Genetic characterization of resistance and virulence genes in *Enterococcus* species from animal isolates in Durban

by

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PREFACE

The experimental work described in this dissertation was carried out in the Discipline of Genetics, School of Life Sciences, College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Westville Campus, Durban, South Africa from October 2017 to June 2018, under the supervision of Dr. M. A. Adeleke and Dr. O. T. Zishiri.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

I certify that the above information is correct

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DECLARATION FOR PLAGERISM

I, Eberechi Phoebe Nnah, declare that:

1. The research reported in this thesis, except where otherwise indicated, is my original research.
2. This thesis has not been submitted for any degree or examination at any other University.
3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
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DECLARATION FOR PUBLICATIONS

DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this thesis (include publications in preparation, submitted, *in press* and published and give details of the contributions of each author to the experimental work and writing of each publication)

Publication 1: **Molecular detection and antibiotic resistance of *Enterococcus* species isolated from animals**

The research reported on is based on the data I Eberechi Phoebe Nnah collected from veterinary clinics and poultry abattoir in Durban. I did most of the experimental work, analyzed the data and wrote the paper. My supervisors conceived the idea and provided guidance on the actual execution of the project including sample collection, microbiology and molecular work, statistical analysis, writing and finalization of the manuscript.

This article was submitted and is undergoing peer review in *South African Journal of Science*.

Publication 2: **Incidence of virulence factors of *Enterococcus* species in chicken and companion animals sampled from Durban, South Africa**

The research reported on is based on the data I Eberechi Phoebe Nnah collected from veterinary clinics and poultry abattoir in Durban. I did most of the experimental work, analyzed the data and wrote the paper. My supervisors conceived the idea and provided guidance on the actual execution of the project including sample collection, microbiology and molecular work, statistical analysis, writing and finalization of the manuscript.

This article is in preparation for submission to *Journal of Animal Biotechnology*.

Signed:

ABSTRACT

Misuse of antimicrobials in animal agriculture has given rise to strains of bacteria that are resistant to multiple antibiotics. *Enterococci* bacteria have emerged among such antibiotic-resistant strains of bacteria and infections due to antibiotic-resistant bacteria is one of the world's critical health challenge. *Enterococci* are gut commensal bacteria but are currently confirmed pathogenic bacteria responsible for so many hospital-acquired infections like urinary tract infections. The aim of this research was to detect the occurrence of *Enterococcus* species in chickens, cats, and dogs; their phenotypic and genotypic resistance to antibiotic drugs and virulence genes. Isolation of *Enterococcus* species was done using microbiological culture methods and confirmed using specific primers through Polymerase Chain Reaction (PCR). Presumptive *Enterococcus* growth on bile esculin agar was positive for 94% of all the isolates. Overall, 77.3% of the isolates were positive for *Tuf* gene (*Enterococcus* genus-specific gene). *Enterococcus faecalis* was detected at a higher frequency (40.4%; $P < 0.05$) compared to *Enterococcus faecium* (8.5%). All the *Enterococcus* isolates were susceptible to High-Level Gentamicin on antimicrobial susceptibility test. *Enterococcus* species in chickens exhibited higher resistance to the antibiotics than the pets. Highest resistance was observed in Quinupristin/Dalfopristin (89.4%) followed by Vancomycin (87.9%), Rifampicin (85%), Ampicillin (76.6%), Erythromycin (72.3%), and Tetracycline (64.5%). Chloramphenicol (24.8%), High-Level Streptomycin Resistance (24.1%), and Ciprofloxacin (14.2%). Eighty-four percent (84%) of the *Enterococcus* isolates expressed multidrug resistance (MDR). Three of the four resistance genes screened were detected: 21.3%, 7.8% and 4.3% for Kanamycin, Streptomycin, and Vancomycin resistance genes respectively. Gentamicin resistance gene was absent in all the isolates. PCR detection of virulence gene showed highest prevalence in *EfaA* gene at 88.7% frequency followed by *GelE* (82.3%), *ccf* (81.6%), Esp (26.2%) and *CylA* (25.5%). All *E. faecalis* and *E. faecium* detected harbored multiple virulence genes. These findings show that chickens, cats, and dogs can be colonized by pathogenic *Enterococci* which harbor resistance and virulence genes and are multidrug resistant. It is therefore important that antibiotics are used prudently in animal husbandry to mitigate emergence and transfer of *Enterococci* pathogens to humans via food chain and direct contact of pets by their owners.

Keywords: *Enterococcus* species; antibiotics; susceptibility test; virulence genes; resistance genes; PCR.

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LIST OF ABBREVIATIONS AND SYMBOLS

AME	Aminoglycoside Modifying Enzymes
ARE	Antimicrobial Resistant Enterococci
bp	base pair
BSAC	British Society for Antimicrobial Chemotherapy
CI	Confidence Interval
CLSI	Clinical and Laboratory Standard Institute
°C	Degree Celsius
DNA	Deoxyribonucleic acid
dH_20	distilled water
Ent. or *E*.	*Enterococcus*
et al	and other people
HGT	Horizontal Gene Transfer
HLA	High-Level Aminoglycoside
HLG	High-Level gentamicin
HLSTR	High-Level Streptomycin
I	Intermediate
MDR	Multi-drug resistance
MGE	Mobile Genetic elements
MHA	Muller Hinton Agar
µg	microgram
min	minutes
VRE	Vancomycin Resistant Enterococci
WEF	World Economic Forum
WHO	World Health Organization
PAI	Pathogenicity Island
PCR	Polymerase Chain Reaction
R	Resistant
rpm	revolution per minute
S	Susceptible
s	seconds
spp.	species
UTIs	Urinary Tract Infections
ml	milliliter

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Background of study

Human population geometrically increases yearly and for this reason, human demands for food especially source of animal protein increases as well. In a bid to solve this problem, antibiotics are used (sub therapeutically and in overdose form) in animal production as growth boosters to increase the animal's muscle yield within the shortest possible time. This overuse of drug in animal care has given rise to many strains of bacteria which are resistant to antibiotics due to increased selective pressure, an example of these bacteria is antibiotic resistant *Enterococcus* bacteria. As the growth of these pathogenic bacteria is not inhibited due to the bacteria's resistance to the drugs administered, they live in the animal and survive even after the animal had been slaughtered and when such animal product is consumed by humans, bacterial infections and other adverse health conditions may follow. These multidrug-resistant bacteria are not only found in the gut of the animals but also in soils, waterbodies and the human environment and can also be transferred to humans through these sources. Infections due to antimicrobial resistant bacteria is one of the major world's health challenges and it is on the increase daily due to treatment failure (Castillo-Rojas *et al*., 2013; Golkar *et al*., 2014).

Enterococci are facultative anaerobic bacteria that are gram-positive, cocci-shaped and non-sporulating (Silva *et al*., 2012). They are now a member of the lactic acid bacteria although initially grouped under D *Streptococcus* bacteria genus; they are found to inhabit the human and animal gastrointestinal tract in a mutual relationship (Hollenbeck and Rice, 2012). They are important bacteria involved in the fermentation of cultured milk products being in the genus of lactic acid bacteria (Buhnik-Rosenblau *et al*., 2013). *Enterococci* can withstand and proliferate in adverse

environmental conditions and are therefore ubiquitously found free-living in waterbodies, plants, foods of animal origin, agricultural, hospital and living environment. Intrinsically, they are found resistant to so many antibiotic drugs and can also pick up and disseminate genes coding for antibiotic resistance to same or other bacteria species and humans (Ozdemir *et al*., 2011; Klibi *et al*., 2013). Chickens, cats, and dogs have been found to harbor *Enterococci* species in their gastrointestinal tract (Warnke *et al*., 2015). More than 40 species of *Enterococcus* have been identified (Murray *et al*., 2013) but *Enterococcus faecalis* and *Enterococcus faecium* are mostly recognized human pathogens (Hollenbeck and Rice, 2012). *E. cecorum, E. gallinarum, E. hirae, E. flavescens/casseliflavus,* and *E. durans* have also been reported in some studies as species of *Enterococcus* bacteria resistant to antibiotics (Brtkova *et al*., 2010; Armour *et al*., 2011; Dolka *et al*., 2016). The focus of this study is on two major species- *Enterococcus faecalis* and *Enterococcus faecium* due to their role in nosocomial infections.

Antibiotics are substances synthesized either naturally or chemically which aid in disease prevention and control in animal production by inhibiting the growth of the harmful microorganism (Lin *et al*., 2015). In human medicine, they are essential drugs used for successful surgical operations and other treatments but are now ineffective due to the emergence of resistance to a variety of antibiotics by bacteria species such as *Enterococci* bacteria (Lin *et al*., 2015). An example is seen in avoparcin. Avoparcin is a feed additive used in boosting the growth of animals and is an example of drugs in glycopeptide class of antibiotics but has been found to be involved in cross-resistance to vancomycin. It has therefore been banned from use in animal agriculture in various parts of the world (Allen *et al*., 2010; Cordero *et al*., 2012; Marinho *et al*., 2016). *Enterococci* bacteria are therefore of great importance to human health due to its intrinsic resistance to antibiotics which is conferred in part by genetic determinants known as resistance

genes carried on their chromosomes. Some of these genes are *aac(6')-Ie-aph (2'')-Ia, Van-A, ant(6')-Ia* and *aph(3')-IIIa* that are known to code resistance for gentamicin, vancomycin, streptomycin, and kanamycin respectively. Antibiotic resistance refers to the inability of an antimicrobial agent to inhibit the growth of microorganisms (Sreeja *et al.*, 2012). Researchers have found *Enterococci* bacteria to be resistant to glycopeptide, aminoglycoside and aminoglycoside classes of antibiotics (Khani *et al*., 2016; Beukers *et al*., 2017). Reports from WEF (World Economic Forum) on the global risk estimated yearly death of 25,000 and 23,000 persons in Europe and United State respectively because of infections due to antibiotic resistance pathogens (Hampton 2013; WEF 2013; WEF 2014; WHO 2014).

In addition to the problem of resistance to antibiotic drugs, *Enterococci* have the ability to harbor virulence genes on its chromosomes which increases the severity of infections caused by these pathogens. They are important factors in the pathogenesis of *Enterococcus* bacteria (Hollenbeck and Rice, 2012). Virulence genes refer to toxic substances chromosomally encoded by a bacterium which facilitates adherence to the host's cell, host colonization, host immune evasion and infection initiation (Jackson *et al*., 2011). Some virulence genes such as *E. faecalis* antigen A (*efaA*), gelatinase (*gelE*), extracellular surface protein (*esp*), sex pheromones (*ccf*) and cytolysin (*cylA*) have been detected in *Enterococci* (Comerlato *et al*., 2013; Medeiros *et al*., 2014).

1.2 Problem statement

Enterococci have been found to perform various important roles which include improving and maintaining intestinal flora balance (probiotics) of animals and humans and enhancing fermentation process of dairy products (Buhnik-Rosenblau *et al*., 2013). However, they are currently identified as one of the leading pathogens of many infections associated with hospital environments partly because of virulence factors they harbor. About 12% of all the acquired

hospital infections are caused by *Enterococcus* bacteria, they are ranked third most common cause of hospital infections globally (Yuen *et al*., 2014). They are known to cause such severe infections as urinary tract infections (UTIs), infections on wound surfaces, endocarditis, peritonitis (Yuen *et al*., 2014; Khani *et al*., 2016). The emergence of antibiotic-resistant *Enterococci* is another major health challenge globally, *Enterococcus* species have developed resistance to so many antibiotics used for treatment in humans and in veterinary services leading to untreatable infections, and death in severe cases (El-Halfawy *et al*., 2017). Prevalence of multi-drug resistant *Enterococci* is on the increase daily, this is in part caused by regular mutations in its genome, misuse of antibiotics and few or no new drugs (Peach *et al*., 2013; Blair *et al*., 2015).

1.3 Justification of study

Ever increasing prevalence of antimicrobial resistance and its accompanying infections caused by *Enterococci* and other bacterial pathogens is a great threat to human health worldwide (Cosentino *et al*., 2010). This, therefore, calls for an urgent need to develop novel antibiotics from time to time. Unfortunately, now that the novel antibiotics are mostly needed pharmaceutical companies are not venturing into the production of novel antibiotics due to lack of new metabolic pathways against targeted bacteria species, lack of funding and low market returns (Coates *et al*., 2011). For a better understanding of the biology of *Enterococci* bacteriaand to provide insights into its intrinsic and pathogenic complex processes, genetic characterization of genetic determinants of virulence traits and antibiotic resistance requires further investigation (Aslam *et al*., 2012; Yuen *et al*., 2014). *Enterococci* antimicrobial resistance ought to be studied using molecular approach. This would help to determine its prevalence and suggest control measures to mitigate human health risk associated with antimicrobial resistant *Enterococci*. Because *Enterococci* possess highly effective mechanisms of gene transfer, it is able to transfer resistance and virulence genes

horizontally to humans when they consume food products from animal origin that are contaminated (Kwon *et al*., 2012; Celik *et al*., 2017). Surveillance studies on the prevalence of *Enterococci* antimicrobial resistance and virulence profiles could help provide information for monitoring and planning intervention programs to control its further spread. Unfortunately, there is still paucity of data on *Enterococci* antimicrobial resistance in South Africa.

1.4 Aims

The aim of this research was to detect the occurrence and prevalence of *Enterococcus* species in chickens, cats and dogs cloacal, rectal and nasal swabs sampled from Durban, South Africa and to further access its phenotypic antimicrobial resistance profiles and genes coding for its antibiotic resistance and virulence traits.

1.5 Objectives

The objectives of this research were:

- to isolate *Enterococcus* bacteria from chickens, cats and dogs cloacal, rectal and nasal swabs using bacteria culture methods presumptively and confirming them via molecular approach using *Tuf* gene (genus specific) and *sodA* genes (species specific) through Polymerase Chain Reaction (PCR).
- to determine the profiles of resistances of the *Enterococcus* isolates to antibiotic drugs using disk diffusion antibiotic susceptibility test.
- to determine the incidence of genes coding for resistance and virulence in the *Enterococcus* species through PCR using various resistance and virulence gene primers.

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CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Enterococci are cocci-shaped, non-sporulating, gram-positive opportunistic bacterial pathogens found especially in immunocompromised individuals (Silva *et al*., 2012). They were known to enhance the fermentation process of milk, milk products, and other fermented food products; and are also found in symbiotic association in the gastrointestinal tracts of human and animals as they eliminate the pathogenic microbes leaving out the beneficial ones (Buhnik-Rosenblau *et al*., 2013). On the contrary, they have emerged among the leading pathogens of many infections acquired in hospital environment because they easily acquire and disseminate genes coding for antibiotic resistance with the aid of plasmids and transposons (Klibi *et al*., 2013) and have the intrinsic ability to become resistant to multiple antimicrobials (Cosentino *et al*., 2010 Khani *et al*., 2016). In addition, they are said to have highly effective resistance mechanism and possess virulence factors which increase the severity of their infections (Kwon *et al*., 2012; Celik *et al*., 2017).

2.2 Overview of Enterococcus bacteria

2.2.1 Classification of *Enterococcus* bacteria

Enterococci bacteria have been classified taxonomically into phylum, class, order, and family as Firmicutes; Bacilli; Lactobacillales and Enterococcaceae respectively (Carrero-Colon *et al*., 2011). Originally, they were grouped under D *Streptococcus* in 1899 by Thiercelin (Fisher and Phillips 2009) and was then classified into fecal *Streptococci* (Enterococci), viridians, dairy *Streptococci* and polygenous *Streptococci* by Sherman in 1937, but he later found out that the fecal *Streptococci* (*Enterococci*) is a member of Lancefield group D *Streptococci* due to the presence of D group antigen (Klein 2003; Foulquie Moreno *et al*., 2006). However, because of the primitive methods

used for classification, Lancefield group D *Streptococci* classification was not done accurately but with the development of genomic methods in 1984, it was reclassified under *Enterococcus* genus through sequencing of 16S rRNA and DNA hybridization (Fisher and Phillips 2009). More than 43 species have been identified under *Enterococcus* bacteria genus but among these species, two which are *Enterococcus faecalis* and *Enterococcus faecium* are the most commonly recognized pathogens of nosocomial infections (Alipour *et al*., 2014). *E. faecium* species are mostly found to be resistant to antibiotics while *E. faecalis* are well known for their pathogenicity due to the abundance of virulence genes (Rathnayake *et al*., 2012). *However, E. cecorum, E. casseliflavus/flavescens, E. hirae, E. durans* and *E. gallinarum* have also been reported as other antibiotics resistant Enterococcus species (Brtkova *et al*., 2010; Armour *et al*., 2011; Dolka *et al*., 2016).

2.2.2 Characteristics of *Enterococcus species*

Enterococci are facultatively anaerobic bacteria which do not form spores and are coccus shaped. When they act on glucose, lactic acid is given out as the primary product of metabolism hence are classified as lactic acid bacteria (LAB). As a member of the LAB, they have the characteristic feature of been catalase negative and gram-positive bacteria, they are known to survive harsh environmental conditions and can grow in 10 °C to 14 °C temperature ranges and high pH of about 9.6 (Araújo and Ferreira 2013). In bile esculin phenotypic test, they have been found to tolerate 6.5% salt concentration and grow in the presence of bile salt (40%), they can also hydrolyze esculin (Pruksakorn *et al*., 2016). *Enterococci* are known to be intrinsically resistant to antibiotics such as clindamycin, erythromycin, cephalosporins and quinolones class of antibiotics and can acquire resistance to glycopeptides, aminoglycosides, ampicillin and β-lactam classes of antibiotics (Celik *et al*., 2017). Intrinsic antibiotic resistance characteristics of *Enterococci* is conferred in part due

to the antibiotic resistance gene borne on their chromosome whereas the acquisition of resistance is triggered by the transfer of resistance genes to their mobile genetic elements (MGEs) from same or other species (Rathnayake *et al*., 2012). They are known to commonly reside in the guts of mammals. Due to their ubiquitous nature, they have been found in abundance in waterbodies, plants, and soils (Cosentino *et al*., 2010).

2.2.3 Importance of *Enterococcus* bacteria

Enterococcus bacteria have been found to play important roles in various ways and in different production processes. For cheeses and sausages, they enhance the flavor and in dairy products they aid the fermentation process and enhance the organoleptic properties (Banwo *et al*., 2013; Klibi *et al*., 2013). They act as probiotics in the gut of humans and animals where they maintain microbial balance and treat such infections as infectious diarrhea. However, they have been recently found to be opportunist pathogens especially in immunocompromised individuals (Avram-Hananel *et al*., 2010). Some studies have reported the use of *Enterococci* as probiotics for the treatment of diarrhea in cattle, pets, poultry and pigs (Bybee *et al*., 2011; Franz *et al*., 2011). Besides, they also produce bacteriocins, bacteriocins are antibiotics produced by *Enterococci* which code for peptides that exert bacteriostatic effects on genetically related species (Banwo *et al*., 2013). Cocolin *et al*., (2007) observed bacteriocin production in *Enterococcus faecium* isolated from dairy products. In addition to the production of bacteriocin is the production of enterocin which in fermentation process serve as starter culture and keeps the food product safe from harmful microorganisms (Javed *et al*., 2011). However, they are currently implicated in many hospital-associated infections such as wound infections, endophthalmitis, endocarditis, bacteremia, urinary tract infections and peritonitis (Silva *et al*., 2012; Comerlato *et al*., 2013; Jimenez *et al*., 2013; Yuen *et al*., 2014; Khani

et al., 2016). They have been found to cause 12% of all the hospital infections and are ranked the third most common nosocomial pathogens (Khani *et al*., 2016).

2.2.4 Causes of antibiotic resistance in *Enterococci*

Antibiotic resistance observed in *Enterococcus* spp. can be attributed to the following: extensive usage of antibiotics in agricultural processes, overuse of the antibiotics by humans and lack of new antibiotics. Lack of regulation of antibiotics use in many countries especially in developing countries has led to the indiscriminate purchase and use of the drugs without the doctor's prescription. Moreover, countries where usage of antibiotics is regulated, people still abuse its use through online order and purchase (Michael *et al*., 2014). Overuse of antibiotics has been found to be directly related to the antimicrobial resistance in *Enterococci* and other bacteria species (Read and Woods 2014).

In different parts of the world, animal agriculture incorporates extensive use of antibiotics for infection treatment, growth, and improvement of animal yield and quality. As these drugs are used incessantly, the bacteria's (pathogenic and commensal) growth is not inhibited, they develop resistance to them because of natural selection and leaves the drugs in unmetabolized form. These bacteria withstand processing heat and are transferred to humans with its accompanying infections when the contaminated food product is consumed as it serves as a reservoir of resistance genes. These antibiotic-resistant bacteria are also transferred and disseminated to soils and water through human and animal waste products (Bartlett *et al*., 2013). Additionally, antimicrobial resistance in *Enterococci* and other bacteria strains is worsen by lack of novel antibiotics. Lack of antibiotics is attributed to high cost of production, and lack of funding for research on the development of new antibiotics by the government (Piddock 2012; Itani and Shorr, 2014). Figure 2.1 illustrates the

relationship involved in the exchange of antibiotic resistance and infections between humans, the immediate environment and animals.

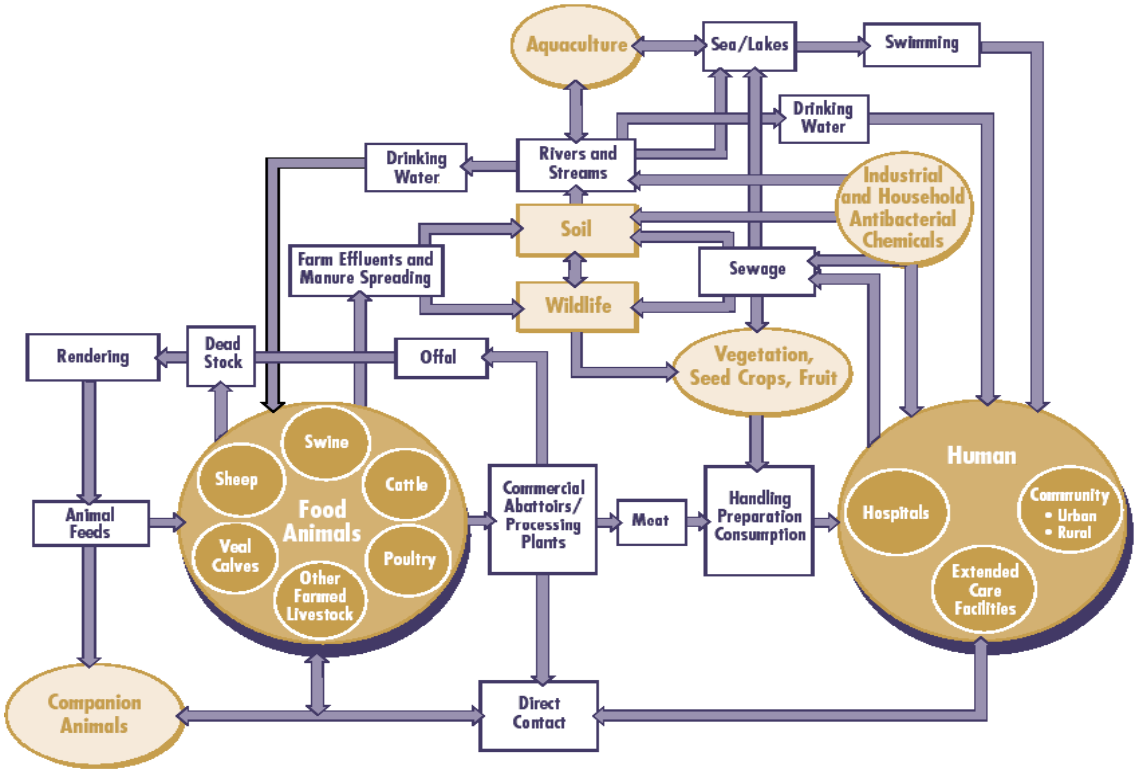

Figure 2.1: Relationship between humans, food animals and companion animals in resistance and virulence gene exchange (Adapted from Bbosa *et al*., 2014)

2.3 Identification of *Enterococcus* species

Proper identification of *Enterococcus* bacteria both at the genus and species level is very important in understanding its pathogenic processes to suggest possible remedies. Various methods of identifying *Enterococcus* bacteria include phenotypic tests, a series of biochemical test and molecular characterization (Schlaberg *et al*., 2012). The first two are time-consuming, limited to identification of few numbers of species because of the biochemical and phenotypic similarities of many *Enterococcal* species but molecular Characterization is more effective and accurate (Li *et*

al., 2012; Schlaberg *et al*., 2012). Repetitive sequence-based polymerase chain reaction and sequencing of the 16S rRNA gene are common molecular methods used in used in the identification of *Enterococcus* species. Non-automatization, high cost, a small volume of test samples and false positive results due to contaminations during gene amplifications are some limitations of 16S rRNA (Sontakke *et al*., 2009; Schlaberg *et al*., 2012). Therefore, *Tuf* gene primers are used for *Enterococcus* genus identification through polymerase chain reaction (Furlaneto-Maia *et al*., 2014; Gaber *et al*., 2015; Hassan and Belal 2016). *Tuf* gene is found to encode the elongation factor EF-TU which participates in peptide chain formation. It also contains the evolutionary genome of *Enterococcus* bacteria and is, therefore, a more efficient method of *Enterococcus* genus identification compared to 16S rRNA gene sequencing (Li *et al*., 2012). *SodA* genes which codes for manganese-dependent superoxide dismutase (Poyart *et al*., 2000) are used for identification of the different species of *Enterococcus* bacteria.

2.4 Classes of antibiotics

Antibiotics are drugs synthesized naturally or chemically which are used for disease prevention in human and animals as well as the elimination of harmful microorganisms (Allen *et al*., 2010). Usage of antibiotics began in 1928 when Alexander Fleming discovered penicillin which is the first antibiotic for the treatment of pneumonia and other bacterial infections due to the high death rate at that time. After the discovery of penicillin came the invent of prontosil, a sulfonamide class of antibiotics by Gerhard Domagk in the year 1935. Prontosil was effective against *Streptococcus* infections (Tortora, 2001). After 1935 many other classes of antibiotic drugs were developed, these drugs had a high lifespan and were very effective as they were found to reduce mortality rate due to bacterial infections (Arakawa, 2000). From the year 1949 to 1980 antibiotic drugs were been rapidly developed but from 1987 till date there had been a serious slowdown in the development

of novel antibiotic drugs (Silver, 2011). The heavy slowdown in the development of antibiotics over the years is due to decreased government funding for the discovery of antibiotics and increased regulatory requests on antibiotics which increased the cost of production thereby minimizing profits for pharmaceutical industries (Coates *et al*., 2011).

Antibiotics are classified on the basis of their mechanisms of action as follows: a) class 1 antibiotics, these are drugs that inhibit the synthesis of bacterial cell wall and it includes tetracyclines, β- lactams, penicillin, glycopeptides, and monobactams; b) class 2 antibiotics, these are drugs that inhibit the synthesis of the 50S and 30S protein. They include streptogramins, macrolides, and aminoglycosides; c) class 3 antibiotics, these are drugs that inhibit the synthesis of RNA and DNA an example is ansamycins; d) class 4 antibiotics, these are drugs that interfere with metabolic processes within bacterial cells (antimetabolites). They include quinolones and fluoroquinolones (Brunton *et al*., 2013). Table 2.1 depicts the representative drugs of these classes of antibiotics and their mechanisms of action.

Table 2.1: Classes of antibiotics and their mechanism of action

Classes of antibiotics	Representative drugs	Mechanism of action
β–Lactam	Phenoxypenicillin, oxacillin, amoxicillin, carbenicillin, piperacillin	Inhibits synthesis of cell wall in bacteria
Penicillins	Penicillin, ampicillin	Inhibits synthesis of cell wall in bacteria.
Monobactams	Aztreonam	Inhibits synthesis of cell wall in bacteria.
Aminoglycosides	Gentamycin, kanamycin, streptomycin	Inhibits synthesis of 30s and 50S proteins.
Glycopeptides	Vancomycin	Inhibits synthesis of cell wall in bacteria.
Ansamycins	Rifampin	Inhibits synthesis of RNA
Macrolides	Clarithromycin, erythromycin-H2O Oleandomycin, roxithromycin Spiramycin, tylosin	Inhibits synthesis of 30s and 50S proteins.
Streptogramins	Quinupristin-Dalfopristin	Inhibits synthesis of 30s and 50S proteins.
Tetracyclines	Chlortetracycline, demolocycline Doxycycline, oxytetracycline Tetracycline	Inhibits synthesis of cell wall in bacteria.
Chloramphenicol	Chloramphenicol	Inhibits the synthesis of proteins.
Quinolones	Oxolinic acid, nalidixic acid, pipemidic acid, flumequine, pefloxacin	Inhibits the replication of DNA.
Fluoroquinolones	Ciprofloxacin, norfloxacin, ofloxacin, enrofloxacin, enoxacin, sarafloxacin, danofloxacin, dofloxacin, lemofloxacin	Inhibits the replication of DNA.

(Adapted from Gothwal and Shashidhar, 2015)

2.5 Antibiotic resistance in *Enterococci*

In the past two decades, antimicrobial resistant strains of bacteria emerged. It has deprived us of the health benefits we derived from the drugs initially (Ferri *et al*., 2017). Antibiotic resistance refers to a phenomenon whereby bacteria species are not susceptible to the antibiotic drugs used against them (Magiorakos *et al*., 2012). The first case of antimicrobial resistance in bacteria species was first observed in *Staphylococcus aureus* against penicillin in 1945 (Capita and Alonso-Calleja,

2013). As *Staphylococcus aureus* developed resistance to penicillin, erythromycin was used alternatively for treatment of *Staphylococcus* infections, but it later developed resistance to it as well. Subsequently, resistance was observed in other antibiotics such as chloramphenicol and tetracycline by multiple drug-resistant bacteria (Ferri *et al*., 2017).

Enterococcus species exhibit intrinsic resistance to several antibiotics but are also able to pick up and disseminate antibiotic resistance genes through their mobile genetic elements (MGEs) (Ventola 2015). *Enterococci* antibiotic resistance also take place due to regular mutations in the genome of the bacteria, which makes the drug to miss its target. Natural selection, a situation whereby bacteria species that are resistant to antibiotics proliferates and displace the susceptible species sets in because of antibiotic resistance (Read and Woods, 2014). *Enterococcus* species have been reported to exhibit resistance to glycopeptide antibiotics, β lactam antibiotics and aminoglycoside antibiotics (Sreeja *et al*., 2012; Khani *et al*., 2016).

β lactam antibiotics are antibiotic drugs that have β lactam ring in its molecular structure, examples include ampicillin, penicillin, and amoxicillin. The resistance of *Enterococcus* species to β lactam antibiotics has been found to be as a result of β lactamase enzyme production and mutations found in penicillin-binding proteins (PBPs). *Enterococcus* species are said to exhibit low resistance to these drugs therefore, β lactams antibiotics are used in combination with aminoglycosides to treat bacterial infections due to *Enterococcus* species (Garrido *et al*., 2014).

Aminoglycosides are antibiotic class active against bacteria species by attaching to the A site of 16S rRNA of the bacteria, they are known to cure enterococcal infections and they include kanamycin, streptomycin, gentamycin, and neomycin antibiotic drugs. Mechanism of resistance of *Enterococcus* species to aminoglycosides involves mutations, pumping out of ribosomal

proteins and modification of the drug target sites by aminoglycoside-modifying enzymes (AMEs). *Enterococcus* species have been reported to be exhibit high (72.37%) resistance to high-level streptomycin antibiotics as well as high-level gentamycin (Padmasini *et al*., 2014).

Tetracyclines are another class of antibiotics used to treat bacterial infections, they function by binding to the 30S ribosome subunit reversibly giving rise to inhibition of protein synthesis by the bacteria. However, *Enterococci* become resistant to this antibiotic using the efflux mechanism which involves pumping out of the drugs from the bacteria. Tetracycline resistance is conferred by *tet* genes (such as tetA and tetB) (Bbosa *et al*., 2014).

Glycopeptides are antibiotics which function by inhibiting the synthesis of bacterial cell wall, examples of drugs that fall under this group of antibiotics are vancomycin and teicoplanin. Resistance mechanism of *Enterococci* to glycopeptide involves mutation in peptidoglycan found in the cell wall, which results in thickened cell wall thereby limiting drug access to D-Ala-D-Ala (D-Alanyl-D-Alanine) peptide which enhances synthesis of peptidoglycan. Of great importance in *Enterococci* glycopeptide resistance is the rise of strains of *Enterococci* that are resistant to vancomycin antibiotics which is more prevalent in intensive care units (ICU's) of many hospitals. About 34-times increase in the incidence of strains of *Enterococci* that are resistant to vancomycin was recorded in the US (Amberpet *et al*., 2016).

2.6 Mechanisms of antibiotic resistance in *Enterococci*

Various mechanisms involved in antibiotic resistance in *Enterococci* are the alteration of the drug target site, efflux (pumping mechanism), impermeability of the drugs to the bacteria cell wall and drug modification by enzymes. Alteration of the drug target site is an antibiotic resistance mechanism in *Enterococci* whereby the drug target site is changed by the bacteria thereby

rendering the drug ineffective. An example of this resistance mechanism is the ribosomal mutation of aminoglycoside-resistant *Enterococci* (Blair *et al*., 2015). Efflux or pumping mechanism involves the export of antibiotics out from the bacterial cell to prevent it from reaching the targeted site of action. This is a resistance mechanism used against fluoroquinolones and tetracycline antibiotics by bacteria species (Lin *et al*., 2015). Impermeability of the drugs to the bacterial cell wall is a mechanism of antibiotic resistance whereby *Enterococci* produce biofilms which cross its cell wall and prevent penetration of the drugs. Biofilm producing bacteria are known to exhibit high resistance to antibiotics, this usually occurs in β lactam drugs resistance. For drug modification, *Enterococci* secret β lactamase enzyme which renders the drugs infective by hydrolyzing β lactam ring of the drugs. Resistance by drug modification is triggered by chromosomal mutation and resistance gene acquisition via plasmids and transposons (Bbosa *et al*., 2014). Figure 2.2 illustrates resistance mechanisms to antibiotics in Enterococci.

Transfer of genes coding for antibiotic resistance is done via vertical and horizontal routes. Transfer of antibiotic resistance gene via vertical route involves the transfer of the resistance genes between cells of same bacterial species while that of horizontal transfer refers to the transfer of resistance genes between two different bacterial species or humans with the aid of mobile genetic elements (Gothwal and Shashidhar, 2015). Processes of horizontal resistance gene transfer include conjugation, transduction, and transformation. Conjugation is a process of horizontal gene transfer that involves exchange of plasmids between two bacterial cells directly in contact with each other in a mating process which are sexually different, transduction refers to resistance gene transfer by bacteriophages into a bacterium while transformation refers to picking up and insertion of naked DNA into a bacteria's genome or plasmid from another bacteria. (Cytryn 2013).

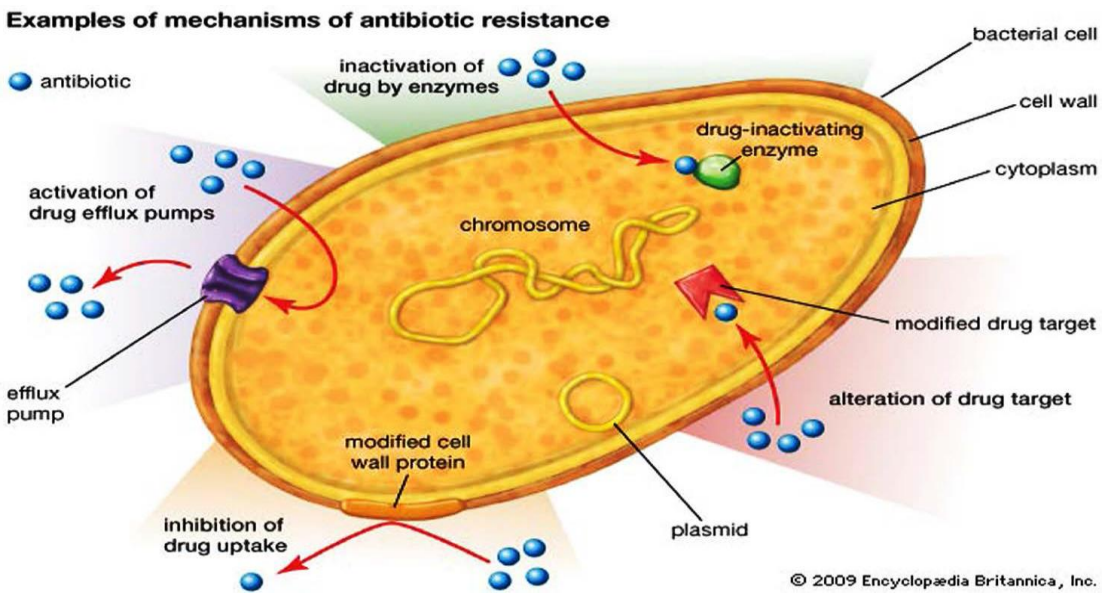

Figure 2.2: Mechanisms of antibiotic resistance (Adapted from Bbosa *et al*., 2014)

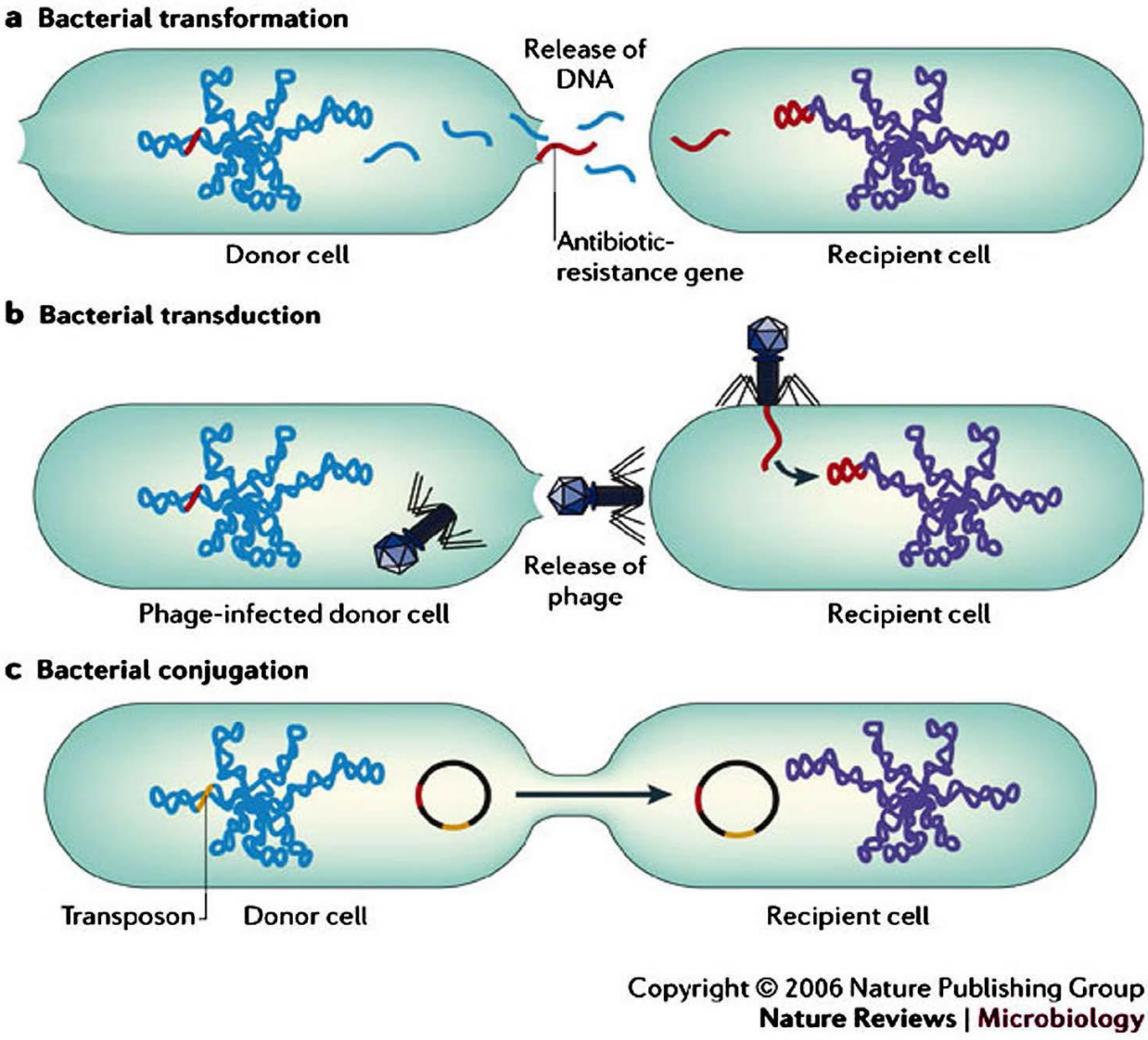

Figure 2.3.: Mechanisms of horizontal gene transfer in bacteria (Adapted from Bbosa *et al.*, 2014)

2.7 Incidence of antimicrobial resistance phenotypes and genotypes in *Enterococci* from chickens, cats and dogs

In animal agriculture, antibiotics are used intensively for growth promotion, prophylactic and therapeutic purposes. Consequently, these drugs are misused as they are administered in an overdosed form and sub-therapeutically leading to selective pressure on the normal commensal

bacteria in the gut of the animals which in turn results to antimicrobial resistant bacteria with accompanying infections (Teymournejad *et al*., 2015). It has been reported that the indiscriminate use of antibiotics corresponds to increase in antibiotic resistance and the resistance phenotypes are conferred by the resistance genes (Beerepoot *et al*., 2011; den Heijer *at al*., 2012). These antimicrobial resistance bacteria pathogens can be transferred to humans when contaminated foods are consumed and the physical contact of pets with their owners (Olsen *et al*., 2012). The occurrence of *Enterococci* has been reported in cats, dogs, and chickens, in a study by Abdel-Moein *et al*., (2017), *E. faecalis* was detected in 3.2% and 5.5% of dog and cat isolates and *E. faecium* 22.2% and 15.8% from dogs and cats. Among the *E. faecium* isolates, 17.5% from the dog and 5.3% were found resistant to ampicillin. All the ampicillin resistant *E. faecium* were multidrug resistant. A research on antibiotic resistance from fecal *Enterococci* isolated from sheep, poultry and beef, 87 of 96 samples were found positive for *Enterococcus* species of which 46% were *E. faecium* and *E. faecalis* 5%. Tetracycline, erythromycin, gentamicin, streptomycin, kanamycin, and ciprofloxacin resistance were observed in the *Enterococcus* isolates. Erythromycin, tetracycline, gentamicin, kanamycin, and streptomycin resistance genes were detected (Klibi *et al*., 2013). In Celik *et al*., (2017), 100% of dog and cat *E. faecium* showed resistance to the following drugs- ampicillin, tetracycline, and penicillin. High resistance rates were also recorded in rifampicin, erythromycin, streptomycin, gentamycin, and ciprofloxacin. In an investigation of the occurrence of antimicrobial resistance of *Enterococcus* from poultry farms by Ngbede *et al*., (2017), 42.8% of the samples were found positive for *Enterococcus*. Low to high rates of resistance was observed in gentamicin, ampicillin, erythromycin, and tetracycline. About 53.1% of all the *Enterococcus* isolates exhibited multiple drug resistance. The following genes: *tetK, tetL, tetM, tetO* and *ermB* coding resistances for tetracycline were detected.

2.8 Virulence genes in *Enterococci*

Virulence genes are toxic substances borne on the chromosomes of bacteria which increases the severity of their infections (Jackson *et al*., 2011). The pathogenesis of *Enterococci* bacteria includes attachment to the host cell, colonization and immune evasion of the host (Kwon *et al*., 2012). *E. faecalis* antigen A -*efaA* gene, gelatinase -*gelE* gene, hyaluronidase -*hyl* gene, aggregation substance -*asa1* gene, cytolysin -*cylA* gene, extracellular surface protein -*esp* gene and adhesion of collagen -*ace* gene are virulence genes found in *Enterococcus* species (Comerlato *et al*., 2013; Medeiros *et al*., 2014). *esp* gene (extracellular surface protein) occupies a large pathogenicity island (PAI) of *Enterococcus* species. It is a cell wall protein that aids in the attachment of *Enterococcus* species to its host during the infection process. *Esp* virulence gene is known to cause infections in the urinary tract and involved in the formation of biofilm. *ace* gene (Adhesion of collagen) is also involved in colonization and adherence of *Enterococci* to proteins of cell matrix of the host during the infection process. The proteins are collagen I, collagen IV and laminin, ace virulence gene is implicated in endocarditis infections and is more prevalent in *E. faecalis*. asa1 gene (Aggregation substance) is known to enhance the aggregation of *Enterococcus* species during conjugation and is induced by sex pheromones, this virulence gene is also more prevalent *E. faecalis* (Schlievert *et al*., 2010). *gelE* (Gelatinase) is a virulence gene found in *Enterococci* bacteria that hydrolyze casein, hemoglobin and gelatin, it is *Enterococci* zinc metalloprotease and extracellular protein (Lindenstrau *et al*., 2011). *Cyl* (Cytolysin) virulence gene is conferred by *cyLS, cylR1, cylLL, cyl1, cylB cylR2, cylM* and *cylA* operons in *Enterococci*, it is known to cause lysis of red cells in the host (Chuang-Smith *et al*., 2010). *E. faecalis* antigen A (*efaA*) is a virulence gene in *Enterococci* that helps it colonize the host and cause infections. It is found to cause peritonitis infection (Kafil *et al*., 2016). Sex pheromones (*ccf*) virulence gene is

found to be responsible for conjugative plasmid transfer between the bacteria cells through horizontal gene transfer (Sava *et al*., 2010). In a study on food animals, *esp, ace*, and *gelE* virulence factors were detected at a frequency of 10.5%, 4.6%, and 11.5% respectively. *Hyl* gene was not detected (Klibi *et al*., 2013). In another study on pets by Celik *et al*., (2017), *efaA* and *gelE* virulence genes were detected at the rate of 13.8% and 11.1% respectively while *esp*, and *ace* genes were not detected. Jimenez *et al*., (2013) detected a high incidence of *ccf* gene amongst Enterococcus faecalis isolates.

2.9 Biofilm formation in *Enterococci*

Biofilms refer to complex structures of biological systems which are produced by microorganisms to enable them to cope with adverse conditions in the environment (Piggot *et al*., 2012). Biofilms are embedded with slimy substances called Extracellular Polymeric Substances (EPS), EPS enables bacteria species to attach to the host's cell and other surfaces. With the aid of biofilm formation, *Enterococcus* species adhere to the host's gut matrix of extracellular proteins and initiate an infection. Biofilms are involved in antibiotic resistance in microorganisms and known to increase the severity of infections by pathogens (Mika *et al*., 2009; Osman *et al*., 2015; Abdullahi *et al*., 2016). About 80% of infections caused by bacteria are due to the formation of biofilm in bacterial pathogens. Additionally, infections due to bacteria biofilms in livestock farms are said to amount to huge economic losses. Biofilm forming bacterial pathogens are known to cause such infections as wound and urinary infections (Garcia and Percival 2011; Zambori *et al*., 2012).

Processes involved in biofilm development are the formation of conditioning film, attachment to cell surfaces, the formation of microcolonies, matrix polymers expression and dispersal of the cells (McDougald *et al*., 2008). Firstly, the conditioning film is formed to serve as a base for attachment

to surfaces. Polysaccharides, glycoprotein and humic compounds are found in the conditioning film, their function is altering the chemical properties of the substratum. Formation of conditioning film is said to be enhanced by tears, blood components, urine and saliva from animals (Percival *et al*., 2011). Attachment to cell surfaces involves reversible and irreversible attachment. Reversible attachment to cell surface refers to weak attachment to the host cell or surfaces while irreversible attachment to cell surface refers to strong or permanent attachment to the host cell or surfaces. Reversible attachment to the cell surface is a mechanism used by bacteria to overcome the scarcity of nutrients in the host. Fimbriae and pili are bacterial structures used for attachment during the development of biofilm (Karatan and Watnick 2009; Abdullahi *et al*., 2016). Attachment to cell surfaces is proceeded by the formation of microcolonies, recruitment and cell division takes place giving rise to formation and growth of microcolonies. Microcolonies are found in production and expression of extracellular polymers which produce aminoglycoside modifying enzymes and beta-lactamase. (Hoiby *et al*., 2010; Qin *et al*., 2012). Finally, colonies of cells formed separates and disperses into many environments. This is said to enhance the ability of pathogens to overcome adverse environmental conditions, promote dissemination of infections and genetic diversity of the bacteria (Percival *et al*., 2011). The formation of biofilm is illustrated in Figure 2.4.

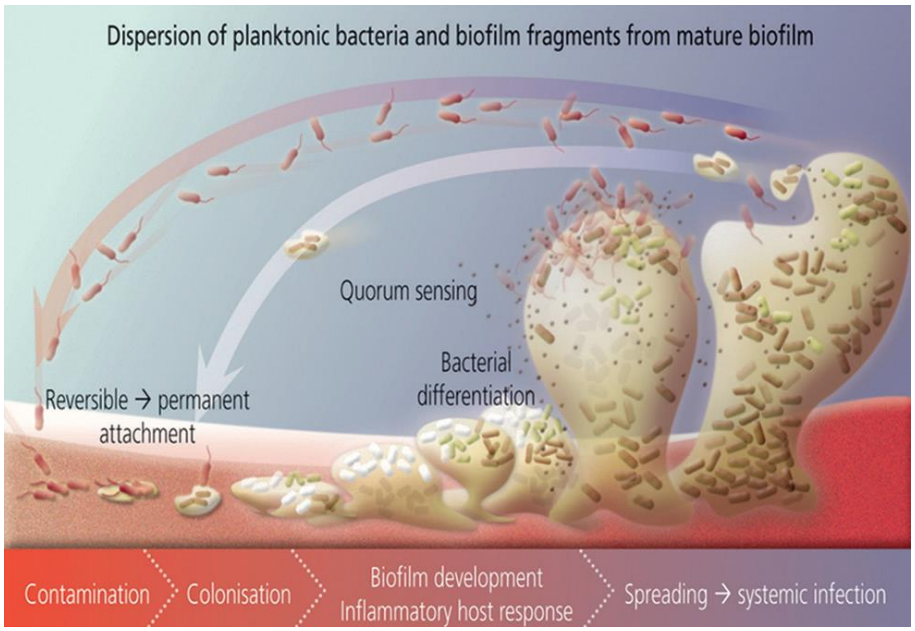

Figure 2.4: Development of biofilm (Adapted from Abdullahi *et al*., 2016)

2.10 Economic importance of antibiotic resistant *Enterococci*

Antibiotic-resistant *Enterococci* pose great health challenge and huge burden economically in a nation. When enterococcal infections are resistant to antibiotics, it makes treatment options expensive to both the individual and the nation because it leads to a longer hospital stay, increased health care cost, long-term disability and loss of productivity (Ventola, 2015). *Enterococci* are pathogens of many hospital-associated infections like the wound, urinary tract and bloodstream infections. Of great importance are infections due vancomycin-resistant strains of *Enterococci*, as vancomycin antibiotics are last alternative antibiotics used for treating severe enterococcal infections in humans making infections more severe and very challenging. VRE (Vancomycin-resistant *Enterococci*) is mostly caused by *Enterococcus faecium* and occasionally *Enterococci faecalis*. It was reported that in the US that two million individuals suffer from bacterial infections due to antibiotic resistance and 99,000 deaths occurs yearly (CDCP, 2013). Thirty (30%) of these overall hospital-acquired *Enterococci* infections in the US is due to VRE infections which is

usually accompanied by 1,300 deaths yearly (CDCP, 2013; Ventola, 2015). A total cost of $20 billion and $35 billion productivity loss was estimated to be incurred because of antibiotic resistance infections (CDCP, 2013; Lushniak, 2014). In Europe, 25, 000 deaths were said to occur yearly while 23,000 deaths occurred yearly in the US because of human health risk associated with antibiotic resistance infections (Hampton, 2013; WEF, 2013; WHO, 2014).

2.11 Antibiotic usage and *Enterococci* antibiotic resistance in South Africa

In Africa, antibiotics are known to be the most commonly used drugs as approximately 90.1% people indulge in self-medication (Kimanga, 2012). Developing countries such as South Africa rely heavily on antibiotics for animal production although information on the volume of its use is limited (Henton *et al*., 2011). In South Africa, antibiotics are mostly used in poultry and pig farming for disease prevention and growth promotion (Eagar *et al*., 2012). Tylosin, macrolides, tetracyclines, sulphonamides, and penicillin are mostly sold (Henton *et al*., 2011). Twelve percent of the drugs mentioned above are administered in water while 68.5% are administered in their feed (Eagar *et al*., 2012). This, however, constitutes public health crises as farmers administer them without veterinarian's prescription (Carlet *et al*., 2012). As South Africa has the highest burden of immune-compromised individuals with HIV and Tuberculosis, these bacterial infections are more severe. (Moyane *et al*., 2013). A study on *Enterococcus* species from dairy cattle in Eastern Cape recorded the prevalence of *Enterococcus* species in 341 of 400 samples of which 100% enterococcal isolates expressed resistance to vancomycin, 99% and 94% resistance rates recorded in erythromycin and streptomycin. The isolates were also found to harbor, *gelE* and *esp* virulence genes at high prevalence rate (Iweriebor *et al*., 2016). In a study in Cape Town South Africa, eight out of 55 patients were colonized with VRE. The VanA gene was detected among all the isolates

except one. More so, four patients were confirmed positive for VRE bloodstream infections (Lochan *et al*., 2016).

Durban is a city located in a province of South Africa called KwaZulu-Natal, made up of industrial communities and intensive farming activities. It is found on the East coast of the nation. The intensive farming system and industrial activities may, however, lead to the emergence and spread of genes coding for antibiotic resistance in humans and animals (Lin *et al*., 2004). Few studies have been done on *Enterococci* antimicrobial resistance but there is limited information in Durban. Of importance is the presence of Beach which makes it well known for recreational activities. Moreover, it has been found that the pollution of this beach by antibiotic-resistant organisms such as *Enterococci* pose a great threat to public health and tourist activities (Mardon and Stretch, 2004).

In conclusion, literatures reviewed show that there is a persistent increase in *Enterococci* antibiotic resistance and other bacteria worldwide. Because of the adverse effects of antibiotic resistance in humans and animals, action needs to be taken to monitor and control its further emergence and spread. There is need therefore to venture into molecular diagnostics to understand the epidemiology of this emerging clinical pathogen and suggest possible solutions to mitigate the adverse health and economic burden associated with antibiotic-resistant infections.

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CHAPTER 3

Manuscript 1

Molecular detection and antibiotic resistance of *Enterococcus* species isolated from animals

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CHAPTER 3

Molecular detection and antibiotic resistance of *Enterococcus* species isolated from animals

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Abstract

Antimicrobial resistant *Enterococci* (ARE) is one of the leading public health crises due to regular mutations in its genome and lack of novel drugs resulting in untreatable infections and death in severe cases. It is, therefore, crucial to monitor the prevalence of ARE to mitigate its adverse effects. Hence, in our study, the purpose was to survey the occurrence of *Enterococcus* antibiotic resistance phenotypes and genotypes from chickens and pets in Durban. Overall, *Tuf* gene was detected in 109 (77.3%) samples. *Enterococcus faecalis* was highly detected in all the animals ($P < 0.05$) at a frequency of 40.4% whilst *Enterococcus faecium* was detected at a lower frequency, 8.5%. Chicken samples showed the highest frequency of resistance to the antibiotics compared to pets. Highest resistance frequency was observed in Quinupristin/Dalfopristin 89.4% followed by Vancomycin 87.9%, Rifampicin 85%, Ampicillin 76.6%, Erythromycin 72.3%, and Tetracycline 64.5%. Chloramphenicol 24.8%, High-Level Streptomycin 24.1%, and Ciprofloxacin 14.2%. Eighty-four (84%) of the *Enterococcus* isolates expressed multidrug resistance. Out the four resistance genes screened, 21.3%, 7.8% and 4.3% for Kanamycin, Streptomycin, and Vancomycin resistance genes respectively were present. These findings show that chickens, cats, and dogs *Enterococcus* isolates harbor resistance genes and were resistant to multiple antibiotics. The study further demonstrated an association between resistance genes and different animal species in this

investigation. It is therefore important that antibiotics be used prudently in animal husbandry to mitigate emergence and transfer of antibiotic resistance zoonotically.

Keywords: Antibiotics; *Enterococcus*; resistance genes; susceptibility; nosocomial infections.

Significance of study:

- Incidence of resistance to antibiotics in *Enterococcus* species from chicken and pets in South Africa is largely unknown.
- High resistance rates to critically important antibiotics used for treatment in human medicine in this study such as rifampicin calls for serious attention.
- Chickens and pets were found to harbor genes coding for antibiotic resistance and there is a possibility of transferring these genes to humans.

Running headline: Antibiotic resistance profiles of *Ent*. spp.

3.1 Introduction

Antibiotics are essential drugs used in medical and veterinary practices for therapeutic and prophylactic purposes. However, its misuse in human therapy and animal agriculture gave rise to multidrug-resistant bacteria in addition to the acquisition of plasmid, pathogenicity island and chromosomal mutations. Misuse of these agents led to evolution of bacteria species with high resistance to the drugs both phenotypically and genotypically (Teymournejad *et al*., 2015). Multidrug-resistant bacteria prevalence is on the increase daily and is a great public health threat worldwide due to infection treatment failure especially in immunocompromised individuals and few or no novel antibiotics (Pesavento *et al*., 2014).

Enterococcus genus group of bacteria have emerged as one of the life-threatening multidrug-resistant pathogens globally. They are opportunistic, gram-positive, cocci shaped, non-sporulating bacteria species (Silva *et al*., 2012). Although known to be gut commensal bacteria of humans and animals and playing several important roles as: improvement of organoleptic quality and shelf life of cheese and fermented products; indicator bacteria; probiotics (Pesavento *et al*., 2014; Barbosa *et al*., 2014; Pieniz *et al*., 2015), they have gained peculiar healthcare attention as they are now confirmed pathogenic agents for such nosocomial ailments as endophthalmitis, peritonitis, endocarditis, urethritis, bacteremia among others (Arias and Murray 2012; Olsen *et al*., 2012). Commonest reported *Enterococcus* species that are causative organisms for nosocomial infections are *Enterococcus faecalis* and *E*nterococcus *faecium* (Alipour *et al*., 2014). This is because of their persistence and survival in extreme environmental conditions (Boehm and Sassoubre 2014). Also, their intrinsic ability to become resistant to antibiotics like beta-lactams and streptogramins (Prieto *et al*., 2016), and their propensity to receive and disseminate antibiotic resistance genes through their mobile genetic elements to antibiotics like glycopeptides, tetracyclines, macrolides,

aminoglycosides and lincosamides through horizontal gene transfer (HGT) process (Hollenbeck and Rice 2012; Higuita and Huyck 2014; Yuksel *et al*., 2015; Prieto *et al*., 2016). *Enterococci* become resistant to antibiotics through the following mechanisms i) alteration of target sites, which has to do with changes in the target sites of the drugs by the bacteria rendering it ineffective and resulting in resistance; ii) impermeability which involves cell wall crossing through biofilm production also promoting antimicrobial resistance; iii) enzymatic modification which involves producing enzymes which inactivate the antibiotics and iv) efflux which involves pumping out of the antibiotics from their cells using pumping mechanisms (Bbosa *et al*., 2014; Blair *et al*., 2015; Lin *et al*., 2015). Additionally, *Enterococci* harbor genes on their chromosomes which are responsible for their intrinsic resistance to antibiotics referred to as antibiotic resistance genes. Antibiotic resistance genes are genes born on the chromosome of bacteria, they code for the production of proteins that inhibit the bacteriostatic effect of antibiotics (Blair *et al*., 2015). Some of these genes are *aac(6')-Ie-aph (2'')-Ia, Van-A, ant(6')-Ia* and *aph(3')-IIIa* genes are known to code resistance for gentamicin, vancomycin, streptomycin, and kanamycin respectively.

It is noted that South Africa is one of the nations that have the high rate of antibiotics usage (Van Boeckel *et al*., 2014). It is very important to monitor the resistance profile of the various antibiotics in bacteria to provide information that can guide Health Practitioners, Veterinarians, and Livestock production industries to judiciously use antimicrobial agents in animals and humans to reduce the rate of development and mitigate the adverse effects of antimicrobial resistance (Cummings *et al*., 2013). More so, the increasing risk of antimicrobial infection transmission via poultry slaughterhouses and direct contact of pets with their owners (Bagcigil *et al*., 2015) reinforces the importance of this study. *Enterococci* antimicrobial resistance has been reported in wastewater, pig farms and clinical environments in South Africa (Iweriebor *et al*., 2015; Molale and

Bezuidenhout 2016; Mahabeer *et al*., 2016). Here we report the prevalence of *Enterococci* antimicrobial resistance phenotypes and genotypes by determining the antibiotic resistance profiles and screening resistance genes from *Enterococcus* species isolated from chickens, cats, and dogs in Durban, South Africa.

3.2 Materials and Methods

3.2.1 Sample collection

Cloacal, rectal and nasal swabs from chickens, cats and dogs were sampled from poultry abattoir and veterinary clinics within Durban metropolis of South Africa between November 2017 and March 2018. One hundred and fifty (150) animals were sampled comprising 50 chickens (30 males and 20 females), 26 cats (10 males and 16 females) and 74 dogs (33 males and 44 females). The samples were collected with sterile swabs and 15 ml sampling tubes containing buffered peptone water on ice storage before taken to the laboratory for analysis. The samples were then analyzed immediately upon arrival to the laboratory.

3.2.2 Isolation of *Enterococcus* species

Isolation of *Enterococcus* species was done using microbiological analysis. This involved enrichment of the samples by adding buffered peptone water (10 ml) to each of the tubes and incubating the samples for a period of 24 hours at 37 °C. This was followed by inoculating 1 ml of the resultant culture into a broth medium (Brain Heart Infusion, BHI) and incubating for a period of 24 hours at 37 °C. Afterward, the culture was inoculated onto Bile Esculin Agar (BEA) (*Enterococcus* selective media) by streaking and grown for 24 hours in an incubator set at 37 °C. *Enterococcus* growth was distinguished by halo black colonies. Pure cultures were then obtained by inoculating and incubating the colonies in the BHI broth medium for a period of 24 hours at 37

°C. DNA extraction and antimicrobial susceptibility tests were done using the resultant culture while the remaining culture was perpetuated using 25% glycerol stock at -80 °C temperature for use in future.

Extraction of DNA for all the *Enterococcus* positive isolates was done using the BHI broth culture according to Ruiz-Barba *et al*., 2005.

3.2.3 Confirmation of *Enterococcus* by Polymerase Chain Reaction

Tuf gene (*Enterococcus* genus-specific gene) presence was confirmed for the presumptive *Enterococcus* species isolates using primer sequences shown in Table 3.1. The polymerase chain reaction was 25 µl volume reaction mix that contained 12.5 µl DreamTaq Green master mix, 5 µl DNA, 5.5 µl dH_20 and 1 µl forward and reverse primers respectively. Amplification of *Tuf* gene was done in a thermocycler with the protocol presented in Table 3.2. After PCR, running of gel electrophoresis was done on 1.5% agarose at 100 volts for a period of 30 min. The bands were visualized using BIO-RAD, ChemiDoc^{TM}MP gel imaging system.

TABLE 3.1: Primer sequences used for *Enterococcus* genus, species confirmation and screening of resistance genes in *Enterococcus* species from chickens, cats, and dogs

Antibiotics	**Target gene**	**Primer sequence (5'→3')**	**Product size (bp)**	**Resistance mechanism**	**Reference**
Gentamicin	*aac (6')-Ie-aph (2'')-Ia*	Forward: CAGAGCCTTGGGAAGATGAAG Reverse: CCTCGTGTAATTCATGTTCTGGC	348	Efflux and target alteration	Vakulenko *et al*., (2003).
Vancomycin	*Van-A*	Forward: GTAGGCTGCGATATTCAAAGC Reverse: CGATTCAATTGCGTAGTCCAA	231	Reprogramming peptidoglycan and biosynthesis	Furlaneto-Maia *et al*., 2014.
Streptomycin	*ant (6')-Ia*	Forward: ACTGGCTTAATCAATTTGGG Reverse: GCCTTTCCGCCACCTCACCG	577	Efflux and target alteration	Sepulveda *et al*., (2007).
Kanamycin	*aph (3')-IIIa*	Forward: GGCTAAAATGAGAATATCACCG Reverse: CTTTAAAAAATCATACAGCTCGCG	523	Efflux and target alteration	Padmasini *et al*., (2014).
	Tuf	Forward: TACTGACAAACCATTCATGATG Reverse: AACTTCGTCACCAACGCGAAC	112		Ke *et al*., (1999).
	SodA (E. faecalis)	Forward: ACT TAT GTG ACT AAC TTA ACC Reverse: TAA TGG TGA ATC TTG GTT TGG	360		Jackson *et al*., (2004).
	SodA (E. faecium)	Forward: GAA AAA ACA ATA GAA GAATTAT Reverse: TGC TTT TTTGAA TTC TTC TTT A	215		Jackson *et al*., (2004).

3.2.4 Detection of *Enterococcus faecalis* and *Enterococcus faecium*

The two species- *E. faecium* and *E. faecalis* was screened in the samples using the *SodA* genes. Primer sequences for these genes are also shown in Table 1. *SodA faecalis* gene amplification was done according to Alipour *et al*., 2014 in a thermocycler using 35 cycles while *SodA faecium* gene amplification was carried out with the protocol presented in Table 2.

3.2.5 Antibiotics susceptibility test

Ten (10) antibiotics: Rifampicin (RD 5 µg), Ampicillin (AMP 10 µg), Chloramphenicol (C 30 µg), Vancomycin (VA 30 µg), Gentamicin (CN 200 µg), Tetracycline (TE 30 µg), Streptomycin (S 300 µg), Ciprofloxacin (CIP 5 µg), Quinupristin/ Dalfopristin (QD 15 µg) and Erythromycin (E 15 µg) were used for susceptibility test using disc diffusion method by Kirby-Bauer. The selection of these antibiotics was based on their extensive use in poultry production, veterinary services, and treatment of human infections. Glycerol stocks of the *Enterococcus* isolates were recovered by inoculating and incubating a loopful of the stocks into the BHI broth. The resultant culture was spread on Muller-Hinton agar (MHA) plates with the aid of a glass spreader. Lastly, the different discs of antibiotics were placed on the MHA and incubated. Zones of inhibition were measured and reported as resistant (R), intermediate (I) and susceptible (S) using the guidelines of CLSI (2016) and BSAC, CLSI stands for Clinical and Laboratory Standards Institute while BSAC stands for British Society for Antimicrobial Chemotherapy. BSAC guideline was used for the high-level aminoglycosides. Multiple drug resistance (MDR) was observed and recorded when an isolate expressed resistance to at least three antibiotics.

3.2.6 Detection of antibiotic resistance genes

Van-A (glycopeptide gene); *aac(6')-Ie-aph(2'')-Ia; ant(6')-Ia* and *aph(3')-IIIa* (aminoglycosides genes) coding resistance for vancomycin, gentamicin, kanamycin and streptomycin were detected.

Table1 shows primer sequences used for the screening of these resistance genes. Amplification for the aminoglycoside resistance genes was done according to Hassan and Belal (2016) procedure in a thermocycler using 35 cycles while amplification for *Van-A* gene was carried out with the protocol presented in Table 2.

TABLE 3.2: PCR amplification protocols for *Tuf, SodA faecium*, and *VanA* genes

Genes	Number of cycles	Initial denaturation	Denaturation	Annealing temperature	Extention	Final Extention
Tuf	34	94 °C for four min	94 °C for one min	53 °C for one min	72 °C for one min	72°C for five min
SodA (faecium)	39	95 °C for four min	95 °C for 30 S	48 °C for one min	72 °C for one min	72 °C for seven min
VanA	35	94 °C for five min	94 °C for 30 S	53 °C for 30 S	72 °C for one min	72 °C for seven min

3.2.7 Statistical analysis

Descriptive statistics were used in reporting frequencies of *Enterococci* bacteria occurrence, antibiotics susceptibility, and resistance genes profiling. Correlation between the resistance genes was performed using Pearson correlation analysis to determine the relationship between the various dependent variables (resistance genes). Significance of occurrence of the resistance genes were tested on chi-square analysis. A model of logistic regression was implemented to detect the association between the binary outcomes (1- presence and 0- absence) of *Enterococcus* and resistance genes and exposure variables (animal species and sex) which are sources of enterococcal isolates. All tests were carried out using IBM SPSS software (version 25).

3.3 Results

3.3.1 Isolation and confirmation of *Enterococcus* species

Presumptive *Enterococcus* growth on bile esculin agar was positive for 141 out of 150 samples; 49 (98%), 25 (96%) and 67 (91%) for chickens, cats and dogs respectively. Figure 3.1 depicts a gel image with 112bp amplicon for *Tuf* gene, 360bp amplicon for *SodA faecalis* and 215bp amplicon for *SodA faecium* demonstrating the presence of *Enterococcus* genus and the two *Enterococcus* species investigated - *Enterococcus faecalis* and *Enterococcus faecium*. Overall as depicted in Figure 3.2, 109 (77.3%) of all the presumptive *Enterococcus* isolates were confirmed positive for *Tuf* gene; 46 (93.9%) for chickens, 16 (64.0%) for cats and 47 (70.1%) for dogs. *E. faecalis* was more prevalent relative to *E. faecium* with an overall prevalence of 40.4% and 8.5% respectively in all the isolates.

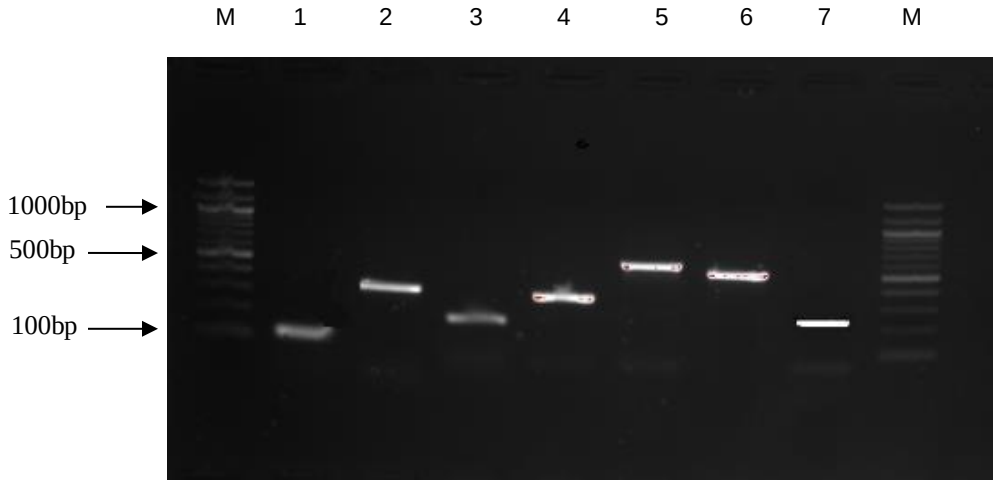

Figure 3.1: Representative gel picture of *Enterococcus* species identification and resistance genes screened from chickens, cats, and dogs
Lanes: M, 100-bp marker; 1, *Tuf*, 112bp; 2, *SodA faecalis* 360bp; 3, *SodA faecium* 215bp; 4, *aac(6')-Ie-aph(2'')-Ia* 348bp; 5, *ant(6')-Ia* 577bp; 6, *aph(3')-IIIa* 523bp; 7, *Van-A* 231bp.

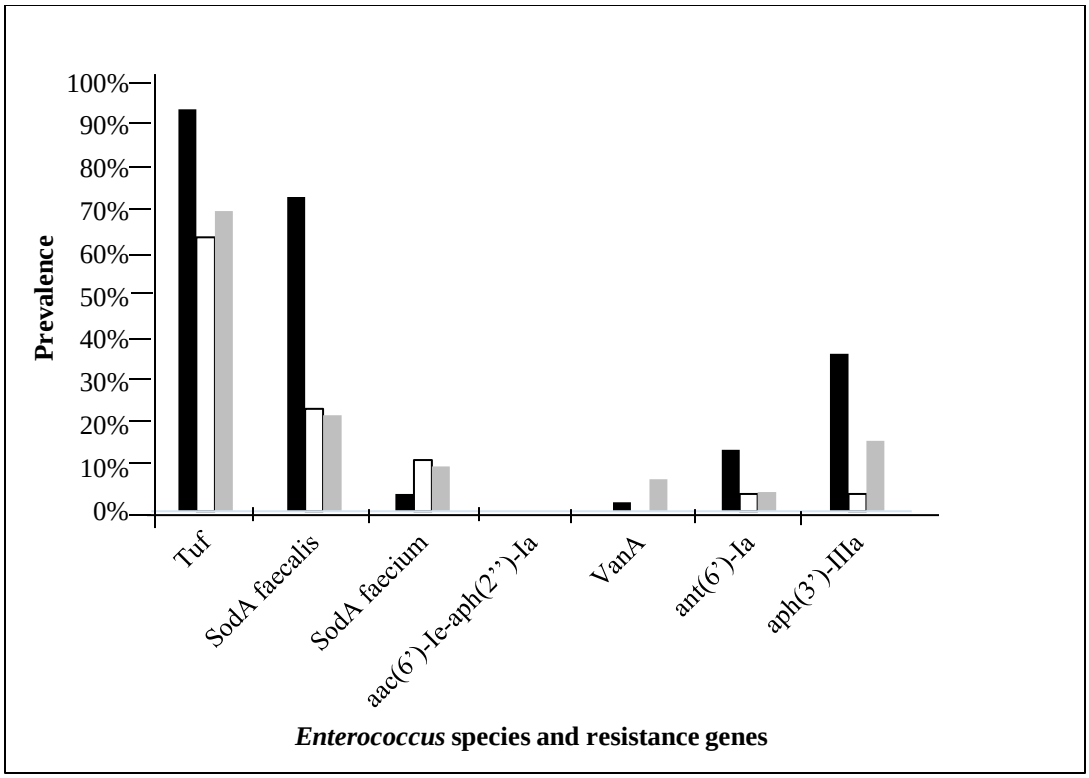

Figure 3.2: Prevalence of *Enterococcus* species and resistance genes isolated from chickens, cats, and dogs

Keywords: ■ Chickens,□ Cats,■ Dogs

3.3.2 Antibiotics susceptibility profiles

The profiles of the susceptibility of *Enterococcus* isolates to different antibiotics tested are presented in Table 3.3. Antimicrobial susceptibility test for 10 antibiotics showed that all *Enterococcus* isolates from all the animal species were found susceptible to High-Level Gentamicin (HLG). Of the overall *Enterococcus* isolates, the highest frequency of resistance was observed in Quinupristin/Dalfopristin (89.4%) followed by Vancomycin (87.9%), Rifampicin (85%), Ampicillin (76.6%), Erythromycin (72.3%), Tetracycline (64.5%), Chloramphenicol 24.8%, High Level Streptomycin (HLSTR) (24.1%) and Ciprofloxacin (14.2%) had the least frequency of resistance.

Table 3.3: Antimicrobial resistance phenotypes in *Enterococcus* species isolated from chickens, cats, and dogs

Antibiotics	**Chicken isolates (n = 49) Number of isolates (%)**			**Cat isolates (n = 25) Number of isolates (%)**			**Dog isolates (n = 67) Number of isolates (%)**			**Total** (n = 141) **Number of isolates (%)**	**Total (n = 141) Number of isolates (%)**	**Total (n = 141) Number of isolates (%)**
	R	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**
RD	49 (100.0)	0 (0.0)	0 (0.0)	17 (68.0)	3 (12.0)	5 (20.0)	54 (81.6)	5 (7.5)	8 (11.9)	120 (85,1)	8 (5.7)	13 (9.2)
AMP	49 (100.0)	0 (0.0)	0 (0.0)	12 (48.0)	0 (0.0)	13 (52.0)	47 (70.1)	0 (0.0)	20 (29.9)	108 (76.6)	0 (0.0)	33 (23.4)
VA	49 (100.0)	0 (0.0)	0 (0.0)	19 (76.0)	0 (0.0)	6 (24.0)	56 (83.6)	0 (0.0)	11 (16.4)	124 (87.9)	0 (0.0)	17 (12.1)
CN	0 (0.0)	0 (0.0)	49 (100.0)	0 (0.0)	0(0.0)	25 (100.0)	0 (0.0)	0 (0.0)	67 (100.0)	0 (0.0)	0 (0.0)	141 (100.0)
S	14 (28.6)	0 (0.0)	35 (71.4)	8 (32.0)	0 (0.0)	17 (68.0)	12 (17.9)	0 (0.0)	55 (82.1)	34 (24.1)	0 (0.0)	107 (75.9)
QD	49 (100.0)	0 (0.0)	0 (0.0)	18 (72.0)	1 (4.0)	6 (24.0)	59 (88.1)	0 (0.0)	8 (11.9)	126 (89.4)	1 (0.7)	14 (0,9)
E	46 (93.9)	2 (4.1)	1 (2.0)	11 (44.0)	7 (28.0)	7 (28.0)	45 (67.2)	15 (22.4)	7 (10.4)	102 (72.3)	24 (1.7)	15 (1.1)
TE	43 (87.8)	5 (10.2)	1 (2.0)	8 (32.0)	2 (8.0)	15 (60.0)	40 (59.7)	4 (6.0)	23 (34.3)	91 (6.5)	11 (0.8)	39 (2.8)
C	25 (51.0)	17 (34.7)	7 (14.3)	2 (8.0)	1 (4.0)	22 (88.0)	8 (11.9)	5 (7.5)	54 (80.6)	35 (2.5)	23 (1.6)	83 (58.9)
CIP	4 (8.2)	4 (8.2)	41(83.7)	8 (2.0)	0 (0.0)	23 (92.0)	14 (20.9)	0 (0.0)	53 (79.1)	26 (1.8)	4 (0.3)	117 (8.3)

RD, Rifampicin; AMP, Ampicillin; VA, Vancomycin; CN, Gentamicin; S, Streptomycin; QD, Quinupristin/ Dalfopristin; E, Erythromycin; TE, Tetracycline; C, Chloramphenicol; CIP, ciprofloxacin; R, Resistant; I, Intermediate; S, Susceptible.

The results of multiple drug resistance (MDR) patterns are reported in Table3.4. Out of 141 isolates that were positive for *Enterococcus*, 119 (84.3%) expressed MDR. There were 28 different MDR patterns but RD/AMP/VA/QD/E/TE was the most predominant pattern (n = 18). Highest MDR (89.8%) was observed in chicken isolates followed by dogs (86.6%), while the least MDR was found in cats (68%).

Table 3.4: Multiple drug resistance patterns of *Enterococcus* species

Antimicrobial resistance patterns	Number of isolates (%)			
	Chicken isolates (n = 49)	**Cat isolates (n = 25)**	**Dog isolates (n =67)**	**Total (%) (n = 141)**
RD, AMP, VA, QD, S, E, TE, CIP	1 (2.0)	1 (4.0)	1 (1.5)	3 (2.1)
RD, AMP, VA, QD, E, TE, C, CIP	1 (2.0)	0 (0.0)	3 (4.5)	4 (2.8)
RD, AMP, VA, QD, S, E, TE, C	6 (12.2)	1 (4.0)	2 (3.0)	9 (7.0)
RD, AMP, VA, QD, E, TE, CIP	1 (2.0)	0 (0.0)	6 (9.0)	7 (5.0)
RD, AMP, VA, QD, S, E, CIP	1 (2.0)	0 (0.0)	0 (0.0)	1 (0.7)
RD, AMP, VA, QD, S, E, TE	5 (10.2)	2 (8.0)	3 (4.5)	10 (7.1)
RD, AMP, VA, QD, E, TE, C	15 (30.6)	0 (0.0)	1 (1.5)	16 (11.3)
RD, AMP, VA, QD, TE, CIP	0 (0.0)	0 (0.0)	2 (3.0)	2 (1.4)
RD, AMP, VA, QD, E, TE	11 (22.4)	0 (0.0)	7 (10.4)	18 (12.8)
RD, AMP, VA, QD, TE, C	2 (4.1)	0 (0.0)	0 (0.0)	2 (1.4)
RD, AMP, VA, QD, E, C	1 (2.0)	0 (0.0)	1 (1.5)	2 (1.4)
RD, AMP, VA, S, QD, E	0 (0.0)	1 (4.0)	3 (4.5)	4 (2.8)
RD, AMP, VA, S, QD, C	0 (0.0)	0 (0.0)	2 (3.0)	2 (1.4)
RD, AMP, VA, QD, TE	0 (0.0)	2 (8.0)	2 (3.0)	4 (2.8)
RD, AMP, VA, S, QD	0 (0.0)	1 (4.0)	1 (1.5)	2 (1.4)
AMP, VA, QD, E, TE	0 (0.0)	0 (0.0)	1 (1.5)	1 (0.7)
AMP, VA, QD, E, TE	0 (0.0)	0 (0.0)	1 (1.5)	1 (0.7)
RD, AMP, E, TE, CIP	0 (0.0)	0 (0.0)	1 (1.5)	1 (0.7)
RD, AMP, VA, QD, E	0 (0.0)	0 (0.0)	7 (10.4)	7 (5.0)
RD, VA, QD, E, CIP	0 (0.0)	1 (4.0)	0 (0.0)	1 (0.7)
RD, VA, QD, E, TE	0 (0.0)	1 (4.0)	4 (6.0)	5 (3.5)
AMP, VA, S, QD, C	0 (0.0)	1 (4.0)	0 (0.0)	1 (0.7)
RD, AMP, VA, QD	0 (0.0)	1 (4.0)	3 (4.5)	4 (2.8)
RD, VA, QD, TE	0 (0.0)	1 (4.0)	2 (3.0)	3 (2.1)
RD, VA, QD, E	0 (0.0)	3 (12.0)	0 (0.0)	3 (2.1)
VA, QD, E, TE	0 (0.0)	0 (0.0)	1 (1.5)	1 (0.7)
RD, VA, QD	0 (0.0)	1 (4.0)	3 (4.5)	4 (2.8)
QD, E, TE	0 (0.0)	0 (0.0)	1 (1.5)	1 (0.7)
Total (%)	**44 (89.8)**	**17 (68.0)**	**58 (86.6)**	**119 (84.3)**

RD, Rifampicin; AMP, Ampicillin; VA, Vancomycin; CN, Gentamicin; S, Streptomycin; QD, Quinupristin/ Dalfopristin; E, Erythromycin; TE, Tetracycline; C, Chloramphenicol; CIP, ciprofloxacin.

.3.3.3 Prevalence of resistance genes in *Enterococcus* species from chickens, cats, and dogs

The frequency of occurrence of the various genes coding for resistance to antibiotics in *Enterococcus* isolates in our study is shown in Figure 3.2. Out of the four resistance genes screened for, three were present. Of the overall, the prevalence rates observed for the genes were 21.3%, 7.8% and 4.3% for *aph(3')-IIIa, ant(6')-Ia* and *Van-A*respectively. PCR amplification of the resistance genes is depicted in the representative gel in Figure 3.1.

3.3.4 Correlation between resistance genes detected in *Enterococcus* species isolated from chickens, cats, and dogs

Correlation coefficients between resistance genes are presented in Table 3.5. The correlation coefficient between *Tuf* gene and *SodA faecalis* (0.343), between *SodA faecalis* and *aph(3')-IIIa* (0.278) and between *ant (6')Ia* and *aph(3')-IIIa* (0.366) were positive, moderate and very highly significant (P<0.001). The results of the chi-square tests for all the resistance genes are presented in Table 3.6, it shows that was significant difference in the prevalence of *Tuf, SodA faecalis* and *aph(3')-IIIa* genes from chickens, cats and dogs sampled in this study. The association between the binary outcome (presence or absence of the resistance genes) in *Enterococcus* and exposure to chickens, cats, and dogs is presented in Table 3.7. This was done to predict the presence of the genes in these animals. The covariates variable species was found to be statistically significant in terms of *Tuf*, *aph(3')-IIIa* and *ant(6')-Ia* prevalence. However, the odds of harboring *Tuf* gene, *aph(3')-IIIa* and *ant(6')-Ia* genes are significantly higher among chickens (OR = 6.292, 95% CI = 1.743, 22.717), (OR = 5.564, 95% CI = 1.242, 24.926) and (OR = 3.131, 95% CI = 1.295, 7.567) when compared with cats and dogs. Other independent variables such as *SodA faecium* and *VanA*

genes were found not to be statistically significant. Sex did not contribute to the occurrence of the genes coding for resistance in chickens, cats, and dogs.

TABLE 3.5: Output of Pearson's correlation analysis for genes coding for antibiotic resistance in *Enterococcus* species isolated from chickens, cats, and dogs

Genes	**Statistical tests**	***Tuf***	***SodA faecalis***	***SodA faecium***	***aac(6')-Ie-aph(2'')-Ia***	***Van-A***	***ant (6')Ia***	***aph(3')-IIIa***
Tuf	Pearson's correlation	1	0.343**	0.105	b	-0.137	0.094	0.033
	Sig. (2-tailed)		0.000	0.217	.	0.104	0.265	0.694
SodA faecalis	Pearson's correlation	0.343**	1	-0.148	b	-0.102	-0.078	0.278**
	Sig. (2-tailed)	0.000		0.081	.	0.228	0.358	0.001
SodA faecium	Pearson's correlation	0.105	-0.148	1	b	0.062	-0.089	-0.096
	Sig. (2-tailed)	0.217	0.081		.	0.468	0.295	0.255
aac(6')-Ie-aph(2'')-Ia	Pearson's correlation	b	b	b	b	b	b	b
	Sig. (2-tailed)	.	.	.	.	.	.	.
Van-A	Pearson's correlation	-0.137	-0.102	0.062	b	1	0.070	0.062
	Sig. (2-tailed)	0.104	0.228	0.468	.		0.412	0.464
ant(6')-Ia	Pearson's correlation	0.094	-0.078	0-.089	b	0.070	1	0.366**
	Sig. (2-tailed)	0.265	0.358	0.295	.	0.412		0.000
aph(3')-IIIa	Pearson's correlation	0.033	0.278**	-0.096	b	0.062	0.366**	1
	Sig. (2-tailed)	0.694	0.001	0.255	.	0.464	0.000	

**. Correlation is significant at the 0.01 level (2-tailed).

b. Cannot be computed because at least one of the variables is constant.

Table 3.6: Chi-square test for the genes coding for antibiotic resistance in *Enterococcus* species from chickens, cats and dogs

Statistical test	**Asymptotic significance (2-sided)**					
	Tuf	***SodA faecalis***	***SodA faecium***	***Van-A***	***ant(6')-Ia***	***aph(3')-IIIa***
Pearson Chi-square	0.002	0.000	0.378	0.183	0.111	0.002

Table 3.7: Binary logistic regression analysis output for the relationship between *Enterococcus* species, resistance genes and exposure variables (animal species and sex)

Genes	Covariate variable	-2Log Likelihood	B (estimate)	SE (standard error)	Wald *P*-value	*OR (Odd ratios)*	95% CI (confidence interval)
Tuf		135.431					
	Sex (ref: female)						
	Male		0.525	0.432	0.225	1.691	0.724 – 3.946
	Species (ref: dogs)						
	Chicken		1.839	0.655	0.005	6.292	1.743 – 22.717
	Cat		-0.214	0.501	0.669	0.807	0.303 – 2.155
SodA faecalis		155.505					
	Sex (ref: female)						
	Male		0.023	0.399	0.954	1.024	0.468 – 2.238
	Species (ref: dogs)						
	Chicken		2.260	0.438	0.000	9.579	4.058 – 22.608
	Cat		0.094	0.555	0.866	1.098	0.370 – 3.259
SodA faecium		79.891					
	Sex (ref: female)						
	Male		0.109	0.615	0.859	1.115	0.334 – 3.726
	Species (ref: dogs)						
	Chicken		-1.020	0.828	0.218	0.361	0.071 – 1.826
	Cat		0.171	0.738	0.817	1.186	0.279 – 5.043
Van-A		44.523					
	Sex (ref: female)						
	Male		0.782	0.894	0.382	0.457	0.079 – 2.638
	Species (ref: dogs)						
	Chicken		-1.284	1.117	0.250	0.277	0.031 – 2.470
	Cat		-18.774	7978.723	0.998	0.000	0.000
ant(6')-Ia		53.673					
	Sex (ref: female)						
	Male		-19.817	4562.566	0.997	0.000	0.000
	Species (ref: dogs)						
	Chicken		1.716	0.765	0.025	5.564	1.242 – 24.926
	Cat		-0.373	1.197	0.755	0.689	0.066 – 7.192

aph(3')-		131.547				
IIIa	**Sex (ref: female)**					
	Male	-0.464	-0.440	0.292	0.629	0.266 – 1.489
	Species (ref: dogs)					
	Chicken	1.141	0.450	0.011	3.131	1.295 – 7.567
	Cat	-1.616	1.076	0.133	0.199	0.024 – 1.636

*****ref: Reference species and sex

3.4 Discussion

This study indicated a high prevalence of *Enterococcus* species. The high prevalence agrees with the results in a similar study in nine European Union countries by de Jong *et al.*, 2018. A higher prevalence (93.7%) was reported by Zhao *et al*., 2012 Furthermore, relatively low prevalence was recorded in the studies of Elal Mus *et al*., 2012 and Ngbede *et al*., 2017 Differences observed in the prevalence of the species across various locations could be attributed to feeding regimes, management practices, veterinary care and environmental conditions of the animals (Ho et al., 2013). *E. faecium* and *E. faecalis* detected in our study have also been reported by other researchers in other nations (Kukanich *et al*., 2012; Kwon *et al*., 2012; Kurekci *et al*., 2016; Ngbede *et al*., 2017; de Jong *et al*., 2018). *E. faecalis* and *E. faecium* in some years back evolved multiple resistance to many antimicrobial agents and are frequently associated with hospital-acquired infections especially urethritis (Arias and Murray 2012; Ben Sallem *et al*., 2016; Paosinho *et al*., 2016). Significant Chi-square test for *Tuf* and *SodA faecalis* genes indicates that the observed difference in the percentages of harboring *Enterococcus* genus, *Enterococcus faecalis* and *aph(3')-IIIa* gene was not due to chance but because there was a difference in the prevalence of this bacterium in the different animal species sampled in this study. This might be because of their propensity to pick up resistance genes to adapt to biotic challenge in the environment.

The highest frequency (89.4%) of antibiotic resistance was observed in Quinupristin/Dalfopristin. In contrast, some researchers recorded a very low resistance rate to this drug (Elal Mus *et al*., 2012; Kurekci *et al*., 2016; Freitas *et al*., 2018). Quinupristin/Dalfopristin is an example of drugs belonging to the class of streptogramin antibiotics. It's a combination of two antimicrobial agents Quinupristin and Dalfopristin, used cure infections due to Vancomycin-Resistant *Enterococci* (VRE) in humans (Higuita and Huyck 2014; WHO 2014). High prevalence of resistance to this antimicrobial agent observed in this study suggests that *Enterococcus* species can be intrinsically resistant to streptogramins class of antibiotics. Our study also recorded high (87.9%) phenotypic vancomycin resistance. This is close to the findings reported by Iweriebor *et al*., 2015 However, previous reports were relatively high compared to the findings from similar research by Kurekci *et al*., 2016; Adesida *et al*., 2017; and Freitas *et al*., 2018. Vancomycin is a glycopeptide mostly combined with beta-lactam antibiotics to cure ailments caused by *Enterococcus* species and other gram-positive bacteria (Choi and Woo 2013). In recent times, the emergence of (VRE) is significantly important in nosocomial pathogens as it is the last resort to treating *Enterococcus* infections in humans. Misuse of avoparcin, a glycopeptide used in animal production could explain the high resistance prevalence observed in vancomycin in this study. Aminoglycosides are also combined with beta-lactams for treatment of enterococcal infections. However, high (76.6%) to low (24.1%) resistance was observed for ampicillin and high-level streptomycin in our study. Resistance to any of these drugs poses a great threat in treatment options as the synergistic bactericidal activity is lost (Bortolaia *et al*., 2015). High antibiotic resistance prevalence of 85% was also observed in rifampicin, an antimicrobial agent widely used to treat tuberculosis in humans and in combination with other antibiotics to cure ailments caused by MDR bacterial pathogens

(Hu *et al*., 2016). It is not normally used for treatment of *Enterococcus* infections (Prichula *et al*., 2016) but *Enterococci* resistance to this drug is said to be triggered by misuse of antibiotics (Teymournejad *et al*., 2015) and acquired resistance from other bacterial species (Bagcigil *et al*., 2015). Our findings are higher compared to a similar study in pet dogs by Zhao *et al*., 2012.

MDR pattern observed in this study is a speculation of antibiotic use in the study location as it could also be because of the acquisition of plasmids, mutations in chromosomes and pathogenicity islands acquisition. This poses a great threat to human health as these antimicrobial agents are also used to treat bacterial infections in humans.

Our study also recorded the presence of resistance genotypes that conferred the phenotypic resistance observed in vancomycin and aminoglycosides. *VanA* resistance gene borne on plasmids of *Enterococcus* species (Bortolaia *et al*., 2015) was found in 4.3% of vancomycin-resistant isolates. *VanA* gene has been reported in a similar study in broilers by Bortolaia *et al*., 2015 This gene has been found to be associated with human infections caused by VRE such as endocarditis (Werner *et al*., 2008; Pinholt *et al*., 2012). *VanA* gene is involved in the substitution of D-Ala-D-Lac(D-Alanyl-D-Lactate) peptide terminal for D-Ala-D-Ala (D-Alanyl-D-Alanine) peptide terminal responsible for the synthesis of peptidoglycan in *Enterococci* cell wall that causes its low affinity with vancomycin drug (Azimian *et al*., 2012). About 21.3% and 7.8% of the isolates resistant to High-Level Aminoglycosides (HLA) possessed *aph(3')-IIIa* and *ant(6')-Ia* antibiotic resistance genes respectively for kanamycin and streptomycin resistance. Enterococcal isolates from poultry and other food-producing animals have been found to harbor these genes (Klibi *et al*., 2013; Hidano *et al*., 2015; Klibi *et al*., 2015). *Enterococci* resistance to HLA is mediated by Aminoglycosides Modifying Enzyme (AME) genes (*aph(3')-IIIa* and *ant(6')-Ia)* which are

acquired horizontally by Mobile Genetic Elements (MGE). The AME genes function by modifying target RNA (Ribonucleic acid) through the production of inactivating enzymes such as nucleotidyltransferase (phosphotransferase-Aph and adenylytransferase-Ant) and acetytransferase (Aac) leading to impermeability of these antibiotics in the bacteria (Sheppard and Gilmore 2002). Pearson's correlation analysis demonstrated a positive correlation (0.278) between *SodA faecalis and aph(3')-IIIa* gene, $P < 0.01$. Also, *aph(3')-IIIa* and *ant(6')-Ia* antibiotic resistance genes were positively correlated (0.366) $P< 0.01$.

Animal species and sex were used as the exposure of interest for harboring *Enterococcus* resistance genes as log odds ratio comparing chickens, cats and dogs along with sex differentiation. Because the population means is a fixed unknown number in different animal species, confidence interval (CI) is expected to vary between different samples. A CI gives the range of values within which there is reasonable confidence that the population difference exists meaning that if this study is conducted 100 times, the true prevalence will fall between the lower and upper limit of 95% confidence interval for targeted genes in this study. All the CI were positive indicating an increase in prevalence in the population sampled for *Enterococcus* species and resistance genes. Because the narrower the CI the more precise the estimate, *Van-A* gene had the narrowest CI relative to other resistance genes and therefore implying highest prevalence precision (95% of the times) which can be more readily applicable for chicken, cat and dog populations sampled.

Odds ratio (OR) values for all the genes in this study fall within their respective CI indicating the correctness of the analysis and validity of the results in measuring the association between the animal species and resistance genes as well as sex and resistance genes. In all, OR was greater than 1 showing a positive association between chickens and prevalence of resistance genes. On the

other hand, OR for gender was generally less than 1 suggesting a negative or protective association between gender and resistance genes. Males had 1.691 times chance of harboring *Tuf* gene while chicken had 6.292 times risk of harboring *Tuf* gene compared to cats and dogs. Also, males had 1.024 times chance of having *SodA faecalis* gene while chickens had 9.579 times compared to cats and dogs. Males were 0.457 times in *Van-A* than females while chicken had 0.277 times relative to cats and dogs. Males were 0.629 times than females for *aph (3')-IIIa* while chicken had 3.131 times compared to cats and dogs.

3.5 Conclusion

This study shows that *Enterococcus* species recovered from chickens, cats, and dogs expressed multidrug resistance to important antibiotic drugs used in both human and animal health care. They also harbored antibiotic resistance genes. There is a possibility of transferring these resistance genes to human via food chain and direct contact with pets representing a potential health risk to humans and animals due to resultant treatment failure, infection severity, and economic burden. Therefore, more surveillance studies on the screening of antimicrobial resistance profiles in *Enterococci* in other food animals such as cattle, goats, sheep in other locations of South Africa should be done with higher sample size. Additionally, antimicrobials should be used judiciously in animal production and veterinary services. Hygienic practices should be highly planned and maintained.

3.6 Statement on animal rights

Animal studies have been approved by the appropriate ethics committee of the University of KwaZulu-Natal (Reference: AREC/051/017M); therefore, they have been performed in

accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

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CHAPTER 4

Manuscript 2

Incidence of virulence factors of *Enterococcus* species in chicken and companion animals sampled from Durban, South Africa

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CHAPTER 4

Incidence of virulence factors of *Enterococcus* species in chicken and companion animals sampled from Durban, South Africa

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Abstract

There is a significant amount of scientific research that has indicated that *Enterococcus* species are among the leading pathogens of infections associated with hospital environment. They are gut commensal bacteria of human and animals but are now recognized as pathogenic bacteria. Their pathogenic ability is partially conferred by their ability to acquire and transfer virulence genes using several mechanisms such as mobile genetic elements. Therefore, the objective of this paper was to detect the incidence of genes coding for virulence in two species of *Enterococcus* bacteria isolatedfrom chicken and companion animals. The totalof 150 cloacal, rectal and nasal swabs sampled from chickens, cats and dogs were analyzed for the isolation of *Enterococcus* species using microbiological culture methods. The isolates were further confirmed to be the *Enterococcus* genus through the amplification of *Tuf* gene using polymerase chain reaction (PCR) and recorded 109 (77.3%) positive isolates. The highest prevalence was detected in chickens (93.9%) followed by dogs (70.1%) and cats (64.0%). *E. faecalis* had a higher prevalence rate compared to *E. faecium*.

Five genes (*EfaA, GelE, ccf, Esp, and CylA*) coding for virulence traits in *Enterococci* were screened using PCR. All *E. faecalis* and *E. faecium* detected harbored multiple (at least three) virulence genes. Overall, *EfaA* (88.7%) was mostly detected followed by *GelE* (82.3%), *ccf* (81.6%), *Esp* (26.2%) and *CylA* (25.5%). A significant association was found between *Tuf, SodA faecalis, CylA, EfaA* and *Esp* genes. This study demonstrates that *Enterococcus* species detected in chickens, cats and dogs could be pathogenic, and these virulence genes with accompanying infections could possibly be transmitted to humans when they consume contaminated chicken meat and via direct contact of pets with their owners.

Keywords: *Enterococcus faecalis*; *Enterococcus faecium*; virulence genes; polymerase chain reaction; infection; chickens; cats; dogs.

4.1 Introduction

Enterococcus are a genus of bacteria that are facultatively anaerobic found ubiquitously in animals, humans, waterbodies and soils (Murray *et al.*, 2013). They are commensal gut bacteria of humans and animals and can be used as probiotics (Yilmaz *et al*., 2017). Conversely, *Enterococcus* species have emerged as healthcare-associated infectious pathogens harboring virulence genes responsible mainly for pathogenicity. More than forty species of the *Enterococcus* genus have been identified. However, *Enterococcus faecium* and *Enterococcus faecalis* are most recognized as pathogens of hospital-acquired infections in humans (Alipour *et* al., 2014). About 90% of *E. faecalis* are implicated in nosocomial infections while 15% of the nosocomial infections are caused by *E. faecium.* In this view, *Enterococcus faecalis* are considered more virulent compared to *E. faecium* while *E. faecium* is mainly characterized by multiple-drug resistance (Silva *et al*., 2012). Virulence genes borne on the pathogenic bacteria's chromosome code for secretion of toxic substances that

increase the severity of infections in its host. These virulence genes alongside resistance genes increase the severity of infections in their hosts (Jackson *et al.,* 2011).

Many virulence genes such as those that promote colonization (Esp-*Enterococcus* surface protein, and *EfaA-Endocarditis* antigen A), those that evade host tissues (cytolysin *cylA* and gelatinase *GelE*) and sex pheromones (*ccf*) which facilitate conjugative plasmid transfer have been reported in *Enterococci* (Chajecka-Wierzchowska *et al*., 2016). To establish an infection, there must be an interaction between the host and the pathogen. *Enterococcus* species with the possession of virulence factors, the first line of action is to attach to the host cell, followed by colonizing the host's tissues, evading the host's immune system and finally initiating an infection (Cosentino *et al*., 2010; Kwon *et al.,* 2012). *Enterococcus* surface protein (*Esp*) located on a large pathogenicity island of *Enterococcus* species enhances its ability to attach to its host, form biofilm and colonize the urinary tract. It also facilitates the exchange of genes responsible for antibiotic using transposons, plasmids and integrons (mobile genetic elements) and found mostly in lineages of strains emanating from hospitals. When the attachment issuccessful, it persists and survives at the infection sites thereby increasing the severity of infections (Van Tyne and Gilmore 2014). Endocarditis antigen (*EfaA*) virulence factor is encoded by *efAfs* (*Enterococcus faecalis* antigen A) and *efArm* (*Enterococcus faecium* antigen A) genes, it aids *Enterococcus* bacteria to attach and colonize the host's cell during the infection process. It is found on afaCBA operon which is controlled by magnesium ions and responsible for peritonitis infection as well as infective endocarditis in animals (Chajecka-Wierzchowska *et al*., 2016; Kafil *et al*., 2016). Cytolysin encoded by chromosomal *cyl* gene plays important role in the production of bacteriocin which exerts bacteriostatic effect against pathogenic microbes in the gut but found to lyse bacterial and eukaryotic cells (Van Tyne and Gilmore 2014). Gelatinase (*gelE*) is a zinc metalloprotease that

hydrolyzes gelatin, haemoglobin, and elastin. Its expression is regulated by *fsr* operon and is implicated in endocarditis infection caused by *E. faecalis*. Sex pheromones *ccf, cob, cpd* are virulence factors responsible for conjugation and transfer of plasmids associated with virulence and antibiotic resistance determinants. Pheromones are peptides which enhance transfer of plasmids during conjugation. When pheromone is secreted, the conjugative operon is induced and after binding with the host, transduction of signal which give rise to genes responsible for aggregation takes place (Chajeck-Wierzchowska *et al*., 2016). *Enterococcus* species are known as causative organisms for infections such as wound infections, bacteremia, endocarditis, and endophthalmitis, which are fatal especially in immunocompromised individuals as a result of mainly HIV/AIDS and cancer (Arias and Murray, 2012).

Of importance also in the pathogenicity of *Enterococci* is the production of biofilms. Biofilms are extracellular polymeric substances secreted by bacteria which help them to attach to the host's extracellular matrix and adapt to unfavorable environmental conditions (Piggot *et al.*, 2012). Formation of biofilm by strains of pathogenic bacteria is found to increase the severity of infection in the host (Abdullahi *et al.*, 2016). Formation of biofilm by *Enterococcus* species is known to cause and increase the severity of infections such as wound infection and urinary tract infections (UTIs) (Garcia and Percival 2011).

Enterococcus spp. are important nosocomial pathogens globally and are ranked the third most common nosocomial pathogens (Werner *et al*., 2013). Chickens, cats, and dogs have been demonstrated to serve as a reservoir of *Enterococcus* virulence genes and humans are at great risk of acquiring these virulence factors through horizontal gene transfer from contaminated foods and direct physical contact by pet owners (Obeng *et al*., 2013; Bagcigil *et al*., 2015). Findings from Olsen *et al*., (2012) showed that *E. faecalis* virulence gene sequences from both human and poultry

Enterococci isolates are similar suggesting that virulence genes in animals can be zoonotically transferred to humans. This, therefore, calls for serious public health concerns. There is a persistent increase in the spread of enterococcal infections due to the imprudent antibiotic usage in medical services and animal husbandry which gave rise to infectiveness of the drugs and severity of the infections now that there are few or no novel antibiotics. Information on virulence factors in *Enterococcus* spp. could help provide insights into its intrinsic pathogenic complex process (Comerlato *et al.*, 2013). These data are needed to understand fully the epidemiology of *Enterococcus* species and plan for intervention programs. However, very little information is available on virulence factors in *Enterococcus* species emanating from chicken and cats and dogs in South Africa. Hence, the objective of this paper was to detect the incidence of genes coding for virulence in *Enterococcus* species from chickens, cats, and dogs in Durban, South Africa.

4.2 Materials and Methods

4.2.1 Sampling and bacteria isolation

This project received approval of the Animal Research Ethics Council (AREC) of KwaZulu-Natal University with Protocol Reference number- AREC/051/017M. A total of 150 swabs were randomly collected from chickens (50), cats (26) and dogs (74). Among the 50 chicken cloacal swabs, 30 were males and 20 females. For the 26 rectal cat isolates, 10 were males and 16 females while the 74 nasal dog isolates comprised 33 males and 44 females. Cats and dog samples were collected from sick animals on a visit to a veterinary clinic while chicken samples were taken from chickens in a poultry abattoir. All samples were collected from Durban metropolitan area in South Africa within November 2017 and March 2018 aseptically with sterile swabs and sampling tubes on ice storage before been transported back to the Department's laboratory for further analysis. On arrival to the laboratory, *Enterococcus* species bacteria were isolated by enriching the samples in

10 ml buffered peptone water following incubation for a period of 24 hours at a temperature of 37 °C. Brain Heart Infusion (BHI) broth was then used for inoculation of 1 ml of peptone from each sample and incubation for a period of 24 hours at 37 °C. The broth culture was then streaked on Bile Esculin Agar (BEA) plates which is an *Enterococcus* selective media and incubated for a period of 24 hours at 37 °C. *Enterococcus* species hydrolyzes esculine in BEA to 6, 7-dihydroxycoumarin which react with ions in the media to form black coloration, an indication of positive test for *Enterococcus* species. Bile salt contained in BEA is an inhibitory agent which eliminates other gram positive and negative bacteria (Lindell and Quinn 1975). Presumptive *Enterococcus* species were morphologically identified by black halo colony growth on the plates. Two or more representative colonies were picked using sterile inoculation loop and inoculated into BHI broth and incubated to obtain pure cultures for DNA extraction and storage for future use in glycerol stocks (25%) at -80 °C.

4.2.2 The extraction of genomic DNA

Extraction of DNA was done using the overnight BHI culture grown at 37 °C for 24 hours following the protocols reported by Ruiz-Barba *et al*., (2005) with some modifications. One thousand (1000) µl of the BHI broth culture was added into a microcentrifuge tube and spinned at 12,000 rpm for five min in a microcentrifuge to obtain pellets. This was followed by discarding the resultant supernatant and washing of the pellets by brief centrifugation using 100 µl volume of sterile ddH_20 (deionized water). After the brief centrifugation, the water was discarded. 500 µl of sterile deionized water was added to the washed pellet and vortexed briefly for 5 s. To the resultant mixture was added 500 µl volume of Chloroform-Isoamyl alcohol (24:1) followed by centrifugation using the conditions stated early on ice to denature the proteins and separate aqueous and organic phases. The upper aqueous layer was taken as the source of DNA template and was

preserved at 4 °C for other applications such as molecular detection of the species and screening of the virulence genes. The DNA template was quantified with Nanodrop Spectrophotometer (Wilmington, Delaware, USA).

4.2.3 Confirmation of *Enterococcus* genus and detection of *Enterococcus* species by polymerase chain reaction (PCR)

Enterococcus species was confirmed to the genus level using *the Tuf* gene on PCR in 35 cycles of reaction using Bio-RAD, T100^{TM} Thermal Cycler (Singapore) as shown in Table 4.1. *Tuf* gene which is specific to *Enterococcus* genus identification codes for elongation factor TU (EF-TU) and also participates in formation of peptide chain (Ke *et al*., 1999). The two *Enterococcus* spp. investigated were detected using *SodA* primers specific to each of them as listed in Table 4.1 using PCR in 35 and 39 cycles of reaction respectively for the two species. The *SodA* gene which is specific to *Enterococcus* species identification codes for manganese-dependent superoxide dismutase (Poyart *et al*., 2000). Amplification for *SodA faecalis* gene was performed in accordance with PCR amplification protocol reported by Alipour *et al*., (2014) while that of *Tuf* gene and *SodA* faecium was performed using the following conditions: four minutes of pre-denaturation at 94 °C and 95 °C; 94 °C and 95 °C of denaturation for 1 minute and 30 s respectively; 1 minute of annealing; 72 °C of prolongation for a period of 1 minute and 72 °C final prolongation for 5 and 7 minutes respectively. PCR reactions were done using 25 µl reaction volume which consisted of 5 µl of genomic DNA, 1 µl each of forward and reverse primers, 12.5 µl green master mix (Thermo-Scientific DreamTaq) and 5.5 µl sterile water. Electrophoresis was run with agarose gel (1.5%) on ethidium bromide (EtBr) to analyze the PCR products at 100 volts for 30 min. The bands were visualized using BIO-RAD, ChemiDoc^{TM}MP Imaging system.

4.2.4 Molecular Detection of the Virulence genes through PCR

Virulence genes (*cylA, efaAfs, Esp, ccf* and *GelE)* coding for cytolysin, *E. faecalis* antigen A, *Enterococcus* surface protein, sex pheromone and gelatinase respectively were detected using primers presented in Table 4.1 through PCR. *cylA* gene was amplified using cycling conditions reported by Jung *et al*., (2017) while that for the rest virulence genes was accomplished with the protocol reported by Eaton and Gasson (2001).

TABLE 4.1: Primer sequences used for *Enterococcus* genus confirmation, species confirmation and virulence genes screening in *Enterococcus* species isolates from chickens, cats, and dogs

Target gene	Function	Sequence (5'→3')	Annealing temperature (°C)	Product Size (bp)	Reference
Tuf	*Enterococcus* genus identification	Forward: TACTGACAAACCATTCATGATG Reverse: AACTTCGTCACCAACGCGAAC	53	112	Ke *et al*., 1999
SodA (*E. faecalis*)	*E. faecalis* identification	Forward: ACT TAT GTG ACT AAC TTA ACC Reverse: TAA TGG TGA ATC TTG GTT TGG	38	360	Jackson *et al*., 2004
SodA (*E. faecium*)	*E. faecium* identification	Forward: GAA AAA ACA ATA GAA GAATTAT Reverse: TGC TTT TTTGAA TTC TTC TTT A	48	215	Jackson *et al*., 2004
cylA Cytolysin	Biosynthesis of cytolysin	Forward: ACTCGGGGATTGATAGGC Reverse: GCTGCTAAAGCTGCGCTT	55	688	Creti *et al*., 2004; Choi and Woo 2013.
efaAfs *E. faecalis* antigen A	Cell wall adhesions that function as endocarditis specific antigen	Forward: GACAGACCCTCACGAATA Reverse: AGTTCATCATGCTGTAGTA	53	705	Eaton and Gasson (2001)
Esp (Enterococcus surface protein)	Aids in host's immune evasion.	Forward: TTGCTAATGCTAGTCCACGACC Reverse: GCGTCAACACTTGCATTGCCGAA	52	933	Eaton and Gasson, 2001
Ccf Sex pheromone	Helps in conjugative plasmid transfer	Forward: GGG AAT TGA GTA GTG AAG AAG Reverse: AGC CGC TAA AAT CGG TAA AAT	52	543	Eaton and Gasson (2001)
GelE gelatinase	Biosynthesis of an extracellular metalloendopeptidase	Forward: ACC CCG TAT CAT TGG TTT Reverse: ACG CAT TGC TTT TCC ATC	53	419	Eaton and Gasson (2001)

4.2.5 Statistical analysis

Prevalence of genes for species identification and virulence was analyzed using descriptive statistics. Chi-square test was performed to determine the significance of all the genes while $P< 0.05$ was considered significant. The data was further analyzed by fitting a classical binomial logistic regression model. The model included the presence (coded as 1) and absence (coded as 0) for each gene used to detect *Enterococcus* species and the virulence factors. All statistical tests were performed using IBM SPSS statistics software version 24 (SPSS, Inc, Chicago, IL, USA).

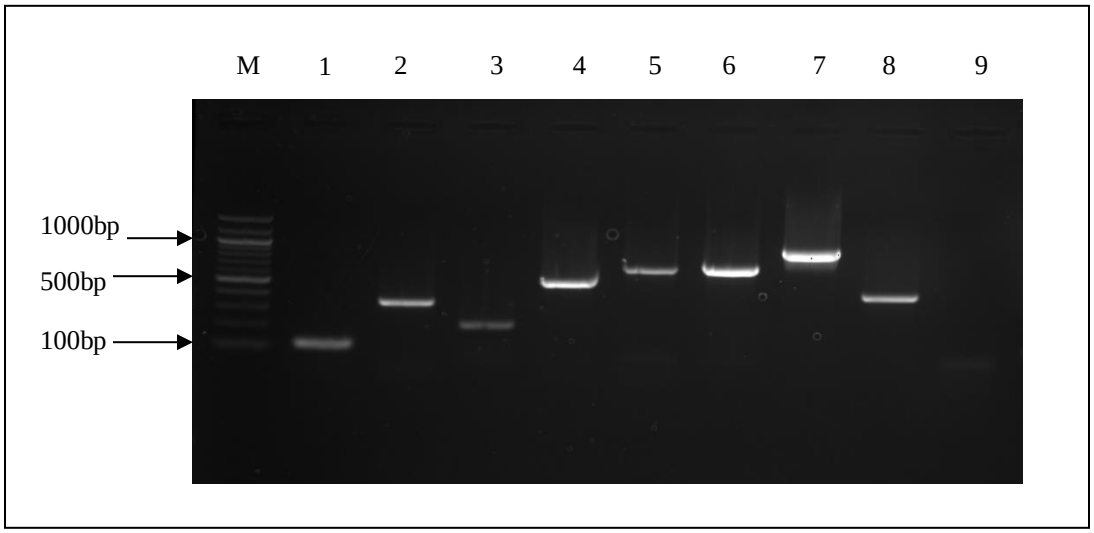

Figure 4.1: Representative gel picture of *Enterococcus* species identification and virulence genes screened from chickens, cats, and dogs

Lanes: M, 100-bp marker; 1, *Tuf*, 112bp; 2, *SodA faecalis* 360bp; 3, *SodAFaecium*215bp; 4, *ccf* 543bp; 5, *cylA*688bp; 6, *EfaA*705bp; 7, *esp*933bp; 8, *gelE* 419bp; 9, negative control.

4.3 Results

4.3.1 Prevalence of *Enterococcus* species in chickens, cats, and dogs

Figure 4.1 shows the representative gel for the confirmation of the *Enterococcus* genus through amplification of the *Tuf* gene. A total of 114 *Enterococcus* isolates were recovered presumptively

on bile esculin agar test from the three-animal species but were confirmed to be 109 (77.3%) *Enterococcus* isolates using PCR; 46 (93.9%), 16 (64.0%) and 47 (70.1%) isolates respectively from chickens, cats, and dogs. From the 109 *Enterococcus* spp. detected, 57 (40.4%) were *E. faecalis* while 12 (8.5%) were *E. faecium* and 40 (28.4%) were other *Enterococcus* species that were not differentiated during this study. Thirty-six (73.5%) *E. faecalis* and 2 (4.1%) *E. faecium* were recovered from chickens, 6 (24.0%) *E. faecalis* and 3 (12.0%) *E. faecium* from cats and lastly 15 (22.4%) *E. faecalis* and 7 (10.4%) *E. faecium* from dogs.

4.3.2 Prevalence of virulence genes in *E. faecalis and E. faecium* detected in chickens, cats, and dogs

It was observed that cat and dog isolates harbored all the five virulence genes screened while chickens harbored only four. *E. faecalis* was found to be the major pathogen in all the animal species compared to *E. faecium.* The Prevalence of all the virulence genes in the three-animal species studied is presented in Figure 4.2; *EfaA* (88.7%) was most prevalent followed by *GelE* (82.3%), *ccf* (81.6%), *Esp* (26.2%) and *CylA* (25.5%).

As depicted in Figures 4.3-4.5, the prevalence of *EfaA* and *GelE* genes was significantly (P<0.001) higher (100%) among *E. faecalis* and *E. faecium* detected in chickens and cats than among *E. faecalis* (14 of 15 [93.3%] and 9 of 15 [60%]) respectively and *E. faecium* (6 of 7 [85.7%] and 4 of 7 [57.1%]) respectively detected in dogs.

The distribution of *ccf* gene was significantly (P<0.001) higher (100%) among *E. faecalis* and *E. faecium* detected in chickens and *E. faecium* detected in dogs than among *E. faecalis* (4 of 6 [66.7%] and 13 of 15 [86.7%]) respectively from cats and dogs and *E. faecium* (2 of 3 [66.7%]) detected in cats.

The *Esp* gene was found in 10 of 15 [66.7%] *E. faecalis* and 4 of 7 [57.1%] *E. faecium* detected in dog isolates whereas it was found in 2 of 6 [33.3%] of *E. faecalis* isolates in cats. *Esp* gene was not detected in chickens and cats *E. faecium* isolates.

Cytolysin virulence factor encoded by *CylA* gene was found in 9 of 15 [60%] *E. faecalis* and 3 of 7 [42.9%] *E. faecium* detected in dog isolates whereas 2 of 36 [5.6%] of *E. faecalis* isolates in chicken and 1 of 6 [16.7%] of *E. faecalis* isolates in cats harbored this gene. *CylA* gene was not detected in chickens and cats *E. faecium* isolates.

Table 4.2 depicts the results of Chi-square test. The relationship between *Tuf* gene, *SodA faecalis* and all the virulence genes (*cylA*, *efaAfs, Esp, ccf*, and *GelE*) and the animal species were statistically significant ($P< 0.05$). However, *SodA faecium* gene was found statistically insignificant ($P> 0.05$).

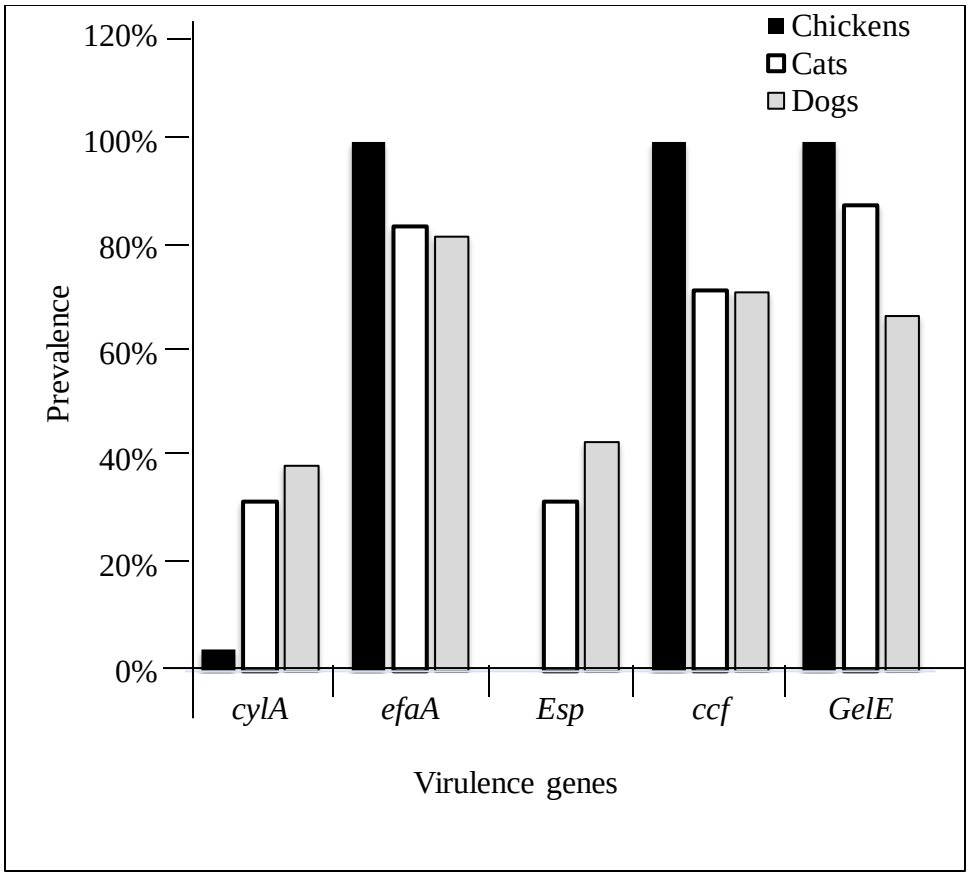

Figure 4.2: Prevalence of virulence genes from *Enterococcus* species isolated from chickens, cats, and dogs

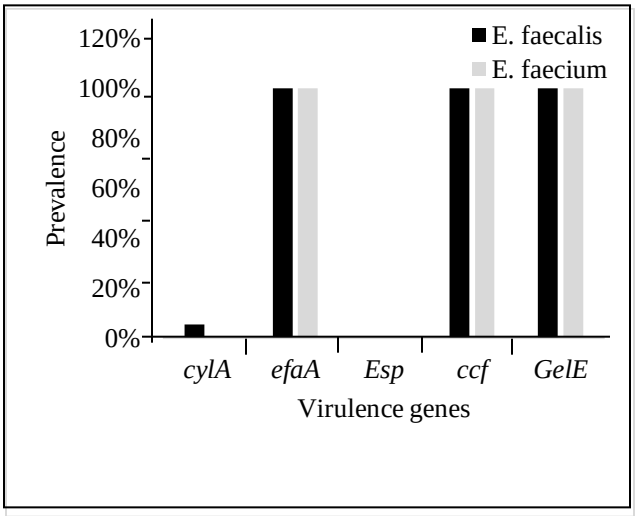

Figure 4.3: Prevalence of virulence genes from chicken isolates per *Enterococcus* species

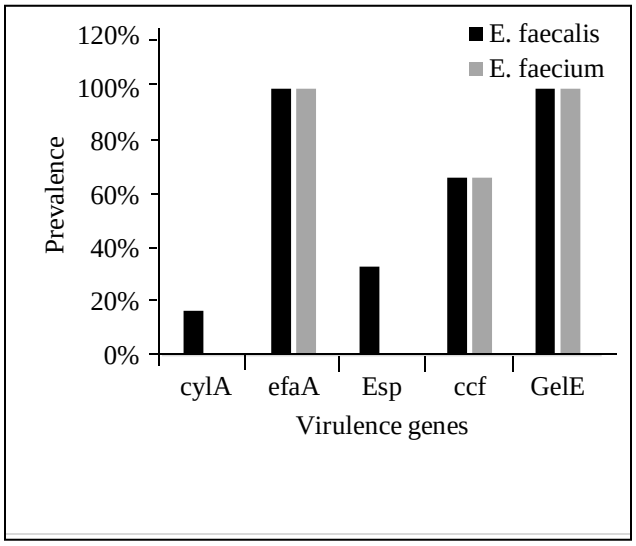

Figure 4.4: Prevalence of virulence genes from cat isolates per *Enterococcus* species

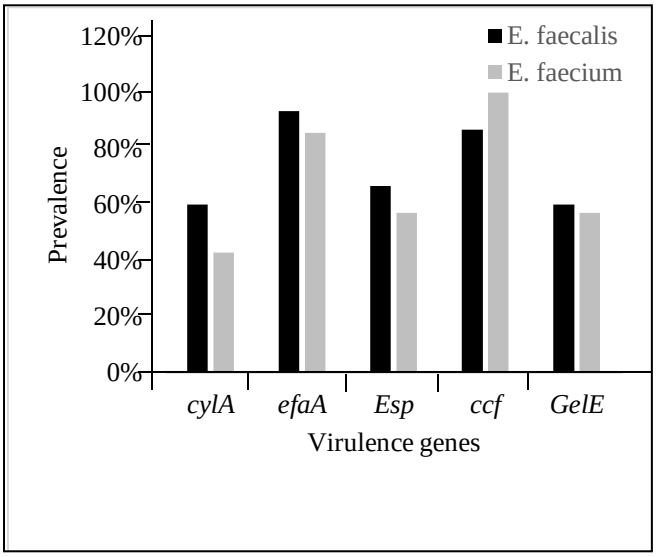

Figure 4.5: Prevalence of virulence genes from dog isolates per *Enterococcus* species

4.3.3 Association of virulence genes with the animal species and gender

The result of binomial logistic regression is presented in Table 4.3. Cats and dogs are less likely (OR: 0.13, 95% CI: -3.66 to -0.70 and OR: 0.16, 95% CI: -3.34 to -0.68 respectively) to harbor *Tuf* gene compared to chickens. Considering gender, females are less likely (OR 0.59, 95% CI: -1.39 to 0.31) to harbor *Tuf* gene compared to males. Cats and dogs are less likely (OR: 0.11, 95% CI: -3.37 to -1.09 and OR: 0.10, 95% CI: -3.15 to -1.43 respectively) to harbor *SodA faecalis* gene compared to chickens. Regarding the effect of gender on *SodA faecalis* gene, females are less likely (OR 0.98, 95% CI: -0.80 to 0.77) to harbor this gene compared to males. Cats and dogs are more likely (OR: 3.29, 95% CI: -0.69 to 3.29 and OR: 2.77, 95% CI: -0.46 to 2.96) in the order listed to harbor *SodA faecium* gene compared to chickens. Considering gender, females are less likely (OR 0.89, 95% CI: -1.34 to 1.12) to harbor *SodA faecium* relative to males. For *cylA* and *Esp* genes, cats and dogs are more likely to harbor these genes compared to chickens while females are more likely (OR 1.99, 95% CI: -0.14 to 1.56 and OR: 2.69, 95% CI: 0.13 to 1.91) respectively to harbor *cylA* and *Esp* genes compared to males. Females are more likely (OR 1.62, 95% CI: -0.47 to 1.44) to harbor this *GelE* gene compared to males. Cats and dogs are less likely to harbor *EfaA and ccf* genes compared to chickens. Regarding the effect of gender on the prevalence of *EfaA* virulence gene, females are more likely (OR: 3.19, CI: 0.00 to 2.33) to harbor this gene than males.

Table 4.2: Chi-square test for virulence genes detected in *Enterococcus* species from chickens, cats, and dogs

Statistical tests	**Asymptotic significance (2-sided)**							
	Tuf	***SodA faecalis***	***SodA faecium***	***cylA***	***efaA***	***Esp***	***ccf***	***GelE***
Pearson Chi-square	0.002	0.000	0.378	0.000	0.008	0.000	0.000	0.000

Table 4.3: Output of binomial logistic regression estimation of association between the log odds of animal species and virulence genes detected in *Enterococcus faecalis* and *Enterococcus faecium*

Genes	Variables	Estimate (B)	Odd ratios (OR) exp. B	Standard error (SE)	95% Confidence interval		*P* value
					Lower limit	Upper limit	
Tuf	Intercept	2.97					0.00
	Species (ref: Chickens)						
	Cats	-2.05	0.13	0.73	-3.66	-0.70	0.01
	Dogs	-1.84	0.16	0.66	-3.34	0.68	0.00
	Gender (ref: male)						
	Female	-0.53	0.59	0.43	-1.39	0.31	0.22
SodA faecalis	Intercept	1.03					0.00
	Species (ref: Chickens)						
	Cats	-2.17	0.11	0.58	-3.37	-1.09	0.00
	Dogs	-2.26	0.10	0.44	-3.15	-1.43	0.00
	Gender (ref: male)						
	Female	-0.02	0.98	0.40	-0.80	0.77	0.95
SodA faecium	Intercept	-3.11					0.00
	Species (ref: Chickens)						
	Cats	1.19	3.29	0.96	-0.69	3.29	0.22
	Dogs	1.02	2.77	0.83	-0.46	2.96	0.22
	Gender (ref: male)						
	Female	-0.11	0.89	0.62	-1.34	1.12	0.86
cylA	Intercept	-3.49					0.00
	Species (ref: Chickens)						
	Cats	2.28	9.78	0.85	0.77	4.24	0.01
	Dogs	2.67	14.44	0.77	1.38	4.54	0.00
	Gender (ref: male)						
	Female	0.69	1.99	0.43	-0.14	1.56	0.11
EfaA	Intercept	19.19					0.99
	Species (ref: Chickens)						
	Cats	-18.17	0.00	1501.30	-0.00	2950.54	0.99
	Dogs	-18.15	0.00	1501.30	-0.00	2950.56	0.99
	Gender (ref: male)						
	Female	1.16	3.19	0.59	-0.00	2.33	0.05
Esp	Intercept	-20.05					0.99
	Species (ref: Chickens)						
	Cats	18.61	>999.99	1499.09	-54.81	461.19	0.99
	Dogs	19.25	>999.99	1499.09	-50.98	479.31	0.99
	Gender (ref: male)						
	Female	0.99	2.69	0.45	0.13	1.91	0.02
GelE	Intercept	19.39					0.99
	Species (ref: Chickens)						
	Cats	-17.68	0.00	1528.73	-3.040.64	3005.28	0.99
	Dogs	-18.91	0.00	1528.73	-3.041.86	3004.05	0.99
	Gender (ref: male)						
	Female	0.48	1.62	0.48	-0.47	1.44	0.32

ccf	Intercept	19.25					0.99
	Species (ref: Chickens)						
	Cats	-18.85	0.00	1509.76	-0.00	2966.61	0.99
	Dogs	-18.74	0.00	1509.76	-0.00	2966.71	0.99
	Gender (ref: male)						
	Female	0.91	2.48	0.48	-0.03	1.87	0.06

4.4 Discussion

This study was carried out to investigate the incidence of virulence factors in *Enterococcus* species emanating from chickens, cats, and dogs. For the detection of *Enterococcus* at genus and species level, there was statistical association between animal species and prevalence of *Tuf* gene and *E. faecalis* with chickens having the higher risk of harboring these genes compared to cats and dogs. This could be attributed to the fact that chickens utilize high energy more than other animals during digestion and so possess a unique digestive system suitable for this function. To effectively carry out this function, the gastrointestinal tract of chicken is composed of diverse kinds of microbes including those of human and animal pathogens which aid in the digestion of food (Stanley *et al*., 2014). Also, the host's genotype may influence the composition of its gut microbiota directly or indirectly. Directly by controlling motility in the gut, secretions and modifying surface of epithelial cells. Indirectly through host's preferences on food. Environmental factors such as feed composition and management practice may also affect the microbiota of an animal (Zhao *et al*., 2013).

As *Enterococci* bacteria are common residents of the gastrointestinal tract of animals and humans, some are commensal and others pathogenic. Upon invasion, the immune system of the host quickly recognizes the pathogenic ones and eliminate them leaving out the commensal ones which in symbiotic association promotes the host's nutrition and health (Silva *et al*., 2012). Host's innate immune Pattern Recognition Receptors (PRR) encodes germline which enables it to recognize

Microbial Associated Molecular Patterns (MAMPS) to induce an immune response (Diacovich and Gorvel 2010). However, pathogenic *Enterococci* evade its host immune response by modifying its peptidoglycan. Peptidoglycan (PG) is found in the cell wall of *Enterococcus* species, a polymer composed of polysaccharides and short stem peptide chains. Modification of the bacteria's peptidoglycan involves disruption of lysozyme catalytic activity by modification of the glycan backbone chain of PG and reduction of immune recognition and response by modifying the PG stem peptide chain (Sukhithasri *et al*., 2013).

Various virulence factors involved in the pathogenesis of *Enterococcus* species was detected in this study. *EfaA* gene was found in *E. faecalis* and *E. faecium* isolates from chicken and cats at a high (100%) prevalence. This agrees with the findings of Jahan and Holley (2014) although the prevalence of *EfaA* gene was detected at a lower frequency (15%) in *E. faecium*. However, results from the previous authors are higher compared to the results of Iseppi *et al*., (2015). *E. faecalis* antigen A encoded by *EfaA* gene is a toxic substance secreted by *Enterococcus* species which aids its attachment to the host cell during the infection process. It has been found to be responsible for infective endocarditis and peritonitis infection in animals (Kafil *et al*., 2016).

GelE gene coding for gelatinase virulence factor was also highly (100%) prevalent amongst *E. faecalis* and *E. faecium* isolates from cats and chickens. Hammad *et al.*, (2014) reported a high (100%) prevalence of this gene in *E. faecalis* isolates but low (14.2 %) prevalence in *E. faecium*. The *GelE* gene is an extra-cellular zinc endopeptidase which helps to hydrolyze collagen, gelatin and small peptides in the bacteria. It increases the severity of endocarditis infection (Medeiros *et al*., 2014).

Furthermore, high (100%) prevalence of *ccf* gene was recorded in *E. faecalis* and *E. faecium* isolates from chickens in the present study. This is consistent with the works of Jimenez *et al*., (2013) amongst *E. faecalis* isolates. However, none of the *E. faecium* isolates in the works of Jimenez *et al*., (2013) harbored this gene. *ccf* is a virulence gene coding for sex pheromone. Sex pheromone facilitates conjugative plasmid transfer between bacteria cells (Sava *et al*., 2010). Sex pheromones transduce signals that induce secretion of genes that promote colonization (Chajecka-Wierzchowska *et al*., 2016). They have also been reported to be responsible for the production of mutagenic substances that induce inflammation in human infection sites (Bhardwaj *et al*., 2008). High prevalence of sex pheromone in this study indicates that the presence of the various virulence genes detected in this study was acquired through horizontal gene transfer by plasmids and transposons in the *Enterococcus* species.

The reason for the absence of *Esp* gene coding *Enterococcus* surface protein in chicken isolates in this study is unclear. However, Olsen *et al*., (2012) detected *Esp* gene in poultry *E. faecalis* isolates. *Enterococcus* surface protein is found resident on *E. faecalis* and *E. faecium* pathogenicity island (PAI) (Seputiene *et al*., 2012). *Enterococcus* surface protein is a cell wall protein that helps in the adhesion of pathogenic *Enterococci* to its host tissue. It damages the cell membrane and facilitates the infection process. It is responsible for enterococcal urinary tract infections and aids *Enterococci* biofilm formation because of its structures, which consist of tandem repeat units (Vankerckhoven *et al*., 2004; Medeiros *et al*., 2013). Cytolysin activator (*cylA* gene) is borne on the plasmid of *Enterococcus* bacteria and increases the severity of endocarditis and endophthalmitis in animals. It is an extracellular protein that is encoded by *cylL1, cylL2, cylA, cylM* and *cylB* operon (Vankerckhoven *et al*., 2004; Medeiros *et al*., 2013). Low prevalence of

cylA gene in this study is in concordance with other findings (Iseppi *et al*., 2015; Boyar *et al*., 2017).

Among the five virulence genes detected, only *cylA* showed a significant association with animal species. Furthermore, there was an association between the prevalence of *EfaA* and *Esp* genes with the female gender of the animal species. Generally, males for both human and animals are more susceptible to infections than their female counterpart. This in part is due to their sex hormone. Sex hormones are estrogen, androgen, testosterone produced by ovaries, testes and adrenal gland which regulate development and functioning of the reproductive organs of the animals. Testosterone in males has been reported to decrease natural killer cells in animals and toll-like receptor 4 involved in pattern recognition receptor and immune response while estrogen found in female animal species enhance innate immune response (Garcia-Gomez *et al*., 2012). Sex pheromones also influence the expression of genes linked with the animal's characteristics to become susceptible or resistant to an infection such as growth, acquisition of virulence factors and bacterial metabolism (Ahmed *et al*., 2010; Garcia-Gomez *et al*., 2012). However, further studies are required to establish the effect of gender in the acquisition of virulence gene because females in some cases in this study were more likely to harbor virulence genes.

4.5 Conclusion

Our study revealed that *Enterococcus faecalis* and *Enterococcus faecium* strains from chicken, cats and dog harbored multiple virulence genes which are critical in the pathogenesis of *Enterococcus* species. Our findings affirm that *E. faecalis* are more pathogenic as it harbored more virulence genes compared to *E. faecium*. A significant association was found between *Tuf*, *SodA faecalis, CylA, EfaA* and *Esp* genes. With the view of adverse effects of enterococcal infections especially in the Nation that has the highest burden of immune-compromised individuals, there is,

therefore, an urgent call for attention to monitoring emerging enterococcal infections in animals as there is the possibility of transferring these genes to humans. Further studies with larger sample size are critical to monitor the incidence of these virulence factors and their roles in other Provinces of South Africa.

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4.7 Conflicts of interest

The authors declare none.

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CHAPTER 5

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

Antibiotics play a significant role in medical science as their use has greatly reduced child mortality. They are crucial in performing successful surgical operations and other important treatments like chemotherapy (Lin *et al*., 2015). However, its misuse in humans and animal husbandry have led to emergence of resistant strains of bacteria thereby hampering the benefits we initially derive from them (El-Halfawy *et al*., 2017). This in turn led to persistent rise in untreatable infections and death in severe cases especially now that there are few or no novel antibiotics (Lin *et al*., 2015). *Enterococci* are commensal gut bacteria of humans and animals but have been recognized as the third most common cause of hospital acquired infections such as urinary tract infections and endocarditis due to its intrinsic ability to become resistant to multiple drugs (Khani *et al*., 2016). They have effective gene transfer mechanisms and are ubiquitously found free-living everywhere. Therefore, they have been found in chickens, cats and dogs posing great health risk to humans as they laterally transfer antibiotic resistance genes and infections to humans through the consumption of contaminated chicken meats and direct contact of pets with their owners (Celik *et al*., 2017). Alongside antimicrobial resistance in *Enterococci* is the acquisition of virulence genes through plasmids, pathogenicity island and transposons which increases the severity of infections (Hollenbeck and Rice 2012). Antibiotic resistance is one of the leading health crises globally with the most hit in developing countries such as South Africa (Golkar *et al*., 2014). Unfortunately, information on prevalence of *Enterococci* antimicrobial resistance is very limited in South Africa. This reinforces the importance of this study on antibiotic resistance profiles and genetic characterization of virulence and resistance genes of *Enterococcus* species.

5.1 General discussion

The aim of this study was to isolate *Enterococcus* species (particularly *Enterococcus faecalis* and *Enterococcus faecium*) from chickens, cats and dogs using microbiological culture methods and molecular detection through polymerase chain reaction to determine the antibiotic resistance profiles and the prevalence of resistance and virulence genes. *Enterococcus* species was highly prevalent (109 of 150 [77.3%]) in the three-animal species investigated. This is in agreement with the findings of de Jong *et al*., (2018). Chicken samples had the highest prevalence of *Enterococcus* species compared to cats and dogs. The reason for this could be that chicken's gastrointestinal tract is composed of many microbes which helps it to digest food as it utilizes high energy during digestion more than other animals (Stanley *et al*., 2014). *Enterococcus faecalis* was predominantly detected than *Enterococcus faecium.* Some researchers reported the predominance of *E. faecalis* over *E. faecium* (Kwon *et al*., 2012; Kurekci *et al*., 2016) while others detected *E. faecium* more than *E. faecalis* (Kukanich *et al.,* 2012; Ngbede *et al.,* 2017). *E. faecalis* and *E. faecium* are recognized as the most pathogenic organisms among other *Enterococcus* species (Ben Sallem *et al.*, 2016; Paosinho *et al.,* 2016).

Antimicrobial susceptibility test on disc diffusion by Kirby Bauer showed a high resistance rate (more than 70%) to quinupristin/ dalfopristin, vancomycin, rifampicin, ampicillin, and erythromycin and lower (60- 14%) resistance rate to tetracycline, chloramphenicol, high-level streptomycin and ciprofloxacin. However, all the isolates were susceptible to high-level gentamicin. Twenty-eight multiple drug resistance (MDR) patterns were observed in 119 of 150 (84.3%) samples with rifampicin/ ampicillin/ vancomycin/ quinupristin- dalfopristin/ erythromycin/ tetracycline (n = 18) pattern mostly prevalent. In all, chicken *Enterococcus* isolates showed the highest rate of resistance to all the antibiotics tested compared to cats and dogs. MDR

pattern observed in this study is a speculation of antibiotic use in the study location as it could also be as a result of the acquisition of plasmids, mutations in chromosomes and pathogenicity islands acquisition. High resistance to these drugs especially rifampicin poses great public health risk to South Africa due to high burden immunocompromised individuals with tuberculosis and HIV/AIDS. Resistance to any these drugs leads to treatment failure and loss of synergistic bactericidal activity (Bortolaia *et al*., 2015).

Polymerase chain reaction (PCR) was used to detect the presence of genes- *aac(6')-Ie-aph(2'')-Ia; aph(3')-IIIa; ant(6')-Ia* and *Van-A* conferring resistance for gentamicin, streptomycin, kanamycin and vancomycin respectively. Out the four resistance genes screened, 21.3%, 7.8% and 4.3% for kanamycin, streptomycin, and vancomycin resistance genes respectively were present. These genes have been reported in other studies (Bortolaia *et al.,* 2015; Hidano *et al.,* 2015; Klibi *et al*., 2015). They are carried on mobile genetic elements (plasmids, transposons) of *Enterococcus* species and could be easily transferred through horizontal gene transfer process to other bacteria species and humans (Jurado-Rabadan *et al.,* 2014). A positive correlation (0.278) was found between *SodA faecalis and aph(3')-IIIa* gene, $P < 0.01$. Also, between *aph(3')-IIIa* and *ant(6')-Ia* genes (0.366) $P< 0.01$.

This study further demonstrated that the *Enterococcus* species detected in chickens, cats and dogs could be pathogenic as they harbored varying number of virulence genes (*cylA, efaAfs, Esp, ccf* and *GelE)* coding for cytolysin, *E. faecalis* antigen A, *Enterococcus* surface protein, sex pheromone and gelatinase respectively which are important in the pathogenesis of *Enterococci* bacteria. *E. faecalis* isolated in all the three-animal species investigated harbored more virulence genes compared to *E. faecium*. Cytolysin encoded by chromosomal *cyl* gene plays important role

in the production of bacteriocin which exerts bacteriostatic effect against pathogenic microbes in the gut but found to lyse bacterial and eukaryotic cells (Van Tyne and Gilmore 2014). *E. faecalis* antigen A encoded by *efaA* gene is a toxic substance secreted by *Enterococcus* species which aids its attachment to the host cell during the infection process (Kafil *et al*., 2016). Of great importance is the pathogenesis of *Enterococci* is the detection *Enterococcus* surface protein. It is found resident on *E. faecalis* and *E. faecium* pathogenicity island (PAI) (Seputiene *et al*., 2012). It is responsible for enterococcal urinary tract infections and aids *Enterococci* biofilm formation (Vankerckhoven *et al*., 2004). The *GelE* gene is an extra-cellular zinc endopeptidase which helps to hydrolyze collagen, gelatin and small peptides in the bacteria. It increases the severity of endocarditis infection (Medeiros *et al*., 2013). *ccf* is a virulence gene coding for sex pheromone. Sex pheromone facilitates conjugative plasmid transfer between bacteria cells (Sava *et al*., 2010). Statistical analysis revealed a significant association between *Tuf, SodA faecalis, CylA, EfaA* and *Esp* genes.

5.2 Conclusions and recommendations

In this study, *Enterococcus* species was mostly detected in chickens and relatively high frequencies in cats and dogs. They were found to be highly resistant to critically important antibiotics and harbored multiple virulence genes which implies that they could be pathogenic. High prevalence of sex pheromone (*ccf*) gene was found which partially signifies that the antibiotic resistance genes and virulence genes present in enterococcal isolates in this study were mainly due to conjugative plasmid transfer. This study shows that *E. faecalis* harbored more virulence traits compared to *E. faecium.* Humans are at great risk of having these pathogens transferred to them due to their continuous relationship with these animals. Therefore, great efforts should be made by the government of South Africa to monitor and control the emergence and spread of antibiotic

resistance and infections due to *Enterococci* and other bacterial pathogens. Genomic research that focuses on unravelling new metabolic pathways for development of novel drugs against targeted bacteria species should be encouraged and funded. Law on prudent use of antibiotics should be enacted and enforced as do other countries. More studies focusing on genome sequencing of *Enterococcus* species should be explored to better understand the biology of this emerging clinical pathogen.

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