The Assessment of Multiple Antibiotic Resistant Enterococci in Communal and Commercial Cattle Faecal Samples and their Water Sources in Mafikeng, North-West Province, RSA

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Submitted in fulfilment of the requirements for the degree of Master of Science Agriculture, Department of Animal Health, Faculty of Agriculture, Science and Technology, North-West University, Mafikeng Campus.

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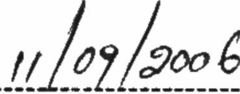
DECLARATION

I declare that, the dissertation for the Degree of Master of Science in Agriculture (Molecular Microbiology) at the North-West University – Mafikeng Campus hereby submitted, has not been submitted by me for a degree at this or any other University. It is my own work, in design and execution, and that all material contained herein has been duly acknowledged.



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DEDICATION

This work is dedicated to the late Dr. Princess Tlou Mmolawa and Mr. Herson Nkidibo Ramatlhape. My grandmother Pauline Maphiello Tladi, my parents Mr. Johannes and Mrs. Jaqueline Ramatlhape, my brothers and sister, Kagiso, Itumeleng, Obakeng and Rebaone.

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ABSTRACT

Enterococcus species are found in faeces of mammals, birds, insects, reptiles, but also soil, plants and water. These bacteria can also be isolated from animal products such as milk, cheese and meat. This study was aimed at isolating *Enterococcus* species from communal and commercial cattle faecal and water samples. A further objective was to determine the antibiotic resistance profiles of the isolates as well as some of the potential factors and mechanisms that could be responsible for their resistance to antibiotics.

A total of 79 cattle faecal and water samples were collected from the communal and commercial farms. Sixty-five faecal samples were collected from commercial (33 healthy and 16 diarrhoeal cattle) and communal (16 healthy cattle) farms. Twelve water samples were collected from the commercial farms and 2 from the communal farm.

From all the samples collected, 129 *Enterococcus* isolates were identified. Isolates, which included *Enterococcus faecium* (*E. faecium*), *Enterococcus avium* (*E. avium*), *Enterococcus durans* (*E. durans*) and *Streptococcus bovis I* (*Sc. bovis I*), were isolated from bovine faeces and water samples, while *E. avium* was only isolated from water at the communal farm. Furthermore, isolates from the healthy and diarrhoeal commercial cattle included *E. faecium*, *E. avium*, *E. durans* and *Sc. bovis I*. *E. faecium* and *E. avium* species were also isolated from the commercial farm cattle water sources. However, *E. faecium* was the predominant species in communal cattle faecal and water samples. On the other hand, *E. avium* was dominant in commercial cattle faecal and water samples.

Multiple antibiotic resistance (MAR) was observed in enterococci from all samples at both farm types. The predominant MAR phenotype that was prevalent in all enterococci species was GEN-SMX-NAL-NIT-KAN-STR. All isolates showed an MAR index above 0.2 (water; 0.58 to 0.68 and faeces; 0.6 to 1.7). Cluster analysis based on antibiotic inhibition zone diameter data, resulted in

dendrograms that showed a similar relationship of *Enterococcus* isolates from the two farms. Between 13% and 50% of *Enterococcus* isolates from cattle faeces and water samples from communal and commercial farms were resistant to vancomycin and oxytetracycline. In general, 11% of all the *Enterococcus* isolates from the cattle faeces was resistant to vancomycin. Thirty one percent of the isolates from cattle water sources were resistant to both drugs. Vancomycin Resistant *Enterococcus* (VRE) genes conveying the *vanC* phenotype were obtained from *E. durans* and *E. avium*. This was an unexpected result. The *tet A*, *tet B* and *tet C* genes were not obtained from any of the *Enterococcus* species.

Further studies on antibiotic resistance should be undertaken especially in rural areas, where farmers could be using over-the-counter medicines such as tetracycline even when it is not necessary. It was speculated in this study that there could be a development of potential reservoirs of antibiotic resistance in farmlands. In order to prevent the distribution of MAR organisms or their transferable resistance genes, a sensible use of antibiotics is necessary in veterinary medicine, animal husbandry and human medicine.

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CHAPTER 1

1.1 INTRODUCTION

Enterococcus species are present in the normal intestinal flora in humans and animals, and are common in environments contaminated by human and animal faecal material (e.g. soil receiving fertilisers of animal origin) as well as in food products derived from animals (Franz *et al*., 1999). Various *Enterococcus* species have been isolated from animal products (Hayes *et al*., 2003; Khan *et al*., 2005). In food-production animals like poultry, cattle and pigs, *E. faecium* is a frequently isolated species (Klein, 2003). Other species like *E. faecalis* and *E. cecorum* can occur in high numbers, whereas, species like *E. gallinarum*, *E. durans/hirae* or *E. avium* are normally present in lower numbers in animals (Mareno and Blanch, 1999). These microorganisms are not regarded as primary pathogens, but due to their ability to obtain resistance determinants to antimicrobial agents, they have developed as nosocomal infections all over the world (Linden and Miller, 1999).

Strains resistant to antibiotics have also developed and have been widely dispersed under the ecological pressure of extensive use of antibiotics as additives to animal feeds and for the treatment of animal diseases (Thatcher and Clark, 1988). Antimicrobial resistance is a major health problem in human and veterinary medicine that has an enormous social and economic consequence (Wray and Wray, 2000; Warnick *et al*., 2001). The use of antimicrobial drugs in food animals may lead to multidrug antibiotic resistant strains of pathogens/non-pathogens, which may be transmitted to humans through food (Davis *et al*., 1999). The entry of glycopeptide resistant enterococci (GRE) of animal origin into the human food chain allows these strains to become established in the human gut and can also favour transfer of their resistance genes to human commensals (van den Bogaard *et al*., 2002).

1.2 PROBLEM STATEMENT

In veterinary medicine antibiotics are used as prophylactic agents, to prevent epidemic spread of infectious animal diseases, to provide high efficiency of animal production, to prevent the transfer of zoonoses from animals to the human population, to warrant safety of food of animal origin and to prevent food-borne diseases (Ungemach, 2000). However, the problem is that, farmers might use such drugs for the control, treatment or prevention of various infections and diseases without consulting the veterinary surgeons and animal health technicians, and thus, end up misusing them (Goldman, 2004).

The use or misuse of antimicrobials can increase the prevalence of antimicrobial resistance of bacteria (Dunlop *et al.*, 1998). Inappropriate use of antimicrobials such as administering sub-therapeutic doses or early discontinuation of treatment further predisposes for resistant bacteria within a farm (McDermott *et al.*, 2002; Roe and Pillai, 2003). However, a lot of factors can influence antimicrobial resistance. These factors differ among farms depending on the health status of herds, farm management and environmental factors. In addition to that, resistance patterns vary widely among farms (van der Wolf *et al.*, 1999; Regula *et al.*, 2003).

Food animals may act as reservoirs of antibiotic resistant enterococci, which can be transmitted to humans (Klein *et al.*, 1998). Transmission of bacteria between animals and man is not only limited to agents of zoonotic diseases. Bacteria that are normally non-pathogenic such as *Enterococcus* species can also be transferred. These could be spread under unhygienic conditions of food handling and slaughtering. Some of these species are opportunistic pathogens and can cause serious health problems.

Therefore, in this study MAR enterococci was assessed in communal and commercial farms around Mafikeng (Nort-West Province, South Africa) in order to aid in monitoring the spread of resistant organisms.

1. 3 AIM AND OBJECTIVES

1.3.1 Aim

The aim of this study was to determine the antibiotic resistance profiles of *Enterococcus* species found in both communal and commercial cattle faeces and in their water sources, and to determine the factors that could be responsible for the observed antibiotic resistant phenotypes.

1.3.2 Objectives

1. To isolate, and characterise *Enterococcus* species from the faeces of cattle and their water sources.
2. To determine the antibiotic susceptibility profiles of *Enterococcus* species to various antibiotics.
3. To compare antibiotic resistance data of these microorganisms from communal and commercial cattle as well as water sources.
4. To determine/characterise mechanisms potentially responsible for selected observed antibiotic resistance phenotypes.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

The genus *Enterococcus* can be identified by phenotypic characteristics such as the ability to grow in brain heart infusion (BHI) broth at 10°C for 7 days, 45°C for 2 days, and in 6.5% NaCl (Mannu *et al.*, 2003; Klein, 2003). Other primary phenotypic methods for identification of this genus include esculine hydrolysis, Gram staining, catalase, haemolytic reactions and pigmentation (Hayes *et al.*, 2003). Commercially prepared analytical kits such as the API 20 Strep and the API system ID32-strep can be used to distinguish various *Enterococcus* species (Mannu *et al.*, 2003).

Enterococci are Gram-positive, non-spore-forming bacteria that grow facultative anaerobically (Peters *et al.*, 2003). They are catalase negative and their major end product, after fermentation of carbohydrates, is lactic acid (Holzapfel and Wood, 1995). These microorganisms are ubiquitous, commensal inhabitants of the gastrointestinal tract of humans and animals (Johnston and Jaykus, 2004). *Enterococcus* species are frequently isolated from environmental sources such as soil, surface waters, raw plants, birds and animal products (Franz *et al.*, 1999; Harwood *et al.*, 2000; Johnston and Jaykus, 2004). They are also common components of the micro floral community of insects and reptiles (Hayes *et al.*, 2003). Their natural ruggedness allows them to persist and spread in the environment (Johnston and Jaykus, 2004).

The *Enterococcus* species are known to be intrinsically resistant to several antibiotics (Murray, 1990). Their natural resistance includes sulphamethoxazoles, aminoglycosides, cephalosporins and mostly clindamycin (Mallon *et al.*, 2002; Peters *et al.*, 2003). Even though enterococci are not regarded as primary pathogens, they have emerged as nosocomial pathogens all over the world

due to their ability to acquire high-level resistance to antimicrobial agents (Linden and Miller, 1999).

These microorganisms are also becoming increasingly resistant to multiple antibiotics (Cetinkaya *et al.*, 2000). Antibiotics used in human medicine belong to the same general classes as those used in food animals. In many cases, if they are not the same compounds, their mode of action is the same. The use of antibiotics in food animals select for bacteria resistant to antibiotics used in humans (Phillips *et al.*, 2004). Antibiotic resistant enterococci of pathogenic and non-pathogenic strains may be transmitted to humans through ill-prepared food due to the use of antimicrobial drugs in food animals (Davis *et al.*, 1999).

2.2 SPECIES DISTRIBUTION OF ENTEROCOCCI IN ANIMALS AND AGRICULTURAL PRODUCTS

Enterococci consist of a bacterial group widely diffused in several habitats. They may also be found in a variety of fermented foods such as cheese sausages and vegetables (Mannu *et al.*, 2002; Leclerc *et al.*, 1996). Several scientists have isolated *Enterococcus* species from animals and agricultural products. Kühn *et al.* (2003) conducted a study on the enterococcal populations in animals, humans and the environment in different geographical regions and in different parts of the food chain. They collected 2868 samples from humans, slaughterhouse carcasses, farm animals, and sewage and surface water in Sweden, Denmark, UK and Spain. Their study showed the most common species to be *E. faecium* (33%), *E. faecalis* (29%) and *E. hirae* (24%). These enterococcal populations differed in their species distribution. They found *E. faecalis* as a dominant species in broilers especially in Spain and Denmark. In Sweden and Denmark *E. hirae* was a predominant species in cattle and pigs whereas *E. faecium* was dominant in pigs from Spain (Kühn *et al.*, 2003). In South Africa, *Enterococcus* species such as *E. faecium*, *E. avium*, *E. durans* and *E. gallinarum* have been isolated from pig faeces in farms around the Mafikeng area (Moneoang, 2003).

Khan *et al.* (2005) isolated predominantly *E. gallinarum* from the milk of cows with mastitis, chicken and turkey litter. The aim of their study was to characterise multidrug-resistant *Enterococcus* species from poultry and dairy farms using molecular typing methods. Peters *et al.* (2003) assessed the species distribution and antibiotic resistance patterns of enterococci isolated from food of animal origin. They conducted their study on 155 samples of animal products in which 416 enterococcal strains were isolated. The predominant isolate was *E. faecalis* followed by *E. faecium* with the least dominant strain being *E. avium*.

Johnston and Jaykus (2004) demonstrated that antibiotic resistant *Enterococcus* species could also be isolated from leafy vegetables and other agricultural products. In that study, 185 *Enterococcus* isolates (97 *E. faecium*, 38 *E. faecalis* and 50 other *Enterococus* species) were identified from fresh produce harvested in the South-Western United States. Hayes *et al.* (2003) conducted a study that aimed at assessing the prevalence and antimicrobial resistance of *Enterococcus* species isolated from retail meats. *E. faecium* appeared as the predominant species in turkey, beef and chicken, while *E. faecalis* was predominant in pork samples from grocery stores in the United States (Hayes *et al.*, 2003).

2.3 *ENTEROCOCCUS* SPECIES DISTRIBUTION IN HUMANS

Enterococcus species are the second most important bacterial genus in hospital infections in European countries (Klare *et al.*, 2003). They have been implicated in infective endocarditic and urinary tract infections in humans for nearly a century (Murray, 1990; Huycke *et al.*, 1998).

Infections caused by *Enterococcus* species may be due to at least 12 species including *E. faecalis*, *E. faecium*, *E. gallinarum*, *E. hirae*, *E. malodoratus*, *E. mundtii*, *E. pseudoavium*, *E. raffinosus*, *E. solitarius*, *E. cecorum*, *E. columbae* and *E. saccharolyticus*. Occasional infections are due to *E. gallinarum*, *E. durans* and *E. avium* and other species such as *E. mundtii*, *E. hirae* (Mundy *et al.*,

2000). The most-frequently isolated *Enterococcus* species in human intestines are *E. faecium* and *E. faecalis* (Klein, 2003; Phillips *et al.*, 2004; Qu *et al.*, 2005). Kaçmaz and Aksoy (2005) isolated 9% *E. faecium* and 78% *E. faecalis* from human specimens.

Qu *et al.* (2005) obtained a total of 106 *Enterococcus* clinical isolates when conducting a study on the implementation of effective infection control measures in a Chinese hospital. The *Enterococcus* species isolated included 48 *E. faecalis*, 46 *E. faecium*, 6 *E. gallinarum*, 1 *E. avium*, 1 *E. hirae* and 4 *E. casseliflavus*. The majority of these isolates were from the abdominal fluid and bile.

In the Republic of South Africa, 184 rectal swabs were obtained from patients identified as being at high risk for glycopeptide resistant enterococci (GRE) in four Johannesburg hospitals. Twenty enterococcal isolates showing various glycopeptide resistance genotypes were recovered. They isolated *E. faecium* that possessed the vancomycin resistant genes *vanA* and *vanB*, *E. gallinarum* and *E. avium* possessed the *vanC1* and *vanA* genes, respectively (von Gottberg *et al.*, 2000).

2.4 THE USE OF ANTIBIOTICS IN FOOD ANIMALS

In livestock production, it is important to limit succession of disease in the animal population, because illness decreases production. Antimicrobial agents are usually used as therapeutic agents against bacterial infections, for treatment of mastitis during lactation or a cure at dry off (Cupáková and Lukášová, 2003). They can be administered to an animal or a group of animals, which show a clinical disease, and also to animals usually as a herd or flock in which morbidity and/ or mortality has exceeded baseline norms (van der Wolf *et al.*, 1999).

Antibiotics can also be used as growth promoters in animal feeds (Phillips *et al.*, 2004). Tylosin, oxytetracycline and other antibiotics are used as growth promoters, but in lower doses than those required for therapeutic use (Cupáková and Lukášová, 2003). Prescott and Baggot (1993) have

shown that growth promoters perform best when animals are in poor health and exposed to unhygienic living conditions. Therefore, it was suggested that if the animal's local environments are improved with overcrowding reduced and infection control techniques introduced, then the need for growth promoters may be removed. Antibiotic use selects not only for resistance in pathogenic bacteria, but also in the endogenous flora of exposed animals (van den Bogaard *et al.*, 2002).

2.5 *ENTEROCOCCUS* ANTIBIOTIC RESISTANCE

Gene transfer systems are an important requirement for the spread of drug resistance in microorganisms (Tomita *et al.*, 2003). Antibiotic resistance in enterococci can arise because of chromosomal mutation or through the acquisition of transferable genetic material such as plasmids and transposons (Mundy *et al.*, 2000). Chromosomal mutations that result in antibiotic resistance produce structural changes in the bacterial cell wall, while transferable resistance tends to code for enzymes that metabolise antibiotics. Transfer of genetic material may produce resistance to several antibiotics simultaneously (Prescott and Baggot, 1988). Enterococci acquire resistance to antibiotics with relative ease and are able to spread the resistance genes to other species (Kühn *et al.*, 2000).

Enterococcus faecium strains possess a broad spectrum of natural and acquired antibiotic resistance (Klare *et al.*, 2003). Drug resistance genes can be transferred by conjugation in various species of Gram-positive bacteria including *Enterococcus* species. The genes conferring conjugative activity are found on plasmids, prophages and transposons (Hugo and Russell, 1992; Murray, 1998).

The tendency of enterococci to acquire MAR traits may result from a variety of distinctly different mechanisms for conjugation. One of the mechanisms involves a broad host range plasmid that can transfer among species of enterococci and other Gram-positive organisms such

as streptococci and staphylococci (Clewell, 1981).

Other resistance genes, including those encoding resistance to erythromycin and kanamycin can also be found on conjugative transposons that contain or are related to Tn*916*. These transposons may have evolved from a Tn*916* ancestor and their emergence suggests the possibility of further spread of resistance among Gram-positive organisms. Enterococci have also developed plasmid and transposon-mediated resistance to tetracycline and erythromycin (Murray, 1998). All antibiotics can select spontaneous resistant mutants and bacteria that have acquired resistance by transfer of transposable elements from other bacteria. These resistant variants as well as species that are inherently resistant to antibiotics can become dominant and spread in host animal populations (Phillips *et al.*, 2004).

2.6 MECHANISM OF ACTION OF VARIOUS ANTIBIOTICS

The most important purpose of using antimicrobial agents for the treatment of infections is to eliminate the pathogen's harmful effects (Ungemach, 2000). Therefore, the antibiotic must bind to a specific target-binding site on the microorganism in order to disrupt a point of biochemical reaction. The concentration of the antimicrobial must also be sufficient to occupy a number of specific active sites on the microorganism for a long period. Active sites are different for different classes of antibiotics (Capitano and Nightingale, 2001). Table 2.1 is a summary of various classes of antibiotics' modes of action and mechanisms of resistance.

Table 2.1: Antibiotic mechanisms of action and resistance mechanisms.

Antibiotic Group	Examples	Target	Active against G (+)	Resistance Mechanism
B-Lactams[a]	Ampicillin	Cell Wall synthesis. Inhibitor- act on penicillin binding proteins (PBP)	√	Penicillin-G impermeable to G (-) Mutations in PBPs Produce β-Lactamase
Aminoglycosides[b]	Gentamycin Kanamycin Streptomycin	Bind to 30S subunit of ribosomes – inhibit protein synthesis	√	Aminoglycoside modifying enzymes Flux mechanisms RNA modifications
Tetracyclines[c]	Tetracycline	Bind to 30S subunit of ribosomes – inhibit protein synthesis	√	Efflux mechanisms 16S mutations
Chloramphenicols[d]	Chloramphenicol	Bind to 50S subunit of ribosomes – inhibit protein synthesis	√	Efflux mechanisms Inactivation by enzymes
Quinolones[e]	Nalidixic acid	Inhibits DNA gyrase synthesis	√	Inhibit the microbial enzyme, DNA gyrase and thus block chromosomal replication
Glycopeptides[f]	Vancomycin	Cell Wall synthesis. Inhibitor	√	Bind to D-alnyl-D-alanine, inhibit transfer of linear glycan acceptor, to the N-acetylmuramylpentapeptide-N-acetylglucosamine

G (+)= Gram positive (Lavigne[f] *et al.*, 2005; Rice[a], 2001; Capitano and Nightingale[a-f], 2001; Cetinkaya[b] *et al.*, 2000; McKessar[f] *et al.*, 2000; Fontana[a] *et al.*, 1994 and Hugo and Russell[c], 1992).

2.6.1 *β*-Lactams

Beta-lactamase production is one of the resistance mechanisms that can occur in enterococci (Murray *et al.*, 1991). Ampicillin resistance can also be caused by the overproduction of the important penicillin-binding protein 5 (PBP5) (Fontana *et al.*, 1994; Table 2.1). *E. faecium*

resistance to ampicillin, for example, is due to an alteration of the PBP5 (Ligozzi *et al.*, 1996). This PBP5-mediated ampicillin resistance is thought to be intrinsic to the enterococci and is usually non-transferable (Rice, 2001).

Epidemiological characteristics of ampicillin-resistant, but vancomycin-susceptible *E. faecium* are not well known (Fortu'n *et al.*, 2002). A few years ago the co-transfer of ampicillin and vancomycin resistance was described and ampicillin resistance was then proposed as a risk factor for the endemic spread of vancomycin-resistant strains (Suppola *et al.*, 1999).

2.6.2 Aminoglycosides

Aminoglycoside resistance is due to the production of aminoglycoside-modifying enzymes, which alter the ribosomal binding site and reduce the uptake or decrease cell permeability (Table 2.1; Prescott and Baggot, 1993; Cetinkaya *et al.*, 2000; Barnhart *et al.*, 2002). Enterococcal therapy is greatly affected by high-level resistant (HLR) enterococci to aminoglycosides. This degree of resistance predicts resistance to synergism between cell wall active agents and the aminoglycoside to which the organism is highly resistant (Murray, 1990).

High-level resistance to streptomycin can be due to a mutation that results in ribosomes resistant to streptomycin inhibition or streptomycin adenylyltransferase. This can also coexist with the genes for HLR to other aminoglycosides. Streptomycin resistance is mainly encountered in *Enterococcus* strains that produce streptomycin adenyltransferase (Cetinkaya *et al.*, 2000), an enzyme known as 3'-phosphotransferase, APH (3')-III. This enzyme can eliminate synergism between cell wall active agents and is responsible for HLR to kanamycin. High-level resistance to gentamycin results from the bifunctional protein (AAC(6')-I/APH(2")), encoded by a single gene with two active sites, one with 6'-acetyltransferase activity and the other with 2"-phosphotransferase activity (Ferretti *et al.*, 1986).

Aminoglycosides penetrate the cell envelope, bind to the receptors on the 30S subunit of the ribosome and induce the misreading of the genetic codes on the messenger RNA (mRNA) template. Incorrect amino acids are then incorporated into the peptide and ribosomal protein where synthesis is inhibited (Prescott and Baggot, 1993; Cetinkaya *et al.*, 2000).

Aminoglycoside-acquired resistance develops from aminoglycoside-modifying enzymes that decrease the ability of the drug to bind to ribosomes (Cetinkaya *et al.*, 2000; Barnhart *et al.*, 2002). Strains resistant through chromosomal mutation to gentamycin are also resistant to streptomycin, neomycin and kanamycin. Moellering *et al.* (1971) demonstrated that two types of streptomycin resistance occur in enterococci. The first one was moderate level resistance (MIC, 62 to 500µg/ml) caused by low permeability and can be overcome with penicillin (it increases the cellular uptake of the aminoglycoside). Secondly, the high level resistance (MIC$\geq$2.000µg/ml) that is ribosomal mediated due to a mutation that results in ribosome resistant streptomycin inhibition, or caused by the production of streptomycin adenylytransferase (Moellering *et al.*, 1971; Murray, 1990). Streptomycin resistance is mainly encountered in *Enterococcus* strains that produce streptomycin adenylytransferase (Cetinkaya *et al.*, 2000).

2.6.3 Tetracycline

Due to the extensive use of tetracycline, resistance to this antibiotic has been observed in a wide variety of bacteria. Most bacteria resistant to this drug have acquired tetracycline resistance genes (Roberts, 1994). This group of antibiotics bind to the 30S subunit of bacterial ribosome (Hugo and Russell, 1992; Table 2.1). The importance of the uptake process in their mode of action is shown by the fact that resistance to tetracycline is associated either with failure to accumulate the antibiotic or to an active efflux system which removes the drug from the cells (Hugo and Russell, 1992). Tetracycline resistant (*tet* genes) are found in pathogens, opportunistic pathogens, members of the normal flora and that can be isolated from humans, animals, food and the environment (Roberts, 1994; Taylor and Chau, 1996).

2.6.4 Glycopeptides

The glycopeptides molecules have a specific affinity for the D-alanyl-D-alanine (D-Ala-D-Ala) portion of peptidoglycan precursors. They bind to this region and inhibit the transfer of the linear glycan acceptor in the wall to the N-acetylmuramylpentapeptide-N-acetylglucosamine on its lipid carrier (Lavigne *et al.*, 2005; Table 2.1). Peptidoglycan assembly is stopped at an earlier stage than transpeptidation (Hugo and Russell, 1992).

Acquired resistance to vancomycin has emerged among enterococci and three phenotypes of vancomycin resistance and have been described as VanA, VanB and VanC. The VanA type strains are typically highly resistant to vancomycin and moderately to highly resistant to teicoplanin. This phenotype is often plasmid or transposon mediated and is inducible. On the other hand VanB encoded by *vanB* gene in the *vanB* gene cluster also stimulates the formation of D-Ala-D-Lac. The VanB phenotype is typically associated with moderate to high levels of vancomycin resistance, but is without resistance to teicoplanin (Murray, 1998).

The VanC phenotype is known to present low-level resistance to vancomycin and is susceptible to teicoplanin. It is an inherent property of *E. gallinarum* and *E. casseliflavus*. This intrinsic property is regarded as non-transferable. It is linked to the presence of species-specific genes *vanC-1* and *vanC-2,* respectively (Dutka-Malen *et al.*, 1992; van den Bogaard *et al.*, 1997). These genes lead to the formation of D-Ala-D-Ser containing cell wall precursors (Murray, 1998; McKessar *et al.*, 2000). Enterococci with *vanC* genes can cause significant infections, but is rare and they have not been implicated in nosocomial outbreaks (Leavis *et al.*, 2004).

Vancomycin resistant enterococci have been found in sewage, from faeces of healthy farm animals and animal products and can be spread through both clonal expansion of resistant enterococci and horizontal transmission of resistance genes (Bates, 1997; Stobberingh *et al.*,

1999). Several types of both acquired and intrinsic glycopeptide's resistance, with either low or high levels of resistance, have been found in enterococci (Klare *et al.*, 2003).

The results of the multiplex-PCR assay of Khan *et al.* (2005) for vancomycin resistance markers revealed the *vanC1* gene in 22 *E. gallinarum* strains. In a study of VRE in pork and poultry it was revealed that 10.2% of *E. faecium* isolates and one strain of *E. durans* had carried *vanA* genotypes. Fifty nine percent *E. gallinarum* had a *vanC1* genotype and 29% *E. casseliflavus* strains presented a *vanC2* genotype (Gambarotto *et al.*, 2001). Low-level resistance to vancomycin is conferred by *vanC* genes (Bell *et al.*, 1998), and it has been known as an intrinsic vancomycin resistance found in *E. casseliflavus*, *E. gallinarum* and *E. flavescens* (Arthur *et al.*, 1996).

2.7 TRANSFER OF ANTIBIOTIC RESISTANCE FROM ANIMALS TO HUMANS

It is hypothesised that glycopeptide-resistant *Enterococcus* strains and other resistant determinants are being introduced into the human population from a number of sources (Wegener *et al.*, 1999; Schwalbe *et al.*, 1999). Such sources significantly enhance the number of potential reservoirs for infection (Vandamme *et al.*, 1996).

The use of antibiotics in food animals selects for bacteria resistant to antibiotics used in humans and these might spread via food to humans and cause human infections (Phillips *et al.*, 2004). Several studies indicated food animals as reservoirs of resistant enterococci that might be transmitted to humans through the food chain and represent a potential risk for consumers (Schouten *et al.*, 1997; Sorensen *et al.*, 2001; Moubareck *et al.*, 2003).

In Europe the food chain has been suspected to be a source of VRE acquired by humans (Sorensen *et al.*, 2001). Consumption of meat has been associated with colonisation by VRE of the gastrointestinal tract of humans. The presence of glycopeptide resistant enterococci in non-

hospitalised humans and people who eat meat has been associated with the spread of VRE from animals (Schouten *et al.*, 1997). Sorensen *et al.* (2001) conducted a study in which healthy human volunteers were given GRE of animal origin to ingest. This study revealed the prolonged presence of GRE of animal origin.

It has been previously investigated and shown that there is the occurrence of horizontal gene transfer between bacteria that colonise livestock and humans (Moubareck *et al.*, 2003; Sorensen *et al.*, 2001). Moubareck *et al.* (2003) demonstrated that various resistance genes could be conjugatively transferred from an *E. faecium* strain of animal origin to that of a human. They conducted their experiment in the gastrointestinal tracts of gnotobiotic mice in the absence of selective pressures. This simply showed that there was gene transfer from animal to human enterococci. Their study suggested that under natural conditions, gene exchange could take place more commonly than previously suspected.

Kühn *et al.* (2003) found low similarities between the enterococcal populations when comparing bacteria of humans to those of animal origin (cattle) as well as between other animal species. Therefore, they concluded that there was a limited exchange of enterococcal strains between certain species.

2.8 METHODS USED TO IDENTIFY MICROORGANISMS, TO GENERATE ANTIBIOTIC RESISTANCE/SUSCEPTIBILITY DATA AND TO IDENTIFY RESISTANT GENES

Typing systems are used to define specific characteristics of the object under investigation. The procedures are specific for various phenotypic or genetic parameters. These procedures can also be applicable to any microbial species and can be genus specific. Typing procedures are the basis for the integration of epidemiology, taxonomy and evolutionary genetics (van Belkum *et al.*, 2001).

Standard biochemical testing kits like VITEK and API 20 STREP can be used to identify *Enterococcus* species. Analytical profile index (API) kits are biochemical test kits that can be used to identify bacteria.

The Kirby Bauer paper disk plate method (Bauer *et al.*, 1966) can be used to determine the resistance of microorganisms to chemotherapeutic agents. Inhibition zone diameter measurements can be obtained by measuring the inhibition zone around the paper disk by means of a ruler.

The measure of degree of the drug resistance for isolates in the MAR groups can be added in order to determine their MAR indices (Krumperman, 1983; Kaspar *et al.*, 1990). According to Ehinmidu (2003) an MAR greater than 0.2 implies that the strain of the bacteria originates from an environment where several antibiotics are used. These traditional identification methods of bacteria or antibiotic resistant bacteria usually take at least 4 days to do after sampling.

Rota *et al.* (1996) used the disk diffusion technique, IZD data, MAR percentages and MAR phenotype patterns for susceptibility testing of *Listeria* species to 12 antimicrobial products. Guan *et al.* (2002) conducted a study in which the objective was to evaluate methods for differentiating *E. coli* isolates of livestock, wildlife, or human origin that might be used to predict the sources of

faecal pollution of water.

Polymerase Chain Reaction (PCR) can be used to screen the *Enterococcus* isolates for resistance genes by using the appropriate primer sets. Rapid and accurate identification of carriers of resistant microorganisms is an important aspect of efficient infection control in hospitals. A duplex real-time PCR assay for rapid detection of ampicillin-resistant *E. faecium* (ARE) was developed (Mohn *et al.*, 2004). They used primers and probes that specifically detected the *D-Ala-D-Ala* ligase gene of *E. faecium* and the modified penicillin-binding protein 5 gene (*pbp5*) carrying the Glu-to-Val substitution at position 629 (Val-629) in a set of 129 tested *E. faecium* strains with known pbp5 sequences. The results of this experiment were obtained after 4 hours. Therefore, it shows that with real-time PCR, carriers can be rapidly identified. Intensified infection control measures can also be started as soon as possible.

CHAPTER 3

MATERIALS AND METHODS

3.1 SAMPLING REGIME AND AREA

Between January and February 2005 a total of 79 cattle faecal and water samples were randomly collected from a communal and commercial farm near Mafikeng (North-West Province, South Africa). The commercial farms used for sampling were the North-West University farm, known as Molelwane near Mafikeng campus, which is 15km north of Mafikeng, and the Rooigrond prison farm, located 20km south east of Mafikeng. All the commercial farm samples were collected from the dairy cattle (Dutch Friesians) where the milk is either sold to the public or given to the prisoners as part of their daily diet. Sampling at communal farms was conducted at Gelukspan, a village 55km south east of Mafikeng.

3.2 GENERAL MICROBIOLOGICAL ANALYSIS

Faecal samples were collected directly from the rectum of animals using clean, sterile arm-length gloves. These were stored in sterile plastic faecal containers. Sterile 500ml bottles were used to collect water from the cattle water troughs. The samples were transported on ice in sealed bags and analysed within 6 hours of collection.

3.2.1 Enrichment

On arrival, 0.5g of the faecal sample was inoculated into 3 ml Nutrient broth and incubated at 37°C for 24 hours. The water samples were not enriched.

3.2.2 Selective plating

After 24 hours of enrichment of faecal samples, 0.1ml of the culture was plated onto *Enterococcus* selective agar and incubated at 45°C for 48 hours. For water samples a membrane filter method was employed (0.45µm pore size filter) on the *Enterococcus* selective agar and incubated at 45°C for 48 hours. Pure cultures were obtained by successive sub-culturing onto *Enterococcus* selective agar, using the streak-plate method. All agar plates were incubated for 48 hours at 45°C. Single pure colonies were then sub-cultured into brain-heart infusion broth and incubated for 2 days at 45°C and 5 days at 10°C. The isolates that showed growth at 45°C and 10°C were grown in 6.5% sodium chloride (NaCl) and brain-heart infusion broth at pH 9.6.

3.2.3 Primary biochemical tests for preliminary identification

3.2.3.1 Gram staining

To characterise the bacteria into Gram-positive or Gram-negative, Gram staining was performed (Cruikshank *et al.*, 1975).

3.2.3.2 Haemolysis test

A colony from the *Enterococcus* selective agar was plated on 5% sheep blood agar in order to check for the presence of haemolysis. *Enterococcus* species were expected to have non-haemolytic zones (Health protection agency, 2004).

3.2.3.3 Catalase test

Catalase test was performed by a cover-slip method. One drop of 3% hydrogen peroxide solution was placed on the microscope slide. A small portion of the colony from the *Enterococcus* selective agar was placed on the centre of the cover-slip. The cover-slip was then inverted and placed on the drop of hydrogen peroxide solution. A bubbling reaction after 10 seconds indicated the presence of catalase (positive reaction) while the absence of bubbling denoted the absence of

catalase (negative reaction) (Health protection agency, 2006). *Enterococcus* species were expected to have a negative reaction (Holzapfel and Wood, 1995).

3.2.4 Secondary biochemical identification by Analytical Profile Index test (API 20 STREP)

API 20 STREP (Bio Merieux, Cedex, France) assists with the identification of Gram-positive cocci. This test was employed according to the manufacturer's instructions to confirm the identification of isolates with reactions typical of *Enterococcus* species.

3.2.4.1 Preparation of API 20 STREP inoculums

After 24 hours of incubation, 5 ml distilled water was aliquoted into the honeycombed wells of the incubation tray to create a humid atmosphere and the strip was then put into the incubation tray. The culture was harvested by using a sterile swab and it was inoculated into 2 ml sterile distilled water (without additives) to make a turbid suspension.

3.2.4.2 Inoculation

The bacterial culture suspension (0.1 ml) was distributed into each cupule of tests VP to LAP. The tube was filled for test ADH only. For tests RIB to GLYG, 0.5 ml of the prepared suspension was mixed with API GP medium and distributed into the tubes of these tests and the cupules of ADH to GLYG were filled with mineral oil. The strips were then incubated at 36°C for 24 hours.

After 24 hours of incubation, one drop of each of reagent VP1 and VP2 was added to the VP cupules. Two drops of NIN were added to HIP. One drop of ZYM A and of ZYM B was added to tests PYRA to LAP. The reactions were read after ten minutes by referring to a reference table. Reactions were interpreted by using the API 20 STREP charts. Positive numbers on the result sheet were added and the analytical profile index programme was used to determine the identity of each isolate.

3.2.5 Bacterial sensitivity test

3.2.5.1 The disk diffusion technique

The Kirby Bauer disk diffusion technique was used (Bauer *et al.*, 1966) to determine the resistance/susceptibility of microorganisms to chemotherapeutic agents (Table 3.1). A single well-isolated colony was removed from an isolation plate by means of a sterile wire loop and inoculated into 5 ml sterile distilled water to make a turbid bacterial suspension. This suspension was swabbed onto Mueller Hinton agar that contained 5% defibrinated sheep blood and left to dry at room temperature for 20 minutes. Paper disks (Mast Diagnostics, UK) with the specific concentration of antibiotics (Table 3.1) were placed on the surface of the agar plates. Plates were then incubated overnight at 37°C.

3.2.5.2 Reading of sensitivity test results

The diameter of the inhibition zone was measured using a transparent ruler and recorded to the nearest millimetre. The inhibition zone measurements were used to interpret whether the isolates were resistant, intermediate or sensitive to antibiotics, using Table 3.1.

3.2.5.3 Multiple antibiotic resistance (MAR) Index and cluster analysis

The inhibition zone data of isolates were subjected to analysis that included determining multiple antibiotic resistance (MAR) indices as well as cluster analysis. The MAR index for each group was determined by the method of Krumperman (1983) and Kaspar *et al.* (1990). This index was the measure of the degree of the drug resistance for isolates in the group. Each host group MAR index was calculated by adding the numbers of drugs to which each isolate was resistant and dividing the resulting number by the product of the antibiotics and the number of isolates tested i.e.

$$\text{MAR Index} = \frac{\text{Number of isolates resistant to all antibiotics in a specific sample population}}{\text{(number of antibiotics tested) x (total number of organisms in sample)}}$$

For cluster analysis, Ward's method and Euclidean distances in the Statistica version 7 software were used and the results expressed as dendrograms. This was done to determine the commonness and resolve differences between the *Enterococcus* isolates.

Table 3.1: The details of antibiotics used in this study.

Class	Antibiotic used	Abbreviations	Concentration	Resistant	Intermediate	Susceptible
Penicillin/ β-lactamase	Ampicillin	AMP	10 µg	≤16	-	>17
Aminoglycoside	Kanamycin	KAN	30µg	≤13	14-17	>18
Nitrofurans	Nitrofurantoin	NIT	100 µg	≤14	15-16	>17
Tetracycline	Oxytetracycline	OXY-TET	30 µg	≤14	15-18	>19
Quinolone	Nalidixic acid	NAL	30 µg	≤13	14-18	>19
Sulphonamide	Sulphamethoxazole	SMX	25 µg	≤10	11-15	>16
Aminoglycoside	Gentamycin	GEN	10µg	≤12	13-14	>15
Chloramphenicol	Chloramphenicol	CHL	30µg	≤12	13-17	>18
Aminoglycoside	Streptomycin	STR	10µg	≤6	7-9	>10
Glycopeptide	Vancomycin	VAN	30 µg	≤14	15-16	>17

Concentrations used as well as the inhibition zone measurements (in mm) that were considered resistant, intermediate and susceptible are shown and were according to the NCCLS (2002). The abbreviations were according to the 2005 instructions to authors for the Journal of Clinical Microbiology (http://jcm.asm.org/misc/itoa.pdf).

3.3 MOLECULAR CHARACTERISATION OF ANTIBIOTIC RESISTANCE

3.3.1 Genomic DNA extraction

Genomic DNA was extracted from the bacterial colonies using a cetyltrimethyl ammonium bromide (CTAB), polyvinyl pyrrolidone (PVP) extraction method (Doyle and Doyle, 1990). An overnight culture of a resistant microorganism was prepared by picking one pure colony from the *Enterococcus* agar media and inoculated into 5 ml Luria Bertani (LB) broth. The culture was incubated overnight at 37°C by shacking (120 rpm). One millilitre of the overnight culture was added into a 1.5 ml microfuge tube and centrifuged at 13 400 rpm for 5 minutes and the supernatant was discarded. Hundred microliters of TE buffer (10mM Tris- HCl, pH 8.0, 1mM EDTA) and 10 µl of lysozyme (10mg/ml) were added to the pellet and incubated at 37°C for 30 minutes.

Hot (65°C) CTAB (Sigma H-6269) isolation buffer (250 µl), 50 µl of PVP (Sigma P-5288) (5% solution) and 10 µl of proteinase K (20mg/ml) were added to the sample, which was then incubated at 65°C in a water bath for 15 minutes. Inverting the microfuge tubes every 3 minutes mixed the contents in the tubes. This mixture was then extracted at room temperature with an equal volume (± 420 µl) of TE buffered phenol: chloroform: isoamyl alcohol (25:24:1). The sample was centrifuged at 13 400 rpm for 5 minutes and the aqueous phase was transferred to a new sterile microfuge tube. An equal volume (± 420 µl) of TE buffered chloroform: isoamyl alcohol (24:1) was used to re-extract the aqueous phase for 10 minutes at room temperature. After centrifugation at 13 400 rpm, the aqueous phase was transferred to a sterile microfuge tube. A 100 µl of 5 M NaCl (final concentration of 1.2 M) and 2 volumes of ice-cold 95% ethanol were added. The samples were incubated at -80°C for 1 hour or -20°C overnight to precipitate the DNA.

To collect the DNA, the sample was centrifuged at 13 400 rpm at 4°C for 5 minutes and the supernatant was discarded. The pellet was washed with 1 ml ice cold 70% ethanol to remove NaCl

and the sample was centrifuged at 13 400 rpm at 4°C for 5 minutes and the supernatant was discarded. Lastly, the pellet was dried under vacuum, dissolved in TE buffer and incubated at 65°C for 1 hour to reconstitute the DNA.

3.3.2 Polymerase Chain Reaction (PCR)

The bacterial 16S rRNA DNA fragments were amplified by PCR and sequenced these for enterococci species classification conformation (Muyzer *et al*., 1995). PCR was also used to characterise isolates for the presence of genes that code for vancomycin resistance (Bell *et al*., 1998). PCR reactions were performed in 25µl volumes containing 50 pmoles of each primer set, 12.5 µl of 2x PCR master mix, 0.2 µl (1 unit) of Taq DNA polymerase, 0.5 µl (50ng) BSA, DNase-RNase free water and between 50 and 100ng of Template DNA. Reagents used were from Fermentas Life Sciences (USA), and supplied by Inqaba Biotech (South Africa). The amplification protocol was performed for 30 cycles with initial denaturing at 95°C for 5 minutes followed by denaturing at 95°C for 30 seconds, annealing temperature at 62°C for 30 seconds for 16S and vancomycin primer sets, elongation was done at 72°C for 1 minute (initial elongation) and 10 minutes (final elongation). Reactions were amplified in a Peltier thermal cycler model PTC-220DYAD™ DNA ENGINE (M. J Research Inc, USA). PCR reactions were analysed by electrophoresis on 1% (w\v) agarose gels (Section 3.3.4). In Table 3.2 a list of all the primers used is given.

Table 3.2: The PCR primer sequence used in this study.

Specificity	Primer	Sequence 5'-3'	Amplicon size
16S[a]	GM57	TACGGGAGGCAGCAG	
	907R	CCGTCAATTCCTTTGAGTTT	550
Van A[b]	VanAF	GTAGGCTGCGATATTCAAAGC	
	VanAR	CGATTCAATTGCGTAGTCCAA	210
Van B[b]	VanBF	GTAGGCTGCGATATTCAAAGC	
	VanBR	GCCGACAATCAAATCATCCTC	310
Van C[b]	VanCI R	AGATTGGAGCGCTGTTTTGTC	
	VanCI F	TGGTATTGGTATCAAGGAAACC	425

(Muyzer[a] *et al.*, 1995 and Bell[b] *et al.*, 1998).

3.3.3 Agarose gel electrophoresis

This was used to determine the quality of genomic DNA extracted, and to determine if PCR had successfully amplified the various fragments. Electrophoresis of DNA samples was performed at room temperature on a horizontal 1% (w/v) agarose gel. Five microliters of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 50% glycerol) was mixed with 10μl of the sample and loaded into the wells of the gel matrix. A final concentration of 0.001μg/ml ethidium bromide was added to the agarose. The DNA was electrophoresed in a Hoefer HE 99X system (Amersham Pharmacia, Biotech, Sweden). The electrophoresis buffer was 1 X TAE (40mM Tris, 1mM EDTA, 20mM glacial acetic acid) and electrophoresis was at constant 80 volts for 1 hour. A 100-bp DNA ladder molecular weight marker (5 μl) was included in each gel to determine the sizes of the products. The DNA was visualised with 420x nm ultra-violet light (Sambrook *et al.*, 1989). Gel images were captured using a Gene Genius Bio Imaging System (Syngene, Synoptics UK) and documented using GeneSnap (version 6.00.22) software.

3.4 EXPERIMENTAL SET UP

A total of 79 samples were collected from three different areas of which one was a communal farm (Gelukspan) and the other two were commercial farms (Molelwane and Rooigrond farms). These samples consisted of cattle faeces and the water from troughs on the farms. A total of 65 cattle faecal samples were collected from both commercial (33 from healthy and 16 from diarrhoeal cattle) and communal (16 healthy cattle) farms. Each commercial farm had 6 water troughs whereas only two were present on the communal farm. Therefore, 12 water samples were collected from the commercial farms and 2 were from the communal farm (Table 3.3).

Table 3.3: The number of samples collected at each farm from different sources.

Area of collection	**Sources and number of sample collected**	
	Cattle water	Cattle faeces
Commercial cattle (Healthy cattle) Rooigrond and Molelwane	12	33
Commercial cattle (Diarrhoeal) Molelwane	-	16
Communal cattle Gelukspan	2	16
	Total=14	**Total= 65**
	TOTAL=79	

The numbers of individual isolates were subjected to calculations of averages and percentages using Microsoft Office Excel statistical software to determine the number of *Enterococcus* isolates from various sample categories that were resistant to antibiotics. The same data was also subjected to cluster analysis using Ward's method and Euclidean distances on Statistica version 7 software and analysis of MAR indices (Section 3.2.5.3).

CHAPTER 4

RESULTS

4.1 SAMPLE DISTRIBUTION AND *ENTEROCOCCUS* SPECIES COMPOSITION

This section reports on the results of the *Enterococcus* species isolated from commercial (Molelwane and Rooigrond) and communal (Gelukspan) cattle farms. Samples from which these isolates were obtained and analysed were the faecal as well as from the water sources of the cattle. The isolates were compared in terms of the species composition and antibiotic resistance profiles. In Table 4.1, the number of samples analysed, the number of isolates obtained, as well as the number and species of further tested isolates are shown.

Table 4.1: The number of samples collected from various sources in communal and commercial cattle farms, the total number of *Enterococcus* isolates, total number tested and the names of *Enterococcus* species obtained.

Area	Sample source	No. samples collected	No. Isolates	Tested *Enterococcus* species	No. Tested	%
Gelukspan communal farm	Healthy cattle	16	17	*E. faecium*	13	76
	Water	2	7	*E. faecium*	5	71
				E. avium	2	29
Molelwane and Rooigrond commercial farms	Healthy cattle	33	41	*E. faecium*	11	27
				E. avium	17	41
				E. durans	8	20
				Sc. bovis I	5	12
	Diarrhoeal cattle	16	44	*E. faecium*	14	32
				E. avium	27	61
				E. durans	2	5
				Sc. bovis I	1	2
	Water	12	29	*E. faecium*	8	28
				E. avium	16	55

Percentages were obtained by dividing the number of tested *Enterococcus* species by the number of isolated *Enterococcus* species and multiplying the dividend by hundred.

Seventy-nine samples from the communal and commercial cattle faeces and their water were analysed for the presence of *Enterococcus* species. After initial enrichment and selective plating at least 4 potential enterococci colonies were sub-cultured and purified on the appropriate solid medium (Section 3.2.2). Only those that were positive for all the primary and secondary identification tests for enterococci were used for further analysis (Table 4.1). The sampling strategy for this study would have included obtaining an equal number of cattle faecal samples from commercial and communal farms (Section 3.1). Two farms from each of these categories were identified. Healthy cattle faecal samples from commercial farms (16 and 17, Molelwane and Rooigrond prison, respectively) were collected and pooled (Table 4.1).

Furthermore, a diarrhoea outbreak during the sampling period at the Molelwane campus gave the opportunity to collect faecal samples from this commercial cattle population. These additional samples expanded the data set of the commercial farms (Table 4.1). On the other hand, samples could only be collected from one communal farm, thus only 16 samples were included in that category. No diarrhoea outbreak was observed within the communal cattle population during sample collection. Only one water sample was collected from each of the water sources. Each commercial farm had 6 water troughs whereas only 2 were present on the communal farm (Table 4.1). A total of 138 *Enterococcus* isolates were identified from the 79 samples. They were all identified to species level (Sections 3.2.3 and 3.2.4) but only 129 of these were further investigated.

In addition to the results in Table 4.1, 8 of the identified isolates were *E. gallinarum* of which 4 were from the communal healthy cattle and the other 4 from the commercial cattle water source samples. The remaining identified isolate was *Sc. acidominimus* and was obtained from the commercial cattle water source. API 20 STREP identification profiles of these two species were, however, doubtful. Zouain and Araj (2001) also used the API 20 STREP identification kit (Bio Merieux, Cedex, France) as well as the software supplied by the manufacturer and received excellent results for all the *Enterococcus* isolates except for the two strains of *E. gallinarum*. Hence, it was decided to continue the study with only the following species: *E. avium*, *E. faecium*, *E. durans* and *Sc. bovis I* as shown in Table 4.1 (*E. gallinuram* and *Sc. acidominimus* were not included in the table as they were not further tested).

Table 4.1 also illustrates that among the 17 *Enterococcus* species isolated from the communal cattle faeces, *E. faecium* was the predominant species. On the other hand, three different *Enterococcus* species were identified among the faecal isolates from commercial farms. The pooled data of the isolates from healthy individuals from the two farms showed that the species composition was as follows: *E. avium* (41%), *E. faecium* (27%), *E. durans* (20%) and *Sc. bovis I* (12%). These results

are depicted in Table 4.1 and Figure 4.1. Figures 4.1 and 4.2 indicate the species composition of the *Enterococcus* species isolated. Figure 4.1 also indicates *Enterococcus* species isolated from commercial healthy and diarrhoeal cattle, whereas Figure 4.2 illustrates the common *Enterococcus* species composition from communal and commercial cattle water sources.

The results in Figure 4.1 also show that the *Enterococcus* species composition in diarrhoeal cattle faeces was generally similar to those of healthy individuals. However, *E. avium* (61%) and *E. faecium* (32%) constituted a greater proportion of the individuals among the diarrhoeal *Enterococcus* species than among the healthy isolates (Figure 4.1). On the other hand, *E. durans* (5%) and *Sc. bovis I* (2%) constituted a smaller proportion among the *Enterococcus* species from diarrhoeal individuals than among the healthy isolates (Figure 4.1).

Figure 4.2 demonstrates the differences in the levels of *E. faecium* and *E. avium* isolates from the communal and commercial cattle water sources. Among the water sample isolates from the communal farm, 71% was *E. faecium* and 29% was *E. avium*. *Enterococcus* isolates from the pooled commercial cattle water sources were identified as 28% *E. faecium* and 55% *E. avium*. The results demonstrated that *E. faecium* constituted a greater proportion of the individuals among communal cattle water sources. High proportions of *E. avium* individuals were obtained from commercial cattle water sources.

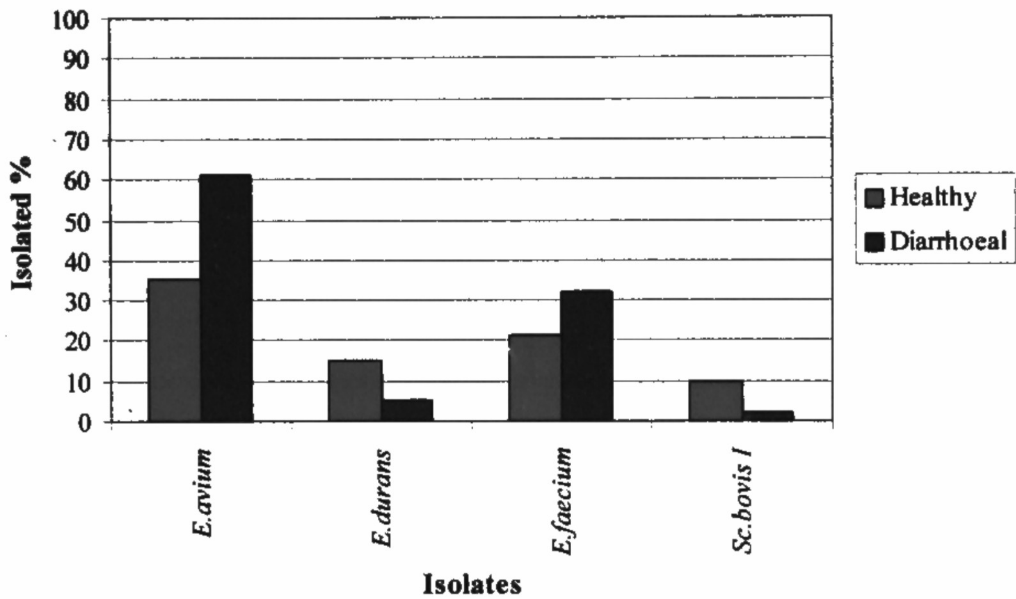

Figure 4.1: Species composition of *Enterococcus* isolates from commercial healthy Cattle.

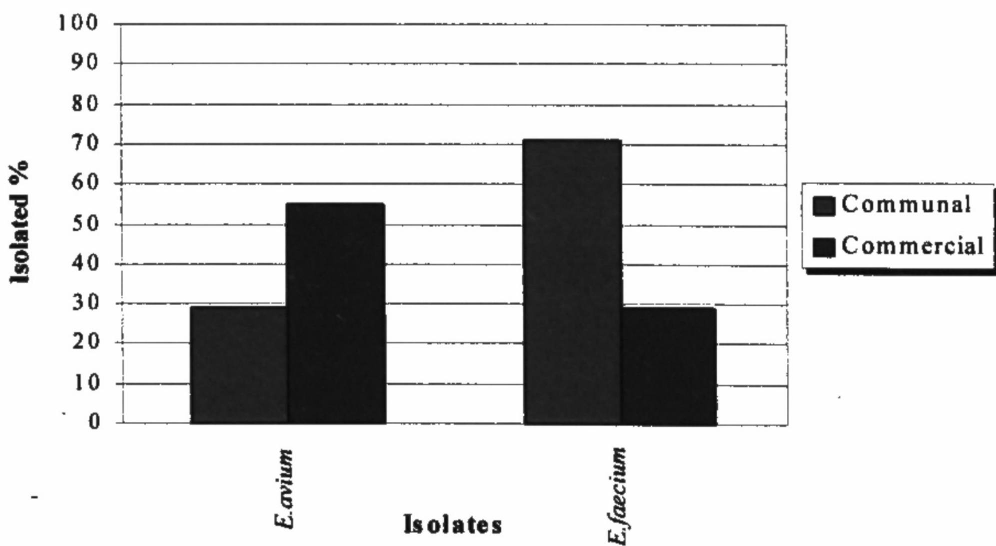

Figure 4.2: Species composition of common *Enterococcus* isolates from communal and commercial cattle water sources.

From the preceding interpretation of the results obtained it is evident that *E. faecium* was present in all the samples. As a result, only *E. faecium* data were used for comparisons where applicable. In other cases appropriate corresponding species data were also included in the analysis.

4.2 ANTIBIOTIC RESISTANCE PROFILES OF *ENTEROCOCCUS* SPECIES

This section reports on the antibiotic resistance profiles of the various *Enterococcus* species isolated during this study (Table 4.1). All the *Enterococcus* isolates were tested against 10 antibiotics (Section 3.2.5, Table 3.1). The antibiotic resistant profile data of the *Enterococcus* species are reported as follows: (i) Section 4.2.1 reports on the antibiotic resistance of *E. faecium* isolates from communal (Gelukspan) and commercial (Molelwane and Rooigrond) cattle faeces. (ii) Section 4.2.2 deals with antibiotic resistance of isolates namely, *E. faecium* and *E. avium* that were dominant in the cattle water sources from the communal and commercial farms. (iii) Antibiotic resistance of *Enterococcus* species isolated from healthy (Molelwane and Rooigrond commercial cattle) and diarrhoeal cattle faeces are reported in Section 4.2.3. Throughout this section, all the *Enterococcus* isolates that showed total resistance to antibiotics in all compared scenarios will not be illustrated in the figures, but is available in the Appendix B1- B13.

4.2.1 Antibiotic resistance of *E. faecium* isolates from communal and commercial cattle faecal samples

The percentages of *E. faecium* isolates from Gelukspan (communal farm), Molelwane and Rooigrond (commercial farms) farms that were resistant to the various antibiotics are presented in Figure 4.3.

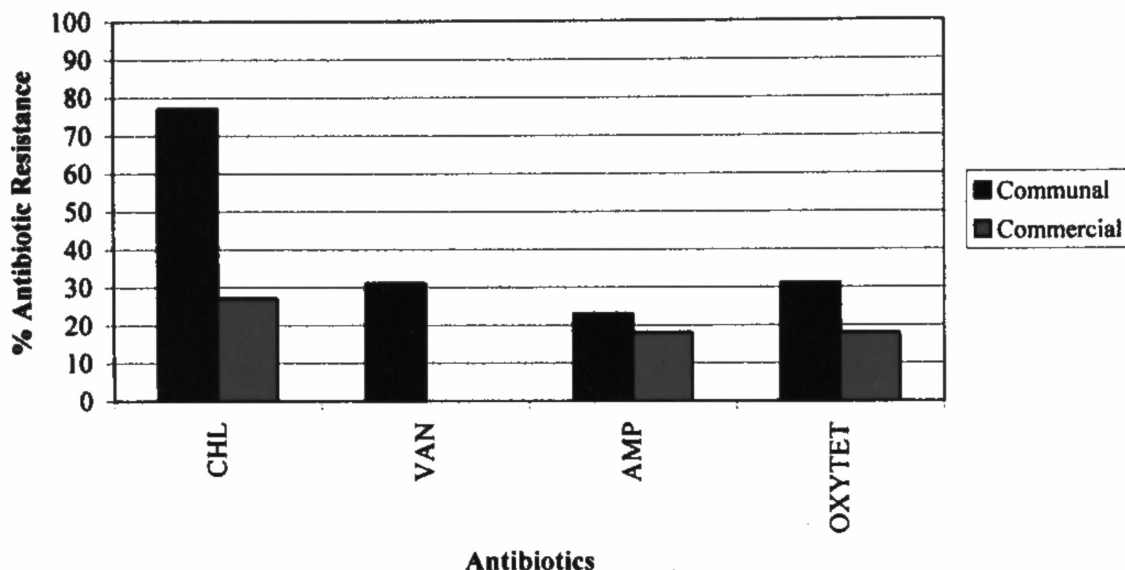

Figure 4.3: Percentages of antibiotic resistant *E. faecium* isolates in communal and commercial cattle faecal samples.

In this section the antibiotic resistance data of the *E. faecium* isolates from communal and commercial cattle (Table 4.1) are presented. The results are summarised in Figure 4.3, which depicts the percentages of *E. faecium* isolates that were resistant to the following antibiotics, CHL, VAN, AMP and OXYTET.

Enterococcus faecium isolates from both sample sets (communal and commercial cattle faecal samples) were all resistant to the following antibiotics: SMX, NAL, NIT, KAN and STR, but it has been previously found that enterococci are naturally resistant to SMX and KAN (Peters *et al.*, 2003). All tested *E. faecium* isolates from the commercial cattle faeces were resistant to GEN, whereas 92% of those isolated from the communal cattle faecal samples was resistant to the same drug (Appendix B1). Furthermore, when comparing percentages of the *E. faecium* species that were resistant to CHL, OXY-TET, VAN and AMP (Figure 4.3), it was evident that more isolates from cattle faecal samples from communal farms were resistant to these antibiotics than those from commercial farms. Seventy-seven percent of *E. faecium* from communal cattle faecal samples was resistant to CHL compared to 27% from commercial farms. Thirty one percent of the isolates from

communal cattle were resistant to OXY-TET and VAN, whereas 18% of isolates from commercial farms were resistant to OXY-TET and all were susceptible to VAN.

The observed differences and similarities in antibiotic resistance profiles between the *E. faecium* isolated from cattle faeces from communal and commercial cattle are further demonstrated in Table 4.2. In this table a summary of the common MAR phenotypes of *E. faecium* isolates from communal and commercial cattle faeces is shown. Thirty-eight percent showed a GEN-SMX-NAL-CHL-NIT-KAN-STR phenotype (mostly emerging from communal cattle faeces). Twenty five percent had the GEN-SMX-NAL-NIT-KAN-STR phenotype, 13% portrayed the GEN-SMX-NAL-NIT-OXYTET-KAN-STR phenotype and 8% showed a GEN-SMX-NAL-CHL-OXYTET-VAN-NIT-AMP-KAN-STR MAR phenotypic pattern.

Table 4.2: Phenotypic MAR results of *E. faecium* isolated from communal and commercial cattle faecal samples.

MAR Phenotype	C1	C2	No. Ob	%
GEN- SMX- NAL-NIT-CHL- OXYTET- VAN-AMP- KAN- STR	2	-	2	8
GEN- SMX- NAL-NIT- CHL-KAN- STR	6	3	9	38
GEN- SMX- NAL-NIT- KAN- STR	1	5	6	25
GEN- SMX-NAL-NIT-OXYTET-KAN-STR	1	2	3	13

C1- Communal; C2- Commercial, No. Ob-Number observed. The percentages were obtained by taking the sum of all *E. faecium* MAR phenotypes from cattle faeces of communal and commercial farms (Appendix C1 and C2) and dividing it by the number of common or highest MAR phenotypes that were obtained from either communal or commercial cattle faeces.

It was also shown that all the *E. faecium* isolates from the communal and the commercial cattle faecal samples had a similar antibiotic resistance trend since they all were carrying the resistant MAR phenotype 'GEN-SMX-NAL-NIT-KAN–STR'. The underlined antibiotics (Table 4.2) within the MAR phenotypes indicate the individual antibiotics that were different when the MAR phenotypes were compared.

4.2.2 Antibiotic resistance of *E. faecium* and *E. avium* isolates in cattle water sources from the communal and commercial farms

This section reports on the antibiotic resistant percentages of the dominant *Enterococcus* species namely, *E. faecium* and *E. avium* (Table 4.1) that were isolated from the cattle water sources from the communal and the commercial farms (the commercial farm water source data was pooled). Once again, throughout this section, all the *Enterococcus* isolates that showed total resistance to antibiotics in all compared scenarios will not be illustrated in the figures, but is available in Appendix B1-B13. Figure 4.4 represents the percentages of *E. faecium* and *E. avium* isolates from the commercial cattle water sources that were resistant to CHL, VAN, AMP and OXYTET.

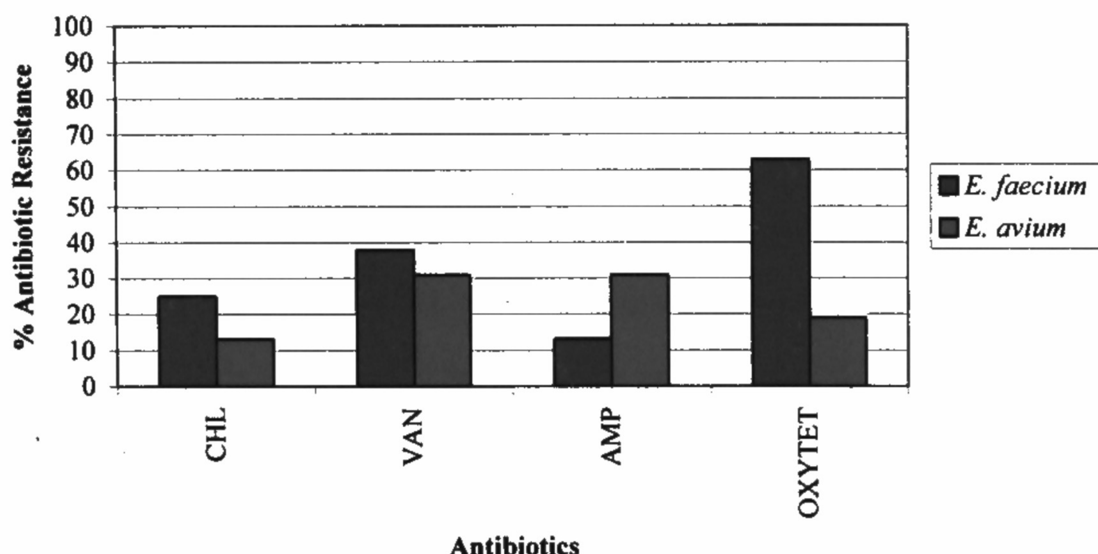

Figure 4.4: Percentages of antibiotic resistant *E. faecium* and *E. avium* isolates from commercial cattle water sources.

All the *E. faecium* isolates from the commercial cattle water sources were resistant to SMX, NAL, NIT, STR, 63% to GEN and 75% to KAN. Seventy-five percent of the *E. avium* isolates was resistant to NIT. On the other hand, all *E. avium* isolates were resistant to GEN, SMX, NAL, KAN and STR (Appendix B13). As shown in Figure 4.4, *E. faecium* and *E. avium* isolates from commercial cattle water sources were resistant to CHL (25% and 13%), VAN (38% and 31%),

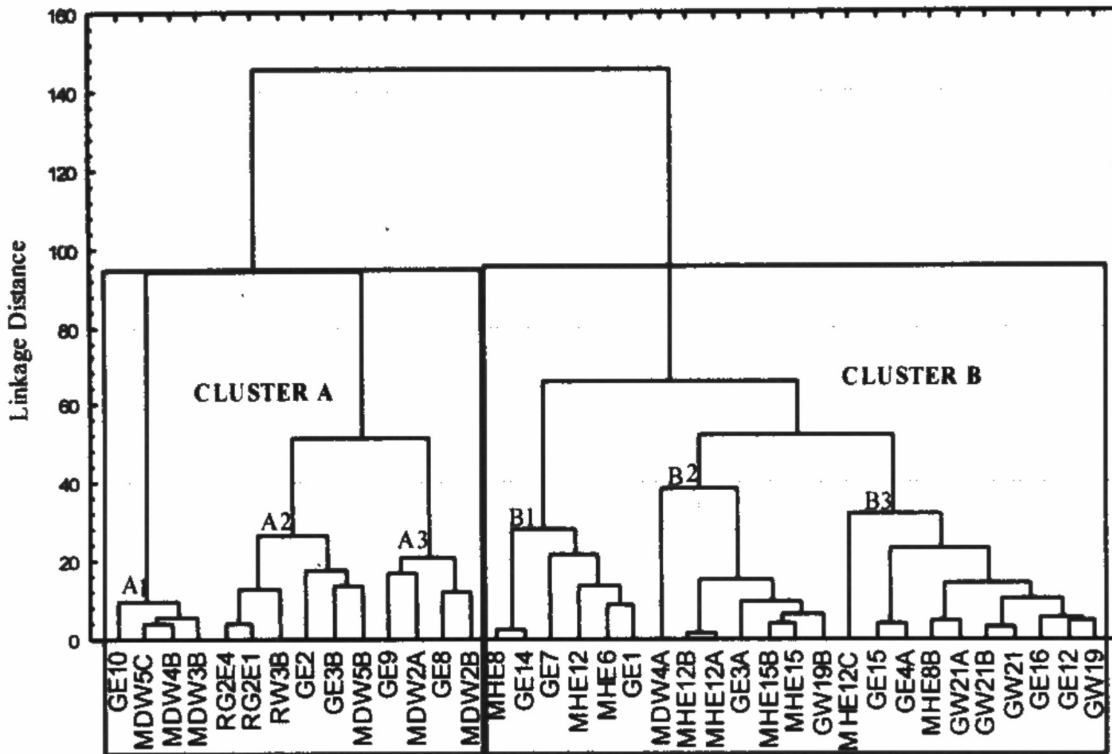

Figure 4.5: Illustration of the relationship of the 37 *E. faecium* isolates from the cattle faecal and water source samples isolated from (Commercial farms) Molelwane, Rooigrond and (Communal farm) Gelukspan.

The relationships of the 37 *E. faecium* isolates from the cattle faecal and water source samples isolated from (Commercial farms) Molelwane, Rooigrond and (Communal farm) Gelukspan were based on the IZD data obtained. Cluster A, with sub-clusters A1, A2 and A3, respectively, had 14 *E. faecium* isolates from the communal and the commercial cattle faecal and water samples. Cluster A1 had a total of 4 cases with 3 predominant *E. faecium* isolates from Molelwane water samples and 1 isolate from Gelukspan cattle faecal samples. Six *E. faecium* isolates were found in sub-cluster A2. Two were from the Gelukspan cattle faeces, 2 from Rooigrond cattle faeces, 1 from Rooigrond and another one from Molelwane commercial cattle water sources. *Enterococcus faecium* isolates from Rooigrond cattle faecal and water samples had similar antibiotic resistance profiles and were all presented in one cluster (cluster A, sub cluster A2). Sub-cluster A3 had 4 *E. faecium* isolates; two emerged from Gelukspan cattle faeces and the other two from Molelwane water sources. There were no *E. faecium* isolates from the Gelukspan cattle water sources and Molelwane cattle faecal samples in cluster A.

Twenty-three *E. faecium* isolates were in cluster B (the larger cluster, with sub-clusters B1, B2 and B3). Sub-cluster B1 had 6 *E. faecium* isolates of which 3 were from Gelukspan cattle faeces and another 3 from Molelwane cattle faeces. The *E. faecium* isolates from sub-cluster B2 were mainly from the commercial farms. Seven *E. faecium* isolates were found in this sub cluster. One *E. faecium* isolate was from Gelukspan cattle faeces and water sources. Four isolates were present from the Molelwane cattle faeces; whereas 1 was from Molelwane cattle water sources. Nine *E. faecium* isolates from Molelwane cattle faecal samples were found in Cluster B and only 1 isolate of *E. faecium* from the Molelwane cattle water sources was found in this cluster. Thirteen *E. faecium* isolates from Gelukspan were present in this cluster, of which 8 were from the cattle faecal samples and 5 emerged from the cattle water. As shown in Figure 4.5, Cluster B did not present any *E. faecium* isolates from the Rooigrond prison farm.

Sub-cluster B3 had a total of 10 *E. faecium* isolates of which 4 were from the Gelukspan cattle faecal and another 4 from cattle water source samples. Two of the isolates were from the Molelwane cattle faecal samples. These were clustered together and it indicates that these isolates did have a link.

Enterococcus faecium isolates from Gelukspan, Molelwane and Rooigrond could not be separated from each other in terms of the antibiotic resistance data, hence they clustered together in clusters A and B eventhough they were from different geographical locations. Thus, Figure 4.5 clearly indicates that the *Enterococcus* isolates from all farms and sources had a relationship. This relationship could be due to the same antibiotic history exposure.

MAR indices were calculated as indicated in Table 4.3. It indicates the measure of the degree of the drug resistance for isolates in the group (Section 3.2.5.3).

Table 4.3: Average MAR indices of *Enterococcus* species isolated from communal and commercial farms.

Sample source	**Isolates**	**MAR Index**	
		Communal	**Commercial**
Cattle faeces	*E. faecium*	0.754	0.682
Cattle drinking water	*E. faecium*	0.58	0.655
	E. avium	0.6	0.663

The average MAR index of the *E. faecium* isolates from the communal and commercial cattle faecal samples were 0.754 and 0.682, respectively (Table 4.3). Average MAR indices of *E. faecium* (0.58 and 0.655) and *E. avium* (0.6 and 0.663) isolates from water were lower than the ones obtained from the faecal samples. This was lower than all the other indices that were observed, although according to Ehinmidu (2003) they could still be classified as high-risk.

4.2.3 Antibiotic resistance of *Enterococcus* isolates from healthy and diarrhoeal cattle faecal samples from commercial populations

Antibiotic resistance of *E. faecium*, *E. durans* and *E. avium* isolates from the faeces of healthy cattle (pooled data of healthy cattle from Molelwane and Rooigrond commercial farms) and the diarrhoeal cattle from Molelwane were compared and the results are presented in Figures 4.6, 4.7 and 4.8 and Tables 4.4, 4.5 and 4.6, respectively.

Figures 4.6, 4.7 and 4.8 are illustration of antibiotic resistant *E. faecium*, *E. durans* and *E. avium* from healthy and diarrhoeal commercial cattle faeces.

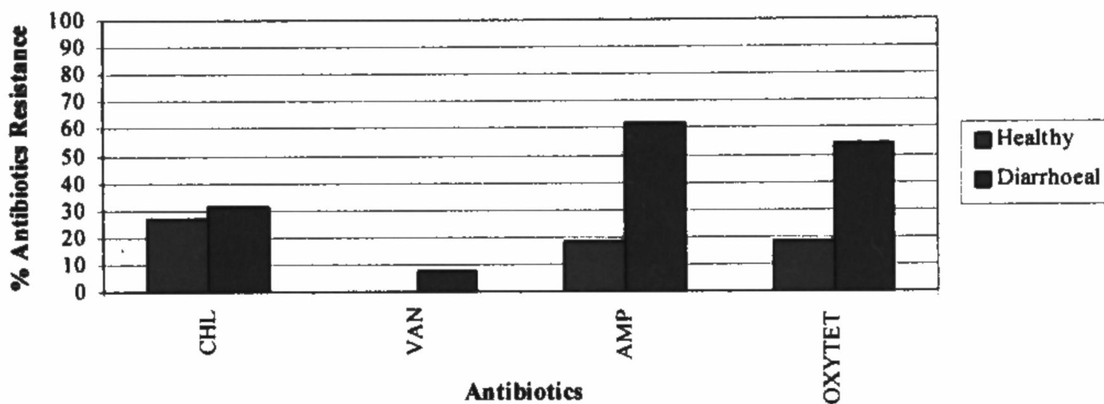

Figure 4.6: *E. faecium* isolates in healthy and diarrhoeal cattle.

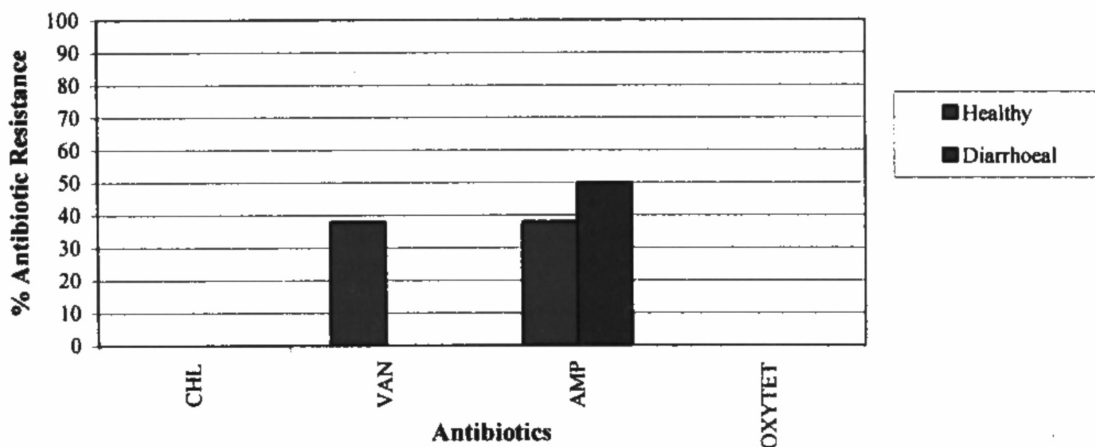

Figure 4.7: *E. durans* isolates in healthy and diarrhoeal cattle.

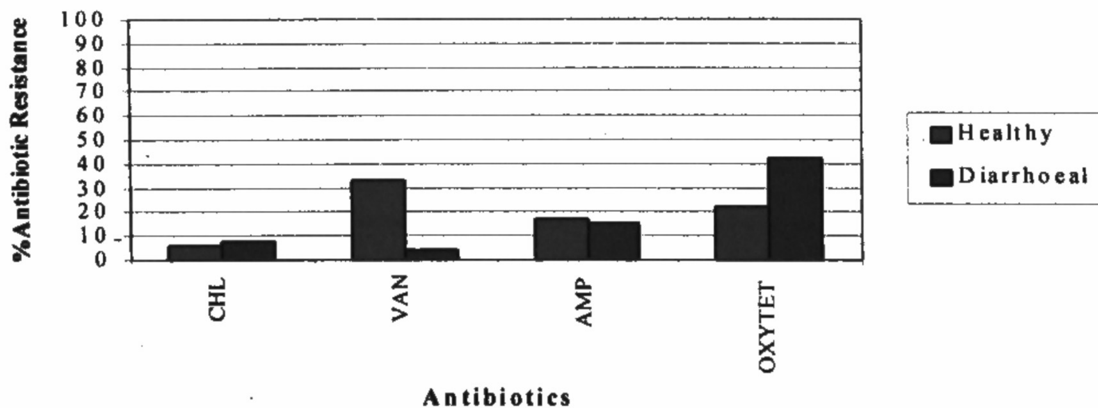

Figure 4.8: *E. avium* isolates in healthy and diarrhoeal cattle.

Table 4.4: MAR phenotypes of *E. faecium.*

MAR phenotype	H.C	D.C	Total No. Observed	%
GEN-SMX-NAL-NIT- KAN-STR	5	5	10	40
GEN-SMX-NAL-NIT-CHL-KAN-STR	3	1	4	16
GEN-SMX-NAL-NIT-OXYTET-KAN-STR	2	1	3	12
GEN-SMX-NAL-NIT-CHL-OXYTET-KAN-STR	-	3	3	12

H.C- Healthy Cattle, D.C- Diarrhoeal Cattle. The percentages were obtained by taking the sum of all *E. faecium* MAR phenotypes of cattle (Appendix C2 and C3) and dividing it by the number of common or highest MAR phenotypes that were obtained from either healthy and diarrhoeal cattle.

Table 4.5: MAR phenotypes of *E. durans.*

MAR phenotype	H.C	D.C	Total No. Observed	%
GEN-SMX-NAL-NIT- KAN-STR	4	2	6	60
GEN-SMX-NAL-NIT-VAN-KAN-STR-AMP	2	-	2	20
SMX-NAL-STR	1	-	1	10
GEN-SMX-NAL-NIT-KAN-STR-AMP	1	-	1	10

H.C- Healthy Cattle, D.C- Diarrhoeal Cattle. The percentages were obtained by taking the sum of all *E. durans* MAR phenotypes of cattle (Appendix C7 and C8) and dividing it by the number of common or highest MAR phenotypes that were obtained from either healthy and diarrhoeal cattle.

Table 4.6: MAR phenotypes of *E. avium.*

MAR phenotype	H.C	D.C	Total No. Observed	%
GEN-SMX-NAL-NIT- KAN-STR	7	12	19	42
GEN-SMX-NAL-OXYTET-KAN-STR	1	4	5	11
GEN-SMX-NAL-KAN-STR	2	1	3	6
GEN-SMX-NAL-NIT-OXYTET-KAN-STR	1	2	3	6

H.C- Healthy Cattle, D.C- Diarrhoeal Cattle. The percentages were obtained by taking the sum of all *E. avium* MAR phenotypes of cattle (Appendix C4 and C5) and dividing it by the number of common or highest MAR phenotypes that were obtained from either healthy and diarrhoeal cattle.

Figure 4.6 illustrates the antibiotic resistance of *E. faecium* isolates from healthy and diarrhoeal cattle faeces. *Enterococcus faecium* isolates from the diarrhoeal cattle had a greater antibiotic

resistance variation compared to the *E. faecium* from the healthy cattle. A relatively high percentage of *E. faecium* isolates from diarrhoeal cattle was resistant to AMP and OXYTET (62% and 54%), compared to 18% (both AMP and OXYTET) from the healthy cattle faecal samples. *Enterococcus faecium* from diarrhoeal and healthy cattle were resistant to CHL (31% and 27%, respectively). All the *E. faecium* isolates from healthy commercial cattle were susceptible to VAN, whereas a small percentage of those isolated from the diarrhoeal cattle (8%) were resistant to VAN.

Enterococcus durans from healthy cattle (38%) were resistant to VAN and AMP, whereas, 50% of these isolates from the diarrhoeal cattle faeces were resistant to AMP and all were susceptible to VAN. Other *E. durans* isolates from both healthy and diarrhoeal cattle faeces were susceptible to CHL and OXYTET. Figure 4.8 clearly illustrates that 6% of the *E. avium* isolates from the healthy cattle were resistant to CHL, 33% to VAN, 17% to AMP and 22% to OXYTET, whereas 8% of these isolates from diarrhoeal cattle showed resistance to CHL, 4% to VAN, 15% to AMP and 42% to OXYTET. Most of the *E. avium* isolates from healthy cattle displayed a higher level of resistance to most of the tested antibiotics compared to isolates from the diarrhoeal cattle.

The predominant MAR phenotype for all the *Enterococcus* isolates was GEN-SMX-NAL-NIT-KAN-STR (Tables 4.4, 4.5 and 4.6). The underlined antibiotics (Table 4.4) within the MAR phenotypes indicate the individual antibiotics that were different when the MAR phenotypes were compared. Two *E. durans* isolates had the MAR phenotype; GEN-SMX-NAL-NIT-VAN-KAN-STR-AMP and one portrayed the following MAR pattern, SMX-NAL-STR (Table 4.5). Only two MAR phenotypes of *E. avium* included OXYTET (Table 4.6). The MAR phenotypes of *E. faecium* and *E. avium* did not include VAN (Tables 4.4 and 4.6), but it was included in one of the MAR phenotypes of *E. durans* (Table 4.5).

The *E. faecium* isolates from healthy cattle showed total resistance to GEN, SMX and KAN, whereas 92% of those isolated from diarrhoeal cattle was resistant to the same antibiotics. *Enterococcus faecium* isolates from both categories of cattle showed total resistance to NAL, NIT and STR (Appendices B2 and B3). *Enterococcus durans* isolated from healthy and diarrhoeal cattle showed total resistance to SMX, NAL and STR. Eighty eight percent of these isolates from healthy cattle were resistant to GEN, NIT and KAN, whereas, the ones from diarrhoeal cattle faecal samples were totally resistant to those three antibiotics. The healthy and diarrhoeal *E. avium* isolates were totally resistant to GEN, SMX and NAL. All *E. avium* isolates from healthy cattle were resistant to KAN and STR, whereas diarrhoeal cattle (85% and 88%) were resistant to the same drugs. Healthy and diarrhoeal cattle (78% and 61%, respectively) were resistant to NIT.

The results for *Sc. bovis I* isolates were not shown in the figures, but can be found in Appendices B8 and B9. *Streptococcus bovis I* was totally resistant to six of the ten tested antibiotics (GEN, SMX, NAL, NIT, KAN and STR) in both healthy and diarrhoeal cattle. Twenty percent of the *Sc. bovis I* isolates from healthy cattle faecal samples was resistant to CHL and AMP, whereas 60% was resistant to OXYTET. All the *Sc. bovis I* isolates from the diarrhoeal animals were found to be totally susceptible to CHL, AMP and OXYTET. These isolates from the two sample sets of cattle (healthy and diarrhoeal) were totally susceptible to VAN. It was evident that the *Sc. bovis I* isolates from the healthy cattle were more resistant to antibiotics than those isolated from the diarrhoeal cattle. Thirty-three percent of the *Sc. bovis I* isolates had a predominant MAR phenotype, GEN-SMX-NAL-NIT-OXYTET-KAN-STR.

The following dendrogram illustrates the relationship of various *Enterococcus* isolates from all the communal (Gelukspan) and the commercial (Molelwane and Rooigrond) cattle faecal and water source samples, including the isolates from the commercial healthy and the diarrhoeal cattle in terms of the antibiotic resistant profile.

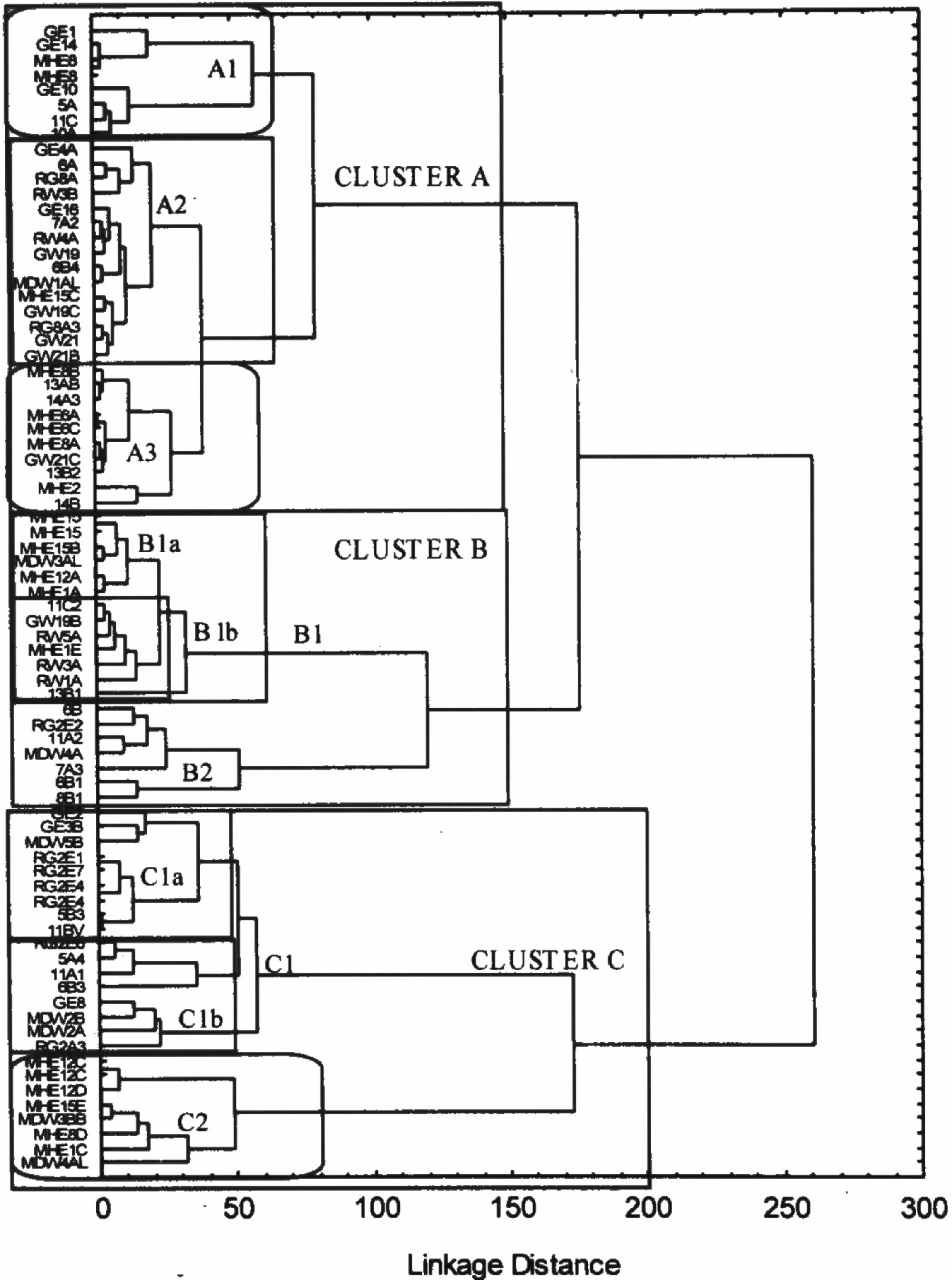

Figure 4.9: Dendrogram illustrating the relationship of the 78 various *Enterococcus* isolates from the all faecal and water samples from Gelukspan, Molelwane and Rooigrond, all the healthy and the diarrhoeal cattle from Molelwane and Rooigrond in terms of the antibiotic IZD data.

Cluster analysis was conducted to determine the relationship of *Enterococcus* species in terms of their antibiotic resistance profiles at all farms. A total of 78 cases of *Enterococcus* isolates were presented in Figures 4. 9 and 4.10. Cases were from healthy cattle faeces, water sources and diarrhoeal cattle from Gelukspan, Molelwane and Rooigrond. Both clusters were divided into 3 clusters (A, B and C). Cluster A appeared to be the largest cluster as it contained 3 sub-clusters (A1, A2 and A3). Cluster B also exhibited 2 sub-clusters (B1 and B2); with two minor sub-clusters (B1a and B1b) presented in cluster B1. Cluster C was divided into sub-clusters C1 and C2, with C1 further divided into C1a and Clb.

When cluster A1 was compared to every other cluster it was characterised by its common *Enterococcus* species that showed the same antibiotic resistant pattern even though the individual isolates were from different farms (Figure 4.9). Fifteen cases of different origins were observed in Cluster A2. These were *E. faecium* 7 (47%), *E. avium* 5 (33%) and *E. durans* 3 (20%). *Enterococcus faecium* was mainly from Gelukspan cattle faeces and water sources. Of the 5 *E. avium* isolates, 2 (40%) were from the diarrhoeal cattle and the other 3 (60%) were from the Molelwane, Rooigrond and Gelukspan cattle water sources. Ten isolates were presented in sub-cluster A3 of which all prevailed from Molelwane, except for only one that was from Gelukspan. The predominant species was *E. avium* of which 60% were from Molelwane. Other individuals consisted of *E. avium* (20%) from other sources, and *Sc. bovis I* (20%).

All the 6 cases presented in cluster B1a were also from Molelwane with *E. faecium* (67%) as the predominant isolate and only 2 (33%) *E. avium* were obtained. Cluster B1b had 7 isolates, of which 5 (71%) were *E. avium*. *Enterococcus avium* (5) still prevailed and only 2 (40%) *E. faecium* were shown in B2.

The predominant species sub-cluster Cla was *E. faecium* (88%). On the other hand, in sub-cluster C1b, *E. avium* (50%) was the predominant species. All these were from all the farms and there was no specific pattern. In sub-cluster C2, *E. avium* (63%) was also the predominant species present. This sub-cluster contained isolates from Molelwane farm. It is therefore shown in Figure 4.9 that the *Enterococcus* isolates from all farms and categories of animals cluster together despite the fact that they originated from different geographical locations. Furthermore, Figure 4.9 illustrated a relationship of species from all sources and from both farms. This could be due to similar history of antibiotic exposure of these isolates.

The MAR index shown in Table 4.7 indicates the measure of the degree of the drug resistance for isolates in species context (Section 3.2.5.3). *Enterococcus faecium* isolates from the diarrhoeal cattle showed a very high MAR index of 1.977. All the other isolates also illustrated high MAR indices (Table 4.7), although these were not as high as the *E. faecium* from the diarrhoeal population.

Table 4.7: Average MAR indices of *Enterococcus* species isolated from healthy and diarrhoeal commercial cattle faeces.

Sample source	Isolates	MAR Index	
		Healthy	Diarrhoeal
Cattle faeces	*E. faecium*	0.655	1.977
	E. avium	0.663	0.581
	E. durans	0.638	0.65
	Sc. bovis I	0.7	0.6

Table 4.8 illustrates the number of *Enterococcus* species isolated from communal and commercial cattle faecal and water samples that were resistant to vancomycin and oxytetracycline.

Table 4.8: Vancomycin and Oxytetracycline resistant *Enterococcus* isolates.

Source	Species	No. Isolated	No. Resistant to vancomycin	%	No. Resistant to oxytetracycline	%
Cattle faeces	*E. faecium*	38	5	13	13	35
	E. avium	44	6	14	15	34
	E. durans	10	3	30	0	0
	Sc. bovis I	6	0	0	3	50
Total		**98**	**14**	**11**	**31**	**32**
Cattle water	*E. faecium*	13	3	23	5	38
	E. avium	18	5	28	3	17
Total		**31**	**8**	**31**	**8**	**31**
Overall Total		**129**	**22**	**17**	**39**	**30**

As shown in Table 4.8, *E. avium* was the predominant species in both cattle faecal (44%) and their water source (18%) samples. Thirty percent of *E. durans* isolates were resistant to vancomycin, but all were susceptible to oxytetracycline. *Enterococcus faecium* and *E. avium* isolates (13% and 14% respectively) were resistant to vancomycin. More *E. faecium* and *E. avium* isolates (35% and 34%) were resistant to oxytetracycline. On the other hand, *E. avium* isolates (28%) from cattle water sources were resistant to vancomycin compared to *E. faecium* isolates (23%). More over, 38% of *E. faecium* isolates were resistant to oxytetracycline compared to 17% of *E. avium* isolates from cattle water sources. Therefore, the data in Table 4.8 clearly shows that more *E. avium* isolates were resistant to vancomycin whereas more *E. faecium* isolates were resistant to oxytetracycline. It is also shown that *Enterococcus* species were more resistant to oxytetracycline compared to vancomycin (Table 4.8).

4.3 MOLECULAR ANALYSIS

All the *Enterococcus* species that were resistant to vancomycin and oxytetracycline were selected and subjected to molecular analysis in order to determine genes that could be potentially responsible for antibiotic resistances. These samples were all subjected to PCR analysis (Section 3.3.3).

The images of the Genomic DNA, 16S and *vanC* amplifications are illustrated in Figures 4.10, 4.11 and 4.12, respectively.

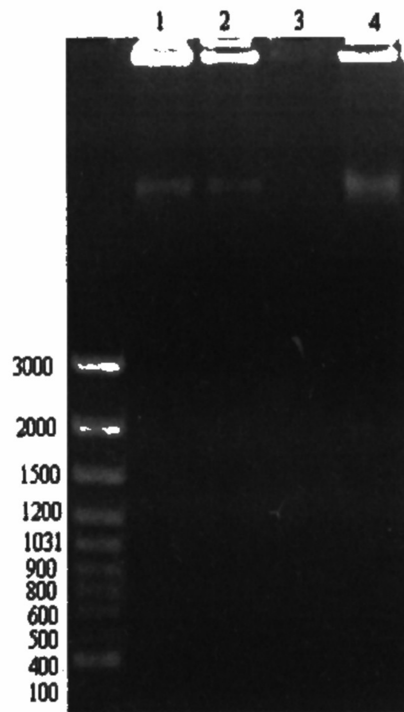

Figure 4.10: Illustration of the genomic DNA of *Enterococcus* isolates.

The genomic DNA of three enterococci, namely, *E. faecium* (Lane 1), *E. avium* (Lane 2) and *E. durans* (Lane 4) are illustrated. A reasonable quantity of the genomic DNA was extracted and this is illustrated by the intensity of the DNA bands. The genomic DNA was of good quality, as it showed no fragmentations of the DNA and no RNA was present (Figure 4.10).

Figure 4.11: Illustration of *Enterococcus* 16S amplification.

The extracted genomic DNA was subjected to 16S PCR amplification. Figure 4.11 illustrates a negative image of ethidium bromide agarose gel showing one of the 16S amplicons of an *Enterococcus* isolate. A pure amplicon of excellent quality was observed and the size of the fragment was approximately 550 base pairs (bp).

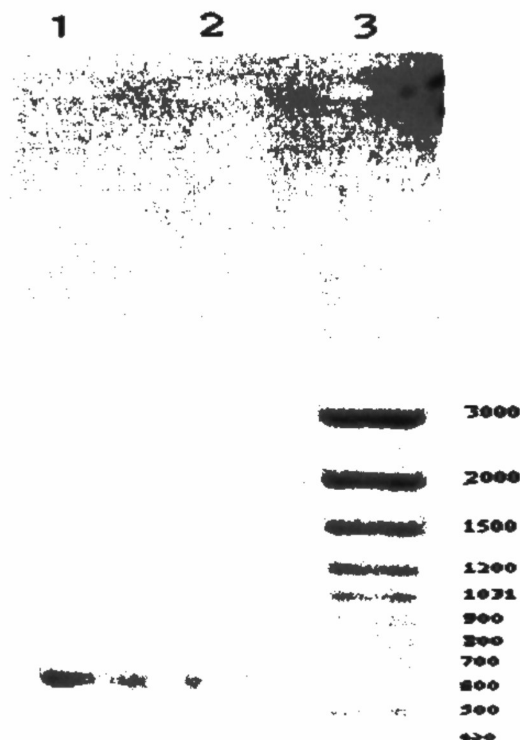

Figure 4.12: Illustration of *Enterococcus vanC* gene amplifications.

The above Figure (Figure 4.12) illustrates the *vanC* gene amplification of *E. avium* (Lane 1) and *E. durans* (Lane 2) at 600bp. Lane 3 illustrates a 100bp marker. According to Bell *et al.* (1998) the expected *vanC* gene fragment size ranges between 447 and 597bp.

Table 4.9 illustrates the number of the resistant vancomycin genotypes that were obtained from different *Enterococcus* species.

Table 4.9: PCR results of resistant vancomycin genotypes.

Species	*vanA*	*vanB*	*vanC*	Non-ABC	Total
E. durans	-	-	2	8	10
E. avium	-	-	1	61	62
E. faecium	-	-	-	50	50
Sc. bovis I	-	-	-	6	6

As illustrated in Table 4.9, none of the *Enterococcus* isolates carried the *vanA* and *vanB* resistant gene. Amongst the entire *E. durans* that were resistant to vancomycin (Table 4.8) only two carried the *vanC* gene and only one, *E. avium* (Table 4.9; Figure 4.12), carried the same gene. The VanC phenotype confers low-level resistance to vancomycin (Arthur *et al*., 1996). All *E. faecium* isolates were negative for *vanA, vanB* and *vanC* genes.

4.4 SUMMARY OF RESULTS

Enterococcus faecium was isolated from the communal cattle faecal and water source samples, while *E. avium* was also isolated from the cattle water sources at this farm. *Enterococcus avium*, *E. faecium*, *E. durans* and *Sc. bovis I* were isolated from the healthy and diarrhoeal commercial cattle faeces. On the other hand, *E. faecium* and *E. avium* were also isolated from the commercial cattle water sources. In general, *E. faecium* was predominant in communal sample sources and *E. avium* in commercial sample sources (Table 4.1).

Multiple antibiotic resistances (MAR) were observed in isolates from all sources from both farms. Other MAR phenotypes were not common between the two species, but all their MAR phenotypes

included the antibiotics GEN, SMX, NAL, KAN and STR. The predominant MAR for *E. durans* was GEN- SMX-NAL-NIT- KAN-STR and the one for *Sc. bovis I* was GEN- SMX-NAL-NIT-OXYTET- KAN-STR. The predominant MAR phenotype that was dominant in all *Enterococcus* species from all sources was GEN-SMX-NAL-NIT-KAN-STR.

All isolates showed an MAR index above 0.2 (water; 0.58 to 0.68 and faeces; 0.6 to 1.7). In general, resistances to OXYTET and VAN were showed by *E. faecium* (38% and 23%) and *E. avium* (17% and 28%), respectively, from all the cattle water sources. On the other hand, *E. faecium* (35% and 13%), *E. avium* (34% and 14%), *E. durans* (0% and 30%) and *Sc. bovis I* (50% and 0%) from the entire cattle faecal sample showed resistance to OXYTET and VAN, respectively. On the whole, 32% of all the *Enterococcus* isolates from the cattle faeces was resistant to OXYTET and 11% to VAN, whereas 31% of the ones isolated from the cattle water sources was resistant to both drugs. Cluster analysis showed relationships of *Enterococcus* isolates from all sources and from both farms. They were all presented in similar clusters irrespective of their different geographical locations. Vancomycin resistant *Enterococcus* (VRE) genes conveying the VanC phenotype were obtained from *E. durans* and *E. avium.*

CHAPTER 5

DISCUSSIONS

5.1 GENERAL DISCUSSIONS

The purpose of this study was to isolate *Enterococcus* species and to determine their antibiotic resistance profiles as well as the factors that could be responsible for their resistance to antibiotics. *Enterococcus* species are normal flora of the gastro-intestinal tract of animals and humans (Panesso *et al.*, 2002; Johnston and Jaykus, 2004). Apart from enterococci having intrinsic resistance to several antibiotics, they can also acquire high-level resistance to a variety of antimicrobials by horizontal transfer of mobile genetic determinants (Petsaris *et al.*, 2005). This study is one of the first comprehensive studies on antimicrobial resistance of *Enterococcus* isolates in cattle faecal and water source samples from the North-West Province, Mafikeng (RSA). It is thus an important study and will provide baseline data on antibiotic resistance patterns among the selected cattle populations.

In this study, *Enterococcus* species were isolated from both communal (Gelukspan) and commercial (Molelwane and Rooigrond) cattle faeces and their water sources. A diarrhoea outbreak at the commercial farm (Molelwane) also gave an opportunity to isolate these species from the diarrhoeal cattle. Species composition and distribution analysis showed that *E. faecium* and *E. avium* were the two predominant isolated species from commercial cattle population studies. On the other hand, *E. faecium* was the predominant species in the communal cattle population studies. *Enterococcus faecium* is frequently identified as the predominant species in faeces of production animals such as poultry, cattle and pigs (Mareno and Blanch, 1999).

Other researchers have previously isolated *E. faecium* from animal products (Hayes *et al.*, 2003; Peters *et al.*, 2003) and from the water sources (Cupáková and Lukášová, 2003; Junco *et al.*, 2001; Švec and Sedláček, 1999). For instance, *E. faecium* was the predominant species from samples of retail raw meat (chicken, turkey, pork and beef) from grocery stores in Iowa (Hayes *et al.*, 2003). In their study, Hayes *et al.* 2003 obtained 65% of *E. faecium* from ground beef. Peters *et al.* (2003) isolated 54 strains of *E. faecium* from food of animal origin in Germany. A study in Czech Republic also showed that *E. faecium* was the predominant species in water environments (Cupáková and Lukášová, 2003). In a study by Švec and Sedláček (1999) it was shown that 21% of the 630 bacterial strains isolated from surface water were *E. faecium*. Junco *et al.* (2001) also isolated *E. faecium* from different water samples. Therefore, this study agrees with the findings of other scientists because herein *E. faecium* was also the predominant isolate from cattle faeces and their water sources.

Enterococcus faecium species were also shown to be common among clinical isolates of enterococci (Huycke *et al.*, 1998; Mundy *et al.*, 2000). Thus, it is speculated that since the animals live and graze around the Gelukspan hospital, they have the potential of spreading *E. faecium* strains into the hospital environment.

In the present study, *E. avium* was also isolated from the communal cattle water sources. However, it was present in high proportions from the commercial cattle, particularly the diarrhoeal cattle faeces and their water sources. In food production animals, species like *E. avium* were previously obtained in lower numbers (Mareno and Blanch, 1999). During the present study, *E. avium* appeared to be the predominant species especially in samples from the diarrhoeal cattle faeces. High proportions of *E. avium* obtained from the samples of these farms could be due to the transfer of *E. avium* strains by poultry or birds that normally eat and drink with these cattle from the same troughs. Rodents and birds could have been responsible for the spread of *E. avium*. These vertebrate

species can also acquire environmental contaminants and pass them on through their excreta to the grazing land or to the feeds of food animals (Phillips *et al.*, 2004).

Enterococcus clinical infections may be due to at least 12 species including *E. faecium*. Occasional infections are due to, among others, *E. durans* and *E. avium* in hospitalised patients (Mundy *et al.*, 2000). Another species isolated from the commercial cattle in the present study was identified as *Sc. bovis I*. The latter species also exists in the alimentary tract of cattle and has been known to cause fever, diarrhoea and abdominal cramps (Kawata *et al.*, 2004). However, it was speculated in this study that these species could have contributed to the outbreak of diarrhoea on the one commercial farm. This aspect was not conclusively established.

The *Enterococcus* species can also be released into the environment by animal and human faecal materials such as fertilisers of animal origin (Harwood *et al.*, 2000). Since they are resistant to chemical and physical stress, enterococci can survive for a long time outside their natural intestinal host (Kühn *et al.*, 2000). Thus, apart from them being indicator microorganisms in water, these species could have been released into the water sources of these animals by the same animals. These animals also drink from the rivers that are normally used by humans for various domestic purposes, such as washing of clothes and bathing (Bezuidenhout *et al.*, 2002).

Cattle are mostly exposed to antibiotics due to the production of food supply, control, treatment or prevention of various diseases. These animals are raised in close proximity and a disease that starts in one animal often runs through many others if not the whole group. Therefore, treatment is necessary in livestock production since it limits progression of disease in the population. On the other hand, this increases the selection, survival and growth of drug resistant strains (Phillips *et al.*, 2004). Resistance to multiple classes of antibiotics can be revealed by *Enterococcus* isolates from animals, (Harwood *et al.*, 2000). They are known to acquire antibiotic resistance with relative ease

and are able to spread the resistance genes to other species (Kühn *et al.*, 2000). In the present study, multiple antibiotic resistances (MAR) have been observed in all the *Enterococcus* isolates from the communal and commercial cattle faecal and water samples. Each isolate showed total resistance to at least three antibiotics. High percentages of *Enterococcus* species were also resistant to other antibiotics.

All *E. faecium* isolates from the communal water sources were susceptible to CHL, OXYTET, VAN and AMP. On the other hand, *Enterococcus* isolates from the communal cattle faecal samples were resistant to the same antibiotics. It is interesting that such results were obtained. They could, however, not be explained. Klare *et al.* (2003) indicated that *E. faecium* possesses a broad spectrum of natural and acquired antibiotic resistances. Enterococci are naturally resistant to SMX due to endogenous sources of folate (Ardino and Murray, 1990: referenced by Flores *et al.*, https://www.moffitt.us.edu/pubs/ccj/v3nl/dept5/dept5.html). All the *Enterococcus* isolates in this study were also resistant to SMX.

Resistance of OXYTET in *E. faecium* from communal cattle faeces could be due to the overuse of this drug by communal farmers in the Mafikeng district. Due to financial constrains many communal farmers prefer to treat their own animals without consulting veterinary surgeons and animal health technicians. Another reason is that this drug is easy to obtain because it is sold as over-the-counter medicine and farmers could be using it even when it is not necessary. Lower proportions (18%) of *E. faecium* from faecal samples were resistant to OXYTET compared to a higher proportion of 63% of the same isolates from the water samples. This could imply that tetracycline is not used a lot for the treatment of these commercial cattle. However, the high proportion of resistance that was obtained from the water sources suggests that the other farm animals could also have contributed to this observation.

Fifty four percent of the *E. faecium* isolates from the commercial diarrhoeal cattle was resistant to OXYTET. It could imply that tetracycline was used during or prior to the period of the diarrhoeal outbreak. Peters *et al.* (2003) found that 18% of the *E. faecium* isolates from food of animal origin were resistant to tetracycline. They speculated that high-level resistance to tetracycline could be due to the high use of this drug in veterinary medicine. In a study by Mannu *et al.* (2003) in Italy, only 1% of *E. faecium* strains from ovine faeces were found to be resistant to tetracycline. In the present study, 80% to 100% of the *E. faecium* isolates from all sources at both farms were resistant to NIT. These results were much higher than the results that were obtained by Mannu *et al.* (2003). In their study only 17.5% of the isolates were resistant to NIT.

Large numbers of *Enterococcus* isolates in the present study were resistant to aminoglycosides. This is a clear indication of growing aminoglycoside resistance. In 1992, Moellering found that *Enterococcus* isolates had multiple resistances to antimicrobial agents including high levels of aminoglycoside resistance. Hayes *et al.* (2003) found that the *Enterococcus* species isolated from the retail meat conveyed a high level of aminoglycoside resistance. Kaçmaz and Aksoy (2005) found that *E. faecium* strains had a higher rate of combined resistance to streptomycin and gentamycin than other species, and that resistance to streptomycin was higher than it was to gentamycin. This trend was also observed in the present study. However, the species that showed the mentioned trend were *E. faecium* and *E. avium*. Only three *E. avium* isolates from the 129 enterococci tested, were susceptible to streptomycin and only four *E. faecium* were susceptible to gentamycin.

Qu *et al.* (2005) obtained similar results as discussed above. Their study investigated the antibiotic resistance of aminoglycoside-modifying enzyme (AME) and high-level gentamycin resistant (HLGR) *Enterococcus* in clinical specimens. They isolated 106 *Enterococcus* and 46 (43.4%) were identified as *E. faecium*. Sixty-eight percent of HLGR isolates were obtained from their study and *E. faecium* (52%) was the predominant species. In the present study all the *E. faecium* isolates from

the communal water sources showed resistance to GEN. Ninety two percent of the *E. faecium* isolates from the communal cattle faeces also showed resistance to the same drug. *Enterococcus faecium* isolates from both communal cattle faecal and water samples were all resistant to STR. High numbers from the communal and the commercial cattle, and their water sources, also showed resistance to gentamycin and streptomycin. These results agree with the fact that *Enterococcus* species are naturally resistant to aminoglycosides (Mallon *et al.*, 2002; Peters *et al.*, 2003). Even though the majority of these isolates were totally resistant to the aminoglycosides, they were not further tested for the presence of the HLGR genes.

Qu *et al.* (2005) found that the majority of HLGR isolates contained the *aac (6')-Ie-aph (2")-Ia* gene that has been previously reported as the main HLGR gene (Ferretti *et al.*, 1986). Further molecular studies should be undertaken to determine and analyse for AMEs or the HLGRs amongst isolates obtained in the present study.

Vancomycin resistant enterococci were isolated from the healthy communal and commercial as well as diarrhoeal cattle faecal samples in this study. Vancomycin resistant enterococci (VRE) have been found from stools of healthy animals (Bates, 1997; Grosso *et al.*, 2000; Kühn *et al.*, 2003). The VRE, especially *E. faecium*, has been found in European commercial animal husbandry in which the glycopeptide avorpacin (AVO) was used as a growth promoter (Klare *et al.*, 2003). Glycopeptide-resistant *E. faecium* has been isolated in clinical samples, but also in environmental samples as well as in wastewaters or sewage treatment plants (Klare *et al.*, 1993). A total of 22 VREs were obtained in the present study, of which fourteen were from cattle faecal samples and eight were from their water sources (Table 4.8). It was observed in this study that high percentages of VREs were obtained from the healthy animals compared to the lower percentage in diarrhoeal cattle faeces.

The VanC phenotype confers low-level resistance to vancomycin and has been known to be an intrinsic vancomycin resistance in *E. casseliflavus* and *E. galllinarum* (Arthur *et al*., 1996, Leavis *et al*., 2004). However, in the present study, vancomycin resistant *Enterococcus* genes conveying the *vanC* genes were observed in *E. durans* and *E. avium*. This is surprising because according to researchers the vancomycin resistant intrinsic genes are not transferable and are related to species-specific genes *vanC1* (*E. gallinarum*) and *vanC2* (*E. casseliflavus*) (Dutka-Malen *et al*., 1992). Lemcke and Bülte (2000) attained the *vanC* genes in a study where they were assessing the occurrence of the vancomycin resistant genes in *Enterococcus* strains isolated from poultry and pork. They isolated 38 *vanC1* genes from *E. gallinarum*, 14 *vanC2* genes from *E. casseliflavus* from poultry isolates. In another study 59% of *E. gallinarum* isolates had a *vanC1* genotype and 29% *E. casseliflavus* strains a *vanC2* genotype (Gambarotto *et al*., 2001). As indicated above, several studies have shown that the *vanC* gene is found in *E. gallinurum* and *E. casseliflavus* and not in *E. avium* and *E. durans* as indicated in this study. Therefore, more studies on *vanC* genes should be undertaken in Mafikeng in order to trace this source of *vanC1* in *E. durans* and *E. avium*. No *vanA* and *vanB* genes were obtained in the present study.

Ampicillin resistant *E. faecium* (ARE) represents a therapeutic challenge especially when combined with aminoglycoside and glycopeptides resistance (Mohn *et al*., 2004). In the present study 8% of the *E. faecium* isolates from the communal cattle faeces presented the following resistant phenotype: AMP, GEN, KAN, STR and VAN. The same resistance pattern was also observed in the *E. faecium* isolates from the healthy and the diarrhoeal cattle faecal samples and from the commercial cattle water sources. The overproduction of the important penicillin binding protein 5 (PBP5) can lead to ampicillin resistance (Fontana *et al*., 1994). Even the *E. faecium* resistance to ampicillin is said to be due to the alteration of the PBP5 (Ligozzi *et al*., 1996).

The MAR indices were calculated to confirm the measure of the degree of the drug resistance for isolates in the group of faecal and water samples. Multiple antibiotic resistance indices of *Enterococcus* species from communal and commercial cattle faeces as well as their water sources were above 0.2. An MAR index that is greater than 0.2 implies that the strain of bacteria originates from an environment where several antibiotics are frequently used (Ehinmidu, 2003). The observation of high MAR indices is of concern.

Cluster analysis showed a similar relationship of *Enterococcus* isolates from both farms in terms of the antibiotic inhibition zone diameter data. The commonness could suggest that there is a similar history of antibiotic exposure to cattle in Mafikeng. This in turn becomes a problem because studies have indicated food animals to be reservoirs of resistant enterococci that could be transmitted to humans through the food chain (Klare *et al.*, 2003; Manie *et al.*, 1999; Phillips *et al.*, 2004).

Equally important is the opportunity of organisms to spread. The presence of glycopeptide-resistant enterococci in 'meat-eating' non-hospitalised humans, showed a food-associated spread of vancomycin-resistant enterococci from animals to humans (Schouten *et al.*, 1997). Moubareck *et al.* (2003) showed that various resistance genes could be conjugatively transferred from an *E. faecium* strain of animal origin to a human strain of the same species in the gastrointestinal tracts of gnotobiotic mice in the absence of selective pressure.

Infections caused by the genus *Enterococcus* in humans include urinary tract infections, bacteraemia, intra-abdominal infections, meningitis, septicaemia and endocarditis (Klare *et al.*, 2003; Huycke *et al.*, 1998; Murray, 1990). In such infections, the combination of an aminoglycoside with penicillin, ampicillin or a glycopeptide are used to obtain a synergistic bactericidal effect. However, strains that are highly resistant to aminoglycosides are no longer susceptible to the combination therapy and are posing another serious problem (Murray, 1999). The

intrinsic resistance of enterococci to many commonly-used antimicrobial agents may have allowed them to accumulate an advantage for further spread of genes encoding high-level resistance to aminoglycosides, tetracycline and vancomycin (Mundy *et al*., 2000).

5.2 CONCLUSION AND RECOMMENDATIONS

In this study it was shown that in communal cattle faeces and the water sources of these cattle, *E. faecium* was the predominant species isolated. This is a cause for concern because *E. faecium* is known to be of clinical significance. The majority of these isolates were also resistant to a large number of antibiotics, including the over-the-counter tetracycline compounds. *Enterococcus avium* was isolated from the communal water sources, but it was present in high proportions in the faeces of healthy and diarrhoeal commercial cattle and their water sources. Other species isolated from the commercial cattle include *E. faecium*, *E. durans* and *Sc. bovis I.* It was evident that high numbers of *Enterococcus* species were resistant to a large number of antibiotics. Vancomycin resistant enterococci (VRE) were also isolated from cattle faeces.

The MAR indices illustrated that there could be a high usage of antibiotics in cattle in Mafikeng. The predominant MAR phenotype that existed in all *Enterococcus* species from all sources was GEN-SMX-NAL-NIT-KAN-STR. Cluster analysis suggest that there is a similar history of antibiotic exposure of cattle in Mafikeng.

These antimicrobial resistance results among *Enterococcus* species have created the following concerns:

1. If antimicrobial resistance continue to develop in food animals, eventually there will be fewer tools to manage diseases in farms.
2. Food animals are reservoirs of antimicrobial resistant bacteria, therefore there is a great potential

of transmission of resistant enterococci by these animals to humans.

On the other hand, antibiotic resistance is unavoidable. There may be measures that can be taken to slow it. Some of these include:

1. Educational programmes

Qualified professionals such as veterinarians, medical doctors, nurses, animal health technicians and pharmacists should aid and teach the communities about the importance of simple public health measures and the more appropriate usage of antibiotics. Basic hygienic measures such as washing of hands, usage of clean equipment and their significance during handling of slaughtered carcases, the importance of properly cooking the meat or pasteurising milk before consumption can aid towards preventing the spread of antibiotic resistant enterococci.

2. Constant surveillance programmes

To minimise the risk of drug resistance transfer and assure food safety and public health, a rigorous screening and surveillance programme for pathogenic/non-pathogenic bacteria is needed.

Molecular typing has been showed to be necessary for understanding the differences in the mode of spread and acquisition of antibiotic resistance among different species of enterococci (Qu *et al.*, 2005; Jackson *et al.*, 2004; Mohn *et al.*, 2004). More studies of this nature should be undertaken and records must be kept in order to aid in monitoring the spread of resistant organisms and their resistant genes.

Future studies should evaluate genes responsible for aminoglycoside, β-lactam and tetracycline resistance in enterococci because a lot of these isolates have shown resistance to these drugs in this study.

Future studies should determine *Enterococcus* resistance in cattle populations and their products in communal farms. Rural communities had been generally neglected by researchers and research institutes. Recently there has been a great concern about the potential development of environmental reservoirs of antibiotic resistance in farmlands (Harwood *et al.*, 2000). A closer contact between communal food animals and the general human population creates risk. This in turn becomes a cause for concern that the human population becomes exposed to large numbers of antibiotic resistant pathogenic or non-pathogenic microflora.

3. Limiting usage of antibiotics

In order to reduce the distribution of MAR enterococci or their transferable resistance genes, a prudent use of antibiotics is necessary in veterinary medicine, animal husbandry and human medicine. Antibiotics should only be used when it is necessary. If antibiotics are used judiciously, an animal's inevitable resistance to the drugs will increase at a much slower pace (Sundlof, 1994). It is also important to note low-level resistance as it might be the first step towards clinical resistance (Phillips *et al.*, 204).

4. Development of new antibiotics

If pharmaceutical industries constantly develop new antibiotics to be used, then there might be a decrease in the level of resistant bacteria.

The assessment of multiple antibiotic resistant enterococci in communal and commercial cattle and their water sources showed that there could be a development of potential reservoirs of antibiotic resistance in farms. Thus, all these recommendations may contribute to reduce the risks of acquiring antibiotic resistant enterococci in Mafikeng, RSA.

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LIST OF APPENDICES

APPENDIX A

Inhibition zone diameter interpretive results of *Enterococcus* isolates

Appendix A1: Gelukspan cattle faecal isolates-*Enterococcus faecium* (Communal farm in Mafikeng).

	Antibiotic disc concentrations																				
	GEN10 µg		SMX 25 µg		NAL 30 µg		CHL 10 µg		OXYTET 30 µg		NIT 100 µg		VAN 30 µg		AMP 10 µg		KAN 30 µg		STR 10 µg		MAR Phenotype
Sample ID	z	Int	z	Int	z	Int	z	Int	z	Int	z	Int	z	Int	z	Int	z	Int	z	Int	
GE 1	10	R	0	R	0	R	10	R	22	S	0	R	15	S	20	S	8	R	0	R	GEN-SMX-NAL-CHL-NIT-KAN-STR
GE 2	10	R	0	R	8	R	20	S	0	R	8	R	22	S	19	S	10	R	0	R	GEN-SMX-NAL-OXYTET-NIT-KAN-STR
GE 3A	10	R	0	R	8	R	10	R	0	R	0	R	15	S	18	S	0	R	0	R	GEN-SMX-NAL-CHL-OXYTET-NIT-KAN-STR
GE 3B	0	R	0	R	0	R	10	R	20	R	12	R	15	S	18	S	0	R	0	R	GEN-SMX-NAL-CHL-NIT-KAN-STR
GE 4A	0	R	0	R	0	R	10	R	20	R	0	R	18	S	18	S	8	R	0	R	GEN-SMX-NAL-CHL-NIT-KAN-STR
GE 7	8	R	0	R	0	R	10	R	22	R	0	R	0	R	18	S	0	R	0	R	GEN-SMX-NAL-CHL-NIT-VAN-KAN-STR
GE 8	0	R	0	R	0	R	0	R	10	R	0	R	14	S	12	I	0	R	0	R	GEN-SMX-NAL-CHL-OXYTET-NIT-KAN-STR
GE 9	0	R	0	R	0	R	0	R	0	R	10	R	10	I	16	S	8	R	0	R	GEN-SMX-NAL-CHL-OXYTET-NIT-KAN-STR
GE 10	16	S	0	R	0	R	21	S	16	R	0	R	14	S	16	S	0	R	0	R	GEN-SMX-NAL-NIT-KAN-STR
GE 12	0	R	0	R	0	R	10	R	22	R	0	R	18	S	18	S	0	R	0	R	GEN-SMX-NAL-CHL-NIT-KAN-STR
GE 14	0	R	0	R	0	R	0	R	22	R	0	R	20	S	20	S	0	R	0	R	GEN-SMX-NAL-NIT-KAN-STR
GE 15	0	R	0	R	0	R	10	R	24	R	0	R	18	S	18	S	8	R	0	R	GEN-SMX-NAL-CHL-NIT-KAN-STR
GE 16	0	R	0	R	0	R	14	I	25	I	0	R	20	S	20	S	0	R	0	R	GEN-SMX-NAL-CHL-NIT-KAN-STR

z-zone of inhibition, Int-interpretation, MAR-multiple antibiotic resistance, S- susceptible, I- intermediate, R- resistant.

Appendix A2: Rooigrond cattle faecal isolates (Commercial farm in Mafikeng).

Sample id	isolate	GEN 10 µg z	GEN 10 µg Int	SMX 25 µg z	SMX 25 µg Int	NAL 30 µg z	NAL 30 µg Int	CHL 10 µg z	CHL 10 µg Int	OXYTET 30 µg z	OXYTET 30 µg Int	NIT 100 µg z	NIT 100 µg Int	VAN 30 µg z	VAN 30 µg Int	AMP 10 µg z	AMP 10 µg Int	KAN 30 µg z	KAN 30 µg Int	STR 10 µg z	STR 10 µg Int	MAR Phenotype
RG2A	*Sc. B*	0	R	0	R	0	R	25	S	0	R	0	R	21	S	26	S	0	R	0	R	**GEN-SMX-NAL-NIT-OXYTET-AMP-KAN-STR**
RG2A3	*Sc. B*	0	R	0	R	0	R	0	R	0	R	0	R	18	S	0	R	0	R	0	R	**GEN-SMX-NAL-NIT-OXYTET-KAN-STR**
RG2A4	*Sc. B*	0	R	0	R	0	R	16	I	28	S	0	R	19	S	23	S	0	R	0	R	**GEN-SMX-NAL-NIT-OXYTET-AMP-CHL-KAN-STR**
RG2E1	*E. F*	0	R	0	R	0	R	15	I	0	R	0	R	15	S	20	S	0	R	0	R	**GEN-SMX-NAL-NIT-OXYTET-KAN-STR**
RG2E2	*E. A*	0	R	0	R	0	R	14	I	10	R	13	R	12	S	22	S	13	R	0	R	**GEN-SMX-NAL-NIT-OXYTET-KAN-STR**
RG2E4	*E. F*	0	R	0	R	0	R	15	I	0	R	0	R	15	S	16	S	0	R	0	R	**GEN-SMX-NAL-NIT-OXYTET-KAN-STR**
RG2E6	*E. A*	0	R	0	R	0	R	20	S	0	R	15	I	23	S	24	S	0	R	0	R	**GEN-SMX-NAL-OXYTET-KAN-STR**
RG2E6p	*E. A*	0	R	0	R	0	R	15	I	0	R	18	I	11	S	20	S	0	R	0	R	**GEN-SMX-NAL-NIT-OXYTET-KAN-STR**
RG2E7	*Sc. B*	0	R	0	R	0	R	15	I	0	R	0	R	15	S	20	S	0	R	0	R	**GEN-SMX-NAL-NIT-OXYTET-KAN-STR**
RG8A	*E. D*	0	R	0	R	0	R	15	I	19	S	0	R	14	S	19	S	0	R	0	R	**GEN-SMX-NAL-NIT-KAN-STR**
RG8A1	*E. D*	0	R	0	R	0	R	17	I	25	S	0	R	17	S	21	S	0	R	0	R	**GEN-SMX-NAL-NIT-KAN-STR**
RG8A2	*E. D*	18	S	0	R	0	R	20	S	19	S	18	I	15	S	21	S	17	I	0	R	**SMX-NAL-STR**
RG8A3	*E. D*	0	R	0	R	0	R	19	S	22	S	0	R	19	S	19	S	0	R	0	R	**GEN-SMX-NAL-NIT-KAN-STR**

Antibiotic disc concentrations

z- zone of inhibition, Int- interpretation, MAR- multiple antibiotic resistance, S- susceptible, I- intermediate, R- resistant , *E. A- Enterococcus avium, E. F- Enterococcus faecium, E. D- Enterococcus durans, Sc. B- Streptococcus bovis.*

Appendix A3: Molelwane healthy cattle isolates.

Antibiotic disc concentrations

Sample id	isolate	GEN 10 µg		SMX 25 µg		NAL 30 µg		CHL 10 µg		OXYTET 30 µg		NIT 100 µg		VAN30 µg		AMP 10 µg		KAN 30 µg		STR 10 µg		MAR Phenotype
		z	Int	z	Int	z	Int	z	Int	z	Int	z	Int	z	Int	z	Int	z	Int	z	Int	
MHE 1	*E.A*	0	R	0	R	0	R	15	I	0	R	14	R	9	R	20	S	0	R	0	R	GEN-SMX-NAL-OXYTET-NIT-VAN-KAN-STR
MHE 2	*Sc.B*	0	R	0	R	0	R	19	S	20	S	0	R	23	S	30	S	0	R	0	R	GEN-SMX-NAL-NIT-KAN-STR
MHE 6	*E. F*	12	R	0	R	0	R	0	R	19	S	0	R	15	I	19	S	0	R	0	R	GEN-SMX-NAL-CHL-NIT-KAN-STR
MHE 8	*E. F*	0	R	0	R	0	R	0	R	24	S	0	R	19	S	21	S	0	R	0	R	GEN-SMX-NAL-CHL-NIT-KAN-STR
MHE 12	*E. F*	10	R	0	R	0	R	11	R	19	S	0	R	16	I	21	S	0	R	0	R	GEN-SMX-NAL-CHL-NIT-KAN-STR
MHE 15	*E. F*	0	R	0	R	0	R	17	I	21	S	10	R	20	S	22	S	0	R	0	R	GEN-SMX-NAL-NIT-KAN-STR
MHE 1A	*E. A*	0	R	0	R	0	R	18	S	25	S	10	R	18	S	25	S	0	R	0	R	GEN-SMX-NAL-NIT
MHE 1B	*E. A*	0	R	0	R	0	R	18	S	25	S	18	S	17	S	20	S	0	R	0	R	GEN-SMX-NAL-KAN-STR
MHE 1C	*E. A*	0	R	0	R	0	R	19	S	25	S	12	R	0	R	0	R	0	R	0	R	GEN-SMX-NAL-NIT-VAN-KAN-STR-AMP
MHE 1D	*E. A*	0	R	0	R	0	R	14	I	22	S	17	S	16	I	21	S	0	R	0	R	GEN-SMX-NAL-KAN-STR
MHE 1E	*E. A*	0	R	0	R	0	R	15	I	21	S	12	R	11	R	23	S	0	R	0	R	GEN-SMX-NAL-NIT-KAN-STR
MHE 6A	*E. A*	0	R	0	R	0	R	17	I	25	S	0	R	19	S	23	S	0	R	0	R	GEN-SMX-NAL-NIT-KAN-STR
MHE 6B	*E. A*	0	R	0	R	0	R	18	S	25	S	0	R	18	S	23	S	0	R	0	R	GEN-SMX-NAL-NIT-KAN-STR
MHE 6C	*E. A*	0	R	0	R	0	R	17	I	25	S	0	R	19	S	24	S	0	R	0	R	GEN-SMX-NAL-NIT-KAN-STR
MHE 6D	*E. A*	0	R	0	R	0	R	12	R	25	S	0	R	18	S	21	S	0	R	0	R	GEN-SMX-NAL-NIT-KAN-STR
MHE 8A	*E. A*	0	R	0	R	0	R	17	I	27	S	0	R	19	S	23	S	0	R	0	R	GEN-SMX-NAL-NIT-KAN-STR
MHE 8B	*E. F*	0	R	0	R	0	R	20	S	27	S	0	R	20	S	20	S	0	R	0	R	GEN-SMX-NAL-NIT-KAN-STR
MHE 8C	*E. A*	0	R	0	R	0	R	15	I	25	S	0	R	19	S	22	S	0	R	0	R	GEN-SMX-NAL-NIT-KAN-STR
MHE 8D	*E. A*	0	R	0	R	0	R	20	S	21	S	0	R	0	R	10	R	0	R	0	R	GEN-SMX-NAL-NIT-VAN-KAN-STR-AMP
MHE 12A	*E. F*	0	R	0	R	0	R	19	S	27	S	10	R	18	S	23	S	0	R	0	R	GEN-SMX-NAL-NIT-KAN-STR
MHE 12B	*E. F*	0	R	0	R	0	R	18	S	27	S	11	R	18	S	24	S	0	R	0	R	GEN-SMX-NAL-NIT-KAN-STR
MHE 12C	*E. F*	0	R	0	R	0	R	15	I	21	S	0	R	15	I	0	R	0	R	0	R	GEN-SMX-NAL-NIT-KAN-STR-AMP
MHE 12D	*E. A*	0	R	0	R	0	R	16	I	24	S	0	R	19	S	0	R	0	R	0	R	GEN-SMX-NAL-NIT-KAN-AMP
MHE 15A	*E. D*	0	R	0	R	0	R	18	S	22	S	0	R	0	R	0	R	0	R	0	R	GEN-SMX-NAL-NIT-VAN-KAN-STR-AMP
MHE 15B	*E. F*	0	R	0	R	0	R	16	I	23	S	10	R	17	S	20	S	0	R	0	R	GEN-SMX-NAL-NIT-KAN-STR
MHE 15C	*E. D*	0	R	0	R	0	R	17	I	21	S	0	R	17	S	17	S	0	R	0	R	GEN-SMX-NAL-NIT-KAN-STR
MHE 15D	*E. D*	0	R	0	R	0	R	16	I	21	S	0	R	18	S	0	R	0	R	0	R	GEN-SMX-NAL-NIT-KAN-STR-AMP
MHE 15E	*E. D*	0	R	0	R	0	R	18	S	18	S	0	R	0	R	0	R	0	R	0	R	GEN-SMX-NAL-NIT-VAN-KAN-STR-AMP
MHE 17A	*E. A*	0	R	0	R	0	R	16	I	25	S	0	R	16	I	19	S	0	R	0	R	GEN-SMX-NAL-NIT-KAN-STR

z- zone of inhibition, Int- interpretation, MAR- multiple antibiotic resistance, S- susceptible, I- intermediate, R- resistant, *E. A- Enterococcus avium, E. F- Enterococcus faecium, E. D- Enterococcus durans, Sc. B- Streptococcus bovis.*

Appendix A4: Molelwane diarrhoeal cattle isolates.

Sample id		GEN 10 µg		SMX 25 µg		NAL 30 µg		CHL 10 µg		O-T 30 µg		NIT 100 µg		VAN 30 µg		AMP 10 µg		KAN 30 µg		STR 10 µg		MAR Phenotype
		z	Int	Z	Int	z	Int	z	Int	z	Int	z	Int	z	Int	z	Int	z	Int	z	Int	
5A	*E. F*	10	R	0	R	0	R	13	I	18	I	0	R	17	S	19	S	0	R	0	R	GEN-SMX-NAL-NIT-KAN-STR
5B	*E. F*	9	R	13	S	0	R	15	I	0	R	0	R	17	S	13	R	0	R	0	R	GEN-NAL-OXYTET-NIT-KAN-AMP-STR
6A	*E. F*	0	R	0	R	0	R	15	I	15	I	0	R	16	I	17	S	0	R	0	R	GEN-SMX-NAL-NIT-KAN-STR
6B	*E. F*	9	R	0	R	0	R	14	I	13	R	11	R	17	S	15	R	17	I	0	R	GEN-SMX-NAL-OXYTET-NIT-AMP-STR
10A	*E. F*	10	R	0	R	9	R	16	I	18	I	0	R	14	R	15	R	10	R	0	R	GEN-SMX-NAL-NIT-KAN-AMP-STR
11A	*E. F*	13	S	0	R	0	R	17	I	11	R	0	R	17	S	13	R	0	R	0	R	SMX-NAL-OXYTET-NIT-KAN-AMP-STR
11C	*E. F*	11	R	0	R	9	R	10	R	15	I	0	R	15	I	17	S	0	R	0	R	GEN-SMX-NAL-CHL-NIT-KAN-STR
14B	*Sc. B*	9	R	0	R	0	R	30	S	18	I	0	R	22	S	25	S	0	R	0	R	GEN-SMX-NAL-NIT-KAN
5A1	*E.D*	6	R	0	R	9	R	14	I	24	S	11	R	18	S	15	R	0	R	0	R	GEN-SMX-NAL-NIT-KAN-AMP-STR
5A2	*E.A*	0	R	0	R	0	R	15	I	0	R	23	S	16	I	24	S	0	R	0	R	GEN-SMX-NAL-OXYTET-KAN-STR
5A4	*E.A*	0	R	0	R	0	R	17	I	0	R	17	S	18	S	24	S	0	R	0	R	GEN-SMX-NAL-OXYTET-KAN-STR
5B2	*E. F*	0	R	0	R	0	R	15	I	0	R	0	R	19	S	19	S	0	R	0	R	GEN-SMX-NAL-NIT-OXYTET-KAN-STR
5B3	*E. F*	0	R	0	R	0	R	12	R	0	R	0	R	18	S	15	R	0	R	0	R	GEN-SMX-NAL-CHL-OXYTET-NIT-KAN-AMP-STR
5B4	*E. F*	0	R	0	R	0	R	12	R	0	R	0	R	18	S	13	R	0	R	0	R	GEN-SMX-NAL-CHL-OXYTET-NIT-KAN-AMP-STR
6B1	*E.A*	0	R	0	R	0	R	26	S	0	R	22	S	16	I	22	S	19	S	14	S	GEN-SMX-NAL-OXYTET
6B2	*E.A*	0	R	0	R	0	R	16	I	0	R	19	S	13	R	0	R	0	R	0	R	GEN-SMX-NAL-OXYTET-AMP-KAN-STR
6B3	*E.A*	0	R	0	R	0	R	9	R	0	R	23	S	16	I	0	R	0	R	0	R	GEN-SMX-NAL-CHL-OXYTET-AMP-KAN-STR
6B4	*E.A*	0	R	0	R	0	R	15	I	23	S	0	R	18	S	14	R	0	R	0	R	GEN-SMX-NAL-NIT-KAN-AMP-STR
7A1	*E.A*	0	R	0	R	0	R	17	I	26	S	0	R	20	S	22	S	0	R	0	R	GEN-SMX-NAL-NIT-KAN-STR
7A2	*E.A*	0	R	0	R	0	R	14	I	23	S	0	R	16	I	19	S	0	R	0	R	GEN-SMX-NAL-NIT-KAN-STR

Antibiotic disc concentration

7A3	*E.A*	0	R	0	R	0	R	14	I	25	S	12	R	19	S	19	S	21	S	16	R	GEN-SMX-NAL-NIT
7A4	*E.A*	0	R	0	R	0	R	15	I	25	S	0	R	18	S	21	S	0	R	0	R	GEN-SMX-NAL-NIT-KAN-STR
8B1	*E.A*	0	R	0	R	13	R	22	S	0	R	20	S	15	I	19	S	20	S	17	R	GEN-SMX-NAL-OXYTET
8B2	*E.A*	0	R	0	R	0	R	14	I	0	R	18	S	16	I	23	S	0	R	0	R	GEN-SMX-NAL-OXYTET-KAN-STR
8B3	*E. A*	0	R	0	R	0	R	15	I	0	R	23	S	15	I	21	S	0	R	0	R	GEN-SMX-NAL-OXYTET-KAN-STR
8B4	*E. A*	0	R	0	R	0	R	15	I	0	R	13	R	19	S	21	S	0	R	0	R	GEN-SMX-NAL-OXYTET-NIT-KAN-STR
11A1	*E. A*	0	R	0	R	0	R	12	R	0	R	10	R	15	I	21	S	0	R	0	R	GEN-SMX-NAL-CHL-OXYTET-NIT-KAN-STR
11A2	*E. A*	0	R	0	R	0	R	16	I	22	S	16	I	16	I	17	S	18	S	0	R	GEN-SMX-NAL-STR
11BL	*E. A*	0	R	0	R	0	R	18	S	24	S	0	R	18	S	17	S	0	R	0	R	GEN-SMX-NAL-NIT-KAN-STR
11BV	*E. F*	0	R	0	R	0	R	12	R	0	R	0	R	19	S	14	R	0	R	0	R	GEN-SMX-NAL-CHL-OXYTET-NIT-KAN-AMP-STR
11C1	*E. A*	0	R	0	R	0	R	13	I	21	S	0	R	16	I	20	S	0	R	0	R	GEN-SMX-NAL-NIT-KAN-STR
11C2	*E D*	0	R	0	R	0	R	16	I	21	S	14	R	15	I	20	S	0	R	0	R	GEN-SMX-NAL-NIT-KAN-STR
11C4	*E. A*	0	R	0	R	0	R	17	I	0	R	0	R	17	S	17	S	0	R	0	R	GEN-SMX-NAL-NIT-OXYTET-KAN-STR
13AB	*E. F*	0	R	0	R	0	R	21	S	25	S	0	R	19	S	21	S	0	R	0	R	GEN-SMX-NAL-NIT-KAN-STR
13B1	*E. A*	0	R	0	R	0	R	22	S	33	S	28	S	19	S	23	S	0	R	0	R	GEN-SMX-NAL-KAN- STR
13B2	*E. A*	0	R	0	R	0	R	15	I	25	S	0	R	18	S	22	S	0	R	0	R	GEN-SMX-NAL-NIT-KAN- STR
13B3	*E. A*	0	R	0	R	0	R	15	I	25	S	11	R	18	S	23	S	0	R	0	R	GEN-SMX-NAL-NIT-KAN-STR
13B4	*E. F*	0	R	0	R	0	R	16	I	25	S	0	R	16	I	15	R	0	R	0	R	GEN-SMX-NAL-NIT-KAN-AMP-STR
14A1	*E. A*	0	R	0	R	0	R	14	I	26	S	0	R	18	S	19	S	0	R	0	R	GEN-SMX-NAL-NIT-KAN-STR
14A2	*E. A*	0	R	0	R	0	R	18	S	26	S	0	R	17	S	13	R	0	R	0	R	GEN-SMX-NAL-NIT-KAN-AMP
14A3	*E. A*	0	R	0	R	0	R	21	S	26	S	0	R	19	S	23	S	0	R	0	R	GEN-SMX-NAL-NIT-KAN- STR
14A4	*E. A*	0	R	0	R	0	R	14	I	23	S	0	R	18	S	21	S	0	R	0	R	GEN-SMX-NAL-NIT-KAN-STR
12BC	*E. A*	0	R	0	R	0	R	14	I	23	S	0	R	18	S	22	S	0	R	0	R	GEN-SMX-NAL-NIT-KAN- STR

z- zone of inhibition, Int- interpretation, MAR- multiple antibiotic resistance, S- susceptible, I- intermediate, R- resistant , *E. A- Enterococcus avium, E. F- Enterococcus faecium, E. D- Enterococcus durans, Sc. B- Streptococcus bovis.*

Appendix A5: Molelwane cattle water isolates.

Sample id	isolate	Antibiotic disc concentration GEN 10 µg		SMX 25 µg		NAL 30 µg		CHL 10 µg		OYXTET 30 µg		NIT 100 µg		VAN 30 µg		AMP 10 µg		KAN 30 µg		STR 10 µg		MAR phenotype
		z	Int	z	Int	z	Int	z	Int	z	Int	z	Int	z	Int	z	Int	z	Int	z	Int	
MDW 1AB	*E.A*	0	R	0	R	0	R	16	I	21	S	0	R	18	S	20	S	0	R	0	R	GEN-SMX-NAL-NIT-KAN-STR
MDW 1AL	*E.A*	0	R	0	R	0	R	16	I	25	S	0	R	17	S	16	R	0	R	0	R	GEN-SMX-NAL-NIT-KAN-STR
MDW 1B	*E.A*	11	R	0	R	0	R	0	R	16	I	0	R	22	S	17	S	0	R	0	R	GEN-SMX-NAL-CHL-NIT-KAN-STR
MDW 2B	*E.F*	0	R	0	R	0	R	0	R	0	R	0	R	18	S	16	R	0	R	0	R	GEN-SMX-NAL-CHL-OXYTET-NIT-KAN-STR
MDW 3AL	*E.A*	0	R	0	R	0	R	15	I	21	S	10	R	16	I	21	S	0	R	0	R	GEN-SMX-NAL-NIT-KAN-STR
MDW 3B	*E.F*	25	S	0	R	0	R	20	S	14	R	0	R	13	R	18	S	0	R	0	R	SMX-NAL-OXYTET-NIT-KAN-STR
MDW 3BB	*E.A*	0	R	0	R	0	R	16	I	21	S	0	R	0	R	0	R	0	R	0	R	GEN-SMX-NAL-NIT-VAN-AMP-KAN-STR
MDW 3BL	*E.A*	0	R	0	R	0	R	23	S	20	S	18	S	0	R	0	R	0	R	0	R	GEN-SMX-NAL-VAN-AMP-KAN-STR
MDW 4A	*E.F*	0	R	0	R	0	R	19	S	17	I	13	R	19	S	23	S	24	S	0	R	GEN-SMX-NAL-NIT-STR
MDW 4AL	*E.A*	0	R	0	R	0	R	26	S	0	R	0	R	0	R	0	R	0	R	0	R	GEN-SMX-NAL-OXYTET-NIT-VAN-AMP-K AN-STR
MDW 4AB	*E.A*	0	R	0	R	0	R	15	I	23	S	0	R	0	R	0	R	0	R	0	R	GEN-SMX-NAL-NIT-VAN-AMP-KAN-STR
MDW 4DL	*E.F*	0	R	0	R	0	R	21	S	16	I	0	R	12	R	17	S	0	R	0	R	GEN-SMX-NAL-NIT-KAN-STR
MDW 4E	*E.F*	12	S	0	R	0	R	21	S	0	R	0	R	18	S	13	R	0	R	0	R	GEN-SMX-NAN-OXYTET-NIT-KAN-STR
MDW 5B	*E.F*	21	S	0	R	0	R	19	S	16	I	0	R	16	I	13	R	0	R	0	R	SMX-NAL-NIT-KAN-STR
MDW 5C	*E.F*	0	R	0	R	0	R	0	R	0	R	0	R	0	R	17	S	0	R	0	R	GEN-SMX-NAL-CHL-OXYTET-NIT-VAN-KAN-STR

z- zone of inhibition, Int- interpretation, MAR- multiple antibiotic resistance, S- susceptible, I- intermediate, R- resistant , *E. A- Enterococcus avium, E. F- Enterococcus faecium, E. D- Enterococcus durans, Sc. B- Streptococcus bovis.*

Appendix A6: Rooigrond cattle water isolates.

Sample id	isolate	GEN 10 µg z	GEN 10 µg Int	SMX 25 µg z	SMX 25 µg Int	NAL 30 µg z	NAL 30 µg Int	CHL 10 µg z	CHL 10 µg Int	OYXTET 30 µg z	OYXTET 30 µg Int	NIT 100 µg z	NIT 100 µg Int	VAN 30 µg z	VAN 30 µg Int	AMP 10 µg z	AMP 10 µg Int	KAN 30 µg z	KAN 30 µg Int	STR 10 µg z	STR 10 µg Int	MAR Phenotype
RW 1A	*E.A*	0	R	0	R	0	R	17	I	11	R	16	I	15	I	20	S	0	R	0	R	GEN-SMX-NAL-OXYTET-KAN-STR
RW 1B	*E.A*	0	R	0	R	0	R	16	I	11	R	9	R	16	I	21	R	0	R	0	R	GEN-SMX-NAL-NIT-OXYTET-KAN-AMP-STR
RW3A	*E.A*	0	R	0	R	0	R	10	I	23	S	13	R	16	I	17	S	0	R	0	R	GEN-SMX-NAL-NIT-CHL-KAN-STR
RW3B	*E.F*	0	R	0	R	0	R	17	I	10	R	0	R	16	I	21	R	0	R	11	R	GEN-SMX-NAL-NIT-OXYTET-KAN-AMP-STR
RW3C	*E.A*	0	R	0	R	0	R	14	I	25	S	16	I	16	I	20	S	0	R	0	R	GEN-SMX-NAL-KAN-STR
RW3D	*E.A*	0	R	0	R	0	R	17	I	26	S	13	R	16	I	20	S	0	R	0	R	GEN-SMX-NAL-NIT-KAN-STR
RW3E	*E.A*	0	R	0	R	0	R	15	I	20	S	15	I	16	I	23	S	0	R	0	R	GEN-SMX-NAL-KAN-STR
RW4A	*E.A*	0	S	0	R	0	R	15	I	22	S	0	R	18	S	19	S	0	R	0	R	GEN-SMX-NAL-NIT-KAN-STR
RW5A	*E.A*	0	R	0	R	0	R	16	I	21	S	17	S	13	R	22	S	0	R	0	R	GEN-SMX-NAL-KAN-VAN-STR

z- zone of inhibition, Int- interpretation, MAR- multiple antibiotic resistance, S- susceptible , I- intermediate, R- resistant , *E. A- Enterococcus avium, E. F- Enterococcus faecium.*

Appendix A7: Gelukspan cattle water isolates.

Sample id	isolate	GEN 10 µg		SMX 25 µg		NAL 30 µg		CHL 10 µg		OYXTET 30 µg		NIT 100 µg		VAN 30 µg		AMP 10 µg		KAN 30 µg		STR 10 µg		MAR Phenotype
		z	Int	z	Int'	z	Int	z	Int	z	Int	z	Int	z	Int	z	Int	z	Int	z	Int	
GW 19	*E.F*	0	R	0	R	0	R	13	I	20	S	0	R	18	S	21	S	0	R	0	R	GEN-SMX-NAL-NIT-KAN-STR
GW 21	*E. F*	0	R	0	R	0	R	18	S	20	S	0	R	20	S	21	S	0	R	0	R	GEN-SMX-NAL-NIT-KAN-STR
GW19B	*E. F*	0	R	0	R	0	R	16	I	20	S	15	I	17	S	21	S	0	R	0	R	GEN-SMX-NAL-KAN-STR
GW19C	*E. A*	0	R	0	R	0	R	18	S	22	S	0	R	16	I	20	S	0	R	0	R	GEN-SMX-NAL-NIT-KAN-STR
GW21A	*E. F*	0	R	0	R	0	R	19	S	27	S	0	R	17	S	24	S	0	R	0	R	GEN-SMX-NAL-NIT-KAN-STR
GW21B	*E. F*	0	R	0	R	0	R	17	I	20	S	0	R	19	S	24	S	0	R	0	R	GEN-SMX-NAL-NIT-KAN-STR
GW21C	*E. A*	0	S	0	R	0	R	15	I	27	S	0	R	18	S	23	S	0	R	0	R	GEN-SMX-NAL-NIT-KAN-STR

Antibiotic disc concentration

z- zone of inhibition, Int- interpretation, MAR- multiple antibiotic resistance, S- susceptible, I- intermediate, R- resistant , *E. A- Enterococcus avium, E. F- Enterococcus faecium.*

APPENDIX B

Antibiotic resistance of *E. faecium* from communal and commercial cattle faecal samples

Appendix B1: Communal cattle faéces.

Antibiotic	GEN	SMX	NAL	CHL	OXY-TET	NIT	VAN	AMP	KAN	STR
Nr/nt	12/13	13/13	13/13	10/13	4/13	13/13	4/13	3/13	13/13	13/13
%	92	100	100	77	31	100	31	23	100	100

Nr/nt= number resistant/number tested.

Appendix B2: Healthy commercial cattle faecal samples.

Antibiotic	GEN	SMX	NAL	CHL	OXY-TET	NIT	VAN	AMP	KAN	STR
Nr/nt	11/11	11/11	11/11	3/11	2/11	11/11	0/11	2/11	11/11	11/11
%	100	100	100	27	18	100	0	18	100	100

Nr/nt= number resistant/number tested.

Appendix B3: Commercial diarrhoeal cattle faecal samples.

Antibiotic	GEN	SMX	NAL	CHL	OXY-TET	NIT	VAN	AMP	KAN	STR
Nr/nt	12/13	12/13	13/13	4/13	7/13	13/13	1/13	8/13	12/13	13/13
%	92	92	100	31	54	100	8	62	92	100

Nr/nt= number resistant/number tested.

Antibiotic resistance of *E. avium* from commercial cattle faecal samples

Appendix B4: Healthy commercial cattle faecal samples.

Antibiotic	GEN	SMX	NAL	CHL	OXY-TET	NIT	VAN	AMP	KAN	STR
Nr/nt	18/18	18/18	18/18	1/18	4/18	14/18	6/18	3/18	18/18	18/18
%	100	100	100	6	22	78	33	17	100	100

Nr/nt= number resistant/number tested.

Appendix B5: Diarrhoeal cattle faecal samples.

Antibiotic	GEN	SMX	NAL	CHL	OXY-TET	NIT	VAN	AMP	KAN	STR
Nr/nt	26/26	26/26	26/26	2/26	11/26	16/26	1/26	4/26	22/26	23/26
%	100	100	100	8	42	61	4	15	85	88

Nr/nt= number resistant/number tested.

Antibiotic resistance of *E. durans* from communal and commercial cattle faecal samples

Appendix B6: Healthy cattle faecal samples.

Antibiotic	GEN	SMX	NAL	CHL	OXY-TET	NIT	VAN	AMP	KAN	STR
Nr/nt	7/8	8/8	8/8	0/8	0/8	7/8	3/8	3/8	7/8	8/8
%	88	100	100	0	0	88	38	38	88	100

Nr/nt= number resistant/number tested.

Appendix B7: Diarrhoeal cattle faecal samples.

Antibiotic	GEN	SMX	NAL	CHL	OXY-TET	NIT	VAN	AMP	KAN	STR
Nr/nt	2/2	2/2	2/2	0/2	0/2	2/2	0/2	1/2	2/2	2/2
%	100	100	100	0	0	100	0	50	100	100

Nr/nt= number resistant/number tested.

Antibiotic resistance of *Sc. bovis I* from communal and commercial cattle faecal samples

Appendix B8: Healthy cattle faecal samples.

Antibiotic	GEN	SMX	NAL	CHL	OXY-TET	NIT	VAN	AMP	KAN	STR
Nr/nt	5/5	5/5	5/5	1/5	3/5	5/5	0/5	1/5	5/5	5/5
%	100	100	100	20	60	100	0	20	100	100

Nr/nt= number resistant/number tested.

Appendix B9: Diarrhoeal cattle faecal samples.

Antibiotic	GEN	SMX	NAL	CHL	OXY-TET	NIT	VAN	AMP	KAN
Nr/nt	1/1	1/1	1/1	0/1	0/1	1/1	0/1	0/1	1/1
%	100	100	100	0	0	100	0	0	100

Nr/nt= number resistant/number tested.

Antibiotic resistance of *Enterococcus faecium* from communal and commercial cattle water sources

Appendix B10: Communal cattle water sources.

Antibiotic	GEN	SMX	NAL	CHL	OXY-TET	NIT	VAN	AMP	KAN
Nr/nt	5/5	5/5	5/5	0/5	0/5	4/5	0/5	0/5	5/5
%	100	100	100	0	0	80	0	0	100

Nr/nt= number resistant/number tested.

Appendix B11: Antibiotic resistance of *Enterococcus faecium* isolates from commercial cattle water sources.

Antibiotic	GEN	SMX	NAL	CHL	OXY-TET	NIT	VAN	AMP	KAN
Nr/nt	5/8	8/8	8/8	2/8	5/8	8/8	3/8	1/8	6/8
%	63	100	100	25	63	100	38	13	75

Nr/nt= number resistant/number tested.

Appendix B12: Antibiotic resistance of *Enterococcus avium* isolates from communal cattle water sources.

Antibiotic	GEN	SMX	NAL	CHL	OXY-TET	NIT	VAN	AMP	KAN
Nr/nt	2/2	2/2	2/2	0/2	0/2	2/2	0/2	0/2	2/2
%	100	100	100	0	0	100	0	0	100

Nr/nt= number resistant/number tested.

Appendix B13: Antibiotic resistance of *Enterococcus avium* isolates from commercial cattle water sources.

Antibiotic	GEN	SMX	NAL	CHL	OXY-TET	NIT	VAN	AMP	KAN	STR
Nr/nt	16/16	16/16	16/16	2/16	3/16	12/16	5/16	5/16	16/16	16/16
%	100	100	100	13	19	75	31	31	100	100

Nr/nt= number resistant/number tested.

APPENDIX C

Multiple Antibiotic Resistance Phenotypes of *Enterococcus* isolates

Appendix C1: *E. faecium* MAR phenotype from Gelukspan cattle.

MAR Phenotype	No. Observed	Percentage
GEN-SMX-NAL-CHL-NIT-KAN-STR	6	46
GEN-SMX-NAL-OXYTET-NIT-KAN-STR	1	8
GEN-SMX-NAL-CHL-OXYTET-NIT-KAN-STR	1	8
GEN-SMX-NAL-CHL-NIT-VAN-KAN-STR	1	8
GEN-SMX-NAL-CHL-NIT-OXYTET-VAN-AMP-KAN-STR	2	15
SMX-NAL-NIT-VAN-AMP-KAN-STR	1	8
GEN-SMX-NAL-NIT-KAN-STR	1	8

Total=13

Appendix C2: *E. faecium* MAR phenotype from healthy Molelwane cattle.

MAR Phenotype	No. Observed	Percentage
GEN-SMX-NAL-CHL-NIT-KAN-STR	3	27
GEN-SMX-NAL-OXYTET-NIT-KAN-STR	2	18
GEN-SMX-NAL-NIT-KAN-STR	5	45
GEN-SMX-NAL-NIT-AMP KAN-STR	1	9

Total=11

Appendix C3: *E. faecium* MAR phenotype from diarrhoeal Molelwane cattle.

MAR Phenotype	No. Observed	Percentage
GEN-SMX-NAL-CHL-NIT-KAN-STR	1	7
GEN-SMX-NAL-CHL-OXYTET-NIT-KAN-STR	3	21
GEN-SMX-NAL-NIT-KAN-STR	5	36
GEN-SMX-NAL-KAN-STR	1	7
SMX-NAL-OXYTET-NIT-KAN-STR	1	7
GEN-SMX-NAL-OXYTET-KAN-STR	1	7
GEN-NAL-OXYTET-NIT-KAN-STR	1	7
GEN-SMX-NAL-OXYTET-NIT-STR	1	7

Total=14

Appendix C4: *Enterococcus avium* Molelwane healthy cattle.

MAR Phenotype	No. Observed	Percentage
GEN-SMX-NAL-NIT-KAN-STR	7	47
GEN-SMX-NAL-KAN-STR	2	13
GEN-SMX-NAL-NIT-KAN-AMP-STR	1	7
GEN-SMX-NAL-NIT-VAN-AMP-KAN-STR	3	20
GEN-SMX-NAL-NIT	1	7
GEN-SMX-NAL-NIT-OXYTET-VAN-KAN-STR	1	7

Total=15

Appendix C5: *Enterococcus avium* Molelwane diarrhoeal cattle.

MAR Phenotype	No. Observed	Percentage
GEN-SMX-NAL-CHL-OXYTET-AMP-KAN-STR	1	4
GEN-SMX-NAL-CHL-NIT-OXYTET-KAN-STR	1	4
GEN-SMX-NAL-NIT-KAN-STR	12	44
GEN-SMX-NAL-KAN-STR	1	4
GEN-SMX-NAL-OXYTET-NIT-KAN-STR	2	7
GEN-SMX-NAL-STR	1	4
GEN-SMX-NAL-NIT	1	4
GEN-SMX-NAL-OXYTET-KAN-STR	4	15
GEN-SMX-NAL-OXYTET	2	7
GEN-SMX-NAL-OXYTET-AMP-KAN-STR	1	4

Total=26

Appendix C6: *Enterococcus avium* Rooigrond healthy cattle.

MAR Phenotype	No. Observed	Percentage
GEN-SMX-NAL-OXYTET-NIT-KAN-STR	1	33
GEN-SMX-NAL-STR	1	33
GEN-SMX-NAL-OXYTET-KAN-STR	1	33

Total=3

Appendix C7: *Enterococcus durans* Molelwane healthy cattle.

MAR Phenotype	No. Observed	Percentage
GEN-SMX-NAL-NIT-KAN-STR	1	33
GEN-SMX-NAL-NIT-AMP-KAN-STR	1	33
GEN-SMX-NAL-NIT-VAN-AMP-KAN-STR	1	33

Total=3

Appendix C8: *Enterococcus durans* Molelwane diarrhoeal cattle.

MAR Phenotype	No. Observed	Percentage
GEN-SMX-NAL-NIT-KAN-STR	2	100

Total=2

Appendix C9: *Enterococcus durans* Rooigrond healthy cattle.

MAR Phenotype	No. Observed	Percentage
GEN-SMX-NAL-KAN-STR	3	75
SMX-NAL-STR	1	25

Total=4

Appendix C10: *Streptococcus bovis I* Molelwane healthy cattle.

MAR Phenotype	No. Observed	Percentage
GEN-SMX-NAL-NIT-KAN-STR	1	100

Total=1

Appendix C11: *Streptococcus bovis I* Molelwane diarrhoeal cattle.

MAR Phenotype	No. Observed	Percentage
GEN-SMX-NAL-NIT-KAN	1	100

Total=1

Appendix C12: *Streptococcus bovis I* Rooigrond healthy cattle.

MAR Phenotype	No. Observed	Percentage
GEN-SMX-NAL-NIT-OXYTET-AMP-KAN-STR	1	25
GEN-SMX-NAL-NIT-OXYTET-KAN-STR	2	50
GEN-SMX-NAL-NIT-OXYTET-AMP-CHL-KAN-STR	1	25

Total=4

Multiple Antibiotic Resistance Phenotypes of *Enterococcus* isolates from cattle water sources

Appendix C13: *E. faecium* MAR phenotype from Gelukspan cattle water sources.

MAR Phenotype	No. Observed	Percentage
GEN-SMX-NAL-KAN-STR	1	20
GEN-SMX-NAL-NIT-KAN-STR	4	80

Total=5

Appendix C14: *E. faecium* MAR phenotype from Molelwane and Rooigrond cattle water sources.

MAR Phenotype	No. Observed	Percentage
GEN-SMX-NAL-NIT-KAN-STR	1	12
GEN-SMX-NAL-OXYTET-KAN-STR	1	12
GEN-SMX-NAL-CHL-OXYTET-NIT-KAN-STR	1	12
SMX-NAL-OXYTET-KAN-STR	1	12
GEN-SMX-NAL-CHL-NIT-OXYTET-VAN-KAN-STR	1	12
SMX-NAL-NIT-KAN-STR	1	12
GEN-SMX-NAL-NIT-STR	1	12
GEN-SMX-NAL-NIT-OXYTET-KAN-STR	1	12

Total=8

Appendix C15: *Enterococcus avium* MAR phenotype from Gelukspan cattle water sources.

MAR Phenotype	No. Observed	Percentage
GEN-SMX-NAL-NIT-KAN-STR	2	100

Total=2

Appendix C16: *Enterococcus avium* MAR phenotype from Molelwane and Rooigrond cattle water sources.

MAR Phenotype	No. Observed	Percentage
GEN-SMX-NAL-CHL-NIT-KAN-STR	2	13
GEN-SMX-NAL-CHL-NIT-OXYTET-KAN-STR	1	6
GEN-SMX-NAL-VAN-AMP-KAN-STR	1	6
GEN-SMX-NAL-NIT-KAN-STR	5	31
GEN-SMX-NAL-KAN-STR	3	19
GEN-SMX-NAL-NIT-VAN- AMP-KAN-STR	2	13
GEN-SMX-NAL-OXYTET-KAN-STR	1	6
GEN-SMX-NAL-NIT-OXYTET-VAN-AMP-KAN-STR	1	6

Total=16

Appendix C17: *E. durans* MAR phenotype from Molelwane and Rooigrond cattle water sources.

MAR Phenotype	No. Observed	Percentage
GEN-SMX-NAL-NIT-VAN-AMP-KAN-STR	1	100

Total=1

Pooled Multiple Antibiotic Resistance Phenotypes

Appendix C18: *Enterococcus faecium.*

MAR Phenotype	No. Observed	Percentage
GEN-SMX-NAL-CHL-NIT-KAN-STR	10	20
GEN-SMX-NAL-OXYTET-NIT-KAN-STR	5	10
GEN-SMX-NAL-CHL-OXYTET-NIT-KAN-STR	5	10
GEN-SMX-NAL-CHL-NIT-VAN-KAN-STR	1	2
GEN-SMX-NAL-CHL-NIT-OXYTET-VAN-AMP-KAN-STR	1	2
SMX-NAL-NIT-VAN-AMP-KAN-STR	1	2
GEN-SMX-NAL-NIT-KAN-STR	16	31
GEN-SMX-NAL-KAN-STR	2	4
GEN-SMX-NAL-NIT-KAN-STR-AMP	1	2
GEN-SMX-NAL-CHL-OXYTET-NIT-VAN-KAN-STR	1	2
SMX-NAL-OXYTET-NIT-KAN-STR	2	4
SMX-NAL-NIT-KAN-STR	1	2
GEN-SMX-NAL-NIT-STR	1	2
GEN-SMX-NAL-OXYTET-KAN-STR	1	2
GEN-NAL-OXYTET-NIT-KAN-STR	1	2
GEN-SMX-NAL-OXYTET-NIT-STR	1	2

Total=51

Appendix C19: *Enterococcus avium.*

MAR Phenotype	No. Observed	Percentage
GEN-SMX-NAL-CHL-NIT-KAN-STR	2	3
GEN-SMX-NAL-CHL-OXYTET-AMP-KAN-STR	1	2
GEN-SMX-NAL-CHL-NIT-OXYTET-KAN-STR	1	2
GEN-SMX-NAL-NIT-OXYTET-VAN-AMP-KAN-STR	1	2
GEN-SMX-NAL-VAN-AMP-KAN-STR	1	2
GEN-SMX-NAL-NIT-KAN-STR	26	43
GEN-SMX-NAL-KAN-STR	6	10
GEN-SMX-NAL-NIT-KAN-AMP-STR	1	2
GEN-SMX-NAL-NIT-VAN-KAN-STR	5	8
GEN-SMX-NAL-OXYTET-NIT-KAN-STR	4	7
GEN-SMX-NAL-STR	2	3
GEN-SMX-NAL-NIT	2	3
GEN-SMX-NAL-OXYTET-KAN-STR	6	10
GEN-SMX-NAL-OXYTET	1	2
GEN-SMX-NAL-OXYTET-AMP-KAN-STR	1	2
GEN-SMX-NAL-NIT-OXYTET-VAN-AMP-KAN-STR	1	2

Total=61

Appendix C20: *Enterococcus durans.*

MAR Phenotype	No. Observed	Percentage
GEN-SMX-NAL-NIT-KAN-STR	6	60
GEN-SMX-NAL-NIT-KAN-STR-AMP	1	10
GEN-SMX-NAL-NIT-VAN-KAN-STR-AMP	2	20
SMX-NAL-STR	1	10

Total=10

Appendix C21: *Streptococcus bovis I.*

MAR Phenotype	No. Observed	Percentage
GEN-SMX-NAL-NIT-KAN-STR	1	17
GEN-SMX-NAL-NIT-KAN	1	17
GEN-SMX-NAL-NIT-OXYTET-AMP-KAN-STR	1	17
GEN-SMX-NAL-NIT-OXYTET-KAN-STR	2	33
GEN-SMX-NAL-NIT-OXYTET-AMP-CHL-KAN-STR	1	17

Total=6

APPENDIX D

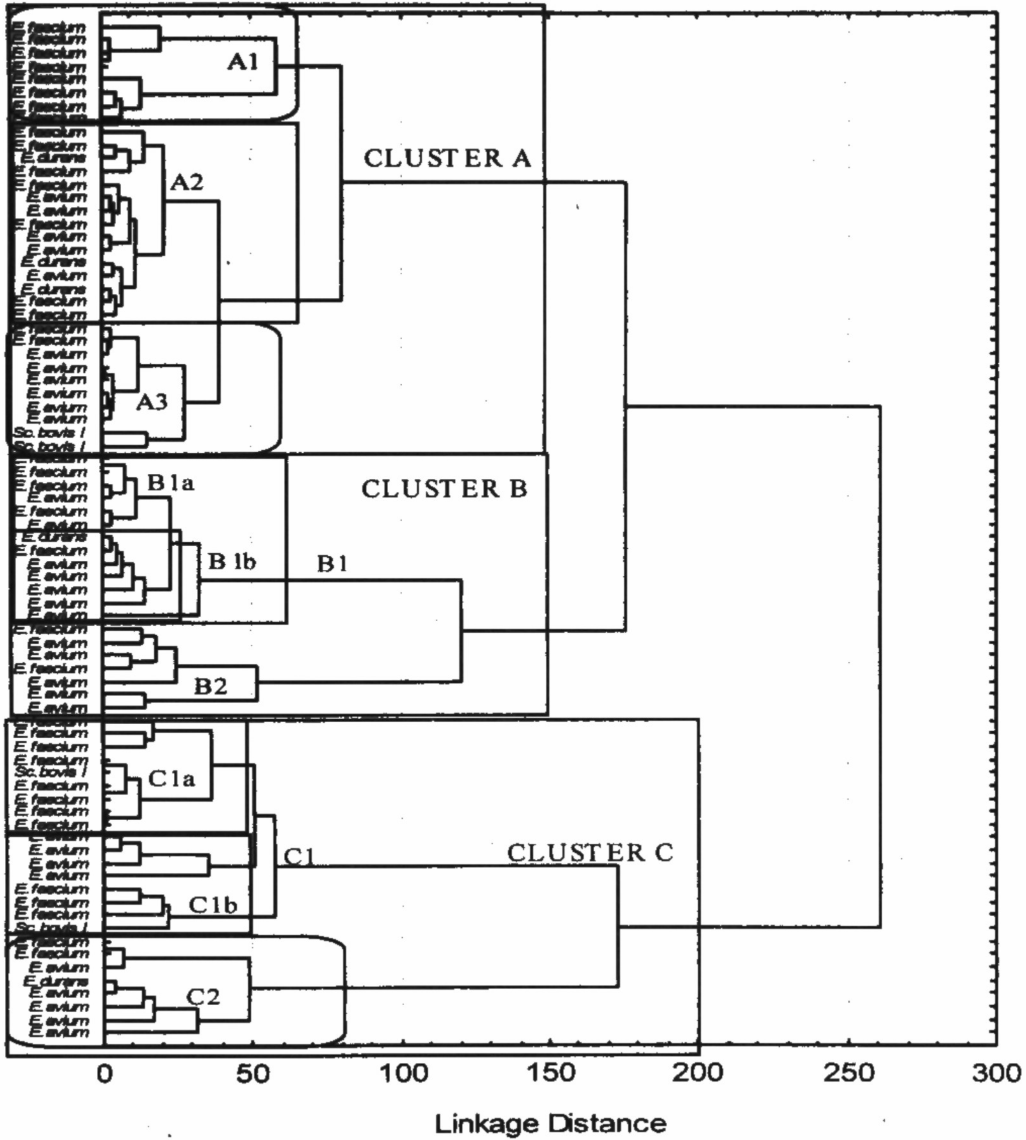
Multiple antibiotic resistance indices

Appendix D1: MAR indices of *E. faecium* from communal and commercial cattle farms.

Area	MAR Index	No. of isolates	GEN	SMX	NAL	CHL	OXYTET	NIT	VAN	AMP	KAN	STR
Communal	0.7	2	-	-	-	-	+	-	*	+	-	-
Cattle faecal samples	0.7	1	-	-	-	+	-	-	+	+	-	-
	0.8	1	-	-	-	-	-	-	*	+	-	-
	0.7	4	-	-	-	-	+	-	+	+	-	-
	0.8	1	-	-	-	-	+	-	-	+	-	-
	1	2	-	-	-	-	-	-	-	-	-	-
	0.7	1	+	-	-	+	*	-	-	-	-	-
	0.6	1	-	-	-	*	+	-	+	+	-	-
Average MAR Index	**0.754**											
Commercial cattle faecal samples	0.7	2	-	-	-	-	+	-	*	+	-	-
	0.7	1	-	-	-	-	+	-	+	+	-	-
	0.7	1	-	-	-	*	+	-	+	+	-	-
	0.6	1	-	-	-	+	+	-	+	+	-	-
	0.7	1	-	-	-	+	+	-	+	+	-	-
	0.6	1	-	-	-	+	+	-	+	+	-	-
	0.7	1	-	-	-	*	+	-	*	-	-	-
	0.6	1	-	-	-	*	+	-	+	+	-	-
	0.7	1	-	-	-	*	-	-	*	+	-	-
	0.8	1	-	-	-	*	-	-	*	-	-	-
Average MAR Index	**0.682**											

Communal cattle water samples	0.6	2	-	-	-	*	+	-	+	+	-	-
	0.6	2	-	-	-	+	+	-	+	+	-	-
	0.5	1	-	-	-	*	+	*	+	+	-	-
Average MAR Index	**0.58**											
Commercial cattle water samples	0.8	1	-	-	-	-	-	-	+	+	-	-
	0.7	1	+	-	-	+	-	-	-	+	-	-
	0.5	1	-	-	-	+	*	-	+	+	+	-
	0.6	1	+	-	-	+	*	-	-	+	-	-
	0.7	1	-	-	-	+	-	-	+	+	-	-
	0.5	1	+	-	-	+	*	-	*	+	-	-
	1	1	-	-	-	-	-	-	-	-	-	-
	0.7	1	-	-	-	*	-	-	*	+	-	-
Average MAR Index	**0.688**											

APPENDIX E



Dendrogram illustrating the relationship of the 78 *Enterococcus* species in terms of the antibiotic IZD data.

APPENDIX F

Solutions and reagents used

1. Media

1.1 Solid media

The following solid media were used for bacterial cultivation.

Mueller Hinton agar (Biolab Merck, Gauteng) [meat infusion 5.0 g/l, casein hydrolysate 17.5 g/l, soluble starch 1.5 g/l, agar 14.0 g/l] was used for antibiotic resistance test.

***Enterococcus* selective agar** (Biolab Merck, Gauteng) selective agar used for the isolation and counting of enterococci belonging to Lance Field Group D [esculin 1.0 g/l, ferric ammonium citrate 0.5 g/l, oxbile 10.0 g/l, peptone 3.0 g/l, sodium azide 0.5 g/l, sodium chloride 5.0g/l, sodium citrate 1.0 g/l, tryptone 17.0 g/l, yeast extract 5.0 g/l, agar 12.0 g/l].

Blood agar base (Biolab Merck, Gauteng) A media used for the isolation and cultivation of pathogenic and non-pathogenic bacteria and also used for the detection of haemolytic organisms [meat extract 10.0 g/l, peptone 10.0 g/l, sodium chloride 5.0 g/l, agar 10.0 g/l].

Triple sugar iron agar (Mast diagnostics, UK) Used for the differentiation of enterobacteriaceae based on hydrogen sulphide production and fermentation of lactose, sucrose and dextrose [meat extraction 4.0 g/l, yeast extract 3.0 g/l, peptone mixture 18.0 g/l, sodium chloride 5.0g/l, lactose 10.0 g/l, sucrose 10.0 g/l, dextrose 1.0 g/l, ferric ammonium citrate 0.3 g/l, sodium thiosulphate 0.3 g/l, phenol red 0.025 g/l, agar 14.0 g/l].

1.2 Liquid media

The liquid media used were:

Nutrient broth (Biolab Merck, Gauteng) ['lab-lemco'powder 1.0 g/l, yeast extracts 2.0 g/l, peptone 5.0 g/l, sodium chloride 5.0 g/l].

Luria Bertani broth (Biolab Merck, Gauteng) used for the cultivation and maintenance of bacteria for generic and molecular studies [tryptone 12.0 g/l, sodium chloride 12.0 g/l, yeast extract 6.0].

Brain heart infusion broth (Biolab Merck, Gauteng) [brain heart infusion solids 17.5g/l, dextrose 2.0g/l, peptone 10.0g/lsodium chloride 5.0g/l and di-sodium phosphate 2.5g/l].

2. Reagents and chemicals

Crystal violet- 10 g crystal violet; 100 ml alcohol; 1% aqeuous ammonium oxalate 400 ml (i.e 1 g in 100 ml/ 4g).

Lugol's iodine – 5 g iodine; 10 g potassium iodide; distilled water.

Carbol fuchsin – Basic fuschin powder 1 g; phenol crystals 4.5 g; alcohol 10 ml; Distilled H_2O 500 ml.

TE -10 mM Tris-Cl, pH 8; 1 mM EDTA

Ethidium bromide -10 mg/ml

Proteinase K – 10mg/ml

Polyvinyl pyrrolidone (PVP) - 5% solution

Chloroform isoamyl alcohol 24:1

Cetyltrimethyl ammonium bromide (CTAB) -2% hexadecyltrimethylammonium bromide, 1.4 MNaCl, 0.2% 2-mercaptoethanol.

Ampicillin stock -5 mg/ml in sterile H_2O, (50µl/ml final concentration).

Tetracycline stock - 12 mg/ml in 70% ethanol. (12 mg/ml final concentration).

Loading buffer – 0.25% bromophenol blue, 0.25% xylene cyanol, 50% glycerol.

TAE – 0.04 M Tris, acetic acid, 0.002 M EDTA.