Genotypic and Phenotypic characterization of enterococci from cow dung and environmental water sources in three selected dairy farms in Amathole District

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Master of Science (MSc) degree in Microbiology

BY

Tanih Godfred Ngu

Department of Biochemistry and Microbiology

Faculty of Science and Agriculture

University of Fort Hare

Alice, 5700

Supervisor: Prof. E. Green

Co- supervisors: Prof. RN Ndip

Prof. AI Okoh

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DECLARATION

I, the undersigned, declare that the dissertation hereby submitted to the University Of Fort Hare for the degree MSc (Microbiology) and the work contained therein is my original work and has not previously, in its entirely or in part, been submitted to any university for a degree.

Signed …….this……..…day of…….……2016.

Supervisor……..

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DEDICATION

This work is dedicated to my lovely parents, Mr. Tanih David Azongho and Mrs Tanih Beatrice Anwi and to my brothers and sisters Dr. Tanih Nicoline, Tanih Kenneth, Tanih Ruth, Tanih Godlove, Tanih Robin, Tanih Claire and Tanih Clinton

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LISTS OF ABBREVIATIONS

AS	Aggregation substance
Cyl	Cytolysin
D-Lac	D-Lactate
D-ser	D-serine
Esp	Enterococcal surface protein
GIT	Gastrointestinal tract
PBP	Penicillin-binding proteins
PCR	Polymerase chain reaction
UV	Ultraviolet Light
UTI	Urinary Tract Infection
RAPD	Random Amplified Polymorphic DNA
MLST	Multi locus Sequence Typing
ALFS	Amplified length Fragment Polymorphism
PFGE	Pulse Field Gel Electrophoresis
FDT	Fort Hare Dairy Trust
MDT	Middledrift Dairy Trust
SDT	Seven Star Dairy Trust

ABSTRACT

Enterococcus species are integral members of the gastrointestinal microfloral of humans, animals, birds, as well as insects. Their presence in water and food has been greatly associated with faecal contamination. This study was aimed at evaluating the incidence of *Enterococcus species* in cow dung and environmental water sources in three commercial dairy farms. In addition, their antibiotic profiles were determined as well as resistance and virulence genes. Furthermore, the genetic relatedness of the isolates was determined by molecular typing method (RAPD PCR).

Three hundred and thirty four water and faecal samples consisting of 117, 116 and 101 were collected from Seven Star Middle Drift and Fort Hare Dairy trusts respectively. Of the 334 samples collected, 289 were of faecal origin and 45 from water sources within the farms. All samples were screened for enterococci using culture base growth media and molecular methods targeting the *tuf* gene. Speciation was done using species-specific primers and the incidences of various species within the farms determined. Furthermore resistance to antibiotics and multidrug-resistant phenotypes were established using the disk diffusion method. Genes coding for virulence and resistance were also determined.

From the samples collected, 313 (289 faecal and 24 water) presumptive enteroccocci were isolated, 305 of 313 (97.45%) were confirmed as *Enterococcus* of which 239 of 305 (78.38%) were identified as *E. hirae*, 15 of 305 (4.92 %) as *E. faecium*, 12/305 (3.93%) as *E. durans*, 6 of 305 (1.97%) as *E. faecalis* and 33 of 305 (10.82%) were unidentified. Out of the five virulence genes that were targeted in the study only *gelE* (71.80% of 219/305) and *ace* (27.2% 83/305) were present in the isolates. Phenotypic resistance to antibiotics was

observed is in all twelve antibiotics tested with multidrug resistance phenotypes detected in some enterococcal isolates most predominant in Seven Star and Middledrift dairy trust. Finally RAPD profiles of the isolates showed high relatedness between the strains from water and cow dung sources in all three commercial dairy farms suggesting possible contamination from cow dung to the water sources or vice versa.

CHAPTER ONE

INTRODUCTION

Enterococci are Gram positive, facultative anaerobic, non-spore forming bacteria that exist predominately as normal flora of the gastrointestinal tract (GIT) of both humans and warm-blooded animals (Foulquié Moreno *et al.*, 2006; Psoni *et al.*, 2006). However, in some cases they are reported as opportunistic pathogens especially in immuno-compromised individuals or persons with prolong broad spectrum antimicbrobial therapy (Castillo-Rojas *et al.*, 2013). They have been reported in several ecological niches most commonly in human and animal faeces.

Cow dung is known to harbour and sustain the growth of a wide variety of microorganisms. Bacteria species reported include *E. coli, Salmonella spp, Campylobacter spp, Enterococcus spp, Shigella spp,* and *Klebsella spp*. *Hepatitis A, Adenoviruses*, *noroboviruses* and *Enteroviruses* are amongs the viruses while the protozaons include *Giardia spp*, *Entamoeba spp*, *Naelgeria spp* and *Cryptosporidium spp* (Stewart *et al.*, 2007). Generally, cow dung consists of a variety of nutrients which support the growth of both pathogenic and non-pathogenic microorganisms (Stewart *et al.*, 2007).

Probably due to cross contamination from human and animals faeces as well as their ability to survive adverse conditions, enterococci have been reported in soil, water, plants, vegetables, foods, dairy products and milk (Ozdemir *et al.*, 2011; Kagkli *et al.*, 2007; Sapkota *et al.*, 2007). In the production of traditional cheese, enterococci serve as starter culture in the fermentation process (Foulquié Moreno *et al.*, 2006). Due to their environmental stability

and as commensal of the GIT, they are used as an indicator of faecal contamination of water (USEPA, 2000).

In recent years, the incidence of enterococcal infections in both humans and animals has increased worldwide with increasing nosocomial-associated enterococcal infections. These incidences may be ascribed to the emergence of genetically modified strains resistant to a variety of antibiotics or exhibiting various virulence factors which were not present in their parental strains (Chigor *et al.*, 2010). According to the survey carried out by the National Nosocomial Infections Surveillance (NNIS), 28% of enterococcal isolates in ICUs of the more than 300 participating hospitals were vancomycin-resistant. In South Africa, Von Gottberg *et al*. (2000) reported the presence of vancomycin resistant enterococci isolated from patients with high risk of infection to glycopeptides resistant bacteria in four hospitals in Johannesburg.

Enterococci have been implicated in several animal diseases. These include diarrhoea in swine, and cattle; endocarditis, septicaemia, arthritis, spondylitis, femoral head necrosis, osteomyelitis, lameness and paralysis in poultry as well as urinary tract infections in rats and rabbits (Bisgaard *et al.*, 2010; Stalker, *et al.*, 2010; Ok *et al.*, 2009). In addition, other studies have reported enterococci as the causative agents in 2-20% of all bovine mastitis (Aarestrup *et al.*, 1995; Jackson *et al.*, 2011).

Antibiotics are being used for treatment of particular diseases or as preventive treatment in animal husbandry (Wegener *et al.*, 1999). The indiscriminate use of these antibiotics in animal farms have been implicated in the development of resistant bacteria in humans and farm animals (Vignaroli *et al.*, 2011). Therefore, the use of antibiotics by veterinary medicine

to improve animal yield is a call for concern, most especially for those used in the treatment of enterococcal infections. In addition, the ability to acquire and transfer antibiotic resistant and virulent genes among *Enterococcus spp* is a major threat to both public health and dairy farming. These bacteria can be transferred from livestock to humans or to other cattle herd through uncooked meat, raw milk or contaminated drinking water (Jackson *et al.*, 2011).

Surface and underground water resources are experiencing a serious drop in quality due to pollution from various human activities such as mismanagement of animals waste (cow dung) and discharge of substances from farmland (Schaper *et al.*, 2002). These activities eventually release enteric pathogens and other resistant bacteria into rivers and streams either through runoffs from the fields or by direct release of poorly treated and untreated sewage effluent from commercial farms and sewage treatment plants.. Faecal contamination of water bodies is a serious problem in most countries. Globally, an estimated four billion cases of diarrhoea occur each year of which 88% are ascribed to unsafe drinking water (WHO, 2008). This problem is even worse in developing countries and particularly in the rural areas where water is inadequately treated or receives no treatment at all. It is also evident that contaminated drinking water, contributes to the death of millions of the poorest people in the world due to water associated diseases (WHO, 2007). This might not be very different with South Africa and the Amatole district in particular.

1.1 PROBLEM STATEMENT

For decades, enterococci have been playing a major role in traditional food processing due to their ability to carry out fermentation especially in foods of animal origin (Foulquié Moreno *et al.*, 2006). Other benefits associated with them include their contribution to ripening and aroma development, probiotics properties and production of antimicrobial substances (Giraffa, 2003). Although this organism is beneficiary, the incidence of enterococcal

infections in animals has increased over the past years leading to diseases such as bovine mastitis, diarrhoea, endocarditis, bacteraemia and septicaemia (Bisgaard *et al.*, 2010; Ok *et al.*, 2009). In addition, the emergence of new strains which could be resistance to multiple antibiotics commonly used in dairy farms is major concern today. Enormous efforts have been vested to better understand the ecology, prevalence and epidemiology of these versatile bacteria. In the Amatole district, different water sources are being used by the local community for various purposes including dairy and irrigation farming. However, currently these water bodies are faced with faecal contamination from unknown sources. Other reports have shown that *Enterococcus* isolated from water sources serve as reservoirs for antibiotic resistance (Ntloko & Okoh, 2014). This study therefore seeks to generate information on the prevalence of *Enterococcus species* in both cow dung and water sources; determine the resistance pattern of antibiotics frequently used in treatment of enterococal infections as well as those used in farm management practices. The virulence associated genes of these species will also be screened in an effort to determine their potential pathogenicity

1.2 HYPOTHESIS

Cow dung and environmental water sources in Amatole District are sources of pathogenic *Enterococcus* species.

1.3 AIM

To evaluate the genotypic and phenotypic characteristics of enterococci isolated from cow dung and environmental water sources in three selected dairy farms in Amatole district

1.4 OBJECTIVES

- To determine the prevalence of *Enterococcus species* in cow dung and water sources in commercial farms
- To determine the antibiotic resistance profile.
- To detect the genes that mediate antibiotic resistance.
- To determine the prevalence of virulence genes.
- To determine the genetic relatedness of the isolates using RAPD PCR

CHAPTER TWO

LITERATURE REVIEW

The genus *Enterococcus* is an important bacteria group that has been associated with several benefits and risks. Their primary existence as enteric bacteria and ubiquitous nature too, make them important in medical, environmental and food microbiology. Traditionally, this bacteria group are being exploited for their fermentation potentials towards the production of handmade cheese, fermented sausages and the improvement of other food of animal origin (Foulquié Moreno *et al.*, 2006). In addition some strains have been reportedly used as starter culture, feed supplements as well as probiotics (Foulquié Moreno *et al.*, 2006). Contrary to their benefits, some enterococcal strains have been reported to harbour virulent and resistant genes and have been implicated with several diseases in humans as well as animals (Bisgaard *et al.*, 2010). Enterococci are good indicators of faecal contamination of surface and marine water. Despite the benefit and risks involved with the genus *Enterococcus*, their use as probiotics remain a matter of controversy.

2.1 TAXONOMY OF ENTEROCOCCI

Enterococci are Gram positive, facultative anaerobic bacteria which exist as single and short chain cocci. They generally belong to the phylum Firmicutes, class Bacilli, and order Lactobacillales and family Enterococcaceae (Carrero-Colón *et al.*, 2011). The family Enterococcaceae has several genera that include *Atopobacter, Catellicoccus, Melissococcus, Pilibacter, Tetragenococcus Enterococcus* and *Vagococcus* (Araújo & Ferreira, 2013). In the past, identification and classification of these bacteria was a major challenge because of the

difficulty to distinguish *Enterococcus* from *Streptococcus*. As such they were classified under the genus *Streptococci* based on phenotypic characteristics. The genus *Enterococcus* was first described by Thiercelin in 1899 and later by Thiercelin & Jouhaud, in 1903. However, by then there was no clear cut distinction between the genus *Enterococcus* and *Streptococcus*. With the advent of newer molecular techniques such as 16S rRNA DNA sequencing, DNA–DNA hybridization and whole protein analysis in 1987 the genus *Streptococcus* was divided into 3 genera namely *Streptococcus, Enterococcus* and *Lactococcus* (Schleifer & Kilpper Balz, 1987).

Presently, this genus is made of 54 species with the most important frequently reported members of this group being *Enterococcus thailandicus, E. italic, E. mundtii, E. hirae, E. gallinarum, E. casseliflavus, E. faecium, E. faecalis, E. durans, E. dispar, E. avium, E. moraviensis, E. haemoperoxidus, E. villorum* and *E. porcinus.* (http://www.bacterio.cict.fr/e/enterococcus.html). However, the classification of species under this genus requires revision and reclassification as some species were reported to be synonymous. A study carried out by Naser *et al.* 2006 on *E. casseliflavus* and *E. flavescens* as well as on *E. saccharominimus* and *E. italicus* revealed that they was close taxonomic relatedness. A partial sequence analysis of *Enterococcus* using three housekeeping genes (phenylalanyl-tRNA synthase alpha subunit (pheS), RNA polymerase alpha subunit (rpoA) and the alpha subunit of ATP synthase (atpA) revealed that *E. flavescens* should be reclassified as *E. casseliflavus* (Naser *et al.* 2006). However, in a study conducted by Jackson *et al.* (2011) *E. casseliflavus* and *E. flavescens* were isolated and characterised as two separate species among other enterococcal species from US dairy cattle. Within the enterococcal species are sub-species (strains) usually denoted by the name of the species plus

a letter and number for example *E. faecium* SF68 (where *E. faecium* indicates the species and SF68 indicates the strain of the species).

2.2 GENERAL CHARACTERISTICS OF ENTEROCOCCI

Enterococcus are oxidase negative, non-spore forming and facultative anaerobic bacteria capable of growing in a broad temperature range (10^{o}C and 45^{o}C), salt concentrations of 6.5% NaCl, pH of 9.6 and 40% bile salts (Foulquié Moreno *et al.*, 2006). They are catalase negative with some species exhibiting pseudocatalase activity (Byappanahalli *et al.*, 2012). In addition, they are good homo-fermentative Lactic Acid Bacteria (LAB) due to their ability to convert glucose to lactic acid (Franz *et al.*, 2011a). They ferment a wide variety of carbohydrates such as D-glucose, D-fructose, lactose, ß-gentiobiose, cellobiose, maltose, D-mannose, galactose, N-acetylglucosamine, salicin and arbutin. Some literatures have reported their resistance to disinfectants such as chlorine (Peter *et al.*, 2012). They are capable hydrolysing L-naphthylamide-β-naphthylamide and aesculin (Facklam, 2002), hence this chacteristics are used in diagnostics laboratory in the identification of enterococci. Most enterococci are non-motile however some species have been reported to be motile (*E. casseliflavus* and *E. gallinarum*).

2.3 ECOLOGY AND SPECIES DISTRIBUTION

Enterococci just like other bacteria being ubiquitous in nature has been isolated from many different ecological niches such as soil, sewages, marine water, faeces, milk, plants, surface water and food of animal origin (Cheese, fermented meat and sausages) (Aslam *et al.*, 2012; Moore *et al.*, 2008; Jurkovic *et al.*, 2006). They are also present in lower concentrations in body secretions and other niches like oropharyngeal secretions, urogenital tracts and wound ulcers. Distribution of enterococcus species varies from one ecological niche to another

(Klein, 2003) and also varies within different countries or geographical locations. Generally, *E. fecalis* and *E. faecium* from human faecal sources remain the most isolated species from different habitats (Kumar Patidar *et al.*, 2013). In animals, *E. hirae, E. durans, E. fecalis, E. faecium, E. avium E. gallinarum* are the most commonly isolated (Ali *et al.*, 2014; Jackson *et al.*, 2009, 2011). *E. mundtii, E. casseliflavus E. fecalis, E. faecium, E. avium* and *E. gallinarum* have been isolated from other sources like rivers, drinking water, sewage water and faeces (Ali *et al.*, 2014; Castillo-Rojas *et al.*, 2013; Ferreira da Silva *et al.*, 2006; Jackson *et al.*, 2011). From birds, isolated species include E. *columbae, E. gallinarum* (Devriese *et al.*, 1993) and finally, species that have been isolated from soil, milk and plants include *E. eurekensi, E. lactis, and E. plantarum* respectively. The existence of diverse enterococal species in different ecological niches apart from faeces is alleged to be linked with cross contamination from faecal material (Ogier & Serror, 2008). Nevertheless, there are diverse opinions on which species is commonly isolated from a particular source.

2.4 ENTEROCOCCI AS PROBIOTICS

Probiotics are live microorganisms which when administered in adequate doses to a host confers significant health benefits (Franz *et al.*, 2011b). It supports the beneficial balance of the autochthonous microbial population of the gastrointestinal tract. The use of LAB (lactic acid bacteria) group in the improvement and preservation of food is a well-established practice. LAB are utilised in food microbiology as probiotics cultures in diverse food products such as yogurt, milk, infant formulas and they possess antimicrobial properties against pathogenic bacteria, yeasts, molds and viruses (Deegan *et al.*, 2006; Settanni & Corsetti, 2008). Beneficial effects of probiotics include: (i) treatment of diarrhoea associated with antibiotic therapy, viral infections, chemotherapy and even foodborne diseases, (ii)

inhibition of pathogenic microorganisms, (iii) strengthening of the intestinal mucosal barrier, (iv) antimutagenic and anticarcinogenic activities, (v) stimulation of the immune system, (vi) prevention of ulcers related to Helicobacter pylori infection and (vii) reduction of serum cholesterol (Franz *et al.*, 2011a). Certain criteria are outline for the selection of a good probiotic cultures by WHO and FAO; (I) ability to survive in the gastrointestinal tract of humans and animals; (ii) bile salt hydrolase activity; (iii) adherence to mucosal or epithelial cells; (iv) exclusion or reduction of pathogenic adherence and (v) antimicrobial activity against pathogens.

Enterococci being part of the LAB group, possess these probiotic potentials as well and are often present in milk due to their ubiquitous nature and resistance to harsh conditions (pasteurization, salt and acids). In addition, it is alleged that they can be used as starter or adjunct culture in the manufacture of fermented dairy products (Foulquié Moreno *et al.*, 2006; Furlaneto-maia *et al.*, 2014).

Some enterococci strains are being utilised as supplements or as animals feed additives due to their commensal status in animals. Two main enterococcal probiotics strains have been established which are *E. faecium* SF68® (NCIMB10415, produced by Cerbios-Pharma SA, Barbengo, Switzerland) and *E. faecalis* Symbioflor 1 (SymbioPharm, Herborn, Germany) (Franz *et al.*, 2011b). They are benefial in the treatment of diarrhoea, antibiotic-associated diarrhoea or irritable bowel syndrome, lower cholesterol levels or to improve host immunity resulting in improve growth in animals. Furthermore, studies have shown that, microencapsulated *E. faecium* SF68 (NCIMB 10415) are capable of reducing the rate of endogenous chlamydiae infection in swine (Pollmann *et al.*, 2005). Kuritza *et al.*, (2011) in (Araújo & Ferreira, 2013) also investigated the effects of *E. faecium* strain in chicken feeds

as probiotics and revealed that, they were capable of reducing the contamination by *Salmonella minnesota* in poultry.

Nevertheless, enterococci are still not generally recognised as safe (GRAS) in the food industry due to their potential to transfer and harbour resistant and virulent genes. Therefore their utilisations as probiotics or starter culture in the production of dairy food still should be discouraged.

2.5 FAECAL INDICATOR BACRTERIA AND WATER POLLUTION

This is a group of bacteria that indicates the occurrence of faecal contaminants in surface water such as thermotolerant coliforms and enterococci. They may or may not cause disease upon ingestion; however their presence in water correlates the presence of some pathogenic bacteria in the water. Coliforms and faecal streptococci are the two major groups of bacteria used as indicator of water pollution by sewage material and the most commonly tested bacteria groups include; total coliforms, faecal coliforms, faecal streptococci, *E. coli* and enterococci (Ashbolt *et al.*, 2001).

Total coliforms are Gram negative, rod shaped, non-spore forming, oxidase negative, facultative anaerobic bacteria which are capable of fermenting lactose to acid and producing Gas within 24 to 48 hours. Faecal coliforms or thermotolerant coliforms are a subgroup of total coliforms that are found specifically in the gut of humans and animals. *Escherichia coli* (ferment tryptophan to produce indole) is an example of a faecal coliform and it ferments lactose to produce acid and gas at 44.5^{o}C within 24 hours.

For the purpose of bacteriological examination of water, faecal streptococci (*Enterococcus species*) till date are recommended for use due to their long survival period in the environment and the ease in which they can be enumerated. The existence of enterococci in water signifies contamination with faeces although occasionally some strains might originate from other sources. The use of indicator bacteria to determine the faecal contamination of surface water was initiated several years ago for the following reasons:

(i) Faecal polluted water, sewage and wastewater contain a variety of pathogenic bacteria and viruses; therefore monitoring each these of pathogenic bacteria routinely was a major challenge.

(ii) Enterococci and *E. coli* exist relatively in large quantity in faeces of animals and humans.

(iii) They exist persistently in the environment without reproducing.

(iv) Isolation and enumeration methods for some these important pathogens are unavailable or very difficult to perform e.g. hepatitis and rotaviruses (Ashbolt *et al.*, 2001).

USEPA recommended the use of enterococci and *E. coli* as indicators bacteria for the monitoring of faecal pollution in marine and freshwaters respectively (Hicks, 2002). Enterococci being a commensal of the GIT of humans and warm-blooded animals, are excreted in faeces together with other pathogens as such they could be used as an alternative to determine the presence of these pathogens. Basically, a faecal indicator should be easily detectable and counted, non-pathogenic, should not multiply under natural conditions and should have a significant correlation with other pathogens (Suzuki *et al.*, 2012).

Water pollution may be categorized into point and non-point source pollution; whereby point source pollution is described as the direct discharged of pollutants into a water body while non-point source pollution is the indirect dislodgement of pollutants into a water body (Ribaudo *et al.*, 1999a). In the United States, non-point source pollution has been identified as one of the major reasons for water pollution with the agriculture sector being the largest contributor (Ribaudo *et al.*, 1999b).

2.6 ANTIMICROBIAL RESISTANCES ON FARM ANIMALS

Generally, bacteria become resistant to antimicrobial agents as a result of close contact with the antimicrobial from either clinical or natural environment. Antimicrobials are usually employed in animal husbandry management practices to improve productivity and decrease morbidity and mortality caused by clinical and subclinical infections (Butaye *et al.*, 2003). The correct use of antibiotics and in appropriate dosage will ensure good health and weight gain of the livestock. In addition, it reduces the transfer of infections among animals or herds. The extensive use of antibiotics on farm animals has been implicated in the emergence antibiotic related drug resistances. The application of the drugs in animals, may lead to a selection of resistant strains of bacteria, which in turn may proceed to infect other animals and subsequently humans (Moyane *et al.*, 2013). Antibiotics such as ciprofloxacins, penicillins, tetracyclines, tylosin, virginmycins, gentamycin and sulphonamides, are some of the drugs used in South Africa on food animals (Moyane *et al.*, 2013). Furthermore, (Henton *et al.*(2011) reported the extensive use of tylosin in South Africa, which is one of 4 growth promoters (tetracyclines, sulphonamides and penicillins) banned in Europe.

2.7 ANTIBIOTIC RESISTANCE IN ENTEROCOCCI

The emergence of antibiotic resistance is a problematic issue in many countries worldwide. The development of antibiotic resistance usually occurs by horizontal transfer of genes mediating resistance between species or closely related genus through conjugative plasmids, transposons, possession of integrons and insertion elements, as well as by lytic and temperate bacteriophages (Teuber *et al.*, 1999). Transposons play an important role in distribution of antibiotic resistance genes thereby contributing to both long-term bacterial evolution and short-term adaptation, enabling rapid responses to environmental change (Scott, T.M. *et al.*, 2002). Enterococci exhibits intrinsic resistance to several antibiotics like cephalosporins, penecillins, low levels aminoglycoside , quinolones and others (Trivedi *et al.*, 2011; Cortés *et al.*, 2006). On the other hand, they may acquire resistance genes via mutation that confer resistance to vancomycin, tetracyclines, erythromycin, clindamycin, chloramphenicol and newer drugs (McBride *et al.*, 2007).

2.7.1 DIFFERENT MECHANISMS OF ANTIBIOTIC RESISTANCE IN ENTEROCOCCI

Enterococci display several mechanisms against different antibiotics (fig 1). *Enterococcus* becomes insensitive to β-lactams antibiotics by inhibiting their affinity to penicillin binding proteins. However, their affinity to PBP5 varies from one β-lactam-agent to another with penicillin being the most active and cephaloporins are the least active. *E. faecium* often exhibit resistance to ampicillin but this occurs rarely in *E. faecalis* (Klare *et al.*, 2001). Arias & Murray, (2009) observed a high level of *E. faecalis* resistance to penicillin and this was associated with β-lactamase activity. Nonetheless, Top *et al.* (2008) confirmed that susceptibility to β-lactams varies between *Enterococcus species*.

Resistance to aminoglycoside can either be intrinsic low resistance due to poor drug permeability across the cell wall or high level resistance due to the acquisition of genes coding for aminoglycoside-modifying enzymes (Arias & Murray, 2012). High level aminoglycoside resistance in enterococci is encoded by acc *(6')Ie-aph (2'').* This gene encodes for enzymes such as 2 phosphotransferase-6-acetyltransferase, 3-phosphotransferase-III, streptomycin adenyltransferase (Sava *et al.*, 2010). This plasmid-borne gene has also been detected in some *Staphylococcus spp.* Strains that produce 2" phosphotransferase-6'-acetyltransferase, are reported to mediate high level resistance to gentamycin, amikacin and kanamycin. High level resistance to streptomycin has been associated with ribosomal mutation which alters the binding target (Arias & Murray, 2012).

Vancomycin a glycopeptide produced by *Streptomyces orientalis* was first manufactured and introduced in the 1950s. Vancomycin functions by interfering with cell wall synthesis leading to breakdown of cell wall. It binds tightly to the D-Alanyl-D-Alanine (D-Ala-D-Ala) end of the pentapeptide chain thereby hiding it from the transpetidase that catalyses the cross-linking in the peptidoglycan synthesis (Sujatha & Praharaj, 2012). Although vancomycins are active against Gram-positive bacteria most Gram-negative organisms are resistant to it. Vancomycins are been recommended in the clinical treatment of severe cases of enterococci infection most especially those associated with multidrug resistant enterococci (Landman & Quale, 1997) Intrinsic resistance to low levels of vancomycin is due to production of D-Ala–D-Ser ending peptidoglycan side chain precursors for which vancomycin has a lower binding affinity compared to the D-Ala-D-Ala side chains (Klare *et al.*, 2001; Top *et al.*, 2008).

Tetracycline inhibits protein synthesis by interfering with the binding of amionoacyl-tRNA to the ribosome. Resistance is encoded by *tetM* that is mostly carried on plasmid. However other

genes also mediate tetracycline resistance which have also been detected in other bacteria (Roberts, 2011)**.** These genes confer resistance by two main mechanisms; mediates active efflux of tetracycline from cells (te*tL* and te*tO*) and protects the ribosomes from inhibition by tetracycline **(**tet*N* and *tetM***)**.

Macrolides is a group of antimicrobials produced by *Streptomyces* spp. Erythromycin and tylosin are members of this group used in the treatment of Gram-positive cocci infections. Macrolides resistance is encoded by the *ermB* gene and is very common among enterococci isolated from farm animals and humans (Jensen *et al.*, 1999) Resistance to quinupristin–dalfopristin (synercid) by enterococci involves several pathways, including drug modification, drug inactivation and drug efflux via the ATP-binding cassette protein macrolide–streptogramin resistance protein (MsrC) (Arias & Murray, 2012).

Linezolid resistances by enterococci have been reported to be mediated by mutation of the domain V of the 23S rRNA which alters the binding of drug to its target site (Arias *et al.*, 2010). Resistance to daptomycin results from the alteration cell membrane proteins like LiaF as well enzymes involved in phospholipid metabolism like cardiolipin (Cls) and glycerophosphoryl diester phosphodiesterase (Arias & Murray, 2012).

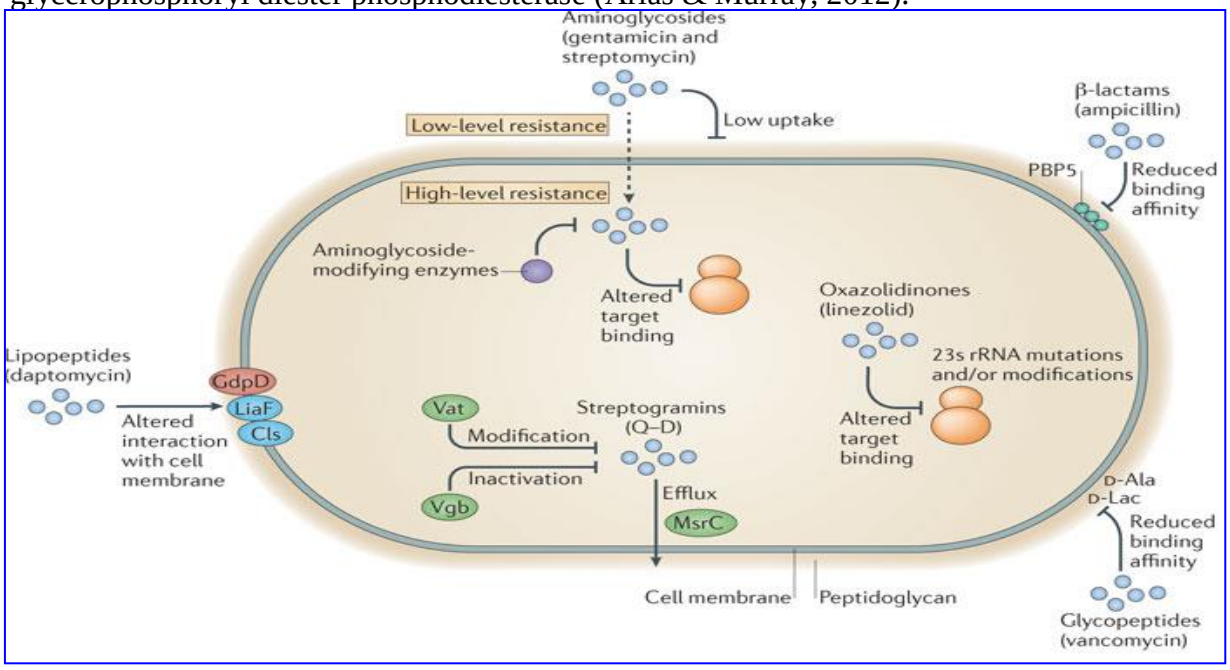

Figure 1: Antibiotic resistance pathways in enterococci (Arias & Murray, 2012)

2.8 VANCOMYCIN RESISTANCE ENTEROCOCCI (VRE)

VRE are a group of enterococci strains that have developed multiple resistances to several antibiotics most especially to vancomycin. They usually originate from hospitals and animal farms through drug selective pressure and are disseminated to other environments through infected persons, animals and contaminated materials. It may also occur via the transfer of resistant genes between bacteria-through the process of conjugation. VRE were first reported in Europe in 1986 in an animal farm and in the United States of America in 1987 in hospital intensive care units (ÇetİnkayaEtAl., 2013). Since then, these strains have spread to different parts of the world and could be found in different sources. The occurrence of VRE in Europe was associated with the use of avoparcin (an analogue of the glycopeptide vancomycin) as growth promoter in animal feed for several years (Fisher & Phillips, 2009). Genotypically, vancomycin resistance in enterococci has been associated with nine genes namely; *vanA, vanB, vanC, vanD, vanE, vanG, vanL, vanM* and *vanN* (ÇetİnkayaEtAl., 2013). These genes confers resistance to vancomycin through the removal of the terminal residue of the peptidoglycan precursor D-alanine which is replaced either by D-lactate (D-lac) for *vanA, vanB* and *vanD*, or v-serine (D-ser) for *vanC, vanE* and *vanG* (Périchon & Courvalin, 2009a). With the exception of *vanC* which is intrinsic in some *Enterococcus* species most of these genes are acquired. *vanA* and *vanB* are the most common variants associated with clinical enterococcal infection. The van alphabet in vancomycin resistant enterococci is described in Table 1. VanA is the most common phenotype of resistance usually located on plasmid and carried on *Tn1546* transposon that is transferable to other enterococcus species (Périchon & Courvalin, 2009b). It is the most predominant phenotype responsible for VRE nosocomial infections. On the other hand, VanB phenotype is chromosomally mediated, inducible and transferable by conjugation. *vanA, vanB, vanC-1* and *vanC2/3* genes are usually isolated from clinical enterococcal isolates (GetachewEtAl., 2009).

Table 1: The "Van Alphabet" (Phenotypes and Genotypes of vancomycins resistant enterococci).

Phenotype	**Genotype (Gene clusters)**	**Vancomycin Resistances**	**Teicoplanin Resistance**	**Type of Resistance**
VanA (common in *E. feacalis* and *E. faecium*)	*vanA* gene cluster	High-level resistance MIC- 64µg- >1000 µg/mL	High-level resistance MIC-16 –512 µg/mL	High level inducible resistance
VanB(common in *E. feacalis* and *E. faecium*)	*vanB* gene cluster	High-level resistance MIC-4- 512 µg/mL	Sensitive MIC≤ 0.5 µg/mL	High level inducible resistance
VanC (*E. flavences*, *E. gallinarum* and *E. casselifavus*)	*vanC1*, *vanC2* and *vanC3* gene clusters	Low level resistance MIC-2-32 µg/mL	Sensitive MIC≤ 0.5 µg/mL	Low level constitutive resistance
VanD	*vanD* gene cluster	Moderate –High level resistance MIC-64 – 256 µg/mL	Low level Resistance- MIC -4-32 µg/mL	Inducible resistance
VanE	*vanE* gene cluster	Low-level resistance MIC-16 µg/mL	Sensitive MIC≤ 0.5 µg/mL	Inducible resistance
VanG	*vanG* gene	Low-level resistance MIC-16 µg/mL	Sensitive MIC≤ 0.5 µg/mL	Inducible resistance
VanL	*vanL* gene cluster	Low-level resistance MIC-8 µg/mL	Sensitive	Inducible resistance
VanM	*vanM* gene	High-level resistance MIC> 256 µg/mL	High level Resistance	Inducible resistance
vanN	*vanN* gene	Low-level resistance MIC-16 µg/mL	Sensitive MIC≤ 0.5 µg/mL	Constitutive resistance

2.9 VIRULENCE AND PATHOGENESIS OF ENTEROCOCCI

Although enterococci are commensals of the GIT, their ability to exchange genetic material with other closely related bacteria allows them to acquire some antibiotic resistance genes making them virulent. Therefore, their ability to cause infections and diseases in humans and animals could be explained by the presence of virulent traits which allow the bacteria to colonise and invade host tissues facilitated by their ability to adhere to host surfaces like the GIT, epithelial cells and extracellular protein (Fisher & Phillips, 2009). The functions of these virulent traits have been highlighted in previously studies. Jurkovic *et al.* 2006 detected three viruelent genes from *E. faecium, E. durans* and *E. faecalis*, isolated from bryndza cheese. Of the 308 species isolated, twenty *E. faecalis* isolates habour the *gelE* gene, *E. faecalis* species had five of the cytolysin genes and four had the *agg* genes. Also other virulence genes coding for adhesion (*ace, efaAfs*), gelatinase (*gelE*), aggregation substances (*agg*) and cytolysin (*cylA,cylB, cylL,cylM*) were detected in the majority of *E. faecalis* isolated from retailed meat in Alberta Canada (Aslam *et al.*, 2012). Virulence factors associated with pathogenicity in enterococci include aggregation substances, enterococcal surface protein, gelatinase, cytolysin operon, *Enterococcus faecalis* antigen, *Enterococcus faecium* antigens and hyaluronidase (Dahlén *et al.*, 2012; Eaton & Gasson, 2001b; Vankerckhoven *et al.*, 2004).

2.9.1 AGGREGATION SUBSTANCES

These are pheromones-inducible surface protein which assists in aggregate formation conjugation to enable the transfer of plasmid. It facilitates the adherence of enterococci with other bacteria or eukaryotic cell surfaces and shown to increase valvular vegetation mass in an animal model of endocarditis (Eaton & Gasson, 2001a). They are encoded by *agg* gene which are located on the plasmids (Hällgren *et al.*, 2009).

2.9.2 ENTEROCOCCAL SURFACE PROTEINS

These are cell wall associated surface proteins, predominantly common among clinical isolates (Shankar *et al.*, 1999). It is encoded by *esp* gene carried on the chromosome and is believed to promote colonization, adhesion and evasion of the immune system. In addition, they play a significant role in antibiotic resistance, biofilm formation and resistance to environment stress (Foulquié Moreno *et al.*, 2006; Hällgren *et al.*, 2009). Interference with the *esp* genes has shown to impair the ability of production of biofilm (Latasa *et al.*, 2006).

2.9.3 CYTOLYSINS

Enterococci are known to produce Cytolysins or hemolysins toxins which exhibit haemolytic properties against eukaryotic cells (horse, rabbit and humans erythrocytes) and bactericidal against other Gram positive bacteria (Fisher & Phillips, 2009). Cytolysins are encoded by a cytolysin operon consisting of five genes: *cylLv -Ls, -M, -B,* and –A. Production of cytolysin significantly aggravate the endocarditis and endophthalmitis in animal models (Vankerckhoven *et al.*, 2004).

2.9.4 GELATINASE

Gelatinase is an extracellular Zn-metalloendopeptidase capable of hydrolysing haemoglobin, gelatin, lactoglobulin, collagen, casein and other bioactive compound (Thurlow *et al.*, 2010). Gelatinase is formed by the *gelE* gene and has been shown to split fibrin, making it important virulence trait among *Enterococcus spp*. In addition, studies have used mouse models to show the contribution of *gelE* in virulence of endocarditis (Thurlow *et al.*, 2010).

2.9.5 HYALURONDINASE

This is a virulence factor found in some strains of *E. faecium* (Vankerckhoven *et al.*, 2004). It is encoded by the *hyl* gene chromosomally located and exhibit same homology as hyalurondinase reported in some Staphylococcus and Streptococcus species (Hynes et al., 2000).

2.9.6 *ENTEROCOCCUS* ENDOCARDITIS ANTIGENS *E. FECALIS* EFAAFS OR *E. FAECIUM* EFAAFM

These are potential virulence determinant reported in *E. fecalis* and *E. faecium*. They are presumed to encode for the production of adhesins-like substances which facilitate the adhesion of enterococcus to surfaces (Vankerckhoven *et al.*, 2004). Enterococci harbouring the gene efaAfs and efaAfm have been isolated from both clinical isolates and food (Eaton & Gasson, 2001a).

2.9.7 COLLAGEN BINDING PROTEIN

These are cell-surface proteins belonging to the microbial surface components recognizing adhesive matrix molecules (MSCRAMM) (Fisher & Phillips, 2009). They are encoded by *ace* genes and may play an important role in the pathogenesis of endocarditis (Koch *et al.*, 2004). The presence and existence of these virulence genes in some strains has made these bacteria to be regarded as pathogenic and infectious. Several diseases in both humans and animals such as bacteraemia, septicaemia, endocarditis, urinary tracts infections and the frequently reported nosocomial infections worldwide have been associated to some pathogenic strains (Ok *et al*., 2009; Bisgaard *et al.,* 2010; Stalker *et al*., 2010).

2.10 DETECTION OF ENTEROCOCCI

2.10.1 CULTURE METHODS

The ability of enterococci to grow under particular conditions and growth requirement has been widely used in the isolation of *Enterococus* from different environments. Due to their significance in environmental, food and clinical samples, the detection and enumeration has become an important issue not only in daily routine practices but also in current research activities. Several culture media have been suggested for the isolation and enumeration of *Enterococcus* from several sources. Choice of media used is usually dependent on the source of isolation of the bacteria. Some commonly used synthetic media include; Brain Heart infusion Agar (BHI agar), Kanamycin Azide Agar (KAA), modified Membrane filter Entercoccus agar (mMF), Bile esculin azide agar (BEA) M-enterococcus agar etc. Culture-based techniques have made it possible to detect these indicator bacteria in water and faeces.

2.10.2 MEMBRANE FILTRATION METHOD

This method is most frequent and routinely used in many research laboratories for the monitoring of water quality. It involves the use of a two-step procedure in which the membrane filter (0.45 µm) is incubated on a selective medium that inhibit the growth of Gram negative bacteria and differentiates enterococci from others. The filter is transferred unto bile esculin azide media and the presence of brown-black precipitate is indicative of enterococci caused by the hydrolysis of aesculin by the enzyme β-glucosidase. By the use of a chromogenic substrate indoxyl β-D-glucoside, EPA has modified this method to a one step making the process easier and more rapid. With the aid of β-glucosidase in the media all *Enterococcus* show blue halo colonies. This method offer several advantages; these includes a lesser time of 24 hours as opposed to 48 hours in other culture methods as well as more distinct colonies easily counted.

2.10.3 USE OF ENTEROLERT MEDIUM

The Enterolert medium contains the substrate 4 methylumbelliferone-β-D-glucoside which is cleaved by β-glucosidase to form the fluorescent product 4-methylumbelliferylone which is seen when exposed UV light of 365 nm allowing for the rapid detection of enterococci from fresh, marine and waste water. This method is very sensitive capable of detecting bacteria at one colony forming unit (CFU)/100 mL within 24 h. It is less laborious and a result obtained within 24 hours and requires very few confirmatory tests.

2.10.4 MOLECULAR METHODS

The genus *Enterococcus* is a complex group of bacteria that has several challenges in the past in their identification and classification. Due to this inherent possibility to misclassify them based on some cultural and biochemical characteristics, molecular methods were initiated to facilitate the identification process. The molecular identification of enterococci is based on the detection and analysis of various gene sequences of the complete genome. Most of the molecular techniques are developed to amplify conserved sequences such as the 16S or the 23S rRNA sequences of the organism.

2.10.4.1 Standard Polymerase chain reaction (PCR).

This technique allows for the production several copies of the original DNA. PCR protocol is part of a routine practice carried out in most research institutes. It involves denaturation of the double stranded DNA, followed by annealing of the primer to the complimentary segment and amplification using DNA polymerase and dNTP to complete the synthesis of the new strand. This process is repeated several times exponentially to obtain several copies of the original DNA. A standard PCR process consists of denaturation, annealing, extension and final extension.

PCR has been used in the confirmation of the genus enterococcus and identification of several *Enterococcus* species from different samples using genus and species specific primers (Jackson *et al.*, 2004; Ke *et al.*, 1999). More so, it has been employed in the screening and amplification of antibiotic resistant genes in some enterococcal isolate like vancomycin, penicillin, gentamycin erythromycin and others.

2.10.5. METHODS OF TYPING ENTEROCOCCI SUBSPECIES

Several molecular techniques have been developed to characterise and type enterococci into subspecies. The choice of the method to be used will depend on what kind of information the results seek to display. The principle behind these methods is based on polymorphism of the whole bacterial genome analysis or DNA pattern. The methods include, Pulsed field gel Electrophoresis (PFGE), Random Amplified Polymorphic DNA (RAPD), Amplified Fragment length polymorphism (AFLP) and Multi-locus sequence typing (MLST). These methods have been employed in several epidemiological studies to differentiate between isolates from food, animal and human using their DNA fingerprints (Lu *et al.*, 2002; Bogaard & Stobberingh, 2000).

2.10.5.1 Pulsed field gel Electrophoresis (PFGE)

Pulsed field gel electrophoresis is a molecular method used in the separation of larger DNA fragments (10 to 800kb) in an electrophoresis with alternating current. This technique involves the use of restriction enzymes such as *SmaI* and *ApaI* capable of cutting through purified genomic DNA at restriction sites. The digested products can be electrophoresed in an alternating electric field producing band pattern or DNA fingerprint unique to the DNA of the isolates. This method has a high discriminatory power and is a suitable tool that has been used in most epidemiological studies. In addition, it has been applied in the interpretation of inter-strains relationship among enterococci from different sources. PFGE was used to sub characterise *E. faecalis* and *E. faecium* isolates and to screen for virulence genes (Hällgren *et al.*, 2009).

2.10.5.2. Random Amplified Polymorphic DNA (RAPD PCR)

This technique is based on the same operational procedure as polymerase chain reaction (PCR). However there are slight modifications; with RAPD a short single and arbitrary synthetic oligonucleotide primer is used to serve as both forward and reverse primer. This primer is able to anneal at multiple sites along the genome during the PCR. The agarose gel electrophoresis displays a spectrum of amplification products that is unique to the DNA template used. RAPD is also an important tool that can be used in epidemiological studies. This technique is mostly utilised in the detection of lactic acid bacteria strains from different environmental sources (Hummel *et al.*, 2007; Riboldi *et al.*, 2008). In addition, it has also been employed in the identification and subtyping of some enterococci strains from various sources. The major limitation of this method is that they is lack comparisons of the DNA band pattern of bacteria species from different laboratories.

2.10.5.3 Amplified Fragment Length Polymorphism (AFLP)

This technique is based on the digestion of the targeted genomic DNA with two restriction enzymes, the amplification of the restricted fragments using PCR and analysis of the amplified fragments using gel electrophoresis. It can be used in the typing of enterococcal sub species.

2.10.5.4 Multi-locus sequence typing (MLST)

This molecular technique involves the characterization of bacteria species using their internal fragments (450-500 bp in length) consisting of seven housekeeping genes. The main concept of this technique is the identification of the genetic variation in the sequence internal fragments of the house keeping genes. For each house-keeping gene, the different sequences present within a bacterial species are assigned as distinct alleles and, for each isolate, the

alleles at each of the seven loci define the allelic profile or sequence type (ST). Therefore, isolate of a species can be unambiguously characterised by a series of seven integers which correspond to the alleles at the seven house-keeping loci. MLST has aid in resolving the confusion in genotype among bacteria species based on the difference in their distinct allelic profile. It has greater advantage over other molecular methods such as RAPD and PFGE in that the allelic profile can be compared with data from a central database. MLST was used in an epidemiological study in Malaysia to determine the genetic relatedness of enterococcal isolates from hospitals (Weng *et al.*, 2013).

The Amatole District is largely a rural settlement with many commercial animals' and dairy farms. The use of antimicrobial to prevent diseases, facilitate growth and consequently mange productivity is a general farming practice employed in most farms within this region. In addition, most streams or rivers which supply these farms are faced with pollution from unknown sources. This study provides information on the potential sources of water contamination. Secondly, the antibiotic and virulence profiles of enterococci isolated from these sources are known which intend provides a potential risk involve if this organisms gets into the human population.

CHAPTER THREE

MATERIALS AND METHODS

3.1 SAMPLING AREA

This study was conducted in three selected commercial dairy cattle farms; Fort Hare dairy trust, Middle drift dairy trust located in Nkonkobe local municipality and Seven Star dairy trust located in the Amahlathi local municipalities. These farms were selected based on their large fresh milk production capacity. Both local municipalities are found under the Amathole District which is one of the seven districts in the Eastern Cape Province of South Africa. The Amathole District is made up of eight local municipalities namely Amahlathi, Great Kei, Mbhashe, Mnquma, Nkonkobe, Nxuba, Buffalo city and Ngqushwa. It has an estimated population of 892,637. Figure 2 is a map indicating sample collection areas in the two local municipalities in which the selected commercial farms are located. Fresh milk from these farms is transported to Clover for further processing and distribution to shops. The various capacities of these commercial dairy farms are listed in Table 2.

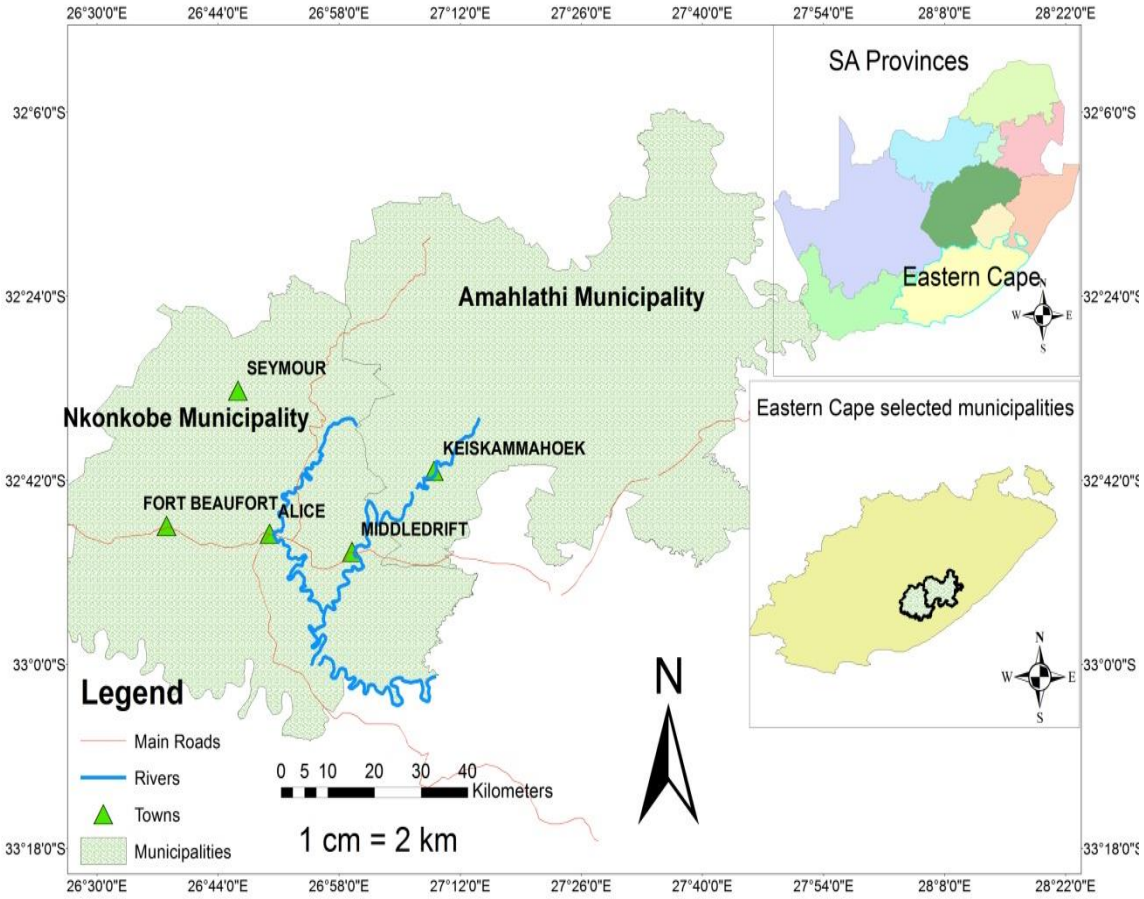

Figure 2: Map showing the two municipalities where this study was carried out

Table 2: Description of the selected farms

DAIRY FARMS	LOCATION (GIS)	LOCAL MUNICIPALITY	NUMBER OF COWS	MILK PRODUCTION/ DAY
Fort Hare dairy trust	32° 47' 0" South, 26° 50' 0" East of Alice town	Nkonkobe	800	1000 L/day
Middle drift dairy trust	32° 49' 0" South, 26° 59' 0" East of Middledrift town	Nkonkobe	600	3280 L/day
Seven star trust	Keiskammahoek town	Amahlathi	400	2000 L/day

3.2 SAMPLING SITES

Samples were collected from three different water facilities (irrigation water, drinking water and waste water) (Figure 3) and from the rectum of the cattle during the process of milking. The farms are arranged in a similar pattern whereby irrigation water (stream) is located downhill (Fort Hare and Middledrift dairy Trusts) and wastewater facilities are located uphill. For Seven Star dairy trusts the irrigation water is piped from a distant stream into a collection chamber from which it is then use for irrigation purposes. Drinking water in all three farms is gotten from piped borne water filled into the trough regular by an automated system. These water facilities were common within the farms and hence provided an appropriate basis to compare the water quality from these sources.

IRRIGATION WATER DRINKING WATER WASTE WATER

Figure 3: Different water sources within the commercial farms

3.3 STANDARD MICROBIOLOGICAL PRACTICES

Faecal and water samples were collected using clean sterile sample containers and arm-length gloves. All media were prepared following manufacturers instruction. Culture media and distilled water were sterilised by autoclaving at 121°C for 15 minutes. All glassware were washed with detergents, rinsed with water and dried at room temperature. Working surfaces were kept clean and disinfected with 70% alcohol before and after work. Inoculation and

other procedure that requires aseptic condition were done in the biosafety cabinet level II. For inoculation, wire loop was flamed until red-hot and then allowed to cool before use.

3.4 SAMPLE COLLECTION

Rectal swabs were collected from dairy healthy cattle during the process of milking and irrigation water, waste water and drinking water also sampled. A total of two hundred and eighty-nine (289) rectal swab samples and forty-five water samples from the different water sources (water from drinking trough, irrigation water (stream or river used for irrigation farming in the farm) and wastewater collection chamber) were collected. Samples were collected over a period of three months (July to September). Water samples were collected in sterile cap scroll bottles, whereas faeces were collected with sterile swabs sticks placed in special cooler boxes at 4^{o}C and transported immediately to the laboratory at the University of Fort Hare for analyses.

Of the 334 samples from Fort Hare dairy trust (FDT), Middledrift dairy trust (MDT) and Seven Star dairy farm (SDT), 289 were of faecal origin consisting of 86 FDT, 101 MDT and 102 SDT and 45 of water origin (drinking water (DW), irrigation water (IW) and wastewater (WW) from within the three farms shown in Table 3.

Table 3: Number of samples collected from each farm from different sources

Sampling Area	Rectal swabs (RS)	Drinking water (DW)	Irrigation water (IW)	Wastewater (WW)	Total samples
Fort Hare Dairy Trust	86	5	5	5	101
Middledrift Dairy Trust	101	5	5	5	116
Seven Star Dairy trust	102	5	5	5	117
TOTAL	289	15	15	15	334

3.5 ISOLATION OF ENTEROCOCCI

On arrival in the laboratory, rectal swab sticks were suspended in sterile nutrient broth and incubated at 37^{o}C for 24 hours. Thereafter an inoculating loop was used to streak from broth onto bile aesculin azide agar plates and incubated for 48 hours at 37^{o}C. Water samples were not enriched; rather they were thoroughly mixed by agitation to avoid sedimentation of the bacteria prior to inoculations on bile aesculin agar plates. Waste water was serially diluted up to 10^{-4}. One hundred microliters of the water sample both the diluted and the undiluted was then inoculated onto bile aesculin agar plates by spread plate method using a spreader and incubated for 48 hours at 37^{o}C. All plates were observed for characteristics black colonies. Discrete presumptive isolates on the agar plates were further streaked on another bile aesculin azide agar plates for purity. All presumptive isolates were processed for confirmatory biochemical and genotypic analyses.

3.6 GRAM STAINING

This was performed as described by Cheesbrough, (2000) to distinguish between Gram positive and Gram negative cocci.

3.7 OXIDASE TEST

This test was done as described by Cheesbrough (2000). Isolated colony from a pure overnight culture plate was picked and rubbed onto the oxidase strip paper and observed for colour change.

3.8 GENOTYPIC CHARACTERIZATION

3.8.1 DNA EXTRACTION

DNA extraction was done as described by Gomez-Duarte, (2009). Overnight broth cultures were centrifuged at 13000 rpm for 10 minutes and supernatant discarded. The pellets were re-suspended in sterile distilled water and well mixed by votexing using a minishaker (Digisystem Laboratory Instruments Inc., New Taipei City, Taiwan). The cells were then lysed by heating for 10mins at 100^{o}C in a MS2 a Dri-Block DB.2A (Techne, SA). The bacteria suspensions were centrifuged at 13 000 rpm for 5 minutes to pellet the cell debris. Thereafter, the lysate supernatant were removed and aliquoted into two sterile 1.5 mL microcentrifuge tubes; one stored at -20^{o}C as backup while the other at 4^{o}C and was used for agarose gel electrophoresis and PCR.

3.8.2 AGAROSE GEL ELECTROPHORESIS

This was performed to detect the presence of DNA from the isolates after extraction and after each PCR amplification. Five microliters of DNA from the isolates were mixed with one microlitre of loading dye (bromophenol blue and xylene cyanol FF; thermoscientific) and loaded into the wells of a 1.5% agarose gel containing 5 µL ethidium bromide. The gels were run in a 1× TAE buffer (40 mM Tris-HCl, 20 mM Na-acetate, 1 mM EDTA, pH 8) and in the case of post PCR separations; 5 µL of 100 bp DNA ladder was included in the gel as a molecular standard. The DNA was then electrophoresed at 100volts for 45 minutes and visualized in a transilluminator (Alliance 4.7 XD-79 System, Uvitec, Cambridge, UK).

3.8.3 MOLECULAR CHARACTERISATION OF ENTEROROCOCCI

The purified DNA was used as a template for the molecular confirmation of *Enterococcus spp*, speciation (using species-specific primers), screening of virulent and antibiotic resistant genes and determination of genetic relatedness of the isolates (by RAPD PCR).

3.8.3.1 CONFIRMATION OF ENTEROCOCCI

This was done by amplifying the genus specific *tuf* gene as previously described (Ke *et al.*, 1999). A 25µl PCR reaction was made comprising of 12.5µL mastermix, 0.5µL of each primer (forward and reverse), 6.5µL of nuclease free water and 5µL of bacterial DNA. Reaction conditions consisted of initial denaturation at 94^{o}C for 2 minutes, followed by 30cycles of amplification at 94^{o}C for 30 seconds each annealing at 55^{o}C for 15 seconds, extension at 72^{o}C for 30 seconds and final extension 72^{o}C for 4 minutes. PCR products were resolved on 1.5% agarose gels as earlier described (section 3.6.2) Primers for confirmatory identification of the genus *Enterococcus* are shown in Table 4.

3.8.3.2 SPECIATION OF CONFIRMED ENTEROCOCCI ISOLATES

PCR was conducted to identify species of *Enterococcus* present among the confirmed isolates using species specific primers as described by Jackson *et al.*(2004) shown in Table 5. PCR ingredients include; 12.5µL KAPA taq mastermix, 1µL of each primer (forward and reverse) and 5.5µL nuclease free water and 5µL DNA template. Following an initial denaturation at 95 °C for 4 min, products were amplified in 30 cycles of denaturation at 95 °C for 30 s, annealing at 52°C (*E. faecalis*, *E. durans* and *E. casseliflavus*) or 48 °C (for *E. faecium* and *E. hirae*) for 1 min, and elongation at 72 °C for 1 min followed by a final extension at 72 °C for 7 min

Table 4**:** PCR primers used for identification of enterococci

Gene	**Primer (5' – 3')**	**product size (bp)**	**Reference**
tuf gene	F - TACTGACAAACCATTCATGATG R - AACTTCGTCACCAACGCGAAC	112	Ke *et al*. (1999)

Table 5: Primer sequences and expected amplicons sizes of targeted enterococci species.

Targeted species	**accession number**	**Primer sequence (5'-3')**	**Amplicon size (b p)**	**References**
E. faecalis	AJ387912	ACTTATGTGACTAACTTAACC TAATGGTGAATCTTGGTTTGG	360	Jackson *et al*. (2004)
E. faecium	AJ387913	GAAAAAACAATAGAAGAATTAT TGCTTTTTTGAATTCTTCTTTA	215	Jackson *et al*. (2004)
E. durans	AJ387911	CCTACTGATATTAAGACAGCG TAATCCTAAGATAGGTGTTTG	295	Jackson *et al*. (2004)
E. casseliflavus	AJ38790	TCCTGAATTAGGTGAAAAAAC GCTAGTTTACCGTCTTTAACG	288	Jackson *et al*. (2004)
E. hirae	AJ387915	CTTTCTGATATGGATGCTGTC TAAATTCTTCCTTAAATGTTG	187	Jackson *et al*. (2004)
E. gallinarum	AJ387915	GCTAGTTTACCGTCTTTAACG TTACTTGCTGATTTTGATTCG	173	Jackson *et al*. (2004)
E. avium	AJ387906	GCTGCGATTGAAAAATATCCG AAGCCAATGATCGGTGTTTTT	368	Jackson *et al*. (2004)

3.8.3.3 DETERMINATION OF VIRULENCE GENES

Five virulent genes were screened among the confirmed and characterised isolates using specific primers and PCR reaction condition were as previously described by Nallapareddy *et al.*(2000) and Eaton & Gasson, (2001) with slight modification (Table 6).

Table 6: Primers sequences of targeted virulent genes and their expected product sizes running from a 5′ to 3′.

Genes	**Primer sequence (5'-3')** **Forward / Reverse**	**Product size**	**References**
Ace	GAGCAAAAGTTCAATCGTTGAC/ GTCTGTCTTTTCACTTGTTTCT	1003	Nallapareddy *et al.* (2000)
Agg	AAGAAAAAGAAGTAGACCAAC / AAACGGCAAGACAAGTAAATA	1553	Eaton & Gasson (2001)
gelE	ACCCCGTATCATTGGTTT / ACGCATTGCTTTTCCATC	419	Eaton & Gasson (2001)
Esp	TTGCTAATGCTAGTCCACGACC / GCGTCAACACTTGCATTGCCGAA	933	Eaton & Gasson (2001)
cyIM	CTGATGGAAAGAAGATAGTAT/ TGAGTTGGTCTGATTACATTT	742	Eaton & Gasson (2001)

3.9 ANTIBIOTIC SUSCEPTIBILITY TESTING

The disc diffusion (Kirby Bauer) technique was employed as described by CLSI, (2014) using Muller Hinton agar. Twelve antibiotics were used in this study and they include; gentamycin (10 µg), Chloramphenicol (10 µg), tetracycline (30 µg), erythromycin (15 µg), kanamycin, penicillin (10 µg), ciprofloxacin (5 µg), vancomycin (30 µg), streptomycin (10 µg), linezolid, Nitrofurantoin (300 µg) and Quinupristin-dalfopristin (15 µg). These antibiotics were chosen based on their clinical importance in the treatment of enterococcal infections in humans and animals. However, results of the aminoglycosides (kanamycin, gentamycin and Streptomycin) were not interpreted because of the lack of guidelines usually provided by CLSI (2014) and (EUCAST, 2013).

The bacterial inoculum was prepared from an overnight incubated pure culture. A sterile loop was used to pick 4-5 colonies which were emulsified in sterile distilled water in a test tube and the turbidity of the suspension adjusted to 0.5McFarland standard ($1.0x\ 10^8$cfu/ml). The optical density of the standard was monitored on regular basis with a spectrophotometer at lamda-wavelength of 625nm and 1cm light path. Sterile swabs were used to inoculate the bacterial suspension onto Muller Hinton agar plates uniformly. The plates were allowed for 5 minutes. Using a sterile antibiotic disc dispenser machine, antibiotic discs were placed onto the surface of the inoculated agar plates and incubated at 37^oC in an inverted position for 24 hours. After incubation, the plates were examined and diameters of zone of inhibition measured and results interpreted using zones of interpretative criteria for *Enterococcus* (CLSI, 2014) as shown in Table 7.

Table 7: Zone of inhibition Interpretative criteria for *Enterococcus spp*. (CLSI, 2014)

Classes of antibiotics	**Antimicrobial Agent**	**Disk Content (µg)**	**Zone diameter Breakpoints, nearest whole mm**		
			S	**I**	**R**
Beta Lactams	Penicillin	10 U	≥ 15	-	≤14
Glycopetide	Vancomycin	30	≥ 17	15-16	≤14
Tetracycline	Tetracycline	30	≥ 19	15-18	≤14
Macrolides	Erythromycin	15	≥ 23	14-22	≤13
Fluoroquinolones	Ciproflaxacin	5	≥ 21	16-20	≤15
Streptogramins	Quinupristin-dalfopristin	15	≥ 19	16-18	≤15
Oxazolidone	Linezolid	30	≥ 23	21-22	≤20
Nitrofutrans	Nitrofurantoin	300	≥17	15-16	<14
Phenicol	Chloramphenicol	30	≥18	13-17	<12

3.9.1 PREPARATION OF MCFARLAND STANDARD

McFarland standards are suspensions of either barium sulfate or latex particles that allow visual comparison of bacterial density. A 0.5 McFarland is equivalent to a bacterial suspension containing between 1×10^8 and 2×10^8 CFU. 0.5 ml aliquot of 0.048 mol/litre $BaCl_2$ (1.175% wt/vol $BaCl_2$; $2H_20$) was added to 99.5ml of 0.18mol/litre H_2SO_4 (1% v/v) with constant stirring to maintain a suspension. The turbidity of the standard was verified by measuring absorbance and equilibrating to between 0.08 to 0.13 absorbance units for the standard. The barium sulfate suspension in 4-6ml aliquots was transferred into screw-cap tubes of the same size as those used in standardizing the bacterial inoculums. Tubes were sealed tightly and stored in the dark at room temperature. Prior to use, vigorous shaking was done for uniform turbidity. McFarland standard was used to compare the turbidity of the inoculum to that of the McFarland before inoculation unto Muller Hinton Agar plate.

3.9.2 SCREENING FOR ANTIBIOTIC RESISTANT GENES

Based on the results obtained from the susceptibility testing, genes encoding for resistance to particular antibiotics in the isolates were screened using previously reported primer sets synthesised by inqaba Bioteh (Pretoria SA). Targeted genes for amplification included; *tetM, tetO, ermA, ermB and blaZ.* Primer sequences and PCR conditions for the amplification of these genes are listed in Table 8 with minor modifications as previously reported (Duran *et al.*, 2012). PCR amplifications were carried out in a final volume of 25 µl consisting of 5 µl of genomic DNA and 20 µl of PCR reaction mixture (12.5 µl mastermix, 0.5 µl of forward and reverse primer each and 6.5 µl of nuclease free water). The amplification process was as follows; initial denaturation step at 95 °C, for 3 minutes, 30 cycles of amplification (denaturation at 95°C for 30 sec, annealing at 54 °C for 30 sec, and DNA chain extension at 72°C for 30 sec) and a final extension at 72°C for 4 min.

Table 8: Primers used for screening resistant genes

Genes	Primer sequences	Product size (bp)	Reference
tetM	5'-AGT GGA GCG ATT ACA GAA-3' 5'-CAT ATG TCC TGG CGT GTC TA-3'	158	Duran *et al.* (2001)
tetK	5'-GTA GCG ACA ATA GGT AAT AGT-3' 5'-GTA GTG ACA ATA AAC CTC CTA-3'	360	Duran *et al.* (2001)
ermA	5'-AAG CGG TAA ACC CCT CTG A-3' 5'-TTC GCA AAT CCC TTC TCA AC-3'	190	Duran *et al.* (2001)
ermB	5'-CTATCTGATTGTTGAAGAAGGATT-3' 5'-GTTTACTCTTGGTTTAGGATGAAA-3'	142	Duran *et al.* (2001)
blaZ	5'-ACTTCAACACCTGCTGCTTTC-3' 5'-TGACCACTTTTATCAGCAACC-3	173	Duran *et al.* (2001)

3.10 RANDOM AMPLIFIED POLYMORPHIC DNA PCR (RAPD)

All *E. hirae* isolates which constitutes the most abundant species were subjected to RAPD PCR to determine the genetic relatedness of the isolates using a random primer M13R2 (Table 9) with PCR conditions as previously reported by Martin *et al.*(2005) with slight modifications. Two hundred and thirty nine isolates were subjected to the PCR and subsequent analysis toward drawing the phylogenetic tree. The PCR reactions were performed in a total volume of 25µL with cycling conditions as follows: initial denaturation at 94°C for 4 minutes; followed by 40 cycles of amplification (94°C/1 min, 56 °C/30 seconds, 72°C/1 min) and final extension at 72°C/5 minutes. The PCR products were resolved in 1.5% agarose gel stained with ethidium bromide and visualized with UV transluminator (ALLIANCE 4.7). The RAPD-PCR band sizes were measured using a ruler and analysed using online software (www.miru-vntrplus).org where 1 was recorded as the presence of a particular band size and 0 as the absence of bands. The similarity of the banding patterns was determined by the software and clusters were formed by the unweighted pair group method with arithmetic averages (UPMGA).

Table 9: RAPD PCR Primer

Primer	**Nucleotide sequence**	**Targeted gene**	**Reference**
M13R2	GGAAACAGCTATGACCATGA	Random	Martin *et al.* (2005)

3.11 STATISTICAL ANALYSIS

Descriptive statistics in SPPS version 22.0 was used to describe the incidences of enterococci, antibiotic susceptibility pattern and virulence genes in the isolates obtained from the three dairy farms.

To calculate the incidences of the species as well as resistant and virulent genes amongst the isolates the following formula was used.

$$\text{Incidences} = \frac{\text{Number of positive isolates}}{\text{Total number of isolates}} \times 100$$

CHAPTER FOUR

RESULTS

4.1 PREVALENCE OF ENTEROCOCCI IN THE THREE COMMERCIAL FARMS

All 289 faecal samples collected from the three farms showed positive growth on bile aesculin azide agar plates with presumptive black colonies whereas only 24 water samples were positive for enterococci (Table 9).

Table 10: Total faecal and water samples, total presumptive isolates and confirmed isolates from the three farms

Samples	Presumptive isolates	Confirmed isolates (%)
Faecal samples 289	289	283 (97.9)
Water samples 45	24	22 (100)
TOTAL 334	**313**	**305 (97.4)**

4.2 MOLECULAR CONFIRMATION OF ENTEROCOCCI

Molecular confirmation by PCR amplification of the *tuf* gene revealed a 97.45% (305/313) of *Enterococcus* species. Figure 4 is a gel image showing some of the confirmed *Enterococcus* isolates.

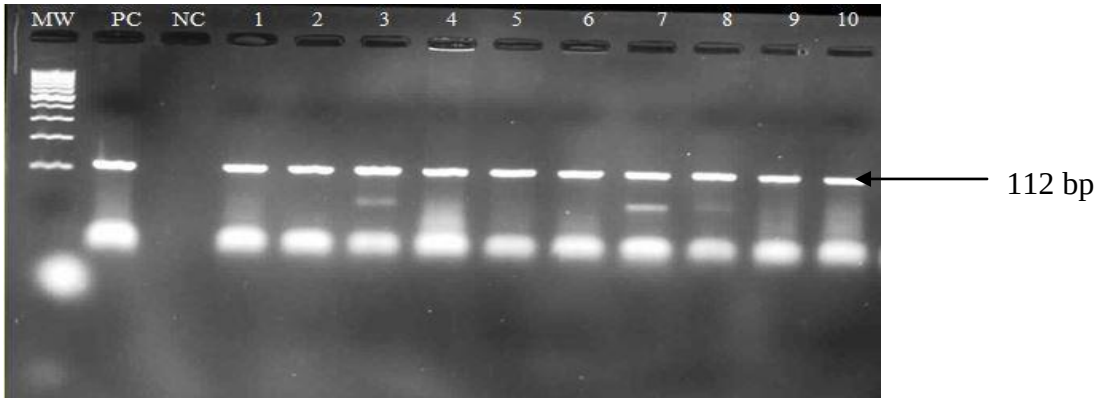

Figure 4: Agarose gel electrophoresis of PCR products of *tuf* gene. MW ladder (100 bp); PC (positive control, ATCC 19433); NC (negative control); and Lanes 1-10 (test isolates).

Out of the 101,116 and 117 samples collected from the farms, 90.1%, 92.3% and 91.5% were confirmed as *Enterococcus* species from Fort Hare dairy, Middledrift Dairy and Seven star dairy respectively (Table 11). All 24 water presumptive isolates collected were confirmed positive by PCR.

Table 11: Percentage of confirmed *Enterococcus spp*

Commercial dairy farms	**Number of samples**	**Percentage of confirmed isolates (%)**
FDT	101	91/101 (90.1)
MDT	116	107/116 (92.3)
SDT	117	107/117 (91.5)
TOTAL	334	305/334 (91.3)

FDT, FORTHARE DAIRY TRUSTS MDT, MIDDLEDRIFT DAIRY TRUSTS and SDT, SEVEN STAR DAIRY TRUSTS

4.3 ENTEROCOCCI SPECIES DISTRIBUTION

Of the 305 confirmed isolates from the three commercial farms (FDT, MDT and SDT), 239 isolates consisted of *E. hirae* (78.36%), 15 of *E. faecium* (4.92%), 12 of *E. durans* (3.93), 6 of *E. faecalis* (1.92%) and 33 were unidentified (10.82%). Table 12 describes the species distribution based on the sources of isolation from the farms and Figures 5, 6, 7, and 8 below show bands of some positive results obtained.

4.3.1 *Enterococcus hirae*

Out of the 305 confirmed isolates 78.36% (239) were identified as *E. hirae*. A representative gel picture of some of the positive isolates is shown in Figure 5.

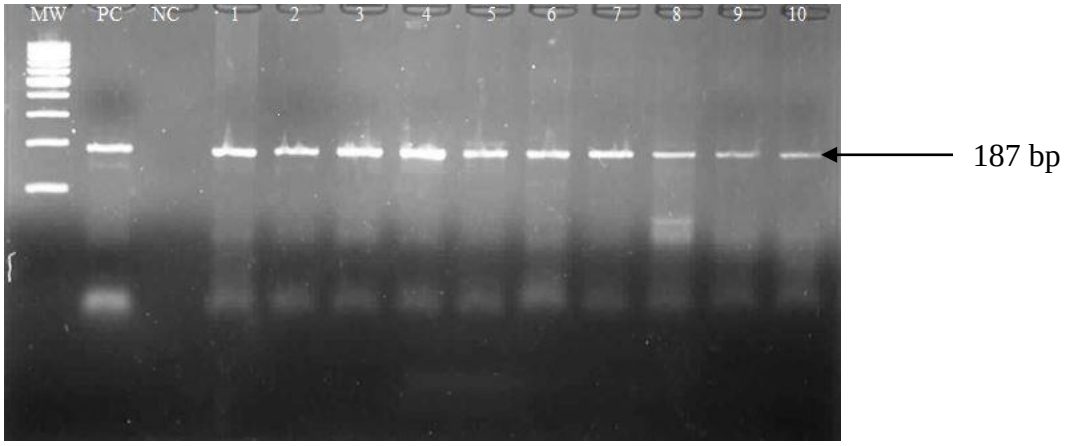

Figure 5: Agarose gel electrophoresis of PCR products of some positive *E. hirae* amplification**.** MW ladder (100 bp); PC (positive control); NC (negative control); and Lanes 1-10 (test isolates).

4.3.2 *Enterococcus faecium*

Out of the 305 confirmed isolates, 4.92 % (15) were identified as *E. faecium*. A representative gel picture of some of the positive isolates is shown in Figure 6.

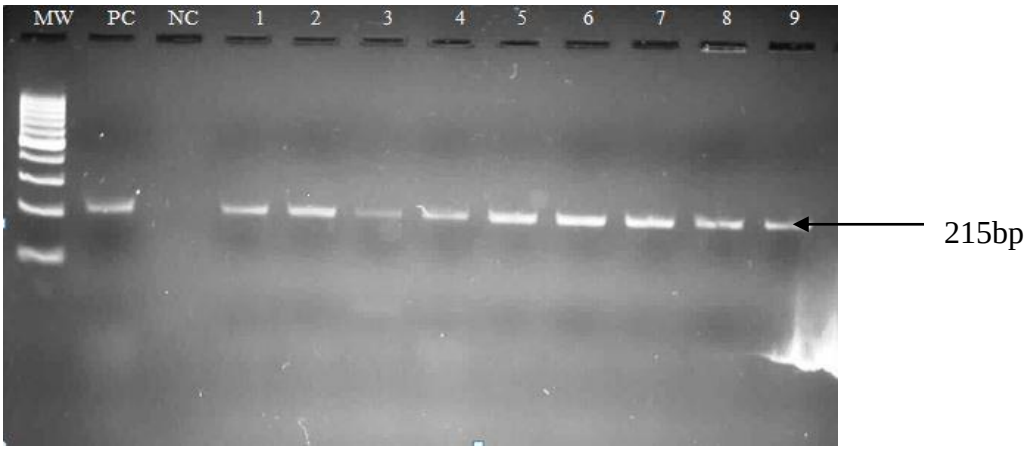

Figure 6: Agarose gel electrophoresis of PCR products of *E. faecium* amplification**.**
MW ladder (100 bp); PC (positive control, ATCC 19433); NC (negative control); and Lanes 1-9 (test isolates).

4.3.3 *Enterococcus durans*

Out of the 305 confirmed isolates, 3.93% (12) were further confirmed as *E. durans* Figure 7 shows some of the confirmed isolates bands.

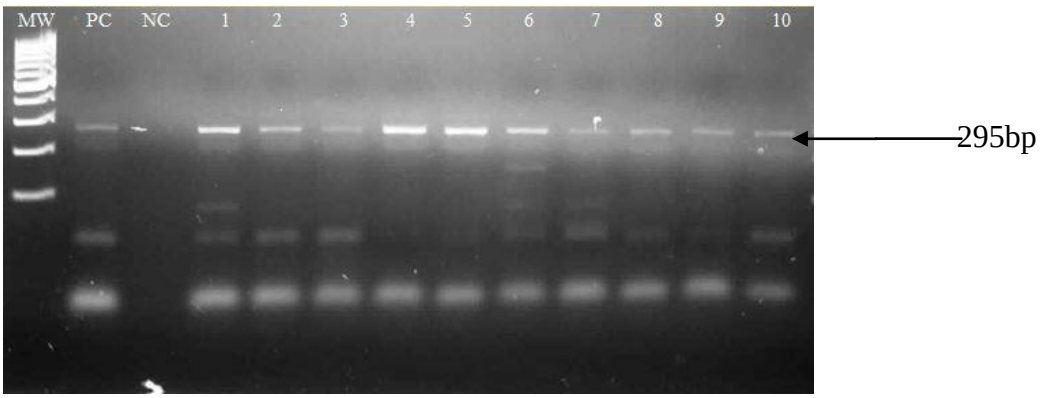

Figure 7: Agarose gel electrophoresis of PCR products of *E. durans* amplification. MW ladder (100 bp); PC (positive control); NC (negative control); and Lanes 1-10 (test isolates).

4.3.4 *Enterococcus faecalis*

Out of the 305 confirmed isolates, only 6 (1.97%) were further confirmed to be *E. faecalis*. Figure 8 shows some of the confirmed isolates bands.

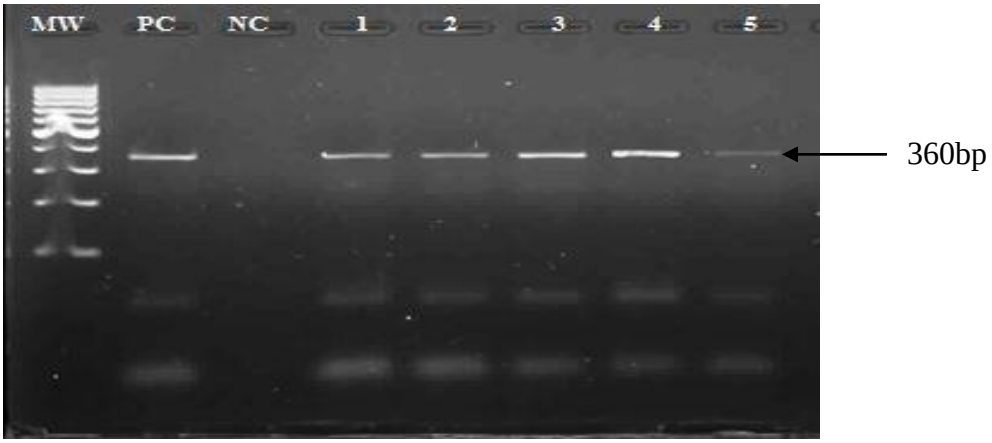

Figure 8: Agarose gel electrophoresis of PCR products of *E. faecalis* amplification.
MW ladder (100 bp); PC (positive control); NC (negative control); and Lanes 1-5 (test isolates).

4.3.5 UNIDENTIFIED ENTEROCOCCUS SPECIES.

Thirty three of the confirmed isolates (10.82 %) were unable to be confirmed for any of the targeted species. Results of the incidences of *Enterococcus* species in the three commercial dairy farms are roughly the same. All species isolated from the sampled sites were common in all three farms. The most predominant species in the three farms from faecal and water samples were *E. hirae*, *E. faecium* and *E durans* (Table 12). We were unable to characterized some of the enterococcocal isolates from faecal and water sources. Therefore they were considered as *Enterococcus* unidentified.

Table 12: Species distribution of enterococci from selected sampled sites within the three commercial farms

Commercial farms	**Sample source**	**No of samples collected**	**Confirmed Isolaates**	***Enterococcus* species**		**Percentage obtained %**
Fort Hare Dairy trust	Rectal Swabs	86	82	*E. hirae* *E. durans* *E. faecium* *E. faecalis* E. unidentified	69 *2* *2* 3 6	84.2 2.4 2.4 3.7 7.3
	Drinking water (DW)	5	1	E. unidentified	1	100
	Irrigation water (IW)	5	3	*E. durans* *E. hirae* E. unidentified	1 1 1	33.3 33.3 33.3
	Wastewater (WW)	5	5	*E. hirae* E. unidentified	4 1	80 20
Middle drift dairy trust	Rectal Swabs	101	101	*E. hirae* *E. durans* *E. faecium* *E. faecalis* E. unidentified	85 3 5 1 7	84.2 3.0 5 1 6.9
	Drinking water (DW)	5	1	E. unidentified	1	100
	Irrigation water (IW)	5	1	E. unidentified	1	100
	Wastewater (WW)	5	4	*E. faecium* *E. hirae* E. unidentified	1 2 1	25 50 25
Seven Star Dairy farm	Rectal Swabs	102	100	*E. hirae* *E. durans* *E. faecium* *E. faecalis* E. unidentified	73 6 7 2 12	73 6 7 2 12
	Drinking water (DW)	5	1	*E. durans*	1	100
	Irrigation water (IW)	5	2	E. unidentified *E. hirae*	1 1	50 50
	Wastewater (WW)	5	4	*E. hirae*	4	100

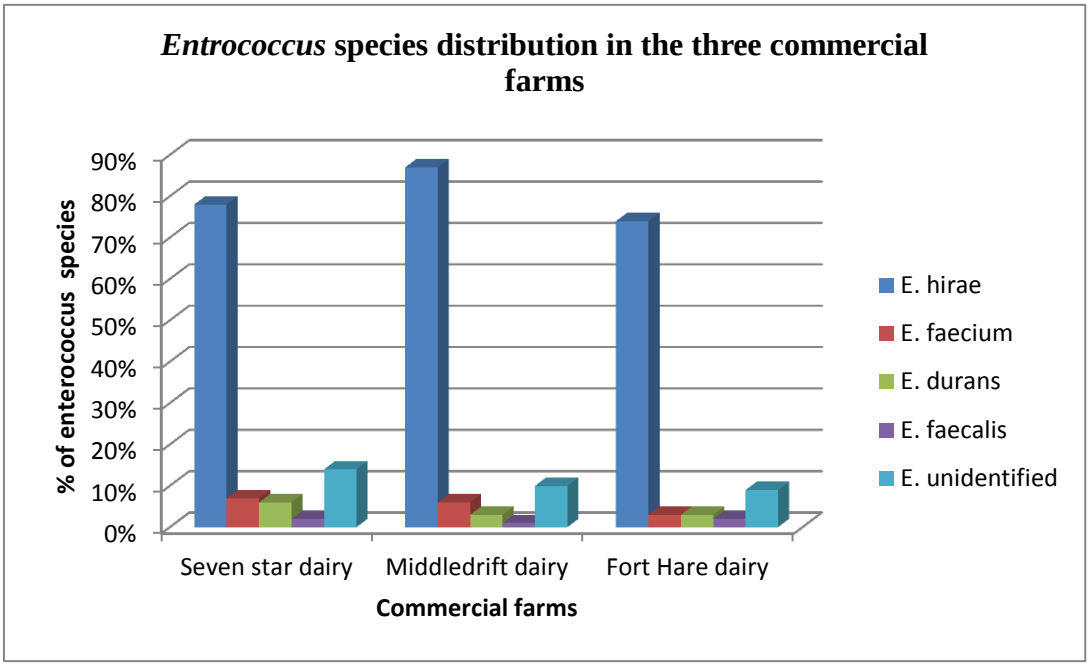

Figure 9: *Entrococcus* species distribution in the three commercial farms

Incidences of *E. hirae* in Middledrift dairy trust were slightly higher than those of Fort Hare and Seven Star dairy trusts (Figure 9). *E. faecium* was slight higher in Seven Star dairy compared to the other farms.

4.4 DETECTION OF VIRULENCE GENES

Of the five virulence genes (*gelE, ace, agg, cylM* and *esp*) that were screened from the 305 enterococcal isolates, only *gelE* and *ace* were amplified (Figures 9 and 10). Generally, there was a high prevalence of *gelE* gene in all three farms compared to the *ace* gene. Also, the results show a slightly higher prevalence in *gelE* genes among isolates from SDT compared to the others farms and higher *ace* genes in FDT enterococcal isolates compared to the other farms (Table 13). Whereas *ace* gene is slightly higher in FDT compared to MDT and SDT.

Table 13**:** Percentage virulent genes in enterococci in the farms

Commercial farms	**Number of isolates**	**Percentage (%) *gelE* gene**	**Percentage(%) *ace* gene**
FDT	91	65/91 (71.4)	41/91 (45.05)
MDT	107	72/107 (67.2)	28/107 (26.16)
SDT	107	82/107 (76.6)	15/107 (14.01)

4.4.1 GELATINASE (*gelE* gene)

In a total of 305 confirmed enterococci isolates, from all three farms, 71.80% (219/305) were observed to harbour the *gel E* gene. Figure 8 shows some of the positive isolates bands.

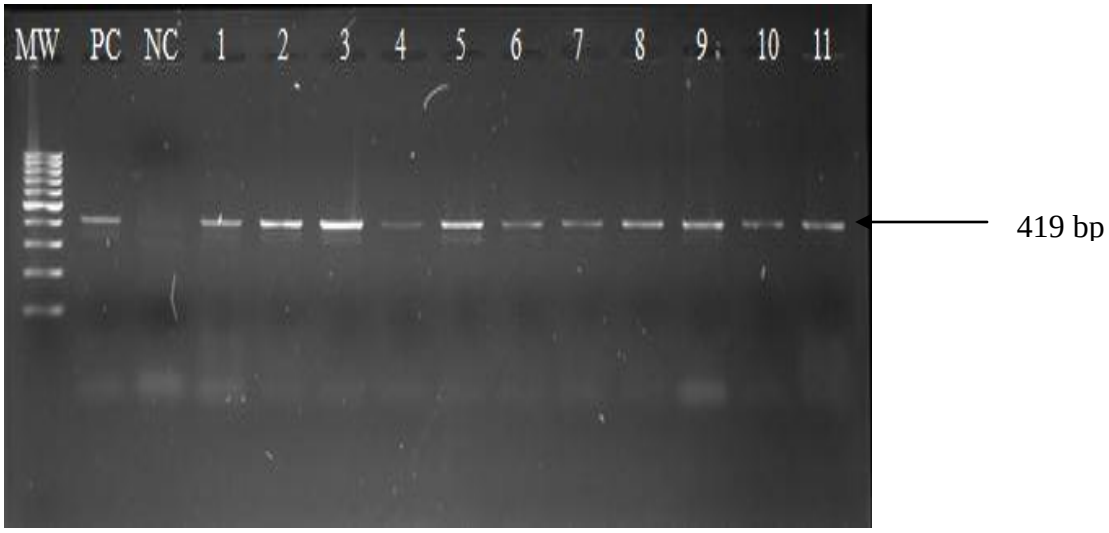

Figure 10: Agarose gel electrophoresis of PCR products of *gelE gene* amplification**.** MW ladder (100 bp); PC (positive control, ATCC 19433); NC (negative control); and Lanes 1-11 (test isolates).

4.4.2 ENTEROCOCCAL SURFACE ADHESION (*ace* gene)

In a total of 305 confirmed enterococci isolates, from all three farms, 27.2% (83/305) were observed to harbour the *ace* gene.

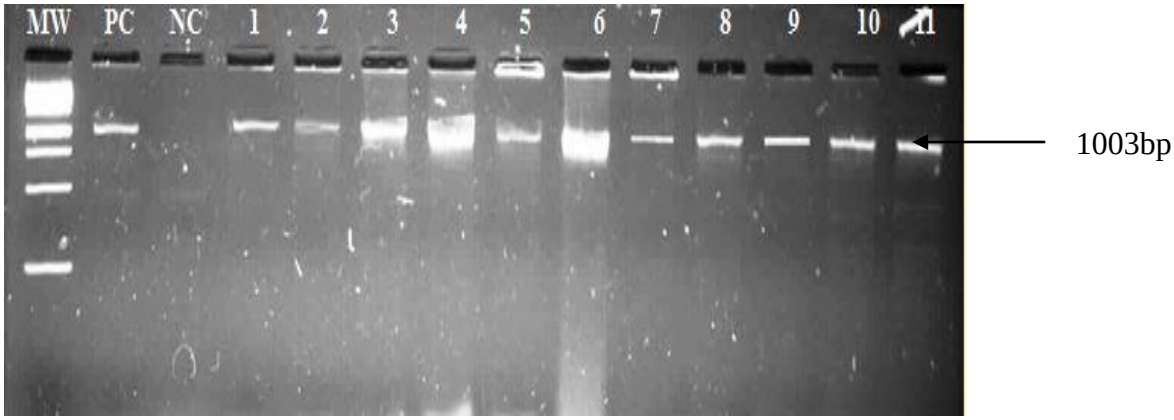

Figure 11: Agarose gel electrophoresis of PCR products of *ace gene* amplification. Lane 1 (1kb MW ladder); Lane 2 positive control (*E. faecalis* ATCC 19433); Lane 3 (negative control); and Lanes 4-12 (test isolates).

4.5 ANTIBIOTICS SUSCEPTIBILITY TESTING

All 305 confirmed isolates were tested against a panel of twelve antibiotics and the results are tabulated based on the three farms in which the study was carried out. Table 14, 15 and 16 summarises the antibiotic susceptibility pattern of *Enterococcus* spp. recovered from Seven Star, Middledrift and Fort Hare Dairy trusts respectively. As mentioned earlier, data on susceptibility pattern to Gentamycin, Kanamycin and Streptomycin in the three commercial farms were not interpreted. However, results obtained show high resistance to these drugs in some isolates (no zones of inhibition) (Appendix 1). The results of the antibiotic susceptibility test on *Enterococcus* species in this section are reflective on the remaining nine antibiotics.

Generally, most enterococcal isolates are susceptible or intermediate to all nine drugs tested, however, some resistances were observed in some isolates to all the nine.

The percentage of *E. hirae* from all three commercial dairy farms which were resistant to either one or more antibiotics is shown in figure 12. The highest resistance was observed in *E. hirae* isolates from Middledrift dairy of 87% and the lowest from Fort Hare dairy trust of 74%.

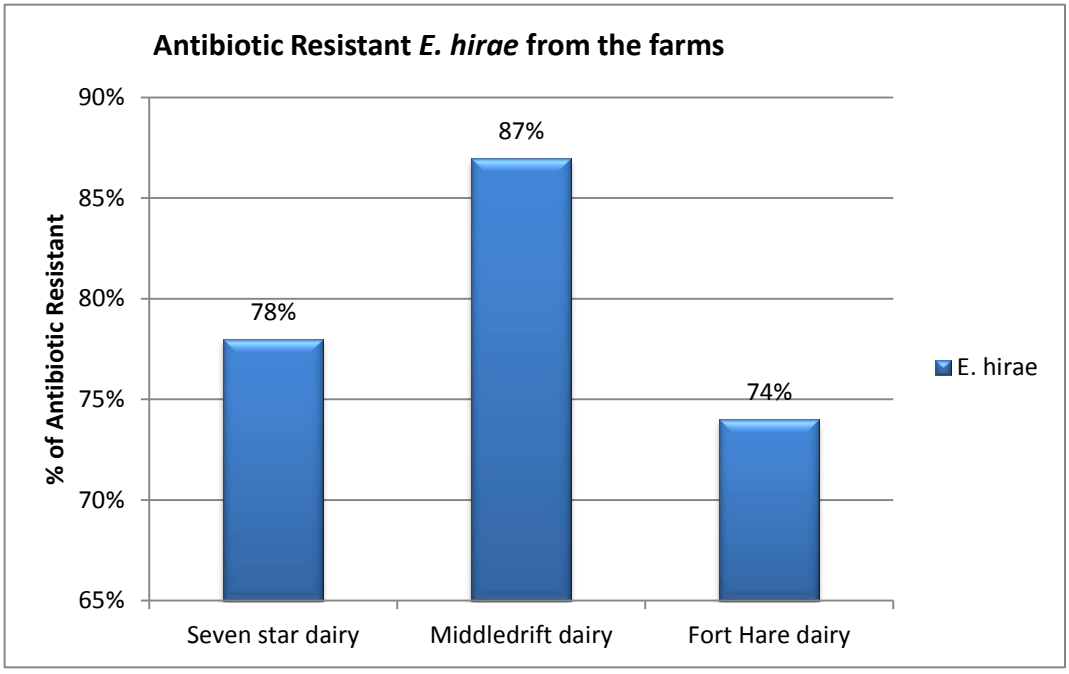

Figure 12: Antibiotic resistant *E. hirae* from the farms

Varied degree of resistances was also observed to either one or more antibiotics in other Enterococcus species. A 6.5% (7/107) of the isolates from Seven Star dairy and 5.6% (6/107) from Middledrift dairy were sensitive to all nine antibiotics tested (appendix 1). No isolate was found in Fort Hare dairy to be sensitive to all nine antibiotics.

All *E. hirae, E. fecaslis, E. durans* and unidentified species isolated from Seven Star dairy trusts were all sensitive chloramphenicol whereas 87.5% of *E. faecium* were sensitive to chloramphenicol. Most isolates were sensitive to ciprofloxacin however minor degrees of resistances were observed among the isolates

Table 14: Antibiotic susceptibility pattern of *Enterococcus* spp. recovered from Seven Star dairy trusts

	Antibiogram percentage (%) response of *Enterococcus species* obtained from (n=107) isolates.														
	***E. hirae* n = 78**			***E. faecium* n= 7**			***E. faecalis* n = 2**			***E. durans n* = 6**			***E.* unidentified n= 14**		
Antimicrobial agents	**R**	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**
Penicillin (10 U)	25.6	0	74.4	28.5	0	71.5	0	0	100	33.3	0	66.7	14.3	0	85.7
Vancomycin (30 µg)	25.6	0	74.4	28.5	0	71.5	0	0	100	33.3	16.7	50	14.3	0	85.7
Ciprofloxacin (15 µg)	1.5	20.6	78.9	0	57.1	42.9	0	0	100	16.7	50	33.3	7.1	0	92.9
Chloramphenicol(10 µg)	0	0	100	14.3	0	85.7	0	0	100	0	0	100	0	0	100
Tetracycline (30 µg)	29.5	0	70.5	42.9	0	57.1	0	0	100	0	0	100	14.3	0	85.7
Erythromycin (15 µg)	19.2	34.6	46.2	28.6	57.1	14.3	0	0	100	16.7	66.6	16.7	7.1	50	42.9
Nitrofurantoin (300 µg)	7.7	6.4	85.9	0	0	100	0	0	100	0	0	100	0	7.1	92.9
Quinupristin /dalfopristin (15 µg)	44.9	20.5	34.6	42.9	28.6	31.5	0	0	100	33.3	16.7	50	28.6	7.1	64.3
Linezolid (30 µg)	32.1	23	44.9	42.9	0	57.1	50	50	0	33.3	0	66.7	28.6	35.7	35.7

Legend: R-Resistant, **I**-Intermediate, **S**-Susceptibility

Table 15: Antibiotic susceptibility pattern of *Enterococcus* spp. recovered from Middledrift dairy trust

	Antibiogram percentage (%) response of *Enterococcus species* obtained from (n=107) isolates.														
	***E. hirae* n = 87**			***E. faecium* n= 6**			***E. faecalis* n = 1**			***E. durans n* = 3**			***E.* unidentified n= 10**		
Antimicrobial agents	**R**	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**
Penicillin (10 U)	11.5	0	88.5	0	50	50	0	0	100	0	0	100	0	0	100
Vancomycin (30 µg)	9.2	3.4	87.4	50	0	50	0	0	100	0	33.3	66.7	0	0	100
Ciprofloxacin (15 µg)	3.4	29.8	66.7	0	33.3	66.7	0	0	100	0	33.3	66.7	0	10	90
Chloramphenicol(10 µg)	2.3	2.3	95.4	0	0	100	0	0	100	0	0	100	0	0	100
Tetracycline (30 µg)	12.6	0	87.4	16.7	0	83.3	0	0	100	0	0	100	0	0	100
Erythromycin (15 µg)	10.3	60.9	27.8	16.7	50	33.3	0	0	100	0	100	0	10	60	30
Nitrofurantoin (300 µg)	4.6	1.1	94.3	0	0	100	0	0	100	0	0	100	0	0	100
Quinupristin /dalfopristin (15 µg)	34.5	17.2	48.3	66.7	0	33.3	0	0	100	66.7	0	33.3	30	10	60
Linezolid (30 µg)	35.6	12.6	51.8	50	0	50	100	0	0	33.3	0	66.7	20	20	60

Legend: R-Resistant, **I**-Intermediate, **S**-Susceptibility

Table 16: Antibiotic susceptibility pattern of *Enterococcus* spp. recovered from Fort Hare Dairy trust

	Antibiogram percentage (%) response of *Enterococcus species* obtained from (n= 91) isolates.														
	***E. hirae* n = 74**			***E. faecium* n= 2**			***E. faecalis* n = 3**			***E. durans n* = 3**			***E.* unidentified n= 9**		
Antimicrobial agents	**R**	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**
Penicillin (10 U)	5.4	0	94.6	0	0	100	0	0	100	33.3	0	66.7	33.3	0	66.7
Vancomycin (30 µg)	6.8	4.1	89.1	0	0	100	0	0	100	33.3	0	66.7	22.2	0	77.8
Ciprofloxacin (15 µg)	5.4	55.4	39.2	33.3	0	66.7	0	50	50	0	0	100	22.2	0	77.8
Chloramphenicol(10 µg)	4.1	5.4	90.5	0	0	100	0	0	100	0	0	100	11.1	0	88.9
Tetracycline (30 µg)	29.7	2.7	67.6	0	0	100	50	0	50	0	0	100	22.2	0	77.8
Erythromycin (15 µg)	18.9	66.2	14.9	0	100	0	0	100	0	0	100	0	22.2	5.6	22.2
Nitrofurantoin (300 µg)	0	0	100	0	0	100	0	0	100	0	0	100	11.1	0	88.9
Quinupristin /dalfopristin (15 µg)	13.5	68.9	17.6	0	0	100	50	0	50	100	0	0	22.2	33.3	44.5
Linezolid (30 µg)	14.8	20.3	64.9	50	50	0	0	33.3	66.7	33.3	0	66.7	22.2	0	77.8

Legend: R-Resistant, **I**-Intermediate, **S**-Susceptibility

Most isolates were resistant to one or more of the 9 antibiotics tested. The highest resistance was observed against quinupristin/dalfopristin (synercid) in isolates from Fort Hare dairy trust 55 of 91 (60.4%), followed by 44 of 107 (41.1%) in Seven star dairy and lastly from 36 of 107 (33.6%) in Middledrift dairy trusts (Table 13). Resistance to linezolid and vancomycin were also observed in isolates from the three farms. About 35.5% of the isolates from Middledrift dairy were resistant to Linezolid compared to 32.7% and 16.5% that were reported in Seven Star and Fort Hare dairy trusts respectively (Table 17). The lowest resistance phenotype to vancomycin was observed in Fort Hare dairy trust and the highest of 24.3% in Seven Star dairy trust. Most of the *Enterococcus species* from faeces and water from the three farms were susceptible to chloramphenicol and ciprofloxacin with only 6 and 11 isolates showing resistance respectively (Table 17).

Table 17: Antibiotic resistance among *Enterococcus* isolates from the three commercial farms

Antibiotics (No of resistant Isolates)	**Seven Star Dairy (n=107)**	**Middledrift Dairy Trust (n=107)**	**Fort Hare Dairy Trust (n=91)**	**Total Percentage of resistance (%)**
Penicillin G (n=44)	26 (24.3%)	10 (9.3%)	8 (8.7%)	14.4
Chloramphenicol (n=6)	1 (0.9%)	2 (1.9%)	3 (3.3%)	2
Linezolid (n=88)	35 (32.7%)	38 (35.5%)	15 (16.5%)	28.9
Erythromycin (n=42)	18 (16.8%)	11 (10.3%)	13 (14.3%)	13.8
Nitrofurantoin (n=10)	6 (5.6%)	3 (2.8%)	1 (1.1%)	3.3
Ciprofloxacin (n=11)	3 (2.8%)	3 (2.8%)	5 (5.4%)	3.6
Vancomycin (n=45)	26 (24.3%)	11 (10.3%)	8 (8.8%)	14.8
Tetracycline (n=66)	29 (27.1%)	12 (11.2%)	25 (25.8%)	21.6
Synercid (n=135)	44 (41.1%)	36 (33.6%)	55 (60.4%)	44.3

Percent resistant was determined by dividing the number of resistant isolates per farm by the total number of isolates per farm.

4.6 MULTIDRUG RESISTANT PHENOTYPE (MDR PHENOTYPE)

Fifty-six multi-drug resistance patterns were obtained in this study from the three commercial dairy farms. The most predominant patterns observed in isolates from Fort Hare and Middledrift dairy trusts was LZD-SYN and for Seven Star dairy is PG-LZD-ERY-VAN-TET-SYN.

Two or more drug resistance (MDR) patterns were observed in most of the resistant enterococcal isolates from all three farms. The most extensive resistance pattern was made up of seven antibiotics *E. faecium* from the Seven Star dairy trust. Different species of enterococci show the same multi-drug resistance pattern to two, three or more antibiotics**.** Nevertheless none of the isolates were resistant to all nine antibiotics tested (Table 18). The largest proportion of multiple drug resistance was observed in *E. hirae* isolates.

Table 18: Multidrug resistant phenotype of enterococci isolates from the three commercial farms

Drug resistance pattern	**No. of resistances**	**Species**	**Total No. observed**	**Commercial farm**
LZD-TET	2	*E. hirae* 2 *E. hirae* 230	2	SEVEN STAR DAIRY
LZD-NIT	2	*E. hirae* 228	1	
TET-SYN	2	*E. hirae* 96,107,108,109	4	
ERY-SYN	2	*E. hirae* 4	1	
LZD-NIT-TET	2	*E. hirae* 245	1	
PG-LZD-ERY-VAN	4	*E. hirae* 47	1	
PG-LZD-ERY-VAN-TET-SYN	6	*E. hirae* 60,98,99,112,113,114, *E.* uniden 105, *E. faecium* 115	8	
PG-LZD-NIT-VAN-TET-SYN	6	*E. hirae* 95	1	
PG-CHL-LZD-ERY-VAN-TET-SYN	7	*E. faecium* 100	1	
PG-LZD-VAN-TET-SYN	5	*E. hirae* 101,102,119 *E.*uniden 104	4	
PG-LZD-NIT-ERY-VAN-TET	6	*E. hirae* 117	1	
PG-LZD-ERY-SYN	4	*E. hirae* 61	1	
PG-LZD-VAN-SYN	4	*E. hirae* 62,116,118, *E. durans* 103	4	
CIP-VAN-SYN	3	*E. hirae 64*	1	
PG-LZD-CIP-VAN	4	*E. hirae 65*	1	
PG-LZD-ERY-VAN SYN	5	*E. hirae* 66,110,111	3	
PG-LZD-NIT-ERY-VAN-SYN	6	*E. hirae* 106	1	
TET-SYN	2	*E. hirae* 145,147	2	
LZD-SYN	2	*E. hirae* 157,168,212,226	4	MIDDLEDRIFT
PG-LZD	2	*E. hirae* 82, 210	2	
LZD-NIT	2	*E. hirae* 81	1	
LZD-ERY-TET	3	*E. hirae* 211	1	
LZD-NIT-SYN	3	*E. hirae* 223	1	
LZD-ERY-VAN	3	*E. hirae* 93	1	
PG-LZD-SYN	3	*E. hirae* 80	1	
PG-LZD-VAN-SYN	4	*E. faecium*, 75,79	2	

PG-LZD-ERY-VAN	4	*E. hirae* 78	1	DAIRY TRUST
PG-LZD-ERY-VAN-SYN	5	*E. hirae 73*	1	
PG-LZD-ERY-CIP-VAN	5	*E. hirae* 86	1	
PG-CHL-LZD-NIT-ERY-VAN-TET-SYN	8	*E. hirae* 88	1	
PG-CHL-LZD-NIT-VAN-SYN	6	*E. hirae* 95	1	
PG-LZD-ERY-VAN-TET-SYN	6	*E. hirae* 87, *E. faecium 92*	2	
ERY-CIP-TET-SYN	4	*E. hirae* 203	1	
TET-SYN	2	*E. hirae* 12,16, 39,41, 129	5	FORT HARE DAIRY TRUST
LZD-SYN	2	*E. hirae* 15,130,131,133,134,135,136	7	
CIP-SYN	2	*E. hirae* 141	1	
VAN-SYN	2	*E. hirae* 37 124	2	
ERY-TET	2	*E. hirae* 200	1	
PG-ERY	2	*E.* uniden 192	1	
ERY-SYN	2	*E. hirae* 26 183	2	
PG-SYN	2	*E.* durans 23, *E. hirae* 43,178	2	
PG-LZD	2	*E.* uniden 308,	2	
TET-SYN	2	*E. hirae* 13,16,39,41,129	5	
PG-LZD-VAN-SYN	4	*E. hirae* 24	1	
PG-LZD-ERY-VAN-SYN	5	*E. hirae* 193	1	
LZD-VAN-SYN	3	*E. durans* 123	1	
LZD-ERY-TET	3	*E.* uniden 121	1	
NIT-VAN	2	*E.* uniden 307	1	
CHL-TET-SYN	3	*E. hirae* 128	1	
CHL-ERY-CIP-SYN	4	*E. hirae* 44	1	
CIP-TET-SYN	3	*E. hirae* 27, 45	2	
ERY-TET-SYN	3	*E. hirae* 32 199	2	
ERY-VAN-SYN	3	*E. hirae* 40, 125	2	
VAN-TET-SYN	3	*E.* uniden 195	1	
LZD-TET-SYN	3	*E. hirae* 35,143	2	

Cip, ciprofloxacin; Ery, erythromycin; Nit, nitrofurantoin; Pen, penicillin; Syn, synercid (quinupristin/dalfopristin); Tet, tetracycline Van, vancomycin; Lzd, linezolid; Chl, chloramphenicol

4.7 AMPLIFICATION OF ANTIBIOTIC RESISTANT GENES

Of the five genes that were targeted using previously published primers, only *blaZ, ermB* and *tetM* were detected in 11.4% (5/44), 26.2% (11/42) and 100% (66/66) of the strains that show phenotypic resistance to the drugs respectively.

4.7.1 PENICILLIN RESISTANT GENE (*BLAZ GENE)*

A total of 11.4% (5/44) of the isolates were positive for the *blaZ gene* and were verified by the generation of a 173 bp amplicon from chromosomal template DNA of isolates 173 bp on agarose gel (Figure 10)

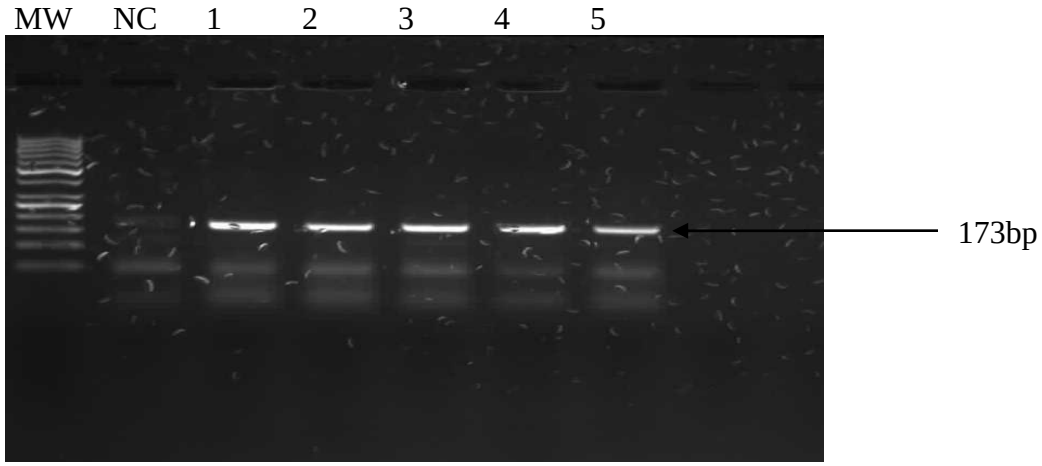

Figure 13: Agarose gel electrophoresis of PCR products of *blaZ* gene amplification. MW ladder (50 bp); NC (negative control); and Lanes 1- 5 (test isolates).

4.7.2 ERYTHROMYCIN *B* RESISTANT GENE (*ERM B* GENE)

Of the 42 isolates that showed phenotypic resistance to erythromycin and screened for *ermB* and *ermA* from the three commercial dairy farms only 11 (26.2%) were positive for *ermB* gene. No amplification was observed for *ermA* gene.

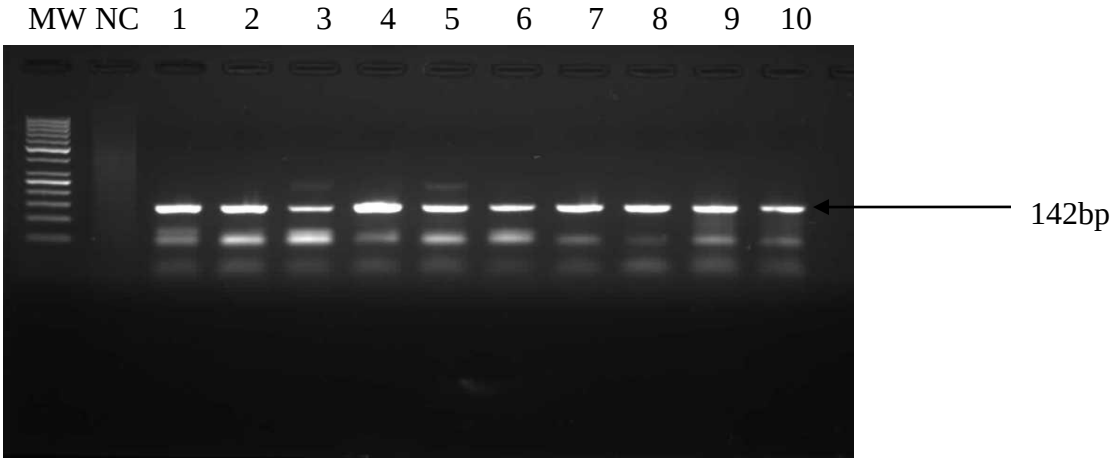

Figure 14: Agarose gel electrophoresis of PCR products of *ermB* gene amplification. Lane 1 MW ladder (50 bp); NC (negative control); and Lanes 1-10 (test isolates).

4.7.3 TETRACYCLINE RESISTANT GENE (*tetM* gene)

Sixty-six isolates showed phenotypic resistance to tetracycline whilst all of the isolates were positive for *tetM* gene (100 %) and negative for *tetK*. Figure 12 is a gel picture showing some of the positive isolates.

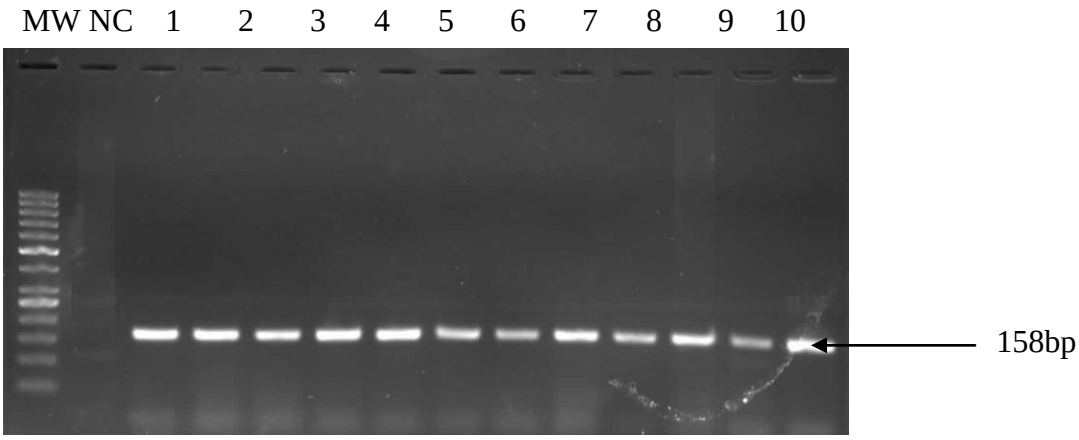

Figure 15: Agarose gel electrophoresis of PCR products of *tetM* gene amplification. MW ladder (50 bp); NC (negative control); and Lanes 1-10 (test isolates)

4.8 RAPD PCR ANALYSIS OF *E. hirae* ISOLATES

Results from the RAPD PCR showed several banding patterns containing multiple amplification products of various sizes ranging from 250 - 480bp. Figure 16 is a representative of some of the different banding patterns that were observed.

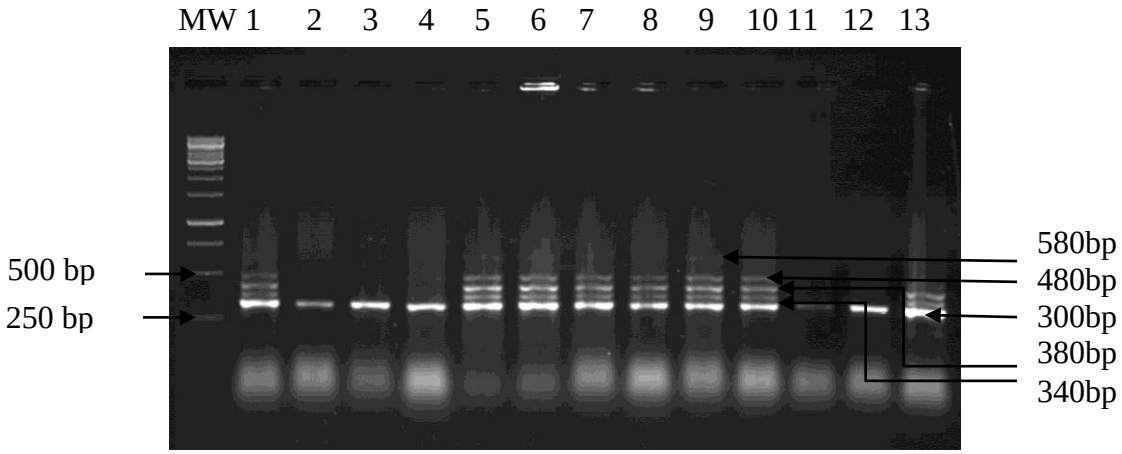

Figure 16: Agarose gel electrophoresis of RAPD PCR products of enterococcal strains. MW ladder (1kb); Lanes 1-13 (test isolates)

Six different banding patterns were obtained (Table 19). Figures 17, 18 and 19 shows the corresponding dendograms that were drawn based on the similarity of the isolates obtained from the dairy farms representing the sources from which they were isolated.

Table 19: Percentage of band patterns in *Enterococcus hirae* from all three farms

Band patterns	**Number of Isolates**	**Percentages**
300bp	95	39.6
300bp, 340bp, 380bp	80	33.5
300bp, 380bp, 420bp	15	6.3
300bp, 380bp	3	1.3
300bp, 340bp	16	6.7
250bp, 300bp	12	5
300bp, 340bp, 380bp, 420bp	12	5
300bp, 340bp, 380bp, 420bp, 480bp	3	1.3
250bp, 300bp, 340, 380bp	3	1.3

Upgma dendogram placed *E. hirae* from seven star dairy trusts into 5 clusters with organisms ranging from 4 to 36 per cluster. Only 2 organisms were not clustered (Figure 17).

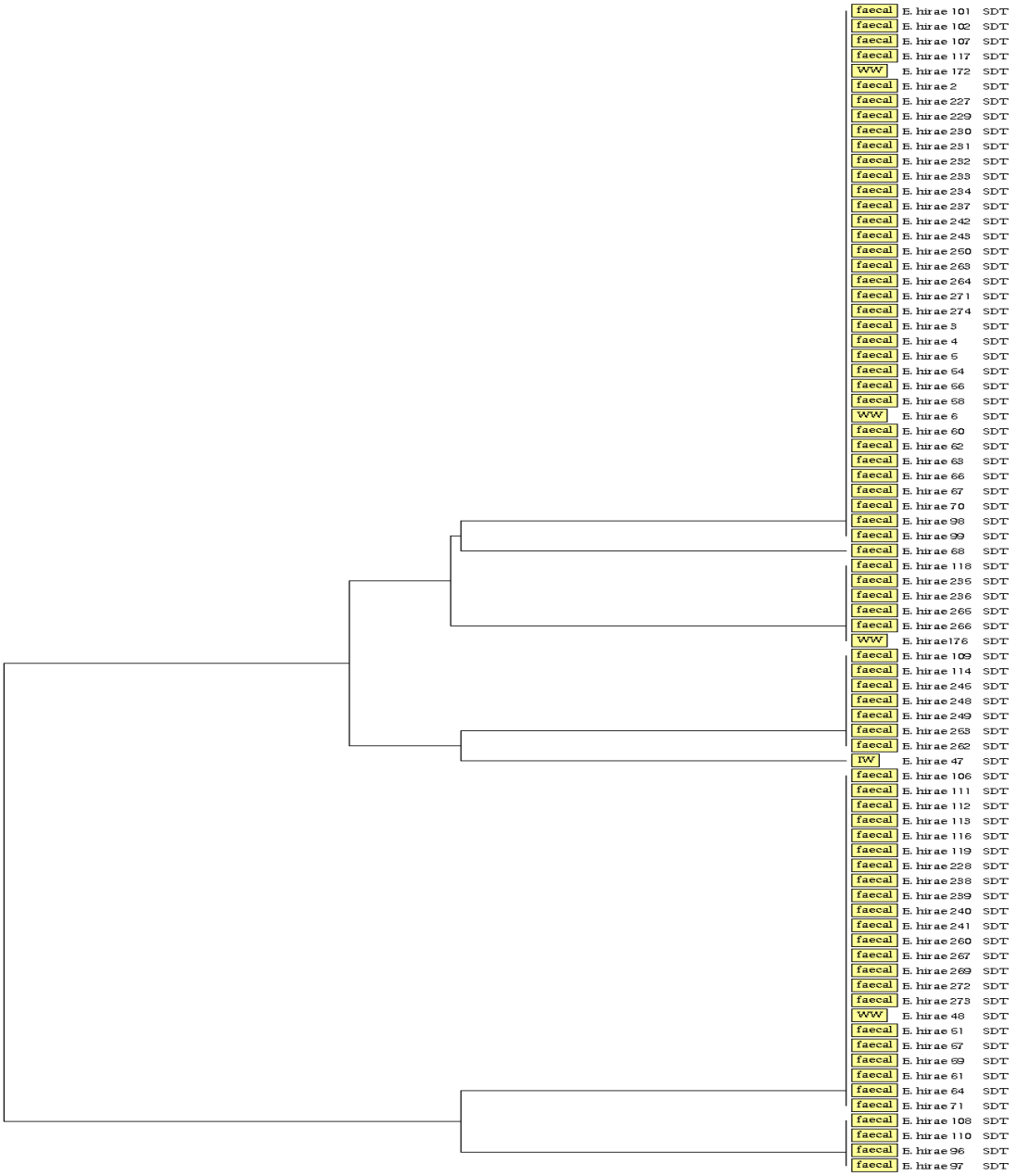

Figure 17: UPGMA dendogram based on the RAPD patterns of enterococcal isolates from faecal and water samples (wastewater, drinking water and Irrigation water) from Seven Star Dairy Trust. This dendogram was constructed with the RAPD patterns of 107 strains using the miru-vntrplus.org program

E. hirae from Middledrift dairy Trust was clustered into 7 clusters with organisms ranging from 2 to 36 per cluster only 2 organisms were not clustered (Figure 18).

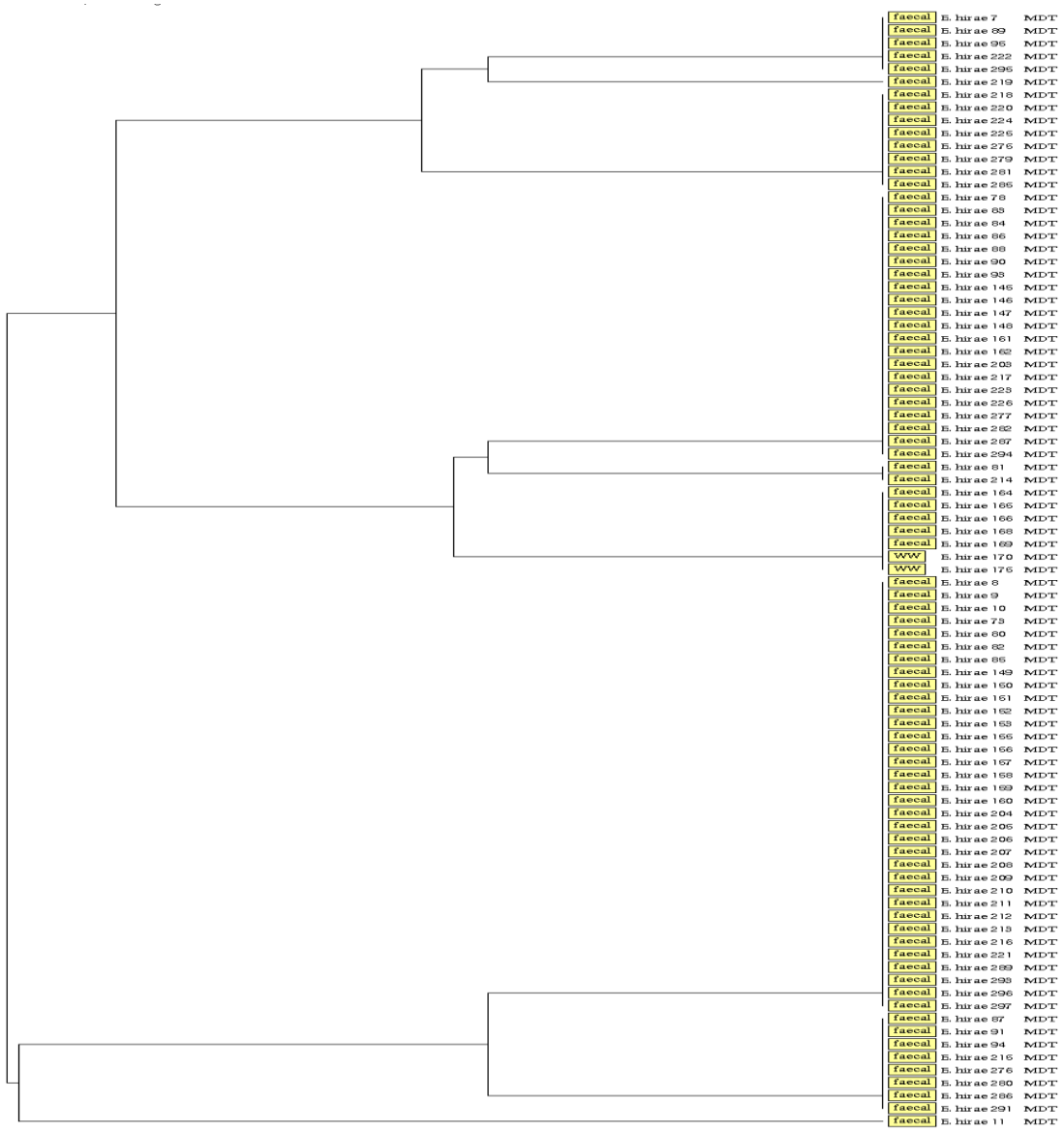

Figure 18: UPGMA dendogram based on the RAPD patterns of enterococcal isolates from faecal and water samples (wastewater, drinking water and Irrigation water) from Middledrift Dairy Trust. This dendogram was constructed with the RAPD patterns of 107 strains using the miru-vntrplus.org programs

Fort Hare dairy Trust *E. hirae* isolates formed 5 clusters having organisms ranging from 2 to 38 per cluster (Figure 19).

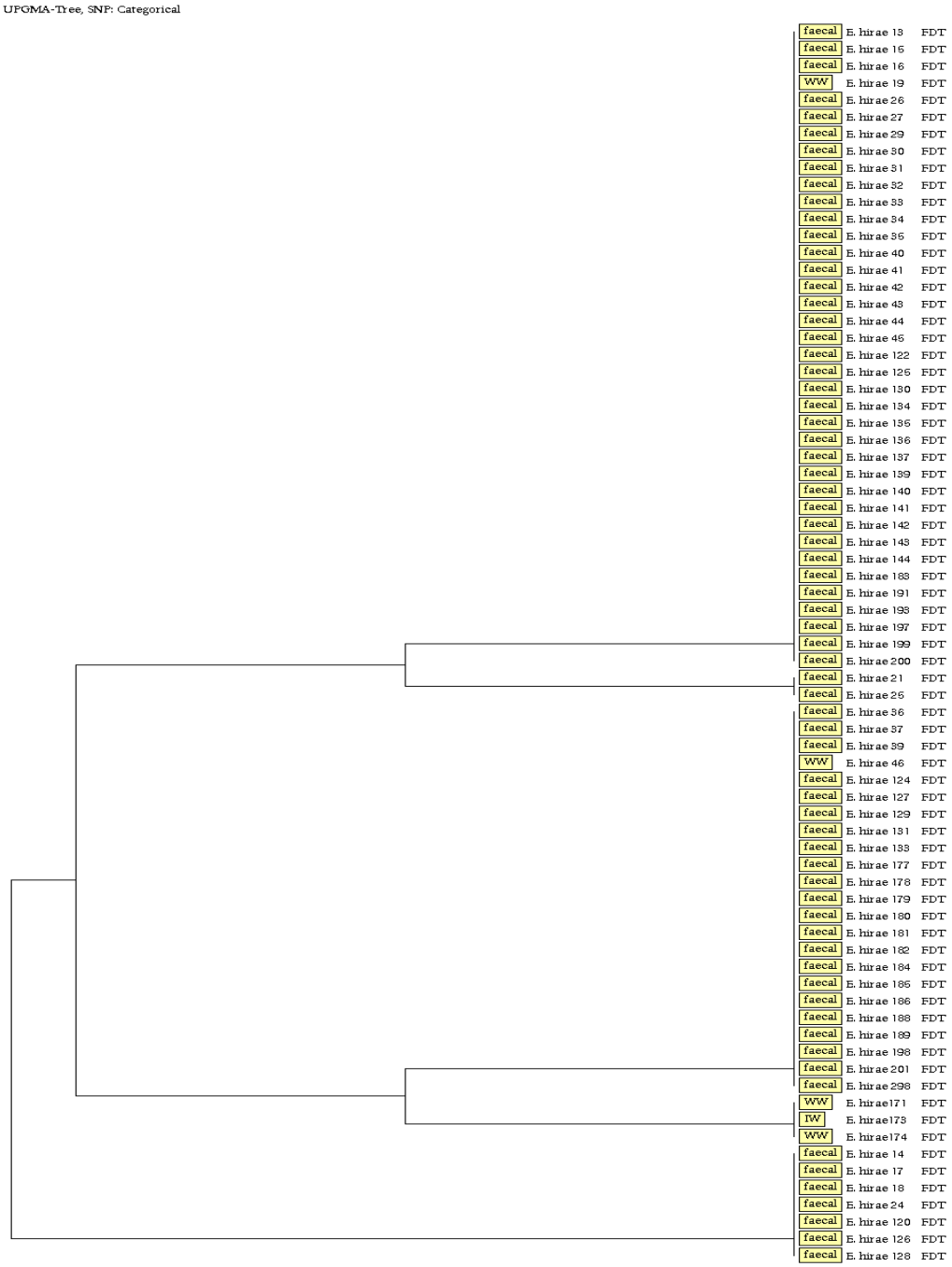

Figure 19: UPGMA dendogram based on the RAPD patterns of enterococcal isolates from faecal and water samples (wastewater, drinking water and Irrigation water) from Fort Hare Dairy Trust. This dendogram was constructed with the RAPD patterns of 91 strains using the miru-vntrplus.org programs

CHAPTER FIVE

DISCUSSION

The genus *Enterococcus* is a diverse group of bacteria that have been reported in a variety of niches with gastro-intestinal tract being their principal habitat. The increasing concern about this organism is their ability to acquire and transfer virulent and antibiotic resistance genes via plasmid- or transposon-mediated genetic transfer mechanisms(Lester *et al.*, 2006). This study provides information about the antibiotic resistance patterns of enterococci from dairy farms as well as their virulent potentials. Previous studies have shown that the occurrence of resistant strains of bacteria in animal farms is due to the overuse of antibiotics in farm management (Bekele & Ashenafi, 2010; Hershberger *et al.*, 2005). The occurrence of resistant and virulent strains of bacteria in farm animals could be detrimental to both the animals and the human population.

The occurrence and distribution of *Enterococcus* species from both water and faeces in all three farms were very similar. All animals from which rectal swabs were obtained were healthy and showed no signs or symptoms of any disease condition. *E. hirae* is the most predominant species recovered from the cattle population in all three commercial farms. This is in line with the results obtained in other studies. Anderson *et al.*(2008) and Jackson *et al.*(2009) also isolated *E. hirae* as the predominant strains from the faeces of cats and cattle respectively. Researchers have associated the existence *E. faecalis, E. faecium, E. hirae E. casseliflavus* and *E. durans* to human and animal faeces as their primary sources (Li *et al.*, 2014; Lanthier *et al.*, 2010; Harwood *et al.*, 2004). A higher incidence (82.4%) of *E. hirae* in faeces was obtained from Fort Hare and

Middledrift dairy trusts whereas in Seven Star Dairy trusts 73% was observed. These results are higher than that reported by Jackson *et al.* (2011) from cattle in different dairy farms in the United States. However, this is in contrast with a previous study conducted by Krause & Khafipour, (2011) in which *E. casseliflavus* was the predominant species isolated from cow dung, a species that was not isolated in this study. Besides, *E. casseliflavus* has been found in the gut of most herbivores in other studies and this has been primarily linked to their association plant and water (Anderson *et al.*, 2008a).

In this study, *E. hirae* was also isolated from irrigation water in Fort Hare and Seven Star dairy trusts (33.3% and 50%) but was completely absent in Middledrift dairy trusts. Fort Hare, Middledrift and Seven Star dairy trust all operate on the same farming style with irrigation water well situated far away from possible contamination by cattle in the farm. However, the reason for the presence of *E. hirae* in the irrigation water could be due to an overflow of wastewater into the irrigation water since irrigation water is located downhill of the wastewater facility. In addition, other animals roaming around the farms utilise the same water that is used by the farms for irrigation and thus could be the source of *E. hirae* species. The wastewater from all three farms contains *E. hirae* with Fort Hare showing 80%, while Middledrift had 50% and Seven Star dairy showing the highest percentage (100%). However, none of the drinking water sources were positive were for this species. The high incidence of *E. hirae* (commensal opportunistic bacteria) in this study is a cause for concern because their pathogenic potentials have not been fully elucidated. A study carried out by Savini *et al.* (2014) shows *E. hirae* as a zoonotic pathogen associated with infection of human umbilical cord. According to their study, it was presumed that the bacteria were acquired by exposure to animals most especially domesticated pets. This

further confirms that *E. hirae* is incriminated in zoonotic diseases which could be pathogenic to humans.

The faecal samples from Fort Hare, Middledrift and Seven Star dairy trusts showed 2.4%, 5% and 7% of *E. faecium* respectively. Most of the water sources were negative for *E. faecium* except for Middledrift dairy trusts wastewater samples which contain 15% of this species. Some strains of enterococci particularly *E. faecalis* and *E. faecium* have been reported as major opportunistic pathogens to both animals and humans. They have been implicated in diseases like urinary tract infections, mammary bovine mastitis, bacteraemia, endodontic, periodontitis and septicaemia (Kumar Patidar *et al.*, 2013; Bisgaard *et al.*, 2010; Ok *et al.*, 2009). Interestingly, the occurrence of these two species was very limited although their pathogenic potential has not been verified. Apart from *E. hirae and E. casseliflavus* other studies have also documented *E. faecalis* or *E. faecium* as the dominant species found in the faeces human and animal and also from water polluted with faeces (Li *et al.*, 2014; Lanthier *et al.*, 2010; Harwood *et al.*, 2004). For instance *E. faecium* was reported as the predominant species isolated from the faeces of chickens in a poultry in Pakistan (Ali *et al.*, 2014). In that study, 66% of the isolates obtained were *E. faecium* of which 39.2% were of faecal origin. Interestingly, this species and others were evident from the faeces and some water samples from our study but in lesser numbers.

E. durans was the least detected from faeces with 2.4% from Fort Hare, 3.0% in Middledrift and 6% from Seven Star dairy trusts. Limited incidences of this species were observed from other water sources from the farms. Fort Hare dairy trusts irrigation water contains 33.3% of *E. durans* whereas it was absent in Middledrift and Seven Star Dairy trusts. This could be attributed to

contamination from other animals or stray cattle which use the stream as a drinking source. This species was also isolated in the drinking water from Seven Star dairy trusts and this could be due to contamination from the cattle in the farms during the process of drinking. Furthermore, enterococci being a commensal of the GIT, their occurrence in faeces are obvious and their ability to withstand adverse environmental conditions and stay for longer periods is well known. In general, the existence of enterococci in the water sources could be ascribed to contamination from non-point sources of pollution such as runoffs and animal or human faeces. In addition, cow dung was often spotted along the banks of the irrigation stream during the three months period of sampling as well as the overflows from the wastewater facility running in to the irrigation stream. The existence of enterococci in the irrigation and drinking water possess a potential hazard to both humans and animals, that uses this source for either drinking or irrigation purposes as this could expose them to other pathogenic microorganisms.

In terms of microbial source tracking techniques and water management development strategy, evaluation of *Enterococcus* species may determine source of faecal contamination as host groups tend to harbour particular species of enterococci (Lanthier *et al.*, 2010). In the case of dairy cattle, studies have confirmed *E. hirae* as the dominant species in faeces (Anderson *et al.*, 2008b; Jackson *et al.*, 2011), although species like *E. durans, E. casseliflavus, E. faecalis, E. faecium, and E. mundtii* could be exist in lesser quantity. The presence of this species in the water sources within the farms signifies possible contamination by cattle dung. In this study we were unable to characterise thirty three of the isolates.

The development of antimicrobial resistance by bacteria to drugs poses a major challenge in both human and veterinary medicine as some of these drugs are commonly used as therapeutics. The susceptibility patterns observed in this study shows that resistance was common to isolates from both faecal and water sources.

Resistance to clinically important drugs such as ciprofloxacins, chloramphenicol vancomycin and gentamycins could also be explained as the transfer of resistant enterococci via irrigation water from the nearby hospital wastewater or from the effluent of the wastewater treatment plant. Iweriebor *et al.*, (2015) suggested that hospital wastewater in Alice Nkonkobe Municipality may contribute to the existence of resistant enterococci in the final effluent of the wastewater treatment plant (WWTP).

Resistances to streptomycin, kanamycin and gentamycin were significantly in most isolates. Enterococci generally are intrinsically resistant to low levels of aminoglycoside. Therefore eradication of enterococci in a disease related condition could be greatly enhanced by a combination therapy of an aminoglycoside and a glycopeptide or betalactams. Nevertheless, higher concentration of aminoglycosides has shown to be effective in the eradication of enterococci (ÇetİnkayaI *et al.*, 2013).

Studies carried out in Europe associated the occurrence of vancomycin resistance enterococci in farm animals to the use of avorparcin an analogue of glycopeptides (Wegener *et al.*, 1999). In contrast to this study no vancomycin related drugs were used in all three farms although

resistances to vancomycin were observed. Therefore there is a need to carry out further studies to determine the source of these resistant bacteria.

Although other researchers (ÇetİnkayaEt al., 2013; Wegener *et al.*, 1999) have shown a high prevalence of glycopeptides and aminoglycosides resistance, the patterns observed in this study could be a reflection of the animals being exposed to antibiotics as growth promoters and for disease prevention which constitute a general operational farm management practice. These practices select for resistant bacteria to the antimicrobials used in the farmlands which may subsequently be disseminated to other bacteria in other ecological niches. Aside from this, the resistances observed could also be as a result of the transfer of resistance genes from other related bacterial species. The penicillins and tetracyclines are drugs of choice used in Middledrift and Seven Star dairy trust for the management diseases which could account for the higher frequency of resistance and multi-resistance trends observed. The indiscriminate use of antibiotics in treatment of animal diseases as well as their incorporation in feeds has been suspected to account significantly to the increase in antimicrobial resistance in pathogenic bacteria isolates (WHO 2000).

Antibiotic resistance genes were amplified from the isolates in relation to the phenotypic resistance observed. The *tetM* gene was identified in all the tetracycline resistant isolates whereas no *tetK* gene was detected in the isolates. Klibi *et al.* (2013a) and Aarestrup, (2000) also detected *tetM* as the most abundant gene found in tetracycline resistant enterococci from meat and animals.

The *ermB* gene was found only in eleven isolates (26.2%) of the forty two erythromycin resistant screened. No *ermA* gene was detected in the isolates. The inconsistency between phenotypic and genotypic pattern could be because only *ermA* and *ermB* genes were verified as opposed to all the erythromycin mediated genes known (Roberts, 2011). The *ermA* and *ermB* genes have been detected in previous studies as the most abundant in erythromycin resistant enterococci isolates (Aarestrup, 2000; Klibi *et al.*, 2013b). In addition *tetM* and *ermB* genes are well known to be associated with conjugative plasmid or transposons Tn916 – 1545 (Bulajic *et al.*, 2015; Teuber *et al.*, 1999).

The pathogenicity of enterococci was investigated by detection of the virulence genes. The existence of these genes in isolates represent a high possibility that isolates would exhibit virulent factors (haemolytic activity, gelatinase, pili) leading to disease conditions if found in host organism. The *gelE* gene which encodes for gelatinase was detected in all three farms (71.4% FDT, 67.2% MDT and 76.6% SDT). Genes play an important role in pathogenicity and therefore the virulence factors which they encode should be verified to determine their significance. However, in this study no phenotypic virulent assay was carried out to determine if the amplified genes in the isolates were expressed in the bacteria. Nevertheless, the high occurrence of *gelE* gene among isolates is quite similar to those reported by others (Klibi *et al.*, 2013b; Medeiros *et al.*, 2014). However, it should be noted at not all isolates which harbour the *gelE* genes may express gelatinase or β-haemolysis activity as a result of the deletion of the *fsr* operon (Lauková *et al.*, 2014) . Adherence factor is encoded by the *ace* gene and is known to promote the attachment of enterococci to host surface. The distribution of this gene in all three farms was as follows; 45.05% FDT 26.16% MDT and 14.01% SDT. Although this gene was less

prevalent in this study, it only supplements the other virulent factors in the disease causing ability of the organism.

The *cylM* (cytolysins operon), *agg* (aggregation substance) and *esp* (enterococcal surface protein) genes that constitute the major virulent genes in the genus *Enterococcus*, were not detected in the isolates. Their absence could signify that the isolates are less pathogenic. However further studies are required to investigate the virulent factors of these isolates.

The determination of the diversity bacteria through molecular typing is an important step in tracing their origin. In references in this regard RAPD PCR has proven to be an efficient and effective method for intraspecies typing of isolates from different sources to determine their genetic relatedness. The dendograms in this study show the relatedness of *E. hirae* isolates from both faeces and water sources (waste water, irrigation water and drinking water) in each of the farms. More clusters (7) were observed in isolates from Middledrift Dairy trust showing a high diversity than in the other two farms containing 5 clusters each. In addition, the phylogenetic relationships seems to indicate that some faecal isolates were similar to the water isolates confirming the possibility of faecal to water sources. However, the discriminating power of RAPD using one primer seems to be low, with the highest clusters being 7 only. It is suggested that the augmentation of RAPD with PFGE will display better results of the relatedness amongisolates investigated.

CONCLUSION AND RECOMMENDATIONS

In this study the incidences of enterococci were confirmed with *E hirae* being the predominant species isolated in both water and faeces. Although studies have referred to this bacteria as a normal floral of the GIT of animals, pathogenic strains may exist that could cause diseases in animals and humans.

Animal's husbandry and dairy farming are well known as a potential source of antbiotic-resistant bacteria due to the overuse of antibiotics as growth promoters. This seems to be substantiated is confirmed in this study where some isolates showed resistance to tetracycline; an antibiotic used in Seven Star and Middledrift dairy trusts. These resistant bacteria could be disseminated to other sources which could pose major threat to public health.

Based on the RAPD PCR, *E. hirae* from different sources (faeces and water) showed a high degree of interspecies similarity confirming the possibility of enterococci from faeces to have been disseminated in to the environmental waters. However, other methods such as PFGE should be used in conjunction with RAPD to increase the discriminating power of RAPD seen in this study.

Based on these, the following recommendations are suggested:

- Resistance of enterococci to antimicrobials should be monitored regularly.
- Dairy farms should be monitored regularly as they could serve as reservoirs for resistant bacteria.

- To prevent MDR in enterococci, veterinarian should ensure the minimal and judicious use of antibiotics in animal farms.
- Antimicrobial surveillance programmes should be created to monitor the occurrence of antimicrobial resistance in food animals and food of animal origin so as to avoid the transfer of these bacteria to humans

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APPENDICES

Appendix 1: Disc diffusion susceptiblity of enterococcus strains isolated from the three commercial farms (zones of inhibition measured in millimetres mm)

Antibiotics / Isolates	Source	PG	C	K	LZD	GM	NI	E	CIP	VA	T	S	SYN
		Seven Star dairy trust (107 Isolates)											
E. faecium 1	faeces	24	24	7	23	13	21	20	20	21	25	15	18
E. hirae 2	faeces	21	18	0	0	12	23	20	20	24	0	17	16
E. hirae 3	faeces	23	19	10	22	15	19	22	24	24	28	18	12
E. hirae 4	faeces	24	19	0	23	11	18	0	27	22	25	17	15
E. hirae 5	faeces	25	23	0	26	11	18	16	20	17	0	17	20
E. hirae 6	water	22	26	15	27	12	26	25	25	25	29	16	14
E. hirae 47	water	0	26	14	0	11	20	0	21	0	22	19	18
E. hirae 48	water	22	21	10	22	12	22	21	20	22	28	18	15
E. faecium 50	faeces	27	23	0	24	14	17	18	20	20	27	18	17
E. hirae 51	faeces	21	21	0	26	0	21	26	21	22	26	17	9
Unidentified 52	faeces	24	22	16	25	15	20	24	23	21	25	19	12
Unidentified 53	faeces	23	24	15	26	17	22	22	23	25	24	19	0
E. hirae 54	faeces	25	22	14	26	14	18	23	22	22	25	19	10
E. faecium 55	faeces	24	19	17	25	11	19	22	22	24	24	18	15
E. hirae 56	faeces	25	22	11	24	0	20	25	20	25	25	20	0
E. hirae 57	faeces	22	22	0	25	0	18	20	20	25	25	19	18
E. hirae 58	faeces	20	20	0	25	11	18	13	23	21	23	16	20
E. hirae 59	faeces	18	22	0	23	0	20	24	20	20	23	18	18
E. hirae 60	faeces	0	21	15	0	11	20	0	26	0	0	22	18
E. hirae 61	faeces	0	24	16	0	10	18	0	27	22	23	20	16
E. hirae 62	faeces	0	27	10	0	13	24	18	27	0	27	20	15
E. hirae 63	faeces	26	24	0	27	12	20	14	16	21	25	21	19
E. hirae 64	faeces	25	20	0	26	13	19	17	0	0	21	20	0
E. durans 65	faeces	0	27	12	0	12	19	17	0	0	21	19	21

E. hirae 66	faeces	0	25	14	0	18	18	0	26	0	21	20	0
E. hirae 67	faeces	22	22	0	22	10	20	25	22	21	0	15	18
E. hirae 68	faeces	20	20	0	25	12	25	26	21	22	25	17	15
E. durans 69	faeces	21	20	0	25	10	20	22	20	15	29	13	19
E. hirae 70	faeces	22	18	0	27	13	22	16	13	17	27	17	15
E. hirae 71	faeces	20	22	9	26	11	22	22	23	22	22	18	19
E.Unidentified 72	faeces	22	20	10	22	14	23	23	23	20	25	20	19
E. hirae 96	faeces	0	25	0	0	15	0	15	24	0	0	18	0
E. hirae 97	faeces	21	24	0	24	13	24	20	21	20	0	19	14
E. hirae 98	faeces	0	0	10	0	11	17	18	23	0	0	21	0
E. hirae 99	faeces	0	25	14	0	16	19	0	25	0	0	20	0
E. faecium 100	faeces	0	0	11	0	12	16	10	25	0	0	21	0
E. hirae 101	faeces	0	28	16	0	10	20	20	25	0	0	20	0
E. hirae 102	faeces	0	23	15	0	15	21	22	30	13	0	20	0
E. durans 103	faeces	0	25	13	0	19	22	21	27	0	22	21	0
E. Unidentified 104	faeces	0	26	0	0	19	19	22	25	0	0	20	0
E. Unidentified 105	faeces	0	25	16	0	11	19	0	24	0	0	20	0
E. hirae 106	faeces	0	18	18	0	13	0	0	24	0	23	21	10
E. hirae 107	faeces	23	25	0	25	0	24	19	22	20	0	20	12
E. hirae 108	faeces	22	24	0	23	0	22	18	20	19	0	15	10
E. hirae 109	faeces	23	24	0	25	13	23	18	19	20	0	16	0
E. hirae 110	faeces	0	27	10	0	17	20	0	25	0	24	22	0
E. hirae 111	faeces	0	27	16	0	10	20	0	27	0	24	21	0
E. hirae 112	faeces	0	26	11	0	19	18	0	21	0	0	21	0
E. hirae 113	faeces	0	23	9	0	16	19	0	22	0	0	19	0
E. hirae 114	faeces	0	28	10	0	18	20	0	21	0	0	21	0
E. faecium 115	faeces	0	29	10	0	16	22	12	22	0	0	21	0
E. hirae 116	faeces	0	26	10	0	14	20	17	22	10	25	21	0
E. hirae 117	faeces	0	23	16	0	10	0	0	20	0	0	18	19
E. hirae 118	faeces	10	27	18	0	12	23	23	27	12	26	22	12
E. hirae 119	faeces	0	28	15	0	18	20	15	28	0	0	21	0
E. hirae 172	water	21	25	15	24	19	21	24	22	18	20	20	15

E. hirae 176	water	23	23	10	22	11	21	20	25	20	24	18	18
E. hirae 227	faeces	27	19	15	22	12	18	24	19	21	21	0	19
E. hirae 228	faeces	17	20	16	19	13	16	22	21	21	23	0	18
E. hirae 229	faeces	24	20	0	21	12	21	25	20	22	24	0	19
E. hirae 230	faeces	25	20	9	19	8	18	20	22	23	0	0	17
E. hirae 231	faeces	20	20	7	22	10	23	25	22	22	26	21	18
E. hirae 232	faeces	20	23	10	25	9	20	26	23	23	25	0	20
E. hirae 233	faeces	17	18	9	22	10	23	23	24	20	25	0	22
E. hirae 234	faeces	20	20	16	24	9	13	20	25	20	20	0	20
E. hirae 235	faeces	20	19	15	25	0	15	25	23	24	23	0	18
E. hirae 236	faeces	30	20	10	22	15	23	25	25	20	28	21	25
E. hirae 237	faeces	25	21	9	24	10	20	23	24	23	21	0	23
E. hirae 238	faeces	32	26	0	25	18	22	28	25	20	30	19	20
E. hirae 239	faeces	29	25	9	21	15	20	25	29	20	26	18	18
E. hirae 240	faeces	27	25	7	19	10	15	23	22	20	20	0	20
E. hirae 241	faeces	29	23	13	21	10	19	25	22	20	27	0	20
E. hirae 242	faeces	29	20	17	22	13	18	25	24	25	25	0	20
E. hirae 243	faeces	30	19	11	25	10	20	28	20	23	27	0	19
E. durans 244	faeces	28	20	15	23	10	18	20	20	20	25	0	18
E. hirae 245	faeces	25	22	13	20	10	13	17	20	23	0	0	19
E. Unidentified 246	faeces	24	26	12	19	14	15	25	23	23	27	14	18
E. faecium 247	faeces	22	25	9	20	13	18	23	20	20	22	13	20
E. hirae 248	faeces	22	23	15	23	12	20	25	21	23	24	0	18
E. hirae 249	faeces	23	25	16	21	13	21	25	23	25	23	0	20
E. hirae 250	faeces	24	26	17	20	13	15	24	22	23	25	21	19
E. faecalis 251	faeces	25	25	16	21	12	18	23	25	20	26	20	23
E. faecalis 252	faeces	25	25	15	0	17	20	25	23	21	27	0	20
E. hirae 253	faeces	20	24	9	23	13	19	24	27	21	22	0	18
E. Unidentified 254	faeces	21	25	10	29	12	15	24	25	20	26	10	21
E. Unidentified 255	faeces	22	21	11	21	11	20	22	27	23	25	12	20
E. durans 256	faeces	23	25	9	25	21	22	23	25	20	25	14	21
E. Unidentified 257	faeces	24	25	10	21	20	23	22	23	20	25	0	18

E. Unidentified 258	faeces	25	23	8	22	12	24	25	22	21	28	0	20
E. Unidentified 259	faeces	26	24	15	19	11	23	24	25	24	27	13	19
E. hirae 260	faeces	22	22	0	25	13	20	26	20	25	24	0	21
E. Unidentified 261	faeces	20	23	14	21	12	22	25	20	23	27	0	21
E. hirae 262	faeces	21	27	0	24	15	23	26	27	22	28	15	22
E. hirae 263	faeces	20	24	16	23	11	18	22	23	21	25	0	20
E. hirae 264	faeces	22	23	0	25	11	21	22	25	23	25	13	20
E. hirae 265	faeces	22	22	9	21	18	18	24	22	22	25	0	21
E. hirae 266	faeces	19	21	16	25	18	19	24	23	21	26	0	20
E. hirae 267	faeces	21	23	15	26	10	19	23	25	21	23	19	18
E. Unidentified 268	faeces	22	22	9	21	15	20	22	26	22	24	14	19
E. hirae 269	faeces	21	22	10	22	19	17	21	24	22	0	15	19
E. faecium 270	faeces	23	21	13	23	17	23	18	18	21	0	0	22
E. hirae 271	faeces	23	24	16	24	12	16	26	20	19	0	15	17
E. hirae 272	faeces	22	25	7	21	12	16	25	19	20	0	16	17
E. hirae 273	faeces	20	22	0	25	11	17	22	21	20	24	20	18
E. hirae 274	faeces	23	24	11	21	11	18	25	21	21	0	21	17
E. durans 301	water	21	21	9	25	12	21	21	17	20	23	20	12
Unidentified 302	water	23	24	15	24	17	20	22	14	24	25	18	21
Middle drift dairy trust (107 Isolates)													
E. hirae 7	faeces	23	22	0	25	12	22	21	23	21	23	15	0
E. hirae 8	faeces	22	21	10	28	10	19	21	21	18	21	20	15
E. hirae 9	faeces	23	20	0	22	15	20	22	25	20	22	20	16
E. hirae 10	faeces	23	22	10	24	0	23	20	25	20	24	19	16
E. hirae 11	faeces	28	26	10	27	12	25	22	25	25	29	21	20
E. faecium 12	water	22	22	0	26	16	17	21	16	24	22	19	0
E. Unidentified 49	water	25	23	12	25	15	22	16	24	23	25	18	14
E. hirae 73	faeces	0	27	15	0	10	20	11	30	0	20	20	0
E. Unidentified 74	faeces	25	24	10	23	18	21	19	26	24	23	20	13
E. faecium 75	faeces	0	25	9	0	11	20	0	18	0	22	19	0
E. Unidentified 76	faeces	30	29	17	27	19	20	27	21	17	28	27	0
E. faecium 77	faeces	22	26	12	26	11	20	18	22	19	30	22	22

E. hirae 78	faeces	0	29	11	0	13	21	12	40	0	27	22	20
E. faecium 79	faeces	0	33	16	0	15	27	26	35	13	30	23	14
E. hirae 80	faeces	0	30	15	0	15	23	24	20	15	26	21	15
E. hirae 81	faeces	20	25	0	20	0	0	18	20	20	25	15	17
E. hirae 82	faeces	0	29	18	15	18	21	20	26	27	36	22	20
E. hirae 83	faeces	24	24	17	22	10	20	20	25	21	26	22	18
E. hirae 84	faeces	22	25	13	21	10	17	24	25	20	25	22	20
E. hirae 85	faeces	21	24	19	26	19	19	22	24	23	26	23	22
E. hirae 86	faeces	0	23	14	0	14	21	11	13	0	0	22	20
E. hirae 87	faeces	0	26	9	0	14	23	11	27	0	0	21	0
E. hirae 88	faeces	0	0	18	0	19	12	0	28	0	0	22	0
E. hirae 89	faeces	21	20	11	23	12	20	19	20	19	24	19	18
E. hirae 90	faeces	21	24	0	23	0	22	16	21	20	0	0	18
E. hirae 91	faeces	15	25	0	22	11	20	14	18	20	0	21	16
E. faecium 92	faeces	0	25	13	0	18	20	0	26	0	22	21	0
E. hirae 93	faeces	25	23	13	0	10	17	12	21	0	23	16	20
E. hirae 94	faeces	20	26	0	22	14	21	17	16	20	27	21	21
E. hirae 95	faeces	0	12	12	0	13	0	18	26	0	0	12	9
E. hirae 145	faeces	21	24	0	21	18	23	21	18	20	25	0	0
E. hirae 146	faeces	26	25	15	25	19	21	25	28	19	10	21	23
E. hirae 147	faeces	20	24	13	24	19	23	24	25	21	24	13	14
E. hirae 148	faeces	24	24	0	25	15	21	22	23	22	24	0	21
E. hirae 149	faeces	25	23	0	23	15	22	22	21	21	25	21	20
E. hirae 150	faeces	23	25	0	23	12	23	21	20	20	25	0	0
E. hirae 151	faeces	20	21	0	21	12	21	19	18	18	21	0	0
E. hirae 152	faeces	20	26	07	0	10	24	19	20	20	21	0	18
E. hirae 153	faeces	22	25	11	23	11	20	21	17	21	27	0	12
E.durans 154	faeces	22	23	09	24	11	21	22	19	16	25	21	0
E. hirae 155	faeces	23	25	0	25	12	20	22	20	22	23	21	19
E. hirae 156	faeces	21	22	0	18	10	23	24	21	23	24	0	20
E. hirae 157	faeces	21	26	15	19	12	22	25	16	22	25	21	0
E. hirae 158	faeces	20	22	13	24	18	22	21	26	21	23	22	0

E. hirae 159	faeces	24	24	10	22	18	21	18	24	25	24	21	15
E. hirae 160	faeces	21	23	0	24	10	21	23	27	19	24	22	11
E. hirae 161	faeces	25	20	0	24	10	20	18	27	20	25	21	12
E. hirae 162	faeces	24	20	15	25	15	23	17	28	21	25	21	11
*E. durans*163	faeces	23	21	13	24	8	21	16	26	18	23	21	0
E. hirae 164	faeces	22	22	19	23	10	23	16	24	22	25	23	21
E. hirae 165	faeces	21	23	14	23	10	20	18	22	20	27	21	0
E. hirae 166	faeces	18	25	15	22	10	22	19	20	19	22	22	15
E. Unidentified 167	faeces	23	21	0	22	13	20	20	22	21	25	21	12
E. hirae 168	faeces	21	22	0	14	18	23	21	25	22	20	22	0
E. hirae 169	faeces	20	19	0	18	18	20	20	21	24	24	21	20
E. hirae 170	water	22	20	18	20	10	21	25	22	20	22	21	22
E. hirae 175	water	21	24	0	23	15	20	15	24	22	21	19	16
E. Unidentified 202	faeces	24	30	16	30	19	21	30	25	22	25	18	22
E. hirae 203	faeces	25	24	17	25	9	23	0	15	20	9	0	15
E. hirae 204	faeces	23	21	14	30	9	20	15	14	23	28	0	20
E. hirae 205	faeces	27	31	13	30	13	23	30	25	25	30	18	22
E. hirae 206	faeces	28	28	09	30	0	28	15	20	29	26	0	25
E. hirae 207	faeces	21	20	12	22	0	20	20	23	21	25	0	0
E. hirae 208	faeces	21	22	0	25	0	21	19	19	20	27	0	10
E. hirae 209	faeces	30	30	0	30	10	21	30	26	20	29	18	24
E. hirae 210	faeces	10	19	0	20	0	15	20	17	20	25	0	17
E. hirae 211	faeces	20	20	17	20	0	22	13	22	20	0	0	17
E. hirae 212	faeces	20	20	0	15	10	19	21	20	23	24	0	10
E. hirae 213	faeces	38	28	0	22	15	22	29	25	20	30	20	20
E. hirae 214	faeces	0	25	17	21	19	18	27	25	0	21	18	19
E. hirae 215	faeces	25	25	10	26	13	23	14	22	0	25	20	23
E. hirae 216	faeces	27	25	15	0	18	19	29	23	19	28	21	27
E. hirae 217	faeces	30	28	16	0	19	22	30	29	20	30	21	21
E. hirae 218	faeces	30	29	10	0	19	22	26	29	20	29	25	30
E. hirae 219	faeces	22	26	18	23	15	20	18	20	19	25	0	13
E. hirae 220	faeces	27	18	0	23	15	22	21	24	23	24	0	20

E. hirae 221	faeces	28	16	12	22	12	21	22	23	24	21	0	18
E. hirae 222	faeces	21	18	11	0	12	18	16	24	20	27	23	19
E. hirae 223	faeces	18	19	18	0	10	0	15	16	20	22	0	12
E. hirae 224	faeces	21	15	15	22	11	22	21	20	20	25	0	20
E. hirae 225	faeces	21	18	17	0	11	20	18	23	18	21	22	14
E. hirae 226	faeces	24	20	0	21	12	19	20	21	21	26	0	14
E. hirae 275	faeces	24	23	13	25	10	19	24	20	19	0	25	18
E. hirae 276	faeces	22	25	16	24	12	18	23	22	20	0	20	17
E. hirae 277	faeces	21	21	13	25	18	19	22	21	20	0	22	18
E. faecium 278	faeces	24	20	12	23	18	23	19	25	19	22	10	20
E. hirae 279	faeces	20	23	0	23	10	21	22	25	10	21	21	20
E. hirae 280	faeces	23	25	15	21	10	20	28	24	23	25	23	22
E. hirae 281	faeces	20	25	14	0	15	20	24	19	22	20	21	19
E. hirae 282	faeces	25	23	15	23	8	23	25	24	22	21	21	23
E. durans 283	faeces	20	25	12	0	10	20	20	22	20	22	22	22
E. Unidentified 284	faeces	24	24	18	23	10	18	22	21	20	24	0	19
E. hirae 285	faeces	25	20	11	29	10	17	21	25	19	22	23	13
E. hirae 286	faeces	26	20	11	21	13	18	20	21	20	23	23	20
E. hirae 287	faeces	25	23	19	25	10	20	0	25	25	27	20	19
E. Unidentified 288	faeces	21	24	9	21	12	19	20	22	23	21	25	21
E. hirae 289	faeces	29	23	0	22	16	20	21	25	22	26	21	20
E. Unidentified 290	faeces	25	23	18	19	13	17	25	25	25	26	22	20
E. hirae 291	faeces	24	22	0	25	18	21	20	24	20	25	22	19
E. faecalis 292	faeces	34	20	0	21	15	23	27	28	20	29	23	20
E. hirae 293	faeces	23	24	12	24	14	23	28	17	21	25	0	23
E. hirae 294	faeces	25	24	0	23	15	20	25	18	23	28	13	20
E. hirae 295	faeces	27	22	15	25	18	20	23	23	20	25	0	17
E. hirae 296	faeces	23	23	14	21	15	20	20	20	20	25	16	20
E. hirae 297	faeces	25	23	15	24	12	26	25	17	20	25	23	25
E. Unidentified 304	water	21	20	12	23	12	20	23	19	20	23	0	17
E. Unidentified 305	water	24	23	18	22	18	21	20	23	22	23	22	19
Fort hare dairy trust													

E. hirae 13	faeces	18	24	0	26	12	24	25	22	21	12	19	0
E. hirae 14	faeces	18	21	0	25	11	21	20	23	21	25	18	0
E. hirae 15	faeces	20	21	0	18	10	18	24	25	22	23	16	15
E. hirae 16	faeces	21	22	13	23	16	20	25	20	17	0	18	15
E. hirae 17	faeces	20	24	16	25	12	21	23	21	20	27	19	12
E. hirae 18	faeces	18	16	13	26	13	20	21	23	21	25	15	13
E. hirae 19	water	20	24	12	25	12	21	20	25	22	26	16	11
E. durans 20	water	25	25	0	26	14	25	20	23	24	25	20	0
E. hirae 21	faeces	25	20	15	26	16	17	11	20	16	26	25	25
E. durans 23	faeces	0	23	14	26	16	20	21	25	22	26	22	0
E. hirae 24	faeces	0	22	15	0	15	22	16	21	0	23	18	11
E. hirae 25	faeces	18	20	12	22	13	22	20	18	21	22	23	12
E. hirae 26	faeces	18	21	18	24	9	18	9	20	20	0	21	11
E. hirae 27	faeces	26	21	11	23	15	20	20	14	22	23	20	0
E. faecium 28	faeces	20	23	11	20	12	20	20	15	20	10	14	20
E. hirae 29	faeces	23	22	19	21	15	22	21	19	21	25	24	22
E. hirae 30	faeces	20	25	9	20	15	25	20	22	20	22	17	17
E. hirae 31	faeces	10	11	0	22	10	20	15	19	20	0	18	20
E. hirae 32	faeces	20	26	18	28	10	20	12	20	21	0	16	0
E. hirae 33	faeces	21	22	0	20	0	17	20	22	20	21	18	16
E. hirae 34	faeces	24	23	0	22	14	19	17	20	21	0	19	10
E. hirae 35	faeces	25	19	12	19	14	20	23	26	20	23	20	15
E. hirae 36	faeces	23	26	15	24	13	19	20	19	0	24	19	0
E. hirae 37	faeces	20	21	0	22	11	17	20	20	20	21	16	17
E. Unidentified 38	faeces	20	21	0	23	15	23	28	21	19	0	20	12
E. hirae 39	faeces	20	22	12	25	15	24	28	23	19	0	21	12
E. hirae 40	faeces	21	20	0	22	10	17	10	15	18	0	14	15
E. hirae 41	faeces	24	22	0	22	17	20	20	16	21	24	16	11
E. hirae 42	faeces	22	21	16	26	12	16	19	26	16	21	25	0
E. hirae 43	faeces	0	21	16	25	10	0	26	23	17	23	25	0
E. hirae 44	faeces	18	9	0	27	10	20	0	9	19	0	0	12
E. hirae 45	faeces	20	13	0	25	13	21	14	11	23	0	21	16

E. hirae 46	water	21	20	0	22	12	20	20	20	20	17	20	21
E. hirae 120	faeces	17	23	0	23	10	22	11	19	17	0	25	19
E. Unidentified 121	faeces	10	26	15	0	11	21	0	27	12	21	20	19
E. hirae 122	faeces	11	28	19	0	17	20``	19	24	11	21	21	0
E. durans 123	faeces	11	27	11	0	0	20	18	26	0	25	19	0
E. hirae 124	faeces	21	21	0	25	17	21	14	20	19	26	20	0
E. hirae 125	faeces	25	26	0	26	13	24	11	21	19	24	20	15
E. hirae 126	faeces	24	25	0	25	0	21	17	16	19	23	25	15
E. hirae 127	faeces	23	23	11	25	12	22	16	19	19	0	21	12
E. hirae 128	faeces	22	0	0	23	17	21	16	17	18	0	22	13
E. hirae 129	faeces	22	26	0	25	11	21	17	20	19	25	22	11
E. hirae 130	faeces	20	25	0	0	15	22	16	22	18	26	23	0
E. hirae 131	faeces	21	25	0	0	13	23	18	24	22	0	0	25
E. faecalis 132	faeces	22	22	0	22	0	21	17	18	18	0	13	0
E. hirae 133	faeces	22	25	12	21	0	23	16	16	19	0	0	11
E. hirae 134	faeces	20	23	0	23	0	20	16	18	17	0	16	12
E. hirae 135	faeces	21	22	0	23	11	22	18	17	16	0	23	11
E. hirae 136	faeces	20	22	0	22	17	21	19	18	18	0	0	0
E. hirae 137	faeces	23	16	18	28	0	20	20	20	18	26	22	20
E. Unidentified 138	faeces	21	16	17	23	17	21	20	21	17	22	24	22
E. hirae 139	faeces	22	27	11	29	13	21	18	21	19	27	21	17
E. hirae 140	faeces	18	21	0	24	12	20	21	20	17	25	21	20
E. hirae 141	faeces	20	13	0	25	17	20	24	15	19	26	19	0
E. hirae 142	faeces	21	16	0	22	0	20	10	18	18	0	0	16
E. hirae 143	faeces	11	26	12	0	17	23	23	23	13	24	16	10
E. hirae 144	faeces	19	21	0	24	13	21	18	21	19	23	22	15
E. hirae 171	water	19	24	11	22	0	24	20	21	19	21	21	0
E. hirae 173	water	20	26	17	24	12	21	20	20	18	21	0	17
E. hirae 174	water	19	22	12	25	17	23	19	26	19	23	0	12
E. hirae 177	faeces	20	25	11	24	11	19	18	21	21	22	11	12
E. hirae 178	faeces	0	20	10	23	15	20	23	20	16	15	0	15
E. hirae 179	faeces	21	22	16	22	0	22	17	19	19	22	0	11

E. hirae 180	faeces	21	20	12	24	0	22	18	19	18	23	0	0
E. hirae 181	faeces	21	21	13	25	0	22	15	19	19	25	0	10
E. hirae 182	faeces	21	20	12	24	0	21	19	21	19	25	0	12
E. hirae 183	faeces	19	22	14	25	13	15	13	20	22	0	10	16
E. hirae 184	faeces	24	24	16	28	13	21	20	22	22	27	22	21
E. hirae 185	faeces	21	26	16	25	0	22	21	20	19	28	0	19
E. hirae 186	faeces	22	22	15	22	12	19	10	27	17	24	25	19
E. faecalis 187	faeces	23	24	13	25	0	18	20	21	19	26	0	12
E. hirae 188	faeces	25	25	9	27	13	22	20	21	23	25	0	18
E. hirae 189	faeces	25	24	15	24	0	23	20	20	24	27	24	19
E. faecalis 190	faeces	26	24	12	25	0	21	20	21	24	30	0	20
E. hirae 191	faeces	24	25	15	28	0	22	18	21	23	28	0	18
E. Unidentified 192	faeces	0	23	15	25	11	23	0	22	22	24	21	20
E. hirae 193	faeces	0	18	10	0	17	22	11	26	0	0	19	0
E. Unidentified 194	faeces	24	25	10	28	0	22	18	21	23	28	0	18
E. Unidentified 195	faeces	0	23	0	0	17	21	19	22	0	21	16	0
E. faecium 196	faeces	17	22	10	28	13	24	22	23	20	27	22	22
E. hirae 197	faeces	19	24	12	28	12	23	21	21	20	24	21	21
E. hirae 198	faeces	20	21	0	22	0	20	0	21	22	24	0	18
E. hirae 199	faeces	21	23	12	22	0	18	0	20	22	0	0	0
E. hirae 200	faeces	21	27	0	27	0	20	0	20	25	0	0	18
E. hirae 201	faeces	24	26	13	24	19	22	19	22	21	26	20	22
E. hirae 298	faeces	25	22	12	25	18	23	20	19	22	21	21	18
E. hirae 299	faeces	26	24	0	28	19	26	25	22	23	22	23	22
E. Unidentified 307	water	21	25	9	25	14	0	22	20	20	25	10	18
E. Unidentified 308	water	0	21	0	0	16	18	18	22	0	24	14	12
E. Unidentified 309	water	22	21	8	28	12	20	23	19	21	23	15	20

PG; penicillin, C; Chloramphenicol, K; Kanamycin, LZD; Linezolid, GM; Gentamycin, NI; Nitrofurantoin, E; Erythromycin, CIP; Ciprofloxacin, VA; Vancomycin, T; Tetracycline, S; Streptomycin, SYN; quinpristin/dalfopristin

Appendix 2: Plates presenting zone of inhibition