=10), *E. faecium* (*n*=3), *E. villorum* (*n*=2), *E. casseliflavus* (*n*=2), *E. faecalis* (*n*=1), *E. durans* (*n*=1), *E. gallinarum* (*n*=1) and *E. thailandicus* (*n*=1) were selected for whole genome sequencing (Table 7.1.). These were selected from an archive of isolates collected between 2004 and 2005, which were previously characterised by PFGE and antimicrobial susceptibility testing (Beukers et al., 2015). At least one representative of each species isolated from bovine faeces was selected, and for *E. hirae* and *E. faecium*, selection was based on maximizing diversity as measured by PFGE profiles as well as selecting isolates that displayed unique antimicrobial resistance profiles.

7.3.2. DNA extraction and sequencing

Genomic DNA was isolated using phenol:chloroform extraction. *Enterococcus* spp. were inoculated into 5 mL brain heart infusion (BHI; BD, Franklin Lakes, New Jersey, USA) broth and grown for 24 h in a shaking incubator (250 rpm; Excella E24 Incubator Shaker, New Brunswick Scientific) at 37°C. To increase cell yield, 150 µL aliquots were inoculated into duplicate tubes containing 6 mL BHI (BD) and grown over 24 h as described above. Cells were harvested by centrifugation at 10,000 × *g* for 5 min into a 2 mL microfuge tube and stored at -20°C until genomic DNA was extracted. For extraction, the pellet was thawed on ice and resuspended in 1 mL of sterile 0.85% NaCl to remove residual growth media. The cells were repelleted by centrifugation (10,000 × *g*) for 1 min and the supernatant decanted. The washed cell pellet was resuspended in 665 µL of $T_{10}E_{25}$ (10 mM Tris-HCl pH 7.5; 25 mM EDTA) and 35 µL of lysozyme (50 mg/mL; Sigma-Aldrich, Co., St. Louis, Mo, USA) was added. The tubes were incubated at 55°C for 60 min as a pre-lysis step. A 175 µL of 5M NaCl, 35 µL of proteinase K (10mg/mL; Sigma-Aldrich) and 44 µL of 20% SDS were added to the suspension and mixed by gentle inversion before being incubated at 65°C for 1-2 h until cell lysis was complete. The lysed cells were extracted once with phenol, once with phenol:chloroform:isoamylalcohol (25:24:1) and twice with chloroform. Ammonium acetate (10 M) was added to the mixture so as to achieve a final concentration of 0.5 M, followed by one volume of isopropanol to precipitate DNA. To encourage precipitation, the tubes were chilled on ice for 10 min before centrifuging at 10,000 × *g* for 10 min. The supernatant was decanted and the DNA pellet washed with 70% ethanol and allowed to air dry before dissolving in 400 µL of TE (10 mM Tris-HCl; 1 mM EDTA). RNase A was added to achieve a final concentration of 30 µg/mL and the mixture was incubated for 20 min at 37°C. Duplicate solutions for each sample were pooled before

performing a second extraction, once with phenol:chloroform:isoamylalcohol and once with chloroform. Ammonium acetate (10 M) was added to the final aqueous solution to achieve a final concentration of 2 M followed by one volume of isopropanol and chilled on ice for 10 min to precipitate DNA. The DNA was pelleted by centrifugation, washed with 70% ethanol, air-dried, dissolved in 100 µL of sterile deionized water and stored at -80°C until genomic library construction.

Genomic library construction was performed using the Illumina Nextera XT DNA sample preparation kit (Illumina, Inc., CA, USA) following the manufacturer's instructions and sequenced on an Illumina MiSeq platform (Illumina). High-quality reads were *de novo* assembled using SPAdes genome assembler version 3.6.0 software (Bankevich et al., 2012) and annotated using Prokka version 1.10 (Seemann, 2014). Multi-locus sequence typing (MLST) was performed using the MLST database (version 1.8) (Larsen et al., 2012).

7.3.3. Comparative analysis

Draft genome sequences of the 21 *Enterococcus* spp. were investigated for the presence of putative virulence genes and ARGs, mobile genetic elements (MGEs), bacteriophage, CRISPR-Cas and secondary metabolite biosynthetic gene clusters. Virulence genes were identified using VirulenceFinder (version 1.5) (Joensen et al., 2014), and ARGs using a combination of ResFinder (version 2.1) (Zankari et al., 2012) and the Comprehensive Antibiotic Resistance Database (CARDs) (McArthur et al., 2012). Results for ARGs were further verified using megaBLAST and hits were manually inspected. Genomes were investigated for integrative conjugative elements (ICEs) by homology searches using BLAST against 466 ICEs downloaded

from the ICEberg database (version 1.0) (Bi et al., 2012). To identify bacteriophage, the contigs of each draft genome were ordered based on alignment against a reference genome (see Appendix 3 Table S7.1.) using progressive Mauve (Darling et al., 2010), and then analysed for the presence of prophage using PHAST (Zhou et al., 2011). CRISPR-Cas were identified using the CRISPRdb (Grissa et al., 2007) and secondary metabolite biosynthetic gene clusters using the Antibiotics and Secondary Metabolite Analysis Shell (antiSMASH) (Medema et al., 2011). All alignments and BLAST searches were performed in Geneious version 9.0.4 (Biomatters, Ltd). Assignment of proteins into Clusters of Orthologous Groups (COGs) was performed using the Integrated Microbial Genomes (IMG) platform (Markowitz et al., 2012). Blast atlases were generated by GView Java package software (Petkau et al., 2010) using both alignment length and percent identity cut-off values at 80%. The GView server (Petkau et al., 2010) was used to perform pan-genome analysis of *E. hirae*.

7.4. Results and Discussion

7.4.1. Sequencing statistics

A summary of the sequencing statistics for the 21 *Enterococcus* spp. genomes can be found in Table 7.1. The genomes ranged in size from 2.60–3.64 Mb with *E. thailandicus* exhibiting the smallest and *E. casseliflavus* the largest genome. There was considerable variation in the size of *E. hirae* genomes, suggesting large differences in the size of the chromosome between strains and/or the presence/absence of plasmids.

7.4.2. Phylogeny

A phylogenetic tree was constructed based on analysis of single-nucleotide polymorphisms (SNPs) of the core genes of all 21 sequenced *Enterococcus* genomes, and using *Enterococcus hirae* ATCC 9790 as an outgroup (Figure 7.1.). The assembled tree was consistent with the PFGE profile dendrogram observed from our previous study (Beukers et al., 2015). As expected, clustering was observed for genomes of the same species further verifying the identity of each species based on previous *groES-EL* spacer speciation (Beukers et al., 2015).

7.4.3. Clusters of orthologous groups (COGs)

Clusters of Orthologous Groups (COGs) are broad functional categories used to assign proteins related by function (Tatusov et al., 2001). Functional categorization of proteins into different COGs (Appendix 3 Figure S7.1.) revealed variation in the functional profile among *Enterococcus* spp., but the percentage of COGs assigned to cell cycle control, cell division, chromosome partitioning; extracellular structures; and intracellular trafficking, secretion and vesicular transport were similar between species. The percentage of COGs assigned to cell motility was greatest for *E. gallinarum* and *E. casseliflavus*, two species of *Enterococcus* that are known to be motile (Palmer et al., 2012). The percentage of COGs for cell motility was low for all other enterococci species, which are known to be non-motile (Devriese et al., 1993). There was little difference in the functional profile between strains of the same species with the exception of the mobilome: prophages, transposons category, in which inter-species variation was observed. Two *E. hirae* strains (*E. hirae* 4 and *E. hirae* 9), two *E. faecium* strains (*E. faecium* 11 and *E. faecium* 12) and an *E. villorum*, *E. faecalis* and *E. casseliflavus* strain

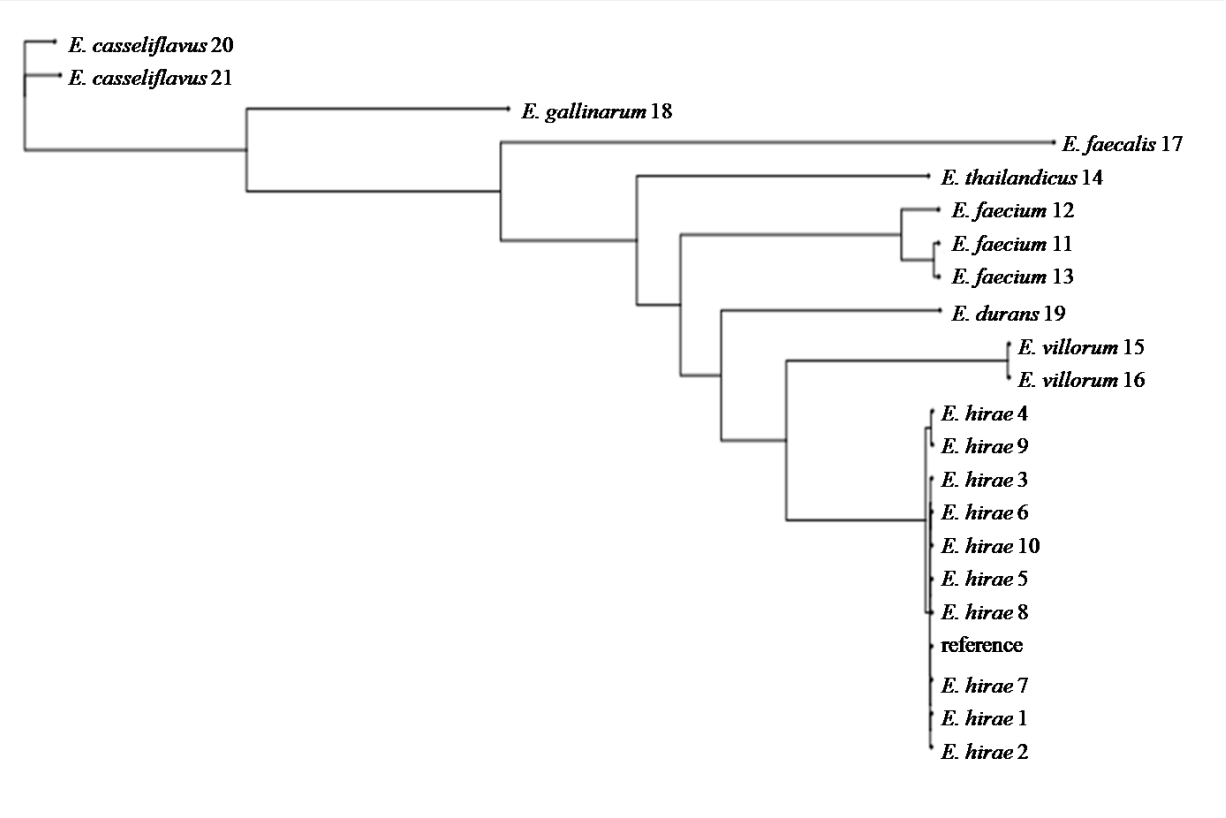

Figure 7.1. Phylogenetic tree constructed based on analysis of single-nucleotide polymorphisms (SNPs) of the core genes of all 21 sequenced *Enterococcus* genomes isolated from bovine faeces using *Enterococcus hirae* ATCC9790 as an outgroup.

(*E. villorum* 16, *E. faecalis* 17 and *E. casseliflavus* 20, respectively) had the greatest percentage of proteins assigned in this category with these proteins being most frequently associated with phage and transposases.

Using the compare genomes function available in the IMG platform, we produced an abundance profile overview of the gene count for different COGs for all 21 *Enterococcus* spp. genomes. Van Schaik et al. (2010) performed a COG-based functional comparison between *E. faecium* and *E. faecalis* in an effort to identify characteristics that distinguished the two species. In their analysis, they identified differences in sugar metabolism for the pentose sugar arabinose. They found COGs responsible for metabolism (COG2160 and COG3957), uptake (COG4213

and COG4214) and degradation (COG3940) of arabinose to be present in *E. faecium* and absent in *E. faecalis*, attributing this to the inability of *E. faecalis* to metabolise arabinose (Deibel et al., 1963). Genes for these COGs, with the exception of COG4214 in *E. faecium* 12, were present in the *E. faecium* strains examined in this study and absent in our *E. faecalis* strain. Genes for these COGs were also present in *E. gallinarum* and *E. casseliflavus* strains suggesting these species of *Enterococcus* also have the ability to metabolise arabinose. Ford et al. (1994) previously documented that strains of *E. gallinarum* and *E. casseliflavus* that they examined were able to metabolise arabinose but demonstrated poor growth compared to *E. faecium*. In the current study, *E. hirae*, *E. villorum*, *E. durans* and *E. thailandicus* all lacked genes for these COGs suggesting that they lacked the ability to metabolise arabinose, an outcome that has been biochemically confirmed by others (Devriese et al., 2002; Farrow and Collins, 1985; Tanasupawat et al., 2008). Arabinose is a subunit of the plant polysaccharide hemicellulose and therefore would be in abundance in the GI tract of cattle (Van Schaik et al., 2010). Despite *E. faecium* being able to utilise arabinose as an energy source, this trait does not appear to provide a competitive advantage for this species to proliferate in the GI tract of cattle, considering *E. hirae* is the predominant species identified (Beukers et al., 2015).

Van Schaik et al. (2010) investigated other COGs involved in the metabolism of carbon sources from plants including COG4677, which is predicted to be involved in the metabolism of pectin, and COG3479, which is involved in the breakdown of coumaric acid and other components of lignocellulose. In our study, COG4677 was present in *E. faecium*, *E. durans* and *E. casseliflavus* and absent from *E. hirae*, *E. thailandicus*, *E. villorum*, *E. faecalis* and *E. gallinarum*, whilst COG3479 was present in *E. hirae*, *E. faecium*, *E. villorum* and *E. durans* and absent from *E. faecalis*, *E. thailandicus*, *E. gallinarum* and *E. casseliflavus*. These authors also

highlighted a number of COGs present in *E. faecalis* that were absent in *E. faecium* including COGs for the utilisation of ethanolamine as a carbon source. Ethanolamine is a phospholipid that can be found in the bovine GI tract (Bertin et al., 2011). In the current study, *E. faecalis* possessed COGs for the utilisation of ethanolamine, which were confirmed to be absent in *E. faecium*. Ethanolamine utilisation has been demonstrated for *E. faecalis* (Florencia Del Papa and Perego, 2008) but not for other *Enterococcus* species. In the current study, these COGs were also identified in *E. gallinarum* suggesting this *Enterococcus* species may also utilise ethanolamine as an energy source but to our knowledge has yet to be demonstrated biochemically. It is clear that different *Enterococcus* spp. have the ability to utilise various carbon sources allowing them to inhabit and survive in many diverse environments, including the GI tract of cattle. From this study, it was not apparent if *E. hirae* possessed specific traits for carbohydrate metabolism that may promote its abundance in the GI tract of cattle over other *Enterococcus* spp.

Van Schaik et al. (2010) also investigated proteins involved in protection against oxidative stress. They identified the enzyme catalase (COG0753) was present in *E. faecalis* and absent in *E. faecium*. Examination of the different *Enterococcus* spp. in this study confirmed catalase to be specific for *E. faecalis* as it was absent from all other species. In the presence of heme, *E. faecalis* exhibits catalase activity (Frankenberg et al., 2002). Catalase production has been speculated to play a role in virulence in pathogenic bacteria including *Staphylococcus aureus* (Clements and Foster, 1999; Kanafani and Martin, 1985). *E. faecalis* can be exposed to oxidative stress as part of the host defence against invasion (Frankenberg et al., 2002). Catalase production may offer some protection against oxidation during invasion, contributing to the virulence of *E. faecalis*. Other mechanisms in *E. faecium* may play a role in the oxidative stress response, including the production of glutathione peroxidase (COG0386) (Van Schaik et al.,

2010). With the exception of *E. faecalis,* this COG was present in all species of *Enterococcus* examined in this study, demonstrating the different strategies *Enterococcus* spp. use to combat oxidative stress.

7.4.4. Multi-locus sequence typing (MLST)

Multi-locus sequence typing (MLST) has been used to study the population structure and evolution of *E. faecium* and *E. faecalis* (Ruiz-Garbajosa et al., 2006; Willems et al., 2005). This technique involves sequencing and analysis of housekeeping genes and assignment of a sequence type (ST) (Homan et al., 2002; Ruiz-Garbajosa et al., 2006). In the current study *E. faecium* 11, *E. faecium* 12 and *E. faecium* 13 were classified as ST214, unknown and ST955, respectively, and *E. faecalis* 17 as ST242 (Table 7.1.). The lack of an assignment of a ST for *E. faecium* 12 suggests there are STs that have yet to be defined within the MLST database. STs can be assigned to a clonal complex (CC) based on their similarity to a central alleic profile (PubMLST, 2016). MLST analysis of the population structure of *E. faecium* has identified that the majority of strains associated with nosocomial infections belong to the Clonal Complex 17 (CC17) (Willems et al., 2005). For *E. faecalis* it appears that two complexes, CC2 and CC9, represent hospital-derived strains (Leavis et al., 2006; Ruiz-Garbajosa et al., 2006). The STs assigned to *E. faecium* and *E. faecalis* identified in the current study have been described previously (Boyd et al., 2015; Camargo et al., 2006; Sun et al., 2009) and are not associated with complexes of hospital-derived strains. There is currently no typing scheme available for other *Enterococcus* spp.

7.4.5. BLAST atlas

A BLAST atlas was constructed for *E. hirae* and *E. faecium* strains using *E. hirae* ATCC 9790 and *E. faecium* DO as reference strains, respectively (Figure 7.2.). Of the *E. hirae* strains, *E. hirae* 7 exhibited the highest relatedness to the reference strain. *E. hirae* 7 and *E. hirae* 8 also shared phage-related genes with the reference strain (Figure 7.2.a). There were few variable regions identified between strains of *E. hirae*, demonstrating similarity in gene content between strains. Likewise, the gene content between strains of *E. faecium* was also highly similar (Figure 7.2.).

7.4.6. Pan-genome analysis

The pan-genome is comprised of three components: i) the core genome, describing genes shared across all strains; ii) the accessory or dispensable genome, describing genes that are present in one or more strains; and iii) unique genes, describing species-specific or strain-specific genes (Tettelin et al., 2005). We proceeded to carry out a pan-genome analysis of *E. hirae* genomes from this study to identify core and unique genes. A core genome consisting of 2,256 genes was identified for the 10 *E. hirae* strains (Figure 7.3.). The core genome of *E. hirae* from this study accounted for approximately 80% of each genome. Genes in the core genome are generally associated with the basic biology and maintenance of the organism (Medini et al., 2005; Tettelin et al., 2005). As expected, the core genome of the 10 *E. hirae* strains accounted for housekeeping genes essential for the basic biology of *E. hirae* such as carbohydrate transport and metabolism; translation, ribosomal structure and biogenesis; amino acid transport and metabolism; and transcription.

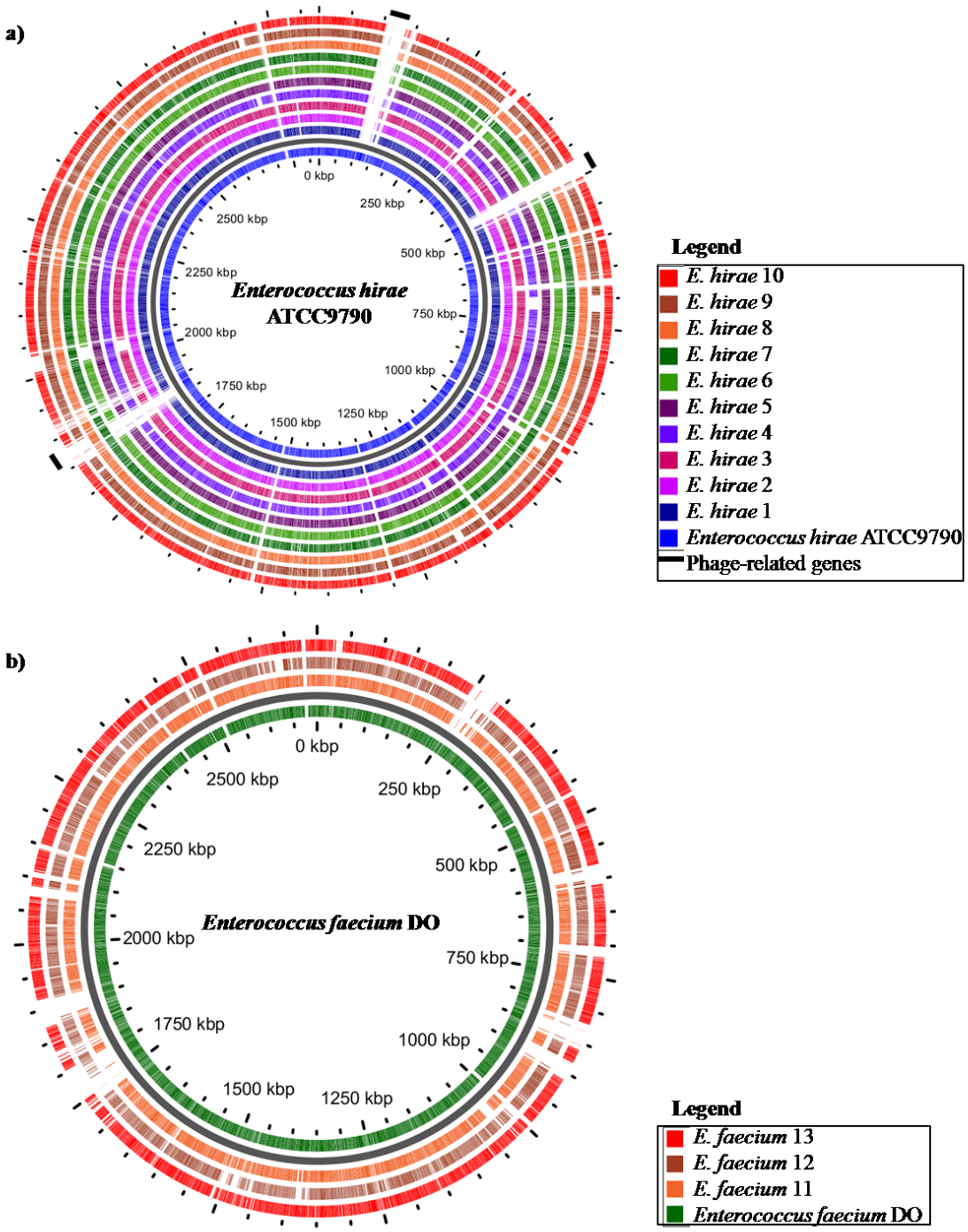

Figure 7.2. a) Blast atlas of *Enterococcus hirae* isolated from bovine faeces mapped against reference sequence *Enterococcus hirae* ATCC9790. Starting from the outer circle: *E. hirae* 10, *E. hirae* 9, *E. hirae* 8, *E. hirae* 7, *E. hirae* 6, *E. hirae* 5, *E. hirae* 4, *E. hirae* 3, *E. hirae* 2, *E. hirae* 1. b) Blast atlas of *Enterococcus faecium* genomes isolated from bovine faeces mapped against reference sequence *Enterococcus faecium* DO. Starting from the outer circle: *E. faecium* 13, *E. faecium* 12, *E. faecium* 11. Blast atlases were generated by GView Java package software (Petkau et al. 2010) using both alignment length and percent identity cut-off values at 80%.

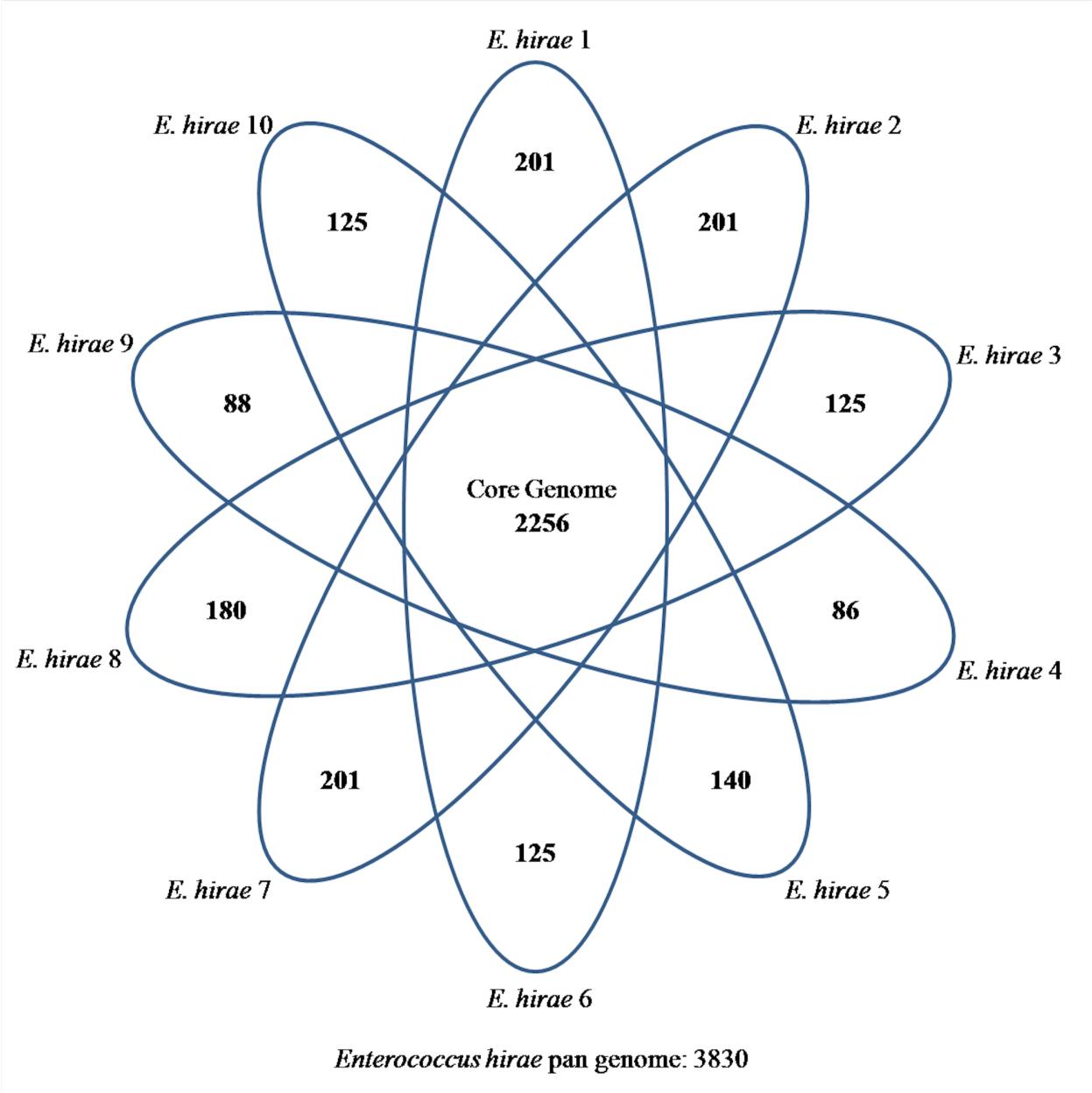

Figure 7.3. Venn diagram showing size of the core genome, pan-genome and number of strain unique CDS in 10 *Enterococcus hirae* genomes isolated from bovine faeces. Petals contain number of unique CDS per strain and core genes are presented in the centre.

7.4.7. Virulence genes

Virulence genes contribute to the pathogenicity of an organism. In this study, virulence genes were only detected in *E. faecium* and *E. faecalis*. All three *E. faecium* strains contained the *efaA* and *acm* genes, whilst *E. faecalis* contained a number of virulence genes including *efaA*,

ace, *ebp* pili genes, *gelE* and *fsrB*. The *acm* and *ace* genes described in *E. faecium* and *E. faecalis*, respectively, are important for facilitating cell wall adhesion to host tissues (Nallapareddy et al., 2003; Rich et al., 1999). The *efaA* gene found in both *E. faecalis* and *E. faecium* also plays a role in adherence to host tissues and is a virulence factor involved in endocarditis (Lowe et al., 1995; Singh et al., 1998). The *ebp* pili genes described in *E. faecalis*, comprising of *ebpA, ebpB* and *ebpC*, assist in adherence and biofilm formation (Nallapareddy et al., 2006). The *gelE* gene also found in *E. faecalis* encodes for gelatinase, which hydrolyses gelatin, collagen, casein and haemoglobin (Su et al., 1991). Its expression is regulated by the two-component *fsr* system, with both *gelE* and *fsr* genes important in biofilm formation (Hancock and Perego, 2004; Nakayama et al., 2006; Qin et al., 2000).

In addition to these virulence genes, a number of bacterial sex pheromone genes were also present in *E. faecalis* including *cad*, *camE, cCF10* and *cOB1*. Certain conjugative plasmids found in *E. faecalis* respond to the secretion of bacterial sex pheromone genes from plasmid-free enterococci, inducing their transfer (Clewell, 1993). Sex pheromone response plasmids have rarely been described in other *Enterococcus* spp. However, there have been a few reported for *E. faecium* (Handweger et al., 1990; Magi et al., 2003). The bacterial sex pheromones detected in the *E. faecalis* genome target the sex pheromone plasmids pAD1, pAM373, pCF10 and pOB1, respectively. Some of these plasmids encode features that can contribute to virulence such as pAD1 and pOB1, both encoding for a bacteriocin and hemolysin, and pCF10, encoding tetracycline resistance (Wirth, 1994). The pheromone cAD1 precursor lipoprotein *cad* gene was detected in all of the *Enterococcus* spp. isolates sequenced in this study, with amino acid identities 98%, 72%, 69%, 67%, 66%, 66%, 64% and 59% for *E. faecalis*, *E. faecium*, *E. durans*, *E. thailandicus, E. hirae*, *E. villorum*, *E. gallinarum* and *E. casseliflavus* respectively, as

compared to the *cad* gene in *E. faecalis* strain FA2-2 (GenBank accession no. AF421355.1). Presence of the cAD1 precursor lipoprotein in these *Enterococcus* spp. increases their potential of receiving the highly conjugative pheromone-responding plasmid pAD1. The hemolysin/bacteriocin (cytolysin) encoded by this plasmid has been shown to contribute to virulence in animal models (Clewell, 2007). Therefore acquisition of this plasmid by these *Enterococcus* spp. could increase their virulence. Further analysis is required to determine if this sex pheromone precursor is able to induce transfer of pAD1 to *Enterococcus* spp. other than *E. faecalis*.

Virulence genes have mostly been characterised in *E. faecalis* and *E. faecium*, with little information available on the nature of these genes in other enterococcal species. A study investigating virulence traits for cytolysin, adhesins and hydrolytic enzymes described the presence of the whole cytolysin operon in *E. durans* and the presence of genes for cytolysin in *E. hirae* and *E. gallinarum*, isolated from cheese and milk. Other virulence genes were also commonly detected in *E. durans*, such as the *esp* gene which is important for adhesion (Semedo et al., 2003). With the exception of *E. faecalis* and *E. faecium*, virulence genes were not detected in the other *Enterococcus* isolates from the bovine GI tract sequenced in this study. The detection of virulence genes is not exclusive to human clinical enterococci. Studies have identified virulence genes in enterococci irrespective of their origin, such as from human and animal hosts, food and the environment (Iweriebor et al., 2015; Jimenez et al., 2013; Semedo et al., 2003). For *E. faecalis* and *E. faecium*, there is usually a greater incidence of virulence genes detected in *E. faecalis* than in *E. faecium* (Eaton and Gasson, 2001; Franz et al., 2001), an outcome that agrees with our study.

7.4.8. Antibiotic resistance genes

Enterococci can exhibit resistance to a number of antibiotics, partly due to their innate resistance to many commonly used antibiotics such as penicillin, but also due to their ability to successfully acquire resistance through horizontal exchange of ARGs on MGEs (Kristich et al., 2014). In this study we screened the 21 *Enterococcus* genomes against the ResFinder and CARDs databases for resistance genes (Table 7.2.).

Genes conferring resistance to vancomycin were only found in the genomes of *E. gallinarum* and *E. casseliflavus*, where the *van*C operon was present. The *van*C operon is intrinsic to these species of *Enterococcus* and provides resistance to low concentrations of vancomycin (Leclercq et al., 1992; Navarro and Courvalin, 1994). Of the isolates examined in this study, only *E. casseliflavus* 20 displayed phenotypic resistance to vancomycin (Appendix 3 Table S7.2.). The intrinsic resistance of *E. casseliflavus* and *E. gallinarum* can provide protection to concentrations of vancomycin as high as 32 µg/mL (Gold, 2001). For disc susceptibility testing, the concentration of vancomycin in the disc was 30 µg (Beukers et al., 2015). This concentration was sufficient to inhibit the growth of *E. gallinarum* 18 and *E. casseliflavus* 21 despite the presence of the *van*C operon in these isolates. However, *van*C in *E. casseliflavus* 20 provided adequate resistance to allow growth of this isolate in the presence of vancomycin. The lack of vancomycin resistance genes in *Enterococcus* isolated from bovine faeces is not surprising as avoparcin, a glycopeptide antimicrobial related to vancomycin, has not been used in cattle in North America (Health Canada, 2002).

Resistance genes to macrolides were present in a number of *Enterococcus* genomes sequenced, a finding that coincides with the fact that cattle were administered tylosin phosphate

in their diets (Beukers et al., 2015). *Erm*(B) confers resistance to macrolide-lincosamide-streptogramin B (MLS_B) antibiotics and was found in isolates of *E. hirae*, *E. faecium*, *E. villorum*, *E. gallinarum* and *E. casseliflavus*. In contrast, *msrC*, a macrolide efflux pump, was only detected in *E. faecium* (Table 7.2.). This is consistent with Portillo et al. (2000) who described *erm*(B) as the predominant gene conferring resistance to erythromycin in *Enterococcus* spp. and *msrC* in *E. faecium*. The presence of these resistance genes corresponded with the phenotypic resistance observed in these isolates (Appendix 3 Table S7.2.). Interestingly, *E. hirae* 6, *E. durans* 19 and *E. casseliflavus* 20 exhibited resistance to macrolides even though no resistance genes to macrolides matched those in either the ResFinder or CARDs databases.

We previously reported that the *E. thailandicus* isolate sequenced in this study exhibited intermediate resistance to erythromycin (Appendix 3 Table S7.2.; Beukers et al., 2015; Beukers et al., 2016). Although there were no obvious macrolide resistance genes present, there were a number of genes identified as having multidrug efflux functions which may have contributed to the observed intermediate resistance to erythromycin (Beukers et al., 2016). There is also the possibility that this phenotype was as a result of an unknown gene that codes for erythromycin resistance.

Genes conferring resistance to high concentrations of aminoglycosides were not detected in any of the genomes analysed. Susceptibility to high concentrations of aminoglycosides was confirmed by the lack of phenotypic resistance to gentamicin and streptomycin (Appendix Table S7.2.). Enterococci are intrinsically resistant to low concentrations of aminoglycosides which is conferred by the genes *aac(6')-Ii*, *aac(6')-Iid* and *aac(6')-Iih* present in *E. faecium*, *E. durans* and *E. hirae*, respectively (Table 7.2.; Costa et al., 1993; Del Campo et al., 2005).

Genes coding for tetracycline resistance were detected in a number of genomes, including *E. hirae*, *E. faecium* and *E. villorum* (Table 7.2.). *Tet*(L) encodes for an efflux protein whilst *tet*(M) and *tet*(O) encode for ribosomal protection proteins (Roberts, 2005). Anderson et al. (2008) found *tet*(O) was the most prevalent gene encoding for tetracycline resistance in enterococci isolated from cattle, a finding that agrees with ours. Anderson et al. (2008) reported *E. hirae* as the predominant species isolated from cattle and *tet*(O) was only associated with *E. hirae* in the current study. Detection of *tet*(M) and *tet*(L) in other isolates is not unexpected as both genes are also frequently detected in enterococci from animals including poultry, pigs, dogs, cats, rabbits, badgers, wildcats and birds (Aarestrup et al., 2000; Poeta et al., 2005; Poeta et al., 2006). Disc susceptibility testing revealed isolates containing *tet*(M) were resistant to doxycycline whilst those containing *tet*(L) or *tet*(O) were susceptible (Appendix 3 Table S7.2.). It is possible that isolates that are sensitive to doxycycline are susceptible to other members of the tetracycline family. In general, bacteria that are resistant to doxycycline are also resistant to other tetracyclines including tetracycline and oxytetracycline (Holzel et al., 2010; Roberts, 2002).

Only a few of the selected genomes contained ARGs to two or more antibiotics. Of particular interest was *E. faecium* 11, which contained at least 11 ARGs as inferred from the analysis of genome sequences (Table 7.2.), including those conferring aminoglycoside, MLS_B, pleuromutilin, streptogramin A, tetracycline and streptothricin resistance.

7.4.9. Mobile genetic elements

Mobile genetic elements (MGEs) play an important role in horizontal gene transfer (HGT) of ARGs within and between bacteria from human and/or animal hosts (Bennett, 2008;

Burrus et al., 2002; Roberts and Mullany, 2011). MGEs include plasmids, transposable elements, prophages and various genomic islands such as integrative and conjugative elements (ICEs) (Wozniak and Waldor, 2010). A number of MGEs have been described in enterococci including transposons, plasmids and bacteriophage (Werner, 2013).

The well-known Tn*3*-like transposon, Tn*917*, which is widely distributed in enterococci was identified in several of the sequenced genomes. Four *E. hirae* strains (*E. hirae* 1, *E. hirae* 2, *E. hirae* 3 and *E. hirae* 4) and one *E. faecium* strain (*E. faecium* 11) had high sequence homology (>95%) to the Tn*917* transposon, previously described in *E. faecalis* (Shaw and Clewell, 1985). All of these strains exhibited erythromycin resistance (Appendix 3 Table S7.2.; Beukers et al., 2015), conferred by the *erm*(B) resistance gene present in Tn*917*. Other distinguishing features of this transposon include a transposase (TnpA) and a resolvase (TnpR) involved in the replicative mode of transposition (Nicolas et al., 2014).

The *erm*(B) gene was present in a number of other genomes including *E. hirae* 5, *E. villorum* 16, *E. gallinarum* 18 and *E. casseliflavus* 21. However, it did not align with the Tn*917* transposon. In *E. hirae* 5, the *erm*(B) gene was found on a contig associated with chromosomal genes. The tetracycline resistance gene *tet*(O) was also found in the vicinity of *erm*(B). Based on sequence information, *erm*(B) in the other three genomes appeared to plasmid mediated. In *E. villorum* 16, the *erm*(B) and *tet*(L) genes were found on contigs associated with a plasmid sequence from an *E. faecium* strain UW8175 (GenBank accession no. CP011830.1). In *E. gallinarum* 18 and *E. casseliflavus* 21, the *erm*(B) gene was found on contigs associated with the plasmid sequence of pRE25 from an *E. faecalis* (GenBank accession no. X92945.2).

The tetracycline resistance genes *tet*(L) and *tet*(M) found in *E. hirae* 1 were located on a contig which shared 21,418 identical bp with the 25,963 bp transposon Tn*6248* of *E. faecium* strain E506 (GenBank accession no. KP834592). The genes responsible for transposition (*tnpA*) and insertion and excision of Tn*6248* (*tndX*) were absent, as was the chloramphenicol acetyltransferase gene (*cat*). This same contig also appeared to be associated with a plasmid sequence in *E. hirae* strain R17 (GenBank accession no. CP015517.1), suggesting this remnant transposon may be on a plasmid.

Integrative conjugative elements (ICEs) are self-transmissible elements that contain modules for their maintenance, dissemination and regulation (Burrus and Waldor, 2004). In major Gram-positive human pathogens (e.g. *Enterococcus* spp., *Staphylococcus* spp. and *Streptococcus* spp.), tetracycline resistance is known to arise from the acquisition of the Tn*916*-family ICE carrying the *tet*(M) gene. The gene synteny in this family of ICE is well conserved, but there are differences in integrase (*int*) and excisionase (*xis*) gene sequences, insertion site specificity, and host range among family members (Ciric et al., 2013; Hegstad et al., 2010; Novais et al., 2012). The Tn*916* ICE was originally identified as an 18-kb conjugative transposon in *E. faecalis* DS16 (Flannagan et al., 1994; Franke and Clewell, 1981). Variants of some Tn9*16*-*tet*(M) members, including Tn*916*, Tn*5397*, Tn*6000* or Tn*5801*, are widely spread among several genera within the Firmicutes, suggesting widespread dissemination of these elements. Many Tn*916*-like ICEs have a broad host range and are responsible for dissemination of tetracycline resistance through *tet*(M) in Gram-positive bacteria associated with humans and animals (Franke and Clewell, 1981; Rice, 1998; Roberts and Mullany, 2011). Recently, almost identical Tn*5801*-like genomic islands have been identified in different Gram-positive species of pet (*Staphylococcus pseudintermedius*) and human (*E. faecalis*, *S. aureus*, *Staphylococcus*

agalactiae) origin, suggesting a horizontal transfer of these elements (De Vries et al., 2016). In our study, two ICEs belonging to the Tn*916*-family were identified in *E. faecium* 11. These ICEs exhibited homology to Tn*916* and Tn*5801*, each harbouring a *tet*(M) variant, and appeared to be located within the chromosome. In Group B *Streptococcus*, the vast majority of Tn*916* and Tn*5801* are inserted into the core genome (Da Cunha et al., 2014). Once inserted in the genome, it is thought that Tn*916* and Tn*5801* are retained, as they impose a minimal impact to the biological fitness of the host bacteria (Celli and Trieu-Cuot, 1998; Da Cunha et al., 2014; Roberts and Mullany, 2011).

A gene cluster *aadE–sat4–aphA-3* encoding resistance to streptomycin, streptothricin and kanamycin, previously described in *E. faecium* (Werner et al., 2001) was also found in *E. faecium* 11 associated with plasmid related contigs. This gene cluster has also been described in Tn*5405* within *S. aureus* (Derbise et al., 1997) and Tn*1545* from *Streptococcus pneumoniae* (Palmieri et al., 2012), suggesting that it is widespread among Gram-positive bacteria.

7.4.10. Bacteriophage

Bacteriophage mediated transduction of antibiotic resistance has been demonstrated in enterococci (Mazaheri Nezhad Fard et al., 2011), and potential virulence determinants have been identified in phage associated with *E. faecalis* (Yasmin et al., 2010). Phage found in enterococci usually belong to the *Podoviridae*, *Siphoviridae* or *Myoviridae*, but others including *Inoviridae*, *Leviviridae*, *Guttaviridae* and *Fuselloviridae* have also been reported (Duerkop et al., 2014; Mazaheri Nezhad Fard et al., 2010).

All *Enterococcus* genomes sequenced contained at least one putative phage, ranging in size from 8.0 to 70.3 kb (Table 7.3.). A total of 37 intact prophages were identified across the 21 sequenced genomes. *E. hirae* and *E. faecium* contained one to three intact prophages, whereas *E. faecalis* and *E. gallinarum* each contained two intact prophages and *E. durans* contained one intact prophage. *E. villorum* and *E. casseliflavus* contained up to four intact prophages whilst no intact prophages were detected in *E. thailandicus*. The intact prophages detected were from the *Siphoviridae*, *Myoviridae* or *Podoviridae* families, with prophage from the *Siphoviridae* family being most prevalent across all species examined (Table 7.3.). Prophages of the *Phycodnaviridae* family was identified in *E. faecium* and *E. villorum*. Its status was intact for only one of the *E. faecium* strains whilst it was questionable or incomplete in the others (Table 7.3.). To our knowledge, phage from the *Phycodnaviridae* family have yet to be described in enterococci species. However, their presence in the rumen microbiome has been reported following metagenomic analysis (Berg Miller et al., 2012).

7.4.11. CRISPR-Cas

Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) genes are a type of adaptive immune response described in bacteria against invading genetic elements such as phage and plasmids (Makarova et al., 2015). A CRISPR locus includes a CRISPR array flanked by various *cas* genes, with the array comprised of short direct repeats alternating with short variable DNA sequences called 'spacers' (Makarova et al., 2015). Three types of CRISPR-Cas systems have been described, distinguished by the presence of different Cas genes namely *cas3* for type I, *cas9* for type II and *cas10* for type III (Makarova et

al., 2011). Recently, two additional types have been proposed to this classification system that includes type IV and type V (Makarova et al., 2015). CRISPR-Cas systems typically described in enterococci are of the type II variety. However, a recent report identified a type I system in *Enterococcus cecorum* (Borst et al. 2015; Katyal et al., 2013).

All *E. hirae* strains contained CRISPR arrays, except for *E. hirae* 8. CRISPR arrays were also detected in *E. thailandicus*, *E. villorum* and *E. durans* (Appendix 3 Table S7.3.). The CRISPR arrays from these genomes were flanked by Cas genes, consisting of *cas9*, *cas1*, *cas2* and *csn2* with the exception of *E. villorum* which lacked the *csn2* gene. CRISPR arrays flanked by these four Cas genes are classified as a type II-A system and are predicted to be functional, indicated by the presence of the core Cas genes *cas1* and *cas2* (Chylinski et al., 2014; Makarova et al., 2011). Following the same nomenclature, the CRISPR-Cas system identified in *E. villorum* would also be classified as a type-II system, but its subtype is unclear.

Multiple CRISPR arrays can often be detected in bacterial genomes. However, not all CRISPR arrays may be accompanied by Cas genes. It is predicted that these arrays lie dormant or that Cas genes from other similar arrays may be sufficient for their activity (Bhaya et al., 2011). Orphan CRISPR arrays (without Cas genes) (Katyal et al., 2013) were identified by the CRISPRdb in a number of genomes, including two *E. hirae* strains and in *E. thailandicus*, *E. faecalis* and *E. durans* (Appendix 3 Table S7.3.). No functional CRISPR arrays were detected for *E. faecium*, *E. faecalis*, *E. gallinarum* or *E. casseliflavus*.

Comparison of CRISPR arrays flanked by Cas genes revealed unique arrays between *Enterococcus* species, but some arrays were shared between strains of the same species (Figure 7.4.). Amongst the nine *E. hirae* strains, only four unique arrays were present. The arrays

identified in *E. villorum* were identical for both strains. The largest array was identified in *E. thailandicus*. Arrays identified in the sequenced *Enterococcus* genomes contained between three and ten direct repeat (DR) sequences, alternating with spacer sequences (Appendix 3 Table S7.4.). A total of 26 unique spacer sequences associated with functional CRISPR arrays and an additional 38 unique spacers were associated with orphan CRISPR arrays were identified (Appendix 3 Table S7.5.).

In enterococci, it is hypothesised that the absence of CRISPR-Cas systems is associated with increased antibiotic resistance in isolates of *E. faecium* and *E. faecalis* (Palmer and Gilmore, 2010). In this study, *E. faecium* 11 lacked CRISPR-Cas and harboured several antibiotic resistance genes, reflecting this association (Table 7.2.; Appendix 3 Table S7.3.). Palmer and Gilmore (2010) detected identities between CRISPR spacer sequences and sequences of known pheromone-responsive plasmids and phage, suggesting CRISPR-Cas systems provided defence against these invading genetic elements. The authors hypothesised that the absence of CRISPR-Cas systems resulted in a compromised genome defence, enabling the acquisition of ARGs on MGEs. Palmer and Gilmore (2010) did not detect spacer sequences with identities to transposons and hypothesised CRISPR-Cas systems may not provide defence against transposons. Several *E. hirae* strains in the current study contained functional CRISPR-Cas systems and the *erm*(B) resistance gene on a Tn*3*-like transposon, supporting this theory.

Functional CRISPR arrays and intact prophage were identified in most of the genomes sequenced in this study, with the exception of *E. thailandicus*. It is not surprising that these genomes contained prophage, as bacteriophage have developed strategies to avoid CRISPR regulation through the development of anti-CRISPR systems to enable integration into the genome (Sorek et al., 2008). In the case of *E. thailandicus*, spacers identified in CRISPR arrays

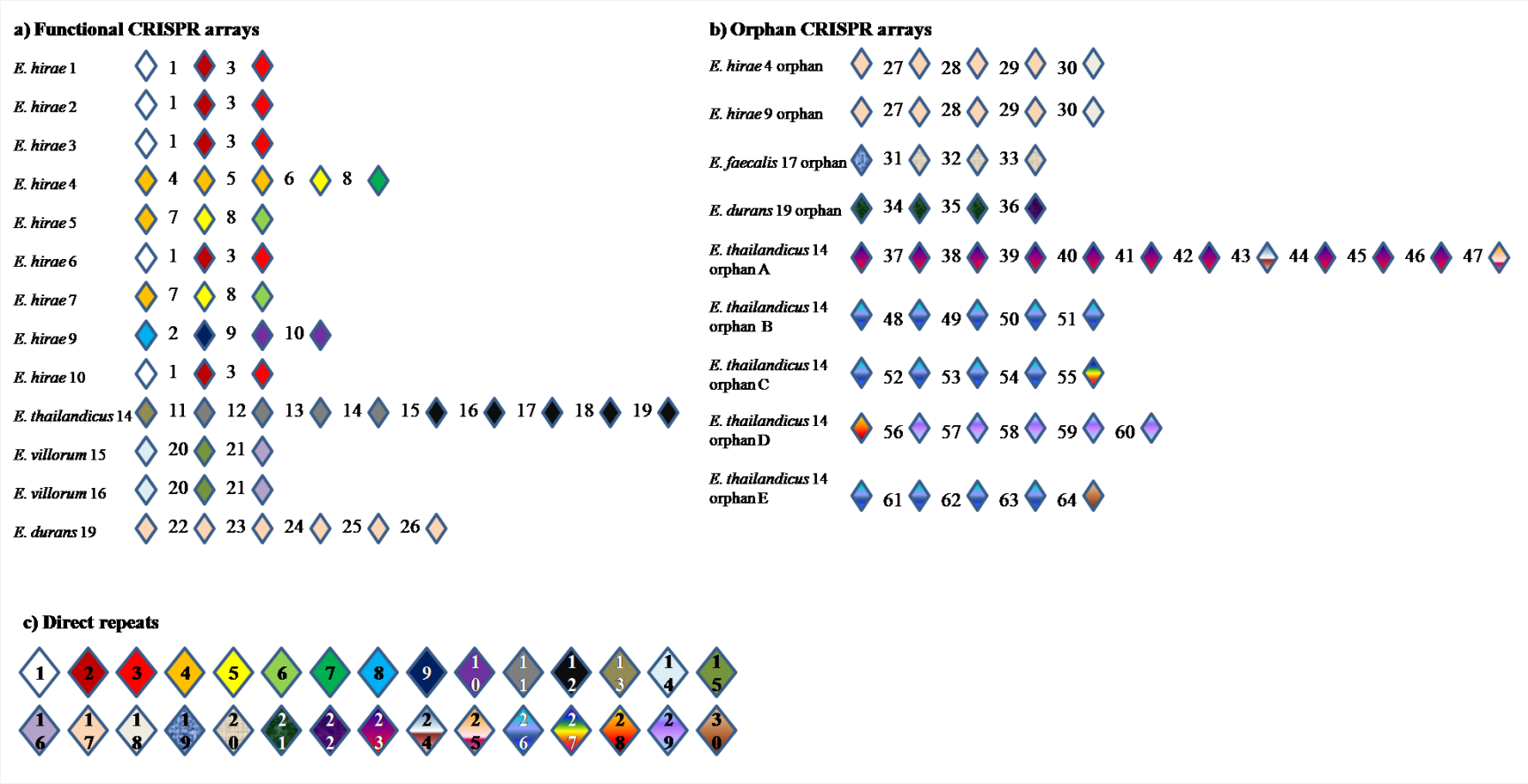

Figure 7.4. Schematic of CRISPR-Cas systems identified in whole genome sequence analysis of 21 *Enterococcus* spp. genomes. a) Functional CRISPR array spacer and direct repeat organization. Diamonds represent direct repeats interspaced with numbers representing spacers. Spacer numbers correlate with sequences displayed in Appendix 3 Table S7.5. b) Orphan CRISPR array spacer and direct repeat organization. Diamonds represent direct repeats interspaced with numbers representing spacers. Spacer numbers correlate with sequences displayed in Appendix 3 Table S7.5. c) Numbered direct repeats. Numbers correlate with sequences displayed in Appendix 3 Table S7.4.

aligned to incomplete prophage sequences with 100% sequence similarity and may possibly explain the lack of intact prophage in this genome. Spacer 60 aligned with both regions 3 and 4 of *E. thailandicus* prophage whilst spacer 12 aligned with region 4. None of the remaining spacers identified in CRISPR arrays had any sequence similarity to identified prophage.

7.4.12. Secondary metabolites

Bacteriocins are ribosomally synthesised antimicrobial peptides produced by Gram-positive and Gram-negative bacteria that have antimicrobial activity against closely related bacteria (Yang et al., 2014). In Gram-positive bacteria, they are classified into three major classes. Class I consists of the heat stable, modified peptides or lantibiotics, Class II describes the heat stable, unmodified non-lantibioitics and Class III consists of large proteins that are heat unstable (Nes et al., 2014; Yang et al., 2014). It is believed the production of bacteriocins by bacteria provides a competitive advantage to their survival in certain ecological niches (Eijsink et al., 2002).

Putative lantibiotics were identified in *E. hirae*, *E. thailandicus* and *E. gallinarum* whilst none were predicted in *E. faecium*, *E. villorum*, *E. faecalis*, *E. durans* or *E. casseliflavus*. Putative class II bacteriocins were identified in seven *E. hirae* strains (*E. hirae* 3, *E. hirae* 4, *E. hirae* 5, *E. hirae* 6, *E. hirae* 8, *E. hirae* 9, *E. hirae* 10), two *E. faecium* strains (*E. faecium* 11, *E. faecium* 13), *E. thailandicus*, *E. villorum*, and *E. durans*. A putative bacteriocin identified in *E. faecium* 11 and *E. faecium* 13 had an amino acid identity of 99% to Enterocin A (Genbank accession no. AAF44686.1). Enterocin A was first described in an *E. faecium* strain isolated from fermented Spanish sausage (Aymerich et al., 1996). Enterocin A inhibits a broad spectrum

of Gram-positive bacteria including species of *Clostridium*, *Propionibacterium*, *Listeria* and *Staphylococcus* (Casaus et al., 1997).

Until recently, terpenes were mainly considered secondary metabolites associated with plants and fungi, and were described in prokaryotes in only a few instances. These compounds serve a number of purposes including acting as antibiotics, hormones, flavour or odour constituents and pigments (Yamada et al., 2012). Since the advent of genomic sequencing, a number of presumptive terpene synthase genes have been discovered in bacteria (Yamada et al., 2015). Putative terpenes were identified in all *E. hirae*, *E. villorum*, *E. gallinarum*, *E. durans* and *E. casseliflavus* genomes sequenced in this study. None were predicted in *E. faecium*, *E. thailandicus* and *E. faecalis* genomes. The role of terpenes in enterococci remains unclear.

7.5. Conclusion

This study has provided valuable insight about genetic differences observed between *Enterococcus* spp. isolated from bovine faeces. We hypothesised that enterococci originating from bovine faeces would lack genes coding for virulence, but would contain MGEs that could promote the dissemination of ARGs. We confirmed the majority of *Enterococcus* spp. isolated from bovine faeces lacked virulence traits. The virulence traits that were identified were primarily associated with *E. faecium* and *E. faecalis*. As *E. faecium* and *E. faecalis* are not the predominant species of the bovine GI tract, the risk of transmission to humans through contamination of food products is likely low. Of most concern perhaps is dissemination of ARGs on MGEs. We identified that both *E. faecium* and *E. hirae* contained the Tn*917* transposon conferring MLS_B resistance suggesting that transfer of ARGs may occur in the bovine GI tract

between *Enterococcus* spp. We also identified two ICE of the Tn*916* family that conferred tetracycline resistance in one isolate of *E. faecium*. As only a small number of isolates were examined in this study it is possible that other enterococci may be present in the bovine GI tract that possess ICE with ARGs. As the cost of genomic sequencing continues to decline, further investigation of ICE using whole genome sequencing will help determine if there are linkages between enterococci isolates from bovine, the surrounding environment and human clinical sources.

7.6. References

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Tables

Table 7.1. Genome characteristics of *Enterococcus* spp. isolated from bovine faeces

Strain	No. contigs	Size (bp)	%GC	Genes	CDSs	ST[a]
E. hirae 1	32	2926392	36.7	2785	2712	-
E. hirae 2	29	2850950	36.7	2678	2631	-
E. hirae 3	81	3088947	36.6	2977	2906	-
E. hirae 4	28	3042973	36.7	2825	2753	-
E. hirae 5	28	2869170	36.8	2741	2670	-
E. hirae 6	62	2966815	36.6	2848	2777	-
E. hirae 7	235	2766361	37.0	2602	2535	-
E. hirae 8	47	2922437	36.7	2801	2730	-
E. hirae 9	47	3178271	36.6	2971	2899	-
E. hirae 10	71	3018341	36.6	2885	2814	-
E. faecium 11	111	2783595	37.9	2719	2648	214
E. faecium 12	182	2712126	38.3	2665	2597	Unknown
E. faecium 13	28	2772865	37.7	2659	2591	955
E. thailandicus 14	17	2603791	36.7	2495	2430	-
E. villorum 15	42	2994157	34.9	2834	2765	-
E. villorum 16	159	3056754	34.9	2907	2837	-
E. faecalis 17	34	2913318	37.3	2788	2729	242
E. gallinarum 18	41	3381991	40.5	3259	3197	-
E. durans 19	43	2931269	37.9	2723	2657	-
E. casseliflavus 20	85	3483586	42.6	3355	3295	-
E. casseliflavus 21	50	3639801	42.2	3436	3375	-

[a]ST: sequence type

Table 7.2. Antibiotic resistance gene profile of *Enterococcus* spp. isolated from bovine faeces. Values represent % pairwise identity

Resistance gene	***aph(3')-III***	***aac(6')-Ii***	***aac(6')-Iid***	***aac(6')-Iih***	***ant(9)-Ia***	***adeC***	***erm*(B)**	***msrC***	***lsa*(A)[a]**	***lsa*(E)**	***lnu*(B)**	***tet*(L)**	***tet*(M)**	***tet*(O)**	***sat4***	***vanC* operon[b]**
Strain																
E. hirae 1				99.5			100					100	96.5			
E. hirae 2				99.5			100									
E. hirae 3				100			100							93.0		
E. hirae 4				98.9			100							100		
E. hirae 5				100			99.5							91.4		
E. hirae 6				100												
E. hirae 7				99.5												
E. hirae 8				100												
E. hirae 9				98.9										96.9		
E. hirae 10				100										92.8		
E. faecium 11	100	100			100	99.9	100	98.9		98.9	99.9	100	95.1		99.0	
E. faecium 12		100				92.9		95.4								
E. faecium 13		99.8				99.8		99.3								
E. thailandicus 14																
E. villorum 15																
E. villorum 16							99.6					100				
E. faecalis 17									99.5							
E. gallinarum 18							98.6									present
E. durans 19			100													
E. casseliflavus 20																present
E. casseliflavus 21							98.6									present

[a]Intrinsic to *E. faecalis*, [b]*van*C operon consists of *van*C, *van*R-C, *van*S-C, *van*XY-C and is intrinsic to *E.gallinarum* and *E. casseliflavus*; GenBank accession numbers for resistance genes: *aph(3')-III* (M26832.1), *aac(6')-Ii* (L12710.1), *aac(6')-Iid* (AJ584701.2), *aac(6')-Iih* (AJ584700.2), *ant(9)-Ia* (JQ861959.1), *adeC* (CP003583.1), *erm*(B) (U86375.1), *msrC* (AY004350.1), *lsa*(A) (AY225127.1), *lsa*(E) (JX560992.1), *lnu*(B) (AJ238249.1), *tet*(L) (M29725.1), *tet*(M) (EU182585.1), *tet*(O) (Y07780.1), *tet*(32) (AJ295238.3), sat4 (U01945.1), *vanC* operon *E. gallinarum* (AF162694.1), *vanC* operon *E. casseliflavus* (EU151753.1)

Table 7.3. Putative prophage detected in *Enterococcus* spp. isolated from bovine faeces

Strain	Region	Length (kb)	Status	#CDS	Putative phage	GC%	Phage family
E. hirae 1	1	44.7	Intact	59	PHAGE_Entero_vB_IME197_NC_028671	35.4	Siphoviridae
	2	49.6	Intact	54	PHAGE_Entero_phiEf11_NC_013696	33.7	Siphoviridae
	3	26.1	Incomplete	32	PHAGE_Lactob_prophage_Lj965_NC_005355	31.0	Siphoviridae
	4	8.0	Incomplete	9	PHAGE_Entero_EF62phi_NC_017732	33.3	Unclassified phage
E. hirae 2	1	46.5	Intact	60	PHAGE_Entero_vB_IME197_NC_028671	35.5	Siphoviridae
	2	52.5	Intact	61	PHAGE_Entero_phiEf11_NC_013696	33.8	Siphoviridae
	3	26.3	Incomplete	32	PHAGE_Lactob_prophage_Lj965_NC_005355	31.0	Siphoviridae
	4	11.2	Incomplete	17	PHAGE_Entero_EF62phi_NC_017732	33.1	Unclassified phage
E. hirae 3	1	33.5	Intact	44	PHAGE_Lactoc_bIL285_NC_002666(14)	33.8	Siphoviridae
	2	15.5	Incomplete	22	PHAGE_Entero_phiEf11_NC_013696(4)	34.7	Siphoviridae
	3	14.7	Incomplete	20	PHAGE_Lister_A006_NC_009815(3)	31.8	Siphoviridae
E. hirae 4	1	11.8	Incomplete	20	PHAGE_Entero_vB_IME197_NC_028671(3)	33.0	Siphoviridae
	2	36.8	Intact	45	PHAGE_Entero_phiFL1A_NC_013646(7)	32.9	Siphoviridae
	3	70.3	Intact	96	PHAGE_Bacill_phBC6A52_NC_004821(11)	36.5	Podoviridae
E. hirae 5	1	31.7	Intact	47	PHAGE_Entero_phiFL2A_NC_013643(7)	33.7	Siphoviridae
	2	17.6	Incomplete	18	PHAGE_Entero_vB_IME197_NC_028671(4)	31.6	Siphoviridae
	3	40.6	Intact	51	PHAGE_Bacill_phBC6A52_NC_004821(13)	35.6	Podoviridae
E. hirae 6	1	25.5	Questionable	40	PHAGE_Lactoc_bIL285_NC_002666(13)	33.8	Siphoviridae
	2	21.2	Questionable	32	PHAGE_Entero_IME_EFm1_NC_024356(3)	33.5	Siphoviridae
	3	17.5	Intact	24	PHAGE_Clostr_phi_CD119_NC_007917(2)	33.3	Myoviridae
E. hirae 7	1	22.1	Incomplete	25	PHAGE_Entero_phiFL3A_NC_013648(6)	35.3	Siphoviridae
	2	34.2	Intact	46	PHAGE_Lister_2389_NC_003291(15)	35.4	Siphoviridae
	3	17.0	Incomplete	21	PHAGE_Entero_vB_IME197_NC_028671(3)	36.1	Siphoviridae
	4	13.1	Incomplete	17	PHAGE_Lactoc_bIL285_NC_002666(13)	33.2	Siphoviridae
E. hirae 8	1	37.6	Intact	51	PHAGE_Lactoc_bIL285_NC_002666(15)	34.5	Siphoviridae
	2	40.5	Intact	55	PHAGE_Lister_2389_NC_003291(16)	34.6	Siphoviridae
	3	25.4	Incomplete	18	PHAGE_Entero_phiEf11_NC_013696(4)	33.4	Siphoviridae
	4	8.0	Incomplete	8	PHAGE_Clostr_c_st_NC_007581(2)	40.1	Myoviridae
E. hirae 9	1	37.8	Intact	57	PHAGE_Entero_vB_IME197_NC_028671(7)	37.1	Siphoviridae
	2	37.2	Intact	45	PHAGE_Entero_phiFL1A_NC_013646(8)	33	Siphoviridae
	3	42.4	Intact	53	PHAGE_Bacill_phBC6A52_NC_004821(11)	35.4	Podoviridae

Table 7.3. Continued

Strain	Region	Length (kb)	Status	#CDS	Putative phage	GC%	Phage family
E. hirae 10	1	36	Intact	45	PHAGE_Lactoc_bIL285_NC_002666(14)	33.6	Siphoviridae
	2	20.9	Questionable	32	PHAGE_Entero_IME_EFm1_NC_024356(3)	33.5	Siphoviridae
E. faecium 11	1	39.7	Intact	56	PHAGE_Lactoc_TP901_1_NC_002747(14)	36.2	Siphoviridae
	2	38.2	Intact	55	PHAGE_Entero_phiFL1A_NC_013646(10)	34.9	Siphoviridae
	3	30.8	Intact	22	PHAGE_Ectoca_siliculosus_virus_1_NC_002687(4)	35.5	Phycodnaviridae
	4	35.6	Questionable	16	PHAGE_Staphy_SPBeta_like_NC_029119(2)	34.8	Siphoviridae
E. faecium 12	1	43.8	Intact	52	PHAGE_Lacto_phigle_NC_004305(10)	36.4	Siphoviridae
	2	18.6	Incomplete	16	PHAGE_Bacter_Sitara_NC_028854(3)	35	Siphoviridae
	3	32.9	Questionable	33	PHAGE_Aureoc_anophagefferens_virus_MM_2014_NC_024697(3)	37.3	Phycodnaviridae
E. faecium 13	1	35.9	Intact	48	PHAGE_Bacill_BCJA1c_NC_006557(11)	35.8	Siphoviridae
E. thailandicus 14	1	26.9	Incomplete	31	PHAGE_Lactoc_bIL285_NC_002666(4)	35.8	Siphoviridae
	2	17.6	Incomplete	17	PHAGE_Lactoc_ul36_NC_004066(6)	36.2	Siphoviridae
	3	24.2	Questionable	25	PHAGE_Lister_B025_NC_009812(8)	37.2	Siphoviridae
	4	28.5	Incomplete	27	PHAGE_Clostr_phiCD27_NC_011398(3)	36.3	Myoviridae
E. villorum 15	1	32.2	Incomplete	23	PHAGE_Staphy_PT1028_NC_007045(2)	34	Unclassified dsDNA phage
	2	11.4	Incomplete	11	PHAGE_Ectoca_siliculosus_virus_1_NC_002687(4)	34.1	Phycodnaviridae
	3	17.7	Incomplete	25	PHAGE_Lactoc_bIL286_NC_002667(2)	33.5	Siphoviridae
	4	45.6	Intact	51	PHAGE_Entero_phiFL3A_NC_013648(6)	32.9	Siphoviridae
	5	52.8	Intact	51	PHAGE_Strept_SM1_NC_004996(15)	34.4	Siphoviridae
	6	30.1	Incomplete	30	PHAGE_Entero_EFC_1_NC_025453(4)	34.9	Siphoviridae
	7	41.2	Intact	50	PHAGE_Lactoc_bIL285_NC_002666(14)	36.1	Siphoviridae
E. villorum 16	1	32.2	Incomplete	23	PHAGE_Staphy_PT1028_NC_007045(2)	34	Unclassified dsDNA phage
	2	11.4	Incomplete	11	PHAGE_Ectoca_siliculosus_virus_1_NC_002687(4)	34.1	Phycodnaviridae
	3	17.7	Incomplete	24	PHAGE_Lactoc_bIL286_NC_002667(2)	33.6	Siphoviridae
	4	38.4	Intact	46	PHAGE_Entero_phiFL3A_NC_013648(6)	32.9	Siphoviridae
	5	51.7	Intact	53	PHAGE_Strept_SM1_NC_004996(15)	34.5	Siphoviridae

Table 7.3. Continued

Strain	Region	Length (kb)	Status	#CDS	Putative phage	GC%	Phage family
E. villorum 16	6	29.4	Intact	33	PHAGE_Lactoc_bIL285_NC_002666(13)	34.9	Siphoviridae
	7	31.8	Incomplete	22	PHAGE_Aureoc_anophagefferens_virus_MM_2014_NC_024697(2)	35.4	Phycodnaviridae
	8	25.4	Incomplete	11	PHAGE_Staphy_SPBeta_like_NC_029119(2)	36	Siphoviridae
	9	25.6	Intact	40	PHAGE_Staphy_SPBeta_like_NC_029119(2)	36.3	Siphoviridae
E. faecalis 17	1	42.2	Intact	56	PHAGE_Entero_phiEf11_NC_013696(20)	36	Siphoviridae
	2	37	Intact	47	PHAGE_Strept_phi3396_NC_009018(6)	35.2	Siphoviridae
	3	17.7	Incomplete	19	PHAGE_Entero_vB_IME197_NC_028671(7)	38.8	Siphoviridae
E. gallinarum 18	1	44	Intact	55	PHAGE_Paenib_HB10c2_NC_028758(5)	38.1	Siphoviridae
	2	28.9	Intact	41	PHAGE_Bacill_BCJA1c_NC_006557(8)	38	Siphoviridae
E. durans 19	1	56.7	Intact	63	PHAGE_Lactoc_TP901_1_NC_002747(11)	36.4	Siphoviridae
E. casseliflavus 20	1	40.7	Intact	51	PHAGE_Entero_EFC_1_NC_025453(10)	39.7	Siphoviridae
	2	38.8	Intact	41	PHAGE_Entero_phiFL3A_NC_013648(5)	40	Siphoviridae
	3	35.2	Intact	27	PHAGE_Bacter_Sitara_NC_028854(4)	39	Siphoviridae
E. casseliflavus 21	1	26.9	Incomplete	40	PHAGE_Lister_LP_037_NC_021787(3)	37	Siphoviridae
	2	27.6	Incomplete	21	PHAGE_Entero_phiFL3A_NC_013648(4)	38.8	Siphoviridae

Chapter 8 – General Discussion

8.1. General Discussion

Antibiotics have been used for more than 60 years in the management of infectious diseases in humans and livestock (Veterinary Medicines Directorate, 2009). The inappropriate use of antibiotics in human medicine and in livestock production has contributed to the emergence of antibiotic resistance. Antibiotic resistance threatens the effectiveness of antibiotics in the treatment of infectious diseases and represents one of the most challenging public health issues in today's society.

It is estimated that over half of the antibiotics produced each year are used in livestock production, with the majority of antibiotics administered subtherapeutically (Schmieder and Edwards, 2012). Subtherapeutic refers to the use of antibiotics at low concentrations in livestock feed or water to prevent disease and improve production efficiency. It has been increasingly recognised that this practice is correlated with the emergence of resistant bacteria. The use of the glycopeptide avoparcin and emergence of glycopeptide-resistant *Enterococcus faecium* was the first example demonstrating a transmission route of antibiotic resistant bacteria from animals to humans (Witte, 2000). Avoparcin was once widely used in Australia and the European Union, but the discovery of an association between avoparcin use and vancomycin resistance eventually resulted in a global ban of avoparcin use in livestock (Casewell et al., 2003; NRA, 2001). Avoparcin was never approved for use in North America (Health Canada, 2002). However, many other antibiotics are still used in livestock production, leading to the possibility of transmission of antibiotic resistant bacteria from animals to humans.

The World Health Organization (WHO) has recognised macrolides as a critically important antibiotic (World Health Organization, 2012). Macrolides are commonly administered to cattle in feedlots for the control of bovine respiratory disease (BRD) and liver abscesses. Macrolides are also used in human medicine and erythromycin is the macrolide antibiotic of choice. The same genes confer resistance to macrolides in cattle production and human medicine leading to concern for the potential spread of macrolide resistant bacteria from cattle to humans.

Enterococci are a commensal of the gastrointestinal tract of both animals and humans (Franz et al., 2011). They are also associated with nosocomial infections in humans (Agudelo Higuita and Huycke, 2014; Poh et al., 2006). Transmission of resistant enterococci from animals to humans has been demonstrated, highlighting the zoonotic potential of enterococci. Vancomycin-resistant *E. faecium* strains sharing identical pulsed-field gel electrophoresis (PFGE) profiles were reported isolated from a turkey farmer and his turkeys (Van den Bogaard, 1997). In another study, vancomycin-resistant *E. hirae* strains with identical PFGE profiles were isolated from a broiler farmer and his broilers (Van den Bogaard, 2002). This suggests transmission of macrolide resistant enterococci from animals to humans could also occur. The zoonotic potential of enterococci, association with nosocomial infections in humans, role as an indicator bacteria, and habitation in the normal gut flora of both animals and humans highlights them as a suitable candidate to study macrolide resistance in beef cattle.

As such, this thesis assessed the potential contribution of antimicrobial use in beef cattle production on antimicrobial resistance by examining differences in the relative abundance of key resistance genes in the resistome of cattle and urban environments using real-time, quantitative PCR. Tylosin phosphate is a commonly used macrolide administered at subtherapeutic levels in the diet of feedlot cattle for the control of liver abscesses. Enterococci were used as an indicator

bacterium to investigate the effects of in-feed administration and withdrawal of tylosin phosphate on macrolide resistance. This study identified a number of enterococci that harboured antibiotic resistance genes (ARGs), which were selected for genomic sequencing and further study using comparative genomics.

Real-time, quantitative PCR (qPCR) has been used in many studies to quantify ARGs in various environments, including in livestock and poultry faeces (He et al., 2014; Mu et al., 2014; Zhu et al., 2013), livestock lagoons (McKinney et al., 2010), and wastewater from urban environments (Marti et al., 2013; Negreanu et al., 2012). Studies such as these have highlighted correlations between antibiotic use and the elevated abundance of ARGs.

It can be argued shotgun metagenomic approaches are slowly replacing molecular techniques such as qPCR in the study of antibiotic resistance (Schmieder and Edwards, 2012). Metagenomic approaches are not limited by the number of resistance genes that can be screened and have the ability to discover novel ARGs, unlike qPCR which is restricted to the study of known resistance genes. Despite its advantages over qPCR, shotgun metagenomics requires complex analysis with high computational power and expertise in bioinformatics (Schmieder and Edwards, 2012). Thus, qPCR is still a useful tool that can provide an approximation of the abundance of ARGs in the environment in order to assess how antibiotic use may be influencing the relative abundance of targeted ARGs within the resistome (Berendonk et al., 2015).

As such, Chapter 4 explored the use of qPCR to determine the resistance gene profile of Canadian beef feedlots and urban environments by quantifying ARGs across five antibiotic classes including the sulfonamides, tetracyclines, macrolides, fluoroquinolones and β-lactams. The relative abundance of ARGs in feedlot cattle faeces was compared to those in feedlot catch

basins, a surrounding waterway and municipal wastewater treatment plants in Alberta to determine how differences in antibiotic use may contribute to different resistance gene profiles. An aspect of the study was collection of data related to antibiotic use from the feedlots sampled, allowing links between antibiotic use and the resistance gene profile to be proposed.

The abundance profiles of the resistance genes quantified in Chapter 4 differed across the environments examined, with sulfonamide, fluoroquinolone and β-lactam resistance genes predominating in urban wastewater whilst tetracycline resistance genes were prevalent in cattle faecal composite samples. The differences in the resistance profiles observed appeared to reflect differences in antibiotic use in cattle versus humans, suggesting the selective pressure of antibiotic use on resistance development. Co-localisation of resistance genes on mobile genetic elements can lead to the co-selection of resistance genes (Chung et al., 1999; De Leener et al., 2004; Giovanetti et al., 2003). This would also influence the abundance profile observed. Furthermore, bacterial composition and diversity can also influence the resistance gene profile, as some ARGs are more common in some bacterial species than in others (Durso et al., 2012; Forsberg et al., 2014). Therefore, there are multiple factors that can influence the selection and persistence of ARGs, highlighting the complex nature of antibiotic resistance development.

Whilst quantifying ARGs can provide a general overview of the resistome in different environments, it is unable to provide an understanding of what may be occurring at a microbiological level. Diverse bacterial populations in the gastrointestinal (GI) tract of cattle and the use of multiple antibiotics in the feedlot can make it difficult to produce a clear idea of the direct impact of antibiotic use on resistance development, and how best we can manage the use of antibiotics in production so as to not reduce their efficacy. Indicator bacteria are therefore used as a tool to interpret the direct impact of antibiotic use on bacterial populations.

Escherichia coli has been used as an indicator bacterium to interpret resistance development to tetracyclines and sulfonamides (Alexander et al., 2008; Sharma et al., 2008). However, this bacterium cannot be used to study macrolide resistance due to its innate resistance (Mao and Putterman, 1968). The results of Chapter 4 highlighted the presence of macrolide resistance genes in composite faecal samples from cattle despite macrolides not being administered at the time of sampling and provided an indication of the relative abundance of macrolide resistance genes in the entire bacterial population. The importance of macrolides in both animal husbandry and human medicine prompted investigation of how macrolide use in the cattle feedlot industry impacts macrolide resistance. To investigate this, enterococci were selected as an indicator bacterium.

Previous studies have demonstrated therapeutic and subtherapeutic administration of macrolides to cattle increases the proportion of erythromycin-resistant (ery^R) enterococci in bovine faeces (Jacob et al., 2008; Zaheer et al., 2013). Prior to slaughter, a withdrawal period is employed to prevent residual antibiotics contaminating meat for consumption. The duration of withdrawal differs depending on the antibiotics used (Compendium of Veterinary Products, 1999). For in-feed macrolides there is no defined withdrawal period. There is limited knowledge regarding how the withdrawal of macrolides prior to slaughter may impact the proportion of ery^R enterococci in bovine faeces. It is expected once antibiotic selective pressure is removed, the proportion of resistance will decrease. If this is the case, antibiotic withdrawal could be an effective control strategy to minimise the potential spread of ery^R enterococci through the food chain.

As such, my second study (Chapter 5) aimed to analyse the effect of in-feed administration of tylosin phosphate to cattle at subtherapeutic levels and its subsequent

withdrawal on macrolide resistance using enterococci as an indicator bacterium. It was demonstrated that administering tylosin phosphate to cattle at subtherapeutic levels increased the proportion of both ery^R and tylosin-resistant (tyl^R) enterococci within the total enterococci population, consistent with observations in previous studies (Jacob et al., 2008; Zaheer et al., 2013). Just prior to its withdrawal, the proportion of ery^R and tyl^R enterococci began to decrease and continued to decrease after tylosin was withdrawn, until there was no difference between the control (administered no antibiotics) and the tylosin phosphate treated groups (administered tylosin phosphate at 11 ppm in the diet). The observed decrease in resistance just prior to the withdrawal of tylosin phosphate from the diet was unexpected. It was predicted that the proportion of ery^R and tyl^R enterococci would decrease after tylosin phosphate was withdrawn from the diet and not before. Stress, age and diet were suggested as potential confounding factors of this observed early decrease in resistance. The difference between control and tylosin phosphate groups was non-existence after tylosin phosphate was removed from the diet, suggesting that this practice has some merit in reducing antibiotic resistance.

Enterococcus hirae has been identified as a predominant species present in the GI tract of cattle (Anderson et al., 2008; Jackson et al., 2010; Zaheer et al., 2013), whilst in humans *Enterococcus faecium* and *Enterococcus faecalis* dominate (Chenoweth and Schaberg, 1990; Noble, 1978). A consistant disparity between the species of *Enterococcus* isolated from cattle and humans may suggest no direct link between the use of macrolides in beef cattle production and the occurrence of ery^R pathogenic enterococci isolated from humans.

In Chapter 5, the species distribution of enterococci in the GI tract of cattle was investigated. *E. hirae* was confirmed as the predominant species present, consistent with observations in previous studies (Anderson et al., 2008; Jackson et al., 2010; Zaheer et al., 2013).

The species diversity of enterococci changed throughout the study, with a greater diversity of enterococci in steers at arrival than at exit from the feedlot. Isolates of *E. faecium* and *E. faecalis*, the two species most commonly associated with nosocomial infections in humans (Ruoff et al., 1990; Sievert et al., 2013; Werner et al., 2008), were isolated at the beginning of the feeding period but were rarely recovered at the end of the experiment. Transitioning of the diet from a forage- to a grain-based diet has been demonstrated to alter the faecal microbiome of cattle (Shanks et al., 2011) and may have created conditions favourable for *E. hirae* to flourish, whilst being detrimental for *E. faecium* and *E. faecalis*. The increasing age of the cattle may have also contributed to the decline in species diversity during the feeding period (Devriese et al., 1992). The species of *Enterococcus* that dominates the GI tract of cattle is clearly different to the species commonly associated with infections in humans. Even though both *E. faecium* and *E. faecalis* can be isolated from the GI tract of cattle, it appears production practices may be reducing their abundance as cattle reach slaughter.

Despite *E. hirae* being the predominant species present in the GI tract of cattle, this species still has the potential to contain and transfer ARGs. Very little is known about the nature and resistance characteristics of enterococci isolated from feedlot cattle. Chapter 5 further investigated the underlying genetic mechanisms conferring resistance to erythromycin in enterococci isolated from bovine faeces, identifying either *erm*(B) or *msr*C or both resistance genes responsible for the observed phenotype. Similar PFGE profiles of ery^{R} *E. hirae* pre- and post-antibiotic treatment suggested that administration of tylosin phosphate selected for ery^{R} enterococci already present in the bovine GI tract.

A number of isolates from Chapter 5 possessed multiple ARGs, including isolates of *E. faecium* and *E. hirae*. This, combined with a lack of genomic sequencing information on

enterococci, excluding *E. faecium* and *E. faecalis*, and from sources other than clinical infections prompted selection of twenty-one *Enterococcus* isolates for whole-genome sequencing and comparative genomic analysis. Chapter 6 and 7 addressed the results from this analysis.

In Chapter 6, a genome note highlighted the key features of the draft genome of an *Enterococcus thailandicus* isolate from bovine faeces following whole-genome sequencing. *E. thailandicus* was first described following isolation from fermented sausage in Thailand in 2008 (Tanasupawat et al., 2008). The identification of *E. thailandicus* in cattle faeces was a unique finding, as this species had yet to be recognised in the bovine GI tract. Its detection can be credited with improvements in characterisation methods towards more molecular based techniques and further verified the suitability of the *groES-EL* spacer region for *Enterococcus* speciation (Zaheer et al, 2012).

Chapter 7 delved deeper into the genus *Enterococcus* by performing a comparative genomic analysis of twenty-one isolates of *Enterococcus* isolated from bovine faeces, and comprising *E. hirae* (*n*=10), *E. faecium* (*n*=3), *Enterococcus villorum* (*n*=2), *Enterococcus casseliflavus* (*n*=2), *E. faecalis* (*n*=1), *Enterococcus durans* (*n*=1), *Enterococcus gallinarum* (*n*=1) and *E. thailandicus* (*n*=1). The analysis examined the presence of putative virulence and ARGs, mobile genetic elements (MGEs), bacteriophage, CRISPR-Cas and secondary metabolite biosynthetic gene clusters. The pertinent findings from this study identified virulence genes in *E. faecium* and *E. faecalis* genomes that corresponded with those found in human clinical isolates, and the identification of MGEs, including the Tn*917* transposon containing the *erm*(B) resistance gene in *E. faecium* and *E. hirae* genomes, and two integrative conjugative elements (ICEs) of the Tn*916* family both containing the *tet*(M) resistance gene in an *E. faecium* genome.

The identification of virulence genes in *E. faecium* and *E. faecalis* isolated from bovine faeces is not surprising. Many virulence genes also play a role in the general survival and colonisation of enterococci in various environments. However, their presence does suggest the potential for them to colonise humans if transmitted from bovine faeces through the food chain. The likelihood of this occurring is likely low considering *E. hirae* is the predominant species present in the bovine GI tract. Virulence factors were not detected in *E. hirae* isolated from bovine faeces. However, there was a pheromone cAD1 precursor lipoprotein *cad* gene detected in *E. hirae* with amino acid identity of 66% to the previously functionally characterised *cad* gene in *E. faecalis* (GenBank accession no. AF421355.1). This sex pheromone induces the transfer of the highly-conjugative, pheromone-responding plasmid pAD1 encoding for a bacteriocin and hemolysin (Wirth, 1994). It is underdetermined if the precursor identified in *E. hirae* is able to induce transfer of pAD1.

A number of MGEs were identified in *Enterococcus* spp. isolated from bovine faeces carrying ARGs. The Tn*917* transposon carrying *erm*(B) was identified in both *E. faecium* and *E. hirae* genomes suggesting transfer of resistance genes on MGEs may occur in the bovine GI tract between different *Enterococcus* spp. Furthermore, the identification of two ICE of the Tn*916* family, Tn*916* and Tn*5801*, both carrying *tet*(M) and conferring resistance to tetracycline in *E. faecium*, indicates ICE are also present in enterococci in the bovine gut. The Tn*916* family of ICE is known to have a wide host range, having been detected in Proteobacteria, Actinobacteria and Firmicutes (Roberts and Mullany, 2009). Several other families of ICE have also been identified in enterococci (Werner et al., 2013). Only a small number of isolates were investigated for this study, therefore it is possible other enterococci present in the bovine gut may contain ICE

carrying multiple ARGs. The prevalence of enterococcal ICEs in the bovine gastrointestinal tract has yet to be fully investigated.

ICEs are capable of acquiring multiple ARGs, which can lead to shared antibiotic resistance as a result of a single genetic transfer event. For example, an ICE identified in *Pasteurella multiocida* conferred resistance to twelve antimicrobials (Michael et al., 2012). The ability of ICE to carry multiple ARGs enabling rapid dissemination of antibiotic resistance is concerning and has implications for both animal and human health by reducing the efficacy of antibiotics. However, in this study the number of multi-drug resistant enterococci was low, despite the inclusion of tylosin phosphate in the diet of feedlot cattle.

8.2. Future Directions

Following on from the work carried out in Chapter 4, it would be beneficial to carry out a metagenomic study of the resistome of urban and cattle environments to enable a more comprehensive study of the resistance genes present. As highlighted earlier, qPCR is limited to the inclusion of known resistance genes. Furthermore, the labour involved increases with number of resistance genes investigated. A metagenomic study could help expand the knowledge gained from Chapter 4 to give a more complete view of the resistome. Further work could also include measuring residual antibiotics in the environments investigated to allow correlations between the resistance genes identified and the presence of antibiotic residues. Characterisation of the bacterial community composition would also allow links between the resistance genes detected and bacteria present to be made. Finally, a longitudinal study would be of value to provide

insight towards seasonal variation that may occur in the resistome of urban and cattle environments.

The number of closed genomes available for the genus *Enterococcus* is limited. There is an imbalance in the availability of *Enterococcus* spp. genomes for analysis, with *E. faecium* and *E. faecalis* genomes from clinical infections comprising the majority. Furthermore, the source of isolates is also biased with a poor representation of strains isolated from healthy humans and non-human sources and from locations excluding Europe and North America. As the cost of genomic sequencing continues to decrease it would be of value to perform further genomic sequencing projects that includes a more diverse range of *Enterococcus* spp. from different origins. In doing so, genetic linkages may be able to be established to determine if there are connections between enterococci isolated from animal and human clinical sources and what role enterococci plays in the dissemination and spread of antibiotic resistance.

While the detection of multidrug resistant enterococci from bovine faeces of cattle administered tylosin phosphate were low, the identification of MGEs such as ICE carrying ARGs indicates potential for resistance genes to spread between organisms. Two ICE were detected in a single genome of an *E. faecium* isolate in Chapter 7, therefore other enterococci may be present in the bovine gut harbouring ICE with resistance genes. Further studies are warranted to investigate the distribution of enterococcal ICE in the bovine GI tract and to determine their prevalence. Furthermore, studies investigating the ability of enterococcal ICE present in the bovine GI tract to be transferred to other organisms should also be carried out. Transfer of Tn*916* conferring tetracycline resistance among strains of *E. faecalis* colonising the intestines of gnotobiotic rats and transfer of Tn*1545* conferring kanamycin, erythromycin and tetracycline resistance between *E. faecalis* and *Listeria monocytogenes* has been demonstrated (Bahl et al.,

2004; Doucet-Populaire et al., 1991). It is therefore plausible enterococcal ICE found in the bovine GI tract may be transferred to other organisms and potentially human pathogens if enterococci containing ICE are disseminated along the food chain.

Although macrolide resistance in enterococci decreased following the withdrawal of macrolides from cattle feed, this is not a reason to become complacent with the use of macrolides in cattle production. Implementation of management practices by farmers that reduce the likelihood of disease spread and decrease the dependence on antibiotics for disease control is becoming increasingly important. For example, investigating alternatives to macrolides for the control of BRD and liver abscesses such as vaccines and use of plant bioactives (Amachawadi and Nagaraja, 2016; Fulton, 2009). Essential oils have been investigated for the control of liver abscesses with varied results. *In vitro* studies have shown essential oils, limonene and thymol at concentrations of 20 or 100 μg/mL and 100 μg/mL, respectively inhibit the growth of *Fusobacterium necrophorum* (Elwakeel et al., 2013). In an *in vivo* study, the inclusion of CRINA, a commercially available mixture of limonene and thymol in the diet of finishing feedlot cattle appeared to reduce the incidence of liver abscesses, but the decline was not statistically significant (Meyer et al., 2009). Both BRD and liver abscesses are polymicrobial, making vaccine development difficult. A number of vaccines are available for BRD control. However, an effacious vaccine for liver abscesses has yet to be developed (Amachawadi and Nagaraja, 2016).

8.3. Concluding Remarks

As the worldwide population continues to grow and the income of low- and middle-income countries rises, the global demand for animal protein will increase leading to increased intensification of food animal production and greater use of antibiotics in agriculture (Center for Disease Dynamics, Economics and Policy, 2015). It is predicted the worldwide consumption of antibiotics by livestock will increase from 63,200 tons in 2010 to 105,600 tons by 2030, unless steps are taken to reduce the need for antibiotics in agriculture (Van Boeckel et al., 2015).

This thesis demonstrated the selective pressure of antibiotics, in particular increased macrolide resistant enterococci following the use of tylosin phosphate in cattle production. The full effects of withdrawing tylosin phosphate from the diet were unclear as the prevalence of macrolide resistant enterococci began to decrease just prior to its withdrawal. Despite this unexpected result, changes to antibiotic use in animal agriculture are important with many authoritarian figures endorsing this.

In May 2015, the World Health Assembly endorsed the Global Action Plan on Antimicrobial Resistance, where it is expected within the next two years all countries will adopt their own national strategies in line with the global plan to address antibiotic resistance (World Health Organization, 2015). In terms of antibiotic use in agriculture, this involves removing the use of antibiotics as antibiotic growth promoters and reducing use of antibiotics in animal production by optimising production through other means such as improving farm hygiene and using vaccines (Center for Disease Dynamics, Economics and Policy, 2015).

Several countries including the European Union Member States, Mexico, South Korea and New Zealand have already enforced bans on antibiotic growth promoters for livestock

production (Center for Disease Dynamics, Economics and Policy, 2015). Presently, there is no ban on the use of antibiotics for growth promotion in the United States or Canada. However, in 2013 the FDA released voluntary guidelines for the withdrawal of medically important antibiotics as growth promoters and in 2014 the Canadian government released similar guidelines (Health Canada, 2014; U.S. Food and Drug Administration, 2013). Australia has had one of the most conservative approaches towards antimicrobial use in livestock production and has many initiatives in place to address antimicrobial resistance including many regulatory restrictions on the prescription and use of antibiotics (Australian Commission on Safety and Quality in Health Care, 2013). Australia is also the only country in the world that has a regulatory measure in place to ban the use of fluoroquinolones in food producing animals (Australian Government, 2015).

One of the major problems of assessing the impact of antibiotic use in agriculture is the limited reliable information on global use. Further, there is limited global information on antibiotic resistance in food animals. Apart from Europe, there have been few instances where the effect of reduced antibiotic use in agriculture on antibiotic resistance has been studied. The ban of avoparcin by the European Union in 1997 provided some insight, with studies comparing resistance prevalence in humans and poultry, indicating a decrease in vancomycin-resistant enterococci (Aarestrup et al., 2001; Klare et al., 1999; Van den Bogaard et al., 2000). It is expected these same trends will be observed as other countries implement similar regulations.

Until these changes take effect, continued surveillance is essential in order to monitor the progress of antibiotic stewardship within all areas of antibiotic use, not just agriculture. There is no doubt antibiotic resistance is a complex issue. Using indicator organisms such as enterococci can aid in understanding the complex relationship between antibiotic use and resistance

development and assist in monitoring progress. However, only time will tell if reduced antibiotic use will have beneficial outcomes for reducing antibiotic resistance over the long term.

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Appendices

Appendix 1

Table S4.1. Summary of samples collected

Sample Type and ID	Type of Production	Average Number of Cattle per pen/catch basin
Feedlot faecal composite		
A (*n*=3)	Conventional	237
B (*n*=3)	Conventional	200
C (*n*=3)	Conventional	371
D_C (*n*=3)	Conventional	251
D_N (*n*=3)	Natural	232
Catch basin		
CB (*n*=5)	n/a	13673
Sewage treatment		
Influent (*n*=2)	n/a	n/a
Effluent (*n*=2)	n/a	n/a
Surface water		
Ephemeral creek (*n*=2)	n/a	n/a

Table S4.2. Summary of antibiotics used at sampled feedlots

Feedlot	Antibiotic family	Antibiotic	Route
A	Tetracycline	Chlortetracycline	In Feed
		Oxytetracycline	Parenteral
	Ionophore	Monensin	In Feed
		Lasalocid	In Feed
	Macrolide	Tylosin	In Feed/parenteral
		Tulathromycin	Parenteral
	Phenicol	Florfenicol	Parenteral
	Cephalosporin	Ceftiofur	Parenteral
	Fluoroquinolone	Enrofloxacin	Parenteral
	Potentiated sulfonamide	Sulfadoxine	Parenteral
	Sulfonamide combination	Sulfanilamide, sulfathiozole, sulfamethazine	Oral Administration
B, C, D_c	Tetracycline	Chlortetracycline	In Feed
		Oxytetracycline	Parenteral
	Ionophore	Monensin	In Feed
		Lasalocid	In Feed
	Macrolide	Tylosin	In Feed/parenteral
		Tulathromycin	Parenteral
		Tilmicosin	Parenteral
	Phenicol	Florfenicol	Parenteral
	Cephalosporin	Ceftiofur	Parenteral
	Fluroquinolone	Enrofloxacin	Parenteral
	Potentiated sulfonamide	Sulfadoxine	Parenteral
	Sulfonamide combination	Sulfanilamide, sulfathiozole, sulfamethazine	Oral Administration

D_C conventional pens at feedlot D

Table S4.3. Primers used in real-time, quantitative PCR analysis

Gene	Primer pair	Sequence (5'-3')	Ann. Temp. (°C)	Amplicon size (bp)	No. cycles	Slope	Intercept point	Eff. (%)	R^2	Reference
16S-rRNA	F	CTCCTACGGGAGGCAGCAGT	60	156	30	-3.4	35.2	97.4	0.999	This study
	R	TTACCGCGGCTGCTGGCAC								
sul1	F	CGCACCGGAAACATCGCTGCAC	55	162	40	-3.6	42.7	88.3	1.000	Negreanu *et al.* 2012
	R	TGAAGTTCCGCCGCAAGGCTCG								
sul2	F	TCCGGTGGAGGCCGGTATCTGG	60	190	40	-3.7	43.0	85.6	0.998	Negreanu *et al.* 2012
	R	CGGGAATGCCATCTGCCTTGAG								
tet(A)	F	GCTACATCCTGCTTGCCTTC	64	210	35	-3.4	38.9	96.3	0.999	Ng *et al.* 2001
	R	CATAGATCGCCGTGAAGAGG								
tet(B)	F	ACACTCAGTATTCCAAGCCTTTG	60	205	40	-3.6	40.8	90.2	0.999	Peak *et al.* 2007
	R	GATAGACATCACTCCCTGTAATGC								
tet(M)	F	TGGACAAAGGTACAACGAGGACGG	64	224	35	-3.5	36.4	94.4	0.998	This study
	R	ACGAGTTTGTGCTTGTACGCCA								
tet(O)	F	ACGGARAGTTTATTGTATACC	53	171	40	-3.4	40.1	95.5	0.999	Aminov *et al.* 2001
	R	TGGCGTATCTATAATGTTGAC								
tet(Q)	F	AGAATCTGCTGTTTGCCAGTG	64	167	35	-3.7	42.4	86.8	0.998	Aminov *et al.* 2001
	R	CGGAGTGTCAATGATATTGCA								
tet(W)	F	GAGAGCCTGCTATATGCCAGC	64	168	35	-3.7	38.4	86.0	0.997	Aminov *et al.* 2001
	R	GGGCGTATCCACAATGTTAAC								
erm(A)	F	CCTTCTCAACGATAAGATAGC	55	207	35	-3.4	37.6	97.2	0.998	This study
	R	ATGGAGGCTTATGTCAAGTG								
erm(B)	F	TTCAATTCCCTAACAAACAGAG	55	161	40	-3.6	45.5	88.5	0.994	This study
	R	TGTTCGGTGAATATCCAAGG								
erm(C)	F	GAGGTGTAATTTCGTAACTGCC	55	189	35	-3.7	38.6	85.0	0.997	This study
	R	TTGCGTATTATATCCGTACTTATG								
erm(F)	F	GCCCGAAATGTTCAAGTTGTCGGTTG	55	164	35	-3.6	38.7	90.3	0.998	This study
	R	TGAAGGACAATGGAACCTCCCAGA								
mef(A)	F	GGAGCTACCTGTCTGGATGG	60	179	40	-3.3	36.3	100.3	1.000	Szczepanowski *et al.* 2009
	R	CAACCGCCGGACTAACAATA								

Table S4.3. Continued

Gene	Primer pair	Sequence (5'-3')	Ann. Temp. (°C)	Amplicon size (bp)	No. cycles	Slope	Intercept point	Eff. (%)	R^2	Reference
qnrS	F R	ATGCAAGTTTCCAACAATGC CTATCCAGCGATTTTCAAACA	60	240	35	-3.5	37.9	91.5	0.998	Marti *et al.* 2013
oqxB	F R	TCCTGATCTCCATTAACGCCCA ACCGGAACCCATCTCGATGC	64	131	35	-3.4	38.4	96.0	1.000	Kim *et al.* 2009
bla_{SHV}	F R	CGCTTTCCCATGATGAGCACCTTT TCCTGCTGGCGATAGTGGATCTTT	64	110	35	-3.5	38.7	94.7	0.999	Xi *et al.* 2009
bla_{TEM1}	F R	TTGGGTGCACGACTGGGT TAATTGTTGCCGGGAAGC	64	504	35	-3.8	36.7	84.8	0.997	Wu *et al.* 2011
bla_{CTX-M}	F R	CTATGGCACCACCAACGATA ACGGCTTTCTGCCTTAGGTT	60	103	35	-3.6	36.9	90.4	0.999	Marti *et al.* 2013

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Appendix 2

A.2.1. Verification of species identity of isolates with unique *groES-EL* intergenic spacer regions

Of the isolates examined for speciation, 36 of these presented unique *groES-EL* intergenic spacer regions not currently deposited in the NCBI database. Of these 36, 5 unique sequences were present (Figure S5.1.). Genetic methods using 16S rRNA, *atpA*, *pheS* and *rpoA* gene sequencing were used to further verify the identity of these *Enterococcus* species. The genes *atpA*, *pheS* and *rpoA* were selected as good candidates for *Enterococcus* species identification due to their high discriminatory power (Naser et al., 2005a; Naser et al., 2005b). The sequences of the primers used for amplification and sequencing of 16S rRNA, *atpA*, *pheS* and *rpoA* genes are listed in Table S5.1. Different primer combinations were used to amplify *atpA*, *pheS* and *rpoA* based on the species each isolate was speculated to be following 16S rRNA amplification and sequence analysis. For *Enterococcus thailandicus,* the primer combinations atpA E. thai; rpoA specific and pheS specific were used, for *Enterococcus villorum,* atpA E. vill; rpoA specific and pheS specific were used, for *Enterococcus faecium,* atpA all; rpoA specific and pheS specific were used and for *Enterococcus casseliflavus* atpA E. cass, rpoA specific and pheS E. cass were used. These primers were designed using *atpA*, *pheS* and *rpoA* partial gene sequences of enterococci species publicly available. For each gene, a 50 μL reaction using 5 μL of DNA template was set up using a final primer concentration of 500 nM, except for atpA all and rpoA all where 1000 nM was used. PCR products were purified using commercial kits and sequenced using both the forward and reverse primers. The reaction conditions were as follows; initial denaturation for 5 min at 95°C, followed by either 35 or 40 cycles of denaturation for 30 s at 94°C, annealing at temperature specified in table for 30 s, extension at 72°C with time specified in table and with a final extension for 10 min at 72°C. Sequence results were BLAST against the non-redundant database to further confirm species identification.

Table S5.1. Primers for 16S rRNA, *atpA*, *pheS*, and *rpoA* amplification and sequencing

Primer name	Primer sequence (5'-3')	Expected product size (bp)	Annealing temperature (°C)	Number of cycles	Extension time	Reference
27F 1492R	AGAGTTTGATCCTGGCTCAG GGTTACCTTGTTACGACTT	~1400	58	35	1 m 30 s	Zaheer et al., 2013
atpA all F atpA all R	GGDYTWGAAAAYGCVATGAGTG CCRAAYTGNGTRAADGCTTC	1070	49	40	1 m	This study
atpA E. thai F atpA E. thai R	GAATGCATGAGTGGTGAGTTGC GCGTAAATGCTTCAAGTTCACGG	1054	59	35	1 m 30 s	This study
atpA E. vill F atpA E. vill R	CGTGCACATGGGTTAGAAAACGC TGTCGCAGCATCTAAATCAGAACC	1098	59	35	1 m 30 s	This study
atpA E. cass F atpA E. cass R	ACGGTTCTTATGGGATGGCA TCTGTGCAGAACCACCAACC	945	58	35	1 m	This study
pheS specific F pheS specific R	CGDACVATGGAAAAACATG CWGCNCCTAARATYTCRATC	337	51	35	40 s	This study
pheS E. cass F pheS E. cass R	GAAGTGCTGATTCGGACCCA CGATCCCTGACATTTCTAAGACG	415	58	35	40 s	This study
rpoA all F rpoA all R	CGTCGTATYYTDYTDTCTTC CCRTCWGTCCADATYTCC	485	48.9	40	40 s	This study
rpoA specific F rpoA specific R	GGTGTACTGCATGAATTCTC CTTTYTCVACCATGATTTCAGC	548	54	35	40 s	This study

Figure S5.1. Alignment of *groES-EL* spacer region of *Enterococcus* species with unique spacer regions isolated from this study deposited in the NCBI database (Accession numbers KP993544, KP993545, KP993546 and KP993547). *Enterococcus casseliflavus* (F1129F 46) displays a 'variant' spacer region that has been reported previously (Tsai et al., 2005 and Zaheer et al., 2012). ATCC strains 19434, 8043 and 25788 were included as comparison. Stop codon of *groES* (TAA) and start codon of *groEL* (ATG) are underlined. Species were verified by sequencing and blast analysis of 16S rRNA, *atpA*, *pheS* and *rpoA*.

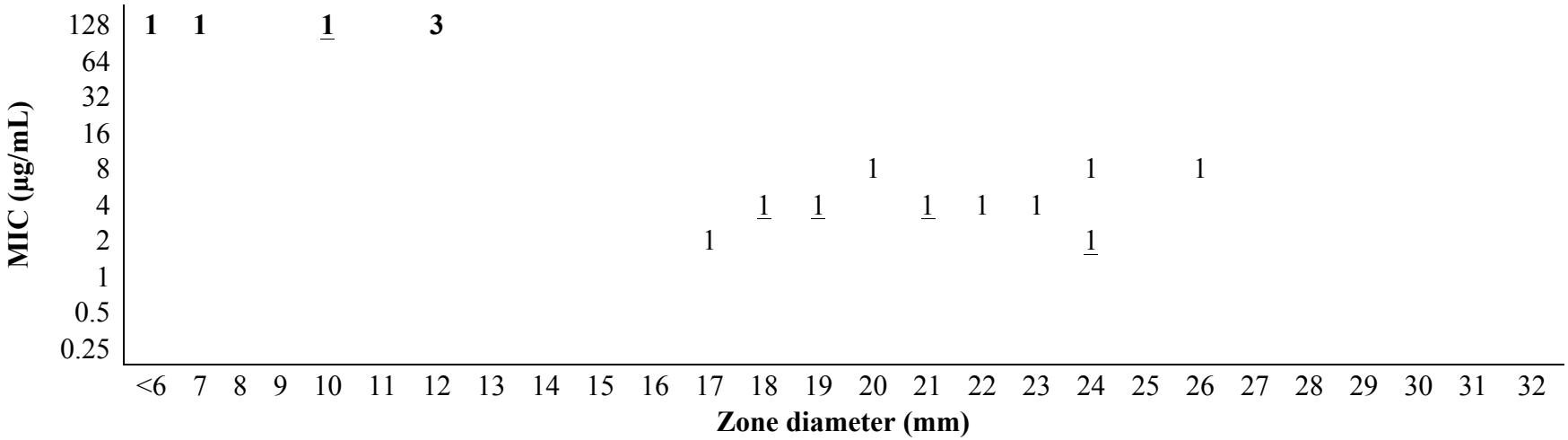

Figure S5.2. Scattergram of MICs versus zone diameters for tylosin. Isolates in bold were confirmed by PCR to have the resistance determinant *erm*(B) and those that are underlined to have the resistance determinant *msrC*.

A.2.2. References

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Appendix 3

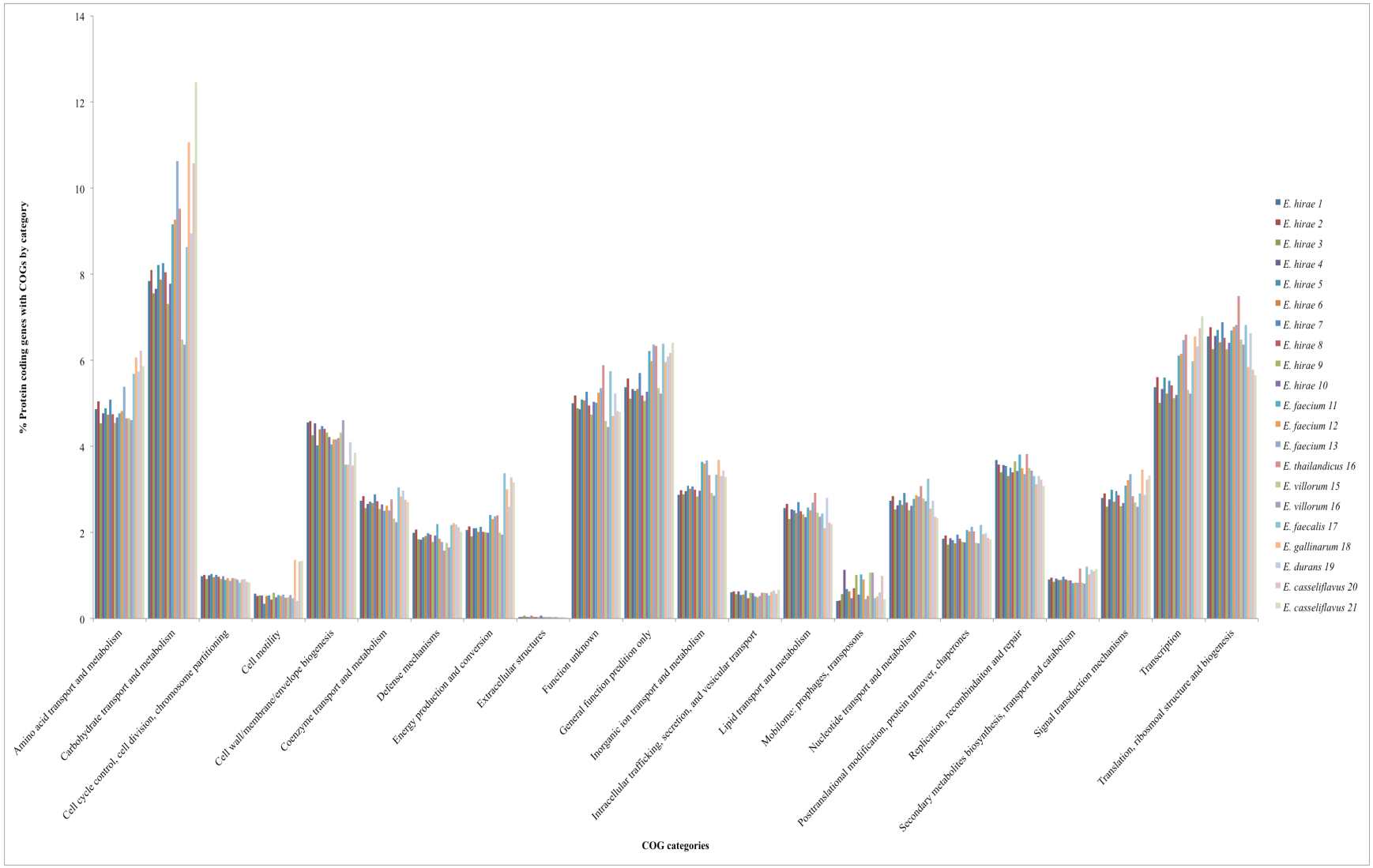

Figure S7.1. Organisation of protein coding genes by Clusters of Orthologous Groups (COGs) category

Table S7.1. Reference sequences for contig ordering using progressive Mauve

Strain	Reference genome name	GenBank accession number for reference genomes
E. hirae 1	*Enterococcus hirae* ATCC 9790, complete genome	CP003504.1
E. hirae 2	*Enterococcus hirae* ATCC 9790, complete genome	CP003504.1
E. hirae 3	*Enterococcus hirae* ATCC 9790, complete genome	CP003504.1
E. hirae 4	*Enterococcus hirae* ATCC 9790, complete genome	CP003504.1
E. hirae 5	*Enterococcus hirae* ATCC 9790, complete genome	CP003504.1
E. hirae 6	*Enterococcus hirae* ATCC 9790, complete genome	CP003504.1
E. hirae 7	*Enterococcus hirae* ATCC 9790, complete genome	CP003504.1
E. hirae 8	*Enterococcus hirae* ATCC 9790, complete genome	CP003504.1
E. hirae 9	*Enterococcus hirae* ATCC 9790, complete genome	CP003504.1
E. hirae 10	*Enterococcus hirae* ATCC 9790, complete genome	CP003504.1
E. faecium 11	*Enterococcus faecium* Aus0085, complete genome	CP006620.1
E. faecium 12	*Enterococcus faecium* Aus0085, complete genome	CP006620.1
E. faecium 13	*Enterococcus faecium* Aus0085, complete genome	CP006620.1
E. thailandicus 14	*Enterococcus faecium* T110, complete genome	CP006030.1
E. villorum 15	*Enterococcus hirae* ATCC 9790, complete genome	CP003504.1
E. villorum 16	*Enterococcus hirae* ATCC 9790, complete genome	CP003504.1
E. faecalis 17	*Enterococcus faecalis* 62, complete genome	CP002491.1
E. gallinarum 18	*Enterococcus gallinarum* strain FDAARGOS_163, complete genome	CP014067.1
E. durans 19	*Enterococcus durans* strain KLDS6.0930, complete genome	CP012366.1
E. casseliflavus 20	*Enterococcus casseliflavus* EC20, complete genome	CP004856.1
E. casseliflavus 21	*Enterococcus casseliflavus* EC20, complete genome	CP004856.1

Table S7.2. Raw antibiogram data from disc susceptibility testing conducted previously (Beukers et al., 2015)

Strain	AMP	DOX	ERY	GEN	LVX	LZD	NIT	Q-D	STR	TGC	TYL	VAN
E. hirae 1	25	14	10	21	23	24	22	19	17	24	6	21
E. hirae 2	26	28	8	22	27	27	19	22	21	23	6	20
E. hirae 3	30	22	12	23	28	27	23	18	21	23	8	21
E. hirae 4	32	16	10	21	23	28	19	20	20	24	7	20
E. hirae 5	32	20	20	22	26	28	19	26	21	24	6	21
E. hirae 6	30	22	14	21	28	27	24	19	18	23	6	21
E. hirae 7	27	27	24	20	23	24	17	25	19	24	20	21
E. hirae 8	31	28	29	21	23	28	25	21	21	21	21	20
E. hirae 9	31	19	24	22	26	24	24	23	20	25	19	21
E. hirae 10	32	22	27	21	27	25	21	22	20	25	24	23
E. faecium 11	32	11	9	27	19	27	21	18	13	26	10	23
E. faecium 12	26	26	14	28	21	27	19	22	27	23	20	20
E. faecium 13	23	28	18	22	16	26	20	21	21	25	20	20
E. thailandicus 14	26	28	18	22	18	27	20	20	20	23	21	20
E. villorum 15	38	28	25	28	28	32	30	29	28	28	22	27
E. villorum 16	40	20	6	24	26	28	28	24	20	27	6	25
E. faecalis 17	23	21	23	19	22	24	23	13	17	20	17	21
E. gallinarum 18	26	28	19	24	21	27	24	21	19	25	11	21
E. durans 19	30	26	13	23	23	25	23	16	20	22	6	23
E. casseliflavus 20	25	27	6	24	17	25	20	14	23	21	7	16
E. casseliflavus 21	26	29	12	23	19	26	25	20	24	24	12	21

AMP, ampicillin; DOX, doxycycline; ERY, erythromycin; GEN, gentamicin; LVX, levofloxacin; LZD, linezolid; NIT, nitrofurantoin; Q-D, quinupristin-dalfopristin; STR, streptomycin; TGC, tigecycline; TYL, tylosin; VAN, vancomycin.

Table S7.3. Presence and absence of CRISPR arrays and intact prophage in *Enterococcus* spp. isolated from bovine faeces

Strain	Functional CRISPR	Type	Orphan CRISPR	Intact prophage
E. hirae 1	Yes	Type II-A	-	Yes
E. hirae 2	Yes	Type II-A	-	Yes
E. hirae 3	Yes	Type II-A	-	Yes
E. hirae 4	Yes	Type II-A	Yes (1)	Yes
E. hirae 5	Yes	Type II-A	-	Yes
E. hirae 6	Yes	Type II-A	-	Yes
E. hirae 7	Yes	Type II-A	-	Yes
E. hirae 8	No	-	-	Yes
E. hirae 9	Yes	Type II-A	Yes (1)	Yes
E. hirae 10	Yes	Type II-A	-	Yes
E. faecium 11	No	-	-	Yes
E. faecium 12	No	-	-	Yes
E. faecium 13	No	-	-	Yes
E. thailandicus 14	Yes	Type II-A	Yes (5)	No
E. villorum 15	Yes	Type II	-	Yes
E. villorum 16	Yes	Type II	-	Yes
E. faecalis 17	No	-	Yes (1)	Yes
E. gallinarum 18	No	-	-	Yes
E. durans 19	Yes	Type II-A	Yes (1)	Yes
E. casseliflavus 20	No	-	-	Yes
E. casseliflavus 21	No	-	-	No

Table S7.4. Direct repeat sequences of CRISPR arrays found in *Enterococcus* spp. isolated from bovine faeces

Direct repeat	Sequence	Direct repeat	Sequence
1	ATCTTCATTCATTCAAAACAA CATAACTCTAAAGC	16	TTACCGTTACTGAATCTTTA AGAGTACAAAAAC
2	TTTTGGAAACATTCAAAACAA CATAGCTCTAAAAC	17	GTTTTAGAGCTATGTTGTTTT GAATGCTTCCAAAAC
3	TTTTGGAAGCATTCAAAACAA CATAGCTCTAAAAC	18	GTTTTAGAGCTATGTTGTTTT GAATGTTTCTGAACT
4	GTTTTAGAGCTATGTTGTTTTG AATGCTTCCAAAA	19	GTGATAGTTTGTTTTTTAAC AACATGGCTCTAAAAC
5	GTTTTAGAGCTATGTTGTTTTG AATGTTTCCAAAA	20	GTTTTGGTACCATTCTAAAC AACATGACTCTAAAAC
6	GTTTTAGTTTTGTGTTATTTTG AGTGCTGACCTCA	21	GTTTTAGAGCTATGTTGTTTT GAATGCTTCCAA
7	GTTTTAGAGTTGTGTTATTTTG AGTGCTGACCTCA	22	GTTTTAGAGCTATGTTGTTTT GAATGTTTCCAA
8	GCATCTTCATTCATTCAAAAC AACATAACTCTAAAGC	23	GTTTTAGAGCTATGTTGTTT AGAATGCTTCCAAAAC
9	CATTTTGGAAACATTCAAAAC AACATAGCTCTAAAAC	24	GTTTTAAAGCTATGTTGTTT AGAATGCTTCCAAAAC
10	TGTTTTGGAAGCATTCAAAAC AACATAGCTCTAAAAC	25	GTTTTAGAACTATGTTGTTT AAAATGCCTCCAACAC
11	GTTTTGGAAGCATTCAAAACA ACATAGCTCTAAAAC	26	GTTTTAGAGCTATGCTGTTT TGAATGCTTCCAAAAC
12	GTTTTGGAAGCATTCGAAACA ACATAGCTCTAAAAC	27	GTTTTAGAGCTATGCTGTTT TGAATGCTTACTTTTG
13	GTTTTGGAAGCGTTCAAAACA ACATAGCTCTAAAAC	28	TTTAGCCAGTCATTCAAAAC AGCATAGCTCTAAAAC
14	TAAGGCTTATTTGAACTTTAA GAGTATAAAAAC	29	GTTTTGGAAGCATTCAAAAC AGCATAGCTCTAAAAC
15	TTACTGTTACTGAATCTTTAA GAGTACAAAAAC	30	GTTTTAGAGCTATGCTGTTT TGAATGCTGACCAAAT

Table S7.5. Spacer sequences of CRISPR arrays found in *Enterococcus* spp. isolated from bovine faeces

Spacer	Sequence	Spacer	Sequence
1	TACCTCTTATCATAGTTGATGAGGTCAGCACTCAAAATAACAC AAAACTAAAACAGCGATCGAGTAAAGCACAACGCTTCTACA	33	GAAAGGTGTCCGAACAGTGTCCGCAAGGCT
2	TACCTCTTATCATAGTTGATGAGGTCAGCACTCAAAATAACACAACTCTAAAACAGCGATCGAGTAAAGCACAACGCTTCTA	34	AACCTGTTTGTGATTATTTATTTAAATTATAGTA
3	AACGACGACGAGTGGTTAGAGCTTGCGAAAG	35	AACTTTGTGGTACTGAATATGTCAGTTTGCTAA
4	CAACCGCATTACCTTCAGAAGAATTTGCATT	36	AACTATTGTCATGGCTGGCGCGAATGGCGGTTT
5	CTAAGGGTTCAACGCAGTACACATTGAGTTT	37	ACATTAGACTAATAAACAATGCTATGGTCA
6	CATCTAAGCGGTAAGTGCTTAGTAGCTTCAA	38	CTTTATCTTATATATTTAGTATACTTTAAA
7	CTTTCGCAAGCTCTAACCACTCGTCGTCGTT	39	TATTGTTTTTGCCTGACAACGTACCCAATA
8	TGTAGAAGCGTTGTGCTTTACTCGATCGCT	40	AGTGATAGACAAAGAAGAATACACGAACAA
9	TTGAAGCTACTAAGCACTTACCGCTTAGA	41	GCACAAAAGGCGAAGAACGTCAAGACCCAA
10	AATGCAAATTCTTCTGAAGGTAATGCGGT	42	CCTGTGAACCGTCCAGAAAATGTGCCGTCT
11	AATTTTTATAATCCTTTGGAATTTCAAAAT	43	AGTCTACATGATAGGTAATACTTTAAATTC
12	GTGCATAACAATTAAGTCAGTGAAAATTGA	44	TAAAGATGTAACAGCTAATTTAGTTGCGTA
13	AGTACCATGACAATGCCTTCATGGCTTTAC	45	AATAGGGGTTCGACTCCTCTACGACCTGTT
14	TACTCAAAATATTTTTCAATTTTGTTTTTG	46	ACTTGTGATAGTGATGTTAGGATCGTGTAT
15	ACTAAGCTTTTCAAATGATTCAATCCTTTT	47	CGGTCATACTTTAGCATTACAAGGCAAATT
16	AGTTGAAATACTTGATTATCTCGTAGATGA	48	TGTGAAAGTTCGTGTGCTAATTCTGATCGG
17	ACACCACCGCCAAGTTCGACAATTACCGAA	49	TTGTTTCATGCTATTCACCGCCTTTATAGT
18	GAGGATATTTTACATTTTAAATTATCCACG	50	TAGCAAGATTGCTCAACCTAAGTCCTGATC

Table S7.5. Continued

Spacer	Sequence	Spacer	Sequence
19	ACTTTTATTGAATCATACACA CTAAACACA	51	GTAGTAGTCAAAAGCTCTT TCCCAATTTTC
20	CAGGTTAAAATCTTAACATTG AGACCCATAATC	52	AATTCTGCTAGGCGTGCCA CCATGTCTGTT
21	AGAGCTAGAAACATTGATAC AGAGCTGATCGTT	53	TGGGACTTGATTTTACCCA CTTGTTAGCTA
22	AGATTGATTATGATGATGCAT TTGACTTGT	54	ATTAGCTAACTTAATTAGT CGTTGTACATT
23	TCATATGATTAATCTCCTTTAT GATATTGT	55	AGTCGTATTAAAGAAAAGC GACTATAGGCT
24	ATTTAGAAAAAAAATAATTA ATCGAGATCA	56	TTAAAGAAAATGCTTCGTG GTCGTGGCTAT
25	AAATGGTTAGTGAAATCATTG AACTAACAA	57	ACAAATTGTTGTGCGTAGT GAATCATATTT
26	TCTTTCGCATGATTGATTCTG CCTCCTCTT	58	GATAAAGAGAGTGGTCAA GTGAAGGTCACT
27	AAATACATGGAAACAAAAGC GCCAGAATCT	59	TTAGAGCTTTTGAATGAAA TTCATTTTGAT
28	GTAAAAAGTTATGGGATCACT TCCATAAAG	60	TGCACGGAATTGATTACTA TGATGTCTCTA
29	TAAAATCAAAGATTTACTTTT CAAAAGGTA	61	TATCCACACGACCAAAGTA ATACCCTAAAT
30	AGCTGCTCTTGAAGAAGCTGA GTATACACC	62	ATTTTTATAAACACTATCAT TGTATACATA
31	TCATCATAGTATCGTAAAACT CTTTCTGGT	63	TCGTCTGGTTTATACATATA TGGATAAGTT
32	TTATTGCTTCACCTATTGGAA TTGTAATTG	64	AACAAGACACTTTAAACGG CTATGTAAATA

A 3.1. References

Beukers, A.G., Zaheer, R., Cook, S.R., Stanford, K., Chaves, A.V., Ward, M.P., and McAllister, T.A. (2015). Effect of in-feed administration and withdrawal of tylosin phosphate on antibiotic resistance in enterococci isolated from feedlot steers. *Front. Microbiol.* 6, 483.

Appendix 4

To whom it may concern,

This is a statement describing our contribution to the published journal article presented in **Chapter 4** of this thesis:

Beukers, A.G., Zaheer, R., Cook, S.R., Chaves, A.V., Ward, M.P., Tymensen, L., Morley, P.S., Hannon, S., Booker, C.W., Read, R.R. and McAllister, T.A. Antimicrobial resistance genes within feedlots and urban wastewater. *PLoS One* (Submitted)

The authors' contributions were as follows:

- A.G.B., R.Z., S.R.C., A.V.C., M.P.W. and T.A.M. designed the study
- A.G.B. conducted the experimental procedures and laboratory analyses
- A.G.B., A.V.C. and M.P.W. analysed and interpreted the data
- A.G.B. drafted the manuscript
- R.Z., S.R.C., A.V.C., M.P.W., L.T., P.S.M., S.H., C.W.B., R.R.R. and T.A.M. critically revised the manuscript

Appendix 5

To whom it may concern,

This is a statement describing our contribution to the published journal article presented in **Chapter 5** of this thesis:

Beukers, A.G., Zaheer, R., Cook, S.R., Stanford, K., Chaves, A.V., Ward, M.P. and McAllister, T.A. (2015). Effect of in-feed administration and withdrawal of tylosin phosphate on antibiotic resistance in enterococci isolated from feedlot steers. *Front. Microbiol.* 6:483

The authors' contributions were as follows:

- A.G.B., R.Z., S.R.C., A.V.C., M.P.W. and T.A.M. designed the study
- A.G.B. conducted the experimental procedures and laboratory analyses
- A.G.B. and K.S. analysed and interpreted the data
- A.G.B. drafted the manuscript
- R.Z., S.R.C., A.V.C., M.P.W. and T.A.M. critically revised the manuscript

Appendix 6

To whom it may concern,

This is a statement describing our contribution to the published journal article presented in **Chapter 6** of this thesis:

Beukers, A.G., Zaheer, R., Goji, N., Cook, S.R., Amoako, K.K., Chaves, A.V., Ward, M.P. and McAllister, T.A. Draft genome sequence of an *Enterococcus thailandicus* strain isolated from bovine feces. *Genome Announc*. 4(4):e00576-16.

The authors' contributions were as follows:

- A.G.B., R.Z., A.V.C., M.P.W. and T.A.M. designed the study
- A.G.B., N.G. and R.Z. conducted the experimental procedures and laboratory analyses
- A.G.B. and R.Z. analysed and interpreted the data
- A.G.B. drafted the manuscript
- R.Z., N.G., S.R.C., K.K.A., A.V.C., M.P.W. and T.A.M. critically revised the manuscript

Appendix 7

To whom it may concern,

This is a statement describing our contribution to the published journal article presented in **Chapter 7** of this thesis:

Beukers, A.G., Zaheer, R., Goji, N., Amoako, K.K., Chaves, A.V., Ward, M.P. and McAllister, T.A. Comparative genomic analysis of *Enterococcus* spp. isolated from bovine feces. *BMC Microbiol*. (Submitted).

The authors' contributions were as follows:

- A.G.B., R.Z., A.V.C., M.P.W. and T.A.M. designed the study
- A.G.B., N.G. and R.Z. conducted the experimental procedures and laboratory analyses
- A.G.B. and R.Z. analysed and interpreted the data
- A.G.B. drafted the manuscript
- R.Z., N.G., K.K.A., A.V.C., M.P.W. and T.A.M. critically revised the manuscript