Genetic analyses of antimicrobial resistance and virulence genes in *Enterococcus* species isolated from livestock production systems in South Africa

by

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PREFACE

The research contained in this dissertation was completed by the candidate while based in the Discipline of Genetics, School of Life Sciences of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Westville, South Africa. The research was financially supported by DAAD/NRF.

The contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

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UNkulunkulu emuhle njalo, Ngiyabonga.

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GENERAL ABSTRACT

Enterococcus species are widely dispersed in the environment this includes soil, water, plants, food and animals. Although *Enterococcus* constitute mostly as a commensal bacterium; over the past years the bacterium has evolved to cause nosocomial infections. The proliferation of this pathogen is attributed to its ability in successfully transferring antimicrobial and virulence genes using several channels such as mobile genetic elements. This study investigated the prevalence of *Enterococcus* spp. in small-scale commercial farms in rural South Africa. The dissemination of virulent *E. faecium* and *E. faecalis* isolates allied with livestock production in the Eastern Cape and KwaZulu-Natal provinces was investigated. A total of 276 samples randomly sampled from livestock and their associated environments (feed, soil and water) were screened for *Enterococcus* spp. using selective media and using DNA molecular methods. *E. faecalis* and *E. faecium* prevalence was confirmed by the amplification of the *tuf* and *sodA* genes. Sixty-one percent of total presumptive isolates were *E. faecalis* (n=61) and only 8% (n=8) were identified as *E. faecium.* The presence of virulence determining factors such as *asa1, ccf, cylA, esp, gelE* and *hyl* was screened in all samples that tested positive for *Enterococcus* species. Presumptive *E.faecalis* and *E. faecium* isolates were mostly recovered from Amandawe (KZN). *E. faecalis* isolates harboured the most virulence genes *asa1* (25%; n=), *ccf* (84%; n=), *esp*(4%;n=), *gelE* (69%; n=) and *hyl* (12%; n=). Whilst *E. faecium* isolates only harboured of *asa1*(12.5%; n=1), *ccf* (100%; n=8), *gelE* (75%;n=6) and *hyl* (25%;n=2). The current study also evaluated the antibiotic resistance profiles and their associated genes in these two species. Antibiotic susceptibility profiles of *E. faecium* and *E. faecalis* were assessed using Kirby-Bauer disk-diffusion assay as per the CSLI guidelines. Erythromycin had the highest occurrence of resistant isolates in both species with 75% (n=6) and 54.1% (n=33) respectively. Isolates were least resistant to ampicillin, with 0.03% resistance in *E. faecalis* and 0% in *E. faecium*. *E. faecalis* had the highest prevalence of Multi Drug Resistance (MDR), exhibiting phenotypic resistance to macrolides, aminoglycoside, tetracyclines and fluoroquinolones. TET-CIP-ERY was the most observed antibiotic resistance pattern. Furthermore, the isolates were screened for *vanA, vanB, vanC1, vanC2/3, aac(6")-aph(2") ,ermA and ermB.* The resistance genes that amplified in *E. faecalis* included *vanB* (8%;n=5), vanC1 (37%;n=23), *vanC2*/3 (37%; n=23), *ermB* (96%;n=58), *ermA* (8%;n=5) and *aac(6")-aph(2") (1.6%*;n=1*)*. The immense dissemination of *E. faecalis* that has potentially pathogenic virulent determinants is a cause for concern in livestock production systems. In addition, faecal contamination from livestock poses a threat to the dissemination of virulent strains. The study demonstrated that *E. faecium* and *E. Faecalis* isolated from livestock and their associated environment were predominantly resistant to macrolides, glycopeptides, tetracyclines and fluroquinolones. In addition to be the first study in South Africa to document the emergence of inducible *vanC* determinants in Vancomycin Resistant Enterococci isolates. .

Keywords: Pathogens; Prevalence; Livestock; Virulence; Resistance; *Enterococcus;* Zoonoses; Communicable .

CHAPTER 1

1. BACKGROUND

Enterococcal species are bacterial species that form part of lactic acid bacterium (LAB) (Fischer and Phillips, 2009). They are cocci that exist in chains or as a single coccus, these cocci are gram positive, non-spore forming and have a low GC content (Nilsson, 2012; Fischer and Phillips, 2009). *Enterococcus* can be detected in the environment; from the soil, water, plants and animals (Hammerum, 2012). Of the 50 enterococcal species that have been described; *Enterococcus faecium* and *Enterococcus faecalis* are of importance as they are identified as part of bacterium that cause nosocomial infections in animals and humans (Torres *et al*., 2018: Zou *et al*., 2011; Ye, 2010).

Enterococcus faecium and *Enterococcus faecalis* are pathogens known to cause several infections such as septicaemia, bacteraemia, meningitis, endocarditis and urinary tract infections in humans and animals (Zou *et al*., 2011). The cocci are part of the microbial community found in both animal and human digestive tracts, and in their colon (Hammerum, 2012; Macovei and Zurek, 2007). The colon has been reported to serve as an antibiotic resistance genes reservoir, it is the site for horizontal intra-inter species gene transfer (Macovei and Zurek, 2007). This is due to the presence of many commensal bacteria that habour mobile genetic elements for genetic exchange (Frolkova *et al*., 2012)

Livestock production systems have been associated with the horizontal transfer of antimicrobial genes from ingested enterococcal species to the microbial community that is in the gut (Haag, 2015; Jackson *et al*., 2012; Sapkota *et al*.,2007). The resistant *Enterococcus* species are selected in animals due to the increased use of antimicrobial agents as growth promoters (Hammerum, 2012). *Enterococcus faecuim* has formed part of the 'ESKAPE' pathogens, these pathogens include *Enterococcus faecuim, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and Enterobacter* species These bacteria acquired the capability to efficiently escape the effects of antimicrobial drugs (Haag, 2015; Jackson *et al*., 2012). These pathogens form part of the "standard indicators" for the Global One Health Approach, which aims at monitoring to eradicating antimicrobial resistance globally (FAO, OIE, 2019)

Enterococcal species recovered from animal feed have not yet been directly identified as the main cause of clinical infections in humans, although consumption of food products namely from livestock that are contaminated by antibiotic resistance enterococcal species may be the possible mode of transfer (Hayes *et al*., 2003). Thus, will in turn result in the transfer or colonization of these antimicrobial resistant species to the host micro-flora (Hayes *et al*., 2003). It has been previously reported that these bacterial species have the ability acquire antimicrobial resistance and virulence determinants, in addition to exhibiting a certain level of resistance to several antibiotics at a time (Lins *et al*., 2013).

It is imperative to understand the ecology, epidemiology, antibiotic resistance and virulence determents of *Enterococcus* species they form part of human and animal gastric bacterial community in addition to inhabiting the environment (soil and water) (Hegstad *et al*., 2012, Dunny *et al.,* 1995). Enterococcal species can acquire new resistance genes through mechanisms such as mobile genetic elements (MGEs) from other bacteria, which will consequently lead to having more pathogenic bacteria that will cause more complex nosocomial infections (Hegstad *et al*., 2012). In addition, *E. faecium* has shown to have an increase in the prevalence (Comerlato *et al*., 2013). The pathogenicity of *E. faecium* is attributed to the efficiency of its virulence determinates found in the large pathogenicity islands (Manson *et al.*, 2010) Whilst *E. faecalis* has been the most recovered strain causing both human and animal infections, it has been reported that 90% of the recovered clinical isolates where *E. faecalis* (Comerlato *et al*., 2013). The species *E. faecalis* has been associated with an increase in virulence, however some *E. faecium* strains are exhibiting multi-drug resistance characteristics (Comerlato *et al*., 2013). It was therefore, imperative to conduct this surveillance study on the Enterococcal species; *E. faecium* and *E. faecalis* as it will assist in determining the resistant profiles found in these two species. This will aid in improving how enterococcal infections are treated in both life stock and humans.

1.1. Aims and objectives

Aims

1.1.1. To determine the role of livestock production systems in the dissemination of the pathogenic *Enterococcus* species.

1.1.2. To determine the occurrence of Vancomycin Resistant Enterococci (VRE) among livestock productions systems in KwaZulu-Natal and Eastern Cape provinces of South Africa.

1.1.3. To determine the interaction of antibiotic resistance and virulence in the prevalence and dissemination of *Enterococcus* species in livestock productions systems in South Africa.

Objectives

1.1.4. To evaluate the prevalence of *Enterococcus* species in livestock production systems by swabbing the animals in farms, faecal samples, slaughterhouses and water samples.

1.1.5. To isolate the presumptive enterococcal species using the enterococcal selective bile esculin azide agar.

1.1.6. To confirm of the presence of *E. faecium* and *E. faecalis* using the *tuf* gene and species specific primers.

1.1.7. To determine the species distribution of *E. faecium* and *E. faecalis* with reference to their antibiotic resistance patterns from isolates recovered from livestock production systems and environmental samples using PCR.

1.1.8. To statistically determine a relationship between the species distribution of *Enterococcus* species *E. faecium* and *E. faecalis* and the source of the isolate using statistical tests.

Justification/Rationale

The data on the prevalence and dissemination of antibiotic resistant *Enterococcus* species in livestock production systems in South Africa is limited, the appropriate identification of these species is necessary. Excluding the fact that they are the third leading cause of nosocomial infections worldwide, for the purposes of treatment it is essential to know which enterococcal species typically cause infections in nosocomial settings (Hammerum, 2012; Jackson *et al*., 2004). In addition, to identifying which virulence and antimicrobial genes aid in the dissemination of these antibiotic resistant species in livestock productions. It has also been scientifically reported that some species of *Enterococcus* have multi drug resistance however resistance to antimicrobial alone does not explain the virulence associated with the bacterium (Ye, 2010). Thus, there is dearth in published literature which describes the effectiveness of the mechanisms of horizontal gene transfer in *Enterococcus* species.

Expected outcomes

This study expects to successfully isolate environmental samples using conventional culture methods to further assess the presumptive isolates using genotypic assays. Previous studies assessed the enterococcal diversity and antimicrobial profiles in meat before and after food preparation. such cases most of the bacterial community has been destroyed. This study will not be limited to assessing antimicrobial profiles of the enterococcal species in food producing animals only. However, the prevalence and antimicrobial profiles of *Enterococcus* species will also be assessed in the environment surrounding livestock productions systems (soil, water and knives) in South Africa. The results of this study will improve the knowledge with reference to the use of antimicrobials in veterinary therapy as it affects the entire production systems. Thus, contributing to the gap in scientific literature.

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CHAPTER 2

LITERATURE REVIEW

2.1. Introduction

Enterococci are gram-positive cocci commonly found in the environment (Ben Said *et al.*, 2016). Recent studies have reported that enterococcal strains are rapidly becoming a cause for concern, as they form part of the pathogens that are responsible for community and hospital acquired infections (Dolka *et al.*, 2017). It is noticeable that species from this genus are commonly resistant to antimicrobials administered to infected animals and humans (Hammerum, 2012). The purpose of this chapter is to review available literature on the dissemination, diversity and pathogenic potential of *E. faecalis* and *E. faecium* in livestock and its associated environment. The current chapter aims to discuss the impact of zoonoses on the prevalence of the virulence and antimicrobial genes in the environment. Enterococci are ubitiqous and are known to have been transferring, in addition to acquiring genetic determinants from other pathogenic bacterium namely; *methicillin resistant Staphylococcus* (Werner *et al.*, 2013) henceforth literature reported on the mechanisms of horizontal gene transfer in this genus will be reviewed in this chapter.

2.2. Dissemination of Antibiotic resistance bacterium in animal husbandry.

Livestock production contributes to food safety and security as well as animal welfare (Rushton, 2015). Globally, it is estimated that per person living there is approximately 190 kilograms of livestock for food consumption (Rushton, 2015). However, livestock farming has changed drastically compared to the traditional methods that were used during the primitive ages (Haag, 2015). Farming is more geared towards low cost and high meat production (Grobler, 2010). At the present moment, there are three types of production systems used to farm livestock. These include industrial systems, mixed farming and grazing systems (Grobler, 2012). With reference to grazing systems livestock can move around the farm freely whilst grazing (Grobler, 2010). However, this type of system has a low production rate compared to the other aforementioned systems. Mixed farming system is regarded as agriculturally friendly as animals and crops are integrated into one system (Jackson *et al.*, 2012). Lastly, the industrial production system is the preferred method of livestock farming as it maximizes production whilst minimizing cost expenditure (Grobler,2012; Jackson *et al.*, 2012; Sapkota *et al.*,2007). Furthermore, from livestock productions systems; food processing industries and slaughterhouses produce meat products that are uniform and have high quality (Rushton, 2015).

The industrial production system is also known as Concentrated Animal Feeding Operations (CAFO's) (Haag, 2015; Jackson *et al.*, 2012; Sapkota *et al.*,2007). CAFO's are production systems in which livestock is raised in artificial and extremely confined conditions (Haag, 2015; Jackson *et al.*,2012). The livestock (this includes cattle sheep and goat) never roam the area, in such a way that they are

bought feed instead of them grazing nor foraging for food, they are usually kept for a period of approximately 45 days of the growing season (Jackson *et al*., 2012). This operation is designed to make the animals gain muscle weight for meat production, the technique is achieved by by minimizing energy expenditure in addition to fitting as many animals as possible in these feedlots (Jackson *et al*., 2012). It is estimated that most livestock are farmed in this manner (Jackson *et al*., 2012). Haag (2015) reported that cattle bought up in such conditions live in pens constantly standing in their own faeces, whilst pigs and chicken live shoulder to shoulder in factories confined to never see the light of day. Such living conditions causes stress in these animals, thus making them vulnerable to diseases.

As a preventative measure, the farms administer subtherapeutic dosage of antibiotics (for example tetracycline, virginiamycin, ampicillin, penicillin, erythromycin, chlortetracycline and sulfamethazine) to the animal feed or water, for preventing diseases (Chattopadhyay, 2014;Jackson *et al*., 2012; Sapkota *et al*., 2007). Some antimicrobials are added in the livestock as feed additives to function as growth promoters, thus increasing feed efficiency (Haag, 2015; Hammerum, 2012). In the United States, the Food and Drug Administration (FDA) reported that over 136 million kilograms of antibiotics sold, approximately 80% of those went to livestock production systems (Haag, 2015). These antibiotics may be administered for prophylactic, therapeutic and metaphylactic measures, however, the use of these drugs creates selective pressure on the microbiota, thus allowing antibiotic resistant (AR) bacteria to colonize the environment (surface water) and the livestock (Jackson *et al*., 2012).

The dissemination of these bacteria can cause public health concern, as individuals (CAFO's employees) who come in direct contact with the livestock faecal matter or any meat product that is contaminated with the AR bacteria will spread it to their respective communities (Jackson *et al*., 2012). In addition, during the transportation of livestock to a slaughterhouse the truck transporting the broiler chickens or swine may be highly contaminated (Jackson *et al*., 2012). In the cases where the bacteria are airborne people travelling along the route of the truck will be exposed to the AR bacteria (Jackson *et al*., 2012). Whilst Macovei and Zurek (2007) documented that house flies from the CAFO's aid in the dissemination of the AR bacterium as they frequent the colonized factories. As afore mentioned, livestock bought up in such conditions are constantly standing in their own faeces. The faeces are collected and used as manure (Haag, 2015). Manure is frequently stored in large outdoor pits then administered to agricultural works as a fertilizer (Sapkota *et al*., 2007). However, because of surface run-offs the manure can affect surface water (rivers, lakes and streams). Due to the prophylactic use of antimicrobials in animal feed, AR bacteria will be selected for in the gut of the livestock. This will lead to manure that is colonized by the selected bacteria (Sapkota *et al*., 2007).

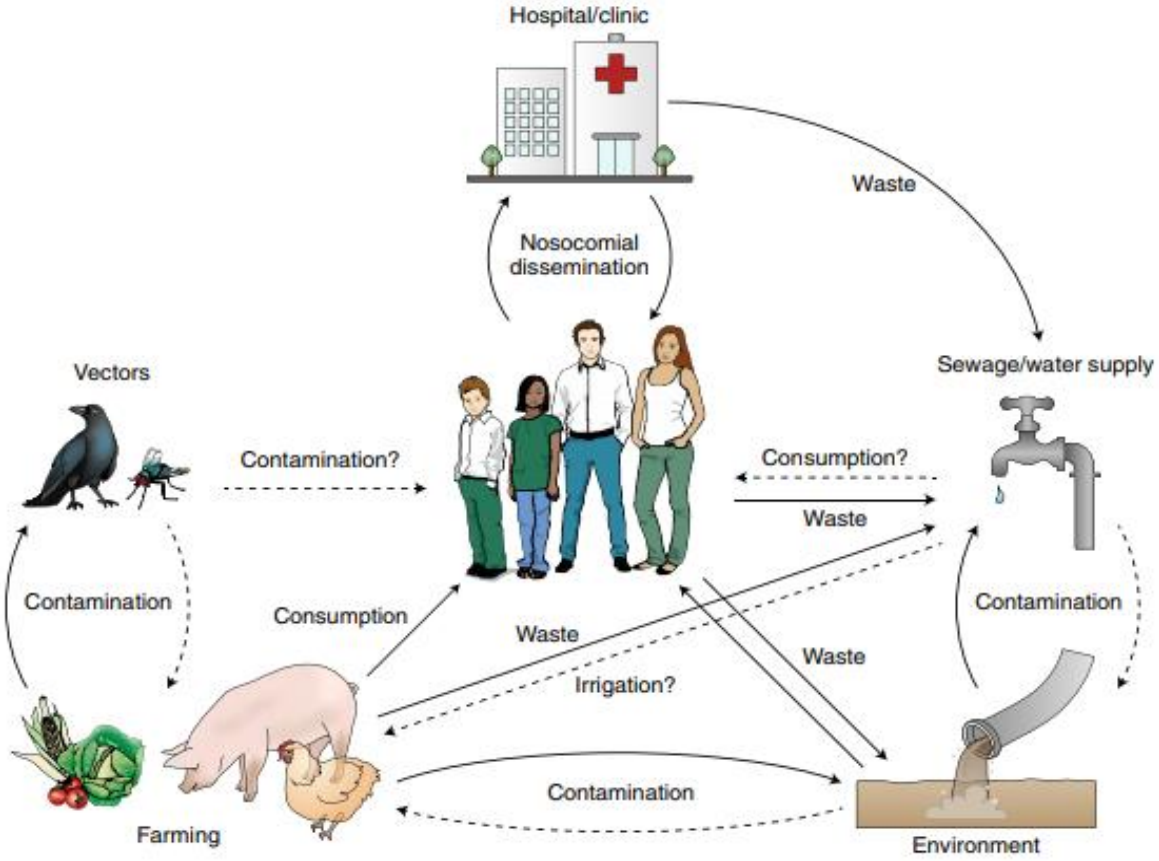

Figure 2.1. Different routes zoonotic bacteria disseminate in the community and the environment (Walsh,2018).

The *Enterococcus* species is one of the many bacteria that predominantly colonizes manure and other livestock associated environments(Haack *et al*., 2015; Ali *et al.*, 2014; Barton, 2014). This is because enterococci can strive in extreme environmental conditions whichincludes extreme salinity and pH, in addition to fluctuating temperatures (Klibi *et al*., 2012). The adverse ability to survive in such conditions has enabled the enterococci to colonize different ecological niches thus spreading between different environments (Klibi *et al*., 2012). Nilsson e*t al*. (2012) highlighted that any disease or infection that can be transmittable naturally between animals and humans or vice-versa whether it be indirect or direct is termed to be zoonotic (figure 2.1). The pathogen can also be transmitted from food to animals, food producing animals to the environment. In reference to foodborne zoonosis, the meat products can be contaminated by faeces from animals during slaughter(Bortolaia *et al.*, 2016; Jaimee & Halami, 2016: Busani *et al.*, 2004). The vegetables may be contaminated from by the manure applied in the fields (Nilsson, 2012). Not only does the transmitted antibiotic resistance bacteria have zoonotic potential, the antimicrobial resistance determinants also have the ability to be transferred to non-pathogenic strains (Nilsson, 2012).

2.3. Structure and Taxonomy of *Enterococcus* Species

Enterococcus species for many years have been assumed to be non-harmful bacteria to animals and humans as this genus produced bacteriocins (Fisher and Phillips, 2009). Due to novel technologies over the years *Enterococcus* has emerged as a nosocomial pathogen (Comerlato *et al*., 2013; Nilsson, 2012; Diarra *et al*., 2010). They are thought to be the third leading cause of urinary tract infections and endocarditis (Comerlato *et al*., 2013). As a result of its emergence as a nosocomial pathogen, there is a need to understand the *Enterococcus* genus including its taxonomy, epidemiology and ecology.

Enterococci is a commensal bacterium that inhabits the gastrointestinal tract of vertebrates and other invertebrates (Nilsson, 2012; Fisher and Phillips, 2009). In addition, the bacteria are found in artificially or naturally fermented food, animal feed and plants (Klein, 2003). These cocci are gram-positive, facultative anaerobes, non-spore forming and catalase negative bacteria (Nilsson, 2012). They occur as a single coccus or in chains (Nilsson, 2012; Fisher and Phillips, 2009). Enterococci produce bacteriocins. Hence they are considered to part of the Lactic Acid Bacteria (LAB) (Nilsson, 2012; Fisher and Phillips, 2009;). LAB's are characterized by low guanine-cytosine (GC) content of less than 50 mol%. Gram-positive and catalase negative bacteria are phenotypically indistinguishable thus identification of *Enterococcus* from other cocci is achieved by elimination of other species traits (Fisher and Phillips, 2009). They have a capability to survive in different conditions for several month this includes high sodium chloride (NaCl) concentrations, temperatures ranging between 5°C - 65° and pH 4.5 – 10 (Nilsson, 2012).

During the 1800's the *Enterococcus* species were part of the *Streptococcus* genus (Fischer and Phillips 2009). In 1937, this genus was classified into four sub-groups by Sherman; namely pyogenous streptococci, viridans, dairy streptococci and faecal streptococci (known as enterococci) (Fischer and Phillips, 2009; Klein, 2003). The term faecal streptococci was used for potentially pathogenic bacteria whereby a patient was observed to be suffering from endocarditis (Klein, 2003). It was indicated that faecal streptococci belonged to the Lancefield group D streptococci (Fischer and Phillips, 2009; Klein, 2003). Consequently, the latter could be distinguished by reactions that are either haemolytic or proteolytic. While different reactions were designed to differentiate between faecal streptococcus and the other subgroups. There was still a certain level of ambiguity with regards to which species belonged to which sub-group.

2.4. Identification of *Enterococcus* species

The enterococci species have been detected in the environment from animals, water, soil and plants, even in the smallest of organisms such as insects (Hammerum, 2012). *E. faecium* and *E. faecalis* cause many nosocomial infections. These include bacteraemia, urinary tract infections and infective

endocarditis (Hammerum, 2012). As nosocomial pathogens they have been associated with a high morbidity and mortality (Hammerum, 2012). The identification of *Enterococcus* species from other cocci and differentiating within the enterococci species has become of primary importance since it causes clinical infections (Chotinantakul *et al.*, 2018; Klein, 2003). As previously alluded, the *Enterococcus* species have been identified using phenotypic characteristics such as the production of gelatinase and haemolysin, hydrolysis of bile salts and biofilm production (Diarra *et al*., 2010). The first step in identification of the species is to distinguish *Enterococcus* from other gram-positive catalase, negative cocci, which is usually achieved using selective media (Chotinantakul *et al.*, 2018). The serological group of *Enterococcus* species according to the Lancefield groupings aids in the conformation of the species (Fischer and Phillips, 2009). Enterococci can grow in the presence of 6.5% sodium chloride (NaCl) at 10°C whilst the other cocci's growth will be inhibited (Nishiyama *et al.,* 2015; Klein, 2003). Gas released from glucose in addition to growth at 45°C indicates that the isolates are of the enterococci genus (Klein, 2003). The species *E. faecium* and *E. faecalis* could be further differentiated by growing the bacteria in the presence of mannitol, sorbitol and arabinose (Chotinantakul *et al.*, 2018).

Selective and elective media are essential for the differentiation of *E. faecium* and *E. faecalis* (Dunny & Berntsson, 2016 Klein, 2003). When both these species are grown on specific media with Tetrazolium-chloride (TTC) *E. faecalis* will reduce TTC strongly showing red colonies whilst *E. faecium* will either reduce TTC weakly or not at all showing pale pink colonies (Madu & Reddy, 2019). The are other several growth media used for enterococcal growth (Domig *et al*., 2003); Columbia agar with 5% defibrinated sheep's blood incubated for 72 hours in conjunction with trypticase soya broth is usually used for antibiotic resistant associated *Enterococcus* (Fisher & Phillips, 2009; Domig *et al*., 2003; Landman *et al*., 1995). The addition of the sheep's blood is applied to assess the haemolysis activity of the enterococcal species, presumptive isolates will have a yellow pigmentation (Domig *et al*., 2003). Another selective media used for enterococcal identification is Bile Esculin Azide agar (Domig *et al*., 2003; Landman *et al*., 1995). Tolerance or hydrolysis of bile indicates the presence of enterococcal strains, in which the growing colonies are black with a black halo (Diarra *et al*., 2010). Although of *Enterobacteriaceae* can colonize in this growth media with colonies having a different pigmentation for example *Klebsiella, Enterobacter* and *Staphylococcus aureus* has black pigmented colonies when cultured in Bile Esculin Azide agar (Domig *et al*., 2003; Landman *et al*., 1995). Overgrowth is unusual in these culture methods (Landman *et al*., 1995). Whilst the use Bile Esculin Azide agar is less sensitive in contrast to TTC media and Columbia agar because it is inexpensive and could be easily used by any molecular laboratory (Landman *et al*., 1995).

The emergence of AR bacteria demonstrates the importance of a more robust approach that provides rapid identification of *Enterococcus* species (Biswas & Micallef, 2017). Unfortunately, the previously alluded methods do not provide a phenotype that is unique for enterococcal strains, also these tests take more than 24-48 hours to obtain results, hence genotypic assays provide a rapid and sensitive approach for the identification of *Enterococcus* species (Song *et al.,* 2019; Biswas & Micallef, 2017). Of the genotypic assays, powerful tools such as Polymerase Chain Reaction (PCR) are used to identify enterococcal strains both within and between genera by targeting a species-specific gene that is conserved in the genus (Li *et al*., 2012). There are numerous conserved genes that can be used for detection of enterococcal strains (Li *et al*., 2012). These include the heat shock protein 60 (HSP60), rRNA species (5S, 16S and 23S), *sodA* gene and *tuf* gene (Li *et al*., 2012). The use of HSP60 and *sodA* gene have been used to detect different species-species regions on unknown *Staphylococcus* strains (Ghebremedhin *et al.,* 2008). Although these genes were specific, the sensitivity of using these genes as detection probes remains unknown, the three rRNA species and *tuf* gene are preferred, as they have high sensitivity (Iweriebor *et al*., 2015; Li *et al*., 2012; Ghebremedhin *et al.,* 2008).

2.4.1. 16s RNA

The ribosomal RNAs are present in all bacterial species, the genes coding for these three rRNA species are highly conserved. In addition, they are found between 5-6 copies per enterococcal cell. Although as afore mentioned the use of ribotyping has high sensitivity with reference to genus specific identification the latter does not apply to species-species identification (Domig *et al*., 2003). It was not until 1984, with the aid of 16S rRNA sequencing and DNA hybridization that the different species where clearly distinguishable from each other (Nilsson, 2012). It was noted that D antigen is present in both *Enterococcus* species and *Streptococcus* (Kosecka-Strojek *et al.,* 2020; Pillay *et al.,* 2018; Werner *et al.,* 2013; Fisher & Phillips, 2009). A study was conducted on the gram-positive genera using 16S rRNA to identify the phylogenetic position of enterococci, streptococci and lactococci species (Figure 2.2.). In the past these were considered to belong to one group. The dendrogram illustrates *Bacillus Subtills-Gruppe* as the most recent common ancestor with the sequence divergence of 10%,the 16S rRNA sequence of *Bacillus subtills-gruppe* have approximately 40% sequence divergence from the enterococci, streptococci and lactococci species(Nilsson, 2012). The *Enterococcus, Streptococcus and Lactococcus* belong to one clade, in addition the dendrogram indicates that *Enterococcus* and *Streptococcus* are two different cocci (Nilsson, 2012). Today over 40 different species have been described as *Enterococcus (*Nilsson, 2012).

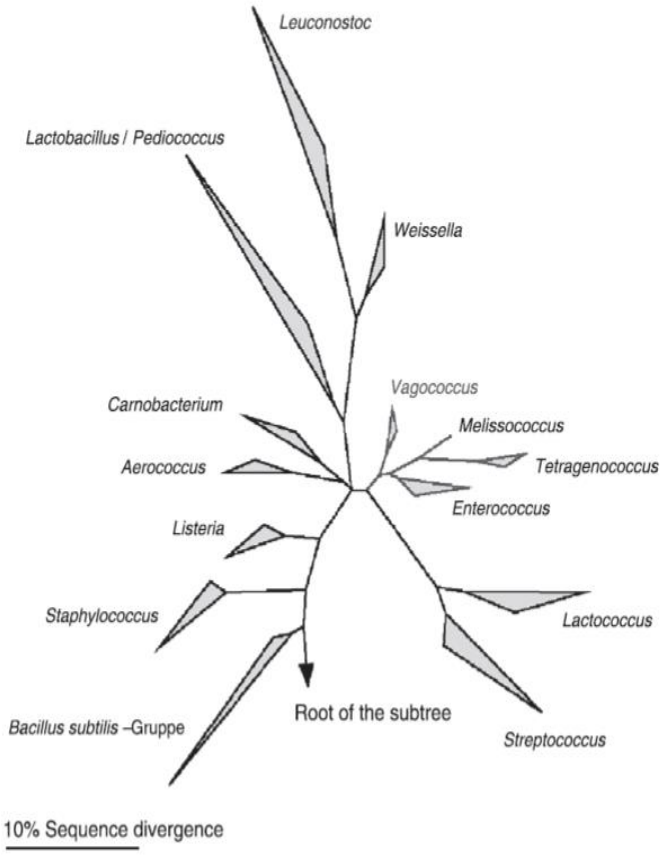

Figure 2.2. The phylogenetic position of the genus *Enterococcus* demonstrated by a 16S rRNA-dendrogram of Gram-positive genera including *Streptococcus* and *Lactococcus* (Klein, 2003).

Of the 40 species that have been described, *E. faecalis* and *E. faecium* were the most disseminated strains in the environment. These strains are widely disseminated in the environment because of their enhanced ability to exchange virulence determinates with other pathogenic strains (Werner *et al*., 2013) With *E. faecalis* being prominent in the human gut and the latter more prominent in different livestock (Nilsson, 2012). Some of the described species includes *Enterococcus haemoperoxidus, Enterococcus hirae, Enterococcus moraviensi, Enterococcus durans, Enterococcus mundtii, Enterococcus porcinus* and *Enterococcus villorum* (Nilsson, 2012).

2.4.2. tuf

When used in genomic assays the *tuf* gene has the ability to distinguish to genus level as it has high discriminating power, because it is a conserved region of the bacterial community (Kosecka-Strojek *et al.*, 2020; Iweriebor *et al.*, 2015; Li *et al*., 2012; Sheu *et al.*, 2009). The *tuf* gene translates a protein known as elongation factor (EF-TU) in the enterococcal genus (Li *et al*.,2012). It functions by synthesizing peptide chains and, it is a GTP binding protein that also acts as mediator during peptide synthesis (Li *et al*., 2012). The transportation of the aminoacyl-tRNAs to their respective A site position in the ribosome is also achieved by the EF-TU, hence why the gene is highly conserved in bacterial

species because it is part of the core genome (Harvey *et al.*, 2019). Phylogenetic relationships of gram positive cocci were successfully distinguished from each other using the *tuf* gene (figure 2.3.). All enterococcal species clustered together in one clade with the bootstrap value of 96. The genus *Staphylococcus* was previously indistinguishable from *Enterococcus* strains, however using the *tuf* gene all *Staphylococcus* and *Enterococcus* strains separated from each other (Li *et al*., 2012)

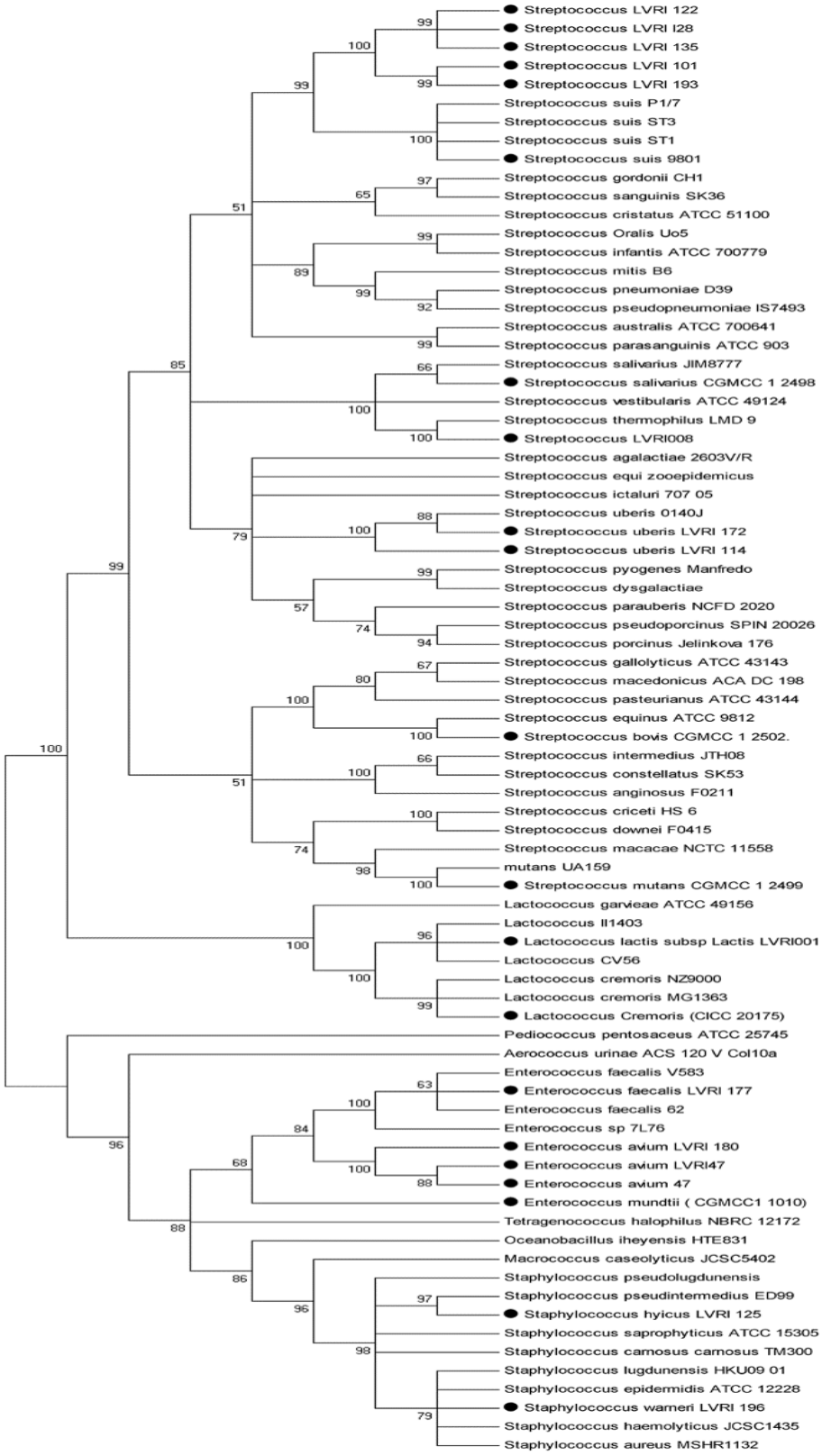

Figure 2.3. Phylogenetic tree based on the *tuf* gene of cocci belonging to the class Bacilli. (Li *et al.*, 2012).

2.4.3. sodA

Partial sequences of the 16SRNA and *tuf* gene can differentiate between streptococci at a genus level (Kosecka-Strojek *et al.*, 2020; Glazunova *et al*., 2009; Sivadon *et al.*, 2005). The use of 16S ribotyping is even more robust in differentiating some of the *Enterococcus* spp. (Frolkova *et al.*, 2012; Glazunova *et al.*, 2009). The detection assay has certain limitations; it cannot discriminate between phylogenetically related bacterial strains (Domig *et al.*, 2003). For *Enterococcus* spp. this includes species like *E. casseliflavus* and *E. gallinarum* (Frolkova *et al.*, 2012). As such new detection assays were required in order to mitigate the problem. Poyart *et al.* (2000) was one of the first studies to sequence partial sequences of manganese dependent superoxide dismutase gene (*sodA*). The study was able to successfully differentiate Gram-positives at a species level. Two decades later *sodA* is still commonly used in molecular identification studies because it is a stringent marker and it is not time-consuming to use in a detection assay (Kosecka-Strojek *et al.*, 2020). The family of superoxide dismutase enzymes catalyses the imbalance of superoxide (O_2^-) to oxygen (O_2) and hydrogen peroxide (H_2O_2) (Schatzman & Culotta, 2018). These enzymes are metalloenzymes that facilitates catalysation using Iron, Magnesium Nickel and Copper as cofactors (Schatzman & Culotta, 2018). The enzymes are important for the pathogenicity of bacteria, as they protect the bacteria from oxidative eruption that is generated by the host (Schatzman & Culotta, 2018). This is the main reason *sodA* is highly conserved in gram-positives. Several studies used *sodA* to identify different enterococcal spp. in livestock. Iweriebor *et al.* (2015) amplified the partial *sodA* sequences to identify *E. faecalis, E. faecium and E cassevilas* from swine isolates recovered from the Eastern Cape, South Africa.

2.5. Molecular detection techniques and Molecular typing

In the 1980's the outbreaks of infection causing bacteria like *Clostridium Difficile* warranted the use of typing methods to distinguish between bacterial strains, this includes methods like serotyping and assessing resistance patterns of the strains (Pérez-Losada *et al*., 2017; Dingle & MacCannell, 2015). These methods had numerous limitations as they highly relied on the phenotypic characterisation of the bacteria, as such they can't accurately distinguish between a large pool of bacterial strains (Magalhães *et al.*, 2014). In addition, the experimental reproducibility of these techniques was fairly low. To mitigate these limitations typing methods became solely based on the genotypic characterization of the strains (Pérez-Losada *et al.*, 2017).

2.5.1. Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction (*ERIC-PCR)*

ERIC-PCR is another form of repetitive sequence based-PCR typing method(Ranjbar *et al.*, 2017). It utilizes the conserved palindromic enterobacterial intergenic sequences in bacterium (Ebumah, 2020; Lucy *et al.*, 2015; Ture *et al*., 2015). These sequences are stretches of 126bp long (Ebumah, 2020). At a nucleotide level they are highly conserved, however the chromosomal positions within species differs greatly (Ferguson *et al.*, 2016; Ture *et al.*, 2015). Due to the varying chromosomal locations between species , this makes ERIC-PCR a good molecular typing tool as it can accurately differentiate between

bacterial strains using primers that anneal to the palindromic sequences that are linked to the intergenic consensus(Lucy *et al.,* 2015; Muñoz-Atienza *et al*., 2016; Zalipour *et al*., 2019) . In addition, ERIC PCR has more added advantages since it is cost effective and not time consuming compared to other typing methods such as Multi-Locus Sequencing (MLST) (Ebumah, 2020). Current studies have been successfully using ERIC-PCR to distinguish multi-resistant bacterium of the same species. Muñoz-Atienza *et al*. (2016) conducted a study on the prevalence and relatedness of *E. faecium* spp. isolated from food products. Their main aim was to clearly distinguish between the fourteen *E. faecium* isolates. In addition to assess the safety of these strains since they were considered as presumptive probiotics. Not only did they employ ERIC-PCR to determine the genetic relatedness of these strains but pulsed-field gel electrophoresis (PFGE), random amplified polymorphic DNA (RAPD), and restriction analysis of amplified16S rDNA(ARDRA) were part of the molecular typing tools used. ERIC PCR was more efficient in distinguishing the genetic relatedness between the fourteen strains as the analysis yielded nine sub-groups hence indicating a high diversity, whilst PFGE and RAPD analysis indicated a low diversity within species (four sub-groups).

2.5.2. Pulsed-Filed Gel Electrophoresis

The use of Pulsed Field Gel Electrophoresis (PFGE) has immensely contributed to molecular epidemiological studies, it is the most commonly used DNA fingerprinting/ typing method for pathogens (Oliver & Jones, 2014; Simner *et al.,* 2014). This assay was first determined by Schwarz *et al*. (1987) who typed the complete chromosomal DNA of *Saccharomyces cerevisiae.* The principle of mechanism for this tool exploits restriction endonucleases and gel electrophoresis techniques to create a DNA fingerprint that is "unique" to a particular pathogen (Peters & Fisher, 2014; Wang *et al.*, 2014; Reading, 2001). The electrical field for PFGE is dissimilar to conventional gel electrophoresis, the electric current passed through the fragmented DNA alternates(Peters & Fisher, 2014). This reorients the DNA fragments at a 120° angle which in turn increases fragment mobility and the accuracy of the band size estimation (Peters & Fisher, 2014). With PFGE the resultant PFGE pattern can elucidate genetic relatedness and lineages (Liu *et al.,* 2015; Peters & Fisher, 2014; Simner *et al.,* 2014). Henceforth, the assay can discriminate at a subspecies level. The ability to discriminate between bacterial strains is based on the sporadic cleaving of whole genomic DNA using specific endonucleases such as *Smal1* (Simner *et al.*, 2014; Wang *et al.,* 2014). The restriction endonucleases will digest the DNA into large varying fragments ranging between 10- 45 base pairs (Peters & Fisher, 2014). When the digested fragments are passed through the electrical field in agarose gel, the observed pattern is termed as the DNA fingerprint (Reading, 2001;Wang *et al*., 2014). PFGE uses similar mechanisms as conventional gel electrophoresis however with pulse field, the assay can separate fragments larger 10mb whilst the latter can only resolve up to 20kb (Peters & Fisher, 2014). There are different types of PGFE such as Contour-Clamped Homogeneous Electric Field and Transverse Alternating Field Electrophoresis (Wang *et al.,* 2014). The comparison of the DNA fingerprints has recently been

standardized to monitor the emergence of foodborne diseases globally. The construction of these databases affords the field of epidemiology to globally compare DNA fingerprints of strains intra- and interlaboratory (Peters & Fisher, 2014; Wang *et al.,* 2014). Daniel *et al.* (2017) investigated the relatedness of *E. faecalis* isolates recovered from farm animals, their environment and the surrounding rivers in Malaysia. The study wanted to establish whether there was a possible relationship between the isolates recovered from the previously mentioned sources. Six groups of farms were sampled, after identification techniques the isolates were digested with Smal1 for PFGE analysis. The generated dendrogram grouped the *E. faecalis* isolates into sixty-three pulsotypes, with forty-four clonal complexes, whilst nineteen isolates were unique. As expected, isolates from the one farm clustered together, also the observed PFGE patterns between poultry and bovine faecal matter in the same farm did not overlap each other. However, poultry isolates recovered from two different farms overlapped each other. This could allude that in their persistence strains of *E faecalis* and *E faecium* could be host specific (Hollenbeck & Rice, 2012; Diarra *et al.,* 2010). Lastly the study noted a high genetic variability in isolates recovered from moving water bodies (wastewater, lakes and rivers). This was not surprising, as the strains were heavily subjected to chemical and physical stress that resulted in inducing evolutionary adaptations to the environment. Dolka *et al*. (2017) aimed to characterize and establish clonal relatedness of pathogenic *E. cecorum* strains from different poultry farms. The isolates clustered into seven pulsotype groups. Three of the pulsotypes their isolates were recovered within the same year. Hence indicating a temporal clustering. In addition, a geographical clustering was noted of the reported E. cecorum outbreak in chicken breeders the cause was due to a unique set of *E. cecorum* isolates that caused an outbreak in the same area at the same time. Although the assay is considered as "golden standard" because of its high discriminating power standardization and reproducibility unfortunately it has some disadvantages associated with it. The assay has been deemed as difficult, time-consuming and costly.

2.5.3. Multi Locus Sequencing Typing (MLST)

The use of typing methods that are reliant on the genetic make-up of an organisms has increased over the past decades. In the genome, regions such as the house keeping genes are essential in comparative genomics (Pérez-Losada *et al.,* 2017). These regions aid in distinguishing differences and recognizing similarities between strains. Multi Locus Sequencing Typing exploits at least six of the well conserved housekeeping genes found in the genome of bacteria (Dingle & MacCannell, 2015; Oliver & Jones, 2014). The housekeeping genes used for MLST analysis in *E. Faecium* and *E. faecalis* differ; with *gdh, gyd, pstS, gki, aroE, xpt,* and *yqiL* used for *E. faecalis* and *atpA, ddl, gdh, purK, gyd, pstS, adk* used for *E. faecium* respectively (Kim *et al.,* 2018; Manson *et al.,* 2010).The precise sequencing of these genes based on the genetic variation of the alleles in each locus will be catalogued (Magalhães *et al.,* 2014). The resultant allelic variation in comparison to other isolates in the database will thus aid in assigning a sequence-type (Yin *et al.,* 2018). MSLT is very

robust. During sequence alignment with other isolate's profiles, even a difference in a single nucleotide will be considered as a unique characterization of that particular strain (Yin *et al.*, 2018). These grouping of observed polymorphisms in each locus will be assigned as sequence type (Dingle & MacCannell, 2015; Magalhães *et al.*, 2014). A study conducted in Tunisia by Ben Said *et al.* (2016) evaluated the prevalence of high-level gentamicin resistance (HLGR) *E. faecalis* and *E. Faecium* isolates present in vegetables, soil and irrigation water. MLST was conducted on ten *E. faecalis* strains and four *E. faecium* strains respectively. The assay classified *E. faecalis* isolates into 3 different sequence types namely; ST2, ST28 and a new sequence type ST528. However, with *E. faecium* isolates two new sequence types were revealed ST885 and ST886 whilst one isolate was not typeable. The allelic combination included *adk, atpA, ddl, gdh, gyd , pstS* and *purK* that was non-typeable. Interestingly the presence of sequence type ST2 is alarming, because previously it has been associated what is considered as a high-risk clonal complex (CC2), this clone was documented to be accountable for the rise in nosocomial infections globally. As such the presence of this sequence type in the environment and vegetables is a cause for concern. The use of MLST has advantages over other typing methods namely; comparative genomic analyses of bacterial strains for phylogenetic and population purposes, because the assay groups strains based on their sequence types, we can determine isolates responsible for hospital outbreaks including strains that are responsible for the persistence of multidrug resistance (Pérez-Losada *et al.*, 2017; Hancock *et al.*, 2014). Hence the tool is essential for epidemiological studies especially in detecting the type of clonal complexes that drive outbreaks in the environment.

2.5.4 .Whole genome sequencing (WGS)

Whole genome sequencing is a high through-put assay that utilizes next generation sequencing applications (Abdelbary *et al.*, 2017). Its principle of mechanism is to sequence the whole genomic DNA of an organism (Abdelbary *et al.*, 2017; Nord *et al.*, 2015). The discriminatory power of this analysis is based on assessing the genomic variations of a whole genome (Yin *et al.*, 2018). The genomic DNA will be fragmented into short reads and aligned into one sequencing library, which will in turn be ran as a single run (Nord *et al.*, 2015; Cavalleri & Delanty, 2012). The library will be compared to already published reference sequences (Abdelbary e*t al.*, 2017; Yin *et al.*, 2018; Zankari *et al.*, 2012). Hence, the use of this assay in the identification of pathogenic strains is quite fundamental as we can determine strains that are genetically divergent due to a mutation in one nucleotide (Zankari *et al.*, 2012). It is for this reason that WGS is ideal for monitoring bacterial outbreaks and being used for clinical diagnostics as it has a short turnaround time and cost effective. Zankari *et al.*, (2012) evaluated the use of WGS in rapidly and accurately identifying bacterial strains that cause UTI from 35 urine samples. Pellets from the urine were cultured for bacteria, whilst the urine samples were sequenced. Upon whole genomic sequencing the present bacteria in the urine were identified as *E. faecalis, E coli* and other bacterial strains. This study validated their results by comparing the presumptive isolates to the sequenced data. Therefore, the study sufficiently demonstrated that the use of WGS in clinical

diagnostics will be beneficial in terms of accurately identifying bacteria in addition to saving cost. The previously mentioned study compared their sequenced data to presumptive isolates, this indicates a need for more bioinformatics databases that can be used concurrently with WGS, also the databases must be easily accessible to the public. Bioinformatical databases and tools are required to analyse large amounts of sequenced data. The use of WGS produces a vast amount of data that can be analysed with the appropriate tools to gather further insights on the type of genetic variability the strains could have. RESFINDER was developed to work in conjunction with sequenced data from WGS (Zankari *et al*., 2012). The tool is a web-based method that utilizes blast for the identification of antimicrobial resistance genes present in sequenced data of a pathogen. Tyson *et al*. (2018) used WGS to predict the presence of antibiotic resistance genotypes and their associated mutations on 197 strains of *Enterococcus* spp. The study employed the use of RESFINDER for sequence analysis.

2.5.5. Pyrosequencing

The Royal Institute of Technology designed an alternative method different to the conventional method of Sanger sequencing (Bharagava *et al*., 2018). Pyrosequencing has recently been the preferred method of sequencing because, it can be automated as such more sequences that are approximately 1000mb long can be generated per run; it does not permit the use of electrophoresis thus it's not costly nor time consuming (Bharagava *et al.*,2018; De Benedictis & De Battisti, 2014). Lastly it is commercially accessible (Bharagava *et al.,* 2018; Simner *et al.*, 2014). Pyrosequencing is unlike Sanger sequencing it is dependent on detecting luminescence after a nucleotide is added and upon the release of the pyrophosphate instead of detecting light after chain-termination (Liu *et al.,* 2015). Compared to Sanger sequencing the limitation of this method is that it is less discriminatory and only small stretches of sequences can be analysed per given time compared to Sanger sequencing (De Benedictis & De Battisti, 2014). Zaheer *et al*. (2012) aimed to construct a high-throughput method that will rapidly identify enterococci in a health care setting including for molecular epidemiological surveillance studies. The study exploited the use of *grosESL* sequences namely; *grosES, grosEL* and intergenic spacer regions within the enterococcal genome. Thirteen enterococcal species were pyro-sequenced by targeting the variable *grosESL* and intergenic spacer regions. After identifying the strains, the study conducted biochemical tests to validate the accuracy of the pyrosequencing identification method, their results agreed with the biochemical tests except for one *E. faecalis* strain that was biochemically categorized as *E. faecium*. The mis-identified strain was subjected to conventional sequencing to validate the pyrosequencing results.

2.6. Mobile genetic elements (MGEs)

The species *E. faecalis* has been documented as a more pathogenic strain than most enterococcal species due to the efficiency of the mobile genetics elements (MGEs) associated with its virulence and pathogenicity (Witte, 2000). *E. Faecalis* can acquire both virulence and antibiotic resistance determents

via horizontal transfer of MGEs, whether it be plasmids, pathogenicity islands and conjugative transposons (Starikova *et al*., 2013). MGEs are transposable elements that contribute to the genomic plasticity by different mechanisms (Hegstad *et al*., 2010). These elements can change the genetic expression through gene disruption by insertions in the coding regions (Hegstad *et al*., 2010). Another possible mechanism is integration in the promoter region of a sequence. The integration can lead to disturbance of the promoter or leading to the enhancement of the existing promoter thus increasing promoter efficiency (Hegstad *et al*., 2010).

2.6.1. Conjugative Plasmids

The plasticity of *Enterococcus* has been heavily influenced by the presence of conjugative plasmids (Werner *et al*., 2013). Pheromone inducing conjugation plasmids are significant in *Enterococcus* species as they are useful in cell-cell signalling which aids in the opportunistic behaviour of enterococcal pathogens (Dunny, 2007). These plasmids are responsible for the distribution of the antibiotic resistance and virulence genes within the bacterial community (Dunny, 2007). The signalling molecules that act as the communicator once in proximity of another cell a activates the synthesis of aggregation substances (AS) (Weaver, 2019). This will promote the Enterococci binding substance (ESB) which is a receptor (Dunny *et al* .,1995). With the use of the ESB-AS binding site, a mating channel will occur between two cells whereby a plasmid will be transferred from donor to a recipient bacterial cell (Dunny *et al.*,1995). Some transferred conjugative plasmid include pCF10 and, pAD1. pCF10 is the most described in enterococcal species (Figure 2.4). The plasmid has many genetic determinants that encode for regulation, adherence, and secretion proteins(Dunny, 2013; Dunny, 2007). This plasmid is less than 70kb and, half of its genetic make-up is for pheromone inducible conjugation (Dunny, 2007). The AS and surface exclusion (SE) proteins which are encoded by the plasmid have typical structures of surface proteins of cocci that are gram positive (Dunny *et al.,* 1995). This includes N-terminal signal sequence and C-terminal wall spanning plus the membrane anchor region (Dunny *et al.,* 1995). Figure 2.5 indicates the multifactorial process occurring at a DNA, protein and cellular level in response to the presence of pheromones in the extracellular matrix of *E, faecalis.* The process is known as the sex-pheromoine inducible conjugative sytem and it is specific to *E. faecalis spp*. (Schiwon *et al.,* 2013; Werner *et al*., 2013; Dunny & Johnson, 2011).

In figure 2.5-**A**, Sex pheromones (i,e. cCf10) are hydrophobic peptides made up of approximately eight amino acids (Weaver, 2019; Dunny & Berntsson, 2016). These peptides are secreted by the lipoprotein of a cell containing conjugative plasmids (Pcf10) in order to initiate a mating response (Dunny & Berntsson, 2016; Dunny & Johnson, 2011). The donor cell secretes a mixture of pheromones (blue circles) and inhibitors (orange circles) at a ratio of 1:80 (Weaver, 2019). The inhibitor peptide competes with the pheromone to bind to *tracA*. When the inhibitor binds to the *Eep,* of which is an intramembrane processing protein. Successful binding of this inhibitor blocks transcription, hence the mating response

will remain uninduced (Dunny & Berntsson, 2016; Clewell *et al.*, 2014; Dunny & Johnson, 2011). The combination of the *Opp* and *traC* enables efficient binding to specific pheromones (Weaver, 2019). At a DNA level 2.5-**B**, the pheromone will bind to *traA* a pheromone response regulator protein, this protein will structurally change upon successful binding. The antisense RNA (aR) will be thus be activated this in turn will initiate down-stream transcription (greenarrows) (Weaver, 2019;Clewell *et al.*, 2014)). Based on the successful binding of the inhibitor or pheromone 2.5-**C,** the mating response will be uninduced or induced (Weaver, 2019).

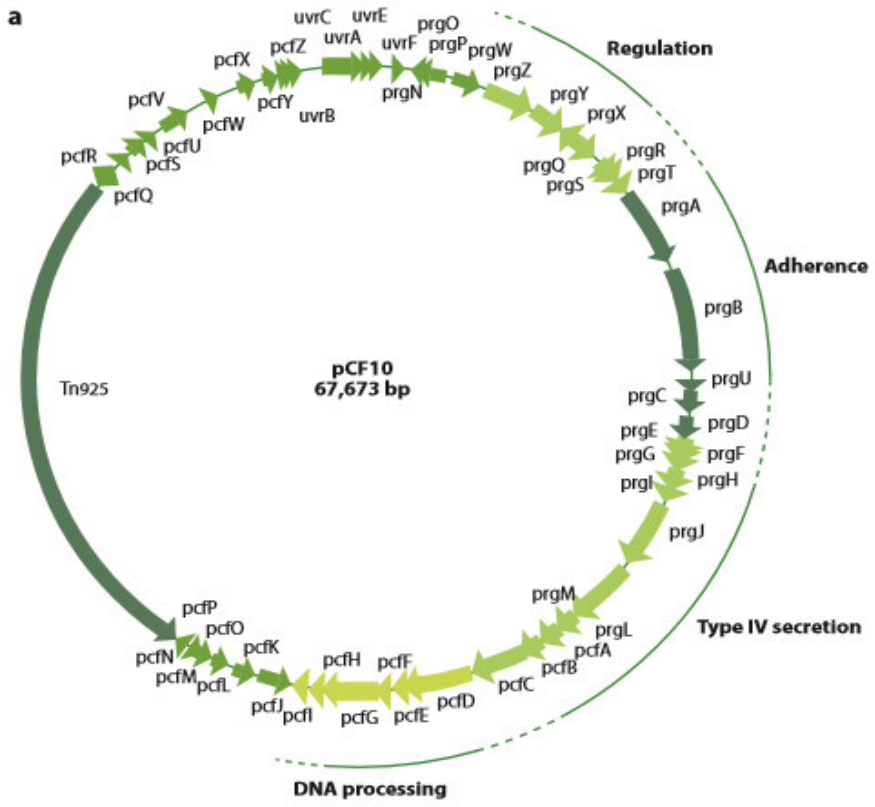

Figure 2.4: The genetic map of *E. faecalis* pCF10 plasmid . The plasmid contains the integrative conjugative transposon *Tn925* and other genetic determinants that aid in pheromone response (Dunny, 2013).

The plasmid has an organizational structure in terms of how these genes are located within the plasmid (figure 2.4). All the regulatory genes are clustered within one section, followed by adherence and type iv secretion encoding genes. The genes located in the regulatory portion of the plasmid are responsible for transcription termination of the IRS1 and not limited to the ORF of *prgR* downstream (Dunny, 2013). The gene *prgR* is responsible for the production of *PrgB* which is also termed as *asa1* (Dunny, 2013). Expression of this gene at the donor cell's extracellular matrix initiates the formation of mating apparatus (Clewell *et al.*, 2014). The adherence module encompasses of four highly conserved genes

namely; *PrgA, PrgB, PrgC* and *PrgU* (Dunny, 2013). These genes facilitate adherence proteins in a sex -pheromone- conjugative system, however the gene *PrgU* is not well documented as to which role it plays in the response mechanism to pheromones (Dunny, 2013). Further downstream of the plasmid are the type IV secretion proteins, prgD-prgM; pcfA-pcfC (Dunny, 2013; Dunny & Johnson, 2011).

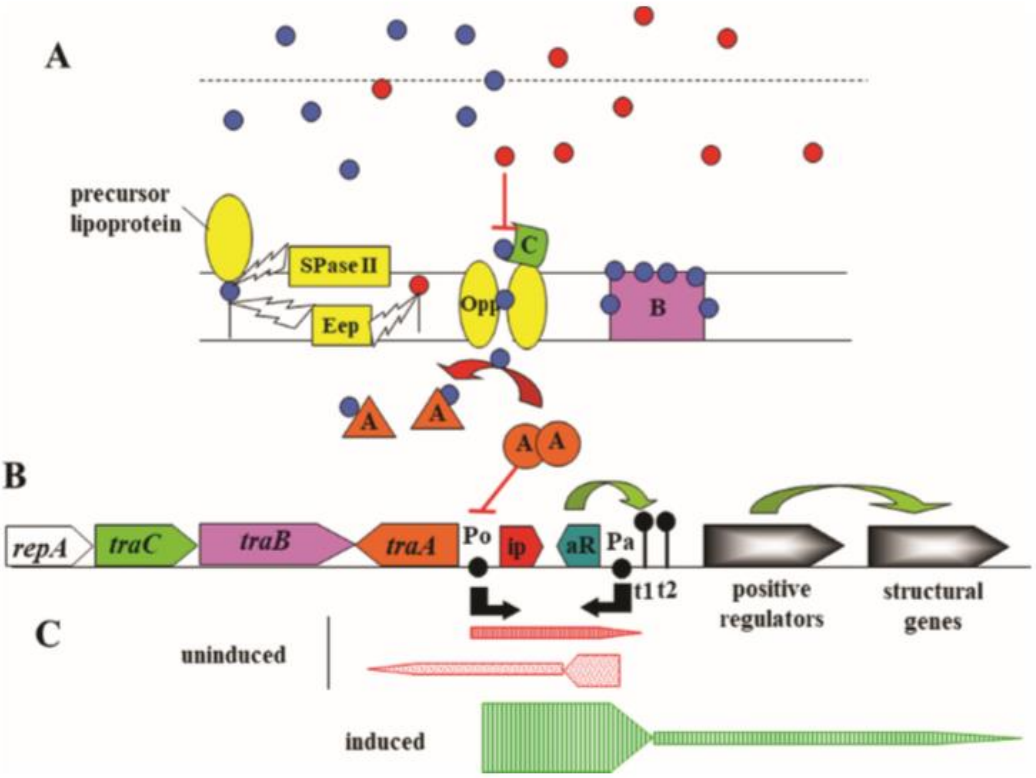

Figure 2.5: Pheromone induced response of the conjugative pCF10 plasmid in *E. faecalis* strains (Weaver, 2019).

2.6.2. Transposons

Transposons in *Enterococcus* can be grouped into three groups namely; composite transposons, intergrative elements and the *Tn3* family of transposons. (Werner *et al.,* 2013). Another example includes conjugative transposons which includes the *Tn916* family which is responsible for transferring tetracycline resistant genes in *Enterococcus* species (Starikova *et al.,* 2013). The family is responsible for the persistence of *tetM* resistant gene in over 80% E. *faecalis* strains obtained from ready to eat food and the gastrointestinal tract (Macovei and Zurek, 2007). This family has been transferred to over 30 different genera of bacteria. The family *Tn6000* and its related elements are only documented in Enterococcal strains (Starikova *et al.,* 2013). In addition, the afore mentioned species has been associated with vancomycin resistance due to the transfer of the transposon *Tn1546* (Iweriebor *et al.,* 2015; Nilsson, 2012; Macovei and Zurek, 2007). It has been documented that the transposon was transferred to *Staphylococcus* aureus isolates thus demonstrating the importance of horizontal gene transfer in the bacterial community of the digestive tract (Macovei and Zurek, 2007). Figure 2.6 illustrates a typical genetic map of a transposon found in *Enterococcus*. This transposon is responsible

for transfer of the *tetM* genetic determinant in bacterium (Werner *et al.*, 2013). The *orf-T* indicates the origin of the transfer. While the purple arrows encode proteins responsible for conjugation (Weaver, 2019). Transposons have regulatory genes that direct a positive or negative regulation, these genes are denoted by the red and blue arrows respectively (Werner *et al.*, 2013). Presence of *Xis* and *Int* proteins facilitate integration and excision. The *Int* protein has two DNA binding domains namely; N-terminal (Int-N) and C-terminal (Int-C) (Weaver, 2019). Both these proteins are tyrosine recombinases. Int-N functions by directing the repeat sequences located at the end of *Tn916* like transposon while Int-C adheres to the target sequence and the transposon terminal. *Xis* protein has been reported to have a dual function, it binds adjacent to Int-N binding site of the transposon (Weaver, 2019; Dunny & Johnson, 2011). As such it actively competes with Int-N, therefore inhibiting excision. (Schiwon *et al.*, 2013; Weaver, 2019). *Clostridium difficile* and *E. faecalis* frequently coexist in hospitalized patients, thus in turn increasing the severity of the nosocomial infections (Weaver, 2019; Schiwon *et al.*, 2013). *C. difficile* and *E. Faecalis* both utilize MGE's to acquire new genetic determinants. Jasni *et al*. (2010) The study assessed the genetic transfer of the *Tn5397* conjugative transposon between *C. difficile* and *E. faecalis.* Transconjugants were selected on antibiotic containing plates. The insertion to *E. faecalis* was determined by the use of single specific primers. *Tn5397* was transferred to *C. difficle* at high mean transfer frequency, from the transconjugant EF20A. The study also reported the reciprocal insertions between the two strains occurred at the same site in both strains. This study shows the capability of *E. faecalis* in transferring genetic elements to strains of a different genus. These are the mechanism that aid in the persistence of resistant genes in the environment.

Conjugative transposons and *Tn3* family transposons are not the only the transposons responsible for the spread of resistant *Enterococcus* (Hegstad *et al.*, 2012). Composite transposons have intracellular mobility due to the insertion sequence copies flanking the transposon (Hegstad *et al.*, 2012). These IS copies act as a unit to accurately move the genetic material (Hegstad *et al.*, 2012). They have been mostly associated with glycopeptide resistance and/or resistance to high levels of gentamicin resistance (Hegstad *et al.*, 2012).

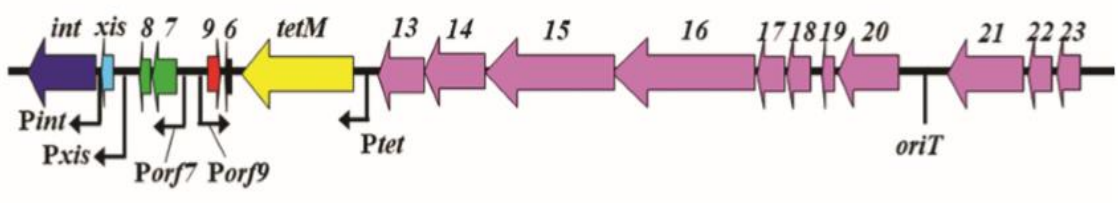

Figure 2.6: *Tn916* transposon genetic organization, with the genetic location of the *Xis* and *Int* binding sites (Weaver, 2019).

2.7. Virulence genes

Most pathogenic strains can be multi-drug resistance, however the resistance to antibiotics alone does not explain the pathogenicity (Diarra *et al*., 2010). Although the virulence factors associated with *Enterococcus* are widely known, the genus continues to be persistent in the envrionment (Ye *et al*., 2012). The virulence factors in the genus are thought to be a multifactorial process that is aided by the expression of certain genes with other different products (Comerlato *et al*., 2004). Some of the virulence determinants in this genus are associated with the prevalence of the pathogenic strains.

The genes include aggregation substances (*asa1*), cytolysin (*cylA*), hyalurodinase (*hyl*), enterococcal surface protein (*esp*), collagen binding protein (*ace*), gelatinase (*gelE*) and pheromones (*ccf*) (Diarra *et al*., 2010; Vankerckhoven, 2004). The genes *asa1, cylA, esp* and *gelE* are found in the species *E. faecalis,* whilst *esp* and *hyl* have been commonly detected in *E. faecium* (Diarra *et al*., 2010). The aggregation substance as mentioned is encoded from a conjugative plasmid and functions by binding the epithelial cells which will in turn initiate aggregation of the bacteria during conjugation (Comerlato *et al*., 2004; Diarra *et al*., 2010; Vankerckhoven *et al*., 2004). It function as a viral factor in clinical infections is documented to increase the adherence in the endocardial cells of the heart (Vankerckhoven *et al*., 2004). Cytosylin is also encoded by plasmid DNA but in some cases its genetic sequence is integrated to the enterococcal chromosome (Vankerckhoven *et al*., 2004). This virulence factor has been linked to worsen the clinical infection endocarditis. Structurally cytosylin has two components namely lysin and activator, these areexpressed by different variants of the cytosylin operon (Vankerckhoven *et al*., 2004). The enterococcal surface protein is responsible for the colonisation of *E. faecalis* in urinary tract infections, mediating in cell-cell primary surface interaction and the formation of biofilm (Comerlato *et al*., 2004). The virulence determinant hyalurodinase is encoded by chromosomal DNA and has been associated with persistence and colonization of *E. faecium* in pneumococcal pneumonia (Vankerckhoven *et al*., 2004). In conclusion, gelatinase has a hydrolytic capacity (zinc metalloprotease) and functions by hydrolysing, gelatine, collagen and small peptides (Vankerckhoven *et al*., 2004; Comerlato *et al*., 2004). It was documented that even though the *gelE* gene is detected, in some instances the enzyme was not expressed (Comerlato *et al*., 2004).

2.8. Antibiotic Resistance Mechanisms and Detection

2.8.1. Antibiotic use in livestock production

For successful therapy, antibiotics work in five different mechanisms such as damaging of functions of the cell membrane, seizing the synthesis of nucleic acids and proteins, inhibition of the cell wall synthesis and the synthesis of folic acid (Mann, 2011). However, due to the persistence of AR in the

bacterial community, it is unlikely that the synthesis of new antimicrobials will be developed to counteract the emergence of resistant bacteria (Aarestrup *et al*., 2001). *Enterococcus* strains are intrinsically resistant to the first line antibiotics as they show low-level resistance to β-lactams and aminoglycosides in addition these strains are resistant to cephalosporins (Hammerum, 2012). As antibiotics were used as growth promoters in animal feed, a study was conducted, and it was documented that *E. faecium* in UK farm animals was resistant to the glycopeptide vancomycin but the antimicrobial drug was never used as a form of therapy or in feedlot in farm animals (Hammerum, 2012). It was then discovered that this was due to the use of avorparcin in feedlot, which promptly selected for vancomycin resistant *Enterococc*i in UK farm animals (Hammerum, 2012). The increasing resistance to vancomycin was concerning as the drug was used to treat life-threating nosocomial infections caused by *E. faecium*, clinical *E. faecium* strains were largely unaffected by ampicillin and gentamicin (Hammerum, 2012). Such instances led to the banning of avoparcin in feedlot in order to mitigate the persistence of vancomycin resistant *Enterococcus* in the UK.

2.8.2. Resistance mechanisms

Low/moderate response to antimicrobials in the bacterium is usually expressed intrinsically from chromosomal DNA (Fisher & Phillips, 2009). However, the resistance mechanism that confers moderate to high resistance in pathogens is typically due to externally acquired genes. Acquired resistance is attributed to sporadic mutations in addition to the attainment of foreign genes (Hollenbeck & Rice, 2012). In *Enterococcus* these genes can confer moderate to high resistance for different classes of antibiotics.

2.8.2.1. β-lactams

In most cases the growth of bacterial cells is depended on the linkage of the enzymatic pentapeptide precursors into peptidoglycan cell wall (Hollenback and Rice, 2012). The enzymes that are responsible for the linkage are known as penicillin binding proteins (PBS) (Hollenback and Rice., 2012). β-lactam antibiotics bind to PBS since they are precursors, the covalent binding will result in the disruption of cell wall formation thus ultimately affecting the cell growth (Hollenback and Rice ,2012). Apoptosis will be initiated due to the formation of oxygen reactive species (ROS). *Enterococcus* shows low affinity to β-lactams. The tolerance has been associated with the expression of the *sodA* gene which will express the enzyme superoxide dismutase (Hollenback and Rice, 2012). Studies have documented *Enterococcus* as being intrinsically resistance to penicillin's (Economou & Gousia, 2015) presence of chromosomal genes which are species-specific constitutively codes for a low-resistance to selected β-lactam antibiotics. *pbp4* is subjective to *E. faecalis* and *pbp5* is expressed in *E. faecium* isolates only (Economou & Gousia, 2015).

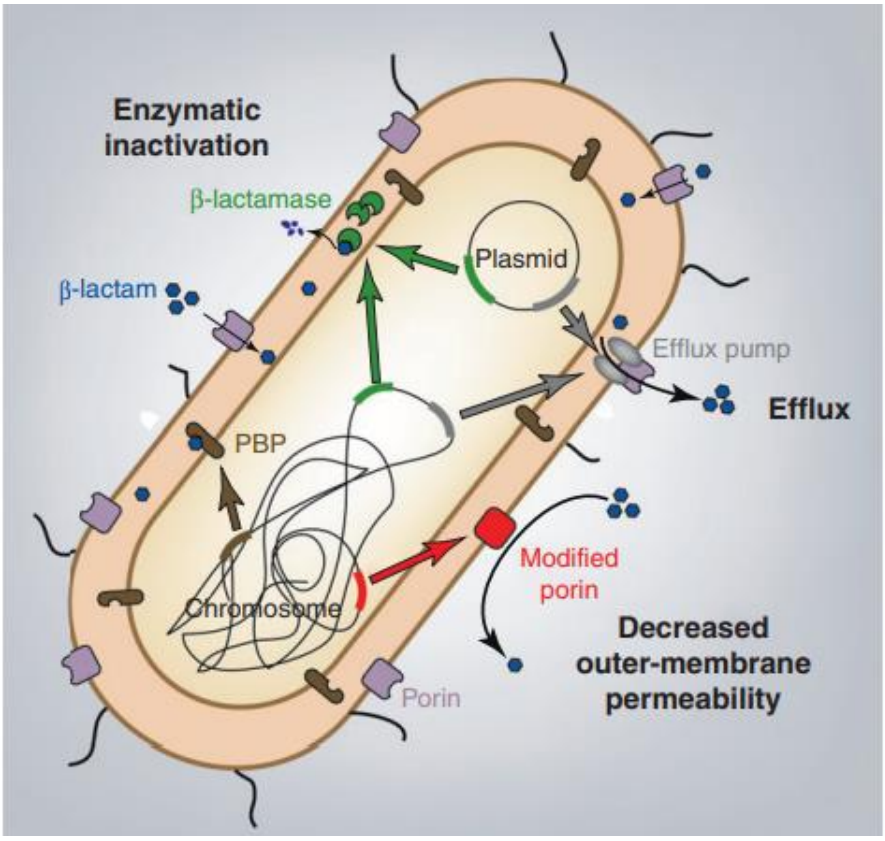

Figure 2.7: Beta-lactam resistance mechanisms in bacterial species (Nordmann *et al.,* 2012)**.**

2.8.2.2. MLS_B (Macrolides, Lincosamides and Streptogramin B)

A majority of MLS_B were used as second line drugs for gram positive bacterium that were resistant to *β*-lactams, however the use of these classes of antimicrobials in *Enterococcus* has declined over the years due to an incline in resistance (Economou *et al.,* 2017). Overall high level resistance to these groups of antibiotics is considered as a MLS_B phenotype (Hollenbeck & Rice, 2012; Zou *et al.*, 2011). The resistance mechanisms in MLS_B includes; modification of target site due to mutation/methylation hence binding of the ribosome is hindered, inactivation of the antibiotic and lastly, with the use of the efflux pump (Leclercq, 2002) (Figure 2.8). The *erm* (erythromycin ribosomal methylase) genes confers high level resistance of *Enterococcus* to MLS_B (Zou *et al.,* 2011). Four clusters of the *erm* genes have been extensively documented namely; *ermA, ermB, ermC* and *ermF*. Though present in *E. faecium ermB* has been widely reported in most *E. faecalis* isolates. The expression of this gene can lead to resistance in macrolides, lincosamides and streptogramin B (particularly quinupristin) (Economou *et al.*, 2017). The 23s RNA which is a constituent of the large ribosomal unit (50s) will be demethylated by the *erm* proteins. Consequently, the methylation of the A2058 residue in domain V will result in unsuccessful binding of erythromycin to its intended target (Leclercq, 2002). The three afore mentioned classes have an overlapping binding site in their respective 23s RNA, hence the resistance to all three classes is explained by the overlap. Other genes confer resistance to macrolides and streptogramin B only (Zou *et al*., 2011). The gene *msrC* is intrinsically expressed in *E. faecium* and encodes for the

ABC efflux pump (Hollenbeck & Rice, 2012). While in *E.faecalis* such resistance is conferred by *Lsa* (Hollenbeck & Rice, 2012). Strains harbouring these genes exhibit low level resistance to streptogramin B and macrolides (Table 1) (Hollenbeck & Rice, 2012). Resistance to macrolides in gram positives is due to the acquisition of two different active efflux pumps namely; ATP-binding-cassette transporter (ABC) and the major facilitator superfamily (MFS) (Leclercq, 2002). The drug quinupristin-dalfopristin is a blend of streptogramin A and B. This blend of antibiotic is approved by the FDA as a therapeutic measure for VRE infections (Hollenbeck & Rice, 2012). In animal husbandry the use of virginiamycin is a cause for concern as the unmonitored use of this streptogramin analogue has led to the selection of enterococci that is resistant. Hence, resistance to the streptogramin blend quinupristin-dalfopristin was observed mostly in environmental samples (Hollenbeck & Rice, 2012). The increase in resistance to this drug is alarming considering the zoonotic potential of *Enterococcus.*

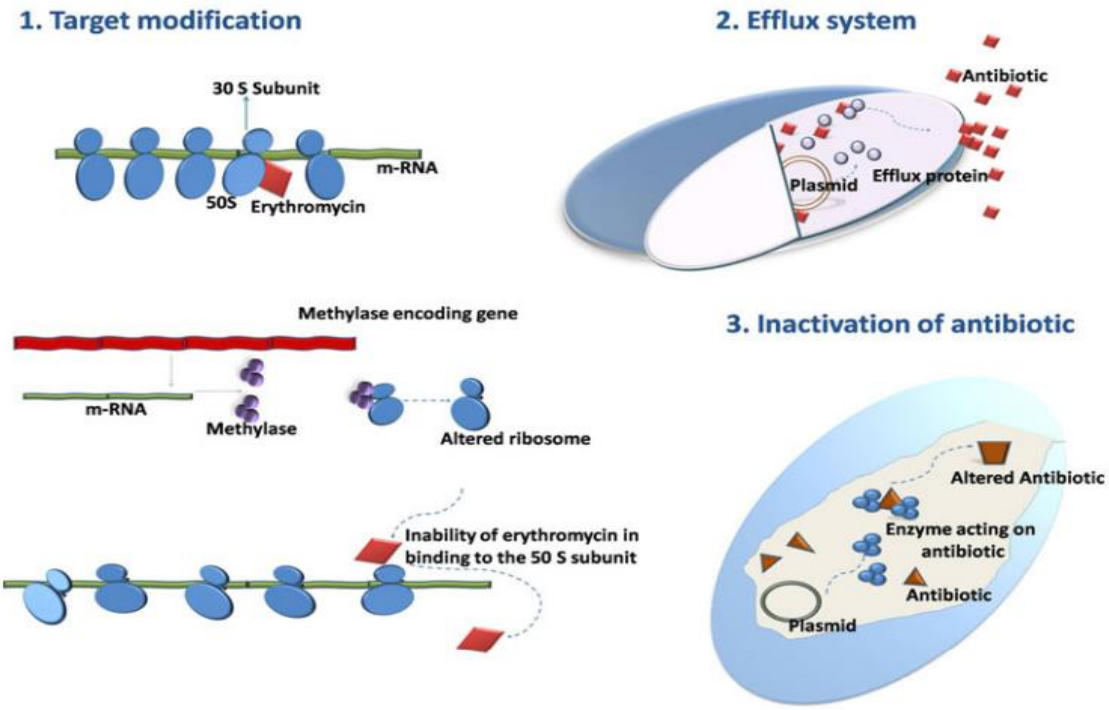

Figure 2.8: Resistance mechanisms associated with Macrolides, Lincosamides and Streptogramin B resistance in bacteria, including Enterococcus species(Thumu & Halami, 2012).

2.8.2.3. Aminoglycosides

Mutually *E. faecium* and *E. faecalis* are documented to be intrinsically resistant to low levels of aminoglycosides (Hollenbeck and Rice, 2012). The resistance mechanism in *E. faecalis* is attributed to the incapability of antibiotic molecules to enter the cell wall of the bacteria because their mode of action is to seize synthesis of the ribosomal proteins (Hollenbeck and Rice, 2012) *aac6'-li* is intrinsic *to E. faecium* and encodes low level resistance to kanamycin and tobramycin by conferring the Aminoglycoside Modification Enzyme (AME) (Diarra *et al.,* 2010; Holelnbeck and Rice,2012). It is

vital to note that resistance can be an acquired expression (Hollenbeck and Rice, 2012). In such instances, resistance occurs due to chance mutations or by acquiring new genetic material as mentioned in section 2.4 (MGEs). These mutations have led to High Level Aminoglycoside Resistance (HLAR) (Hollenbeck and Rice, 2012). There are three resistance mechanisms to aminoglycosides namely; (1) enzymatic modifications, (2) transportation modifications, and (3) ribosomal modifications (Diarra *et al.*, 2010) as illustrated in figure 2.9. The presence of the bifunctional enzyme encoded by the gene *aac(6")-aph(2")* confers high level resistance to all aminoglycosides excluding streptomycin (Werner *et al.*, 2013;Klibi *et al.*, 2012). This gene is clinically important as it is the most disseminated variant in *Enterococcus* species. Studies have documented its presence in enterococci of arising from meat samples (Werner *et al.*, 2013) High Level Gentamicin Resistance (HLGR) is commonly due to the expression of these bifunctional enzymes. The simultaneous phosphorylation and acetylation of the 2'hydroxy and 6'hydroxyl positions respectively in gentamicin results in its inability to bind to the 30s ribosomal subunit. (Hollenbeck and Rice, 2012). Although minor, other genes are responsible for HLGR and its respective analogues. These include other AME's namely; *aph (2")-Ib*, *aph(2")-Ic*, and *aph(2")-Ie*, with some commonly observed in livestock animals (Hollenbeck & Rice, 2012; Diarra *et al.*, 2010). High Level Streptomycin Resistance (HLSR) is also of clinical concern. Its resistance mechanism functions by modification/alteration of the ribosome due to the expression of genes *Ant(6')-Ia* and *Ant(3")* (Economou & Gousia, 2015; Hollenbeck & Rice, 2012). A study conducted indicated that aminoglycosides are not effective alone (Diarra *et al.*, 2010). Thus, a synergistic attempt in conjunction with the cell wall active agents (β-lactams) results in cell death (Hollenback *et al.*, 2012). The increase in HLAR can cause a burden in the health sector as serious enterococcal infections can't be treated due to high concentration of aminoglycosides being rendered inactive.

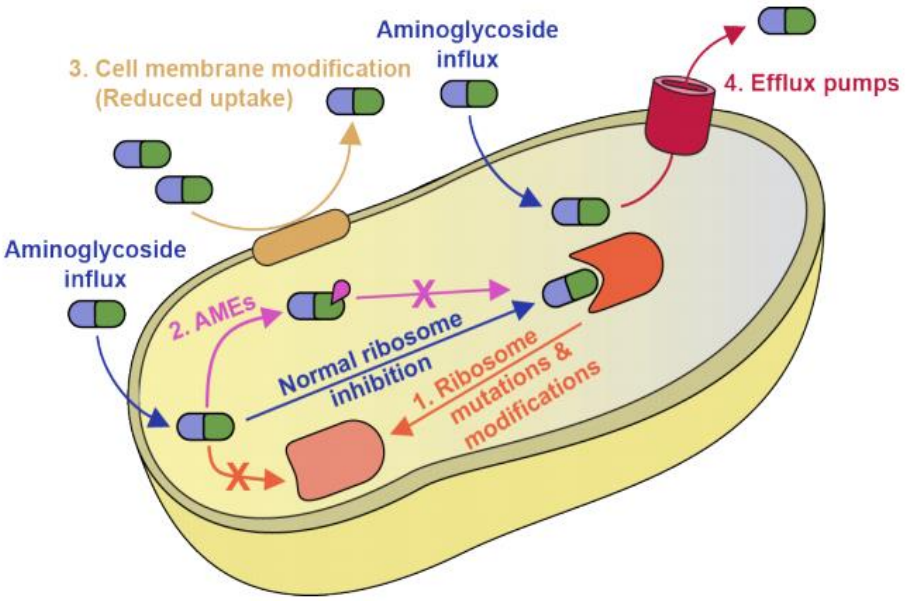

Figure 2.9: Resistance mechanisms to aminoglycosides namely; (1) enzymatic modifications, (2) transportation modifications, and (3) ribosomal modifications

2.8.2.4. Glycopeptides

In South Africa the first clinical outbreak of vancomycin resistance enterococci (VRE) was documented in 1997 leading to an increase of VRE outbreaks in paediatric wards of South African hospitals (Lochan *et al*., 2016). The persistence of these strains in commensal and clinical settings is due to an acquired expression of the *van* operon that codes for glycopeptide resistance (Faron *et al.*, 2016). The operon comprises of two regulator genes namely; *vanS* and *vanR*, a D-lactate dehydrogenase gene (*vanH*), and D-Ala-D-Ala dipeptidase gene (*vanX*). Lastly, a mutable ligase of which ten gene clusters have been identified namely; *vanA, vanB, vanC, vanD, vanE, vanF, vanG, vanM, vanN* (figure. 2.10). (Tatsing Foka *et al.*, 2018; Faron *et al.*, 2016). The resistance mechanism of the operon is to form an alternative cell wall (Ahmed & Baptiste, 2018). With vancomycin susceptible *Enterococcu*s in the presence of vancomycin, it will bind to the membrane's pentapeptide precursor at the D-Ala- D-Ala- terminal, consequently blocking cell wall synthesis (Faron *et al*., 2016). The modification observed in VRE's occurs due to the two-component regulator genes *vanS* and *vanR.* During cellular membrane damage these genes will receive a stimulus about the disruption, several genes will be activated downstream (Faron *et al.*, 2016; Fisher & Phillips, 2009). The reduction of pyruvates to D-Lac in preparation for ligation (*vanA/B*) is achieved by *vanH*. Furthermore, *vanX* will cleave the D-Ala-D-Ala pentapeptides, hence depleting the pool pentapeptide precursors the glycoproteins can bind, the newly ligated D-Ala-D-Lac- pentapeptides have little affinity to vancomycin (Faron *et al.*, 2016). Lastly, the expression of *vanY* ensures that already synthesised D-Ala- D-Ala-pentapeptides are cleaved from the cell membrane. Some variants of the ligase gene clusters are well documented in literature. *vanA* was intensively studied in clinical and environmental strains, this gene confers high level glycopetide resistance (Ahmed & Baptiste, 2018; Hollenbeck & Rice, 2012). Its common carrier species are *E. faecalis* and *E. faecium faecium* (Ahmed & Baptiste, 2018). The high resistance to vancomycin is due to the pool of D-Ala- D-Lac- pentapeptides synthesizes 1000x more compared to other van ligases, whilst resistance to teicoplanin occurs due to the presence of the *vanZ* gene present in the *vanA* operon (Faron *et al.*, 2016). The resistance mechanism *of vanZ* is still misunderstood. The phenotypic expression of *vanA* is inducible and highly transferable as it is commonly located in the transposon *Tn1547* (Wada *et al.*, 2019). Other van ligases confer moderate to high resistance to vancomycin namely *vanB*. This gene is less prevalent compared to *vanA,* however it's majorly documented in *E. faecium* isolates. The reduction in resistance is due decreased production of D-Ala- D-Lac- pentapeptides thus having a pool of pentaglycan that still has an affinity to vancomycin. *vanB* carrying isolates are susceptible to teicoplanin due to the absentia of vanZ (Faron *et al.*, 2016). Although *vanB* is genetically similar to *vanA, vanB* uses homologs of *vanH* and *vanX*, hence the difference in rate of expression (Ahmed & Baptiste, 2018). However, phenotypic expression is inducible as in *vanA.* Although the *van* operon

confers acquired resistance to vancomycin and teicoplanin some of the ligase are chromosomally located in *Enterococcus*. *vanC* is intrinsically expressed in the species *E. casseliflavus, E. flavescens* ans *E. gallinarum* (Ahmed & Baptiste, 2018). It is commonly thought as a selective marker for the afore mentioned species of the van genes. *vanC* is considered to be the less virulent genotype (Fisher & Phillips, 2009). The gene is constitutively expressed and confers no resistance to teicoplanin (Faron *et al.*, 2016). The difference in the rate of resistance conferred by these genes aids in identifying the severity of VRE. Glycopeptides are imperative for therapeutic measures since vancomycin is a drug of choice for the patients who have infections caused by pathogenic *Enterococcus* or other pathogenic bacteria (*Staphylococcus*) (Ahmed & Baptiste, 2018; Torres *et al.*, 2018; Faron *et al.*, 2016). Hence the rise in VRE is concerning, as these species transfer genetic material quite easily.

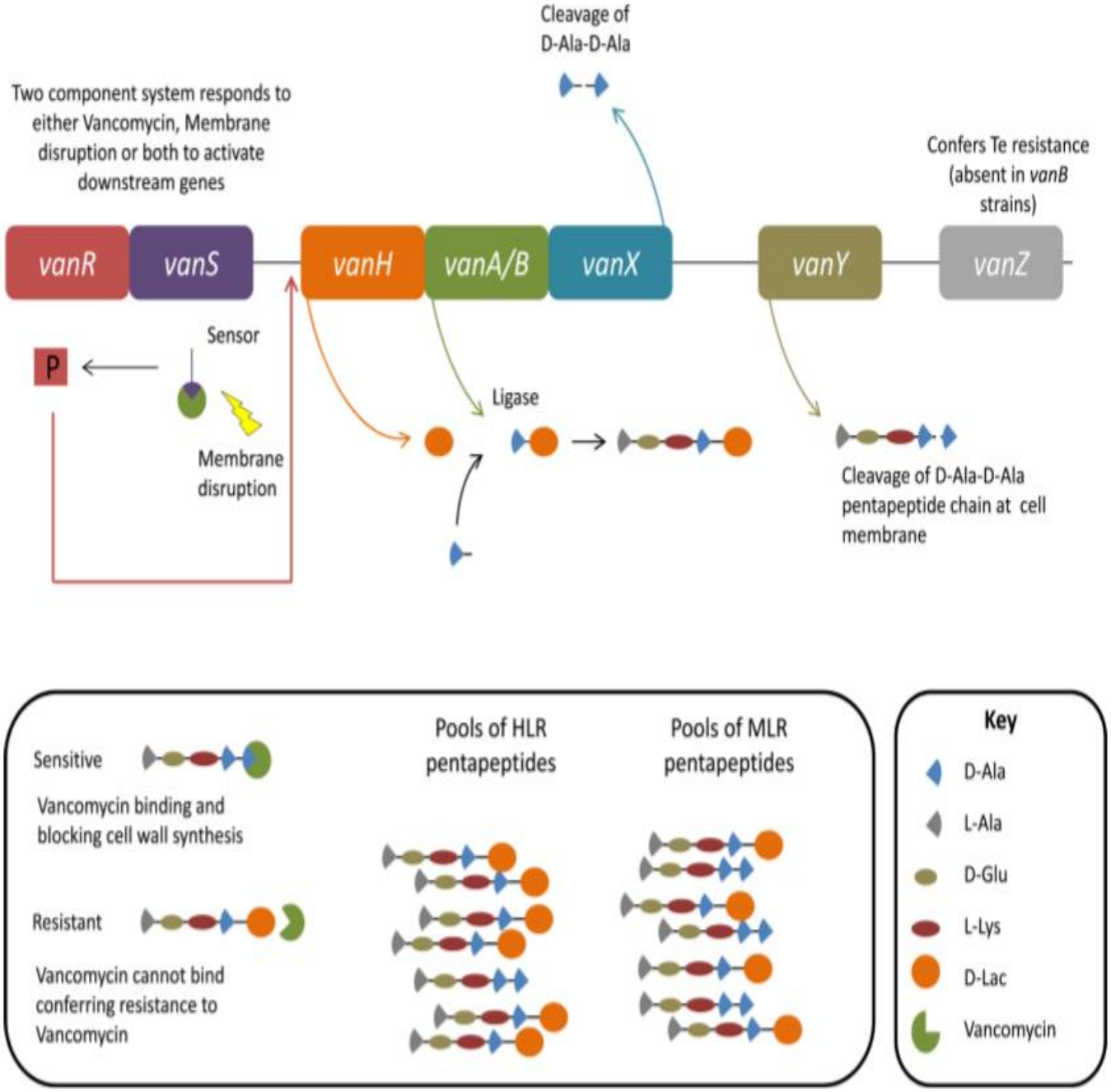

Figure 2.10. The acquisition of vancomycin resistance (glycopeptide) by *Enterococcus* achieved with the aid of modifying the pentapeptide precursor by replacing the D-ala terminal with either a D-lac or D-ser terminal (Faron *et al.*, 2016).

Table 2.1: Summary on the resistance mechanisms associated with Enterococcal species and the type of MGE they get transferred with (Hollenback and Rice, 2012).

Resistant Antibiotic		Resistant mechanism	Enzyme associated with the mechanism	Phenotype	Type of MGE
Aminoglycosides		Low cell wall permeability	-	Has low-level aminoglycoside resistance	Intrinsic
		Ribosome mutation	-	Has high-level aminoglycoside	Sporadic
		Aminoglycoside modifying enzyme (AME)	Aac(6')-Ii	Both tobramycin and kanamycin resistance exhibits low-level of resistance	Intrinsic
		AME	Aph(3')-IIIa	Has low-level kanamycin resistance	pJH1
		AME	Ant(4'')-Ia	Low-level resistance to kanamycin, tobramycin, amikacin and neomycin	plP810
		AME	Aph(2'')-Ia-Aac(6')Ie	High-level gentamicin resistance	Tn5281
		AME	Ant(3'')-Ia	High-level streptomycin resistance	pR538–1
β-lactams and cephalosporins	PBP4/5 production			Low-level penicillin resistance; moderate to high-level cephalosporin resistance	Intrinsic
	PBP4/5 point mutation	-		High-level ampicillin and imipenem resistance	Sporadic
	Altered cell wall	L, D-transpeptidase		β-lactam resistance	Intrinsic
	Destruction of β-lactam ring	β-lactamase on bla genes		β-lactam resistance	Tn552 and others
Glycopeptides	Synthesis of alternative cell wall	VanA, VanH, VanY, VanX, VanR, VanS		Resistance to vancomycin +/2 teicoplanin depending on the phenotype	Tn1546, Inc.18
Lincosamides	ABC-efflux pump	MsrC		Low-level resistance to streptogramin B compounds	Intrinsic
	Altered ribosome	ErmA		MLS_A phenotype	Tn554

2.9. *Enterococcal* surveillance in South Africa

2.10. Conclusion

Enterococci are very ubitiqous in nature, as such they are widely disseminated in the environment (Weaver, 2019). South Africa is a developing country, which creates an increase in industries for livestock rearing (Lochan *et al.*, 2016; Marc Mendelson, 2015). Currently there is a boom in poultry production (Molechan *et al.*, 2019), this literature review indicated how mass rearing of livestock could have a potential impact in the environment. Based on the pathogenicity of *E. Faecalis* and *E. faecium* it is recommended that the Department of Agriculture and Forestry intervene into the communities by creating outreach programs that will equip the population with the necessary knowledge about the dangers of a looming pathogenic outbreak due to simple processes like adding antibiotics in feed for prophylaxis and metaphylaxis measures. A grey area was noticeable in-terms of small-scaled farms, not a lot of studies are focusing on these farms as drivers of antibiotic resistance. WHO's One health approach in mitigating antimicrobial resistance will be hindered due to an un-holistic approach of epidemiological studies. As far as we can affirm the is a need for robust studies in African countries. Lastly, the review identified that the genotypic identification of *Enterococcus* species and employing molecular techniques for virulence and antibiotic resistance surveillance studies is crucial in understanding the pathogenicity of communicable and non-communicable pathogens.

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CHAPTER 3

Detection of virulence genes associated with *E. faecalis* and *E. faecium* recovered from isolates in livestock production systems in South Africa

3.1. Abstract

The study investigated the role of livestock production systems in the dissemination of the virulent *E. faecium* and *E. faecalis* in the Eastern Cape and Kwa- Zulu Natal provinces of South Africa. A total of 276 isolates were randomly sampled from livestock and their associated environment (feed, soil and water) and screened for *Enterococcus* spp. using culture and DNA molecular methods. The *tuf* and *sodA* genes were used to screen for the prevalence of *E. faecalis* and *E. faecium* respectively. Confirmed isolates were further screened for the virulence genes namely; *asa1, ccf, cylA, esp, gelE* and *hyl.* A total of 152 (55.07%) isolates were confirmed to be presumptive *Enterococcus* spp. Sixty-one percent of the isolates were confirmed to be *E. faecalis* and only 8% were identified as *E. faecium.* The remaining thirty-one percent includes other *Enterococcus* spp. such as *E. gallinarum.* Isolates were predominantly recovered from Amandawe (KZN) with *E. faecium* accounting to 62.5% of total prevalence of *E. faecium* in both provinces. This will contribute to the increase in communicable infections as *E. faecium* commonly exhibits resistance to common antibiotics. The gene *cylA* was not present in all *E. faecalis* and *E. faecium* isolates. Furthermore, five of the six screened genes were present in the *E. faecalis* isolates. The gene *ccf* was predominantly found in both species with a 100% (n= 8) prevalence in *E. faecium* and 84% (51) prevalence in *E. faecalis.* The prevalence of the screened virulence genes was not statistically ($p>0.05$) different from each other. The *gelE-ccf-asa1* was the most observed virulence gene pattern whilst *esp* had the lowest prevalence as it only appeared in poultry and cattle. The association with the type of host species and the prevalence of *gelE* was statistically significant ($p<0.05$). None of the categorical variables could significantly ($p>0.05$) predict the absence or presence of the virulence determinants. The immense dissemination of *E. faecalis* that has potentially pathogenic virulent determinants is a cause for concern in livestock production systems due to their zoonotic potential, untreatable nosocomial infections might persist in hospitals. Faecal contamination from livestock poses a threat to the dissemination of virulent strains, hence it can be harmful to the environment since *Enterococcus* are reservoir for virulence determinants.

Keywords: Pathogens; Mobile Genetic Elements; Communicable Infections ; Zoonosis; Environment

3.2. Introduction

Enterococcus is a commensal bacterium that colonizes the gastro-intestinal tract (GI) of animals and humans (Micallef *et al.,* 2013; Arias *et al.,* 2012). Commensal *Enterococcus* have been regarded as the "good" bacteria, because they constitute approximately 1% of the gut natural flora in livestock and humans (Lebreton *et al*., 2014). In addition, it has been demonstrated that enterococci have low levels of virulence since they are natural colonizers of the gut (Biswas & Micallef, 2017; Micallef *et al*., 2013; Nallapareddy *et al*., 2005). There are some other strains of this genera that are used as probiotics whilst some strains have been used in the food sector for decades as starters for fermented foods (Arias *et al*., 2012; Opera and Zervos, 2007). Some strains of *E. faecium* were used as starter cultures for Feta cheeses, the presence of enterococci in dairy positively affects the fermentation process because they form part of Lactic Acid Bacteria (LAB) (Chattopadhyay, 2014). However, upon further investigation, *enterococcal* species have been termed as second-rate pathogens that cause first rate clinical problems (Opera and Zervos, 2007). Some species of this genus have emerged as clinically important strains due to their pathogenicity (Micallef *et al*., 2013). These strains are quite noticeable for causing urinary tract infections (UTI), endocarditis and other infections of the skin and soft tissue (Micallef *et al*., 2013). *Enterococcus faecalis* (*E. faecalis*) and *Enterococcus faecium* (*E. faecium*) mainly cause community and hospital acquired infections. They're noticeable for having antibiotic resistance to clinically important antibiotics namely, streptomycin, gentamycin penicillin (Micallef *et al*., 2013).

The ability of virulent *Enterococcus* spp. to disseminate outside the GI tract of the host (livestock or human beings) is based on their capability to acquire virulent factors and antibiotic resistance genes from other enterococcal species in addition to resisting antibiotics (Arias *et al*., 2012). Enterococci are widely disseminated in animals, humans, soil, plants and water (Riboldi *et al*., 2008; Opera and Zervos, 2007). In farm and community settings contamination of the soil and water with virulent *Enterococcus* species is mostly due to animal faecal matter contaminating the soil and water respectively (Opera and Zervos, 2007). Farmers use surface water as drinking water for their livestock and for irrigation purposes; this includes water from rivers, ponds and tap water. It has been noted that farmers hardly test for antimicrobials and pathogens in surface water (Callahan *et al*., 2018; Salaheen *et al*., 2015; Arias *et al*., 2012). A high diversity of pathogenic bacterium and clinically relevant antibiotics were identified in Maryland (USA) (Callahan *et al.,* 2018). In some settings enterococcal species are used as bioindicators for faecal contamination in recycled water (Micallef *et al*., 2013). The use of ponds and lakes as drinking water for livestock without assessing the water aids in the dissemination of pathogenic bacteria such as *Enterococcus*, as it has been documented that feed and water can be the source of bacterial contamination (Salaheen *et al.,* 2015).

The increase in the pathogenicity of *Enterococcus* has a major impact in food security of the community, thus in turn affecting the economical state of the country (Salaheen *et al*., 2015). Foodborne *enterococci* prevalence has drastically increased over the years (Salaheen *et al*., 2015). It is documented that the exposure to foodborne multi drug resistance *Enterococcus* in the environment and community is under

researched (Micallef *et al*., 2013). In terms of the pathogenesis of *Enterococcus* spp. it is noticeable that several species have host- specific characters (Opera and Zervos, 2007). A study documented that isolates recovered from the farmer had genetic similarity with isolates recovered from one of his farm animals. The communicable strains were *E. faecium* spp. (Opera and Zervos, 2007). Upon further research it was noticeable that these strains were epidemiologically related to each other. Most, if not all livestock predominantly harbour the species; *E. durans, E. Hirae, E. faecalis* and *E. faecium* (Opera and Zervos, 2007). The specie *E. faecalis* is commonly found in poultry (Donado-Godoy *et al.*, 2014). However, in both clinical and farm settings it was noticeable that the host- specific properties of these different species was greatly influenced by the lifestyle of the host, based on the previous use of antimicrobials, age and the host's die (Oprea & Zervos, 2007). Hospitalized patients that were treated for UTI's and endocarditis mostly had a compromised immune system or during their hospital stay invasive devices like catheter's where used.

The extended use of antimicrobials in food producing animals causes multi-drug resistance in most pathogenic strains such as enterococci, however the resistance to antibiotics alone does not explain the virulence of the bacteria (Diarra *et al.*, 2010). *Enterococcusus* spp. are notoriously known to have the ability to acquire or exchange virulence determinants amongst the genera (Hwang *et al.*, 2011). The genetic exchange is achieved through MGE's such as inducible conjugative plasmids or intergrative transposons (Werner *et al.*,2013). It was probable that there is a genetical similarity between enterococcal strains that are from animal origin and the clinically important strains including those that cause nosocomial infections (Ngebde *et al.*, 2016). Although virulence factors of *E. faecalis* are non-pathogenic in the commensal sense, clinical enterococcal strains have a pathogenic potential to unhealthy hosts with a weak immune system as afore mentioned (Hwang *et al.*, 2011). This due the exchange of conjugative plasmids in vivo between enterococcal isolates (Opera and Zervos, 2007). A study reported that enterococci are mostly responsible for transferring virulence factors and resistance genes to other gram-positive spp. This includes *Listeria* spp., the successful transfer was facilitated by mobile genetic elements (Niederhausen *et al.*,2004). The most notable used transposon in the genus for genetic exchange is the *Tn1546* that houses most virulence factors in *Enterococcus* spp. (Niederhausen *et al*.,2004).

Enterococcus species does not yield any effective pro-inflammatory toxins; however, it has genes that are translated to adhesion proteins, these proteins mediate adhesion to the host tissues (Arias *et al*., 2012). Other virulence determinants include secreted factors, these are the proteins that are secreted to the extracellular matrix and they are associated with the pathogenesis of *Enterococcus* (Arias *et al.*, 2012). Of these determinants the best described factors include adhesins, pheromones and aggregation substances (Opera and Zervos, 2007). Enterococcal surface protein coded by the gene *esp* aids *Enterococcus* spp. in the colonization of the hosts cells mostly when infecting the urinary tract of immuno-suppressed hosts (Hwang *et al*., 2011). Cytolysin has lytic activity against other gram-positive

bacteria. The gene clusters *cylB, cylA* and *cylM* encodes for haemolytic activity in enterococci. Cytolysin can mediate virulence to other gram positives and vertebrates lysing their haemolytic ecology (Hwang *et al*., 2011; Opera and Zervos, 2007). The pheromone mating response to recipient cells uses aggregation substance *asa1 to* facilitate the adhesion of the donor recipient cells. This aids in the successful transfer of conjugative plasmids within genus (Opera and Zervos, 2007). Gelatinase has an enzymatic activity that hydrolyses casein, haemoglobin and gelatine of the host. The protein is coded by the gene *gelE* (Opera and Zervos, 2007).

Gelatinase, haemolysins, aggregation substances and enterococcal surface protein have been widely reported in clinical enterococcal strains, in addition there has been an alarming prevalence of these virulence determinants in food producing animals (Hwang *et al*., 2011). Most of virulence factors where best described in *E. faecalis*. As previously alluded, these genes are mostly located in the conjugative plasmid (Hwang *et al*., 2011; Opera and Zervos, 2007). Horizontal gene transfer between species is easily facilitated due to a couple of virulence factors such as *asa1*. The exchange of genetic material between *Enterococcus* species could be achieved via a narrow-host pheromone responsive plasmid, transposons and/or broad host range plasmid (Opera and Zervos, 2007). Hence the noticeable increase in the prevalence of the virulence determinates (*esp, asa1, gel* and *cylA*) in *E. faecium* (Opera and Zervos, 2007). Although past studies indicated that *E. faecium* relatively had less virulence factors compared to *E. faecalis* (Hwang *et al*., 2011; Opera and Zervos,2007). The sudden incline can be attributed to the mobile genetic elements of this bacteria and its transconjugation efficiency (Opera and Zervos, 2007). Globally, studies based on the prevalence and virulence of *Enterococcus* in livestock and the environment are limited. Against this background, this study aims to investigate the prevalence of the *Enterococcus* genus in livestock and its associated environment from South African small-scaled farms. In addition, to assess the dissemination of virulence genes in the two clinically important enterococcal strains. The dissemination of the putative virulence genes in *E. faecalis* and *E, faecium* will be probed with the use of conventional PCR.

3.3. Materials and Methods

3.3.1. Ethical Clearance

The study was approved by the Animal Research Ethics Committee of the University of Kwa-Zulu Natal (Reference numbers AREC/051/017M, AREC 071/017 and AREC014/018). The field sampling protocols, samples collected from animals, and the research were conducted in full compliance with Section 20 of the Animal Diseases Act of 1984 (Act No 35 of 1984) and were approved by the South African Department of Agriculture, Forestry and Fisheries DAFF (Section 20 approval reference number 12/11/1/5).

3.3.2. Study area

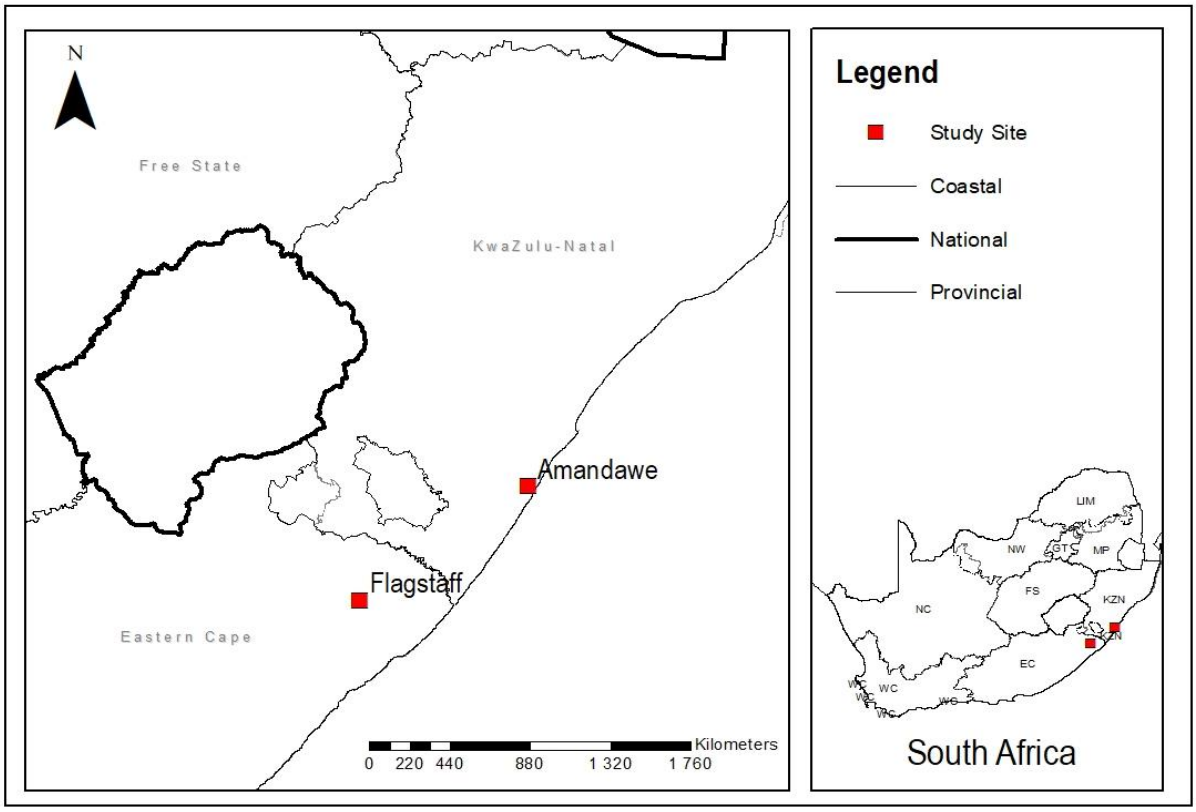

Figure. 3.1. Geographical map of the sampled farms in South Africa

3.3.3. Sampling

Two hundred and seventy-six samples were obtained aseptically from livestock farms in South Africa using simple random sampling(figure. 3.1.). The livestock production farms were small scaled commercial farms in KwaZulu Natal (Amandawe) and the Eastern Cape ((Flagstaff). Samples were recovered from pigs *(n = 34*), cows (*n= 19*), chickens (*n = 80*), ducks *(n=10*), horses (n=*5*), goats (n= *47*) and sheep *(n =22*) respectively. The sampling frame was reflective to the type livestock that was present in the farm. Point of sampling in animals encompassed of rectal, oral and faecal swabs. Environmental samples included samples from water (*n =19*), feedlot (*n = 9*) and the soil (*n =33*). Samples were obtained using sterile cotton swabs. Swabs were immersed in 10 ml of 0.1% (w/v) peptone water. Upon sampling, the samples were transported to the laboratory at the University of KwaZulu-Natal and were stored in ice for immediate processing

3.3.4. Isolation of Enterococcus species

A total of 1ml of the peptone water (with sample) was inoculated into 10ml of Brain Heart Infusion broth (BHI) for enrichment and incubated for a duration of 18-24 hrs at 37°C. The growing culture was streaked onto Bile Aesculin Azide agar and further incubated for a period of 18-24hrs at 37°C. Presumptive isolates for *Enterococcu*s species had a black/brownish dew drop phenotype. Only one

colony per plate was streaked onto Trypticase Soy Agar (TSA) to be further incubated for a period of 18 hours at 37°C for glycerol stock preparation (Iweriebor *et al*., 2015.)

3.3.5. Identification and species verification of Enterococcus species

Genomic DNA was extracted using the boiling method (Riberio *et al*.,2016**).** Molecular confirmation of the isolates was achieved by polymerase chain reaction (PCR) with the use of *Enterococcus* genus-specific primers *tuf* gene and the strain *E. faecalis* ATCC 1943 served as a positive control. A 25 µl reaction volume; with 12.5µl of Dream Taq Green Master mix (Thermo Scientific), 1µl of each of the forward and reverse primers (Inqaba Biotech), 5.5 µl of Nuclease free water (Thermo Fischer) and 5µl of template DNA was used. The t*uf* gene (table 3.1.) amplified in 35 cycles with an initial denaturing step at 94 ℃ for 4 min, Optimum conditions for amplification were denaturation at 94 ℃ for 60 s, annealing at 53 ℃ for 60 s, extension at 72 ℃ for 60 s, and final extension at 72 ℃ for 5 min. Furthermore, of the isolates confirmed to be *Enterococcus* spp. another 25 µl monoplex PCR reactions were conducted using species-specific primers (table 3.1.) to identify *Enterococcus faecalis* and *Enterococcus faecium* respectively. With optimum conditions of initial denaturation at 95 ℃ for 4 min in 30 cycles. Denaturation at 95 ℃ for 30 s, annealing temperatures of 52 ℃ and 48 ºC at 1 min respectively. Extension occurred at 72 ℃ for 60 s, and a final extension of 72 ℃ for 7 min. A non-template control was run with every reaction, without the template DNA, but with 2 µl of Nuclease Free Water. For all PCR reactions the BioRad Thermocycler was used (BioRad, Carlifonia, United States of America). Amplicons were electrophorized in a 1.8% agarose gel at 110 Volts for 45 min. Amplicons were visualized under UV light using Bio ChemiDoc imagining system (BioRad, Carlifonia, United States of America)

Table 3.1. PCR primers that were used for the identification of *Enterococcus* species.

Gene	Primer Sequence (5'-3')	Amplicon size (bp)	Reference
tuf gene	Ent1 TACTGACAAACCATTCATGATG Ent2 AACTTCGTCACCAACGCGAAC	112	Ke *et al*., 1999
E. faecium ATCC19434	FM1 GAAAAAACAATAGAAGAATTAT FM2 TGCTTTTTTGAATTCTTCTTTA	215	Jackson *et al.*, 2004
E. faecalis ATCC 19433	FL1 ACTTATGTGACTAACTTAACC FL2 TAATGGTGAATCTTGGTTTGG	360	Iweriebor *et al*., 2015

3.3.6. Virulence Genes Screening

A monoplex a mplification was conducted on the virulence genes in table 3.2. A 25 µl reaction volume; with 12.5µl of Dream Taq Green Master mix (Thermo Scientific), 1µl of each of the forward and reverse primers (Inqaba Biotech), 5.5 µl of Nuclease free water (Thermo Fischer) and 5µl of template DNA was used to amplify the genes *ccf* and *gelE*. The gene *gelE* amplified in 30 cycles with an initial denaturing step at 95 °C for 15 min. Optimum conditions for amplification were denaturation at 94 °C for 60 s, annealing at 56 °C for 60 s, extension at 72 °C for 60 s, and final extension at 72 °C for 10 min. For the *ccf* gene, amplification occurred in 35 cycles. An initial denaturing step at 94 °C for 4 min. denaturation at 94 °C for 60 s, annealing at 51.3 °C for 60 s, extension at 72 °C for 60 s, and final extension at 72 °C for 5 min. For the genes *asa1*, *cylA1*, *esp* and *hyl* a multiplex reaction was run using a 10 µl reaction volume; with 5 µl of Dream Taq Green Master mix (Thermo Scientific), 0.2 µl of each of the forward and reverse primers (Inqaba Biotech) for reach gene, 1.4 µl of Nuclease free water (Thermo Fischer) and 2µl of template DNA. Initial denaturing step at 95 °C for 15 min, for 35 cycles. Optimum conditions for amplification were denaturation at 94 °C for 60 s, annealing at 56 °C for 60 s, extension at 72 °C for 90 s, and final extension at 72 °C for 10 min.. The amplicons were visualized under UV light using Bio ChemiDoc imagining system (BioRad, Carlifonia, United States of America)

3.3.7. Statistical Analysis

The association between virulence genes and the variables associated with livestock production systems (location, sample site and host) were analyzed using the Fischer's exact test. Furthermore, direction of the relationship and the correlation between the screened genes was investigated using the Pearson's correlations test. The Binary logistic regression null model was implemented to investigate the effect and association of the virulence genes with the animal host, location and sample site, at a 95% confidence level. All three statistical tests were analyzed from the *E. faecalis* data set and tests were considered significant at $p < 0.05$. Lastly, a Mann-Whitney U test was used to analyse whether the prevalence of the virulence genes was different between the two species namely *E. faecalis* and *E. faecium.* Statistical evidence was deemed significant when $p > 0.05$. The IBM Statistical Package for Social Sciences (SPSS), version 25.0 (IBM Cooperation, New York) was the statistical software used of analysis.

Table 3.2. Primers used for the surveillance of virulence genes associated with *Enterococcus* spp.

Gene	Description	Primer Sequence (5'-3')	Amplicon size (bp)	Reference
asa1	Aggregation substance one	ASA 11 GCACGCTATTACGAACTATGA ASA 12 TAAGAAAGAACATCACCACGA	375	Ye, 2010

cylA	Cytolysin	Cyl I ACTCGGGGATTGATAGGC Cyl Iib GCTGCTAAAGCTGCGCTT	688	Iweriebor *et al*., 2015
esp	Enterococcal surface protein	ESP 14F AGATTTCATCTTTGATTCTTGG ESP 12R AATTGATTCTTTAGCATCTGG	510	Ye, 2010
gelE	Gelatinase	GEL 11 TATGACAATGCTTTTTGGGAT GEL 12 AGATGCACCCGAAATAATATA	391	Seputiene *et al*., 2012
hyl	Hyaluronidase	HYL n1 ACAGAAGAGCTGCAGGAAATG HYL n2 GACTGACGTCCAAGTTTCCAA	276	Vankerckhoven, *et al*., 2004
ccf		5'-GGG AAT TGA GTA GTG AAG AAG-3' 5'-AGC CGC TAA AAT CGG TAA AAT-3'	543	Pillay *et al*., 2018

Table 3.3. The prevalence of *E. faecalis* and *E. faecium* isolated from livestock production systems in the provinces of Eastern Cape and KwaZulu Natal in South Africa

	Flagstaff										**Amandawe**										**Total**
	E. faecalis					***E. faecium***					***E. faecalis***					***E. faecium***					
	Oral	Faecal	Water	Soil	Feedlot	Oral	Faecal	Water	Soil	Feedlot	Oral	Faecal	Water	Soil	Feedlot	Oral	Faecal	Water	Soil	Feedlot	
Pig	4	2	0	3	1	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	12
Cow	0	0	0	1	0	0	0	0	0	0	0	4	1	0	0	0	2	0	0	0	8
Chicken	0	0	0	0	0	0	0	0	0	0	5	6	2	2	0	0	0	0	0	0	15
Broiler Chicken	0	0	0	0	0	0	0	0	0	0	7	5	0	0	0	0	0	0	0	0	12
Duck	0	1	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	3
Horse	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Goat	1	0	0	1	0	0	0	0	0	0	4	9	0	0	0	2	0	0	0	0	17
Sheep	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
Total	6	3	0	6	1	0	3	0	0	0	16	24	3	2	0	2	3	0	0	0	**69/276**

3.4. Results

3.4.1. Prevalence of Enterococcus species

A total of 276 samples, sampled from livestock productions systems were screened for *Enterococcus* spp. using selective media (Bile Esculin Azide agar) and Polymerase Chain Reaction (PCR). Based on the colonies which were brown and had a black halo phenotype, 152 (55.07%) isolates were confirmed to be presumptive *Enterococcus* spp. The genus specific *tuf* gene amplified in 100 (36.23%) isolates of the 152 presumptive isolates, with a band size of 112bp. Furthermore, of the isolates confirmed to be *Enterococcus* spp. using molecular amplification of the *sodA gene* for species identification of *Enterococcus faecalis* and *Enterococcus faecium*. A total of 61 (61%) isolates were confirmed to be *E. faecalis* with a band size of (360bp, Figure 3.8); the isolates were distributed as follows; goat = 14**,** broiler chicken =12, chicken =11, pig = 6, cow = 4, duck =1, sheep =1 and the environment (soil, water and feedlot) = 12 (table 3.3.). Whilst 8 (8%) isolates with a band size of 215bp (Figure 3.8.) were identified as *E. faecium.* The *E. faecium* isolates had the lowest prevalence in both provinces, interestingly 75% of the recovered isolates were sampled from faecal matter. The remaining 25% emanated from oral samples of a goat (n=2). The distribution of the *E. faecium* isolates was as follows; pig = 2, duck =2 and cow =2 (table.3.3). Isolates were predominantly recovered from Amandawe (KZN) with *E. faecium* accounting to 62.5% (*n=5*) of total prevalence of *Enterococcus faecium* in both provinces. The same unequal distribution was observed in *E. faecalis* isolates, as 73.8% (*n* =45) of the isolates were also recovered from Amandawe samples.

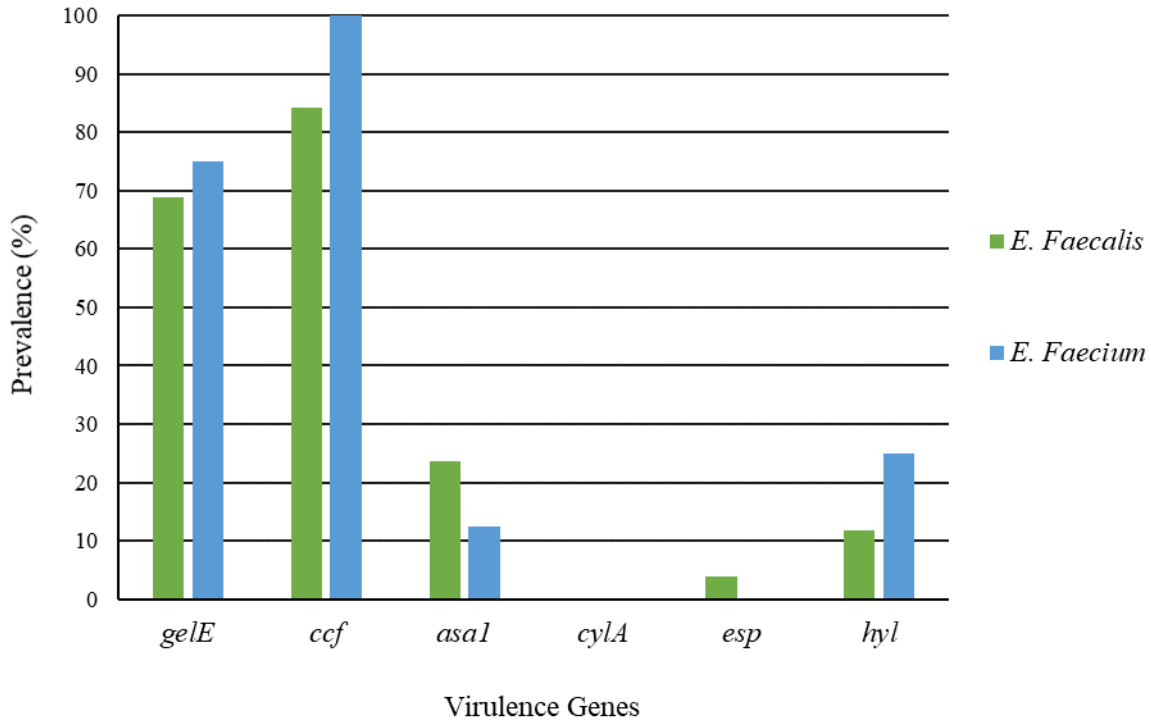

Figure 3.2. The prevalence (%) of virulence genes in the species *E. faecalis* and *E. faecium* isolated from livestock production systems in South Africa

3.4.2. Surveillance of the virulence determinants associated with Enterococcus spp.

A collective of 6 *Enterococcus* spp. associated virulence genes were screened; *gelE, ccf, asa1, esp, hyl* and *cylA*. Of the screened virulence genes, *cylA* was not present in all *E. faecalis* and *E. faecium* isolates. Figure 3.2. illustrates that of the two species, *E. faecium* had the highest prevalence of *gelE, ccf,* and *hyl* with percentages of 75%, 100% and 25% respectively. The gene for enterococcal surface protein (*esp*) was not present in *E. faecium* isolates. It was noticeable that 5 of the 6 screened genes were present in the *E. faecalis* isolates, thus excluding *cylA.* Although *E. faecium* isolates had the highest prevalence of the virulence determinants, *E. faecalis* had 83.3% prevalence of the screened genes. The gene *ccf* was predominantly found in both species with a 100% prevalence in *E. faecium* and 84% prevalence in *E. faecalis*. *gelE* was the second commonly found virulence determinant in *Enterococcus* spp. whilst *esp* had least occurrence in both species with percentages of 4% for *E. faecalis* and 0% in *E. faecium*. A Mann-Whitney U test was used to analyse whether the prevalence of the virulence genes was different between the two species. Table 3.4. indicates the prevalence of the screened virulence genes namely; *gelE, ccf, asa1, esp and hyl.* The prevalence of these genes wasnot statistically different from each other ($p > 0.05$).

Table 3.4. The p-values from Mann-Whitney U test indicating the distribution of the virulence determinants between *E. faecalis* and *E. faecium*.

Virulence gene	p-value
gelE	0.289
ccf	0.193
asa1	0.628
esp	0.524
hly	0.211
cylA	-

*p< 0.05, not significantly different (2-tailed).

3.4.3. Detection of virulence genes in specific animal hosts and the environment from E. faecalis isolates.

The frequency of the virulence determinants differed between the two species based on where the isolates were recovered from not limited to type of animal host or environmental factors (soil, water and feedlot). Figure 3.3. shows the prevalence of the virulence genes in *E. faecalis* isolates recovered from Flagstaff and Amandawe. *gelE-ccf-asa1* was the most observed virulence gene pattern. It was also noticeable that the genes *gelE* and *ccf* were present in almost all isolates that were recovered from the animal hosts; namely pig, duck, cow, country farmed chicken, broiler chicken, goat and sheep. The isolates recovered from the horses had 0% prevalence of any virulence determinants. In addition, isolates from the environment had the presence of *ccf* (4.9%) and *gelE* (6.6%) genes only. *hyl* was only documented in isolates recovered from broiler chickens, country farmed chickens and goats. Interestingly these isolates recovered from these hosts had the highest prevalence of the other virulence genes excluding *cylA.* Virulence factor *esp* had the lowest prevalence as it only appeared in isolates recovered from cows and chickens. Between broiler chickens and country farmed chickens both hosts had the highest prevalence of virulence genes. Yet with the isolates recovered from country farmed chickens all the virulence determinants were present, which includes *esp* (1.6%), *asa1* (6.6%) and *hyl* (6.6%). Whilst broiler chickens had zero presence of *esp*, *hyl* (1.6%) and *asa1* (4.9%) genes were present. The Fisher Exact test was used to analyse any association between the virulence genes and variables associated with the livestock production system (location, animal, host and sample site). Frequencies of the virulence genes in figure 3.3 hints an association between the prevalence of the genes with the type of host and sample site the isolates were recovered from. Association with the type of host species and the prevalence of *gelE* was statistically significant (p< 0.005). For the rest of the genes, there is however, not enough statistical evidence to support the association between the virulence genes and the variables associated with the livestock production systems (p >0.05, table 3.5.).

As afore mentioned, the genes *gelE* and *ccf* were present in almost all isolates that were recovered from the animal hosts. These genes were prevalent equally in their respective host; duck (1.6%),

cow (9.8%), country farmed chicken (19.7%), broiler chicken (18.0%) and sheep (1.6%) (figure 3.3.). From the isolates recovered from the environment the prevalence of *ccf* was 4.9% and *gelE* 6.6%. It appeared that the prevalence of these two genes was similar in most *E. faecalis* isolates. Yet, the two genes did not have significant relationship based on the Pearson Correlations Test ($p > 0.05$, table 3.6.). Substantial evidence suggests a negatively weak relationship between the genes *ccf* and *hyl* ($p < 0.05$, table 3.6) thus in agreement with the high prevalence of *ccf* compared to *hyl* in the recovered *E. faecalis* isolates (figure 3.2). There was a strong significantly positive relationship between the genes *asa1* and *esp* ($p < 0.001$, table 3.6). Lastly, binary logistic regression was used to evaluate the effect sample site, animal host and location has on the five virulence genes. None of the categorical variables could significantly predict the absence or presence of each of the virulence determinants ($p > 0.05$, table 3.7.).

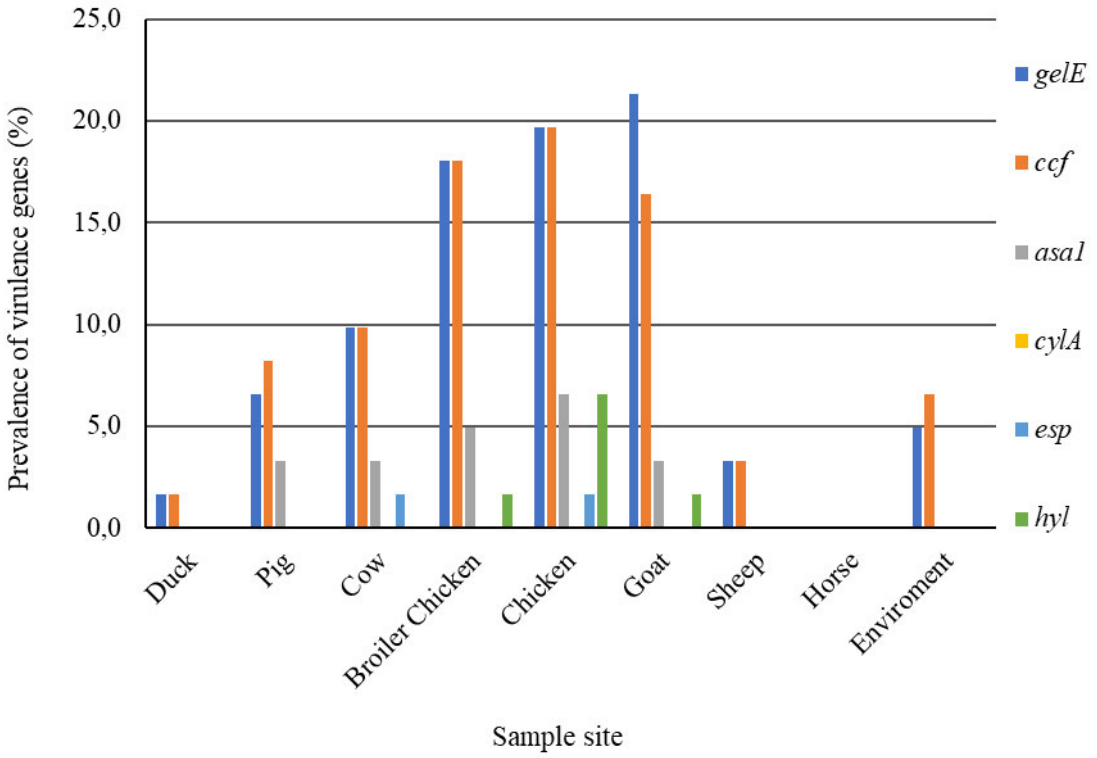

Figure 3.3. The prevalence (%) of the virulence genes associated with *E. faecalis* isolated from livestock and their surrounding environment (water, soil and feed).

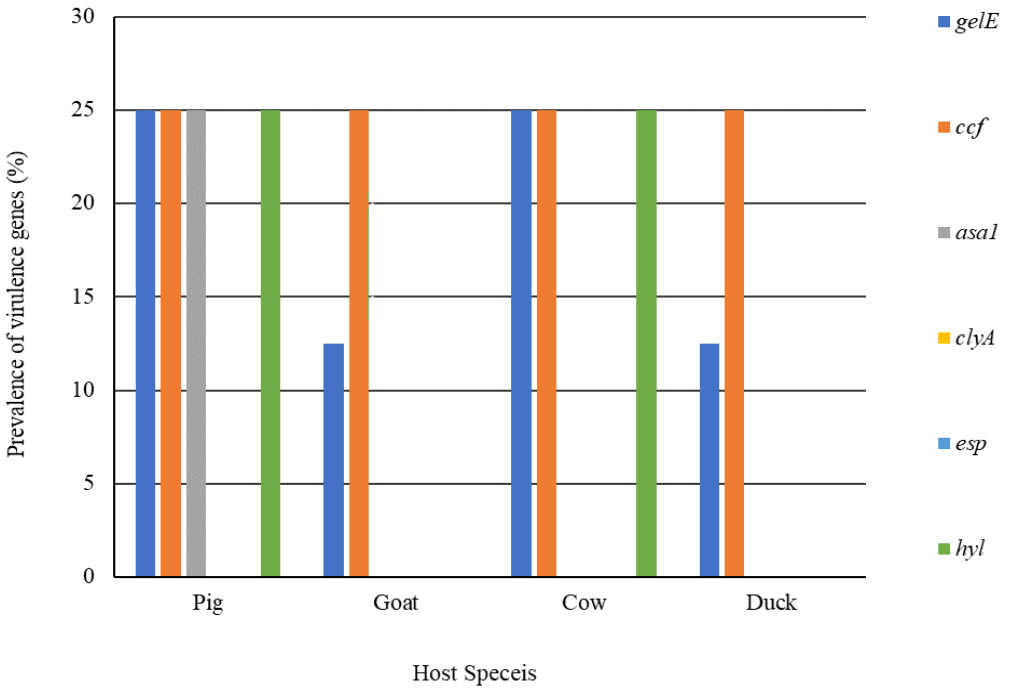

Figure 3.4. The prevalence (%) of the virulence genes associated with *E. faecium* isolated from livestock and their surrounding environment (water, soil and feed).

Table 3.5. Fischer's exact test p-values indicating the relationship between the virulence determinants and the different variables associated with livestock production systems in *E. faecalis.*

Variable	**Genes**					
	gelE	***ccf***	***asa1***	***cyla***	***hyl***	***esp***
Location	0.070	0.052	0.270	-	0.499	0.166
Host Species	0.024*	0.554	0.272	-	0.422	0.257
Sample Material	0.687	0.511	0.777	-	0.673	0.616

*p< 0.05, significant (2-tailed).

Table 3.6. Pearson correlation test p-values indicating the relationship between the virulence determinants in *E. faecalis*

	gelE	***ccf***	***asa1***	***esp***	***hyl***
gelE	1	.099	-.210	-.156	-.227
		.449	.104	.230	.079
ccf	.099	1	.018	.107	-.275*
	.449		.893	.413	.032
asa1	-.210	.018	1	.460**	.114
	.104	.893		.000	.384
esp	-.156	.107	.460**	1	-.075
	.230	.413	.000		.565
hyl	-.227	-.275*	.114	-.075	1
	.079	.032	.384	.565	

* Correlation is significant at the 0.05 level (2-tailed).

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

Table 3.7. Binary logistic regression null model illustrating the effect and association of virulence genes in *E. faecalis* with animal host, location and sample.

		B	**SE**	**p-value**	**Exp (*B*)**	**95% CI**	
						Lower	**Upper**
gelE	Location	-1.234	0.909	0.174	0.291	0.49	1.728
	Sample site	0.156	0.177	0.378	1.169	0.826	1.652
	Host	-0.136	0.515	0.791	0.873	0.318	2.396
ccf	Location	20.116	9963.54	0.998	54.732.	0.000	-
	Sample site	0.053	0.468	0.642	1.243	0.803	1.385
	Host	0.218	0.139	0. 701	1.055	0.461	3.113
asa1	Location	0.691	0.748	0.356	1.995	0.455	8.644
	Sample site	0.001	0.402	0.999	1.001	0.687	2.201
	Host	-0.119	0.131	0.364	0.888	0.455	1.148
hyl	Location	-0.926	1.194	0.438	0.396	0.038	4.113
	Sample site	-0.237	0.509	0.642	1.267	0.632	1.240
	Host	-0.122	0.172	0.479	0.855	0.467	3.436
esp	Location	1.775	1.432	0.215	5.902	0.356	97.45
	Sample site	-0.44	0.257	0.885	0.957	0.579	1.583
	Host	-0.50	0.765	0.948	0.951	0.212	4.260

3.4.4 Detection of virulence genes in specific animal hosts and the environment from E. faecium isolates.

From all 8 recovered *E. faecium* isolates, the virulence determinates were only present in pigs, cows, ducks and goats. As illustrated in figure 3.4., isolates of pig origin had the genes *gelE, asa1* and *hyl.* In addition, the presence of *asa1* was only prevalent in this host. Whereas in goats, the isolates only had the presence of *gelE* and *ccf* though *esp* was not present in the in *E. faecium* isolates.

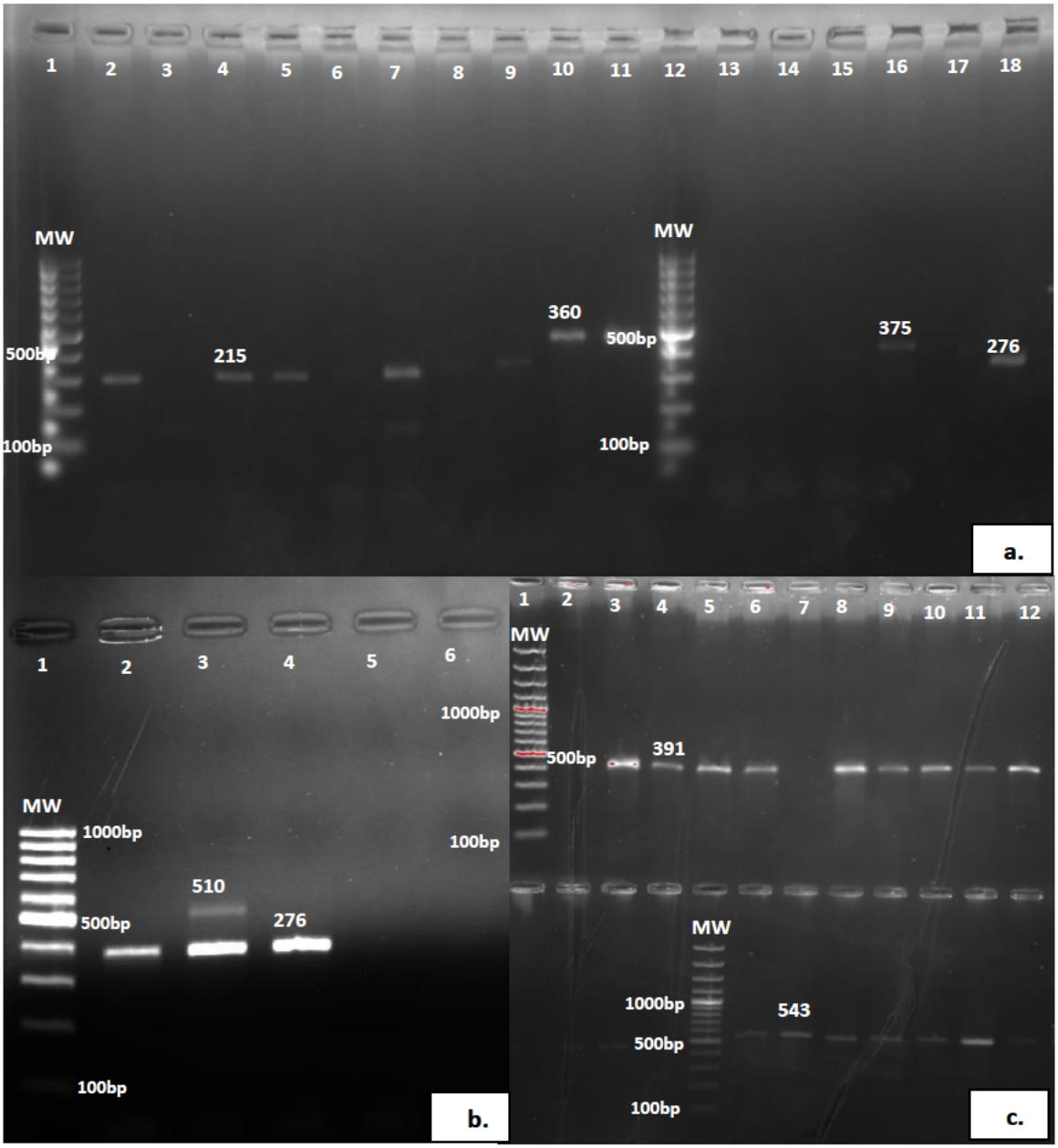

Figure 3.5. The molecular detection of *Enterococcus* spp. and its associated virulence genes using PCR methods and 1.8% agarose gel. **a.)** MW indicates the molecular ladder (100bp plus, GenePlus, Fisher Scientific, Sweden). The species *E. faecalis* (Lane 4) and *E. faecium* (Lane 5) were detected with amplicon sizes 360bp and 215bp respectively. The genes *hyl* and *asa1* illustrated at lanes 16 and 18 amplified band sizes of 276bp and 375bp. **b.)** *esp* amplified at 510bp. **c.)** Whilst for ccf and *gelE* 543bp and 391bp amplicons were observed.

3.5. Discussion

Enterococcus spp. recovered from healthy livestock are important as they could be considered as indicators for AMR in bacteria (Donado-Godoy *et al*., 2014; Diarra *et al*., 2010). The Global One

Health Approach aims to monitor the occurrence of AMR in clinical, food-producing animals, environmental and retail meat strains (WHO,2018). Nevertheless, it is understood that the dissemination of *Enterococcus* spp. is mainly based on the pathogenesis of these species, of which it is thought to be a multifactorial process (Donado-Godoy *et al.*, 2014).

In this study, *E. faecalis* was more prevalent at 61% compared to *E. faecium,* where only 8% of the isolates amplified for the species-specific gene (*sodA*). The current studies results are inconsistent with most livestock studies who observed *E. faecium* isolates being the most recovered species (Molechan *et al.*, 2019; Ngebde *et al.*, 2016; Iweriebor *et al*., 2015 Diarra *et al*., 2010; Dogru *et al*.,2010). Nevertheless, the obtained results are not surprising because it has been previously reported that *E. faecium* accounts to 5-10% and *E. faecalis* accounts to 90- 95% of overall *Enterococcus* spp. in the GI of animals and humans (Skowron *et al*., 2014). Furthermore, the vast dissemination of *E faecalis* rather than *E. faecium* in the current study can be explained since *E. faecalis* strains are documented to harbour the most virulence factors. These factors , aid in the dissemination of this specie as it has increased adaptability to colonize animals and their environment (Klibi *et al*., 2013; Yeong *et al.*, 2011; Dogru *et al*.,2010). Lastly, the differences observed between the current study and other livestock studies could also be attributed to the different methods used to isolate the bacteria or due to the geographical inconsistencies (Klibi *et al*., 2013).

Enterococci isolated from different sources (namely clinical samples, livestock, retail meat and the environment) are known to harbour virulence determinants. These elements are mostly responsible for the escalation of *E. faecalis* and *E. faecium* as opportunistic pathogens (Ngbede *et al*.,2016; Lins *et al*.,2013). The pathogenicity of these species in animals and humans is noticeable by colonizing of the gut, adhering to different proteins of extracellular matrix in the host and the capability to bind to the cells of the epithelial of the oral cavity and urinary tract (Fisher and Phillips, 2009). Consequently, it is imperative to understand the interactions between bacteria and their respective virulence determinant as this will aid in creating new therapeutic measure that will not require the use of antimicrobials (Lins *et al*., 2013) Other authors also observed a high prevalence of these genes. Ngebede *et al*.(2016) and Yılmaz *et al. (*2016) did not differentiate between species , while Thus *et al*. (2019) screened for *gelE* from pig carcasses and pork meat they documented a low prevalence (14.6%).

Previously *E. faecalis* was thought to harbour more virulence determinants compared to *E. faecium,* this includes genes that facilitates adherence, cytolysin and pheromone mechanisms (Giraffa, 2002). This supplements the current study, as a maximum of five virulence genes were detected excluding *cylA* in *E. faecalis* isolates. *cylA* did not amplify in any of *E. faecium* isolates. The current findings of this study differ from other authors who documented a prevalence of this gene (Foka

and Ateba, 2019; Yılmaz *et al.,* 2016; Hwang *et al*.,2011). Cytolysin which has eight coding variants including *cylA, cylB* and *cylM* (Matlou *et al*., 2019). The study's gene of interest codes for cytolysin protein production, which aids the bacteria to evade the immune systems of the host by means of macrophage destruction (Aşgın and Taşkın, 2019; Foka and Ateba, 2019). Though similarly to our study, Yang *et al*. (2018) and Song *et al*. (2018) didn't report any occurrence of *cylA*.

The virulence pattern *gelE-ccf-asa1* was the most observed pattern in *E. faecalis* isolates. Nowakiewicz *et al*. (2016) is in agreement with our findings, as they documented *gelE-ccf* in most of their *E. faecalis* isolates recovered from poultry. However, they documented a low prevalence of the aggregation substance (*agg*). The current study screened for *asa1*, a gene that mediates the production of aggregation substances that facilitate contact during conjugating when cells are adhered to the eukaryotic cells (Aşgın and Taşkın, 2019; Foka and Ateba, 2019). The minor inconsistences can be explained by screening for different genes that code for aggregation substances. Alternatively, other studies observed different genetic patterns (Yang *et al*., 2018; Foka and Ateba, 2019; Maasjost *et al*., 2019).

Gelatinase production in *E. faecalis* has been frequently documented in other studies (Ngebede *et al*., 2016; Yılmaz *et al*., 2016; Nowakiewicz *et al*.,2016; Dogru *et al*.,2010). It functions by hydrolysing various proteins in eukaryotes; not limited to gelatine, haemoglobin, collagen, insulin and haemoglobin (Yang *et al*., 2019). In addition, for successful conjugative transfer for plasmid lacking species, the enterococci will secrete sex pheromones to encourage mating from a plasmid carrying bacterium (Pillay *et al.*,2018). Both these are important for the pathogenesis of *Enterococcus* spp. (Foka and Ateba, 2019). The genes *gelE* and *ccf* were predominant across both species. Moreover, both genes were disseminated in all animal hosts this includes swine, cattle, broiler, goat, sheep, duck, and country farmed chicken with the exception of horses. Lastly, the genes were also recovered from the environment. Similarly, Hwang *et al*. (2011), Molechan *et al*. (2019), Pillay *et al*. (2018) and Yilmaz *et al*. (2016) reported the same findings. Although the genes occurred in the same frequencies across the different hosts and their associated environment, there was no substantial statistical evidence supporting a relationship between these two genes ($p > 0.05$). This differs from Pillay *et al*. (2018) they documented significant association with regards to the presence or absence of *ccf* and *gelE*. Interestingly *gelE* was associated with the type of host species the isolate was recovered from ($p < 0.05$), thus the occurrence of *gelE* is dependent on the type of animal host.

Enterococcal surface protein is transcribed by the *esp* gene, in *E. faecalis* and *E. faecium*, the gene is expressed from the Pathogenicity Islands (PAI) (Aşgın and Taşkın, 2019). It is commonly linked to the invasion, adhering and evading mechanisms of the aforementioned *Enterococcus* species

(Aşgın and Taşkın, 2019; Fisher and Phillips, 2009). This virulent determinant is of critical importance as it has been associated with nosocomial infections in humans, whilst in animal husbandry it has been documented that it aids *E. faecalis* to adhere and persist in slaughterhouse surfaces (Yılmaz *et al*., 2016; Fisher and Phillips, 2009). In the current study *esp* was the least detected gene (4%) in addition to being prevalent only in cattle and poultry (broiler and country farmed). Several studies agreed with our findings (Song *et al*., 2019; Tatsing Foka *et al*., 2019; Nowakiewicz *et al*., 2017; Yılmaz *et al*., 2016). However, Hwang *et al*. (2011) reported a high occurrence of *esp* in isolates recovered from swine and poultry in Korea. The considerable difference between the current study might be due to a selective pressure. As previously mentioned, the *esp* gene does not only aid in persistence of the bacteria but also confers resistance to environmental stresses (Song *et al*. 2018). It is understood that attainment of virulence genes increases in similarly to the increment in AR. (Aşgın and Taşkın, 2019). In terms of climatic condition Korea has harsher winter compared to South Africa, hence the selection of *Enterococcus* spp. that can persist in the environment irrespective of temperatures being less favourable.

A positive correlation between the genes *asa1* and *esp* was observed, ($p<0.05$). It is probable that the prevalence of *asa1* can explain the presence of *esp. asa1* is a surface protein which increases aggregation during conjugation, the expression of this gene is pheromone inducible (Lins *et al.*,2013). *esp* has been thought to have a role in biofilm production (Heinkens *et al*., 2007). Although *asa1'*s importance as an adhesion protein to promote the adhesion of the bacteria to surfaces (Comerlato *et al.*,2013). The positive correlation between these two genes can be explained as Comerlato *et* al. (2013) documented the prevalence of *asa1* from biofilm producing clinical isolates. Furthermore, the *esp* gene has been documented to be a marker for large PAI's, in addition the expression of *asa1* in *E. faecalis* is mostly expressed from pheromone-induced plasmids that are PAI oriented (Seputiene *et al*., 2012). The current study also screened for the gene responsible for the expression of hyaluronidase; which functions by degrading hyaluronic acid resulting in tissue damage of the host (Aşgın and Taşkın, 2019). *hyl* is commonly detected in *E. faecium* isolates, as observed from the current study at 25% compared to a 6.6% in *E. faecalis.* Our results are consistent with Zou *et al*. (2011) who documented 6.84% *hyl* in E. *faecalis* from swine and Foka and Ateba (2019) documented *hyl* at 33.4 in *E. faecalis* and 4.01% in *E. faecium* from isolates recovered from cattle in North West, South Africa, Massjost *et al.* (2019) did not detect presence of hyl in both *E. faecium* and *E. faecalis*.

Lastly, the current study aimed to investigate the role of broiler chickens, country farmed chickens and their associated environment in the dissemination of putative virulence determinates in addition to understanding the difference in the selection of virulence genes between the aforementioned poultry types. Molechan *et al*. (2019) explained that the poultry industry is booming, hence it is

important to assess and understand the microbial quality linked to intensive poultry production and the health risks associated with farming poultry. The current study documented that both broilers and country-farmed chickens harboured the most virulent genes compared to other hosts, the intensive broiler chicken's environment promotes the exchange of genetic materials through MGE's as the animals are reared in close proximity to each other. This is a production system in which livestock are raised in artificial and extremely confined conditions (Haag, 2015; Jackson *et al*.,2012). The current study observed that *E. faecalis* recovered from broilers was less virulent compared to country farmed chickens which are reared in a grazing system, whereby the livestock can move around the farm freely whilst grazing. (Grobler, 2010). Interestingly, the genes *gelE, ccf, hly, esp* and *asa1 were* more disseminated in country farmed chickens. Our results differs from Kasimoğlu Doğru *et al*. (2009) who reported *E. faecalis* strains recovered from intensive broiler being more virulent than strains from country farmed chickens. In South Africa although the use of antimicrobial drugs was banned in 1997, some of these drugs are still being used for animal husbandry (Tasting *et al*., 2018). This can aid in the co-selection of strains that may harbour these putative genes. It was previously mentioned that *esp* aids in the persistence of this species in the environment hence the virulence observed in *E. faecalis* recovered from country farmed could be explained. Different studies have reported a synergistic virulent interface between *E. faecalis* and other pathogens (Seputiene *et al*., 2012). Furthermore, it was documented to be more virulent than *E. faecium* thus posing a risk to the health sector and the environment. The dissemination of virulent *E. faecalis* in the environment was observed in the current study. *gelE* (4.9%) and *ccf* (6.6%) were the only genes detected. There is limited research on the impact of livestock feed, livestock management, and the use of faecal matter as manure on the survival of pathogenic strains in the environment (Salaheen *et al*., 2015).

3.6. Conclusion

E. faecium and *E. faecalis* were recovered from livestock and their associated environment from South African farms (KwaZulu Natal and the Eastern Cape). The vast dissemination of *E. faecalis* that has potentially pathogenic virulent determinants is a cause for concern in livestock and public health as these genes are responsible for the persistence and infectiousness of *Enterococcus* species. Virulence studies on livestock and its associated environment are limited with most studies focusing only on the pathogenicity of vancomycin resistant *Enterococcus*. This study successfully identified the prevalence of *E. faecalis* and *E. faecium* in livestock production systems using molecular techniques. In addition, to determining the putative virulence genes associated with the pathogenicity of *Enterococcus* spp. The current study determined that *gel-ccf-hyl* patterns are more frequent in *E. faecalis* species. In addition to highlighting that the prevalence of *gelE* could be predicted by the type of host species. Furthermore, what seemed to stand out was that *E. faecalis* harboured more virulent genes compared to *E. faecium*. While the virulence determinants are

considered harmless in commensal strains, clinical strains have a high pathogenic potential to immune compromised individuals It is important to note that faecal contamination from colonized livestock poses a threat to the dissemination of virulent strains hence it can be harmful to the environment as explained in the current study. Control strategies that are targeted at mediating the spread of virulence determinants in livestock associated *Enterococcus* spp. are necessary as these species are serve as a virulence reservoir for pathogenic clinical strains.

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CHAPTER 4

Prevalence of antimicrobial resistance genes in *E. faecalis* and *E. faecium* and the dissemination of *vanC* outside *E gallinarum* and *E. casseliflavus* in South African livestock production systems

4.1. Abstract

The study aimed to assess the prevalence of antibiotic resistance genes and their dissemination in *Enterococcus* species from livestock production systems in South Africa. In addition, to determine the occurrence of *vanC* determinants in vancomycin resistant *E. faecalis* and *E. faecium* isolates recovered from livestock and its associated environment. Antibiotic susceptibility profiles of *E. faecium* and *E. faecalis* were assessed using Kirby-Bauer disk-diffusion.Moreover, the isolates were screened for *vanA, vanB, vanC1, vanC2/3, aac(6")-aph(2") , ermA* and *ermB* using simplex PCR. Erythromycin had the highest occurrence of resistant isolates in both species with 75% (n= 6) and 54.1% (n= 33) respectively. Isolates were least resistant to ampicillin, with 0.03% (n=2) resistance in *E. faecalis* and 0% (n=0) in *E. faecium*. The study also screened for high-level aminoglycoside resistance, overall phenotypic resistance to gentamycin was 7.2% (n=5) whereas, streptomycin was 17.4% (n=12). *E. faecalis* had the highest prevalence of Multi Drug Resistance (MDR), exhibiting phenotypic resistance to macrolides, aminoglycoside, tetracyclines and fluoroquinolones. Out of the 23 MDR patterns observed; TET-CIP-ERY was the most observed antibiotic pattern. *vanA* was not present in either species whilst *vanB* (n =5 ;8%) was prevalent in *E. faecalis* only. The genes *vanC1* and *vanC2/3* in *E. faecium* were 38% (n=3) then 37% (n=23) in *E faecalis. aac(6")-aph(2")* was detected at a low prevalence (n=;2%) in *E. faecalis* isolates only. Broilers were the only host that harboured all 6 genes; *vanB* (n=1; 1.6%), vanC1 (n=4;6.6%), *vanC2*/3 (n=7;11.5%), *ermB* (n=11;18.0%), *ermA* (n=2 ;3.3%). There was a significant ($p<0.05$) association of *vanC1* with the studies location ($p<0.001$) and sample sites ($p<0.05$). Lastly, *ermB* was categorically influenced by the sample site whereby the isolate was recovered from [Expβ (10.531), β (2.354) $p<0.05$]. There hasn't been enough paucity of studies on the extent of antimicrobial resistance and resistant patterns of *Enterococcus* in the animal husbandry sector in South Africa. This study reports that *E. faecium* and *E. Faecalis* isolated from livestock and their associated environment were predominantly resistant to macrolides, glycopeptides, tetracyclines and fluroquinolones. In addition to be the first study in South Africa to document the emergence of inducible *vanC* determinants in VRE isolates. The study suggests intense surveillance and monitoring strategies for *Enterococcus* spp. because of their ability to horizontally transfer constitutive genes.

Keywords: Acquired resistance ; Animal husbandry ; Intrinsic; One Health Approach

4.2. Introduction

Antimicrobial Resistance (AR) poses a great threat to the healthcare sector globally (Lochan *et al*., 2016). The antimicrobial resistance strategy framework of South Africa suggests that the country has a great burden of infectious diseases (Mendelson and Matsoso, 2015) which implies a surge in immuno-compromised individuals who can be easily infected by antibiotic resistant bacteria like the 'ESKAPE' pathogens. These bacteria have acquired the ability to evade the effects of antibacterial drugs. *Enterococcus faecuim, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and Enterobacter* species form part of the ESKAPE pathogens (Haag, 2015; Jackson *et al*., 2012). *Enterococcus* species are gram-positive anaerobes and are mostly commensal bacteria as they are found in the gut of animals and humans (Klibi *et al*., 2012; Micallef, 2013). *Enterococcus faecalis* (*E. faecalis*) and *Enterococcus faecium* (*E. faecium*) are two of the most prevalent commensal species with *E. faecium* accounting to 5-10% and *E. faecalis* accounting to 90- 95% of overall *Enterococcus* spp. in the GI tracts of animals and humans (Skowron *et al*., 2014) However, these enterococcal spp. have also been found to disseminate in the environment (soil, water and plants) (Iweriebor *et al*., 2015).

E. faecalis and *E. faecium* have the capacity to disseminate outside the GI tract based on the micro-organisms genome that confers ability to resist antimicrobials (Arias *et al*., 2012) hence the surveillance studies of these clinically important species. in the environment are essential (Lochan *et al.*,2016). The two species have been documented to be the causing agent for urinary tract infections (UTI), endocarditis and other infections such as skin and soft tissue in addition to being notoriously known to cause community acquired infections (CAIs) (Micallef, 2013). Studies suggested that a genetic similarity exists between clinically important strains and animal strains (Ngbede *et al.*, 2016). Not only do these two enterococcal spp. cause infection in humans, they also can cause diarrhoea in swine, myositis or pancreatitis in cattle and lastly sepsis in poultry (Skowron *et al*., 2014). Since these are food producing animals it is important to note that food security is a growing concern globally (Dweba *et al*., 2019) A noticeable rise in organic farming has been documented in the food market, particularly the United States of America (Salaheen, 2015). In addition, the African continent also accounts for an escalation in farming because it is mostly an agro-ecological continent with climate conditions that favour animal husbandry (Ngbede *et al.*, 2016). The rise in farming, particularly livestock farming, poses a question of how frequent zoonosis aids in community acquired infections in reference to these two *Enterococcus* species.

The United States Centre for Disease Control and Prevention (CDC) estimated that 2.5 billion cases of infections and 2.7 million deaths were caused by zoonoses globally (CDC, 2018). Recent studies have investigated the human bacterial infections arising from food-producing animals (Abat *et al*.,

2016). *Enterococcus* strains as previously mentioned are typical commensals in livestock, however these strains maybe transmitted to humans through various paths (Bortolaia *et al*., 2016: Hammerum, 2012). This includes direct contact with livestock or through ingesting of food contaminated by the bacteria (Bortolaia *et al*.,2016; van Bogaard *et al.,* 2000). On first sight it appears that *E. faecalis* and *E. faecium* from the environment and livestock can survive and colonize the gut bacterial community. This notion was supported by a study where these enterococcal spp. where genetically similar in isolates recovered from both animals and humans of the same geographical area (Werner *et al*., 2013). This suggests that enterococci of animal origin has the capability to colonize humans (Haack *et al.,* 2015; Hollenbeck & Rice, 2012; Ruzauskas *et al*., 2009). The dissemination of resistance bacteria of animal origin (commensal) and/or zoonotic bacteria can be disseminated not only by direct contact with livestock but also through food products from slaughterhouses (van Bogaard *et al* 2000). Furthermore, studies have documented that the more commensal antibiotic resistant bacteria (ARB) in food producing animals, the greater the probability of the ARB transferring (horizontal gene transfer) the resistance genes into the bacteria in the environment (van Bogaard *et al* 2000).

The selective pressure and prevalence of ARB can be ascribed to numerous factors such as the horizontal transfer of resistance genes from commensals to pathogenic strains (Daniel *et al*., 2017 Klibi *et al*., 2014;). The increase in the dissemination of these genes could be through unmonitored use of antibiotics in livestock, humans and faecal contamination in irrigation water (Matlou *et al*., 2019; Daniel *et al*., 2017). Livestock, especially in small scale farms utilize lakes and rivers for drinking water thus the use of these aquatic systems without assessing the microbial quality aggravates the dissemination of pathogenic bacteria in the community. It has been reported that feed and animal drinking wells are a haven for bacterial contamination (Salaheen, 2015; Skowron *et al*., 2014). The capabilities of *Enterococcus* spp. surviving outsides GI tract and adequately disseminate across the environment makes them good reservoirs of virulence and antibiotic resistance genes (KlibI *et al*., 2014). These genes could be intrinsically or externally expressed; those expressed intrinsically are chromosomally located whilst most that confer antibiotic resistance are located in plasmids, transposons and other Mobile Genetic Elements (MBE) alike (Torres *et al*., 2018; Ahmed and Baptise, 2017;Hollenbeck and Rice, 2012).

Acquired resistance initially occurs when antibiotic susceptible bacteria due to a sudden event such as horizontal gene transfer or mutations resistance to antibiotics is achieved. (Tasting *et al*., 2018). The increase in the resistance to aminoglycosides and glycopetides is mostly attributed to acquired resistance (Skowron *et al*., 2014). The transfer of large Pathogenicity Islands explains the increase in the acquisition of the previously mentioned antibiotics (Starikova *et al*., 2013; Werner *et al*., 2013; Hegstad *et al.,* 2010)In South Africa although the use of avorpacin was banned in 1997, the vancomycin analogue is still being used for animal husbandry (Tasting *et al*., 2018). This

is quite alarming since vancomycin is used to treat severe enterococcal infections as it functions by blocking cell wall peptidoglycan synthesis (Skowron *et al*., 2014). The glycopeptide resistance is encoded by the *van* genotypes *(vanA, vanB, vanC, vanD, vanE, vanF, vanL, vanM* and *vanN*) although constitutively expressed *vanA/B* are normally found in *E. faecalis* and *E. faecium* (Tasting *et al*.,2018; Werner *et al*., 2013). However, the *vanC* determinants are intrinsically expressed in *E. gallinarum* hence for the last decade this gene has been used for identifying the previously mentioned species; being that it was thought to be chromosomally located (Ahmed and Baptise, 2017; Schaweiger *et al*.,2012). Furthermore, aminoglycosides are important antibiotics as gentamycin and streptomycin have a synergistic effect in combination with cell-wall active substance (Werner *et al*.,2013). High-level aminoglycoside resistance in *Enterococcus* is a rising problem. There are three commonly known aminoglycosides resistance mechanisms transport altercation, ribosomal target modification and enzymatic modification (Diarra *et al*.,2010). High-level gentamycin and streptomycin resistance have been documented to be increasing in poultry, this poses a threat to public health since there has been a rise in the consumption of poultry (Molechan *et al*., 2019).

Globally, there is an increase in demand for animal protein, in the BRICS countries (Brazil, Russia, India, China and South Africa) consumption of antibiotics by livestock is expected to increase by seven times the current consumption (van Boeckal *et al*.,2014). The increase in the consumption of animal protein is not only attributed to how the BRICS countries are economically developing but for cultural and religious purposes there is a high demand for livestock (Anderson *et al.*, 2020) Furthermore, Ngebde *et al.* (2016) alluded that there is a genetic similarity between enterococcal strains that are of animal origin and strains that are clinically important, however, there is insufficient evidence suggesting that resistance enterococci from animal origin can cause infections in humans. In addition, it is noticeable that exposure of foodborne multi drug resistant *Enterococcus* in the community and the environment is under researched (Micallef, 2013). It is therefore, important that surveillance studies of antibiotic resistance in all settings are conducted especially in *E. faecalis* and *E. faecium* which are known to be hotspots for antibiotic resistance genes and can easily transfer genetic determinants to other bacterium. The study aims to assess the dissemination of antibiotic resistance genes and its dissemination in *Enterococcus* species recovered from livestock productions systems in South Africa. Moreover, to determine the occurrence of *vanC* determinants and VRE from *E. faecalis* and *E. faecium* isolates recovered from South Africa.

4.2. Materials Methods

4.2.1 Ethical Clearance

The study was approved by the Animal Research Ethics Committee of the University of Kwa-Zulu Natal (Reference numbers AREC/051/017M, AREC 071/017 and AREC014/018). The field

sampling protocols, samples collected from animals, and the research were conducted in full compliance with Section 20 of the Animal Diseases Act of 1984 (Act No 35 of 1984) and were approved by the South African Department of Agriculture, Forestry and Fisheries DAFF (Section 20 approval reference number 12/11/1/5).

4.2.2. Sampling

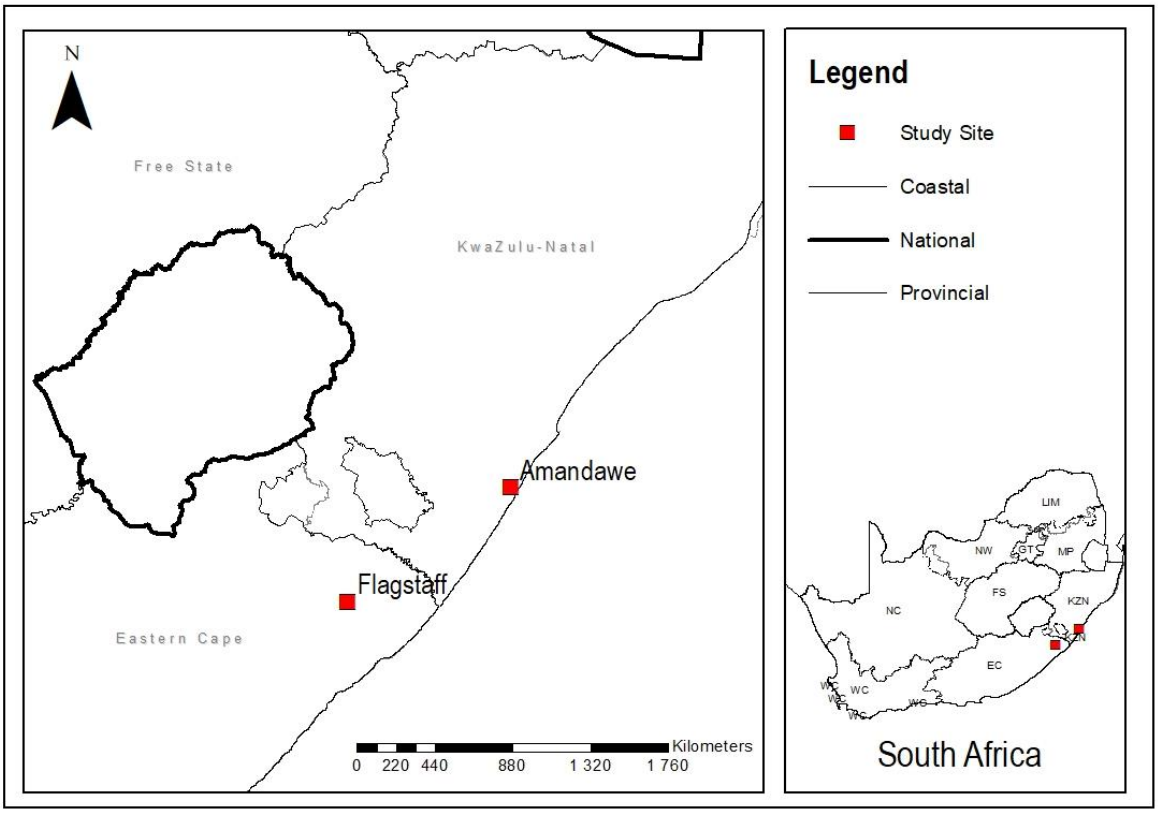

Figure 4.1. Geographical map of the sampled farms in South Africa

Two hundred and seventy-six samples were obtained aseptically from livestock farms in South Africa (figure 4.1.) from pigs *(n = 34*), cows (*n= 19*), chickens (*n = 80*), ducks *(n=10*), horses (n=*5*), goats (n= *47*) and sheep *(n =22*) respectively. Rectal, oral, faecal, water (*n =19*), feedlot (*n = 9*) and soil (*n =33*) samples were obtained using sterile cotton swabs. Swabs were immersed in 10 ml of 0.1% (w/v) peptone water. Upon sampling the samples were transported to the laboratory in the University of KwaZulu-Natal stored in ice for immediate processing.

4.2.3 Isolation of Enterococcus species

A total of 1ml of the peptone water (with sample) was inoculated into 10ml of Brain heat infusion broth (BHI) for enrichment and incubated for a duration of 18-24 hrs at 37°C. The growing culture was streaked onto Bile Aesculin Azide agar and further incubated for a period of 18-24hrs at 37°C.

Presumptive isolates for *Enterococcu*s species had a black/brownish dew drop phenotype. Only one colony per plate was streaked into Trypticase Soy Agar (TSA) to be further incubated for a period of 18 hours at 37°C for glycerol stock preparation (Iweriebor *et al*., 2015.)

4.2.3. Identification and species verification of Enterococcus species.

Genomic DNA was extracted using the boiling method (Riberio *et al*.,2016). Molecular confirmation of the isolates was achieved by polymerase chain reaction (PCR) with the use of *Enterococcus* genus-specific primers *tuf* gene (Table 2) with the strain *E. faecalis* ATCC 1943 serving as the positive control. A 25 µl reaction volume; with 12.5µl of Dream Taq Green Master mix (Thermo Scientific), 1µl of each of the forward and reverse primers (Inqaba Biotech), 5.5 µl of Nuclease free water (Thermo Fischer) and 5µl of template DNA was used. The t*uf* gene (*table 4.1*.) was amplified with an initial denaturing step at 94 ℃ for 4 min, for 35 cycles. Optimum conditions for amplification were denaturation at 94 ℃ for 60 s, annealing at 53 ℃ for 60 s, extension at 72 ℃ for 60 s, and final extension at 72 ℃ for 5 min. Furthermore, of the isolates confirmed to be *Enterococcus* spp. Another 25 µl PCR reactions were conducted using species-specific primers (table 4.1.) to identify *Enterococcus faecalis* and *Enterococcus faecium* respectively. With optimum conditions of initial denaturation at 95 ℃ for 4 min for 30 cycles. Denaturation at 95 ℃ for 30 s, annealing temperatures of 52 ℃ and 48 ℃ at 1 min respectively. Extension at 72 ℃ for 60 s, with a final extension of 72 ℃ for 7 min. A non-template control was run with every reaction, without the template DNA, but with 2 µl of Nuclease Free Water. All PCR reactions were carried out using the BioRad Thermocycler (BioRad, Carlifonia, United States of America). PCR products were electrophorized in a 1.8% agarose gel at 110 Volts for 45 min. Then proceeded to be visualized under UV light using Bio ChemiDoc imagining system (BioRad, Carlifonia, United States of America)

Table 4.1. PCR primers that was be used for the identification of *Enterococcus* species.

Gene	Primer Sequence (5'-3')	Amplicon size (bp)	Reference
Tuf gene	Ent1 TACTGACAAACCATTCATGATG Ent2 AACTTCGTCACCAACGCGAAC	112	Ke *et al*., 1999
E. faecium ATCC19434	GAAAAAACAATAGAAGAATTAT FM2 TGCTTTTTTGAATTCTTCTTTA	215	Jackson *et al.*, 2004

E. faecalis	FL1 ACTTATGTGACTAACTTAACC	360	Iweriebor *et al*.,
ATCC 19433	FL2 TAATGGTGAATCTTGGTTTGG		2015

4.2.4. Antibiotic Susceptibility

Antibiotic susceptibility profiles of *E. faecium* and *E. faecalis* were analysed per Clinical and Laboratory Standards Institute (CLSI) guidelines for antibiotic concentrations (2018). The inoculum was standardized by choosing isolated colonies to be resuspended in BHI for 0.5 McFarland standards. Kirby-Bauer disk-diffusion (Thermo Scientific) was used with antibiotics discs impregnated with specific concentration of antimicrobials and placed on Muller Hinton agar (MHA). The inhibition zones were scored immediately after 18–24 hours at 37° C incubation. Strains *E. faecalis ATCC 19433* (Iweriebor *et al*., 2015) and *E. faecium ATCC19434* (Iweriebor *et al*., 2015) were used as control strains. Susceptibility profiles of the following antibiotics were tested; ampicillin (10µg), teicoplanin (30µg), vancomycin (30µg), penicillin G (10 units), chloramphenicol (30µg), tetracycline (30 µg), ciprofloxacin (5µg) and erythromycin (15g). In addition, high aminoglycoside resistance in *Enterococcus* spp. was tested for, using the antibiotics gentamicin and streptomycin.

4.2.5. Antibiotic Resistance Genes

Monoplex PCR reactions were conducted on the resistance genes in table 4.2. A 25 µl reaction volume; with 12.5µl of Dream Taq Green Master mix (Thermo Scientific), 1µl of each of the forward and reverse primers (Inqaba Biotech), 5.5 µl of Nuclease free water (Thermo Fischer) and 5µl of template DNA was used to amplify all the genes except the van genes. The gene *ermA* was amplified with an initial denaturing step at 93 °C for 3 min and 35 cycles. Optimum conditions for amplification were denaturation at 93°C for 60 s, annealing at 52°C for 60 s, extension at 72 °C for 60 s, and final extension at 72 °C for 3 min. Conditions for *ermB* gene amplification were an initial denaturing step at 94 °C for 1 min, for 35 cycles. Optimum conditions for amplification were denaturation at 94 °C for 60 s, annealing at 55 °C for 60 s, extension at 72 °C for 60 s, and final extension at 72 °C for 7 min. For the genes *vanA, vanB, vanC2/3* the same PCR conditions were used. 10 µl reaction volume; with 5 µl of Dream Taq Green Master mix (Thermo Scientific), 1 µl of each of the forward and reverse primers (Inqaba Biotech) for each gene, 1 µl of Nuclease free water (Thermo Fischer) and 2µl of template DNA. Initial denaturing step at 94 °C for 3 min, for 35 cycles. Optimum conditions for amplification were denaturation at 94 °C for 60 s, annealing at 56.5 °C for 60 s, extension at 72 °C for 60 s, and final extension at 72 °C for 10 min. Lastly, the gene *vanC1* was amplified with an initial denaturing step at 94 °C for 1min and 30 cycles. Optimum conditions for amplification were denaturation at 94°C for 5min, annealing at 54°C for 60 s, extension at 72 °C for 60 s, and final extension at 72 °C for 10 min. PCR products were

electrophorized in a 1.8% agarose gel at 110 Volts for 45 min. Then proceeded to be visualized under UV light using Bio ChemiDoc imagining system (BioRad, Carlifonia, United States of America).

4.2.6. Statistical analysis

The association between resistance genes and the variables associated with livestock production systems (location, sample site and host) were analyzed using the Fischer's exact test. Furthermore, the direction of the relationship and correlation between the screened resistance genes was assessed by the use of the Pearson's correlations test. Lastly, a Binary logistic regression null model was used to investigate the effect and association of antibiotic resistance genes with animal host, location and sample at a 95% confidence level. All statistical tests were analyzed from the *E. faecalis* data set. The IBM Statistical Package for Social Sciences (SPSS), version 25.0 (IBM Cooperation, New York) was the statistical software used of analysis. All tests were considered significant with a critical $p < 0.05$.

Table 4.2. Primer sequences used for AMR surveillance in *Enterococcus* isolates recovered from livestock production systems.

Gene	Description	Primer Sequence (5'-3')	Amplicon size (bp)	Reference
vanA		VANA1 GGGAAAACGACAATTGC	732	Lin *et al*., 2013
		VANA2 GTACAATGCGGCCGTTA		
vanB		VANB ATGGGAAGCCGATAGTC	635	Lin *et al*., 2013
		VANB2 GATTTGCTTCCTCGACC		
vanC1		VANC1-1 GGTATCAAGGAAACCTC	822	Lin *et al*., 2013
		VANC1-2 CTTCCGCCATCATAGCT		
vanC2/3		C2F-CTAGCGCAATCGAAGCACTC	582	Iweriebor *et*
		C2R-GTAGGAGCACTGCGGAACAA		*al*.,2015
ermA	Ribosomal methylase	TCTAAAAAGCATGTAAAAGAA	645	Zou *et al*., 2011
		CTTCGATAGTTTATTAATATTAGT		
ermB	Ribosomal methylase	GAAAAGGTACTCAACCAAATA	216	Padmasini *et al*.,
		AGTAACGGTACTTAAATTGTTTAC		2014

aac(6')-le-aph(2'')-la	Aminoglycoside modifying enzyme	GCAAATGGTGTAGGTAAGACAACT ATCATGTGATGTAAACAAAAT	369	Padmasini *et al*., 2014

4.3. Results

4.3.1. Prevalence of Enterococcus species

A total of 69 isolates recovered from livestock and its associated environment in Amandawe and Flagstaff were confirmed to be *E faecalis* (61) and *E faecium* (8). The samples were distributed as follows in *E Faecalis*; goat = 14, broiler chicken =12, chicken =11, pig = 6, cow = 4, duck =1, sheep =1 and the environment (soil, water and feedlot) = 12. While eight of *E faecium* isolates recovered were distributed in goats (2) ,cows (2) , pigs (2) and ducks (2).

4.3.2. The Distribution of antibiotic resistance patterns and the antibiotic susceptibility profiles of E. faecalis and E. faecium.

The Kirby Bauer Disk Diffusion assay was conducted according to the CLSI guidelines (2018) to screen for the antibiotic susceptibility profiles, resistance patterns and determining multidrug resistance isolates based on isolate phenotype. Erythromycin had the highest occurrence of resistant isolates in both of *E. faecium* and *E. faecalis* with 75% (n=6) and 54.1% (n=33) resistant isolates respectively. Furthermore, in 61 of the confirmed *E. faecalis* isolates 40.9% (n=25) were resistant to vancomycin. The isolates were least resistance to ampicillin, with 0.03% (n=2) resistance in *E. faecalis* isolates and zero occurrence of ampicillin resistance in *E. faecium* isolates. Ciprofloxacin had the highest prevalence of intermediate susceptibility in both *E. faecium* and *E. faecalis* isolates, with 62.5% (n=5) and 52.5% (n=32) isolates exhibiting an intermediate susceptible phenotype. The study also screened for high-level aminoglycoside resistance, the overall phenotypic resistance to gentamycin was 7.2% (n=5) whereas streptomycin was 17.4% (n=12) respectively. Intimidate susceptibility for these two antibiotics was 10.1% (n=7) and 0% respectively (Table 4.3.). *E. faecalis* had the highest prevalence of Multi Drug Resistance (MDR) with 27 (44.2%) isolates exhibiting phenotypic resistance to more than two antibiotic classes predominantly macrolides, aminoglycoside, tetracyclines and fluoroquinolones. In addition, only 2 (25%) *E. faecium* isolates were classified as MDR (Table 4.4). It was noticeable that TET-CIP-ERY was the most observed antibiotic pattern, with 4 *E. faecalis* isolates exhibiting this phenotype. Furthermore 2 of *the E. faecalis* isolates showed high MDR, with an antibiotic pattern that is resistant to all classes of antibiotics screened for in this study namely; aminoglycosides, macrolides, penicillin, tetracyclines, phenecols, fluoroquinolones and glycopeptides. Lastly 23 MDR phenotypes were observed.

Table 4.3. *The antibiotic susceptibility profiles of E. faecalis and E. faecium isolates recovered from livestock production systems is South Africa*

Antibiotic	**Antibiotic Susceptibility Profiles**								
	E. faecalis (*n* = 61)			*E. faecium* (*n* = 8)			**Total (*n* = 69)**		
	I	**S**	**R**	**I**	**S**	**R**	**I**	**S**	**R**
Ampicillin (10µg)	0 (0%)	59 (96.7%)	2 (0.03%)	0 (0%)	8 (100%)	0 (0%)	0 **(0%)**	67 **(97.1%)**	2 **(2.8%)**
Teicoplanin (30µg)	16 (26.2%)	40 (65.5%)	5 (8.2%)	0 (0%)	8 (100%)	0 (0%)	16 **(23.1%)**	48 **(69.6%)**	5 **(7.2%)**
Gentamicin (120µg)	0 (0%)	57 (93.4%)	4 (6.6%)	0 (0%)	7 (87.5%)	1 (12.5%)	7 **(10.1%)**	57 **(82.6%)**	5 **(7.2%)**
Streptomycin (300µg)	0 (0%)	50 (81.9%)	11 (18.0%)	0 (0%)	7 (87.5%)	1 (12.5%)	0 **(0%)**	57 **(82.6%)**	12 **(17.4%)**
Vancomycin (30µg)	21 (34.4%)	15 (24.6%)	25 (40.9%)	4 (50%)	3 (37.5%)	1 (12.5%)	25 **(36.2%)**	18 **(26.1%)**	26 **(37.7%)**
Penicillin G (10U)	0 (0%)	49 (80.3%)	12 (19.7%)	0 (0%)	8 (100%)	0 (0%)	0 **(0%**)	57 **(82.6%)**	12 **(17.4%)**
Chloramphenicol (30µg)	13 (21.3%)	35 (57.3%)	13 (21.3%)	0 (0%)	7 (87.5%)	1 (12.5%)	13 **(18.8%)**	42 **(60.9%)**	14 **(20.3)**
Tetracycline (30g)	9 (14.8%)	23 (37.7%)	29 (47.5%)	1 (12.5%)	3 (37.5%)	4 (50%)	10 **(14.5%)**	26 **(37.7%)**	33 **(47.2%)**
Ciprofloxacin (5µg)	32 (52.5%)	12 (19.7%)	17 (27.9%)	5 (62.5%)	2 (25%)	1 (12.5%)	37 (**53.6%)**	14 **(20.3%)**	18 **(26.1%)**
Erythromycin (15g)	20 (32.3%)	7 (11.5%)	33 (54.1%)	2 (25%)	0 (0%)	6 (75%)	22 **(31.9%)**	7 **(10.1%)**	39 **(56.5%)**

Table 4.4. The distribution of antibiotic resistance pattern amongst *E. faecalis* and *E. faecium* isolates

Number of Antibiotic Classes	**Antibiotic Pattern [a]**	***E. faecalis* (*n* =61)**	***E. faecium* (*n*= 8)**
3 Classes	VAN-TET-ERY	2	1
	VAN-PEN-ERY	1	0
	VAN-PEN-TET	1	0
	VAN-CIP-ERY	1	0
	GEN-VAN-ERY	1	0
	TET-CIP-ERY	4	0
	TEC-TET-ERY	2	0
	CHL-CIP-ERY	1	0
	AMP-VAN-PEN-ERY	1	0
4 Classes	STR-VAN-TET-ERY	3	0
	STR-VAN-CIP-ERY	1	0
	VAN-TET-CIP-ERY	1	0
	AMP-STR-CHL-ERY	1	0
	STR-CHL-CIP-ERY	1	0
	GEN-STR-CHL-TET-ERY	0	1
5 Classes	GEN-VAN-PEN-TET-ERY	1	0
	GEN-CHL-CIP-TET-ERY	1	0
	TEC-PEN-TET-CIP-ERY	1	0
	VAN-PEN-TET- CIP-ERY	1	0
	GEN-VAN-CHL-TET-ERY	1	0
	STR-VAN-PEN-CIP-ERY	1	0
6 Classes	STR- PEN-CHL-TET-CIP-ERY	1	0
7 Classes	STR-VAN-PEN-CHL-TET-CIP-ERY	2	0
Total	**23**	**27 (44.2%)**	**2(25%)**

[a] AMP: Ampicillin, CHL: Chloramphenicol, CIP : Ciprofloxacin, ERY: Erythromycin, GEN: Gentamycin, PEN: Penicillin, STR: Streptomycin, TEI: Teicoplanin, TET: Tetracycline, VAN: Vancomycin

4.3.3. Antibiotic Resistance genes

The dispersion of the AR genes was screened for using Polymerase Chain Reaction. From the seven screened genes in *E. faecalis* and *E. faecium*, four of those genes were *van* genes *(vanA, vanB,*

vanC1, vanC2/3). Although the *vanA* gene was not present in both species, the other three were present with *vanB* (8%; n=5),figure 4.3.) being prevalent in *E. faecalis* only. The *vanC* variants were equally prevalent in both spp. isolates; *vanC1* and *vanC2/3* in *E. faecium* was 38% (n=3) then 37% (n=23) in *E faecalis* (figure 4.3.). *aac(6")-aph(2")* was detected at a low prevalence 2% (n=) in *E. faecalis* isolates only. Whilst *ermB* was highly prevalent, occurring at a high prevalence of 96% (n= 49) in *E. faecalis* and 88% (n=7) in *E. faecium* (figure 4.3.). Upon further investigation, (figure 4.4). indicates that the genes *ermB* and *vanC2/3* were disseminated across all sample sites. However, Pearson correlation indicates a significant relationship that is negative between these two AR genes (R= -0.253, <0.05, table 4.6.) in *E. faecalis*. Moreover, the correlation between *ermA* and *aac(6")-aph(2")* is a significantly positive relationship with a critical p-value (R= 0.568, p <.0.001, table 4.6). Lastly there was not enough statistical evidence to support the relationship of the other AR genes based on the Pearson Correlations Test (p >0.05, table 4.6.).

The broiler chickens harboured all 6 genes (excl. *vanA*); *vanB* (n=1; 1.6%), vanC1 (n= 4; 6.6%), *vanC2*/3 (n=7; 11.5%), *ermB* (n=11; 18.0%), *ermA* (n=2; 3.3%). Furthermore, *aac(6")-aph(2")* was only detected in one isolate recovered from this host (n=1 ;1.6%). Upon further analysis it appeared that *vanC1 > vanC2/3*,

though in broiler chickens only the prevalence of *vanC2/3* was greater. Isolates recovered from ducks and sheep had the lowest prevalence of the AR genes, with *vanC1* and *ermB* being the only present AR genes (figure 4.4.). For *E. faecium vanc2/3, ermB, vanC1* were the only genes detected from the recovered isolates (figure 4.5.). Sample sites from the environment had no isolates that harboured any AR genes. The three previously mentioned genes were detected in pigs, goats and ducks. In the host species duck and cow the prevalence of *vanC1* and *ermB* was equal with 25% (n=2)prevalence for the cows and 12.5% (n=1) prevalence in duck (figure 4.5.).

The Fisher Exact test was used to analyse any association between the AR genes and the variables associated with the livestock production systems (location, animal, host and sample site). Enough statistical evidence supports the association of *vanC1* with studies location and sample sites of the isolates p <0.001 and p <0.05 (table 4.5). Binary logistic regression was used to evaluate the effect/ variation sample site, animal host and location has on the five virulence genes. The variation was tested at 95% confidence interval. Table 4.7. *vanC1* was categorically affected by location (Flagstaff/Amandawe) with an odds ratio (Expβ) of 19.235 and a slope (β) of 2.95 (p<0.001). Furthermore, *ermB* was categorically influenced by the sample site whereby the isolate was recovered from [Expβ (10.531), β (2.354) p <0.05]

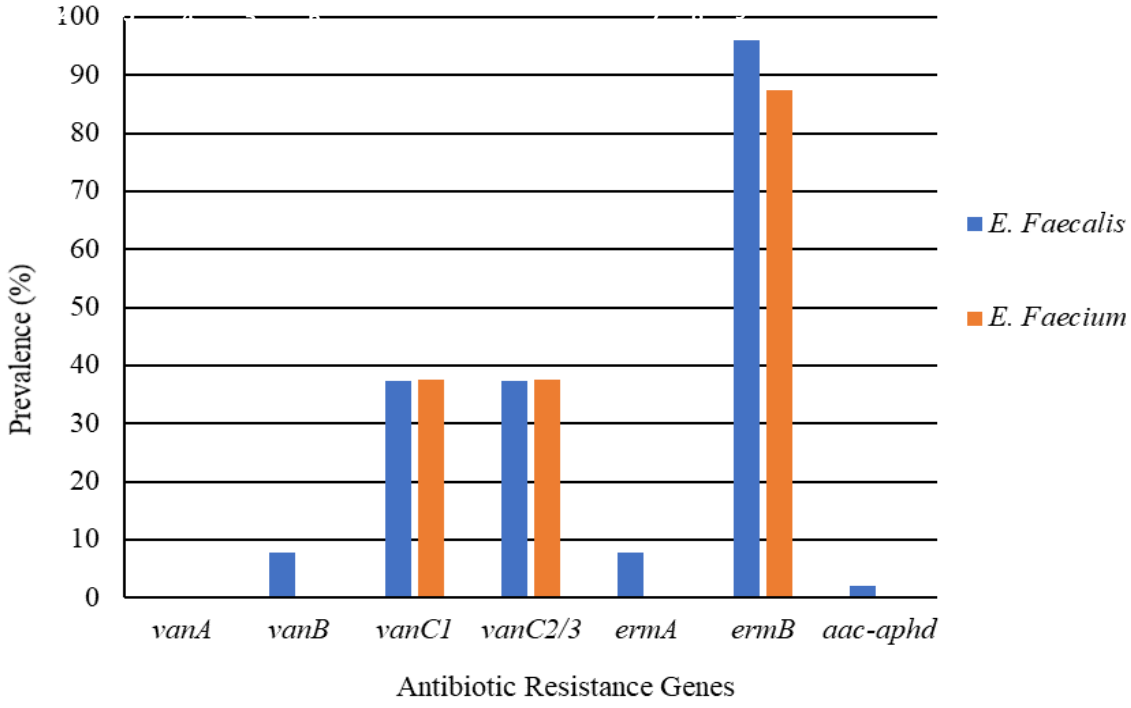

Figure 4.3. The percentage prevalence (%) of antibiotic resistance genes in *E. faecalis* and *E. faecium* isolated from livestock production systems in South Africa

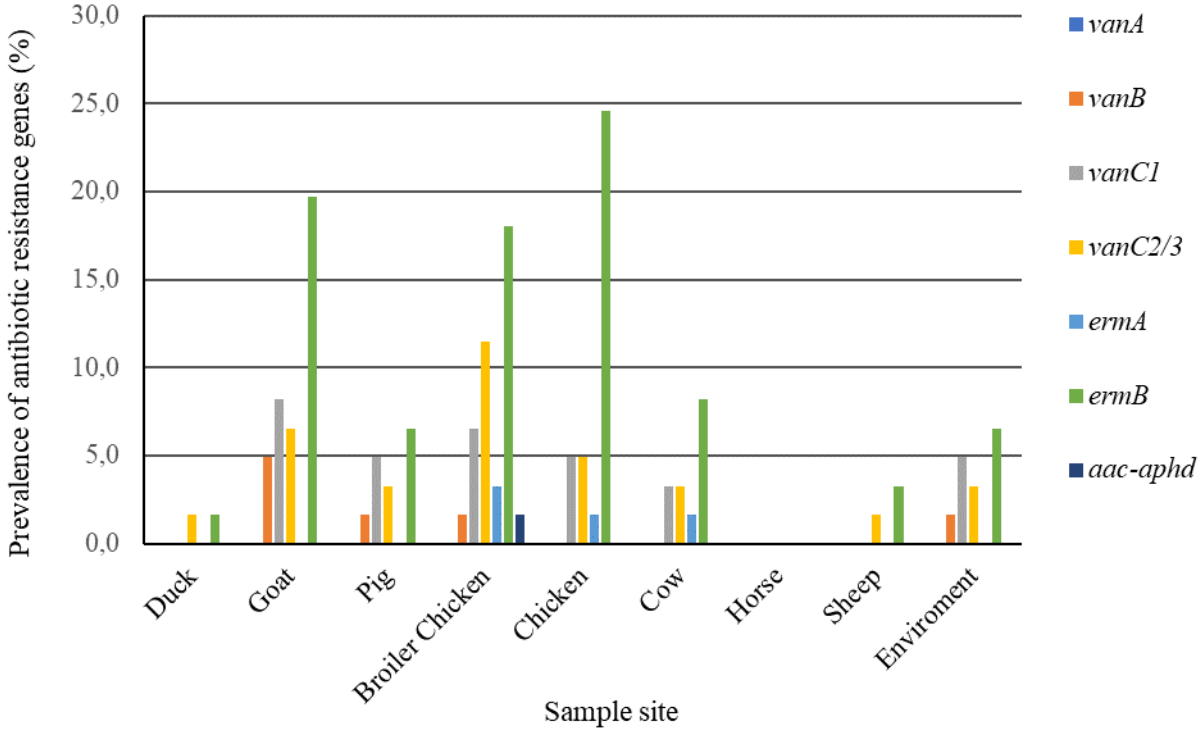

Figure 4.4. The percentage prevalence (%) of the antibiotic resistance genes associated with *E. faecalis* isolated from livestock and their surrounding environment (water, soil and feed)

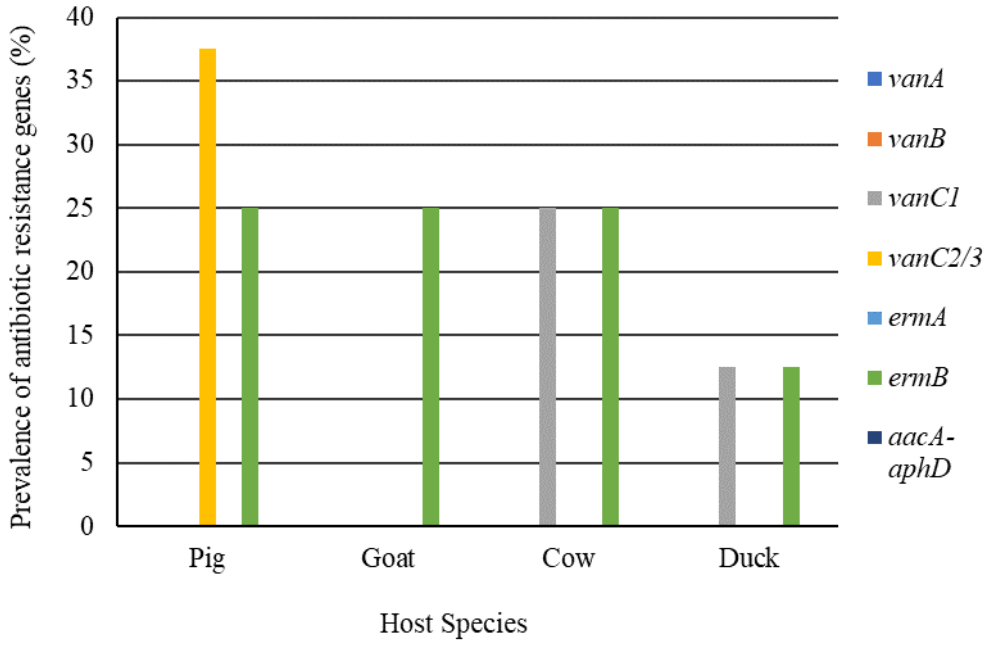

Figure 4.5. The prevalence (%) of the antibiotic resistance genes associated with *E. faecium* isolated from livestock and their surrounding environment (water, soil and feed) **.**

Table 4.5. Fischer's exact test p-values indicating the relationship between the antibiotic resistance genes with the variables associated with livestock production systems in *E. faecalis*

Variable	**Genes**						
	vanA	***vanB***	***vanC1***	***vanC 2/3***	***ermA***	***ermB***	***aac(6")-aph(2")***
Location	-	0.356	0.000*	0.198	0.560	0.499	0.738
Host Species	-	0.745	0.053	0.539	0.485	0.187	0.656
Sample Material	-	0.623	0.031*	0.910	0.837	0.934	0.311

*p < 0.05, significant (2-tailed).

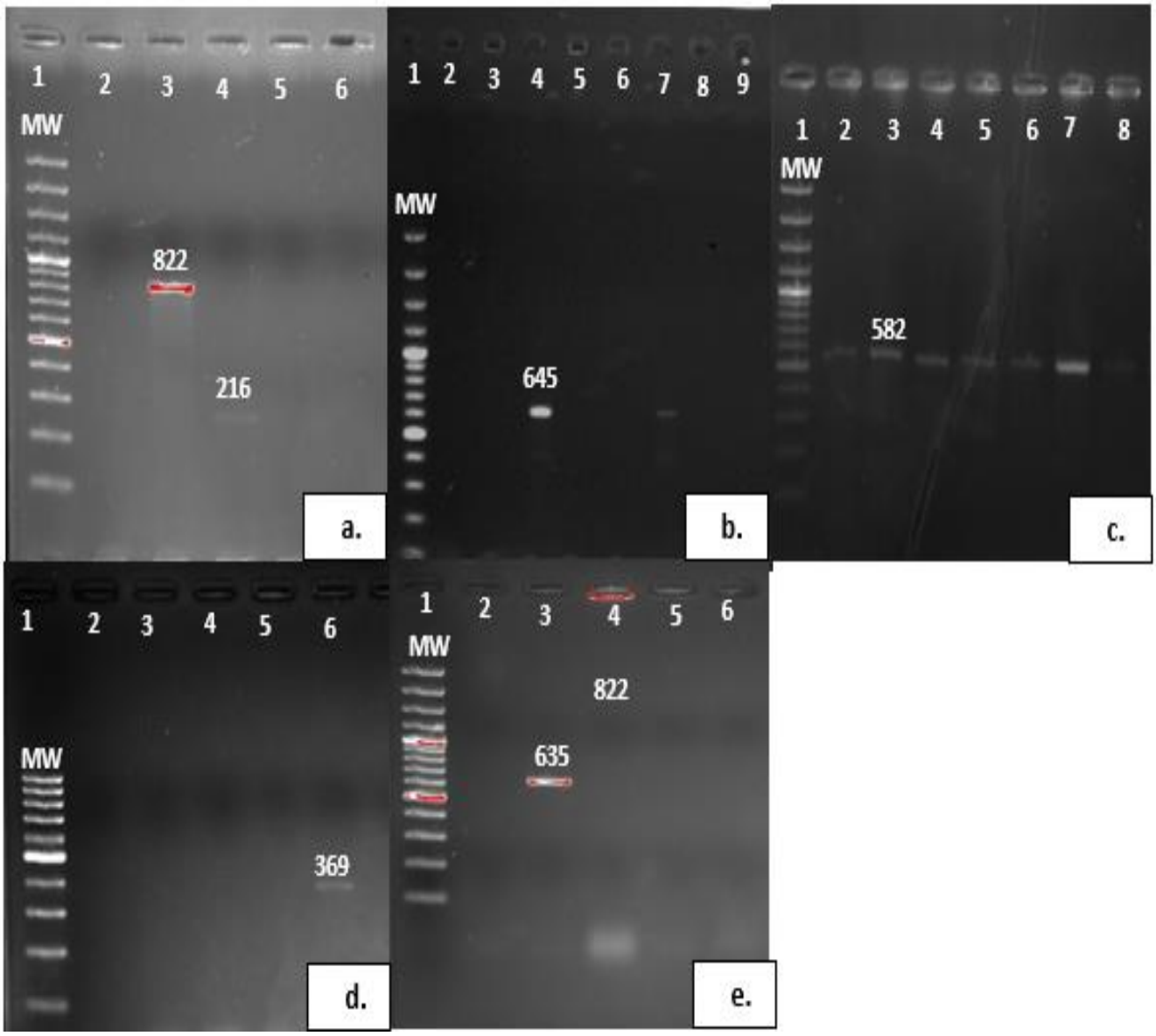

Figure 4.2. The molecular surveillance of antibiotic resistance genes using PCR methods and 1.8% agarose gel. **a.)** MW indicates the molecular ladder (100bp plus, GenePlus, Fisher Scientific, Sweden). The genes *vanC1* and *ermB* amplified in lanes 3 and 4 respectively. **b.)** Lane 4 illustrates the presence of *ermA* (645bp). **c.)** *vanC2/3* was detected in lane 3 with a 582bp amplicon. **d.)** *aac(6")-le-aph(2")* amplified *at 369bp* **e.)** Presence of *vanB* was indicated in lane 3 with a 635bp amplicon.

Table 4.6. Pearson correlation test p-values indicating the relationship between the antibiotic genes screened in *E. faecalis.*

	vanB	***vanC1***	***vanC2/3***	***ermA***	***ermB***	***aac(6")-aph(2")***
vanB	1	-.133	.016	-.075	.109	-.043
		0.305	.905	.565	.403	.744
vanC1	-.133	1	.011	.145	-.096	.172
	.305		.934	.265	.462	.185
vanC2/3	.016	.011	1	.174	-.253*	.192
	.905	.934		.179	.049	.138
ermA	-.075	.145	.174	1	.075	.568**
	.565	.265	.179		.565	.000
ermB	.109	-.096	-.253*	.075	1	.043
	.403	.462	.049	.565		.744
aac(6")-aph(2")	-.043	.172	.192	.568**	.043	1
	.744	.185	.138	.000	.744	

* Correlation is significant at the 0.05 level (2-tailed).

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

Table 4.7. Binary logistic regression null model illustrating the effect and association of antibiotic resistance genes in *E. faecalis* with animal host, location and sample

		B	**SE**	***p*-value**	**Exp (B)**
vanB	Location	-18.920	9978.811	998	2.000
	Sample site	-.271	.596	.649	.762
	Host	1.162	.202	.459	1.162
vanC1	Location	2.957	.906	.001*	19.235
	Sample site	-.190	.363	.600	1.200
	Host	.228	.148	.127	1.253
vanC2/3	Location	.668	.764	.382	.513
	Sample site	-.148	.338	.661	.863
	Host	.079	.111	.474	1.083
ermA	Location	-18.937	9979.496	-	-
	Sample site	.312	.725	.667	1.336
	Host	-.166	.242	.492	.547

ermB	Location	1.034	1.481	.485	2.811
	Sample	2.354	1.152	.041*	10.531
	Host	.127	.194	.514	1.135
aac-	Location	-17.685	10019.87	.999	0.00
aaphD	Sample site	2.77	1.158	.810	1.317
	Host	-.103	.407	.800	.902

*p < 0.05, significant (2-tailed).

4.4. Discussion

Antibiotic resistance pathogens are a growing concern globally(CDC, 2017; Van Den Bogaard & Stobberingh, 2000), the dissemination of antibiotic resistant *Enterococcus* spp. is more worrisome because the genus is known for exchanging genetic determinants within enterococci and also with other bacteria (Hegstad *et al*., 2010; Werner *et al*., 2013) The current study investigated the prevalence of antibiotic resistance in *E. faecalis* and *E. faecium.* Both *E. faecalis* and *E. faecium* isolates had a high resistance to macrolides (erythromycin) and tetracyclines (tetracyclines).This was anticipated since both these antibiotic groups such as tylosin were mostly used for prophalyxis or as a growth promoter in livestock production systems (Zou *et al*., 2011). These studies are in concordance with the study's findings as they also observed high macrolide/ tetracycline resistance in isolates recovered from farms (Ngebde *et al*., 2016; Klibi *et al*.,2013; Seputiene *et al*., 2012). In contrast ciprofloxacin, which is part of fluroquinolones had the highest prevalence of intermediate susceptibility in both *E. faecium* and *E. faecalis* isolates, with 62.5% and 49.2% respectively. Intermediate susceptibility may indicate a latent escalation of resistance to clinically relevant antibiotics. Seputiene *et al*. (2012) and Iweriebor *et al*. (2015) indicated that the lack of policies regulating the usage of antimicrobials that are analogs to clinically relevant antibiotics like fluroquinolones (namely advocin), lead to a high prevalence of *Enterococcus* isolates that are resistant to ciprofloxacin. It is important that we monitor intermediate susceptibility profiles as they indicate which antibiotic resistance may pose risks in the near future. It's imperative to note that even though this is a veterinary study on livestock, *Enterococcus* is disreputably known for transferring genetic determinants to commensal bacteria (Beshuru *et al*., 2017) as such this can cause a public health risk, especially in terms of MDR *Enterococcus*.

This current study also demonstrates that *E. faecalis* had more isolates that were MDR compared to *E. faecium* isolates with a prevalence of 44.2% and 25% respectively. Although the obtained results could be affected by the low incident rate of *E. faecium* with only eight isolates recovered from both farms (Flagstaff and Amandawe). Beshuru *et al.* (2017) and Ngebde *et al*. (2016) agree with our findings while Diarra *et al*. (2010) observed that MDR was more common in *E. faecium* isolates (66.7%) than *E faecalis* isolates. MDR is when a bacterial isolate has phenotypic resistance to more than three antibiotic classes. In the present study two (0.032%) *E. faecalis* isolates were

resistant to all seven of the screened classes. These two isolates had the same antibiogram (STR-VAN-PEN-CHL-TET-CIP-ERY). Interestingly both isolates were recovered from broiler chickens with one isolate being of oral and faecal origin. Molechan *et al*. (2019) findings were dissimilar to the current study's results. Although they screened resistance to ten antibiotics classes in poultry production from South Africa, they only reported MDR to only six antibiotics classes, with an antibiogram TET-ERY-CIP-AMP-CHL-GEN-STR. The current study is important as it reports resistance to significant antibiotics that are clinically relevant to treat enterococcal infections in humans. As a result, the study advises that contaminated faecal matter of broiler chickens must be appropriately handled to mitigate environmental impact and a public health risk (Diarra *et al*., 2010).

As previously mentioned, the use of antibiotics in the environment poses a further public health risk. This is attributed to creating a selective pressure in the resistance of certain antibiotics (Ngbede *et al*., 2016). Resistance to antimicrobials in bacteria is mostly due the resistance genes found chromosomally or in plasmids (Weaver, 2019). Seven resistance genes were screened in all 69 isolates using molecular techniques. The high prevalence of erythromycin resistance was mostly attributed to the presence of the *erm* genes in *Enterococcus. erm* genes encode the expression of the ribosomal methylase. This methylase enzyme facilitates methylation of the 23S RNA thus high resistance to macrolides, lincosamides and streptogramin B (MLS_B) is explained by the presence these *erm* determinants (Diarra *et al*., 2010; Zou *et al.*,2010). The present study screened for *ermB* and *ermA* resistance determinants. *ermB* was the most detected AR gene across all farms with percentages of 80.3% (*E. faecalis*) and 87.5% (*E. faecium*). Similar results were obtained by other studies (Molechan *et al.*, 2019; Iweriebor *et al*.,2015; Klibi *et al*., 2013; Seputiene *et al*., 2012;Diarra *et al*., 2010; Zou *et al*., 2010). *ermA* seems to be in scarcity in both *E faecalis* and *E. faecium* isolates. In the present study it was only detected in *E. faecalis* isolates in a low prevalence percentage of 6.56%. It was mostly recovered in country farmed chickens, cows and in broiler chickens. Although Zou *et al*. (2010) documented 47.4 % of *E. faecalis* isolates from swine in China with the *ermA* gene other studies based in Tunisia (Klibi *et al*., 2014; Klibi *et al*., 2013) did not detect the gene, it is important to note that *ermA* is not commonly detected in *E. faecalis* strains (Schwaiger and Bauer 2008). Consequently, erythromycin resistance observed in the present study is mostly due to the presence of the *ermB* gene.

The present study analysed the prevalence of *ermB* with the associated variables in livestock production systems in *E. faecalis* isolates from South African farms. Although in most studies (Molechan *et al*., 2019; Iweriebor *et al*., 2015; Klibi *et al*., 2013; Seputiene *et al*., 2012; Diarra *et al.*, 2010; Zou *et al*., 2010) and including the current study it was observed that *ermB* was extensively disseminated in livestock production systems irrespective of its location, type of host and sample site. The association was not significant between *ermB* and its prevalence ($p > 0.05$).

However according to the regression model, the prevalence of *ermB* could be significantly predicted by the type of sample site (oral, faecal, water, soil and feed) the isolates were recovered from (β= 2.354, SE= 1.52, Exp(β) =10.531, p<0.05). Furthermore, there was a negative correlation between *ermB* and *vanC2/3* (*R* = - 0.253, p<0.05). The negative relationship might be ascribed to the location of these genes in the isolates. *ermB* is commonly located in transposoons whilst *vanC* and its determinants are intrinsic to the *spp. E gallinarum* and *E. casseliflavus* (Hollenbeck and Rice, 2012).

In the current study the negative relationship may be attributed to *ermB* being expressed from the *Tn3*-familiy (*Tn917*) which is commonly described in *Enterococcus* spp. typically in *E. faecalis* strains (Beukers *et al.*, 2017). Whilst the prevalence of *vanC2/3* could be expressed due to sporadic mutations based on the insertion of elements like Integrative Conjugative Elements (ICE), these ICE elements are self-transmissible (Beukers *et al.*, 2017). Most studies have documented *vanC* and its determinants as a species-specific gene for *E gallinarum* and *E. casseliflavus* (Ahmed and Baptise, 2017; de Moura *et al*., 2013; Schwaiger *et al*., 2012; Hollenbeck and Rice, 2012), quite a few studies have documented the presence of these genes outside the previously mentioned spp. Schwaweiger *et al*. (2012) reported *vanC* from pigs, de Garnica *et al*. (2012) from ewe milk, Moura *et al*. (2013) from broilers, Maseru *et al*. (2013) water samples from the river and lastly Sun *et al*. (2012) from clinical samples. This study to the best of our knowledge is the first to report of these genes from *E. faecalis* and *E. faecium* isolates in a South African livestock setting. The study indicated that dissemination of *vanC1* could be significantly predicted by the location the isolates were predicted from (β= 2.957, SE= 0.906, Exp(β) =19.235, p< 0.01). The regression model was further supported as there was a significant association between the prevalence of *vanC1* its location and sample site (p< 0.05). Based on the reported evidence; if a gene is constitutively expressed (namely *vanC1*and *vanC2/3*) in strains that are intrinsically resistant (*E. gallinarum*) it does not automatically prevent the transfer of the gene to other enterococcal spp. (Ahmed and Baptise, 2017 Schwaiger *et al*., 2012). Hence the *E. faecalis* and *E. faecium* isolates could have acquired *vanC* determinants through the MGE's from *E gallinarum* (Sun *et al*., 2012). It is imperative to reiterate that *E. gallinarum* is commonly recovered from animals thus could explain why there is an increase in *vanC* determinants being detected outside *E. gallinarum* and *E. hirae*.

The *van* genes confer resistance to vancomycin and teicoplanin. *vanA* and *vanB* are commonly found in *E. faecalis* and *E. faecium* strains (Torres *et al.*, 2018). *vanA* confers high resistance to both glycopeptides (Ahmed and Baptise, 2017), this gene was not detected in the current study. Similar results were obtained by most livestock production studies (Diarra *et al*., 2019; Iweriebor *et al*., 2015; Klibi *et al*., 2014; Diarra *et al*., 2010). Whilst *vanB* confers moderate to high resistance to vancomycin but is susceptible to teicoplanin (Ahmed and Baptise, 2017), it was prevalent at a

percentage of 9.84% for *E. feacalis* and not detected in *E. faecium*. Upon further analysis *vanB* was highly prevalent in broiler chickens (1.6%) although Iweriebor *et al*. (2015) also detected *vanB* in swine. However, Diarra *et al*. (2010) and Molechan *et al*. (2019) Klibi *et al* (2014) did not detect any presence of the *van* determinants. The study indicates that broiler chickens harbour AR genes compared to any other host including chickens that are country farmed. There has been an increase in the commercial farming of chickens in South Africa (Molechan *et al*.,2019) thus an incline in resistant enterococcal strains that have pathogenic PAI should be expected. In addition, the current study observed 37.7% VRE isolates of which most harboured only the *vanC* determinants. *vanC* encodes low resistance to vancomycin thus the phenotypic resistance of the VRE isolates in this study could not explained by the presence of *vanC* but other *van* genes that the study didn't screen for (*vanD* for *E. faecium*; *vanG* for *E. faecalis*).

Recent studies have been assessing high level resistance to aminoglycosides due to the growing concern on the increase in resistance of clinically relevant antibiotics. The current study screened for resistance to high concentrations of streptomycin and gentamycin. These aminoglycosides are used simultaneously in synergistic treatments of enterococcal infections (Ngebde *et al*., 2016). Hence once isolates are resistant to one type, the treatment is rendered useless. An equal proportion of gentamycin (HLGR) and streptomycin (HLSR) resistance was observed in *E. faecium* (12.5%). However, with *E. faecalis* 18.0% of the recovered isolates were resistant to streptomycin and only 6.6% resistance to gentamycin. Said and Abdelmegeed. (2019) indicated that HLGR was commonly observed compared to HLSR in enterococcal infections. Consequently, this study screened for *aac(6")-aph(2")* which is commonly associated with high level aminoglycoside resistance (Klibi *et al*.,2013). Furthermore, it has been documented that livestock serve as a reservoir for this gene (Torres *et al*., 2018). However, *aac(6")-aph(2")* was only detected in one *E. faecalis* isolates recovered from faecal matter of broiler chickens. Therefore, the observed HLGR in the current study is not explained by this gene. The expressed HLGR phenotype could be explained by other acquired Aminoglycosides Modification Enzymes (AME's) such as *aph (2")-Ib*, *aph(2")-Ic*, and *aph(2")-Ie* of which some of these genes are commonly cited in livestock animals (Torres *et al*.,2018; Diarra *et al*., 2010,). In addition, Diarra *et al*. (2010) explained the lack of detection of *aac(6")-aph(2")* could be attributed to the mutations such as deletions, insertions and/or loss of gene.

4.5. Conclusion

The BRICS countries have been identified as major consumers of antibiotics; the South African antimicrobial resistance strategy framework indicated a gap in the existing body of literature. There hasn't been enough studies on the resistant rates and patterns of *Enterococcus* in the animal husbandry sector in South Africa.. Consequently, there's a shortfall in data of resistant pathogens that are responsible for communicable infections. This study documented that *E. Faecium* and *E.*

Faecalis isolated from livestock production systems in South Africa were predominantly phenotypically resistant to macrolides, glycopeptides, tetracyclines and fluroquinolones. It has been documented how easily resistance to the previously mentioned classes if present in MGE's of E*nterococcus* can be transferred to other pathogens be it commensals or nosocomial strains. Hence, the observed resistance and presence of AR genes observed could easily be transferred to environmental strains causing an AR gene reservoir without the presence of a selective pressure. The study also highlighted the emergence of inducible *vanC* determinants in VRE isolates. As a result, the study suggests intense surveillance and monitoring strategies for *Enterococcus* spp. since they are capable of horizontally transferring constitutive genes.

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CHAPTER 5

GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1. Literature Review

The route of transfer of VRE was explained as such that primary transmission of VRE strains encompassing the *Tn1546* originates from wildlife to humans and not the other way around (Giraffa, 2002). The literature review identifies the missing links of *Enterococcus* and its resistance mechanisms. The review also details on how the virulence genes aids in the persistence of *E. faecalis* and *E. faecium*. in addition to clearly explaining the phylogeny and identity of *Enterococcus*

5.2. Prevalence of antimicrobial resistance genes in *E. faecalis* and *E. faecium* and the dissemination of *vanC* outside *E gallinarum* and *E. casseliflavus* in South African livestock production systems.

The current study successfully isolated two *Enterococcus* spp. from livestock and their associated environment using selective agar and species-specific primers. *E. faecalis (*61%) was widely disseminated across both farms compered to *E. faecium* (8%). The inconsistencies observed between this study and other livestock studies could be attributed to the different methods used to isolate the bacteria or to the geographical variations (Klibi *et al*., 2014). Furthermore, the pathogenicity of *E. faecalis* and *E. faecium* was investigated by screening for six virulence factors namely; *gelE, ccf, esp, hyl, cylA*, and *asa1*. *E. faecalis* had a high variability in terms of the virulence determinants and it has been understood that the presence of these virulence genes are a strain's specific character. Although the world is going towards organic farming, the present study indicates that even country farmed chicken and its associated environment have the potential of being a reservoir for virulent enterococcal strains. Thus *E. faecalis* and *E. faecium* of livestock and their associated origin aids in increasing virulence in the community.

5.3. Detection of virulence genes associated with *E. faecalis* and *E. faecium* recovered from isolates in livestock production systems in South Africa

This study robustly screened for AR genes in *Enterococcus* species across a wide range of hosts and environmental surfaces. Most enterococci studies were limited in terms of only scrutinizing a small fraction of communicable to nosocomial infections. This study is the first to report on the dissemination of *vanC* and its variants outside the species *E. gallinuim/ E. hirae* in South African livestock production systems. The use of antibiotics in developing countries is hardly monitored. The high prevalence of macrolide and tetracycline resistance is attributed to the use of their analogues, which are currently cheaper hence they are commonly used in absentia of a vetinary prescription (Ngbede *et al*., 2016). Regulation of antibiotic use in animal feed or for metaphylaxis should be regulated as enterococci are notorious for intrinsically transferring genetic elements in

the commensal bacteria. Arias *et al*. (2010) is in agreement with the present study as they have documented that although macrolides and tetracyclines are not a drug of choice for treating enterococcal infections, they are necessary for the treatment of another bacterium namely *Streptococcus pneumoniae*. Since it is known that *Enterococcus* spp. are very adaptive and are notoriously known to transfer their MGE's to other pathogens the surveillance of resistant *Enterococcus* spp. is of clinical importance as these genes will be selected for in animal husbandry settings. In addition, new measures to treat antibiotic resistance bacterium should be further investigated for the future as means of adequately preparing for the emergence of non-treatable pathogens.

5.4. Implications of the study

The current study contributes new knowledge and data regarding the prevalence and dissemination of antibiotic resistant *Enterococcus* species in livestock productions in South Africa. In addition, the current study aligns with WHO's One health approach in mitigating antimicrobial resistance. The two sampled farms were small-scaled farms which are a norm in South Africa. The study successfully identified the dissemination of virulence genes associated with livestock and its environment. Furthermore, this study highlights on the contribution of putative virulent genes in broiler and country farmed chicken productions. The latter is considered to be a safer route (virulent genes reservoir) of rearing chickens, however this study demonstrates the rising potential of virulent enterococcal strains disseminating in this type of environment. Also, of importance as far as we can affirm the current study is the first to document the emergence of *vanC* determinants in *E. faecalis* and *E. faecium* from South African livestock production systems perspective. Lastly, the study reiterated the impact of MDR enterococci in the environment as it was observed that the two species were resistant to the drugs used for synergistic treatment. South Africa is a country that is most burdened by immunocompromised individuals. Considering the zoonotic potential of *Enterococcu*s and its ability to efficiently transfer genetic determinants, the current study alludes to the type of genes that might aid in the persistence of Hospital Acquired Infection's (HAI's) in the near future.

5.5. Recommendations

It is imperative that surveillance studies are not only limited to one type of host or sample site. This study recommends that a broad and robust approach in surveillance should be implemented in order to comparatively assess true patterns and trends on the prevalence and dissemination of zoonotic pathogens. It is also recommended that clonal screening and sequencing be part of surveillance studies in order to get a better understanding of the frequency of horizontal gene transfer and how related are the recovered isolates from livestock and those that cause communicable infections.

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