MOLECULAR EPIDEMIOLOGY OF ANTIBIOTIC-RESISTANT *ENTEROCOCCUS SPP.* FROM FARM TO FOOD-PRODUCTION CHAIN IN INTENSIVE POULTRY PRODUCTION IN KWAZULU-NATAL, SOUTH AFRICA

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A dissertation submitted in fulfilment of the requirements for the degree of Master of Medical Science (Medical Microbiology) in the School of Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal.

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January 2019

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A dissertation submitted to the School of Laboratory Medicine and Medical Sciences, College of Health Science, University of KwaZulu-Natal, Westville Campus, for the degree of Master of Medical Science (Medical Microbiology).

This is a dissertation by manuscript with an overall introduction and final summary.

This is to certify that the content of this dissertation is the original research work of Miss Chantal Molechan, supervised by;

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DECLARATION

I, Miss **Chantal Molechan**, declare as follows:

1. That the work described in this dissertation has not been submitted to UKZN or any other tertiary institution for purposes of obtaining an academic qualification, whether by myself or any other party.

2. That my contribution to the project was as follows:

    - The research reported in this dissertation, except where otherwise indicated, is my original work

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ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to the following people for their role in this research:

- Professor Sabiha Yusuf Essack, for her enthusiastic guidance and immense intellectual leadership throughout the study.
- Dr. Linda Bester, for her patience, guidance and willingness to teach.
- Mr. Daniel Gyamfi Amoako, for his highly appreciated mentorship. Thank you for always expressing confidence in my abilities, all the while encouraging and assisting my growth as a scientist.
- Dr. Akebe Luther King Abia for his kind support and assistance during difficulties in the study. Thank you for allowing me opportunities to learn and grow.
- Biomedical Resource Unit (BRU) colleagues and friends especially Estelle Ramchuran, Nongcebo Malinga and Anou Somboro for all the motivation and support.
- My family, for their patience and support throughout all my academic endeavours.
- Lastly, my best friend and pillar of strength, Letitia Moodley. Thank you for sharing in this journey every step of the way.
- I would like to acknowledge the financial support from the National Research Foundation.

Any omissions and shortcomings that may be identified in this work remain the sole responsibility of the researcher.

C. Molechan

Durban

January 2019

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LIST OF ABBREVIATIONS AND ACRONYMS

ABR Antibiotic resistance

AGPs Antibiotic growth promoters

AMEs Aminoglycoside-modifying enzymes

AMR Antimicrobial resistance

API Analytical profile index

AS Aggregation substance

AST Antimicrobial susceptibility testing

BRICS Brazil, Russia, India, China and South Africa

CC Clonal complex

CLSI Clinical and Laboratory Standards Institute

CRISPR Clustered Regularly Interspaced Palindromic Repeats

Cu Copper

EU European Union

FAO Food and Agricultural Organization of the United Nations

FDA Food and Drug Administration

GAP Global Action Plan

GIT Gastro-intestinal tract

HLGR High-level gentamicin resistant

IS Insertion sequences

LS_AP Lincosamides, Streptogramin A and Pleuromutilins

MDR Multidrug-resistant

MLS Macrolides, Lincosamides and Streptogramins

MLS_B	Macrolides, Lincosamides and Streptogramin B
MLST	Multilocus sequence typing
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSCRAMMs	Microbial surface components recognizing adhesive matrix molecules
NTC	No template control
PBPs	Penicillin-binding proteins
PCR	Polymerase chain reaction
PFGE	Pulse-field gel electrophoresis
QD	Quinupristin-dalfopristin
REP-PCR	Repetitive element palindromic-PCR
VREfm	Vancomycin-resistant *E. faecium*
VRE	Vancomycin-resistant *enterococci*
WHO	World Health Organization
Zn	Zinc

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SUPPLEMENTARY MATERIAL

A Compact Disc (CD) with the electronic copy of this dissertation in portable digital format (pdf).

ABSTRACT

Extensive antibiotic use in intensively-farmed poultry exerts selection pressure for the emergence of multidrug-resistant pathogens. The aim of this study was to determine the antibiotic resistance and virulence profiles of *Enterococcus* spp. along the farm to food-production chain continuum in an intensive poultry system in the uMgungundlovu District in Kwazulu-Natal, South Africa.

A total of 187 samples along the poultry farm to food-production chain continuum (litter, faeces, transport, holding, abattoir and retail meat) were evaluated for the presence of *Enterococcus* spp. Molecular confirmation by PCR, targetting the genus- (*tuf*) and species-specific (*sodA*) genes was undertaken. Susceptibility profiles were assessed by Kirby-Bauer disk diffusion against the WHO-AGISAR recommended panel of antibiotics for *Enterococcus* spp. using CLSI guidelines. Antibiotic resistance and virulence genes were detected using real-time PCR. Genetic relatedness between isolates across the continuum was evaluated by REP-PCR.

Of 134 isolates identified across the continuum, with a prevalence of 72%, molecular speciation confirmed the isolates as *E. faecalis* (36%), *E. faecium* (31%), *E. gallinarum* (2%) and other *Enterococcus* spp. (31%). Resistance to tetracycline (80%), erythromycin (71%), nitrofurantoin (17%), ampicillin (15%), streptomycin (15%), chloramphenicol (11%), ciprofloxacin (5%), tigecycline (4%), gentamicin (4%), teicoplanin (3%) was observed among *Enterococcus* spp. but no vancomycin resistance (0%). *E. faecium* displayed 24% resistance, and 21% were of intermediate susceptibility to quinupristin-dalfopristin. Twenty-one percent (21%) of *E. faecalis* and 100% of *E. gallinarum*, also showed intermediate susceptibility to vancomycin. Forty-three percent (43%) of *E. faecium* were multidrug-resistant (MDR) (resistant to 1 or more antibiotics in 3 or more antibiotic classes). The most frequently observed antibiotic resistance genes, associated with the phenotypic profiles, were *tetM* (76%) and *ermB* (67%) with a smaller percentage noted for *aph(3')-IIIa* (12%) and *vanC1* (1%). Virulence genes *efaAFs* (100%), *cpd* (96%) and *gelE* (81%) were more frequently detected in *E. faecalis*. The cell wall adhesin (*efaAFm*) was more common in *E. faecium* (100%) and other *Enterococcus* spp. (71%). Clonality evaluated by REP-PCR revealed that isolates along the continuum are highly

diverse with major REP-types often consisting of isolates from the same sampling point in the continuum.

This study highlights MDR *Enterococcus* in the poultry food production chain with isolates harbouring both resistance and virulence genes. This can serve as a reservoir for the potential transfer of these genes from poultry to humans through the farm to food-production chain continuum. The findings underscore the need for routine antibiotic resistance surveillance in food animals.

Molecular epidemiology of antibiotic-resistant *Enterococcus spp.* from farm to food-production chain in intensive poultry production in KwaZulu-Natal, South Africa

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

The misuse of antibiotics in intensive animal farming is a major driving force for the emergence of antibiotic-resistant bacteria globally. It is estimated that the global annual consumption of antibiotics in farmed chickens is approximately 148 mg antibiotic per kg of animal produced (Van Boeckel et al., 2015). The selective pressure exerted by antibiotics can engender multidrug resistance, including by co-selection (Tello et al., 2012). This is a major concern since multidrug-resistant (MDR) pathogens have emerged that are resistant to last resort antibiotics, leaving limited options for treatment (Cai et al., 2012; Venter et al., 2017b). *Enterococcus* spp., commonly found in nature and microbiota of humans and animals, have emerged as one of the most notorious global nosocomial pathogens (Guzman Prieto et al., 2016). Vancomycin was among the preferred treatments for resistant enterococcal infections. However, the emergence of vancomycin-resistant enterococci (VRE) has been observed globally. The high prevalence of MDR VRE has implications for human health as vancomycin is among the antibiotics of last resort for the treatment of infection caused by Gram-positive bacteria (Schmidt, 2011). There is currently limited data in South Africa based on the molecular epidemiology of enterococci in poultry. Furthermore, no studies have been conducted to investigate this along the farm to food-production continuum. There is, therefore a need to delineate the burden of antibiotic-resistant *Enterococcus* spp. in poultry to inform evidence-based measures for its containment.

1.2 Literature review

1.2.1 Antibiotic resistance

In 1928, Sir Alexander Fleming stumbled upon a fungal metabolite called penicillin (Fleming, 1929). Within 12 years of its discovery, Fleming and Howard Florey devised a method to harvest this substance that marked the dawn of the golden age of antibiotics.

An antibiotic can be defined as a natural or synthetic compound that can inhibit the growth and proliferation of bacteria (WHO, 2015). Due to this desirable property, the use of antibiotics proved to be a powerful tool with which infections could be overcome.

Since the discovery of penicillin, many other antibiotics were discovered including cephalosporins, carbapenems, aminoglycosides, macrolides and fluoroquinolones which provided a stepping stone to revolutionising medicine. However, the time after the 1980s presents a discovery void with no novel antibiotics discovered (WHO, 2014). Despite this, there has been a significant increase in the use (and misuse) of antibiotics for medical and agricultural purposes which has led to antibiotic resistance (ABR).

Microorganisms have mastered adaptation mechanisms involving an array of evolutionary advantages that allow them to respond rapidly to changing environments (Venter et al., 2017b). Most antimicrobials are produced by other microorganisms; however, the speed at which bacteria develop resistance against these antimicrobials is astonishing. Antimicrobial resistance (AMR) occurs when microorganisms such as bacteria, fungi, viruses, and parasites are no longer affected by compounds that previously had lethal action against them (FAO, 2016a). There is little doubt that human activity has played a significant role in pushing this evolutionary boundary (Sun et al., 2012; Venter et al., 2017b). Although there is significant misuse and/or indiscriminate use of antibiotics in the healthcare setting, it is equally important to note that the indiscriminate use of antibiotics in animal husbandry has contributed considerably to the emergence of antibiotic resistance. In the USA, animal husbandry has been shown to consume twice the quantity of medically important antibiotics as humans (O'Neill, 2015). Several other countries have been reported to include many last resort antibiotics in animal feed (Van Cuong et al., 2016). The term "last resort antibiotic" refers to the last line of effective antibiotics against resistant bacteria. Resistance against last resort antibiotics is a serious threat as minimal treatment options remain (Cai et al., 2012; Venter et al., 2017b). The consequences of ABR are numerous, including failure to treat infections which leads to prolonged infections and higher rates of mortality (FAO, 2016b). In Europe and USA, infections caused by resistant bacteria have claimed up to 50 000 lives with hundreds of thousands of more deaths in other parts of the world (O'Neill, 2015). In 2013, it was

estimated that approximately 214 000 global neonatal deaths were due to resistant sepsis infections (Laxminarayan et al., 2016). For healthcare systems, this means that antibiotics and other drugs that were previously effective are now inadequate and have lost their value (WHO, 2015), consequently increasing healthcare costs. There are no consistent global patterns of ABR. Although each country experiences different major issues, ABR is an issue that every country should concern themselves with, irrespective of their income levels (O'Neill, 2014).

1.2.2 Use of antibiotics in food animals

It is estimated that the global annual consumption of antibiotic for use in farmed chickens is approximately 148 mg antibiotic per kg of animal produced (Van Boeckel et al., 2015). This consumption is often for prophylactic or metaphylactic use as a preventative measure against outbreaks among the animals housed in close proximity (Van Boeckel et al., 2015; Venter et al., 2017b). However, the estimation of the total annual global consumption of antibiotics in agriculture is merely an estimation due to insufficient surveillance and data collection in many countries. Despite several international recommendations put forward by the World Health Organization (WHO) for surveillance of antibiotic resistance in food-producing animals, only a few countries have made initiatives; this excludes South Africa (WHO, 2014).

The primary issue to be addressed is not the residual antibiotics in meat but rather, the selective pressure exerted by the antibiotics leading to the emergence of MDR pathogens. There is a general notion that the use of antibiotics that are not employed in the clinical setting are acceptable for use in agriculture as growth promoters and feed additives (Poole, 2001). However, this is not the case since resistant pathogens can express efflux pumps capable of evading various classes of compounds which can lead to multidrug resistance including those used in both animal feed and healthcare. For this reason, the use of antibiotics prophylactically and as growth-promoters should be reassessed (Venter et al., 2017b).

The use of antibiotics for prophylaxis is defined as the administration of these compounds to healthy animals for the prevention of infectious disease. When administered through

water and feed, this method may not be as effective as believed due to variation in the consumption by individual animals and the number of animals exposed (FAO, 2016a). Metaphylaxis is defined as the use of antimicrobials at therapeutic doses administered to all animals in a group where only a few animals have experienced infection. This acts as a treatment regime for those animals that are infected as well as a preventative measure for the unaffected animals that may still be at risk (FAO, 2016a). Like prophylaxis, metaphylaxis can be used to medicate large groups of animals through water or feed (FAO, 2016a). On the other hand, therapeutic antibiotics are administered to treat active bacterial infection in a single affected animal. While even a single dose of an antibiotic can stimulate ABR within the microbiota of that animal, the repeated and prolonged use of antibiotics to treat recurrent infections poses a risk factor for the emergence of ABR (Kohanski et al., 2010a; Andersson & Hughes, 2014). The use of antibiotics for therapeutic purposes should only be long enough to ensure the elimination of infection in afflicted animals. The use of antibiotic growth promoters (AGPs) as feed additives has been shown to change the gut microbiota of animals as well as promote the transfer of resistance within the animal and environmental microbiome (You & Silbergeld, 2014). AGPs are administered at sub-therapeutic concentrations to groups of animals through drinking water or feed for long durations to advance growth rates (Wielinga et al., 2014). There is inconclusive evidence on whether AGPs provide a significant improvement to animal development and the mechanism behind growth promotion is not well understood (Lee et al., 2012; FAO, 2016a). Without appropriate biosecurity measures, the effect of AGPs could be as low as 1% (Laxminarayan et al., 2016).

1.2.3 Consumption of antibiotics in intensive food-animal production

The three main animal production systems that exist include land-based intensive systems, land-based extensive systems and organic systems (FAO, 2016a). Land-based intensive systems involve large numbers of animals housed at high densities usually indoors. This intensive livestock production method usually involves the use of antibiotics as prophylaxis against infectious diseases for large populations for extended durations (FAO, 2016a). Livestock bred for intensive production tend to lack the variability of their microbiota and instead share similar colonization patterns with particular bacterial species (Schokker et al., 2014). Considering this, as well as the close

proximity of livestock in such a production system, it is likely that antibiotic-resistant populations of bacteria could outcompete susceptible ones. For these reasons, it is possible that intensive production systems that are run with a lack of biosecurity and livestock health are faced with the risk of being colonized with pathogenic species of resistant bacteria (Zhu et al., 2013a; FAO, 2016a). The expansion of intensive systems is expected to increase in future due to the growing global demand for livestock products. However, there is potential to reduce the need for antibiotics (and therefore a reduction in ABR) in intensive production through biosecurity (FAO, 2016a). Land-based extensive systems involve extensive livestock farming that requires low inputs generating low outputs. This system may require lower levels of antibiotics, thereby reducing ABR. However, considering that this farming method involves high numbers of free-range livestock, this may result in high transmission rates of commensal and pathogenic bacteria (from the environment/soil) which may not occur as often in intensive systems (FAO, 2016a, 2013). Organic production systems generally prohibit the use of antibiotics for prophylactic and metaphylactic use, and alternative therapeutics are preferred. Vaccines are encouraged for the prevention of disease and infection (Mazurek et al., 2013; FAO, 2016a). However, different countries stipulate varying levels of acceptable antimicrobial or antibiotic use in organic production systems (FAO, 2016a). Studies observing the levels of ABR in livestock raised through conventional production compared to organic production revealed that conventional methods showed higher ABR levels (Mazurek et al., 2013). In a summary report drawn up in 2015 by the US Food and Drug Administration (FDA), it was stated that the domestic sales and distribution of antibiotics approved for use in food animals showed an increase of 24% from 2009 to 2015 (FDA, 2015). The significant quantities of antibiotics consumed by agriculture are likely one of the key driving forces of ABR (Robinson et al., 2016a).

Countries such as Brazil, Russia, India, China and South Africa (BRICS) have adopted cost-efficient intensive livestock production systems to meet consumer demands. However, these intensive livestock productions rely on antibiotics to maintain animal health and productivity. Therefore these transitioning countries are contributing to the rise of ABR (Van Boeckel et al., 2015). Intensive livestock production suggests direct and indirect contact between animals and humans involved in the production system or

residing within the vicinity, thereby influencing the transfer of ABR genes between animals, humans and the environment (Rushton, 2010; Van Boeckel et al., 2015). Moreover, this system of production generates a substantial amount of waste, much of which is disposed of on nearby land which raises the potential of dispersing ABR genes to environmental bacteria (Hong et al., 2011). In this way, resistance genes and resistant bacteria can spread to farm workers who have the potential to serve as vectors for transmission into their communities (FAO, 2016a).

MDR bacteria have been isolated from food animals in these BRICS countries as well as in other developing countries where the use of antibiotics as growth promoters are not regulated (Maron et al., 2013; Zhu et al., 2013b; Van Boeckel et al., 2015). The absence of clear regulations and guidelines on the use of antibiotics in agricultural and livestock in low and middle -income countries may contribute to irrational use (Van Boeckel et al., 2015). The patterns of antibiotic use in agriculture vary from one country to another due to many factors including regulatory framework and guidance, financial stability, the degree of import and export, population size, culture, education and expertise (FAO, 2016a). It is important to realise that no country alone can successfully combat ABR by acting in isolation. The ease of intercontinental travel today creates an avenue for antibiotic-resistant pathogens to spread globally (O'Neill, 2014). The global trade of agricultural products has also influenced the dissemination of bacteria and therefore the spread of ABR globally (Finley et al., 2013). Many countries are under pressure to intensify agricultural production rates to meet export demands. However, international trade requirements may influence more cautious use of antibiotics in exporting countries. Despite this apparent caution, importing countries are still at potential risk of importing resistant bacteria selected for by the antibiotics used in the exporting country (FAO, 2016a). The developing economies of BRICS are likely to contribute to changing global patterns. In order to meet the export demands and explore new global markets, there is likely to be an increase in antibiotic use to maintain livestock health and productivity (FAO, 2016a). In this way, the global spread of ABR can occur rapidly. Furthermore, live animals are also traded for breeding or slaughter purposes, and this presents another pathway of transmission since livestock harbour a reservoir of bacteria in their gastrointestinal tract which is continually mixing and being excreted (FAO, 2016a).

1.2.4 One health approach

In 2015, the WHO released a Global Action Plan (GAP) on AMR (WHO, 2015). The WHO has formed a tripartite alliance with the Food and Agricultural Organization of the United Nations (FAO) as well as the World Organization for Animal Health (OIE) to combat the burden of ABR (This tripartite has since been expanded to include the United Nations Environmental Programme). The GAP emphasises the need for an effective "one health" approach which includes coordination of numerous international sectors, including human and veterinary medicine, agriculture, finance, environment, and well-informed consumers (WHO, 2015). The One Health approach recognises that there are interlinking connections between the health of people, animals and the environment (Robinson et al., 2016a). Antibiotics used in animal health are often analogues of those used in human health, which in turn can serve as a driving force for the dissemination of resistance determinants between animals and humans through the environment. Human health generally takes priority with the global burden of ABR estimated in several reports (O'Neill, 2016; WHO, 2014), including specific reports such as those reporting the estimated 111 523 neonatal deaths in 2013, resulting from sepsis, that occurred in five of the leading countries in which neonatal deaths occur (India, China, Democratic Republic of Congo, Nigeria and Pakistan) (Laxminarayan et al., 2016). In contrast, such attention has not been drawn to the global burden of ABR in animal health with exact figures and data unavailable. However, the global estimation of antibiotic consumption in animals seems to exceed that of humans, suggesting that animal production is a significant driver of ABR (Robinson et al., 2016a). Unfortunately, the global crisis of ABR is often questioned, as there are those that argue that animal-associated infections are rarely seen in humans (Chang et al., 2015). However, there is evidence that is connecting ABR in animals to ABR in the clinical setting (Robinson et al., 2016b). The use of antibiotics in animal feed at sub-therapeutic doses, for prolonged durations, provides the ideal conditions for the selection of drug-resistant pathogens (Robinson et al., 2016a). Resistance genes arising in animals can be conveyed to human-adapted pathogens or the natural gut microbiota through food, people or the environment. Numerous infections that occur in people are derived from the natural human gut microbiota. Environmental microbes, due to their ubiquity, may also serve as reservoirs of resistance genes for animal and human pathogens. This might be influenced by antibiotic residues dumped into the

environment originating from the clinical, pharmaceutical and agricultural industry (Robinson et al., 2016a). Additionally, a significant proportion of microbial biomass is present in the soil, which is also a source from which many antibiotics have been discovered and used in medicine (D'Costa et al., 2007). The soil microbial biomass has been responsible for the production of various antibiotics, globally, for an estimated 2 billion years (D'Costa et al., 2011). It is, therefore, proposed that soil can also serve as a reservoir of ABR where resistance is naturally occurring. However, the link between resistance human-made, clinical antibiotics and naturally produced antibiotics in soil remains unclear (Woolhouse et al., 2015).

1.2.5 Intrinsic and Acquired Resistance

The genomes of bacteria are comprised of chromosomal DNA and accessory genetic elements such as plasmids, transposons, insertion sequence common regions, integrative and conjugative elements, gene cassettes and integrons (Stokes and Gillings, 2010). Chromosomal DNA encodes the genetic information dictating the life cycle of the bacterium whereas accessory genetic elements are expressed under specific circumstances where they may confer a survival advantage, such as antibiotic resistance. Chromosomal DNA is inherited vertically by all members of the bacterial progeny while the genetic elements can be transmitted to other bacteria. Due to this, ABR can be inherited intrinsically or via acquired resistance (Courvalin, 2008; Prescott, 2008).

Intrinsic resistance is generally shared by all bacteria of the same genus or species as a result of chromosomal genes that are associated with the physiological characteristics of the bacteria. It can also be described as natural resistance. As a result of environmental stress, bacteria are also able to initiate adaptive resistance which can activate ABR phenotypes. This type of resistance can lead to active efflux mechanisms or diminished permeability of an antibiotic (Courvalin, 2008). The distinguishing factor between adaptive resistance and intrinsic resistance is the fact that the adaptive resistance disappears in the absence of the stimulus. Acquired resistance allows bacteria to develop resistance using a variety of methods including vertical transmission and horizontal gene transfer. Vertical transmission of resistance happens when mutations occur in the structural or regulatory chromosomal genes which are then inherited by the bacterial

progeny (Courvalin, 2008). Structural genes may undergo mutations in the regions that encode antibiotic targets, such as proteins against which antibiotics act. These target mutations depend on the mode of action of an antibiotic compound, and regulatory mutations can occur spontaneously, often altering gene expression (Courvalin, 2008).

Horizontal gene transfer can be described as a mechanism of gene transmission which may occur via one of the following events: (1) transformation, whereby a competent bacterial cell can incorporate free DNA into the cell; (2) transduction, where a bacteriophage acts as a vector transporting DNA from one bacterial cell to another and finally, (3) conjugation, which involves the transfer of DNA from a donor to a recipient bacterial cell via cell-to-cell contact (Courvalin, 2008; Kelly et al., 2009). Horizontal gene transfer can arise through the transfer of a single resistance determinant or multiple resistance determinants that are incorporated into mobile genetic elements. Mobile genetic elements refer to determinants such as gene cassettes which insert into integrons which in turn combine into transposons and these transposons incorporate into plasmids (Kelly et al., 2009). This dynamic action of integration and transposition allows for the union of multiple resistance determinants into a single genetic element with the ability to regulate gene expression through rearrangement (Amábile-Cuevas, 2013; Mazel, 2004). The mobility of integrons is also influenced by the presence of antibiotics and these genetic elements are often co-expressed from one promoter enabling activation of various resistance determinants (Mazel, 2006).

1.2.6 *Enterococcus* spp.: commensals and pathogens

Enterococci are natural inhabitants of the gastrointestinal tract (GIT) of humans and animals. They can also be found in food, water and soil. Enterococci may account for >0.1% of a human adult microbiota and can be considered both commensals and opportunistic pathogens (Schloissnig et al., 2012; de Lastours et al., 2017). There are approximately 57 species of *Enterococcus* to date (http://www.bacterio.net/*Enterococcus*.html). These Gram-positive facultative anaerobes have a particular ability to survive under harsh conditions such as high salt concentrations, various pH values and temperatures ranging from 10°C to more than 45°C (Arias & Murray, 2012), making it challenging to control the spread of pathogenic strains.

Enterococcus spp. are the second leading cause of nosocomial infections, globally (Khan et al., 2015). *Enterococcus gallinarum* and *Enterococcus casseliflavus* are responsible for a minority of clinical infections while *Enterococcus faecium* and *Enterococcus faecalis* are among the most notorious nosocomial pathogens (Giraffa, 2014). These microorganisms are well known for being the common cause of endocarditis and a range of other infections including pelvic infections, neonatal infections and urinary tract infections (UTIs) (Arias & Murray, 2012). In the global context, approximately 10% of adult bloodstream infections can be attributed to enterococci with about 65-70% being due to *E. faecalis,* while *E. faecium* accounts for approximately 25% (Falcone et al., 2015). It is also estimated that 10% of neonatal bloodstream infections are caused by enterococci, with meningitis being an occasional resultant complication (Haslam & St. Geme, 2018). Patients that are of particular risk for invasive enterococcal infection include immune-compromised individuals, haemato-oncology and transplant patients (Humphreys, 2014).

E. faecalis and *E. faecium* may be grouped into distinct clonal complexes (CC) based on their enhanced ability to disperse in nosocomial environments. Furthermore, *E. faecium* can be further distinguished into clades: isolates that are hospital-adapted (clade A, such as CC17) and those that are commensals (clade B) (Palmer et al., 2012). It is of particular interest to discern whether clinical isolates of *E. faecium* and *E. faecalis* are genetically similar than isolates from animals. It has been shown that clinical clonal complex isolates such as CC5, CC2 and CC17 are not only confined to nosocomial settings since these strains were also identified in pigs (Freitas et al., 2011).

1.2.7 Agricultural contribution to the rise of ABR in *Enterococcus*

A large proportion of antibiotics are used for food animal production. In 1988, the chemically structural analogue of vancomycin called avoparcin was introduced as an AGP in chickens and pigs in Denmark. Studies conducted in 1995 reported that VRE were present in about 80% chickens that were exposed to avoparcin, whereas no VRE were detected among organically farmed chickens (Aarestrup, 1995). This was an indication that the use of avoparcin as an AGP influenced the rise of VRE (Aarestrup et al., 1996). In 1995 Denmark implemented a ban on the use of avoparcin as a growth

promoter, even so, VRE remained present in pigs at a frequency of 20% for some time after this period (Aarestrup et al., 2000a; DANMAP, 2014). It was only after the subsequent ban of tylosin (a macrolide) that the persistence of VRE decreased (Aarestrup et al., 2000a). This can be explained by the fact that the genes encoding vancomycin and tylosin resistance are located in close proximity on the same plasmid, hence co-selection (Aarestrup et al., 2000a). In Europe, the use of vancomycin in the clinical setting was limited while the use of avoparcin was approved as an AGP. Conversely, in the USA, avoparcin was banned as an AGP while vancomycin was in high demand for clinical use. However, the prevalence of VRE incidence was confined among clinical populations in the USA compared to Europe. It is likely that VRE were confined to hospitals in the USA while VRE from animal sources were able to penetrate human communities and therefore enter the hospital setting (Wielinga et al., 2014; Coque et al., 1996).

In Sweden, avoparcin was used in food animals for a short duration between the late 1970s and early 1980s after which it was quickly banned along with all growth promoters (Nilsson, 2012). In subsequent years, the prevalence of VRE increased from 1% in 2000 to 40% in 2005, for reasons that are unknown (Nilsson et al., 2009). Jansson et al. (2012) sought to investigate the persistence of vancomycin-resistant *E. faecium* (VREfm) within the barns of 12 Swedish poultry farms, between flocks, and to decipher if day-old chicks, poultry feed and forklift trucks were a source of contamination. The results concluded that 75% of environmental samples from 9 farms were positive for VREfm before cleaning and disinfection while 31% of farms were VREfm-positive following cleaning and disinfection. Furthermore, pulse-field gel electrophoresis (PFGE) analysis revealed similar, or indistinguishable banding patterns of isolates between farms investigated, suggesting clonal dissemination via transport crates, farm workers or even water as vectors for transmission. It was also noted that broiler feed and hatcheries were not a significant source of VREfm while strains were found on two forklift trucks' tyres despite disinfection. PFGE banding patterns showed that isolates from the farms and the forklift were indistinguishable, strongly indicating the forklift as a source of contamination between farms. This study brings attention to the need for VRE surveillance to estimate the risk of transmission not only between farms but the possibility of these clones also

entering the community and clinical setting where crucial resistance determinants may circulate.

Virginiamycin, a streptogramin antibiotic has been used across the world, including the European Union (EU), as a feed additive, growth promoter and preventative measure against coccidiosis and enteritis in chickens. In 1997, vancomycin resistant *E. faecium*, also showing quinupristin-dalfopristin, resistance was isolated from chicken samples and a hospital patient in the United Kingdom (Woodford et al., 1997). However, the streptogramin quinupristin-dalfopristin was not approved in the United Kingdom for clinical use at the time despite its activity against vancomycin-resistant *E. faecium*. Quinupristin-dalfopristin resistance found outside the clinical setting was suggested to be associated with the use of virginiamycin in food animals (Hammerum et al., 2010; Aarestrup et al., 2000b). Following several studies linking streptogramin resistance genes isolated from food animals and hospital patients, in 1998 the EU banned the use of virginiamycin (Hammerum et al., 2010). Still, the use of virginiamycin for growth promotion and therapy continues in several countries across the world.

Gentamicin has been used in the EU for therapy in food and companion animals. However, the emergence of high-level gentamicin resistance in poultry, several other food and companion animals and healthy humans quickly drew attention (Hammerum et al., 2010) because gentamicin and other aminoglycosides are used in combination therapy with β-lactams to combat VRE infections (Hammerum et al., 2010). In past studies, it was shown that the genes responsible for high-level gentamicin resistance in humans were also present in the meat of food animals (Donabedian et al., 2003).

Heavy metals used as growth promoters in animal feed can also favour the selection and spread of antibiotic resistance. Copper (Cu) and zinc (Zn) are commonly used in animal feed, at higher concentrations than metabolically required, as a substitute for antibiotics (Monteiro et al., 2010). Resistance to Cu in enterococci is commonly attributed to the presence of the plasmid-borne *tcrB* gene, which has a link to the erythromycin resistance gene *ermB* and glycopeptide resistance gene *vanA* (Hasman & Aarestrup, 2002). This is

indicative that Cu-resistant enterococci may serve as a reservoir and risk for the spread of erythromycin and vancomycin resistance genes.

1.2.8 Mechanisms of antibiotic resistance in *Enterococcus* spp.

Enterococci have intrinsic resistance to numerous antibiotics including cephalosporins, lincosamides and streptogramins in addition to low-level aminoglycoside resistance (Hollenbeck & Rice, 2012). Additionally, these resilient microorganisms are equipped to acquire an array of antibiotic resistance genes via horizontal gene transfer or sporadic mutations.

Invasive infections caused by *Enterococcus* spp. are often treated with a combination of a β-lactam antibiotic (e.g., penicillin) and an aminoglycoside (e.g., gentamicin) or a glycopeptide such as teicoplanin or vancomycin (Hammerum et al., 2010). Vancomycin is among the last resort antibiotics against Gram-positive bacteria (Schmidt, 2011). The rise in antibiotic resistance among enterococci is a cause for concern, especially with the rise of VRE which is mainly associated with *E. faecium* (Santajit & Indrawattana, 2016). Furthermore, *Enterococcus* spp. may also serve as a reservoir of vancomycin resistance genes to more pathogenic Gram-positive bacteria such as methicillin-resistance *Staphylococcus aureus* (MRSA) (Chang et al., 2003). Mechanisms of resistance to enterococcal-relevant antibiotics are discussed in the following section.

1.2.8.1 Resistance against cell wall inhibitors

β-lactams

Penicillins are β-lactams that have bacteriostatic activity against enterococci and when combined with aminoglycosides, provide a synergistic and bactericidal effect. Ampicillin and penicillin are among the most potent agents within the β-lactam arsenal of antibiotics that are active against enterococci. Their mechanism of action is through the inhibition of cell wall biosynthesis. The bacterial cell wall is composed of peptidoglycan which serves as an “armour” enabling bacteria to endure mechanical stress. Peptidoglycan consists of cross-linked glycan chains of alternating N-acetylglucosamine, and N-acetylmuramic acid (Sychantha et al., 2018) Penicillin-binding proteins (PBPs) are the workhorses of

cell wall biosynthesis as they catalyse the polymerisation and crosslinking of glycan chains through transglycosylation and transpeptidation, respectively (Sauvage et al., 2008). All enterococci produce at least five PBPs. *E. faecalis* and *E. faecium* possess six putative PBP genes (Sahare & Moon, 2014; Duez et al., 2004) Intrinsic tolerance of enterococci to β-lactams can be attributed to the presence of chromosomally encoded *pbp5* which allows low binding affinity for ampicillin and cephalosporins (Sifaoui et al., 2001). *E. faecium* harbours *pbp5* on an operon with two other genes: *psr*, a repressor of PBP synthesis and *ftsW* which interacts with PBP3 of *Escherichia coli* (Rice et al., 2001). High-level penicillin resistance can be attributed to mutations in the PBP5 receptor region, resulting in a decreased binding affinity for the drug (Suleyman & Zervos, 2016).

The presence of β-lactamase activity against ampicillin, through disruption of the β-lactam ring, has been documented in both *E. faecalis* and *E. faecium* (Murray, 1992). This system of resistance (initially described in *Staphylococcus aureus*) can be attributed to the presence of the gene *blaZ* which encodes a β-lactamase as part of an operon consisting of *blaR1* and *blaI* which encode a signal transducer and a repressor, respectively (Hackbarth & Chambers, 1993; Sarti et al., 2012). The *blaZ* gene is expressed constitutively and at low levels in enterococci. During routine susceptibility testing, where the enterococcal inoculum concentration is $\sim 1 \times 10^5$ cells per ml, the enzyme is produced at a negligible level and hence the culture tests susceptible. However, during infection, where the inoculum concentration is high, the enzyme is present at higher levels resulting in resistance (Miller et al., 2015).

Ampicillin resistance is more common in *E. faecium* as opposed to *E. faecalis,* and its' prevalence in poultry is often geographically dependent. In Denmark, the prevalence of ampicillin-resistant *E. faecium* is more frequent in imported broiler meat, while in the USA it is more frequent in turkey meat rather than chicken meat (Bortolaia et al., 2016). Martins et al., (2015) investigated the presence of ampicillin-resistant *E. faecium* from non-clinical sources in Angola. Resistant isolates were recovered from poultry with one isolate showing a PBP5 sequence similar to previously isolated clinical strains. This suggests that the use of β-lactams in the animal sector provided selective pressure for the dispersion of resistance genes.

Glycopeptides

Glycopeptides such as vancomycin, teicoplanin and other derivatives are among the critical antibiotics used for the treatment of Gram-positive bacterial infections (Kristich et al., 2014). Glycopeptides act by inhibiting peptidoglycan biosynthesis by forming complexes with the D-Ala-D-Ala peptide terminus of peptidoglycan precursors located on the outer surface of the cell. This obstructs cell wall biosynthesis enzymes from using these precursors which ultimately compromises the integrity of the cell wall (Kristich et al., 2014). Enterococci evade the action of glycopeptides by altering the drugs' target, usually by substituting the D-Ala-D-Ala termini with D-Ala-D-Lactate or D-Ala-D-Ser. Such substitutions significantly reduce the binding affinity of glycopeptides to the peptidoglycan precursors, thereby allowing cell wall biosynthesis to continue unhindered. Substitution with D-Ala-D-Lac results in a 1000-fold decrease in binding affinity for vancomycin while substitution with D-Ala-D-Ser only results in a 7-fold decreased binding affinity, and therefore a lower resistance level to vancomycin (Cattoir & Leclercq, 2013). Resistance operons which are generally encoded on mobile genetic elements allow production of these altered peptidoglycan precursors. Glycopeptide resistance may also be encoded in chromosomal DNA as part of the core genome of enterococci (Kristich et al., 2014).

There are nine gene clusters that have been identified in enterococci that confer glycopeptide resistance: *vanA*, *vanB*, *vanC*, *vanD*, *vanE*, *vanG*, *vanL*, *vanM* and *vanN* (Raza et al., 2018). The *vanA* and *vanB* are the most commonly observed clusters in clinical isolates of enterococci (Tzavaras et al., 2012). The *vanA* phenotype confers high-level resistance to both vancomycin and teicoplanin. The *vanA* determinant is often plasmid-borne on the transferable transposon Tn*1546* or similar transposons. The *vanA* gene functions in conjunction with several other genes such as *vanR*, *vanS*, *vanH*, *vanX*, *vanY* and *vanZ* to confer resistance (Raza et al., 2018). VanR and VanS form a two-component regulatory system which is transcribed from one promoter while the remaining genes are transcribed from a second promoter. *vanR* and *vanS* encode a response regulator and a sensor kinase, respectively. The VanS sensor kinase identifies a stimulus that signals the presence of vancomycin in the environment which prompts activation and autophosphorylation of VanS on the cytoplasmic side of the protein. The

phosphoryl group can be transferred from the conserved histidine region of VanS to a conserved aspartate residue on VanR. This results in the dimerisation of VanR which therefore enhances VanR binding to the two promoters on the *van* operon, ultimately leading to up-regulated transcription of both *van* resistance and regulatory genes (Depardieu et al., 2005). *vanA* encodes a ligase that forms the D-Ala-D-Lac dipeptide while *vanH* encodes for a dehydrogenase that converts pyruvate to lactate. Both these gene products play crucial roles in the production of modified peptidoglycan precursors to evade the action of glycopeptides. In order to eliminate any remaining normal peptidoglycan precursors, the *vanX* and *vanY* genes encode gene products to allow for efficient removal: *vanX* encodes dipeptidase that cleaves D-Ala-D-Ala residues while VanY encodes a D,D-carboxypeptidase. The function of the *vanZ* determine remains poorly understood (Kristich et al., 2014).

vanB confers moderate to high level of resistance to vancomycin only. This phenotype is still susceptible to teicoplanin. The *vanB* determinant can be located on Tn*5382* or Tn*1549* type transposons which may be harboured on plasmids or form part of the core genome (Kristich et al., 2014). The arrangement of the *vanB* genes is similar to *vanA* since it also contains two promoters and seven open reading frames. However, while *vanB* encodes for a two-component system (consisting of $VanR_B$ and $VanS_B$), the way this signalling system functions is different from *vanA*. The *vanS* and $vanS_B$ show minimal sequence similarity in the N-terminal domain responsible for stimulus recognition. *vanB* encodes homologs of VanH, VanX and VanY (Kristich et al., 2014; Depardieu et al., 2007). However, the *vanB* operon lacks the *vanZ* gene and instead encodes a protein designated VanW which has not clearly been defined (Kristich et al., 2014).

The *vanC*-type resistance gene is an intrinsic, chromosomally-encoded gene commonly found in *E. gallinarum* and *E. casseliflaus* which produces the modified peptidoglycan precursor D-Ala-D-Ser (Reynolds & Courvalin, 2005). The *vanC1* gene is a feature of *E. gallinarum* whereas *vanC2/3* is found in *E. casseliflavus*. This feature confers low-level vancomycin resistance through the production of D-Ala-D-Ser using the VanT serine racemase (Meziane-Cherif et al., 2015). Studies have shown that the *vanC* phenotype is not limited to *E. gallinarum* and *E. casseliflavus* as many reports show its presence in *E.*

faecalis and *E. faecium* (Schwaiger et al., 2012; Nishiyama et al., 2015). Tzavaras et al. (2012) investigated the prevalence of VRE in broiler production after the ban of avoparcin and their link to human clinical VRE in Greece. It was shown that 22.6% of isolates were VRE-positive and the *vanA* gene was the predominant resistant determinant recovered from VRE originating from broiler sources. Despite the ban of vancomycin analogues such as avoparcin, VRE seemed to persist without glycopeptide selective pressure. This suggests that the extensive use of other antibiotics may create a selective pressure for the co-selection of glycopeptide resistance determinants, enabling the persistence of VRE; such as the genetic link between macrolide and vancomycin resistance (Tzavaras et al., 2012). This study further reported the results of PFGE which indicated that VRE from broiler and human sources were clustered according to their source which strongly suggested against the clonal spread of isolates.

Daptomycin

Daptomycin is a lipopeptide antibiotic that disrupts the structure of the bacterial cell membrane. The bacterial cell membrane serves as a structural and functional component by regulating cellular processes that involve interaction between the cell's external and internal environments. Daptomycin acts by inserting into the bacterial cell membrane in the presence of calcium ions and oligomerizes to form a pore structure that jeopardises the integrity of the bacterial cell membrane. This leads to reduced functionality of the bacterial cell membrane as many processes become disrupted causing leakage of ions and ultimately leading to cell death (Miller et al., 2015; Steenbergen et al., 2005). Enterococci appear to be less susceptible to this antibiotic as compared to staphylococci (Cantón et al., 2010). In 2011, Arias and colleagues reported that mutations in the following genes were necessary for daptomycin resistance: genes encoding a putative cell membrane protein, LiaF and a GdpD-family protein involved in phospholipid metabolism. LiaF is thought to form part of the three-component regulatory system designated LiaFSR (lipid-II interacting antibiotics) which functions in stress response of the cell envelope to antibiotics (Arias et al., 2011). Since strains of *E. faecium* are commonly multidrug-resistant, daptomycin is often used as the first line of defence. Therefore, daptomycin resistance appears to be more prevalent in *E. faecium* compared to *E. faecalis*. The genetic basis of daptomycin resistance in *E. faecium* appears to be different from *E. faecalis*

(Miller et al., 2015). Whole genome analysis of a clinical daptomycin-resistant strain of *E. faecium* revealed that resistance was associated with mutations in eight genes, including those involved with phospholipid metabolism and associated with *E. faecalis* resistance (Tran et al., 2013). The eight genes responsible for resistance include those encoding YycFG proteins which form part of a two-component regulatory system (*yyc* system). The YycF serves as the response regulator while YycG functions as the histidine kinase sensor. This system functions to modulate cell wall homeostasis during cell division. The authors of the study speculate that the Yyc system in *E. faecium* is an alternative pathway to the LiaFSR to signal cell envelope changes due to antibiotics, and therefore it is associated with the rise of daptomycin resistance (Tran et al., 2013). However, the LiaFSR system has also been implicated in daptomycin resistance in *E. faecium*. Analysis of genomes of several *E. faecium* isolates showed that the most frequently encountered mutations associated with resistance lay within the *liaFSR* genes followed by mutations in the YycFG system (Diaz et al., 2014).

Very few studies focus attention on daptomycin resistant enterococci in poultry, possibly since there are several debates whether daptomycin is clinically superior to linezolid for treatment of VRE (Chuang et al., 2016). Furthermore, no distinct clinical breakpoints have been established for daptomycin resistance in enterococci (de Jong et al., 2018). Nevertheless, it is essential to monitor the resistance situation in food animals to help predict future possibilities, since this drug is among the last line of clinical treatment for VRE. Few studies that have included daptomycin in the panel of antibiotics, when investigating the prevalence of antibiotic resistance in poultry, have reported no daptomycin resistance in the enterococcal isolates recovered (de Jong et al., 2018; Aslam et al., 2012).

1.2.8.2 Resistance against antibiotics that hinder protein synthesis

Aminoglycosides

Aminoglycosides have concentration-dependent bactericidal activity and target the 16S rRNA of the 30S ribosome (Hermann, 2007). This class of drugs are actively transported into the cell cytosol where they reach the 30S ribosome target, leading to disruption of protein synthesis and ultimately cell death (Feldman et al., 2010). Aminoglycosides are

often coupled with β-lactam antibiotics, such as ampicillin, which increase the cell wall permeability and thereby increases the aminoglycoside uptake thereby enhancing the death of enterococci (Chow, 2000). While intrinsic low-level aminoglycoside resistance genes reside within the genome of enterococci, high-level resistance is often attributed to the acquisition of genes encoding aminoglycoside-modifying enzymes (AMEs) (Padmasini et al., 2014; Costa et al., 1993) in tandem with the reduced uptake of the antibiotic. Enzymatic inactivation of the drug occurs through covalent modification of the aminoglycoside thereby causing reduced binding affinity for the ribosomal target. AME's can be classified into three groups: N-acetyltransferase (AAC - responsible for catalysis of acetylation of an amino group), O-adenylyltransferase (ANT – catalyses adenylation of hydroxyl groups) and finally O-phosphotransferase (APH – for the phosphorylation of hydroxyl groups) (Shete et al., 2017). Those of highest concern are those that hinder the activity of gentamicin and streptomycin since these are commonly used for the synergistic treatment of enterococcal infections (Hollenbeck & Rice, 2012). High-level gentamicin resistance is generally mediated by *aac(6')-Ie-aph(2")-Ia* while high-level streptomycin and kanamycin resistance is mediated by *aph(3')-IIIa* (Kobayashi et al., 2001). Enterococci are also able to modify the antibiotic target, such as ribosomal RNA (rRNA) through the activity of the methyltransferase enzyme designated EfmM (Galimand et al., 2011). This enzyme acts by methylating a specific cytidine residue of the 16S rRNA in *E. faecium* which hinders the binding of kanamycin and tobramycin, thereby conferring resistance (Galimand et al., 2011).

Choi & Woo (2013) investigated high-level gentamicin-resistant (HLGR) *E. faecalis* from chicken meat in Korea and demonstrated that all HLGR strains harboured the *aac(6')-Ie-aph(2")-Ia* gene. Multilocus sequence typing (MLST) revealed that several sequence types corresponded to clinical sequence types in the MLST database. This suggests that chicken products could be a reservoir of HLGR enterococci that may circulate through the food chain.

Oxazolidinones

Linezolid is a synthetic, bacteriostatic agent that inhibits protein biosynthesis in Gram-positive bacteria. Its mechanism of action is through blockage of the *23S rRNA* site

required for docking of aminoacyl-tRNA in the A site of the ribosome (Leach et al., 2007) Bacterial resistance to linezolid may arise through point mutations in genes encoding the *23S rRNA* which serves as the primary drug binding site of the ribosome. Enterococci encode several copies of the *23S rRNA* gene which can be correlated with the resistance phenotype. Specifically, the level of resistance has been correlated with the number of *23S rRNA* genes possessing a G2576U mutation in the domain V of *23S rRNA* (Long et al., 2010). Mutations in ribosomal proteins such as L3 and L4, initially described in staphylococci may also contribute to linezolid resistance (Locke et al., 2009; Chen et al., 2013). Enzymatic modification of the *23S rRNA* through methylation of an adenine residue due to a methylase denoted as Cfr has been found in clinical isolates of *E. faecalis* and some strains of *S. aureus*. The *cfr* gene is harboured on a plasmid and often associated with the mobile genetic element, IS*256* (Hennig & Ziebuhr, 2010). The V IS*256* has been implicated in the transfer of antibiotic resistance genes as well as influencing the activation of expression of existing resistance determinants (Hennig & Ziebuhr, 2010). In 2015 in China, Wang et al. reported on the novel plasmid-borne ABC transporter gene *optrA* that conferred multiple resistance to oxazolidinones and phenicols. The authors also observed that the novel gene shared residence with the *fexA*, on a conjugative plasmid, which confers resistance to phenicols. This novel resistant determinant was more prevalent in *E. faecalis*, and *E. faecium* isolates from food animals (pigs and chicken) as compared to those of human origin. This study demonstrated the importance of testing not only for *cfr* and *23S rRNA* mutations but also for the *optrA* gene. However, *optrA* is not limited to its discovery in China and has also been reported for the first time in poultry meat in America (Cavaco et al., 2017). The authors of this American study aimed to reevaluate previously isolated linezolid-resistant enterococci of poultry origin that were devoid of the *cfr* gene and 23S rRNA mutations. This re-examination of the whole genome sequencing data revealed the presence of *optrA*.

Macrolides, lincosamides and streptogramins (MLS)

These are three functionally similar but chemically distinct antibiotics. MLS antibiotics fall under the group of 50S ribosome inhibitors and exert inhibition against the initiation of protein translation or by hindering translocation of peptidyl-tRNAs, thereby halting the elongation of a nascent peptide chain (Kohanski et al., 2010b). Macrolides such as

tylosin are often recommended for veterinary use in the treatment of respiratory infections in cattle, swine and poultry (Marosevic et al., 2017).

Enterococci often present with cross-resistance to macrolides, lincosamides and streptogramin B, known as the MLS_B phenotype. This is commonly associated with methylase genes, such as *ermB*, which dimethylates an adenine residue on 23S rRNA which inhibits binding of the three drugs (Portillo et al., 2000; Weisblum, 1995). Macrolide resistance is also conferred by efflux pump systems encoded by *mef* and *msr* genes (Portillo et al., 2000). The MLS_B phenotype conferred by the *erm* genes provides resistance to quinupristin, which is a streptogramin B, while dalfopristin (streptogramin A) retains its activity. In addition to this phenotype, there exists the LS_AP phenotype which confers resistance to lincosamides, streptogramin A and pleuromutilins. Pleuromutilins are a class of protein inhibitors that share ribosomal binding sites with the MLS antibiotics (Novak & Shlaes, 2010). While *E. faecalis* shows intrinsic LS_AP resistance with the production of an ABC homologue, this phenotype may be selected *in vivo* in *E. faecium* upon exposure to quinupristin-dalfopristin (QD) (Dowzicky et al., 2000). *E. faecium* exhibits resistance to streptogramin B antibiotics through the expression of efflux pumps encoded by *msrC* genes (Portillo et al., 2000). A recent study that investigated the genetic basis of resistance in *E. faecium* in France showed that a mutation in the *E.faecium* ABC transporter gene, *eatA*, conferred LS_AP-type resistance to susceptible strains (Isnard et al., 2013). This gene is thought to be unique *E. faecium* strains and shows amino acid sequence similarity to proteins conferring LS_AP-type resistance in other Gram-positive bacteria (Isnard et al., 2013).

QD is a combination of semisynthetic streptogramins, streptogramin A (dalfopristin) and streptogramin B (quinupristin). QD shows effective activity against *E. faecium* including vancomycin-resistant strains (Hershberger et al., 2004). The dalfopristin component induces a ribosomal conformational change which exposes the high-affinity binding site for quinupristin, thereby inducing irreversible inhibition of the ribosome (Canu & Leclercq, 2001). QD-resistance can arise through one of several mechanisms: enzymatic modification of the antibiotic, efflux of the drug out of the cell via ATP-binding proteins and alteration of the antibiotic target site. A streptogramin A resistance gene (*vat* or *vga*)

is needed to confer resistance to QD. Streptogramin A resistance genes, encoding acetyltransferase, commonly harboured by *E. faecium* include *vatD* and *vatE*. It has been reported that *vatE* has been commonly recovered from poultry farms, retail poultry and human origin (Donabedian et al., 2006). Also, *E. faecium* harbours lactonases, VgbA and VgbB, which cleaves the ring structure of streptogramin B thereby conferring resistance (Korczynska et al., 2007). *E. faecalis* displays intrinsic resistance to QD owing to the presence of the *lsa* gene (lincosamide and streptogramin A resistance) which is thought to encode a putative ATP-binding protein (Singh et al., 2002). Although the exact mechanism of resistance is not well understood, it is assumed that it is the result of active efflux of the drugs (Singh et al., 2002).

Tetracyclines

Tetracyclines are broad-spectrum antibiotics active against a wide range of Gram-positive and Gram-negative bacteria, in addition to several other microorganisms. Tetracyclines primarily target the ribosome and act by preventing the attachment of charged aminoacyl-tRNA to the ribosomal acceptor A-site. This inhibits protein synthesis by hindering the addition of new amino acids to the nascent peptide chain (Chopra & Roberts, 2001). The low cost of these antibiotics, especially chloro-tetracyclines, has prompted widespread veterinary use for the treatment of a variety of infections (Chopra et al., 1992). Bacteria have adopted four main mechanisms of tetracycline resistance: efflux, ribosomal protection, degradation of the drug and rRNA mutations with efflux being the most prevalent mechanism (Nguyen et al., 2014). Ribosome protection proteins, of which 12 have been identified, bind to the tetracycline target site on the ribosome thereby hindering the drug's mode of action. Tetracycline degradation can be achieved by two rarely encountered genes encoding mono-oxygenases (*tetX* and *tet37*). Finally, mutations within 16S rRNA confer reduced binding affinity for tetracycline to the ribosome (Nguyen et al., 2014). Efflux pumps may be encoded by genes such as *tetK* or *tetL* while ribosomal protection proteins are encoded by *tetM, tetO*, *tetT*, *tetS and tetW*. Resistance to tetracyclines is commonly mediated by *tetM* and *tetL* in isolates of food animal origin (Rizzotti et al., 2009; Chopra & Roberts, 2001; Frazzon et al., 2010). It has been reported that the co-existence of *tetM* and *tetL* enhances antibiotic resistance against doxycycline (Schwaiger et al., 2009). Tetracycline resistance is commonly harboured on transposons,

such as *tetM* which is harboured on Tn*916*. The Tn*916* transposon only harbours *tetM* while Tn*1545* harbouring *tetM* is associated with *ermB*, conferring erythromycin resistance as well (Rice, 1998). In a study carried out by Vignaroli et al. (2011) in Italy, enterococcal isolates from pig and chicken meat and faeces were investigated for the presence of multiple antibiotic resistance genes and their potential to transfer to human strains. More than 80% of isolates were co-resistant to tetracycline and erythromycin, harbouring the *tetM* and *ermB* genes, which corresponds to the common locations of these genes on transposons. Co-transfer of *vanA* and *ermB* were mainly detected in *E. durans* isolates of faecal origin suggesting residence on the same transposon. These findings were the first to report such resistance patterns in *E. durans* of farm animal origin and raised concerns for possible co-transfer of such resistance genes to clinically relevant enterococcal strains (Yahav et al., 2011).

1.2.8.3 Inhibition of folic acid metabolism

Quinolones

The founding member of the quinolones class of antibiotics was nalidixic acid, discovered in 1962, with use limited to the treatment of urinary tract infection (Lesher et al., 1962). Due to its narrow spectrum of activity, newer compounds were sought which led to the synthesis of fluoroquinolones. This led to the introduction of norfloxacin followed by ciprofloxacin. Quinolones primarily target bacterial topoisomerases – DNA gyrase and DNA topoisomerase IV (Hooper, 1998). These enzymes catalyse double-strand break in DNA to allow another strand to pass through the break and after that seal the break (Aldred et al., 2014). Gyrase is composed of GyrA and GyrB subunits which are homologous to ParC/GrlA and ParE/GrlB subunits of topoisomerase IV, respectively (Aldred et al., 2014). Quinolone resistance is commonly attributed to amino acid substitutions in *gyrA* and *parC* genes (Drlica & Zhao, 1997). Since the 1980s, fluoroquinolones have been a popular choice for treatment of UTIs caused by *E. faecalis*. However, this has led to an increase in fluoroquinolone-resistant *E. faecalis* (Lee, 2013). The frequent use of fluoroquinolones in animal husbandry for prophylactic and therapeutic purposes has also contributed to the rise of resistance to these drugs (Chang et al., 2015). The emergence of high-level ciprofloxacin-resistant (HLCR) strains of *E. faecalis,* and *E. faecium* has been on the rise with the ease of spread within the clinical

setting (Woodford et al., 2003; Valdezate et al., 2009). In 2015, an investigation into the antibiotic-resistant characteristics of HLCR *E. faecium* and *E. faecalis* isolated from fresh produce, and clinical faecal samples from Gyeonggi Province in Korea were compared. The aim was to identify relatedness between the two sample groups and determine the occurrence of *gyrA* and *parC* mutations in the isolates as well as the sequence type. The study confirmed mutations within *gyrA* and *parC*, from both sample groups, suggesting the importance of such mutations in fluoroquinolone resistance. Also, these isolates proved to be multidrug resistant and harboured virulence genes suggesting that HLCR isolates are highly virulent (Kim & Woo, 2017). Sequence type analysis revealed two main CC's: CC17 and CC87, which were mainly related to outbreaks and clinical samples. These results should be considered when investigating HLCR enterococci from retail meat such as poultry in terms of antibiotic-resistance and virulence potential. Plasmid-borne resistance to fluoroquinolones as a result of *qnr* genes are generally reported in *Enterobacteriaceae*. These genes encode pentapeptides which hinders fluoroquinolone activity against DNA gyrase. A study conducted in Italy investigating the role of *qnr*-like genes in intrinsic resistance of *E. faecalis* to fluoroquinolones showed that such a gene exists in the genome of *E. faecalis* V583. Inactivation experiments showed a two-fold decrease in resistance to fluoroquinolones while overexpression led to four- to nine-fold increase in resistance. This suggested that *qnr*-like genes may serve as a new mechanism of enterococcal fluoroquinolone resistance (Arsène & Leclercq, 2007).

1.2.9 Virulence factors and Pathogenesis of *Enterococcus* spp.

Virulence can be described as the capacity of a microorganism to infect, colonise and cause disease within a host (Schroeder et al., 2017). Genetic virulence factors may influence the physical (flagella, biofilm, adhesions) and biochemical attributes (cell surface modifying enzymes, toxins) of a bacterium. On the virulence hierarchy, *Enterococcus* species of clinical origin dominate followed by isolates of food origin (Busani et al., 2004). The factors that contribute towards virulence of enterococci include the extent of GIT colonization, adherence to extracellular matrix proteins and the ability to adhere to the urinary tract or oral epithelial cells (Fisher & Phillips, 2009). The most studied enterococcal virulence factors include cytolysins, hydrolytic enzymes, aggregation substances, plasmid-encoded pheromones, cell-wall carbohydrate and

capsular polysaccharide, extracellular surface proteins and adhesins implicated in biofilm formation (Giraffa, 2014). Antibiotic resistance determinants may be categorised as a subtype of virulence factors owing to their ability to enhance bacterial persistence or chronic disease within the host (Emaneini et al., 2016).

Secreted factors

A variety of proteins produced by enterococci have been implicated in their virulence. A proportion of *E. faecalis* strains produce cytolysin, a toxin that can lyse several types of eukaryotic cells including immune cells and red blood cells (Cox et al., 2005). Cytolysin, produced by *Enterococcus* spp., is a bacteriocin that is active against the majority of Gram-positive bacteria and also falls under the class of antibiotic peptides (Van Tyne et al., 2013; Willey & van der Donk, 2007). Cytolysin production is encoded by an operon that may be located on the chromosome within a pathogenicity island, or on a pheromone-responsive plasmid (Shankar et al., 2002; Ike et al., 1990). This operon consists of six genes involved in cytolysin biosynthesis and two encoding regulatory genes. Cytolysin comprises of a large and small oligopeptide subunit, encoded by $cylL_L$ and $cylL_S$ respectively, which undergo extensive post-translational modification to form active toxin subunits (Van Tyne et al., 2013). In the presence of target cells, these subunits interact to produce a pore in the target cell membrane, where the $CylL_L$ subunit has a higher binding affinity to the cell membrane leading to an accumulation of free $CylL_S$ which in turn triggers quorum sensing auto-induction leading to up-regulation of the cytolysin operon (Coburn et al., 2004). The CylM protein is responsible for dehydration of the subunits which is thought to be the initial post-translational modification process (Gilmore et al., 1994). Following this modification by CylM, the subunits are then secreted and trimmed by CylB followed by further processing by CylA serine protease which generates the active toxin (Gilmore et al., 1990; Segarra et al., 1991).

The protease gelatinase (GelE) is responsible for degrading host tissue and altering the host immune system. It also plays a role in eliminating misfolded proteins and activates autolysin (Waters et al., 2003). The absence of GelE results in a significant decrease in biofilm formation, translocation across intestinal cells and reduced virulence in endocarditis (Thurlow et al., 2010; Arias & Murray, 2012). Elucidation of *gelE* has

revealed that it is activated by a quorum-sensing system in *E. faecalis* that is encoded within the *fsr* locus, which is comprised of three genes: *fsrA*, *fsrB* and *fsrC* (Qin et al., 2000; Hancock & Perego, 2004). Expression of *gelE* is controlled by FsrB, a transmembrane protein that is ultimately regulated by the *fsr* locus. The absence of GelE results in a significant decrease in biofilm formation, translocation across intestinal cells and reduced virulence in endocarditis (Thurlow et al., 2010; Arias & Murray, 2012). Both GelE and Cyl are often recovered equally from isolates in patients with clinical infections and stools of healthy individuals. This demonstrates that potential enterococcal virulence determinants may also be harboured by strains colonizing the gastrointestinal tract of healthy individuals (Solheim et al., 2009). The GelE protease is also capable of increasing the intestinal permeability of the enteric epithelium, ultimately leading to intestinal inflammation (Maharshak et al., 2015). Zou et al. (2011) investigated the occurrence of erythromycin resistance and virulence genes in *E.* faecalis from pigs in China. Statistical analysis led the authors to draw a significant correlation between the presence of *gelE* and the erythromycin resistance gene, *ermB*, in enterococcal isolates from swine livestock (Zou et al., 2011). This study suggested that enterococci from pigs may serve as potential reservoirs of resistance and virulence genes.

Cell surface determinants

Aggregation substance proteins (AS proteins), commonly found in *E. faecalis*, appear to play a significant role in contributing to the spread of antibiotic resistance genes in addition to enhanced pathogenesis. Genes encoding AS proteins are located on pheromone-responsive plasmids such as pAD1, pPD1 and pCF10, that also carry antibiotic resistance genes (Waters & Dunny, 2001). These proteins promote clumping of *E. faecalis* cells and facilitate the transfer of plasmids in liquid media (Arias & Murray, 2012). Other cell surface proteins include endocarditis and biofilm-associated pili (Ebp), which are common among *E. faecalis* with significance in biofilm formation and pathogenesis in endocarditis models (Gao et al., 2010; Heikens et al., 2011). *E. faecium* often carries four or more putative pilus loci, two of which are known to produce pili and one which shows homology to the *E. faecalis* Ebp loci (Hendrickx et al., 2009; Sillanpää et al., 2010; Sillanpaa et al., 2008). It has been shown that Ebp and AS may be

systematically co-expressed to enhance densely packed biofilm formation (Afonina et al., 2018).

Microbial surface components recognising adhesive matrix molecules (MSCRAMMs) play a vital role in the initial stages of enterococcal infection by potentially binding components of the host extracellular matrix. The best-described adhesins are the collagen adhesins, Ace and Acm found in *E. faecalis* and *E. faecium* respectively. Like AS and Ebp proteins, these adhesins are anchored in the cell wall and contain LPxTG-like motifs. LPxTG-type surface proteins contain a LPxTG-like motif which becomes enzymatically cleaved and immobilizes the surface protein to the cell-wall peptidoglycan (Hendrickx et al., 2009). The folding patterns of Ace and Acm are similar to immunoglobulins and have a striking similarity to the *S. aureus* collagen adhesion (Cna) (Hendrickx et al., 2009). The Ace collagen and laminin adhesion is activated in the presence of collagen or serum and acts by "embracing" collagen molecule after docking (Nallapareddy & Murray, 2006). Part of the cell wall-associated proteins from the WxL family is enterococcal leucine-rich-repeat-containing protein (ElrA) that is thought to be involved in virulence. Brinster et al. (2007) characterised the ElrA protein and noted that in a mouse peritonitis model, the disruption of the *elrA* gene in *E. faecalis* showed decreased virulence potential through impaired ability to infect macrophages.

Other cell surface determinants include polysaccharides which are integral components of the cell surface of Gram-positive microorganisms and enable pathogenesis and evasion of phagocytosis (Arias & Murray, 2012). Some strains of *E. faecalis* contain capsular polysaccharide locus (*cps*) consisting of 8-9 genes. Enterococcal polysaccharide antigen (Epa) is a cell wall antigen that is often recovered from sera of patients with *E. faecalis* infections (Xu et al., 1997). The biosynthesis of this antigen is encoded by the *epa* locus. The disruption of the genes within this locus results in impaired biofilm formation, increased susceptibility to neutrophil-mediated cell death, and attenuation in peritonitis models (Xu et al., 2000).

Sex pheromones

Sex pheromones which are generally chromosomally-encoded include *cpd*, *cob*, *ccf* and *cad*, are a strategy used by enterococci to accumulate plasmids (Clewell et al., 2002). A single enterococcal strain may secrete several different pheromones. Pheromone production by a recipient cell promotes the conjugative transfer of plasmids between the donor and recipient cells (Chandler & Dunny, 2004). Sex pheromones appear to be a significant contributing factor for enterococcal virulence while also participating in the dissemination of antibiotic resistance. The *ccf* gene has been implicated in activating conjugation of the pCF10 plasmid, which spreads tetracycline resistance genes (Akhtar et al., 2009).

Other virulence factors

Large transferable plasmids that are non-pheromone-responsive are commonly associated with clinical strains of *E. faecium* and contribute to virulence (Arias et al., 2009; Panesso et al., 2010). It has been shown that the transfer of a mega-plasmid from a clinical strain of *E. faecium* to a commensal strain, increased the virulence of the commensal strain in a mouse peritonitis model (Arias et al., 2009). Although the exact roles of genes harboured on these plasmids are not well defined, there is an indication that multiple genes in conjunction with each other offer increased virulence potential (Panesso et al., 2011). Some stress response proteins influence virulence, such as Gls24 which aids in resistance of *E. faecalis* to bile salts. *E. faecalis* also produces three types of peroxidase that neutralise reactive oxygen species, which act as mediators of bacterial death through phagocytosis. These include NADH peroxidase (Npr), Ahp (an alkyl hydroxyperoxide reductase) and a thiol peroxidase (Tpx) (La Carbona et al., 2007). Tpx offers the most effective protection against phagocytosis (La Carbona et al., 2007).

1.2.10 Mobile genetic elements

While identification of antibiotic resistance and virulence genes provides valuable insight into the mechanisms of resistance and virulence, it is equally important to understand their association with mobile genetic elements to predict the risk of acquisition and dissemination of these genes. There is little doubt that genetic exchange has enabled the

rise of *Enterococcus* as a nosocomial pathogen. Bacterial mobile genetic elements can be categorised as bacteriophages, plasmids, transposons, integrons and elements within elements (Stokes & Gillings, 2011). These elements play a significant role in the plasticity of enterococcal genomes through interactions with similar genomic elements and facilitate chromosomal and plasmid DNA rearrangements (Manson et al., 2010a). The genomes of *E. faecalis* and *E. faecium* may contain as much as 25% and 38% of foreign DNA or potential mobile genetic elements, respectively (Paulsen et al., 2003; Lam et al., 2012). However, not all mobile genetic elements that are obtained by bacteria can be expressed; genes from Gram-positive bacteria can be transferred to Gram-negative bacteria, but the converse is not possible due to limitations in heterologous gene expression (Courvalin, 2008). Insertion sequences, on the other hand, are short portions of DNA encoding their transposition and can alter gene expression by altering their level of transcription (Depardieu et al., 2007). The mobility of these sequences can occur at random, making the emergence of resistance through gene activation challenging to predict (Courvalin, 2008). Resistance genes frequently share residence on the same genetic element as virulence factors which provide the bacterial host with an enhanced, multidrug-resistant phenotype (McCarthy & Lindsay, 2012).

Plasmids

A plasmid can be described as extrachromosomal DNA capable of autonomous replication that can be maintained in consecutive bacterial generations. Plasmids may be grouped as "narrow" or "broad host range" depending on host initiation factors or if all essential proteins are encoded on the plasmid (Jain & Srivastava, 2013). While clinical strains of enterococci possess an array of plasmids, the most notable are the pheromone-responsive plasmids and the broad host range plasmids (Kristich et al., 2014).

The best characterised pheromone-responsive plasmids include pAD1 and pCF10 (Christie et al., 1987; Ehrenfeld & Clewell, 1987). These types of plasmids encode a response to sex pheromones (extracellular peptides) which are produced by potential recipient bacterial cell, thereby initiating conjugation. Chromosomally encoded peptide pheromones will specifically induce their cognitive pheromone-responsive plasmids: cCf10 induces pCF10 while cAD1 induces pAD1 (Dunny & Berntsson, 2016). The donor

cell (harbouring the pheromone-responsive plasmid) produces cell aggregation substances (AS) on the cell surface which facilitates cell-cell contact for conjugation and DNA transfer (Kozlowicz et al., 2006; Wardal et al., 2010; Yagi et al., 1983). Pheromone-responsive plasmids are more commonly associated with *E. faecalis* as opposed to *E. faecium* (Gilmore et al., 2013). Such plasmids often harbour both virulence and antibiotic resistance determinants (Manson et al., 2010b).

The pBRG1 pheromone-responsive plasmid which harbours the *vanA* operon has been shown to be transferrable from *E. faecium* LS10 to *E. faecalis* laboratory strains and is responsive to cCF10 pheromones (Magi et al., 2003). Vancomycin resistance associated with pheromone-responsive plasmids encoding the *vanB* operon has also been described in *E. faecalis* (Zheng et al., 2009). Two pheromone-responsive plasmids, pMG200 and pMG201, associated with the VanB-type determinants were described in *E. faecalis* NKH15. The pGM200 plasmid was shown to encode vancomycin resistance, bacteriocin production and response to pheromone cCF10. On the other hand, pMG201 encoded resistance to erythromycin, production of cytolysin virulence factors and response to pheromone cAD1 (Zheng et al., 2009). Sequencing of pMG200 revealed that a conjugative transposon, Tn*1549*-like element, contained the *vanB*-type determinant. It also revealed that this pheromone-responsive plasmid encoded a homologue to the negative regulatory gene of the pCF10 plasmid and a homologue of the positive regulatory gene of the pAD1 plasmid (Zheng et al., 2009). This indicates that the diversity of genetic arrangement of such housekeeping genes could be a consequence of genetic recombination between different pheromone-responsive or non-pheromone-responsive plasmids (Zheng et al., 2009).

The “broad host range plasmids”, also known as incompatibility (Inc18) plasmids, are another class of enterococcal plasmids responsible for dissemination of antimicrobial resistance genes. Two plasmids of the Inc18 group have been well characterised in *Streptococcus* and *Enterococcus* spp.; pIP501 and pAMβ1. These plasmids encode multiple antimicrobial resistance genes against macrolides, lincosamides and streptogramin which can be transferred to other bacterial species (Zhu et al., 2010). The frequency of transfer of the Inc18 group of plasmids is significantly lower than that of

pheromone-responsive plasmids (Kristich et al., 2014). These plasmids employ a toxin-antitoxin post-segregational killing system to ensure the maintenance of the plasmid even in the absence of selective pressure (Hayes, 2003).

1.2.11 Role of plasmids in the dissemination of antibiotic resistance

Plasmids encoding antibiotic resistance determinants may persist in isolates despite lack of selective pressure. This has been demonstrated in enterococcal isolates from Danish poultry where the use of avoparcin has been banned since the mid-1990s. Although there has since been a decline in VRE in Danish poultry as indicated in the DANMAP 2008 and 2010 reports (DANMAP, 2008, 2011), a recent study has reported the persistence of vancomycin-resistant *E. faecium* (Leinweber et al., 2018). Leinweber et al. (2018) revealed that these isolates contained a non-conjugative pVEF4-like plasmid, designated pVEF4_A, harbouring the transferable *vanA* gene cluster within transposon Tn*1546*. Persistence of such plasmids in the absence of selective pressure is believed to be as a result of the three-component *ω/ε/ζ* toxin-antitoxin system or the *prgOPN* gene cluster which is thought to stabilise plasmids through toxin-antitoxin-independent mechanisms (Sletvold et al., 2008; Li et al., 2011). Further work revealed that the presence of the conjugation-encoding plasmid pHLSA might play a significant role in the transfer of resistance-conferring plasmid, pVEF4_A. Antibiotic resistance determinants are often clustered on plasmids which can lead to co-selection of resistance. Tremblay et al. (2011) reported on the antibiotic resistance determinants and plasmid co-localization of tetracycline and macrolide genes in *E. faecium* and *E. faecalis* in poultry flocks in Canada. This study was the first to report co-localization of *ermB* and *tetO* genes on a low molecular weight plasmid harboured by MDR *E. faecalis* of poultry origin in Canada. Furthermore, conjugation experiments proved that this plasmid could confer antibiotic resistance to other bacteria. This study also noted tetracycline resistance even though these drugs are not often used in poultry operations in Canada, suggesting that the use of MLS_B antibiotics could enable maintenance of tetracycline resistance through co-selection (Tremblay et al., 2011).

Transposons

The most largely studied enterococcal transposons include those that encode antimicrobial resistance determinants, virulence factors and ease of transfer. Enterococcal transposons can be grouped under three main categories: composite transposons, Tn*3*-family transposons and conjugative transposons (Kristich et al., 2014). Composite transposons have been commonly associated with high-level gentamicin resistance and *vanB1*-type resistance (Hegstad et al., 2010). Their mobility is encoded by insertion sequences (IS elements). These IS elements, encoding enzymes required for their transposition, are identical sequences that may be directly orientated or inverted repeats that flank either side of composite transposons. The Tn*5281* transposon confers resistance to all aminoglycosides except for streptomycin. Resistance can be attributed to a bifunctional aminoglycoside-altering gene, *acc*-6'/*aph*-2'', which is unique to Tn*5281*-like elements (Ubukata et al., 1984; Courvalin et al., 1980; Hodel-Christian & Murray, 1991). The Tn*5281* element encoded on *E. faecalis* pBEM10 plasmid is flanked by two copies of IS*256*-like sequences at one terminus. This transposon is almost identical to *S. aureus* Tn*4001* and *S. epidermidis* Tn*4031* except that both these composite transposons are flanked by single copies of IS*256* elements at each terminus (Hodel-Christian & Murray, 1991).

The Tn*5385* composite transposon, identified in *E. faecalis*, is an example of a mobile element able to confer multi-resistance through the incorporation of other putative transposons within the larger element. Two of the known transposons include Tn*4001* which confers resistance to gentamycin and Tn*5384* which is encoded between the IS*256* terminus of Tn*4001* and another IS*256* located 26kb away (Rice et al., 2009). The determinants for resistance to gentamycin, macrolides and mercury is encoded on the region between these two IS elements. An additional element Tn*5381*, which is a Tn*916*-like conjugative element, is also encoded on the larger Tn*5385* element and appears to be identical to Tn*552*; a staphylococcal transposon mediating β-lactamase resistance (Rice et al., 2007).

The most notable Tn*3*-family of transposons is the Tn*1546* element which encodes the *vanA*-type operon (Arthur et al., 1993). Similar to the expression mechanisms of related

enterococcal Tn*3*-family of transposons, the Tn*1546* expression is induced upon exposure to glycopeptides (Arthur et al., 1993). The *vanA* operon is almost always found in association with the Tn*1546* element. However, more variants are being observed in clinical isolates and are thought to have emerged as a result of the insertion of IS elements within transposons. Tn*1546* may be encoded chromosomally or on transferable plasmids. Transmission of vancomycin resistance to *S. aureus* has been implicated solely with Tn*1546*-like elements in association with Inc18-type plasmids (Zhu et al., 2010).

Antibiotics can act as initiators of resistance genes movement. Some transposons, such as Tn*3*, are mobilised in the presence of the antibiotic to which they convey resistance. For example, the enterococcal transposon Tn*917* described initially in *E. faecalis* DS16, confers resistance to MLS_B and related antibiotics. Upon exposure to erythromycin, this Tn*917* element shows enhanced mobility (Courvalin, 2008). The determinant for the MLS resistance is encoded by the *erm* gene which translates into a methylase which alters 23S rRNA (Shaw & Clewell, 1985). According to the sequence analysis carried out by Perkins et al., (1984), and Horinouchi et al. (1981), there appears to be almost perfect sequence homology between a region of Tn*917* and the *erm* gene located on pAM77. It is hypothesised that the homology is a result of abortive transposition or recombination with a Tn*917*-like element. On the other hand, Tn*917* may have evolved from a pAM77 element incorporating transposition functions (Shaw & Clewell, 1985; Perkins & Youngman, 1984; Horinouchi & Weisblum, 1981). The *Tn917* element displays increased transposition in cells exposed to sub-inhibitory concentrations of erythromycin (Courvalin, 2008; Shaw & Clewell, 1985; Tomich et al., 1979). Experiments carried out by Shaw et al. (1985) using mRNA analysis revealed that *E. faecalis* DS16 exposed to erythromycin produced increased Tn*917*-specific transcripts compared to those that were not exposed to the drug. This could be attributed to the increased induction of resistance. While Tn*917* also shows significant homology with the *S. aureus* Tn*551* transposon, the MLS resistance of the latter element is expressed constitutively (Perkins & Youngman, 1984; Shaw & Clewell, 1985).

Conjugative transposons mobilize through excision from their point of origin and insert into their target sites without replication (Celli & Trieu-Cuot, 1998). These elements

display the remarkable ability to transfer between enterococcal chromosomes and show a broad host range (Carias et al., 1998). Tn*916* was the first conjugative transposon described, encoding the *tetM* gene conferring tetracycline and minocycline resistance. Tn*916*-like elements are the most common vectors for the spread of *tetM* genes amongst Gram-positive bacteria with the ability to also disseminate to Gram-negative bacteria (Swartley et al., 1993). More recently, Scornec et al. (2017) reported that several antibiotics could activate the transfer of Tn*916* despite the lack of cognate antibiotic resistance genes located on the transposon. These included spectinomycin of the aminoglycosides, tetracycline and doxycycline of the tetracyclines and lincomycin and clindamycin of the lincosamides. It is of concern that activation of mobility of Tn*916* can be induced by a broad range of antibiotics since this implies that the dissemination of resistance determinants is not entirely dependent on the cognate antibiotics to which this element encodes resistance (Scornec et al., 2017).

An expansion of the Tn*916*-family of transposons is the Tn*5382* element which harbours the *vanB* operon. (Carias et al., 1998). Sequencing data of various enterococcal strains suggest that Tn*5382* and closely related elements are the fundamental structures that harbour the *vanB* operon (Lu et al., 2005; Bjørkeng et al., 2011). A study carried out by Carias et al. (1998) provided a possible explanation for the frequent association of vancomycin and high-level ampicillin resistance observed in clinical strains of *E. faecium*. This study reported the transfer of vancomycin, ampicillin and tetracycline resistance from *E. faecium* C68 to *E. faecium* GE-1 which proved to be associated with the uptake of a region of DNA harbouring the Tn*5382* element and *pbp5* gene. In 2010 Jasni et al. conducted the first study that demonstrated the reciprocal genetic exchange of Tn5397, encoding tetracycline resistance, between *E. faecalis* and *Clostridium difficile*. Although the laboratory conditions provided the ideal situation of genetic exchange between the organisms, as opposed to the natural environment, these findings emphasize the importance of continual ABR monitoring.

In an attempt to characterise erythromycin and tetracycline-resistant *E. faecalis* in retail chicken meat in China, Kim et al. (2018) reported that a significant proportion of isolates showed simultaneous resistance to both erythromycin and tetracycline. Furthermore,

most isolates simultaneously harboured the corresponding resistance genes; *ermB* (erythromycin resistance), *tetM* and/or *tetL* (tetracycline resistance). Investigating for the presence of transposons revealed that 5.8% of isolates were positive for the Tn*916*/*1545* element. Despite the low occurrence noted in this study, such findings should receive more attention for future surveillance studies regarding the risk associated with horizontal gene transfer of resistance genes in enterococci (Kim et al., 2018).

1.2.12 CRISPR-Cas and its role in the acquisition of mobile genetic elements

Clustered Regulatory Interspaced Short Palindromic Repeats (CRISPR) is a system that enables bacteria to evade infection by bacteriophages and foreign mobile genetic elements. CRISPR is characterised by the presence of short palindromic repeats separated by spacer sequences. These DNA arrays are often flanked by CRISPR-associated genes such as *cas* (Barrangou & Marraffini, 2014). The CRISPR system works in the following way: when a foreign mobile genetic element is introduced into a cell, a segment of this foreign DNA may be incorporated into the CRISPR array, serving as a genetic memory bank of encountered foreign DNA. There are three main types of CRISPR-Cas systems (type I, II and III), each with its specific *cas* gene (Makarova et al., 2011). The Type II systems associated with *cas9*, which include CRISPR1-Cas and CRISPR3-Cas, are often encountered in *E. faecalis* (Palmer & Gilmore, 2010). A third CRISPR system has been observed in almost all sequenced *E. faecalis* genomes and is called CRISPR2, an orphan locus devoid of *cas* genes (Palmer & Gilmore, 2010; Hullahalli et al., 2015). The genomes of MDR bacteria/pathogens are larger than those of commensals due to the acquisition and maintenance of mobile genetic elements. An investigation conducted by Mikalsen et al. (2015) using DNA microarrays to detect CRISPR-Cas elements in clinically relevant lineages of *E. faecalis* and *E. faecium* revealed that there was a complete lack of these elements in all *E. faecium* isolates tested. In contrast, the majority of *E. faecalis* ST40 strains showed evidence of CRISPR-Cas sequences, hence, a putative functional system. These strains also harboured several acquired antibiotic resistance genes contrary to the reports made by Palmer and Gilmore, (2010) that MDR enterococci were devoid of CRISPR-Cas elements (Mikalsen et al., 2015). Furthermore, it has recently been shown that some *E. faecalis* strains have the potential to attenuate their existing CRISPR systems

which ultimately aids in the uptake of beneficial mobile genetic elements (Hullahalli et al., 2018).

1.2.13 Prevalence of antibiotic-resistant enterococci in food animals and the implications for human health

Although enterococci are natural commensals in animals, ABR strains can be potentially transmitted to humans via direct contact with animals or indirect contact with contaminated meat products. While zoonotic transmission of ABR enterococci mainly affects farm workers, veterinarians or slaughterhouse workers, the population as a whole can be affected through contact or consumption of contaminated meat (Marshall & Levy, 2011). Although the risk of zoonotic disease is rare, more attention should be drawn to the fact that ABR strains may serve as a reservoir of resistance determinants for more pathogenic microorganisms of the GIT. It is known that antibiotic resistance determinants have the potential to be transferred to microorganisms of the same or different strain/species. The use of critically important, clinical-related antibiotics in food animals has implications for human health since this can create a selective environment for the emergence of new MDR pathogenic strains. Noble et al. (1992) reported that genes conferring vancomycin-resistance have the potential to be transmitted to other Gram-positive pathogens such as *S. aureus* which raises an alarm in terms of the health concerns for vancomycin and methicillin-resistant *S. aureus*.

Abat et al. (2016) investigated the unusual increase in *E. faecalis* community-acquired urinary tract infections across various medical institutes within the Provence-Alpes-Côte d'Azur region of France. It was speculated that this outbreak was linked to zoonotic transmission, as a result of *Enterococcus*-contaminated poultry meat. This study drew parallels with an investigation carried out in Vietnam which linked poultry as a possible reservoir for *E. faecalis*-associated urinary tract infections in humans where identical or closely-related strains types were isolated from chickens and infected patients (Poulsen et al., 2012).

In a Canadian study carried out by Aslam et al. (2012), characterization of ABR and virulence genotypes of *Enterococcus* spp. was carried out using samples of retail meat

(beef, chicken, pork and turkey). MDR *E. faecalis* was predominant in poultry meat as opposed to beef and pork. The lack of VRE isolation in this study was attributed to the fact that avoparcin was never approved for use in animal feed in Canada (Aslam et al., 2012). This study emphasised the importance of *E. faecalis* as a reservoir of resistance and virulence genes and their potential to be transmitted to humans by exposure to contaminated, undercooked meat. It also suggested that clinically relevant resistance genes were more likely to be recovered from retail poultry meat. Therefore, poultry meat potentially plays an important role in the spread of resistance genes to consumers through handling meat or ingestion of undercooked meat contaminated with enterococci (Aslam et al., 2012).

1.2.14 Prevalence of ABR and virulence enterococci in South Africa

In a study carried out by Iweriebor et al. (2015), faecal samples from two piggery farms located in the Eastern Cape, South Africa, were collected to determine the antibiotic resistance and virulence profiles of *Enterococcus* species. The predominant species was *E. faecium* (37.5%). All enterococcal isolates were reported to be resistant to most of the antibiotics tested against them including vancomycin. The isolates also showed resistance to at least two different classes of antibiotics, with approximately 93.8% of isolates displaying resistance to five or more antibiotics. Isolates showed high levels of resistance to antibiotics commonly used in the two farms: Penicillin (91%), erythromycin (98.72%) which is often selected for by the use of tylosin, and ciprofloxacin (77.5%). In correspondence to the antibiotic-resistance phenotypes observed, the following genes were identified: *str*A (streptomycin resistance), *erm*B (erythromycin resistance), *van*B, *van*C1 and *van*c2/3 (vancomycin resistance). The high prevalence of MDR VRE raises serious concerns for human health since vancomycin is among the last resort antibiotics against Gram-positive bacteria (Schmidt, 2011). The same authors reported a high prevalence of MDR enterococci in dairy cattle within the same vicinity of the piggery farms (Iweriebor et al., 2016). Once again, the predominant species proved to be *E. faecium* (52.94%). All isolates were reported to be resistant to vancomycin followed by a high aminoglycoside resistance (neomycin [91%] and streptomycin [94%] as well as macrolide resistance (erythromycin [99%)]. The authors further suggested that tylosin use in dairy cattle production may have led to the high levels of erythromycin resistance due

to the similarity of the target site of these drugs. Pillay et al. (2018), reported that a predominance of *E. faecalis* (80%) followed by *E. faecium* (10%) in chicken cloacal samples originating from a poultry farm within KwaZulu-Natal. When compared to enterococcal isolates from companion animals and livestock, it was revealed that the highest incidence of virulence genes, >80% *gelE* and >80% *ccf*, was detected in enterococci of poultry origin. This is of interest since pathogenicity is not solely determined by antibiotic resistance but in conjunction to virulence factors.

1.2.15 Justification for study

To date, there have been limited studies carried out in South Africa on the prevalence and antibiotic resistance profiles of *Enterococcus* spp. in poultry. Many studies conducted worldwide focus on the molecular epidemiology of enterococci in poultry production, however, no data is available in South Africa regarding the relationships between enterococci isolates along the farm to fork continuum. This is an important route of investigation to determine the molecular epidemiology of antibiotic-resistant *Enterococcus* spp. and their possible routes of poultry meat contamination from production and slaughter through to retail chicken meat. There is thus a need to quantify the burden of antibiotic-resistant *Enterococcus* spp. in the poultry industry across the farm to fork continuum in order to inform evidence-based measures for its containment.

1.3 Aim

The aim of this study was to delineate the molecular epidemiology of antibiotic-resistant *Enterococcus* spp., from farm to fork, in chickens from an intensive farming system in uMgungundlovu District, KwaZulu-Natal.

1.4 Objectives

- To isolate *Enterococcus* spp. along the farm to food-production chain continuum from an intensice poultry farming system in the uMgungundlovu district, KwaZulu-Natal.
- To determine the antibiotic resistance profiles of *Enterococcus* spp. isolated from an intensive poultry farming system.
- To identify the genes responsible for resistance.
- To identify virulence genes harboured by the isolated enterococci.

- To determine clonal similarities using repetitive element palindromic PCR (REP-PCR).

1.5 Study outline

This study aimed to investigate the molecular epidemiology of antibiotic-resistant *Enterococcus* spp. from farm to food-production chain, in an intensive poultry production farm in the uMgungundlovu District, KwaZulu-Natal. The research is presented in three chapters as follows:

Chapter 1 provides the background, literature review, rationale for the study as well as the aims and objectives.

Chapter 2 provides information about the investigations undertaken and the findings presented in the form of a manuscript prepared for Science of the Total Environment

Chapter 3 presents the conclusions, limitations and recommendations for this study.

1.6 Summary of methodology

Ethical considerations

Ethical approval was obtained from the Animal Research Ethics Committee (Reference: AREC 073/016PD) and the Biomedical Research Ethics Committee (Reference: BCA444/16) of the University of KwaZulu-Natal (Appendices 1 and 2). The study was further placed on record with the South African National Department of Agriculture, Forestry and Fisheries (Reference: 12/11/1/5 (879)). Human samples were obtained from participants 18 years or older upon explicit, voluntary, verbal informed consent as per the participant information leaflet (Appendix 8). All information disclosed by the farm was treated as confidential as per the memorandum of understanding (MOU) between the Antimicrobial Research Unit (ARU) and the farm.

General methodology

This study describes the molecular epidemiology of antibiotic-resistant *Enterococcus* spp. from farm to food-production chain in intensive poultry production in KwaZulu-Natal, South Africa. Samples along the poultry farm to food-production chain continuum

(litter and faeces, transport, holding, abattoir and retail meat) were evaluated for the presence of *Enterococcus* spp. Molecular confirmation by PCR, targetting the genus- (*tuf*) and species-specific (*sodA*) genes was undertaken. Susceptibility profiles were assessed by Kirby-Bauer disk diffusion using the WHO-AGISAR recommended panel of antibiotics for *Enterococcus* spp. Antibiotic resistance and virulence genes were detected using real-time PCR. Genetic relatedness between isolates across the continuum was evaluated by REP-PCR.

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CHAPTER TWO

This dissertation is in a manuscript format (as is the requirement of the College of Health Sciences at the University of KwaZulu-Natal) as follows

Chantal Molechan, Daniel G. Amoako, Akebe Luther King Abia, Linda A. Bester, Sabiha Y. Essack; Molecular epidemiology of antibiotic-resistant *Enterococcus spp.* from farm to food-production chain in intensive poultry production in KwaZulu-Natal, South Africa. To be submitted to Science of the Total Environment.

Contributions

- Ms Chantal Molechan, as the investigator, worked on the study design with the assistance of supervisors, developed the protocols for the study, executed the laboratory work and wrote the manuscript.
- Mr Daniel Gyamfi Amoako and Dr. Akebe Luther King Abia assisted with laboratory protocols, facilitated data acquisition and analysis of the study
- Dr. Linda Bester, as co-supervisor, designed the study, facilitated data acquisition, laboratory work and data analysis, and contributed to the writing and critical revision of the manuscript.
- Sabiha Y. Essack, as the principal supervisor, co-conceptualized the study, guided the literature review and ethical clearance application, facilitated data collection and analysis and undertook a critical revision of the manuscript.

Molecular epidemiology of antibiotic-resistant *Enterococcus spp.* from farm to food-production chain in intensive poultry production in KwaZulu-Natal, South Africa

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Running title: Molecular epidemiology of antibiotic-resistant *Enterococcus* spp. from farm to food-production chain in poultry.

Abstract

Background: The poultry industry is among the main suppliers of meat protein worldwide. Extensive antibiotic use in intensively-farmed poultry exerts selection pressure for the emergence of multidrug-resistant pathogens. The aim of this study was to determine the antibiotic resistance and virulence profiles of *Enterococcus* spp. along the farm to food-production chain continuum in an intensive poultry system in the uMgungundlovu District in Kwazulu-Natal, South Africa. **Methods:** A total of 187 samples along the poultry farm to food-production chain continuum (litter, faeces, transport, holding, abattoir and retail meat) were evaluated for the presence of *Enterococcus* spp. using selective media, biochemical tests, API 20 Strep kits followed by molecular confirmation by PCR, targetting the genus- (*tuf*) and species-specific (*sodA*) genes. Resistance profiles were assessed by the Kirby-Bauer disk diffusion method against the WHO-AGISAR recommended panel of antibiotics for *Enterococcus* spp. using CLSI guidelines. Real-time PCR was used to detect antibiotic resistance and virulence genes. Clonal similarities and differences between isolates across the continuum were evaluated by REP-PCR. **Results:** One hundred and thirty-four (134) isolates were recovered across the continuum with a prevalence of 72%. Molecular screening confirmed 36% of the isolates as *E. faecalis*, 31% as *E. faecium*, 2% as *E. gallinarum* and 31% as other *Enterococcus* spp. Resistance to tetracycline (80%), erythromycin (71%), nitrofurantoin (17%), ampicillin (15%), streptomycin (15%), chloramphenicol (11%), ciprofloxacin (5%), tigecycline (4%), gentamicin (4%), teicoplanin (3%) was observed among *Enterococcus* spp., but no vancomycin resistance (0%). *E. faecium* displayed 24% resistance, and 21% were of intermediate susceptibility to quinupristin-dalfopristin. Twenty-one (21%) of *E. faecalis* and 100% of *E. gallinarum*, showed intermediate susceptibility to vancomycin. Forty-three percent of *E. faecium* were multidrug-resistant (MDR) (resistant to one or more antibiotics in three or more antibiotic classes). The most frequently observed antibiotic resistance genes, associated with phenotypic resistance, were *tetM* (76%) and *ermB* (67%) while a small percentage was noted for *aph(3')-IIIa* (12%) and *vanC1* (1%). Virulence genes *efaAFs* (100%), *cpd* (96%) and *gelE* (81%) were more frequently detected in *E. faecalis*. Cell wall adhesin (*efaAFm*) was more common in *E. faecium* (100%) and other *Enterococcus* spp. (71%). Clonality evaluated by REP-PCR revealed that isolates along the continuum are highly diverse with major REP-types often consisting of isolates from the same sampling point. **Conclusion:** This study highlights MDR *Enterococcus* in the poultry food production chain with isolates harbouring both resistance and virulence genes which can serve as a reservoir for

the potential transfer of these genes from poultry to humans through the farm to food-production chain continuum. The findings underscore the need for routine antibiotic resistance surveillance in food animals.

Keywords: Antibiotic-resistance; *Enterococcus* spp.; multidrug resistance; farm to food-production chain; intensive poultry farming.

1. Introduction

Antibiotic resistance (ABR) is a global concern resulting in increased mortality rates and healthcare costs. While microorganisms are experts at adaptation, there is little doubt that human activity has influenced their evolution (Sun et al., 2012). Agricultural antibiotic consumption is a significant driving force for ABR. It is estimated that approximately 148 mg/kg of antibiotics are used for farmed chickens globally (Van Boeckel et al., 2015). In South Africa, the poultry industry is among the leading suppliers of meat protein, with chicken being in high demand across all income groups (Greenberg et al., 2017; DAFF, 2017). Intensively-farmed poultry increases the risk of dissemination of respiratory and intestinal bacteria (Greenberg et al., 2017) and antibiotics are thus generally used for prophylactic or metaphylactic treatment to minimise the risk of such outbreaks amongst these closely housed animals (Van Boeckel et al., 2015; Venter et al., 2017a). Antibiotics are administered to improve poultry health, growth performance and minimise the risk of enteric disease. However, the use of antibiotics as growth promoters has come under scrutiny with concern for the emergence of multidrug-resistant bacteria (MDR) (M'Sadeq et al., 2015) as antibiotic growth promoters (AGPs) are often used at sub-therapeutic concentrations within feed or water supplies for prolonged durations (Wielinga et al., 2014). The major issue of antibiotic use in poultry is not the residual drugs in the meat, but rather the potential of these antibiotics being a driving force in the emergence of MDR pathogens. AGPs have the potential to alter the microbiome of food-animals and contribute to the enhanced transfer of ABR within the animal and environmental microbiome (You & Silbergeld, 2014).

Enterococcus spp. often serve as indicator bacteria for the surveillance of ABR in food-animals since they are naturally occurring in both humans and animals (WHO, 2017b). Additionally, these microorganisms can be used for monitoring ABR to antibiotics active against Gram-positive bacteria (WHO, 2017b). While generally considered commensals, *Enterococcus* spp. have emerged among the leading causes of nosocomial infections (Sievert et al., 2013). The World Health Organization (WHO) has recently listed vancomycin-resistant enterococci (VRE) among the crucial resistant bacteria in the "global priority list of antibiotic-resistant bacteria for the research and development of new antibiotics" (WHO, 2017a). Among the genus, *Enterococcus faecium* and *Enterococcus faecalis* are the most prevalent nosocomial pathogens responsible for approximately 10-15% and 80-90% of infections, respectively

(Giraffa, 2014). *Enterococcus gallinarum* and *Enterococcus casseliflavus* are responsible for a minority of clinical infections (Monticelli et al., 2018).

Enterococci have intrinsic and acquired resistance to an array of antibiotics. Invasive infections caused by these microorganisms are often treated with a combination of a β-lactam antibiotic and an aminoglycoside; alternatively, glycopeptides such as teicoplanin or vancomycin are used (Hammerum et al., 2010). Although vancomycin is among the last resort treatment options against Gram-positive pathogens, there has been an increase in VRE globally (Santajit & Indrawattana, 2016; Remschmidt et al., 2018). This is a major call for concern as VRE may serve as a reservoir of ABR determinants for more pathogenic Gram-positive microorganisms such as vancomycin-resistant *Staphylococcus aureus* (VRSA) (Chang et al., 2003).

The use of critically important, clinically relevant antibiotics in food animals has the potential to create a selective environment for MDR pathogenic strains. Additionally, despite rare zoonotic transmission of disease, it is important to note that resistant enterococcal strains may serve as a reservoir of resistance genes for more pathogenic bacteria in the gut (Bortolaia et al., 2016). Enterococci may contaminate poultry meat at the slaughter (abattoir) and post-slaughter level (retail meat) through handling involving cross-contamination with poultry faecal matter (Bortolaia et al., 2016). While zoonotic transmission of ABR enterococci mainly affects farm workers, veterinarians or slaughterhouse workers, the population as a whole can be affected through contact or consumption of contaminated meat (Marshall & Levy, 2011).

The success of pathogenic enterococci is not solely due to their ABR profiles, but also in conjunction with virulence determinants. *Enterococcus* spp. may host an array of virulence factors including aggregation substance (*asa*1), cytolysin (*cyl*A), gelatinase (*gel*E), enterococcal surface proteins (*esp*), cell wall adhesins (*efaA*), among many others (Vidana et al., 2016; Eaton & Gasson, 2001). There is currently limited data on the molecular epidemiology of enterococci in poultry in South Africa. Furthermore, no studies have been conducted to investigate this along the farm to food-production chain continuum. There is, therefore, a need to ascertain the antibiotic resistance of *Enterococcus* spp. in poultry with the goal to encourage implementation of measures for its containment. This study delineated the molecular epidemiology of antibiotic-resistant patterns *Enterococcus* spp., from farm to food-production chain, in chickens from an intensive farming system in uMgungundlovu District, KwaZulu-Natal.

2. Methodology

2.1 Ethical clearance

This project forms part of a broader study for which ethical approval had been obtained from the Animal Research Ethics Committee (Reference: AREC 073/016PD) and the Biomedical Research Ethics Committee (Reference: BCA444/16) of the University of KwaZulu-Natal. The study was further placed on record with the South African National Department of Agriculture, Forestry and Fisheries (Reference: 12/11/1/5 (879)).

2.2 Study population and sampling

This study was conducted over seven weeks, between August and September 2017 at an intensive poultry production system in the uMgungundlovu District of KwaZulu-Natal, South Africa, using the farm to food-production chain approach as recommended by the WHO-AGISAR (WHO, 2017b) as follows:

Growth period: Litter and faecal samples were collected from Cobb breed chickens weekly over five weeks (*n*=100). Block sampling was used to ensure representation of the entire flock within the poultry house where total litter (*n*=10) and total faecal (*n*=10) samples were pooled together weekly. Faeces and litter from the pooled samples (1 g each) were inoculated separately into 40 ml of tryptone soya broth (TSB) (Oxoid, Hampshire, England) and incubated at 37 °C for 2 h with shaking at 100 rpm. Following incubation, 1 ml of each culture was inoculated into 9 ml of TSB supplemented with 6.5% NaCl and incubated at 37 °C for 24 h with shaking at 100 rpm. During Week 2, human hand swabs and nasal swabs of farm employees (*n*=8, respectively) were pooled and processed as described. Human samples were obtained from participants 18 years or older upon explicit, voluntary, verbal informed consent as per the participant information leaflet (Appendix 8)

Transport and holding: Sterile swabs (*n*=10) of holding areas (transport crates) and the truck (*n*= 10) were used to randomly sample truck and crate surfaces that were exposed to the target flock. This was collected during transportation of the target flock to the slaughterhouse. Swab samples were pooled into 40 ml of TSB and processed as above.

Slaughter: Upon the sacrifice of the flock at the abattoir, 40 ml of carcass rinsate was collected in a sterile tube as water streamed down several carcasses, 4 ml was inoculated into 36 ml of TSB and processed as above.

Post-slaughter: Caeca from 10 chickens were removed, and 1 g of caeca from each chicken was inoculated into 5 ml TSB, homogenised and pooled into a sterile tube to a final volume of 50 ml. One millilitre of this homogenate was inoculated into 9 ml of TSB and processed as above.

Retail meat: Neck, thigh and whole chicken carcass portions (n=10, respectively) were each rinsed in 10 ml of 0.9% sterile saline solution, which were then pooled together to give a final volume of ~ 100 ml for each portion. Four millilitres of each homogenate were inoculated into 36 ml of TSB and processed as above.

House rinsate: Prior to disinfection of the chicken housing, in preparation for introduction of the new flock, one millilitre of the flock house rinsate was collected from random points as the house was hosed down. One millilitre was inoculated into 9 ml of TSB and processed as above.

All samples were transported in a cooler box at 6 °C and processed within 4 hours from the time of collection.

2.3 Isolation and identification of *Enterococcus*

2.3.1 Phenotypic Determination of *Enterococcus*

All 24 h cultures were sub-cultured by spread plating 100 µl onto Bile Esculin Azide agar (Himedia, Mumbai, India). Plates were incubated for 24 h at 37 °C, and brown-grey colonies surrounded by black halos were considered presumptive enterococci. Identified colonies were further streaked onto Bile Aesculin agar (Lab M, Lancashire, UK), and incubated at 37 °C to obtain pure colonies. Presumptive colonies were then streaked onto 5% Sheep Blood agar (Oxoid, Hampshire, England) for characterisation of haemolysis, and Tryptone Soya Agar (TSA) (Oxoid, Hampshire, England) for further phenotypic characterisation. The biochemical characterisation of the isolates included catalase activities using 3% H_2O_2, oxidase strips (Sigma Aldrich, St. Louis, USA) and the Gram string test (Gregersen, 1978). Further confirmatory biochemical characterisation was performed using API 20 Strep kits (Biomerieux SA, Marcy I'Etoile, France). Isolates showing percentage identification >80% by API were further analysed for molecular confirmation. *S. aureus* American Type Culture Collection

(ATCC) 29213 and *E. faecalis* ATCC 29212 were used as controls. Presumptive enterococci were stored in 10% glycerol stock solution at -80 °C until further processing.

2.3.2 Molecular confirmation of isolates

Stock cultures were resuscitated on TSA plates incubated at 37 °C for 24 h. DNA was extracted using the heat lysis method as previously described (Englen & Kelley, 2000). A multiplex polymerase chain reaction (PCR) was performed to confirm isolates at the genus and species level. Genus-specific and species-specific primer used in all the reactions were as previously described (Jackson et al., 2004; Ke et al., 1999) (**Table 1**). Two PCR reaction mixtures, both containing the *Enterococcus* genus-specific primers, were set up for different primer sets as follows: group 1: *E. faecalis* and *E. faecium*; group 2: *E. gallinarum*; group 3: *E. casseliflavus*. Each reaction was performed in a total volume of 15 µl consisting of 8 µl of DreamTaq Green PCR Master Mix (2X) (Thermo Scientific, Lithuania), 0.5 µl of each primer pair (final concentration of 10 µM of each primer, except for *E. faecium* and *E. casseliflavus* where 16 µM of the species primer was used), 2.5 µl of template DNA and 1.5 µl of nuclease-free water. The following thermal cycling conditions used: initial denaturation at 95 °C for 4 min, 30 cycles of denaturation at 95 °C for 30 s, amplification at 46.1 °C for 1 min, elongation at 72 °C for 1 min and a final extension at 72 °C for 7 min. All reactions were carried out in a T100 ™ thermal cycler (Bio-rad, South Africa). All reactions included a positive control (**Table 1**) and a "no template control (NTC)". The PCR products were electrophoresed at 90 V on a 1.8% gel run in Tris-borate-EDTA (0.5X) containing 0.5 µg/ml ethidium bromide and visualized using the Gel Doc™ XR+ imaging system (Bio-rad, South Africa).

Table 1: List of genus- and species-specific primers and control strains used in this study

Control Strain	Primer	Primer sequence 5'-3'	Product size (bp)	Reference
***E. faecalis* ATCC 51299**	ENT1	TACTGACAAACCATTCATGATG	112	(Ke et al., 1999)
	ENT2	AACTTCGTCACCAACGCGAAC		
***E. faecalis* ATCC 51299**	FA1	ACTTATGTGACTAACTTAACC	360	(Jackson et al., 2004)
	FA2	TAATGGTGAATCTTGGTTTGG		
***E. faecium* ATCC 35667**	FM1	GAAAAAACAATAGAAGAATTAT	215	
	FM2	TGCTTTTTTGAATTCTTCTTTA		
***E. gallinarum* Field strain (NHLS)**	GA1	TTACTTGCTGATTTTGATTCG	173	
	GA2	TGAATTCTTCTTTGAAATCAG		
***E. casseliflavus* ATCC 700327**	CA1	TCCTGAATTAGGTGAAAAAAC	288	
	CA2	GCTAGTTTACCGTCTTTAACG		

*Field strains were provided by the National Health Laboratory Services (NHLS), South Africa

2.4 Antimicrobial susceptibility testing

Susceptibility to different antibiotics was determined using the Kirby Bauer disk diffusion method, on Mueller Hinton agar (Oxoid, Basingstoke), according to the Clinical and Laboratory Standards Institute (CLSI, 2017) recommendations. The European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2017) guidelines were used for those antibiotic breakpoints absent from the CLSI guidelines. The antibiotic panel selected for screening enterococcal isolates included those stipulated in the World Health Organization Advisory Group on Integrated Surveillance of Antimicrobial Resistance (WHO, 2017b). The following antibiotics were used: ampicillin (10 µg), vancomycin (30 µg), teicoplanin (30 µg), erythromycin (15 µg), tetracycline (30 µg), ciprofloxacin (5 µg), nitrofurantoin (300 µg), chloramphenicol (30 µg), linezolid (30 µg) and tigecycline (16 µg). The susceptibility profile of quinupristin-dalfopristin (15 µg) was only reported for *E. faecium* since it is only clinically relevant for this enterococcal species. All antibiotics were purchased from Oxoid, Basingstoke, United Kingdom. *S. aureus* ATCC 25923 was used as the control strain as recommended by CLSI guidelines. High-level aminoglycoside resistance was determined using gentamicin (120 µg) and streptomycin (300 µg) discs on Mueller-Hinton agar with *E. faecalis* ATCC 29212 as the control strain.

2.5 Detection of antibiotic resistance and virulence genes

Real-Time PCR was used to detect antibiotic resistance and virulence genes using the primers listed in **Table 2** and **Table 3***,* respectively. The reactions were carried out in a total volume of 10 µl made up of 5 µl of Luna® Universal qPCR master mix (New England Biolabs), 0.5 ul of each forward and reverse primer set (final concentration, 0.5 µM each), 3 µl of template DNA and 1 µl of nuclease-free water. The optimised cycling conditions were as follows: a hot-start activation at 98 °C for 50 s followed by 30 cycles of denaturation (95 °C for 50 s), annealing (55 °C for 1 min) and extension (72 °C for 1 min). A final extension was achieved at 72 °C for 5 min. The melt-curve was subsequently achieved by a pre-melt step at 95 °C for 15 s followed by ramping the melting temperature from 60 °C to 95 °C at a ramp rate of 0.15 °C/s. All reactions were carried out in a QuantStudio 5 Real-Time PCR System (ThermoFisher Scientific, South Africa). Each PCR assay included a positive and a No Template Control (NTC). The positive control strains for each gene are listed in **Table 2** and **Table 3**. The NTC was made up of the PCR mix with template DNA replaced by nuclease-free water.

Table 2: List of antibiotic resistance genes primers.

Gene	Description	Positive control Strains	Primer sequence (5'-3')	Reference
tetK	Tetracycline resistance	*S. aureus* clinical strain *	F- TTAGGTGAAGGGTTAGGTCC R- GCAAACTCATTCCAGAAGCA	(Aarestrup et al., 2000a)
tetM		*S. aureus* clinical strain*	F- GTTAAATAGTGTTCTTGGAG R- CTAAGATATGGCTCTAACAA	
ermB	Erythromycin resistance	*E. faecalis* ATCC 51299	F- CTATCTGATTGTTGAAGAAGGATT R- GTTTACTCTTGGTTTAGGATGAAA	(Martineau et al., 2000)
vanA	Vancomycin resistance	*E. faecium* ATCC 700221	F- CATGAATAGAATAAAAGTTGCAATA R- CCCCTTTAACGCTAATACGATCAA	(Kariyama et al., 2000)
vanB		*E. faecalis* ATCC 51299	F- GTGACAAACCGGAGGCGAGGA R- CCGCCATCCTCCTGCAAAAAA	
vanC1			F- GGTATCAAGGAAACCTC R- CTTCCGCCATCATAGCT	
vanC2/3		*E. casseliflavus* ATCC 700327	F- CGGGGAAGATGGCAGTAT R- CGCAGGGACGGTGATTTT	
aac(6')-Ie-aph(2'')-Ia	Gentamicin resistance	*E. faecalis* ATCC 51299	F- CAGGAATTTATCGAAAATGGTAGAAAAG R- CACAATCGACTAAAGAGTACCAATC	(Padmasini et al., 2014)
aph(3')-IIIa	Streptomycin resistance	*E. faecalis* ATCC 51299	F- GGCTAAAATGAGAATATCACCGG R- CTTTAAAAAATCATACAGCTCGCG	

*All clinical strains were provided by the National Health Laboratory Services (NHLS)

Table 3: List of virulence genes primers

Gene	Description	Control Strains	Primer sequence (5'-3')	Reference
gelE	Gelatinase production	*E. faecalis* ATCC 29212	F- ACCCCGTATCATTGGTTT	(Eaton & Gasson, 2001)
			R- ACGCATTGCTTTTCCATC	
cpd	Sex pheromone	*E. faecalis* ATCC 51299	F- TGGTGGGTTATTTTTCAATTC	
			R- TACGGCTCTGGCTTACTA	
cylB	Cytolysin	*E. faecalis* ATCC 29212	F- ATTCCTACCTATGTTCTGTTA	
			R- AATAAACTCTTCTTTTCCAAC	
cylA		*E. faecalis* ATCC 29212	F-TGGATGATAGTGATAGGAAGT	
			R- TCTACAGTAAATCTTTCGTCA	
efaAfs	Cell wall adhesins	*E. faecalis* ATCC 29212	F- GACAGACCCTCACGAATA	
			R- AGTTCATCATGCTGTAGTA	
efaAfm		*E. faecium* ATCC 35667	F- AACAGATCCGCATGAATA	
			R- CATTTCATCATCTGATAGTA	

2.6 Clonality

Repetitive extragenic palindromic-PCR (REP-PCR) was carried out using the $(GTG)_5$ primer as described by Versalovic et al. (1994). DNA extraction was performed using the GeneJET Genomic DNA purification kit (ThermoFisher Scientific) according to the manufacturer's guidelines. PCR was carried out in a total volume of 25 µl consisting of 12.5 µl of DreamTaq Green PCR Master mix (2X) (ThermoFisher Scientific), 1 µl of 5 µM $(GTG)_5$ primer, 10.5 µl nuclease-free water and 1 µl of template DNA. The PCR cycling conditions were carried out as described by Tan et al. (2018). Amplicons were electrophoresed on a 1% agarose gel at 75 V for 3 h in 1 X Tris-acetate-EDTA (TAE) buffer. A Quick-load® 1 kb DNA ladder (New England Biolabs) was used as the molecular weight marker. The gels were stained in 0.5 µg/ml ethidium bromide solution and visualised using the Gel Doc™ XR+ imaging system (Bio-Rad, South Africa). The resultant electrophoretic patterns were analysed using Bionumerics software version 6.6 (Applied Maths NV, Belgium) using Dice coefficient and clustering analysis through unweighted pair group with arithmetic averages (UPGMA) using 1% tolerance and 0.5% optimisation.

3. Results

3.1 Prevalence of *Enterococcus* spp. along the farm to food-production chain continuum

A total of 134 enterococcal isolates were recovered along the farm to food-production chain continuum with 69% confirmed to the species level, and 31% confirmed to the genus level by PCR (**Figure 1**). Molecular screening confirmed 36% of the isolates as *E. faecalis* (*n*=48), 31% as *E. faecium* (*n*=42), 2% as *E. gallinarum* (*n*=2) and 31% as other *Enterococcus* spp. (*n*=42). No *E. casseliflavus* isolates were identified. *E. faecalis* dominated the isolates found in Week 1 and 3, human samples, retail meat and abattoir samples while *E. faecium* was dominant at Week 4, in caecal and transport crate samples. Other *Enterococcus* spp. dominated at Week 5 and within the house after the flock was removed. No *Enterococcus* isolates were recovered from "truck" at the transport sampling point nor "thigh" at the retail product level.

3.2 Antibiotic susceptibility tests

Zone diameters were interpreted and reported as intermediate or resistant using the CLSI breakpoints or otherwise stated (CLSI, 2017). Disk diffusion revealed resistance to tetracycline (80%), erythromycin (71%), nitrofurantoin (17%), ampicillin (15%), streptomycin (15%),

chloramphenicol (11%), ciprofloxacin (5%), tigecycline (4%), gentamicin (4%), teicoplanin (3%) among *Enterococcus* spp., but no vancomycin resistance (0%) (**Table 4**). *E. faecalis* showed the highest rate of resistance to the following antibiotics: erythromycin (90%), tetracycline (88%), streptomycin (27%), chloramphenicol (23%), tigecycline (10%) and gentamicin (6%) as compared to the other species. *E. faecium* and *Enterococcus* spp. dominated the resistance profiles for nitrofurantoin (19% and 31%, respectively). The highest prevalence of intermediate susceptibility across all species investigated was observed for ciprofloxacin (62%). The susceptibility profile of quinupristin-dalfopristin was only reported for *E. faecium* since it is only clinically relevant for this enterococcal species, where 24% of isolates were resistant, and 21% were of intermediate susceptibility. All *E. faecium* isolates were completely susceptible to vancomycin and gentamicin. However, 21% of *E. faecalis* showed intermediate susceptibility to vancomycin. **Figure 2** depicts the resistant profiles of the *Enterococcus* isolates along the farm to food-production chain continuum. The occurrence of isolates resistant to tetracycline was observed throughout all the sample points of the continuum. The highest rates of resistance were noted for tetracycline and erythromycin (>75%) for isolates originating from faeces and litter (Week 1-5), human, abattoir, and retail meat. **Figure 3** illustrates the prevalence of *Enterococcus* isolates showing intermediate susceptibility along the farm to food-production chain continuum. It can be noted that the occurrence of intermediate susceptibility to ciprofloxacin spans across all sample points at prevalences of 100%, 71%, 71%, 67%, 67% and 29% for the house, transport crates, retail meat, caecal samples, human samples and abattoir samples respectively. Collectively, 37% of all enterococci were multidrug-resistant (MDR) (resistant to one or more antibiotics in three or more antibiotic classes). Separated by species, 43% percent of all *E. faecium* (*n*=18), 37% of all *E. faecalis* (*n*=17) and 33% of all *Enterococcus* spp. (*n*=14) were MDR. A total of 25 antibiograms were observed (**Table 5**). *E. faecalis* and *E. faecium* showed 11 antibiograms, each. The other *Enterococcus* spp. showed seven antibiograms while *E. gallinarum* did not display any MDR phenotype.

3.3 Detection of antibiotic resistance genes

The prevalence of antibiotic resistance genes in the enterococcal isolates was as follows: *tetM* (76%), *ermB* (67%), *aph(3')-IIIa* (12%) and *vanC1* (1%) (**Table 6**). Most isolates that were resistant or showed intermediate susceptibility to erythromycin harboured the *ermB* gene. A total of 81% and 52% of *E. faecalis* and *E. faecium* contained the *ermB* gene, respectively. This

gene was present in 69% of *Enterococcus* spp. Phenotypic tetracycline resistance appeared to be closely related with the presence of *tetM*. No *tetK* gene was detected in isolates showing phenotypic tetracycline resistance. The *tetM* gene was more prominent in *E. faecium* (86%), closely followed by *E. faecalis* (79%) and *Enterococcus* spp. (62%). All *E. gallinarum* isolates tested positive for the presence of a vancomycin resistance gene in accordance with the phenotypic profile. However, this was expected since the species is known for its inherent resistance via the *vanC1* gene. None of the *van* genes tested was observed in *E. faecalis* or *Enterococcus* spp. although selected isolates showed intermediate susceptibility to vancomycin. The *aph(3')-IIIa* gene was more commonly detected in *E. faecalis* (23%) as compared to *E. faecium* (7%) and *Enterococcus* spp. (5%) which corresponds to the high-level streptomycin resistance detected in the corresponding isolates. The *aac(6')-Ie-aph(2'')-Ia* gene, known for conferring gentamicin resistance, was not detected in any of the 134 enterococcal isolates.

3.4 Detection of virulence genes

The overall prevalence of virulence factors detected among the isolates were as follows: *efaAFm* (54%), *efaAFs* (37%), *cpd* (37%), *gelE* (33%), *cylA* (3%) and *cylB* (3%) (**Table 7**). *E. faecalis* showed the highest frequency of virulence genes compared to any other species with the following distributions: 100% *efaAFs* (*n*=48), 96% *cpd* (*n*=46), 81% *gelE* (*n*=39), 6% *cylA* (*n*=3) and 6% *cylB* (*n*=3). *E. faecium* showed 100% frequency for the presence of *efaAFm* (*n*=42) while no *cylA* or cylB was detected. The most prevalent virulence factor for *Enterococcus* spp. was the cell wall adhesion gene *efaAFm* at 71% (*n*=30). No virulence factors were detected for *E. gallinarum.*

3.5 Repetitive Element Palindromic PCR (REP-PCR)

The DNA fingerprints in the dendrograms generated by REP-PCR helped distinguish evolutionary relationships between all *E. faecalis* (*n*=48) and *E. faecium* (*n*=42) isolates along the farm to food-production chain continuum (**Figure 4** and **Figure 5** respectively). Isolates were grouped based on a similarity of ≥ 70%, in relation to selected phenotypic resistance and corresponding genetic determinants as tested in this study. Major REP-types were further defined for those isolates showing a similarity of ≥ 90%. *E. faecalis* was grouped into 20 REP-types designated A-T. It was observed that 22% (11/48) of *E. faecalis* isolates were grouped into 4 major REP-types: F (*n*=2), J (*n*=2), L (*n*=4) and T (*n*=3). Of note, isolates from different

sampling points did not fall into the same major REP-types except for major cluster L consisting of isolates originating from Week 2 hand and Week 4 faeces samples. *E. faecium* was grouped into 17 REP-types designated A-Q. A total of 29% (*n*=12) *E. faecium* fell into 4 major REP-types: F (*n*=2), G (*n*=2), J (*n*=2) and N (*n*=6). The largest clonal cluster showing a similarity index of >90% was N4 consisting of 4 isolates originating from caecal samples. Furthermore, this major clonal cluster had >70% similarity index relationship with Week 3 isolates originating from litter samples (REP-type N and N1). REP-type F consisted of isolates from Week 4 litter and Week 5 faeces (F and F1, respectively) while REP-type K consisted of isolates originating from Week 5 litter and house (K and K1), respectively. This was contrary to *E. faecalis* REP-types that were less diverse in the source of isolates categorised in the REP-types.

4. Discussion

Given the importance of the poultry industry regarding the supply of meat protein globally, understanding the microbial quality of poultry using a farm to food-production chain approach could give a broad picture of the health risk that may be associated with this valuable protein source. In the current study, *Enterococcus* spp. were isolated at different sampling points within the farm to food-production chain continuum. A substantial percentage of these isolates were multidrug resistant (37%), and up to 54% of isolates carried one or more virulence factors that could potentially enhance their pathogenic potential in humans, under appropriate conditions.

Although there was a fluctuation of species-dominance across the sampling continuum, this study revealed an overall predominance of *E. faecalis* strains (36%) followed by *E. faecium* (31%), and undifferentiated species of *Enterococcus* (31%). A very small proportion of *E. gallinarum* (2%) was detected. These findings are consistent with other poultry studies conducted in different countries that reported a higher incidence of *E. faecalis* in poultry meat and its associated environment (Furtula et al., 2013; Hidano et al., 2015; Hasan et al., 2018; Tyson et al., 2018). Although a limited number of studies within Africa have reported on the prevalence of *Enterococcus* spp. in poultry, most of the studies within the continent suggest that the incidence of *Enterococcus* in poultry is dominated by *E. faecium* strains (Bekele & Ashenafi, 2010; Ngbede et al., 2017). On the contrary, within South Africa where there is a particular paucity of related information, one study reported that *E. faecalis* was the most dominant species recovered from poultry cloacal samples which further corresponds to the

findings of the current study (Pillay et al., 2018). Such scarcity of information makes it difficult to draw sound conclusions, and this underscores the importance of including *Enterococcus* spp. in surveillance programs. Since *E. faecalis* is known to require extensive vitamins and amino acids for optimal growth (Lebreton et al., 2014), the absence of this species at Week 5 could be attributed to alterations of the feed/feed-additives. Furthermore, it is suggested that gut-colonisation of chickens is age-dependent where *E. faecalis* dominates early colonisation but the introduction of AGPs, such as tylosin, allows *E. faecium* to thrive (Kaukas et al., 1987; Lebreton et al., 2014). Finally, at the mature stage of chicken growth, other species such as *E. cecorum* may displace both *E. faecalis* and *E. faecium* (Lebreton et al., 2014). These observations could explain the predominance of undifferentiated *Enterococcus* spp. (13%) followed by *E. faecium* (4%) noted at Week 5 in the current study.

Due to potentially undesirable outcomes such as the antibiotic resistance associated with the use of antibiotics in food animals, the European Union has banned the use of most of these chemotherapeutic agents as growth promoters in the food-animal industry (Eagar et al., 2012). Regrettably, many of these antibiotics are still approved for use in South and are listed under the Stock Remedies Act No. 36 of 1947, indicating that the use of antimicrobials in South Africa is not as prudent (Eagar et al., 2012). Enterococcal isolates in this study displayed the highest prevalences of resistance (≥50%) to tetracycline (80%) and erythromycin (71%). High levels of tetracycline and erythromycin resistance may be attributed to extensive use of tetracycline and macrolide analogues for food animals which can create a selective environment for subsequent resistance (Eagar et al., 2012). The highest prevalence of intermediate susceptibility, across all species investigated, was observed for ciprofloxacin (62%). Ciprofloxacin resistance can be related to the application of analogues such as enrofloxacin administered for treatment of poultry (Eagar et al., 2012). Compared to *E. faecalis*, *E. faecium* is intrinsically more drug-resistant and therefore more difficult to treat (Higuita & Huycke, 2014). The findings of the present study revealed that all *E. faecium* isolates were susceptible to the clinically relevant antibiotics, vancomycin and gentamicin. However, it is concerning that the highest frequency of MDR phenotypes occurred for *E. faecium* (43%), displaying diverse antibiograms. These observations highlight the need to include a broader range of antibiotics, other than vancomycin, for surveillance programs. Consumption of poorly cooked or raw poultry contaminated with such MDR bacteria could result to severe public health consequences.

Additionally, 24% of *E. faecium* isolates were resistant, and 21% were of intermediate susceptibility to quinupristin-dalfopristin which is concerning considering that this is among the last resort antibiotics for the treatment of vancomycin-resistant *E. faecium* (Rossolini et al., 2014). While resistance to quinupristin-dalfopristin in poultry settings is assumed to be linked to agricultural use of virginiamycin, it is possible that cross-resistance may occur due to genetic linkage to genes conferring resistance to other drug classes such as macrolides (Bortolaia et al., 2016). It is known that *ermB* can induce cross-resistance to streptogramin B (Isnard et al., 2013), which could explain the intermediate susceptibility noted for quinupristin-dalfopristin. *E. faecalis* showed 21% intermediate susceptibility to vancomycin. Glycopeptides and their analogues are generally not included for veterinary use in South Africa (Eagar et al., 2012), suggesting that low-level cross-resistance resulting from other drug classes may be possible. There appeared to be some variations between resistance profiles and the presence of corresponding resistance genes. Majority of the *E. faecalis* (81%) isolates harboured the *ermB* gene while only 52% of *E. faecium* tested positive. This suggests that other mechanisms of macrolide resistance such as the presence of the *ermA* or *msrC* genes could have been involved as previously reported (Seputiene et al., 2012; Thumu & Halami, 2014). Most isolates that showed resistance to tetracycline were positive for the *tetM* gene. This corresponds with other findings, where among other tetracycline resistance genes such as *tetL* or *tetO*, the *tetM* gene was more frequently recovered from poultry sources (Kim et al., 2018; Hidano et al., 2015). The *tetM* gene is often harboured in the bacterial chromosome, residing within conjugative transposons of the Tn*916* or Tn*1545* family, with *tetM* in Tn*1545* often associated with *ermB* which has implication for cross-resistance (Cauwerts et al., 2007). The *aph(3')-IIIa* gene, although detected at a low level, was present in the majority of isolates showing resistance. Molecular detection did not confirm the presence of any of the tested *van* resistance genes (*vanA*, *vanB*, *vanC1* and *vanC2/3*) in *E. faecalis* while *vanC1* was confirmed in *E. gallinarum* isolates, as would be expected from these intrinsically resistant species (Hollenbeck & Rice, 2012). However, it should be noted that nine gene clusters that confer glycopeptide resistance have been identified in enterococci: *vanA*, *van*B, *vanC*, *vanD*, *vanE*, *vanG*, *vanL*, *vanM* and *vanN* (Raza et al., 2018). Given that not all the genes were tested in the current study, the tested isolates could still carry some of the resistance genes. Therefore, further studies involving a more comprehensive panel of genes or whole genome sequencing should be carried out to better understand the resistance mechanisms of these resistant isolates within the poultry industry.

Several virulence factors have been attributed to the pathogenic potentials of *Enterococcus* spp. The findings of this study show that all *E. faecalis* and *E. faecium* were positive for their respective cell wall adhesin genes, *efaAFs* and *efaAFm*. There was also a high prevalence of gelatinase (*gelE*) and sex pheromone (*cpd*) genes, and to a lower extent, cytolysin (*cylA* and *cylB)* genes detected in *E. faecalis*. Similar results have been reported in poultry-based studies. Choi & Woo (2013) reported 100% prevalence of *gelE* and *efaA* in all high-level gentamicin-resistant *E. faecalis* from poultry in Korea. More recently, Kim et al. (2018) reported the prevalence of *gelE* (95.3%) and *efaA* (80.5%) genes in antibiotic-resistant *E. faecalis* from poultry samples in Korea as did Pillay et al. (2018) reporting a high prevalence (>80%) of *gelE* in enterococci from poultry samples as compared to livestock or companion animals in South Africa. MDR enterococci possessing both virulence factors and an array of antibiotic resistance genes have the potential to be more effective as opportunistic pathogens (Chajęcka-Wierzchowska et al., 2017). Furthermore, it is vital to understand the association between virulence and antibiotic resistance genes with mobile genetic elements in order to predict the risk of dissemination. It has been shown that enterococcal isolates of poultry origin were able to transfer both antibiotic resistance and virulence genes to recipient strains (Kim et al., 2018). This highlights the risk of *Enterococcus* from poultry serving as a reservoir for resistance and virulence genes.

Determination of clonality was carried out using REP-PCR which revealed that the isolates along the farm to food-production chain continuum were very diverse with 20 REP-types (A-T) designated for *E. faecalis* and 17 REP-types (A-Q) for *E. faecium*. Regarding *E. faecalis*, during Week 1-2, the clones did not appear to establish themselves into major clusters across the continuum, except for a single isolate from Week 2 human hand and Week 4 faecal sample. However, during Week 3 and 4, isolates originating from faeces samples formed a definitive REP-type cluster (T-T5) with similar phenotypic profiles. It is interesting to note that REP-type O consisted of isolates from Week 2 human isolates that were >70% genetically related to those originating from the abattoir. Similar findings have been reported by Tan et al. (2018) where enterococcal clones of human (farmer) and swine origins showed high similarity by pulse-field gel electrophoresis. This may suggest that human handling of poultry at the slaughterhouse may contribute to enterococcal contamination. More isolates fell within the major REP-types (29%) for *E. faecium* with the largest clonal cluster belonging to major REP-type N originating from caecal samples. Furthermore, this major clonal cluster had >70% similarity index relationship with Week 3 isolates originating from litter samples (REP-type N

and N1), indicating possible circulation of these isolates within the flock and along the different stages of the production process.

5. Conclusion

To the best of our knowledge, this is the first study in South Africa that investigated the molecular epidemiology of antibiotic-resistant *Enterococcus* spp. using the farm to food-production chain approach. This study highlights the high prevalence of MDR *Enterococcus* isolates harbouring both resistance and virulence genes in diverse permutations and combinations which can serve as a reservoir for the potential transfer of these genes from poultry to humans through the farm to food-production chain continuum. The findings also underscore the need to include *Enterococcus* spp. in food animal antibiotic resistance surveillance programs. Such information is critical to inform decision making regarding the food safety of poultry, specifically the design and implementation of appropriate measures that could aid in the prevention of potential health risks associated with its consumption, especially in developing countries.

Author contributions: Co-conceptualised the study: All. Performed the laboratory work: CM. Analysed the data: CM, DGA, LB and ALK. Vetting of the results: All. Wrote the paper: CM. Undertook critical revision of the manuscript: All.

Acknowledgment: The research reported in this publication was funded by the *WHO Advisory Group on Integrated Surveillance of Antimicrobial Resistance (AGISAR)* Research Project: "Triangulation of Antibiotic Resistance from Humans, the Food Chain and Associated Environments - A One Health Project" (**Reference ID: 204517**) and the South African Research Chairs Initiative of the Department of Science and Technology and National Research Foundation of South Africa (Grant No. 98342). We express our gratitude to Professor Olarniran Ademola of the School of Life Sciences, University of Kwa-Zulu Natal (UKZN), Durban, South Africa, for granting us access to Bionumerics software version 6.6 (Applied Maths NV, Belgium) to construct dendrograms. **Disclaimer:** Any opinion, finding and conclusion or recommendation expressed in this material is that of the author(s) and the NRF does not accept any liability in this regard.

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Transparency Declaration: Professor Sabiha Essack is the chairperson of the Global Respiratory Infection Partnership sponsored by an unconditional educational grant from Reckitt and Benckiser, UK

Table 4: Susceptibility profile of all isolates recovered from the farm to food-production chain continuum

	Susceptibility profile													
	E. faecalis (*n*=48)			*E. faecium* (*n*=42)			*Enterococcus spp.* (*n*=42)			*E. gallinarum* (*n*=2)			Total R (*n*=134)	Total I (*n*=134)
	I	R	S	I	R	S	I	R	S	I	R	S		
Tetracycline	1 (2%)	42 (88%)	5 (10%)	1 (2%)	34 (81%)	7 (20%)	0	29 (69%)	13 (31%)	0	2 (100%)	0	80%	1%
Erythromycin	4 (8%)	43 (90%)	1 (2%)	16 (38)	24 (57%)	2 (5%)	13 (31%)	28 (67%)	1 (2%)	0	0	2 (100%)	71%	25%
Quinupristin-Dalfopristin	-	-		9 (21%)	10 (24%)	23 (55%)	-	-	-	-	-	-	24%	21%
Nitrofurantoin	2 (4%)	2 (4%)	44 (92%)	10 (24%)	8 (19%)	24 (57%)	10 (24%)	13 (31%)	19 (45%)	0	0	2 (100%)	17%	16%
Ampicillin	-	7 (15%)	41 (85%)	-	5 (12%)	37 (88%)	-	8 (19%)	34 (81%)	-	0	2 (100%)	15%	-
Streptomycin	-	13 (27%)	35 (73%)	-	4 (10%)	38 (90%)	-	3 (7%)	39 (93%)	-	0	2 (100%)	15%	-
Chloramphenicol	19 (40%)	11 (23%)	18 (40%)	12 (29%)	3 (7%)	27 (64%)	4 (10%)	1 (2%)	37 (88%)	0	0	2 (100%)	11%	26%
Ciprofloxacin	31 (65%)	0	17 (35%)	31 (74%)	4 (10%)	7 (17%)	19 (45%)	1 (2%)	22 (52%)	2 (100%)	0	0	5%	62%
Tigecycline	-	5 (10%)	43 (90%)	-	1 (2%)	41 (98%)	0	0	42 (100%)	-	0	2 (100%)	4%	-
Gentamicin	-	3 (6%)	45 (94%)	-	0	42 (100%)	-	2 (5%)	40 (95%)	-	0	2 (100%)	4%	-
Teicoplanin	1 (2%)	2 (4%)	45 (94%)	2 (5%)	0	40 (95%)	0	2 (5%)	40 (95%)	0	0	2 (100%)	3%	2%
Vancomycin	10 (21%)	0	38 (79%)	0	0	42 (100%)	0	0	42 (100%)	2 (100%)	0	0	0%	9%

I= Intermediate susceptibility; R= Resistant; - = no breakpoints available, therefore not tested.

Table 5: Multidrug-resistant profiles of *Enterococcus* isolates.

Antibiogram	***E. faecalis* (*n*=48)**	***E. faecium* (*n*=42)**	***Enterococcus* spp. (*n*=42)**
ERY-CHL-QD	0	1	0
NIT-TET-ERY	0	0	4
NIT-TET-ERY-AMP	0	1	2
NIT-TET-ERY-CIP	0	2	0
NIT-TET-ERY-QD	0	2	0
NIT-TGC-TET-ER	0	1	0
TEC-NIT-ERY-CHL-GEN-STR	1	0	0
TEC-NIT-ERY-CHL-STR	1	0	0
TEC-NIT-TET-ERY	0	0	1
TEC-TET-ERY-GEN-STR	0	0	1
TET-ERY-AMP	2	2	4
TET-ERY-AMP-CHL-GEN-STR	2	0	0
TET-ERY-AMP-QD	0	1	0
TET-ERY-AMP-STR	1	0	0
TET-ERY-CHL	1	0	0
TET-ERY-CHL-STR	5	0	0
TET-ERY-CHL-STR-QD	0	1	0
TET-ERY-CIP	0	2	0
TET-ERY-CIP-AMP-CHL-GEN-STR	0	0	1
TET-ERY-QD	0	2	0
TET-ERY-STR	0	3	1
TGC-TET-ERY	1	0	0
TGC-TET-ERY-AMP	1	0	0
TGC-TET-ERY-AMP-STR	1	0	0
TGC-TET-ERY-CHL-STR	1	0	0
Total (*n*=49; 37%)	17 (35%)	18 (43%)	14 (33%)

Abbreviations: AMP; ampicillin, ERY; erythromycin, CHL; chloramphenicol, CIP; ciprofloxacin, GEN; gentamicin, NIT; nitrofurantoin, Q-D; quinupristin-dalfopristin, STR; streptomycin, TEC: teicoplanin, TET; tetracycline, TGC; tigecycline.

Table 6: Prevalence of ABR genes in *Enterococcus* spp.

Antibiotic resistance gene	***E. faecalis* (*n*= 48)**	***E. faecium* (*n*= 42)**	***E. gallinarum* (*n*= 2)**	***Enterococcus* spp. (*n*= 42)**	**Total (*n*= 134)**
tetM	38 (79%)	36 (86%)	2 (100%)	26 (62%)	76%
ermB	39 (81%)	22 (52%)	0 (0%)	29 (69%)	67%
aph(3')-IIIa	11 (23%)	3 (7%)	0 (0%)	2 (5%)	12%
vanC1	0 (0%)	0 (0%)	2 (100%)	0 (0%)	1%
tetK	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0%
vanA	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0%
van B	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0%
vanC2/3	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0%
aac(6')-Ie-aph(2'')-Ia	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0%

Table 7: Prevalence of virulence factors among *Enterococcus* spp.

Virulence genes	***E. faecalis* (*n*=48)**	***E. faecium* (*n*=42)**	***E. gallinarum* (*n*=2)**	***Enterococcus* spp. (*n*=42)**	**Total No. (*n*=134)**
efaAFm	0 (0%)	42 (100%)	0 (0%)	30 (71%)	72 (54%)
efaAFs	48 (100%)	1 (2%)	0 (0%)	1 (2%)	50 (37%)
cpd	46 (96%)	1 (2%)	0 (0%)	2 (5%)	49 (37%)
gelE	39 (81%)	2 (5%)	0 (0%)	3 (7%)	44 (33%)
cylA	3 (6%)	0 (0%)	0 (0%)	1 (2%)	4 (3%)
cylB	3 (6%)	0 (0%)	0 (0%)	0 (0%)	3 (3%)

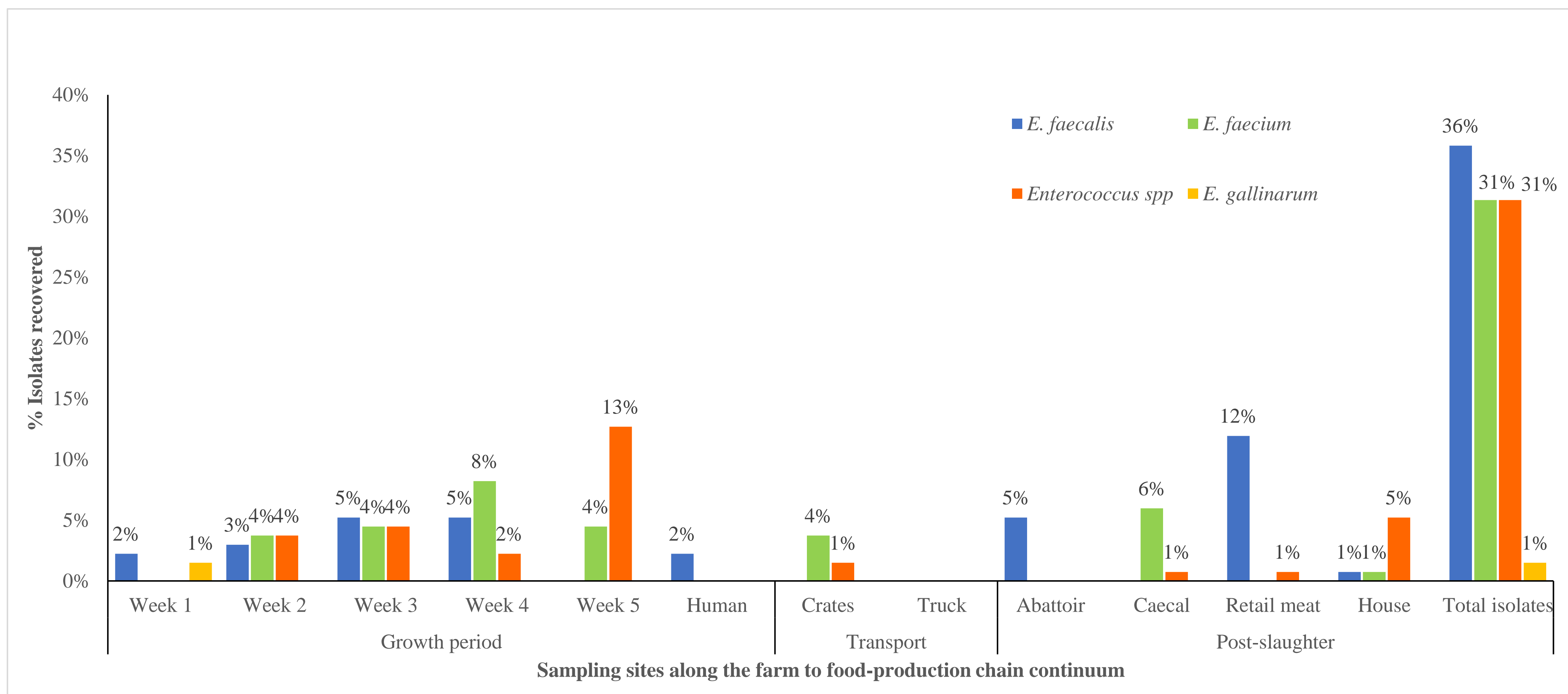

Figure 1: Distribution of *Enterococcus* spp. along the sampling points of the farm to food-production chain continuum.

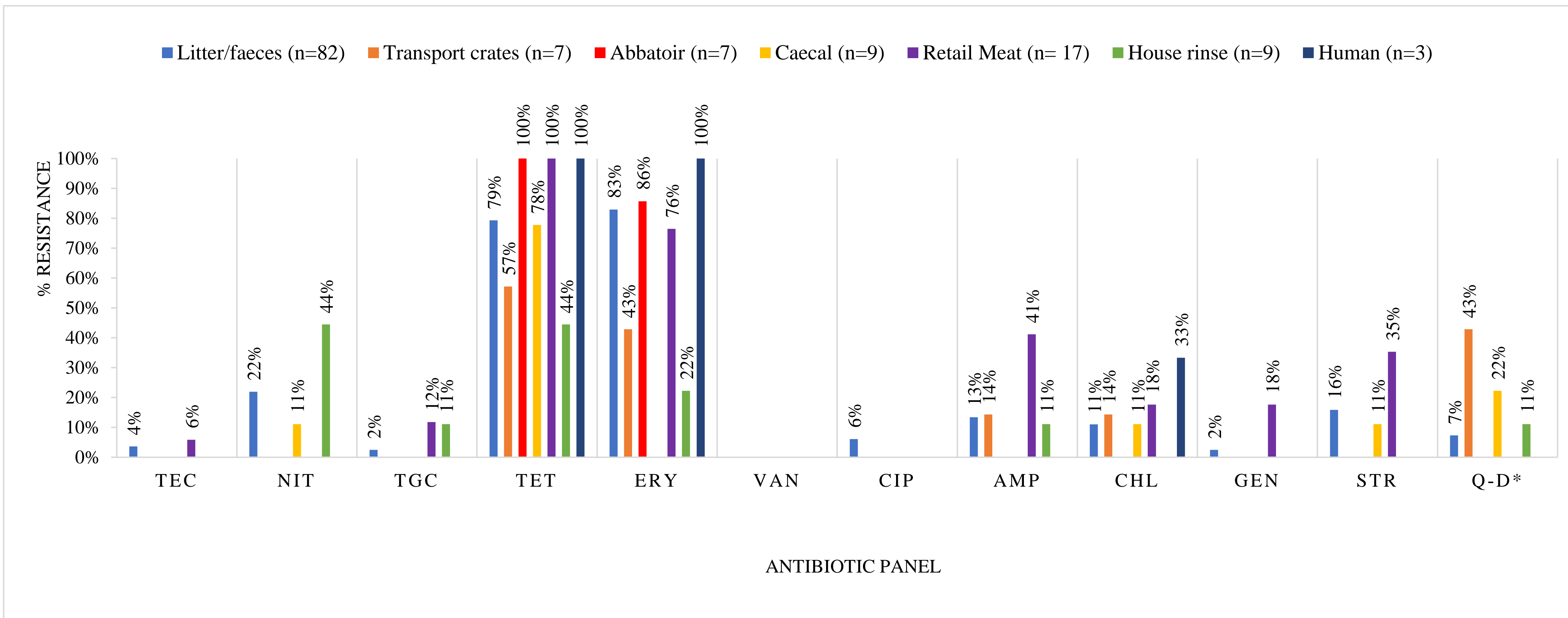

Figure 2: Prevalence of all *Enterococcus* spp. showing resistant profiles along the farm to food-production chain continuum. *Q-D is reported for *E. faecium* isolates only.

Abbreviations: TEC: teicoplanin; NIT: nitrofurantoin; TGC: tigecycline; TET: tetracycline; ERY: erythromycin; VAN: vancomycin; CIP: ciprofloxacin; AMP: ampicillin; CHL: chloramphenicol; GEN: gentamicin; STR: streptomycin; Q-D: quinupristin-dalfopristin

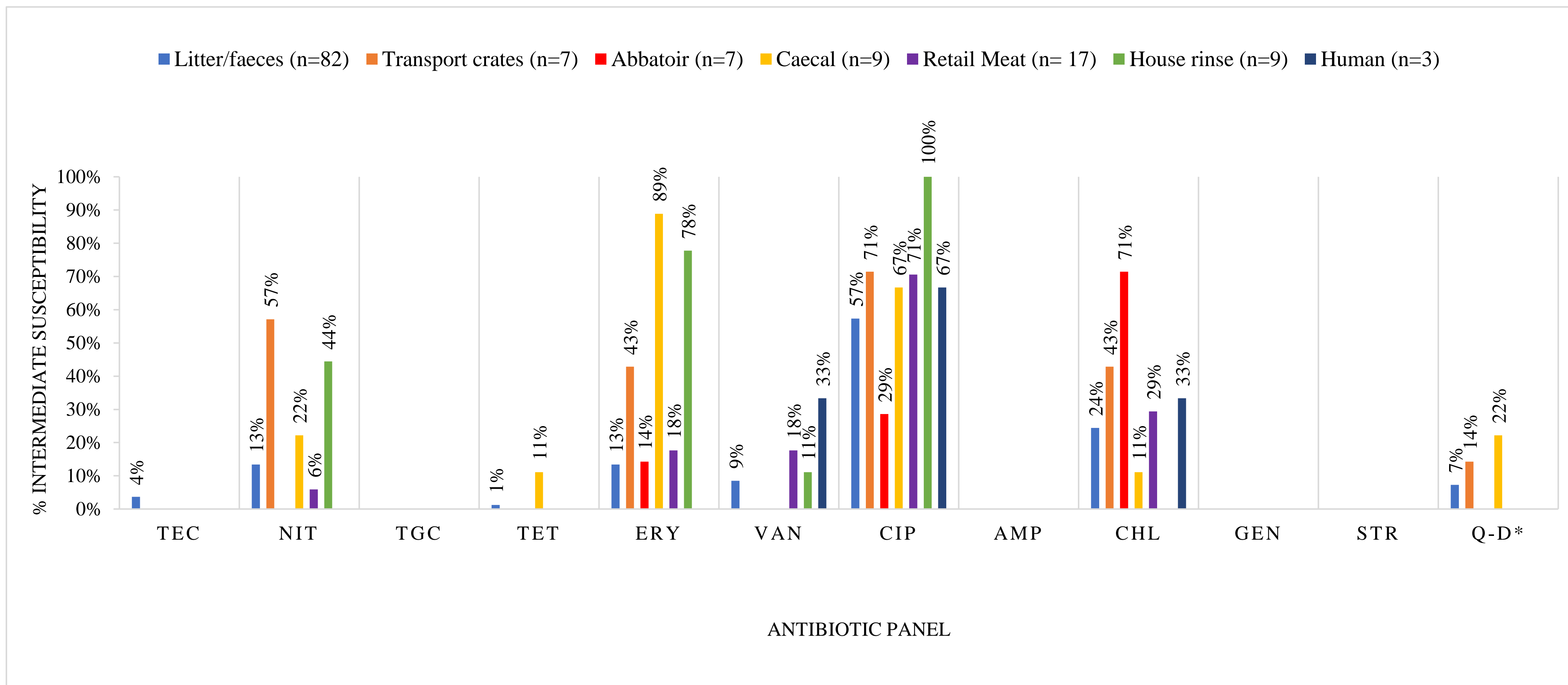

Figure 3: Prevalence of all *Enterococcus* spp. showing intermediate susceptibility along the farm to food-production chain continuum. *Q-D is reported for *E. faecium* isolates only.

Abbreviations: TEC: teicoplanin; NIT: nitrofurantoin; TGC: tigecycline; TET: tetracycline; ERY: erythromycin; VAN: vancomycin; CIP: ciprofloxacin; AMP: ampicillin; CHL: chloramphenicol; GEN: gentamicin; STR: streptomycin; Q-D: quinupristin-dalfopristin

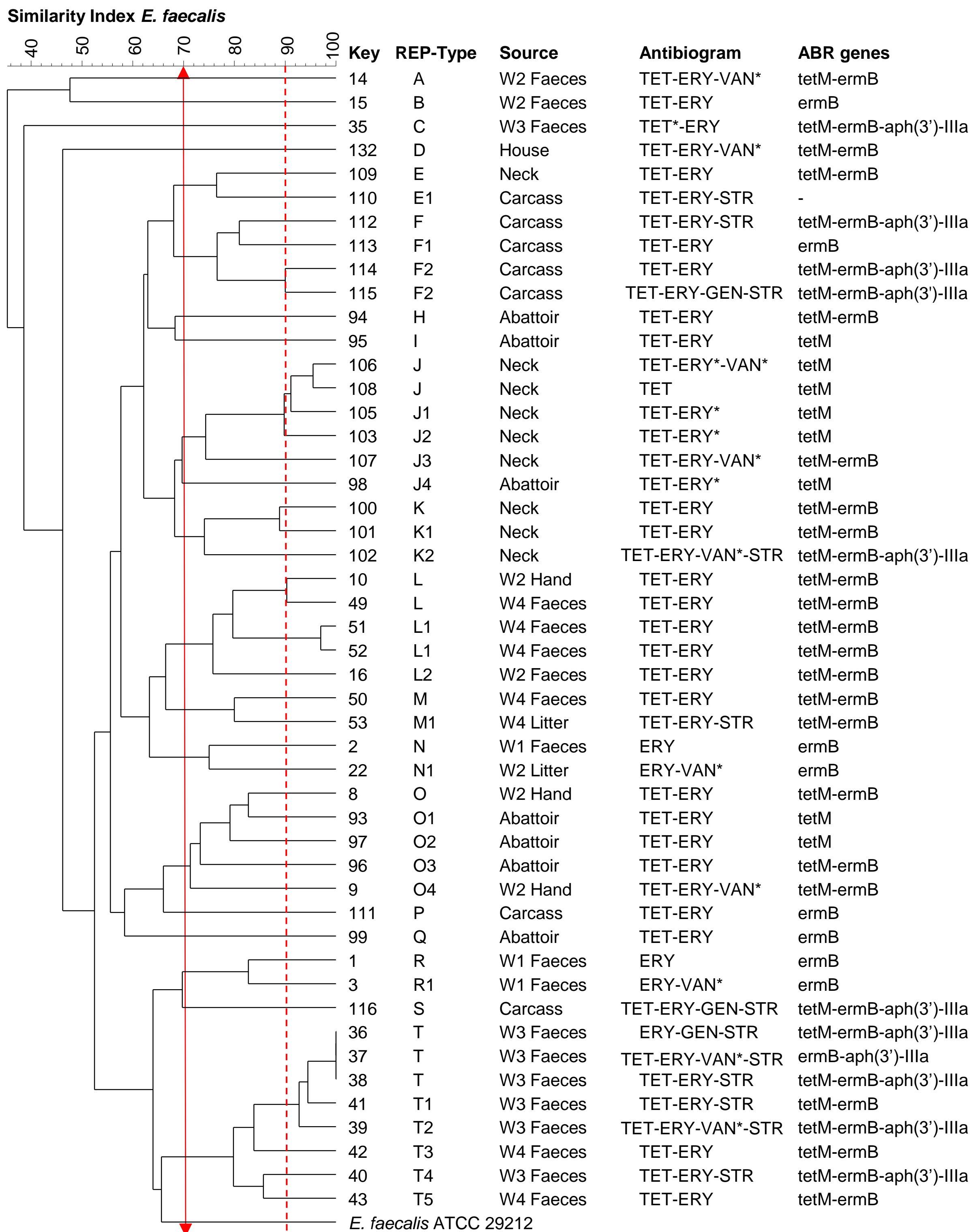

Figure 4: Dendrogram showing REP-type groups of *E. faecalis* isolates, based on the similarity index, recovered along the farm to food-production chain continuum. *E. faecalis* ATCC 29212 was used as the quality control strain. The solid red line indicates the REP-type cut off while the dashed red line indicates the major REP-type cut off. Abbreviations: ERY: erythromycin; TET: tetracycline; VAN: vancomycin: STR: streptomycin. * indicates intermediate susceptibility.

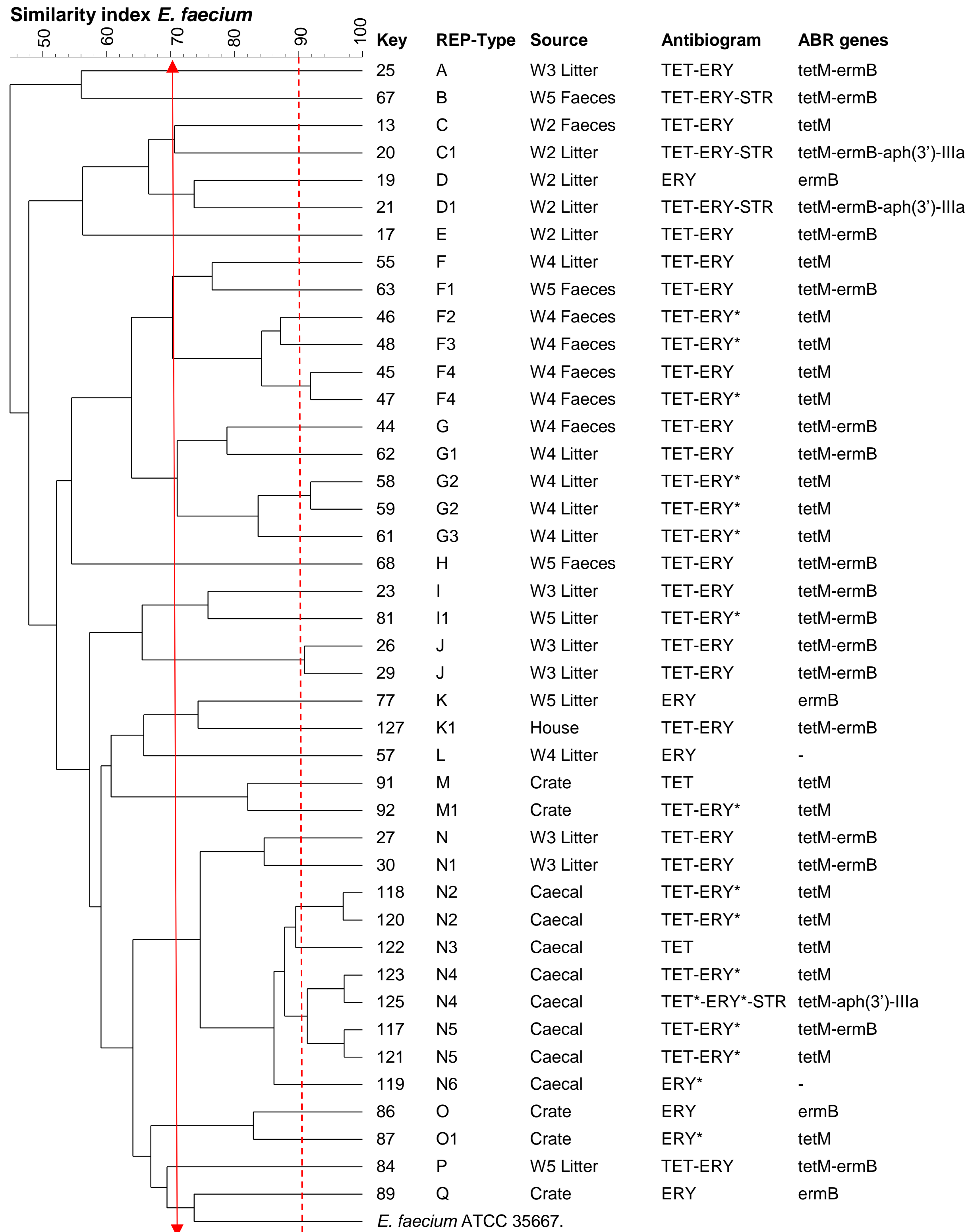

Figure 5: Dendrogram showing REP-type groups of *E. faecium* isolates, based on the similarity index, recovered along the farm to food-production chain continuum. *E. faecium* ATCC 35667 was used as the quality control strain. The solid red line indicates the REP-type cut off while the

dashed red line indicates the major REP-type cut off. Abbreviations: ERY: erythromycin; TET: tetracycline; VAN: vancomycin: STR: streptomycin. * indicates intermediate susceptibility.

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CHAPTER 3

This study describes the antibiotic resistance profiles, antibiotic resistance and virulence genes and assesses the genetic relatedness of 134 *Enterococcus* isolates from an intensive poultry-production farm in the uMgungunglovu in Kwazulu-Natal, South Africa.

3.1 Conclusions

The following conclusions were drawn in reference to the objectives of the study:

- *Enterococcus* isolates were successfully isolated and cultured, from the following farm to food-production chain sampling sites: Week 1-5 litter and faeces, human (hands), transport crates, abattoir, retail meat (neck and whole carcass), caecal and final house rinsate.
- One-hundred and thirty-four isolates were successfully identified using API and PCR.
- The antibiotic susceptibility profile obtained using Kirby-Bauer disk diffusion revealed the following resistance profiles for *Enterococcus* isolates: tetracycline (80%), erythromycin (71%), nitrofurantoin (17%), ampicillin (15%), streptomycin (15%), chloramphenicol (11%), ciprofloxacin (5%), tigecycline (4%), gentamicin (4%), teicoplanin (3%) but no vancomycin resistance (0%). *E. faecalis* showed resistance to the following antibiotics: erythromycin (90%), tetracycline (88%), streptomycin (27%), chloramphenicol (23%), tigecycline (10%) and gentamicin (6%) as compared to the other species. *E. faecium* and *Enterococcus* spp. dominated the resistance profiles for nitrofurantoin (19% and 31%, respectively).
- The highest rate of intermediate susceptibility across all species investigated was observed for ciprofloxacin (62%). *E. faecium* showed 24% resistance to quinupristin-dalfopristin while 21% were intermediate. All *E. faecium* isolates were completely susceptible to vancomycin and gentamicin. However, 21% of *E. faecalis* showed intermediate susceptibility to vancomycin.

- Forty-three percent of *E. faecium* (*n*= 18) were multidrug-resistant (MDR) while 37% percent of *E. faecalis* (*n*= 17) and 33% of *Enterococcus* spp. (*n*= 14) were MDR. A total of 25 MDR antibiograms were observed.
- The overall prevalence of antibiotic resistance genes were as follows: *tetM* (76%), *ermB* (67%), *aph(3')-IIIa* (12%) and *vanC1* (1%). The *tetK*, *vanA*, *vanB*, *vanC2/3* and *aac(6')-Ie-aph(2'')-Ia* genes were not detected
- The overall prevalence of virulence factors detected among the isolates were as follows: *efaAFm* (54%), *efaAFs* (37%), *cpd* (37%), *gelE* (33%), *cylA* (3%) and *cylB* (3%).
- Dendrograms generated by REP-PCR helped distinguish evolutionary relationships between all *E. faecalis* and *E. faecium*.
- *E. faecalis* was grouped into 20 REP-types, with ≥70% relatedness, designated A-T and 22% of *E. faecalis* isolates were grouped into 4 major REP-types displaying ≥ 90% relatedness: F (*n*= 2), J (*n*= 2), L (*n*= 4) and T (*n*= 3).
- *E. faecium* was grouped into 17 REP-types, with ≥70% relatedness, designated A-Q and 29% of *E. faecium* fell into 4 major REP-types with ≥ 90% relatedness: F (*n*= 2), G (*n*= 2), J (*n*=2) and N (*n*= 6). The largest major REP-type cluster originated from caecal samples. Furthermore, this major clonal cluster had >70% similarity index relationship with Week 3 isolates originating from litter samples indicating possible circulation of these isolates within the flock and along the different stages of the production process.

3.2 Limitations

- The study was limited to one poultry-production farm, which is not a true representation of the prevalence of ABR *Enterococcus* spp. throughout KwaZulu-Natal or South Africa.
- All isolates showing intermediate susceptibility should be interpreted with caution as it cannot be concluded if such isolates were susceptible or resistant as the intermediate susceptibility profile does not necessarily imply the presence of a resistance mechanism. Thus, they should be considered potentially resistant.
- Statistical analysis should be explored to draw conclusive correlations between phenotypic antibiotic resistance and the relevant antibiotic resistance genes.

3.3 Future recommendations

That the following recommendations are made based on the findings of this study:

- Molecular mechanisms such as the genes encoding quinupristin-dalfopristin resistance (*vatD* and *vatE*) in *E. faecium* need to be investigated.
- Molecular detection of the Tn*916* or Tn*1545* transposons should be investigated as *tetM* is often associated with *ermB* in transposon Tn*1545*.
- Additional mechanisms of tetracycline resistance should be investigated such as efflux pumps encoded by *tetL* or other ribosomal protection proteins such as *tetO*, *tetT*, *tetS and tetW*.
- Macrolide efflux pump systems encoded by *mef* and *msrC* should be investigated for erythromycin resistance.
- Other genes known to confer resistance to vancomycin (*vanD*, *vanE*, *vanG*, *vanL*, *vanM* and *vanN*) should be considered for isolates showing intermediate susceptibility in addition to carrying out MICs.
- Additional virulence genes should be studied to gain further insight into the virulence potential of isolates, e.g. biofilm-association pili (*epb*), aggregation substance (*asa*), adhesins (*ace*) and collagen adhesins (*acm*).
- Other clonal typing methods such as PFGE or multilocus sequence typing (MLST) should be used to gain clearer insight into clonal complexes.
- Whole-genome sequencing should be considered for a more accurate representation of isolates' genomic profile.
- Further studies including more poultry-production systems should be investigated to provide a better representation of ABR in *Enterococcus* spp. along the farm to food-production chain continuum in South Africa.

APPENDICES

Appendix 1: Biomedical Research Ethics Committee (BREC) approval letter

17 March 2017

Prof SY Essack
Department of Pharmaceutical Sciences
School of Health Sciences
essacks@ukzn.ac.za

Dear Prof Essack

Title: One Health approach to the containment of antibiotic resistance.
Degree: Non-degree
BREC Ref No: BCA444/16

CLASS APPROVAL

The Biomedical Research Ethics Committee (BREC) has considered the abovementioned application at a meeting held on 13 September 2016.

The study was provisionally approved by BREC pending appropriate responses to queries raised. Your responses dated 28 February 2017 to queries raised on 19 September 2016 have been noted and approved by the Biomedical Research Committee at a meeting held on 14 March 2017.

This approval is valid for one year from **17 March 2017.** To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

Pg. 2/...

Biomedical Research Ethics Committee
Professor J Tsoka-Gwegweni (Chair)
Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Telephone: +27 (0) 31 260 2486 **Facsimile:** +27 (0) 31 260 4609 **Email:** brec@ukzn.ac.za

Appendix 2: Animal Research Ethics Committee (AREC) approval letter

23 November 2016

Dr John Osei Sekyere
School of Health Sciences
Westville Campus

Dear Dr Osei Sekyere

Protocol reference number: AREC/073/016PD
Project title: Genomic insights into the Molecular Epidemiology, Evolution, Resistance Mechanisms, and persistence of Veterinary and Food Antibiotic-Resistant Bacteria isolated from South Africa

Full Approval – Research Application

With regards to your revised application received on 11 October 2016. The documents submitted have been accepted by the Animal Research Ethics Committee and **FULL APPROVAL** for the protocol has been granted.

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 23 November 2017.

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

....

Prof S Islam, PhD
Chair: Animal Research Ethics Committee

/ms

Cc Dean & Head of School:Dr Mahmoud Soliman
Cc Academic Leader Research: Professor Mershen Pillay
Cc Registrar: Mr Simon Mokoena
Cc NSPCA: Ms Jessica Light

Animal Research Ethics Committee (AREC)
Ms Mariette Snyman (Administrator)
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Appendix 3: Department of Agriculture, Forestry and Fisheries (DAFF) record

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/5 (878)

Professor Sabiha Yusuf Essack
Antimicrobial Research Unit
College of Health Sciences
University of KwaZulu-Natal
Tel: 031 260 7785
E-mail: ESSACKS@ukzn.ac.za

Dear Prof Essack,

RETROSPECTIVE APPLICATION UNDER SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984) FOR: "MOLECULAR EPIDEMIOLOGY OF LIVESTOCK-ASSOCIATED METHICILLIN RESISTANT STAPHYLOCOCCUS AUREUS (LA-MRSA) AND EXTENDED-SPECTRUM BETA-LACTAMASE-PRODUCING ENTEROBACTERIACEAE (ESBL-PE) IN PIGS AND EXPOSED WORKERS IN CAMEROON AND SOUTH AFRICA"

Your application received on 30 August 2018 for a Section 20 permit for the above mentioned study refers. Unfortunately, Section 20 approval cannot be given retrospectively for a study that has already proceeded or concluded, but we hereby take note of the information divulged in the application.

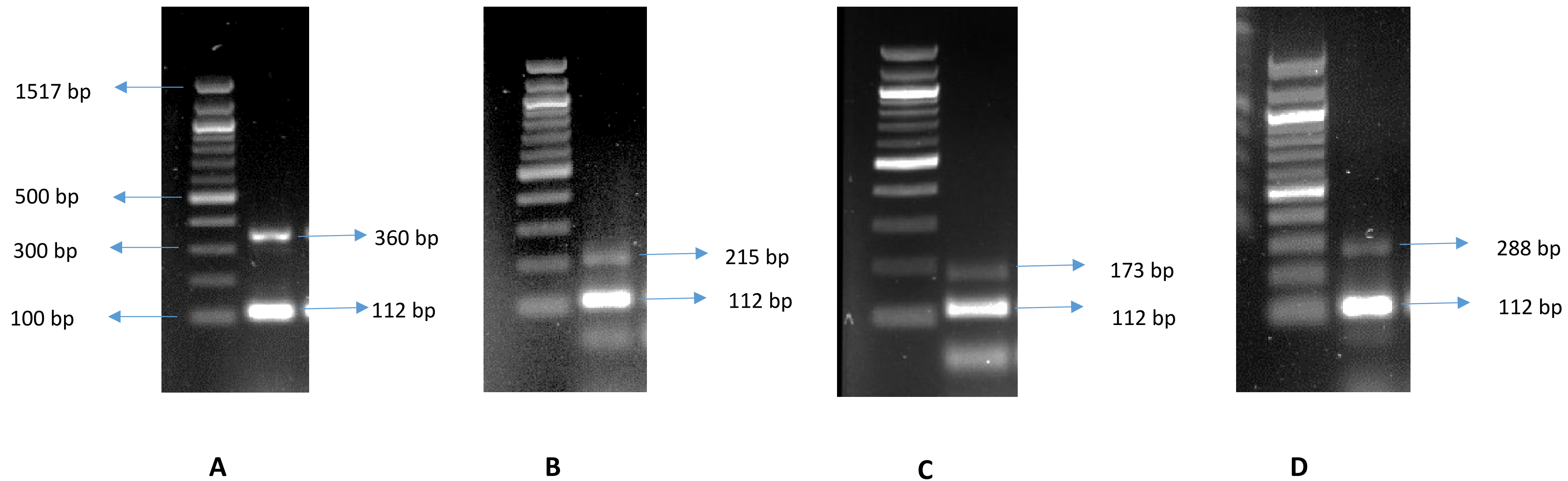
We take note of the letter of apology dated 28 August 2018, as well as the letter dated 13 September 2018 where you undertake to comply with Section 20 of the Animal Diseases Act, 1984 (Act No. 35 of 1984) and relevant processes in future (including obtaining valid Veterinary Import Permits where required).

Kind regards,

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2018 -09- 25

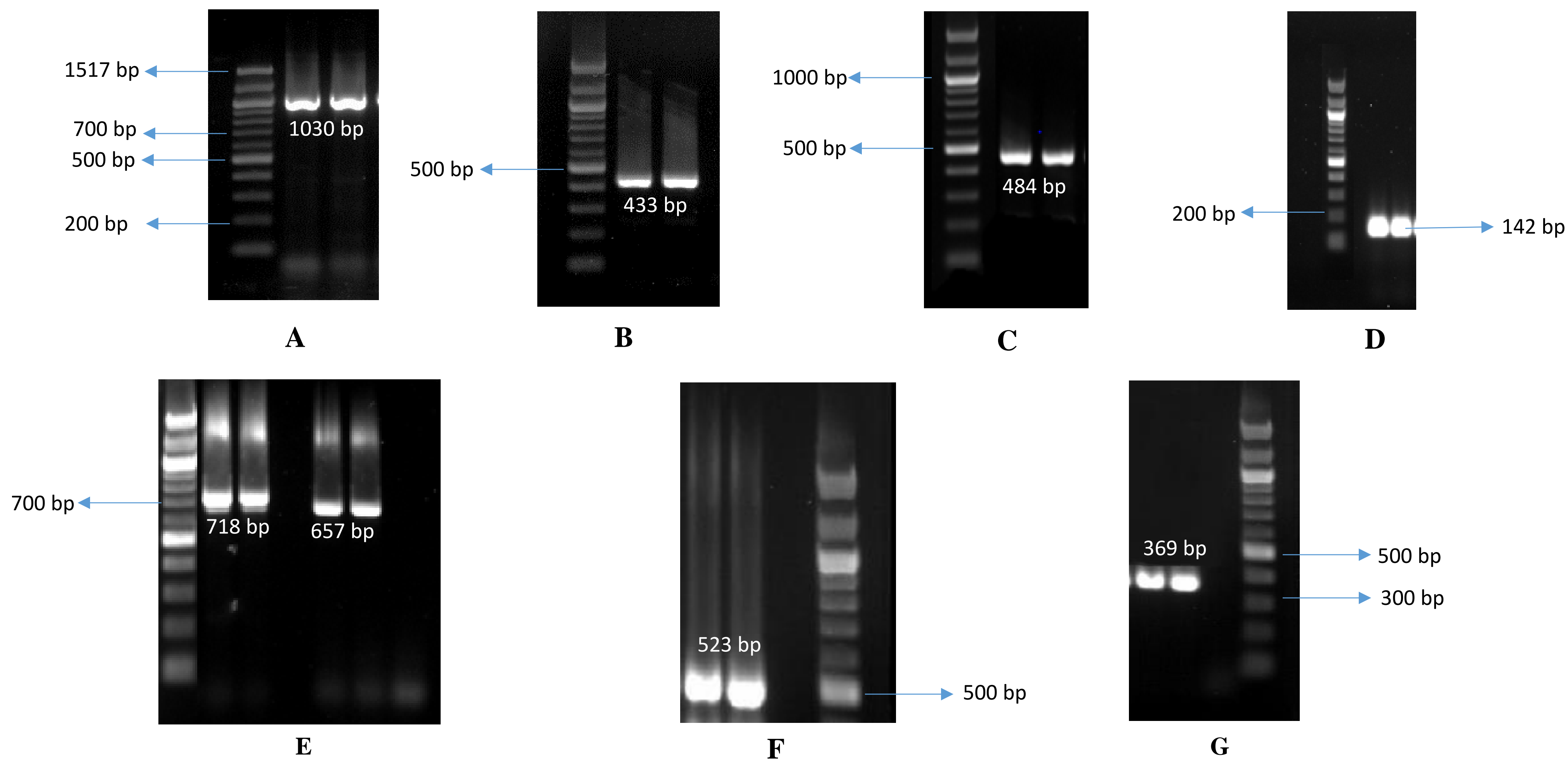
– 1 –

Appendix 4: Agarose gel electrophoresis banding patterns showing the molecular confirmation of the *Enterococcus* genus and species



A; *E. faecalis* ATCC 29212 *sodA* (360 bp) and *tuf* (112 bp), **B**; *E. faecium* ATCC 35667 *sodA* (215 bp) and *tuf* (112 bp), **C**; *E. gallinarum* clinical strain *sodA* (173 bp) and *tuf* (112 bp); **D**; *E. casseliflavus* ATCC 70327 *sodA* (288 bp) and *tuf* (112 bp). All PCR products were run alongside a 100 bp DNA molecular weight marker (NEB Quick-Load®, Massachusetts, USA).

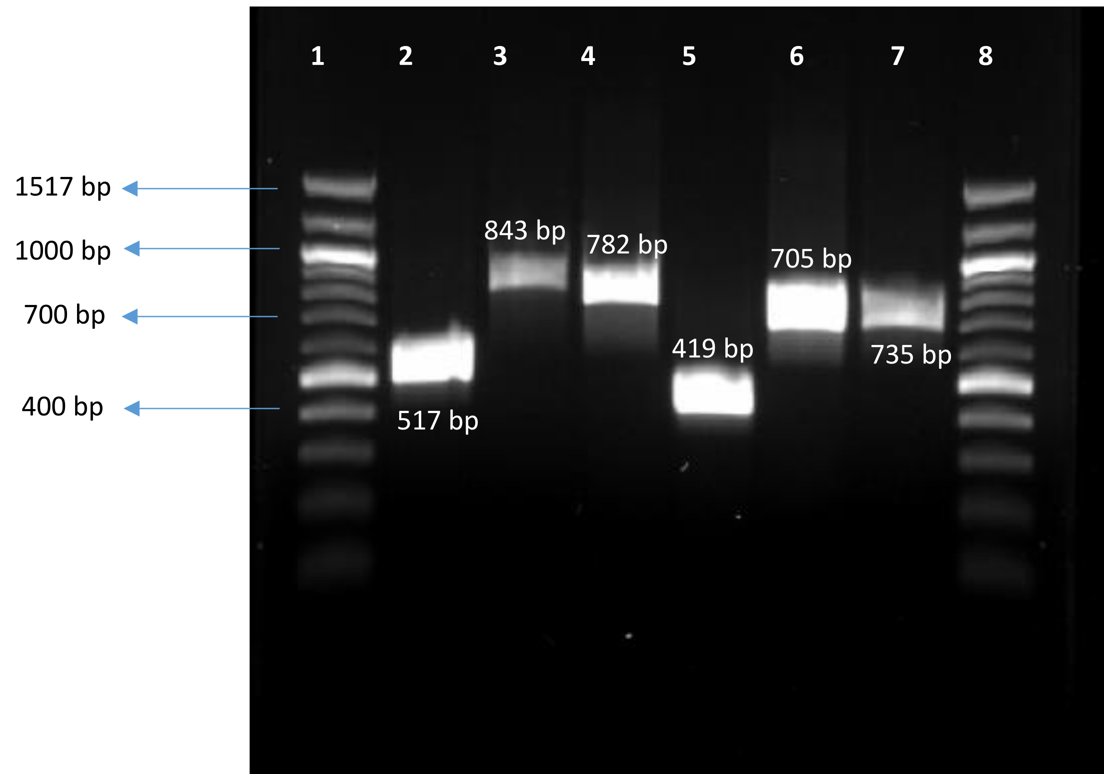
Appendix 5: Agarose gel electrophoresis banding patterns of real-time PCR amplification of antibiotic resistance genes



A; *vanA* amplified from *E. faecium* ATCC 700221, **B**; *vanB* from *E. faecalis* ATCC 51299, **C**; *vanC2/3* from *E. casseliflavus* ATCC 70327, **D**; *ermB* from *E. faecalis* ATCC 51299, **E**; *tetK* (718 bp) and *tetM* (657 bp) from *S. aureus* clinical strain and *E. faecalis*

ATCC 29212, respectively, **F**; *aph(3')-IIIa* from *E. faecalis* ATCC 51299, **G**; *aac(6')-Ie-aph(2'')-Ia* from *E. faecalis* ATCC 51299. All PCR products were run alongside a 100 bp DNA molecular weight marker (NEB Quick-Load®, Massachusetts, USA).

Appendix 6: Agarose gel electrophoresis banding patterns of real-time PCR amplification of virulence genes



Lanes 1 and 8; 100 bp DNA molecular weight marker (NEB Quick-Load®, Massachusetts, USA), Lane 2; *cylA* (*E. faecalis* ATCC 29212), Lane 3; *cylB* (*E. faecalis* ATCC 29212), Lane 4; *cpd* (*E. faecalis* ATCC 51299), Lane 5; *gelE* ((*E. faecalis* ATCC 29212), Lane 6; *efaAFs* (*E. faecalis* ATCC 29212), Lane 7; *efaAFm* (*E. faecium* ATCC 35667).

Appendix 7: Table of raw data

			Antibiotic panel													Genotypic evaluation	
ID	**Isolate**	**PCR speciation**	**TEC**	**NIT**	**TGC**	**TET**	**ERY**	**VAN**	**CIP**	**AMP**	**CHL**	**GEN**	**STR**	**Q-D**	**Antibiogram**	**ABR genes detected**	**Virulence genes**
T1	1	*E. faecalis*	S	S	S	S	R	S	S	S	S	S	S		ER	*ermB*	*gelE-cpd-efaAFs*
T12b	2	*E. faecalis*	I	S	S	S	R	S	S	S	S	S	S		*TEC* ER	*ermB*	*gelE-cpd-efaAFs*
T2	3	*E. faecalis*	S	S	S	S	R	I	S	S	S	S	S		ER *VAN*	*ermB*	*gelE-cpd-efaAFs*
T2b	4	*E. gallinarum*	S	S	S	R	S	I	I	S	S	S	S		TE *VAN CIP*	*tetM-vanC1*	-
Ta	5	*E. gallinarum*	S	S	S	R	S	I	I	S	S	S	S		TE *VAN CIP*	*tetM-vanC2*	-
VRE+W2F3	6	*Enterococcus* spp	S	S	S	R	R	S	S	S	S	S	S		TE ER	*tetM-ermB*	-
VRE+W2F5	7	*Enterococcus* spp	S	S	S	R	R	S	S	S	S	S	S		TE ER	*tetM-ermB*	-
H9	8	*E. faecalis*	S	S	S	R	R	S	S	S	S	S	S		TE ER	*tetM-ermB*	*gelE-cpd-efaAFs*
H10	9	*E. faecalis*	S	S	S	R	R	I	I	S	R	S	S		TE ER *VAN CIP* CHL	*tetM-ermB*	*gelE-cpd-efaAFs*
H11	10	*E. faecalis*	S	S	S	R	R	S	I	S	I	S	S		TE ER *CIP CHL*	*tetM-ermB*	*gelE-cpd-efaAFs*
W2F12	11	*Enterococcus* spp	S	S	S	S	R	S	S	S	S	S	S		ER	*ermB*	*efaAFm*
W2F4	12	*Enterococcus* spp	S	S	S	R	R	S	S	S	S	S	S		TE ER	*tetM-ermB*	*efaAFm*
W2F5	13	*E. faecium*	S	S	S	R	R	S	I	S	I	S	S	R	TE ER CIP CHL QD	*tetM*	*efaAFm*
W2F2*	14	*E. faecalis*	S	S	S	R	R	I	I	S	I	S	S		TE ER *VAN CIP CHL*	*tetM-ermB*	*gelE-cpd-efaAFs*
W2F3*	15	*E. faecalis*	S	S	S	R	R	S	S	S	I	S	S		TE ER *CHL*	*ermB*	*gelE-cpd-efaAFs*
W2F5*	16	*E. faecalis*	S	I	S	R	R	S	I	S	S	S	S		NIT TE ER CIP	*tetM-ermB*	*gelE-cpd-efaAFs*
W2L1	17	*E. faecium*	S	R	S	R	R	S	S	S	S	S	S	I	NIT TE ER CIP QD	*tetM-ermB*	*efaAFm*

ID	Isolate	PCR speciation	TEC	NIT	TGC	TET	ERY	VAN	CIP	AMP	CHL	GEN	STR	Q-D	Antibiogram	ABR genes detected	Virulence genes
W2L4	18	*Enterococcus* spp	S	S	S	R	R	S	S	S	S	S	S		TE ER	*tetM-ermB*	-
W2L1*	19	*E. faecium*	S	S	S	S	R	S	I	S	S	S	S	S	ER *CIP*	*ermB*	*efaAFm*
W2L3*	20	*E. faecium*	S	I	S	R	R	S	I	S	I	S	R	I	*NIT* TE ER *CIP CHL* STR *QD*	*tetM-ermB-aph(3')-IIIa*	*efaAFm*
W2L4*	21	*E. faecium*	S	S	S	R	R	S	I	S	I	S	R	S	TE ER *CIP CHL* STR	*tetM-ermB-aph(3')-IIIa*	*efaAFm*
W2L6*	22	*E. faecalis*	S	S	R	S	R	I	S	S	I	S	S		ER *VAN CHL*	*ermB*	*gelE-cpd-efaAFs*
W3L1	23	*E. faecium*	I	S	S	R	R	S	S	R	S	S	S	S	*TEC* TE ER AMP	*tetM-ermB*	*efaAFm*
W3L3	24	*Enterococcus* spp	S	I	S	R	R	S	I	S	I	S	S		*NIT* TE ER *CIP CHL*	*tetM-ermB*	*efaAFm*
W3L2	25	*E. faecium*	S	S	S	R	R	S	I	S	I	S	S	R	*TE ER CIP CHL* QD	*tetM-ermB*	*efaAFm*
W3L4	26	*E. faecium*	S	R	S	R	R	S	I	S	S	S	S	R	NIT TE ER *CIP* QD	*tetM-ermB*	*efaAFm*
W3L5	27	*E. faecium*	S	I	S	R	R	S	I	S	S	S	S	I	*NIT* TE ER *CIP QD*	*tetM-ermB*	*efaAFm*
W3L6	28	*Enterococcus* spp	S	S	S	R	R	S	S	S	I	S	S		TE ER *CHL*	*tetM-ermB*	*gelE-cpd-efaAFs*
W3L7	29	*E. faecium*	S	I	S	R	R	S	S	S	S	S	S	S	*NIT* TE ER CIP	*tetM-ermB*	*efaAFm*
W3L8	30	*E. faecium*	S	S	S	R	R	S	I	R	I	S	S	I	TE ER *CIP* AMP *CHL QD*	*tetM-ermB*	*efaAFm*
W3L12	31	*Enterococcus* spp	S	R	S	S	I	S	S	S	S	S	S		NIT *ER*	*ermB*	*efaAFm*
W3F11	32	*Enterococcus* spp	S	R	S	S	R	S	S	S	S	S	S		NIT ER	-	-
W3F1	33	*Enterococcus* spp	S	I	S	R	R	S	S	S	S	S	S		*NIT* TE ER	*tetM-ermB*	*efaAFm*
W3F3	34	*Enterococcus* spp	S	S	S	R	R	S	I	S	S	S	S		TE ER CIP	*tetM-ermB*	*efaAFm*

ID	Isolate	PCR speciation	TEC	NIT	TGC	TET	ERY	VAN	CIP	AMP	CHL	GEN	STR	Q-D	Antibiogram	ABR genes detected	Virulence genes
W3F7*	35	*E. faecalis*	R	R	S	I	R	S	I	S	R	S	R		*TEC NIT TE ER CIP CHL STR*	*tetM-ermB-aph(3')-IIIa*	*cpd-efaAFs*
W3F10*	36	*E. faecalis*	R	R	S	S	R	S	I	S	R	R	R		*TEC NIT ER CIP CHL GEN STR*	*ermB-aph(3')-IIIa*	*cpd-efaAFs*
W3F3*	37	*E. faecalis*	S	S	R	R	R	I	I	S	R	S	R		*NIT TGC TE ER VAN CIP CHL STR*	*tetM-ermB-aph(3')-IIIa*	*cpd-efaAFs*
W3F4*	38	*E. faecalis*	S	S	S	R	R	S	I	S	R	S	R		TE ER CIP CHL STR	*tetM-ermB-aph(3')-IIIa*	*cpd-efaAFs*
W3F8*	39	*E. faecalis*	S	S	S	R	R	I	I	S	R	S	R		*TE ER VAN CIP CHL STR*	*tetM-ermB-aph(3')-IIIa*	*cpd-efaAFs*
W3F9*	40	*E. faecalis*	S	S	S	R	R	S	I	S	R	S	R		*TE ER CIP AMP CHL STR*	*tetM-ermB-aph(3')-IIIa*	*efaAFs*
W3F6*	41	*E. faecalis*	S	S	S	R	R	S	I	S	R	S	R		TE ER CIP CHL STR	*tetM-ermB*	*cpd-efaAFs*
W4F9	42	*E. faecalis*	S	S	S	R	R	S	S	S	S	S	S		TE ER	*tetM-ermB*	*gelE-cpd-efaAFs*
W4F15	43	*E. faecalis*	S	S	S	R	R	S	I	S	I	S	S		TE ER *CIP CHL*	*tetM-ermB*	*gelE-cpd-efaAFs*
W4F18	44	*E. faecium*	S	S	S	R	R	S	S	S	I	S	S	I	TE ER CIP *CHL QD*	*tetM-ermB*	*efaAFm*
W4F23	45	*E. faecium*	S	S	S	R	R	S	S	S	S	S	S	S	TE ER	*tetM*	*efaAFm*
W4F31	46	*E. faecium*	S	S	S	R	I	S	S	S	S	S	S	S	TE *ER*	*tetM*	*efaAFm*
W4F37	47	*E. faecium*	S	R	R	R	I	S	I	S	S	S	S	S	NIT TGC TE ER CIP	*tetM*	*efaAFm*
W4F39	48	*E. faecium*	I	R	S	R	I	S	I	S	S	S	S	S	*TEC NIT TE ER CIP*	*tetM*	*efaAFm*

ID	Isolate	PCR speciation	TEC	NIT	TGC	TET	ERY	VAN	CIP	AMP	CHL	GEN	STR	Q-D	Antibiogram	ABR genes detected	Virulence genes
W4F1*	49	*E. faecalis*	S	S	S	R	R	S	I	S	I	S	S		TE ER *CIP CHL*	*tetM-ermB*	*gelE-cpd-efaAFs*
W4F2*	50	*E. faecalis*	S	S	S	R	R	S	I	S	I	S	S		TE ER *CIP CHL*	*tetM-ermB*	*gelE-cpd-efaAFs*
W4F3*	51	*E. faecalis*	S	S	S	R	R	S	I	S	I	S	S		TE ER *CIP CHL*	*tetM-ermB*	*gelE-cpd-efaAFs*
W4F4*	52	*E. faecalis*	S	S	S	R	R	S	I	S	I	S	S		TE ER *CIP CHL*	*tetM-ermB*	*gelE-cpd-efaAFs*
W4L10	53	*E. faecalis*	S	S	S	R	R	S	I	S	S	S	R		TE ER *CIP* STR	*tetM-ermB*	*gelE-cpd-efaAFs*
W4L11	54	*Enterococcus* spp	S	S	S	R	R	S	I	S	S	S	S		TE ER *CIP*	*tetM-ermB*	*efaAFm*
W4L13	55	*E. faecium*	S	S	S	R	R	S	I	S	S	S	S	S	TE ER *CIP*	*tetM*	*efaAFm*
W4L14	56	*Enterococcus* spp	S	I	S	R	R	S	I	R	S	S	S		NIT TE ER CIP AMP	*tetM*	*efaAFm*
W4L20	57	*E. faecium*	S	S	S	S	R	S	I	S	S	S	S	R	ER *CIP* QD	-	*efaAFm*
W4L21	58	*E. faecium*	S	S	S	R	I	S	S	S	I	S	S	S	TE *ER CIP*	*tetM*	*efaAFm*
W4L22	59	*E. faecium*	S	S	S	R	I	S	I	R	S	S	S	S	TE *ER CIP* AMP	*tetM*	*efaAFm*
W4L24	60	*Enterococcus* spp	S	S	S	R	R	S	S	S	S	S	S		TE ER	*tetM-ermB*	-
W4L26	61	*E. faecium*	S	S	S	R	I	S	I	S	S	S	S	S	TE *ER CIP*	*tetM*	*efaAFm*
W4L25	62	*E. faecium*	S	R	S	R	R	S	I	R	S	S	S	S	NIT TE ER CIP AMP	*tetM-ermB*	*efaAFm*
W5F1	63	*E. faecium*	S	S	S	R	R	S	I	R	S	S	S	R	TE ER *CIP* AMP QD	*tetM-ermB*	*efaAFm*
W5F2	64	*Enterococcus* spp	S	R	S	R	R	S	I	R	S	S	S		NIT TE ER CIP AMP	*tetM-ermB*	*efaAFm*
W5F3	65	*Enterococcus* spp	S	I	S	S	I	S	I	S	S	S	S		*NIT ER CIP*	*ermB*	*efaAFm*
W5F4	66	*Enterococcus* spp	S	S	S	S	I	S	I	S	S	S	S		ER CIP	*ermB*	*efaAFm*
W5F5	67	*E. faecium*	S	S	S	R	R	S	I	S	R	S	R	R	TE ER *CIP* CHL STR QD	*tetM-ermB*	*efaAFm*
W5F7	68	*E. faecium*	S	I	S	R	R	S	I	S	S	S	S	S	*NIT* TE ER *CIP*	*tetM-ermB*	*efaAFm*

ID	Isolate	PCR speciation	TEC	NIT	TGC	TET	ERY	VAN	CIP	AMP	CHL	GEN	STR	Q-D	Antibiogram	ABR genes detected	Virulence genes
W5F8	69	*Enterococcus* spp	S	I	S	R	R	S	R	R	R	R	R		NIT TE ER CIP AMP CHL GEN STR	*tetM-ermB*	*efaAFm*
W5F10	70	*Enterococcus* spp	S	R	S	R	R	S	S	S	S	S	S		NIT TE ER	*ermB*	*efaAFm*
W5F14	71	*Enterococcus* spp	S	R	S	R	R	S	S	S	S	S	S		NIT TE ER	*tetM-ermB*	*efaAFm*
W5F16	72	*Enterococcus* spp	S	I	S	R	R	S	I	R	S	S	S		*NIT TE ER CIP AMP*	*tetM*	*efaAFm*
VRE+ W5F7	73	*Enterococcus* spp	R	R	S	R	R	S	S	S	S	S	S		TEC NIT TE ER	-	-
W5L2	74	*Enterococcus* spp	S	S	S	R	R	S	S	S	S	S	R		TE ER STR	*tetM-ermB-aph(3')-IIIa*	*efaAFm*
W5L4	75	*Enterococcus* spp	S	S	S	S	R	S	S	S	S	S	S		ER	*ermB*	*efaAFm*
W5L5	76	*Enterococcus* spp	S	S	S	S	R	S	S	S	S	S	S		ER	-	*gelE*
W5L6	77	*E. faecium*	S	S	S	S	R	S	I	S	I	S	S	S	ER *CIP CHL*	*ermB*	*efaAFm*
W5L7	78	*Enterococcus* spp	S	R	S	R	R	S	S	R	I	S	S		NIT TE ER AMP CHL	*tetM-ermB*	*efaAFm*
W5L8	79	*Enterococcus* spp	S	S	S	R	R	S	S	R	S	S	S		TE ER AMP	*tetM-ermB*	-
W5L9	80	*Enterococcus* spp	S	S	S	R	S	S	I	S	S	S	S		TE CIP	*tetM*	-
W5L10	81	*E. faecium*	S	S	S	R	I	S	I	S	S	S	S	S	TE ER CIP	*tetM-ermB*	*efaAFm*
VRE+W5L1	82	*Enterococcus* spp	S	R	S	S	I	S	S	S	S	S	S		NIT *ER*	-	-
VRE+W5L2	83	*Enterococcus* spp	S	R	S	R	R	S	S	S	S	S	S		NIT TE ER	*tetM-ermB*	*efaAFm*
VRE+W5L4	84	*E. faecium*	S	R	S	R	R	S	S	S	S	S	S	I	NIT TE ER CIP *QD*	*tetM-ermB*	*efaAFm*
VRE+W5L5	85	*Enterococcus* spp	S	R	S	R	R	S	I	S	I	S	S		NIT TE ER *CIP CHL*	*tetM-ermB*	*efaAFm*
Crate 8	86	*E. faecium*	S	I	S	S	R	S	I	S	I	S	S	R	NIT ER CIP CHL QD	*ermB*	*efaAFm*

ID	Isolate	PCR speciation	TEC	NIT	TGC	TET	ERY	VAN	CIP	AMP	CHL	GEN	STR	Q-D	Antibiogram	ABR genes detected	Virulence genes
Crate 9	87	*E. faecium*	S	I	S	S	I	S	I	S	I	S	S	I	*NIT ER CIP QD*	*tetM*	*efaAFm*
Crate 10	88	*Enterococcus* spp	S	S	S	R	R	S	I	R	S	S	S		TE ER CIP AMP	*ermB*	*efaAFm*
Crate 12	89	*E. faecium*	S	I	S	S	R	S	I	S	R	S	S	R	ER *CIP* CHL QD	*ermB*	*efaAFm*
Crate 1	90	*Enterococcus* spp	S	S	S	R	I	S	S	S	S	S	S		TE *ER*	*tetM-ermB*	*efaAFm*
Crate 2	91	*E. faecium*	S	I	S	R	S	S	S	S	S	S	S	S	*NIT* TE	*tetM*	*efaAFm*
Crate 3	92	*E. faecium*	S	S	S	R	I	S	I	S	I	S	S	R	TE *ER CIP CHL* QD	*tetM*	*efaAFm*
Abattoir 16	93	*E. faecalis*	S	S	S	R	R	S	S	S	S	S	S		TE ER	*tetM*	*gelE-efaAFs*
Abattoir 17	94	*E. faecalis*	S	S	S	R	R	S	S	S	I	S	S		TE ER *CHL*	*tetM-ermB*	*cpd-efaAFs*
Abattoir 20	95	*E. faecalis*	S	S	S	R	R	S	S	S	I	S	S		TE ER *CHL*	*tetM*	*gelE-cpd-efaAFs*
Abattoir 22	96	*E. faecalis*	S	S	S	R	R	S	I	S	I	S	S		TE ER CIP CHL	*tetM-ermB*	*gelE-cpd-efaAFs*
Abattoir 23	97	*E. faecalis*	S	S	S	R	R	S	S	S	I	S	S		TE ER CHL	*tetM*	*gelE-cpd-efaAFs*
Abattoir 3*	98	*E. faecalis*	S	S	S	R	I	S	S	S	S	S	S		TE ER	*tetM*	*gelE-cpd-efaAFs*
ABA 3	99	*E. faecalis*	S	S	S	R	R	S	I	S	I	S	S		*TE ER CIP CHL*	*ermB*	*gelE-cpd-efaAFs*
Neck 9	100	*E. faecalis*	S	S	S	R	R	S	I	S	I	S	S		TE ER CIP CHL	*tetM-ermB*	*gelE-cpd-efaAFs*
Neck 10	101	*E. faecalis*	S	S	S	R	R	S	S	S	S	S	S		TE ER	*tetM-ermB*	*gelE-cpd-efaAFs*
Neck 11	102	*E. faecalis*	S	S	S	R	R	I	I	S	R	S	R		TE ER VAN CIP CHL STR	*tetM-ermB-aph(3')-IIIa*	*gelE-cpd-efaAFs*
Neck 12	103	*E. faecalis*	S	S	S	R	I	S	I	S	S	S	S		TE *ER CIP*	*tetM*	*gelE-cpd-cylA-cylB-efaAFs*
Neck 1	104	*Enterococcus* spp	R	S	S	R	R	S	I	S	S	R	R		TEC TE ER CIP GEN STR	*tetM-ermB-aph(3')-IIIa*	*gelE-cpd-efaAFs*

ID	Isolate	PCR speciation	TEC	NIT	TGC	TET	ERY	VAN	CIP	AMP	CHL	GEN	STR	Q-D	Antibiogram	ABR genes detected	Virulence genes
Neck 3	105	*E. faecalis*	S	S	S	R	I	S	I	S	S	S	S		TE *ER CIP*	*tetM*	*cpd-cylA-cylB-efaAFs*
Neck 4	106	*E. faecalis*	S	S	S	R	I	I	S	S	S	S	S		TE *ER VAN*	*tetM*	*gelE-cpd-efaAFs*
Neck 5	107	*E. faecalis*	S	S	S	R	R	I	S	S	I	S	S		*TE ER VAN CHL*	*tetM-ermB*	*gelE-cpd-efaAFs*
Neck 1*	108	*E. faecalis*	S	S	S	R	S	S	I	S	S	S	S		TE *CIP*	*tetM*	*gelE-cpd-cylA-cylB-efaAFs*
Neck 6	109	*E. faecalis*	S	S	S	R	R	S	I	S	S	S	S		TE ER CIP	*tetM-ermB*	*gelE-cpd-efaAFs*
Carcass 6*	110	*E. faecalis*	S	S	R	R	R	S	I	R	I	S	R		TGC TE ER CIP AMP CHL	*tetM-ermB-aph(3')-IIIa*	*gelE-cpd-efaAFs*
Carcass 10	111	*E. faecalis*	S	S	S	R	R	S	I	R	S	S	S		TE ER *CIP* AMP	-	*gelE-cpd-efaAFs*
carcass 14	112	*E. faecalis*	S	S	S	R	R	S	I	R	I	S	S		TE ER CIP AMP CHL	*ermB*	*gelE-cpd-efaAFs*
Carcass 9*	113	*E. faecalis*	S	S	S	R	R	S	S	R	S	S	R		TE ER AMP	*tetM-ermB-aph(3')-IIIa*	*gelE-cpd-efaAFs*
Carcass 1	114	*E. faecalis*	S	S	R	R	R	S	I	R	I	S	S		TGC TE ER CIP AMP CHL	*ermB*	*gelE-cpd-efaAFs*
Carcass 8	115	*E. faecalis*	S	S	S	R	R	S	S	R	R	R	R		TE ER AMP CHL GEN STR	*tetM-ermB-aph(3')-IIIa*	*gelE-cpd-efaAFs*
Carcass 5*	116	*E. faecalis*	S	I	S	R	R	S	I	R	R	R	R		NIT TE ER CIP AMP CHL GEN STR	*tetM-ermB-aph(3')-IIIa*	*gelE-cpd-efaAFs*
Caecal 9	117	*E. faecium*	S	I	S	R	I	S	I	S	I	S	S	I	*TE ER CIP AMP QD*	*tetM-ermB*	*gelE-cpd-efaAFs-efaAFm*

ID	Isolate	PCR speciation	TEC	NIT	TGC	TET	ERY	VAN	CIP	AMP	CHL	GEN	STR	Q-D	Antibiogram	ABR genes detected	Virulence genes
Caecal 10	118	*E. faecium*	S	S	S	R	I	S	I	S	S	S	S	I	TE *ER CIP QD*	*tetM*	*gelE-efaAFm*
Caecal 11	119	*E. faecium*	S	S	S	S	I	S	I	S	S	S	S	S	*ER CIP*	-	*efaAFm*
Caecal 12	120	*E. faecium*	S	R	S	R	I	S	I	S	S	S	S	S	NIT TE *ER CIP*	*tetM*	*efaAFm*
Caecal 13	121	*E. faecium*	S	S	S	R	I	S	I	S	S	S	S	S	TE *ER CIP*	*tetM*	*efaAFm*
Caecal 14	122	*E. faecium*	S	S	S	R	S	S	S	S	S	S	S	S	TE	*tetM*	*efaAFm*
Caecal 5	123	*E. faecium*	S	I	S	R	I	S	I	S	S	S	S	S	*NIT* TE *ER CIP*	*tetM*	*efaAFm*
Caecal 6	124	*Enterococcus* spp	S	S	S	R	I	S	S	S	S	S	S		TE *ER*	*tetM*	*efaAFm*
Caecal 7	125	*E. faecium*	S	S	S	I	I	S	S	S	R	S	R	S	*TE ER* CHL STR	*tetM-aph(3')-IIIa*	*efaAFm*
House 4	126	*Enterococcus* spp	S	I	S	S	I	S	I	S	S	S	S		*NIT ER CIP*	-	*efaAFm*
House 5	127	*E. faecium*	S	R	S	R	R	S	I	S	S	S	S	R	NIT TE ER *CIP* QD	*tetM-ermB*	*efaAFm*
House 6	128	*Enterococcus* spp	S	I	S	S	I	S	I	R	S	S	S		*NIT ER CIP* AMP	-	*efaAFm*
House 8	129	*Enterococcus* spp	S	I	S	S	I	S	I	S	S	S	S		*NIT ER CIP*	-	*efaAFm*
House 9	130	*Enterococcus* spp	S	R	S	S	I	S	I	S	S	S	S		NIT *ER CIP*	*tetM-ermB*	*efaAFm*
House 3	131	*Enterococcus* spp	S	I	S	S	I	S	I	S	S	S	S		*NIT ER CIP*	-	*efaAFm*
House 11	132	*E. faecalis*	S	S	R	R	R	I	I	S	S	S	S		*TGC TE ER VAN CIP*	*tetM-ermB*	*gelE-cpd-efaAFs*
House 12	133	*Enterococcus* spp	S	R	S	R	I	S	I	S	S	S	S		NIT TE *ER CIP*	*tetM*	*efaAFm*
House 13	134	*Enterococcus* spp	S	R	S	R	I	S	I	S	S	S	S		*NIT TE ER CIP*	*tetM-ermB*	*efaAFm*

Appendix 8: Farm personnel consent forms

Participant Information Leaflet – Farm/Abattoir Personnel

You are being asked to volunteer to participate in a research study entitled "One Health Approach to the Containment of Antibiotic Resistance" conducted under the auspices of the South African Research Chair in Antibiotic Resistance and One Health, funded by the Department of Science and Technology via the National Research Foundation (NRF) over a 5-year period 2016-2020.

Your participation is completely voluntary. Please read the following information about the project. If there is anything in this Consent Document that you do not understand, be sure to ask study personnel to explain that portion of the study. If you voluntarily agree to participate, please indicate this verbally and/or sign in the appropriate box below.

This study is being conducted at University of KwaZulu-Natal. The overall purpose of this study is to investigate antibiotic resistance in bacteria isolated from poultry production, including the personnel that work in the production system from farms to abattoirs to butchers and supermarkets. This is called the **"farm-to-fork" protocol**.

Male and female employees in the profession-related to poultry 18 years and older are eligible to participate. We are asking you to take part in this study because you are member of this category of person. The study involves taking a sample/swab of your nares and hands over the course of the to undertake bacteriological investigations.

Participation in this study is completely voluntary. If you decide not to participate there will not be any negative consequences. Please be aware that if you decide to participate, you may stop participating at any time and you may decide not to provide any samples.

The investigators believe that the risks or discomforts to you and your animals are minimal.
You will not receive any payment for your participation in this study. Your participation will provide information to improve practices in the poultry food production systems.

Please do not hesitate to contact the following person should you have any queries or concerns related to your voluntary participation:

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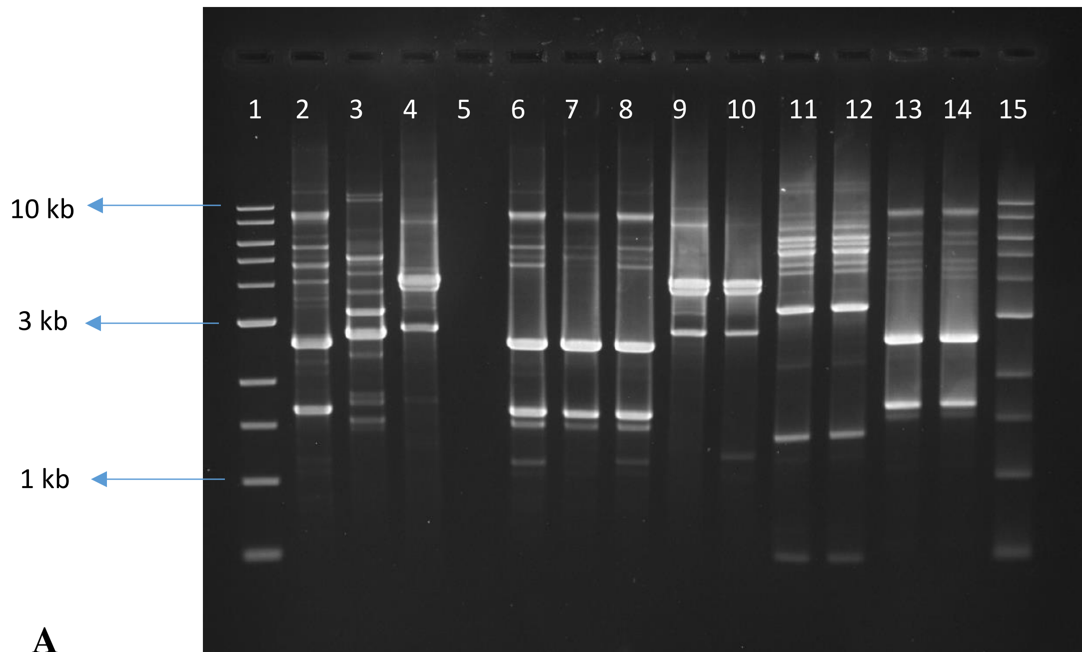
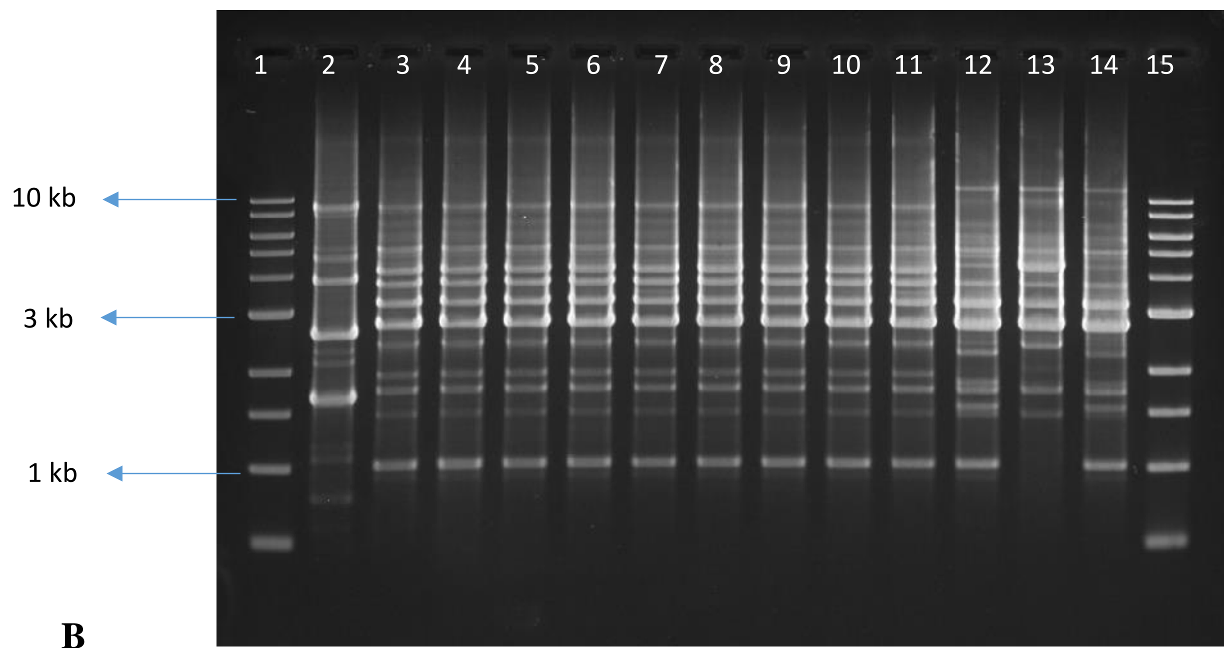
We thank you for your invaluable time and your assistance.

Appendix 9: REP-PCR agarose gel electrophoresis images



A: lane 1: 1 kb DNA molecular weight marker (NEB Quick-Load®, Massachusetts, USA), Lane 2: *E. faecalis* ATCC 29212, Lane 3: *E. faecium* ATCC 35667, Lane 4: *E. gallinarum* clinical strain, Lane 4: No DNA control, Lane 5 – 14: isolate ID 1-9, Lane 15: 1 kb DNA molecular weight marker



B: lane 1: 1 kb DNA molecular weight marker (NEB Quick-Load®, Massachusetts, USA), Lane 2 - 14: isolate ID 116-128, Lane 15: 1 kb DNA molecular weight marker.