DETECTION OF ANTIBIOTIC RESISTANCE AND VIRULENT GENES IN ENTEROCOCCI ISOLATED FROM WATER SAMPLES IN ZARIA, KADUNA STATE, NIGERIA.

BY

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DEPARTMENT OF MICROBIOLOGY,
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NIGERIA

APRIL, 2015

DECLARATION

I hereby declare that the work in the thesis entitled **'Detection of antibiotic resistance and virulent genes in Enterococci isolated from water samples in Zaria, Kaduna state, Nigeria'** was performed by me in the Department of Microbiology under the supervision of ProfsS.A. Ado and J.B Ameh.

The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at any other institution.

Kashim, Zainab Abimbola

_____ _____ _____

Name of Student Signature Date

CERTIFICATION

This thesis entitled, ‘‘DETECTION OF ANTIBIOTIC RESISTANCE AND VIRULENT GENES IN ENTEROCOCCI ISOLATED FROM WATER SAMPLES IN ZARIA, KADUNA STATE, NIGERIA’’ by **Zainab Abimbola, KASHIM** meets the regulations governing the award of the degree of Master of Science of Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

Prof. S.A. Ado

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Chairman, Supervisory committee	Signature	Date

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DEDICATION

This thesis is dedicated to God Almighty Allah for making this work a successful one. To my dear parents Alhaji and Hajiya Kashim, my lovely husband Dr Abdulhakeem Bello whose words of encouragement, prayers, support kept me going all the way.

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ABSTRACT

The present study was conducted to isolate, identify and analyse the distribution of Enterococci,carry out antibiotic susceptibility test and molecularly detect the presence of some virulent and antibiotic resistantgenes in the Enterococci isolated from water samples in Zaria, Kaduna state. A total of two hundred and forty water samples were collected from different sources including wells, boreholes, tap and vended water (mairuwa). A total of34*Enterococci*were isolated, characterized and identified. The prevalence of*Enterococcus avium* and *Enterococcus gallinarum* in water were 58% and 24% respectively while *Enterococcus mundtii and Enterococcus cecorum* had 12% and 6%each. The mean frequency distribution was calculated using the one way ANOVA and based on this, the highest source of contamination was from wells, while vended water (mairuwa) had the least. Zaria city had the highest Enterococci isolates (44%) followed by Samaru (35%) and then Sabon gari (21%). Isolates had resistanceto the following antibiotics, Ampicillin 79% (10µg), Vancomycin 62%(30µg), Erythromycin 53%, (15µg), Chloramphenicol 12%, (10µg), Ciprofloxacin 12% (30µg), Tetracycline 12%, (12µg) and Gentamicin 3% (10µg). The result of the molecularscreening for *van A,van B* and *cyl A* genes showed the absence of all the genes in the isolates.The result of the present study indicatesthat the contamination of water with Enterococci could predispose to various health issues and pose serious therapeutic challenges due to the presence of resistance to antibiotics.The provision of proper water management systems and treated safe water supply to the communities cannot be overemphasized.

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CHAPTER ONE

1.0 INTRODUCTION

Bacteria of the genus *Enterococcus*or enterococci (formerly the 'faecal' or Lance field group D streptococci) are ubiquitous microorganisms, but have a predominant habitat of the gastrointestinal tract of humans and animals. *Enterococcus* is a genus of lactic acid bacteria of the Phylum Firmicutes. Enterococci are Gram-positive cocci that often occur in pairs (diplococci) or short chains, and are difficult to distinguish from streptococci on physical characteristics alone (Gilmore *et al.*, 2002) but they have been distinguished by their ability to hydrolyze esculin in the presence of bile, grow in 6.5% sodium chloride, demonstrate pyrrolidonyl arylamidase and leucine aminopeptidase, and react with group D antiserum. Enterococci are hardy facultative anaerobic organisms capable of cellular respiration in both oxygen-rich and oxygen-poor environment (Fisher and Phillips, 2009). Though they are not capable of forming spores, enterococci are tolerant to a wide range of environmental conditions which include extreme temperature (10-45°C), pH (4.5-10.0) and high sodium chloride concentrations (Ryan and Ray, 2004).

Enterococci belong to the normal flora of the gastrointestinal tract of humans and animals and they are less commonly found at other anatomical sites, such as the vagina and mouth. Under normal circumstances they are harmless commensals, and are even believed to have positive effects on a number of gastrointestinal and systemic conditions (Pangallo*et al*., 2004; Benyacoub *et al*., 2003). However, when the commensal relationship with the host is disrupted, enterococci can become opportunistic pathogens and cause invasive diseases (Pangallo*et al*., 2004). Though not

as virulent as other Gram-positive organisms, they can lead to a variety of clinical syndromes including endocarditis, bacteraemia, meningitis, wound and urinary tract infections, and they are associated with peritonitis and intra-abdominal abscesses (European Antimicrobial Resistance Surveillance System, 2007).Enterococcal species known to cause human infections include *Enterococci faecium, Enterococcusfaecalis, Enterococcus avium, Enterococcus gallinarum, Enterococcus casseliflavus, Enterococcus durans, Enterococcus raffinosus and Enterococcus mundtii* (De Perio *et al*., 2006).

Community water systems obtain water from two sources: surface water and ground water. People use surface and ground water every day for a variety of purposes, including drinking, cooking, and basic hygiene, in addition to recreational, agricultural, and industrial activities. According to the United States Environmental Protection Agency (EPA), the majority of public water systems (91%) are supplied by ground water; however, more persons (68%) are supplied year-round by community water systems that use surface water (EPA, 2007). Surface water is water that collects on the ground or in a stream, river, lake, reservoir, or ocean. Surface water is constantly replenished through precipitation, and lost through evaporation and seepage into ground water supplies. According to the EPA, 68% of community water system users received their water from a surface water source, such as a lake (EPA, 2007).Ground water, which is obtained by drilling wells, is water located below the ground surface in pores and spaces in the rock, and is used by approximately 78% of community water systems in the United States, supplying drinking water to 32% of community water system users (EPA, 2007). Water sources in populous countries have now serve as reservoirs of antimicrobial-resistant pathogenic microbes due to indiscriminate use of

antimicrobials in human and veterinary medicine and addition of faecal contamination through point source as well as non-point sources, storm drain infrastructure and malfunctioning septic trenches (Ahmed *et al.,* 2005).

The presence of enterococci as an indicator of faecal contamination has been used in the management of recreational water quality standards as it correlates best with the incidence of swimming-related illnesses (USEPA, 2003). Enterococci, the 'indicator' of water quality, correlates best with the incidence of gastrointestinal diseases as well as prevalence of other pathogenic microorganisms (Pushpa*et al*., 2009) in water samples.

Ground water can become unusable if it becomes polluted and is no longer safe to drink. Contaminated ground water can be as a result of microbial contamination (faecal contamination from feedlots), high concentrations of naturally-occurring contaminants, such as arsenic and radon (depends highly on the geology of the soil surrounding the well), local land use practices (fertilizers and pesticides), problems with the integrity of nearby on-site septic systems (Moore *et al*., 2008).

1.1 Statement of research problem

Over the last decades, enterococci, formerly viewed as organisms of minimal clinical impact, have emerged as important hospital-acquired pathogens in immunosuppressed patients and intensive care units. Enterococci do not possess the common virulence factors found in many other bacteria, but they have a number of other characteristics, such as the resistance to antimicrobial agents, that may contribute to their virulence and make them selective opportunistic pathogens (Pangallo *et al*., 2004).

Enterococci are widely distributed in nature and spread from faecal contamination and persist due to their high tolerance to the various environmental conditions. In water samples enterococci are used as indicators of faecal pollution (Tejedor *et al*., 2001; Cupáková and Lukášová, 2003). The assurance of the microbiological quality of environmental water used as a source for recreational water is a global issue. However, several studies of both recreational and drinking water samples suggested that enterococci are more relevant indicators of faecal contamination than other coliforms and *E. coli* (Grammenou *et al*., 2006; Kinzelman *et al*., 2003).With increasing antibiotic resistance, enterococci are recognized as feared nosocomial pathogens that can be challenging to treat (Pangallo *et al*., 2004)

1.2 Justification of the study

Globally, over one million people have no access to safe drinking water and 2.6 billion people lack adequate sanitation. This leads to 1.8million people dying every year from water and sanitation related diarrhoeal diseases, 90% being children under 5years, mostly in developing countries (WHO, 2004). Growth and nutrition among young children are also adversely affected by contaminated water supplies, poor hygiene and inadequate sewerage (Cheesebrough, 2004).

Total and faecal coliforms have been used widely for many years as indicators for determining the sanitary quality of water. In recent years, scientists have studied more ways that coliform ecology, occurrence and resistance to stress differs from that of many of the pathogenic microorganisms for which they are a proxy (Scott *et al*., 2002). Therefore, alternative indicators to coliforms have been proposed including *E. coli*, *Enterococcus* sp and *Clostridium perfringens* (Stevens *et al*., 2003). The

enterococci tend to persist longer in the environment than coliforms and are approximately an order of magnitude less numerous than faecalis coliforms and *E. coli* in human faeces, but are still sufficiently numerous to be detected after significant dilution (Stevens *et al*., 2003).

Previous epidemiological studies have demonstrated a correlation between the concentration of enterococci in surface waters and an increase in swimmer-associated gastroenteritis (He and Jiang, 2005). Certain members of the genus, particularly *E. faecalis* (Farrell *et al*., 2003) and *E. faecium*, are becoming increasingly important as opportunistic pathogens (Low *et al.*, 2003). Environmental water quality studies may benefit from focusing therefore, on a subset of *Enterococcus* spp that are consistently associated with sources of faecal pollution including domestic sewage, that poses significant human health risks.

Recent efforts have focused on the development of methods for the characterization enterococci (Coque *et al.*, 1998; Zdrasgas *et al.*, 2008). However, compared to current methods, there is a need to develop and apply more robust, rapid, and cost-effective techniques that can be used to study the population and understand the mechanism of action of virulence in Enterococci.This can be done by studying the effect, presence and expression of virulence determinants in environmental enterococci. Only then can we determine the possible risk to human health when exposed to environmental enterococci (Zhu *et al.*, 2009).

1.3 Aim of the study

The aim of this work was to isolate enterococci species from water samples in Zaria Kaduna stateand to detect antibiotic resistance and virulent genes from such isolates.

1.4 Objectives of the study are to:

1. Isolate *Enterococcus* spp from the water samples and identify them biochemically.
2. Determine antibiotic susceptibility of the isolates.
3. Detect antibiotic resistance and virulent genes in the*Enterococcus* spp isolated.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Overview of Enterococci

The term enterocoque, emphasizing the intestinal origin of a newly recognized Gram-positive coccus, was encountered in a French paper published in 1899 (Murray, 1990). Because of their cell shape, staining characteristics and lack of catalase, enterococci were considered members of the genus *Streptococcus* until the early 1990s. The name *Streptococcus faecalis*, which came to be used for the most common species, emphasized the relationship of these organisms to faeces. Some enterococci were also noted to be β-haemolytic, at least on some types of blood. Based on Lancefield's serological classification, enterococci were considered salt - tolerant group D streptococci. Through the use of DNA hybridization and 16S rRNA sequencing, it was established that the species *Streptococcus faecium* and *Streptococcus faecalis* were sufficiently distinct from the other streptococci to be designated another genus: *Enterococcus*(Foulquie-Moreno *et al.,* 2006). This means that the D group antigen is found in both streptococci and enterococci. Nine species were transferred from the *Streptococcus* groups and now *Enterococcus* includes 28 species (Foulquie-Moreno *et al.*, 2006). The molecular data that were collected using 16S rRNA sequencing of *Streptococcus* enabled the construction of a 16S rRNA-dendrogram (fig. 2.1) showing the relationship between *Streptococcus*, *Enterococcus* and *Lactococcus* species. This method also allowed the grouping of *Enterococcus* spp.

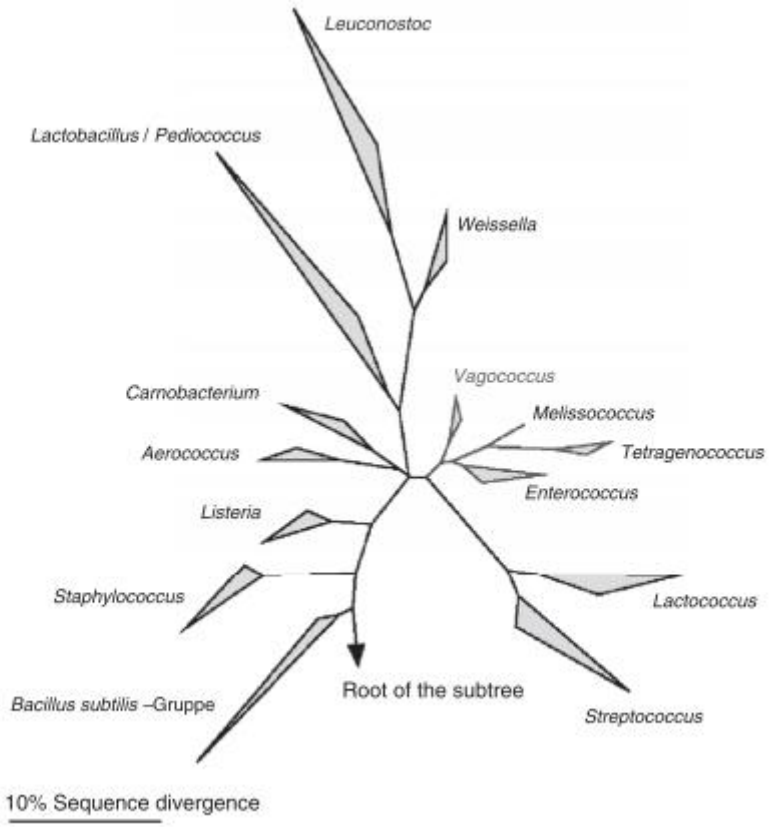

Fig 2.1: 16S rRNA dendrogram of phylogenetic position of *Enterococcus* species (adapted from Klein, 2003).

2.2 Description of the Genus

By Biochemical Characteristics Enterococci are Gram-positive, facultatively anaerobic cocci that typically grow in pairs or short chains. They are catalase negative, although a weak pseudocatalase is occasionally seen. Most strains have the ability to grow in the presence of 6.5% sodium chloride, at 10 °C and at 45 °C and at pH 9.6 and can survive at 60 °C for 30 min. Enterococci are capable of hydrolyzing esculin in the presence of bile, and most strains react with group D antisera. Some react also with group Q antisera. The emergence of vancomycin resistance in enterococci has increased the importance and difficulty of differentiating enterococci from intrinsically

vancomycin-resistant Gram-positive cocci, for example, *Leuconostoc* spp. or *Pediococcus* spp., which also may be isolated from human specimens. Most enterococci possess the enzyme pyrrolidonyl arylamidase and are able to hydrolyze L-pyrrolidonyl-α-naphthylamide (PYR), although some species such as *Enterococcus cecorum*, *Enterococcus columbae*, *Enterococcus pallens* and *Enterococcus saccharolyticus* are negative (Facklam *et al*., 2002). This reaction is very useful for differentiating enterococci from most streptococci, *Leuconostoc* spp. and pediococci. It has been known for decades that enterococci have complex growth requirements. *E. faecalis* has been shown to grow well on Davis minimal medium (consisting of salts, citrate, thiamine, glucose and agar) supplemented with additional vitamins (biotin, calcium pantothenic acid, pyridoxine, nicotinic acid, riboflavin and folic acid) plus 20 amino acids (Murray *et al*., 1993).

2.3 Ecology of Enterococci

Enterococci are usual inhabitants of the gastrointestinal (GI) tracts of warm-blooded animals and are also found in insects and plants, such as seen in works by some researchers who have indicated that they are also found in cattle, pigs (Manero *et al*., 2002), dogs (Wheeler *et al*., 2002) horses (Wheeler*et al*., 2002) rabbits (Poeta *et al*., 2005) chickens (Hammerum *et al*., 2000), sheeps, swine and wild birds (Poeta*et al*., 2005). Species that are also known to be typically associated with plants are the yellow-pigmented *E. mundtii* and *E. casseliflavus*(Salminen *et al*., 2004).

E. faecalis is usually more common in human faeces than *E. faecium*, averaging 105–107 and 104–105 colony-forming units (CFU) per gram, respectively, but *E. faecium* predominates especially in the hospital setting (Murray, 1990; Suppola *et al*.,

1996). Enterococci are less frequently found at other sites such as vagina, skin, oral cavity and dental plaque.

2.4 Classification of Enterococcus Genus

Historically, enterococci were classified as group D streptococci (Salminen *et al.*, 2004). In 1984, after DNA-DNA and DNA-RNA hybridization studies, enterococci were separated from the genus Streptococcus and were given formal genus status (Ogier and Serror, 2008). The following species of enterococci can be distinguished after comparative sequence analysis of 16SrRNA genes (Salminen *et al.*, 2004).

1. *Enterococcus faecium* group: *E. Faecium, E. durans, E. hirae, E. mundtii, E. villorum, E. canis, E. azikeevi*
2. *Enterococcus avium* group: *E. avium, E. malodoratus, E. pseudoavium, E. raffinosus, E. glivus*
3. *Enterococcus gallinarum* group: *E. gallinarum, E. casseliflavus, E. flavescens*
4. *Enterococcus dispar* group: *E. dispar, E. asini, E. pallens*
5. *Enterococcus saccharolyticus* group: *E. saccharolyticus, E. sulfures*
6. *Enterococcus cecorum* group: *E. cecorum, E. columbae*
7. *Enterococcus faecalis group: E. faecalis, E. haemoperoxidus, E. moraviensis, E. ratti*

E. faecium and *E. faecalis* causes 80-90% of human enterococcal infections. Factors contributing to their pathogenesis is their resistance to a wide variety of antibiotics and

possessing of virulence factors that allow an infecting strain to invade the host (Eaton and Gasson, 2001), but the use of enterococci has been suggested as an alternative to *Escherichia coli* for monitoring freshwater quality by the United States Environmental Protection Agency and the Australian National Health and Medical Research Council (NHMRC, 2008; USEPA, 2003).

Enterococcus mundtii – *Enterococcus mundtii* is a coccoid, gram positive, non-motile, yellow pigmented lactic acid bacterium and belongs to the family Enterococcaceae. It was discovered in 1986 isolated from cow teats, hands of milkers, soil and plants. Although, this specie has rarely been isolated from environmental or human sources, it has been found to be implicated in human infections (Achim and Patricia, 1991; Tomomi *et al*., 2005), thus classifying them as pathogenic organisms.

Enterococcus gallinarum – *Enterococcus gallinarum*are gram positive, non-motile organisms that usually occur in pair or short chains. They have been implicated in a variety of infections in humans, especiallyimmunocompromised persons. They are shown to colonize the intestinal tracts of both hospitalized and non-hosptalised individuals with overall rates of colonization that range from 5.7% (Toye *et al*., 1997) to 12.1% (Van Horn and Rodney, 1998). They have also been found to be isolated from poultry farms (CDC,2005).

Enterococcus avium – This species of *Enterococus*is commonly found in birds, usually non-pigmented, non-motile and mostly occur in pairs or short chains. They rarely cause infections in human and in some cases can be vancomycin-resistant (Rosata *et al*., 1995).

Enterococcus cecorum – This specie is 1-1.3µm in diameter, non-motile and nonspore-forming, whose colonies are circular and non-pigmented. It is originally isolated from poultry intestines and rarely caused human disease, although, few reports show its implication in some diseases in human (Po-ren *et al*., 2000; Greub *et al*., 1997)

2.5 Epidemiology of Enterococci

In 2005, there were 7066 reported cases of bacteraemia caused by *Enterococcus* species in the UK, an 8 % increase from 2004, with the Health Protection Agency (2007) stating that 'an increase in a bacteraemia causing pathogen like this has not been observed for some time'. Twenty-eight per cent of all cases were antibiotic resistant (Health Protection Agency, 2007). The risk of death from vancomycin-resistant enterococci (VRE) is 75 %, compared with 45 % for those infected with a susceptible strain (Bearman and Wenzel, 2005). These figures are mirrored in the USA. Over a 15year period there was a 20-fold increase in VRE associated with nosocomial infections reported to CDC's National Nosocomial Infections Surveillance (NNIS) (National Nosocomial Infections Surveillance, 2004).

This dramatic increase in antibiotic resistance of *Enterococcus* species worldwide highlights the need for a greater understanding of this genus, including its ecology, epidemiology and virulence. The differences in the genomes of *E. faecalis* and *E. faecium* were assessed in a study using competitive DNA hybridization (Shanks *et al.*, 2006). *E. faecalis*-specific sequences compared with those of *E. faecium* mainly encoded surface-exposed proteins. Overall 6.4 % of the *Enterococcus* genome is associated with cell-surface proteins and 22.6 % of the differences between the two

species are found in these genes. This variation is thought to have implications in the species avoiding different host immune responses (Shanks *et al.* 2006).

2.6 Survival in External Environments

Enterococci are found in diverse range of habitats. They normally reside in the human and animal gut and are introduced to the environment by means of faeces and subsequently disseminated to diverse niches. Enterococci are the third most important agents in nosocomial infections. Their survival in external environments is linked to their exceptional ability to cope with harsh environmental conditions including heat shock, acid pH and detergents (Bradley and Fraise, 1996; Rince *et al*., 2002). *E. faecalis* is able to develop adaptive responses towards diverse type of stress. Analysis of protein synthesis during the incubation of exponentially growing cells of *E. faecalis* exposed to sub-lethal stresses led to the finding that 167 proteins were over expressed. Six of these are induced by at least six different stress conditions and play an important physiological role in specific stress responses. These general stress proteins have been named as Gsp62 to Gsp67 (Rince *et al*., 2002). Studies on mRNA revealed transcriptional induction of gene gsp 65 in response to heat shock, acid pH, detergents, ethanol, sodium chloride and hydrogen peroxide (Rince *et al*., 2002). This heat resistant property allows enterococci to survive heat-based decontamination processes and thus to go on to cause nosocomial infections.

2.7 Virulence of Enterococci

It was recognized early, that enterococci were less virulent than staphylococci, pneumococci and group A streptococci and were even called facultative parasites. The introduction and widespread use of antibiotics, many of which had no or poor activity

against enterococci (i.e. cephalosporins), was soon followed by an increase in the incidence of enterococcal infections. However, there were many reports of infections before the antibiotic era, which illustrates that these organisms are able to produce infection in normal hosts, particularly endocarditis and pelvic, intra-abdominal and urinary infections. Virulence factors associated with the pathogenesis of enterococcal infections in humans are currently under active investigation. Most infection is thought to be endogenous, by translocation of the bacteria through the epithelial cells of the intestine, which then cause infection via lymph nodes and thus spread to other cells within the body (Franz *et al.*, 1999). Known and putative virulence factors in enterococci are: Cytolysin, pheromones, aggregation substance (AS), protease, lipoteiochoic acid, hyaluronidase, AS 48, haemolysin, gelatinase (Jett *et al*., 1994), enterococci surface proteins (esp), adhesion to collagen from *E. faecalis* (Ace) (Budzik and Schneewind, 2006), biofilm production, ebp (Singh *et al*., 2007), MSCRAMMs (Sillanpää *et al*., 2004), resistance to phagocytosis by neutrophils (Arduino *et al*., 1994, Rakita *et al*., 2000), secreted antigens SagA (Teng *et al*., 2003) and Acm (Nallapareddy *et al*., 2003), quorum sensing (Nakayama *et al*., 2001), and production of hydrogen peroxide (Moy *et al*., 2004). Animal models have been used to demonstrate the effect of these virulence factors (Dupont *et al*., 1998). Enterococci have failed to cause chronic or severe infections after inoculation into subcutaneous tissue or peritoneal cavity in laboratory animals, which has been used as the main argument of enterococci being non-pathogenic organisms despite this the enterococci have emerged as a major nosocomial pathogen over the last decades (Jett *et al*., 1994).

In addition, enterococci are known to transfer mobile genetic elements that often contain genes for antibiotic resistance and/or other virulence factors to other

enterococci species, Gram-positive and Gram-negative species (Donelli, 2004). Antibiotic resistance is considered as an important pathogenicity property of enterococci (Foulquié-Moreno *et al*., 2006). *Enterococcus* species with the highest virulence are medical isolates, followed by food isolates and then starter strains (Busani *et al.*, 2004; Ben Omar *et al.*, 2004). Antibiotic resistance alone cannot explain the virulence of enterococci and their ability to cause infection and disease. Enterococci have other factors that determine their virulence, such as ability to colonize the gastrointestinal tract which is the normal habitat and ability to adhere to a range of extracellular matrix proteins including thrombospondin, lactoferrin and vitronectin, urinary tract epithelia, oral cavity epithelia and human embryo kidney cells which is achieved by the production of adhesins such as aggregation substance, enterococcal surface protein (Esp) and the adhesin of collagen from *E. faecalis* (Ace). Furthermore, *Enterococcus* spp among other factors have the ability to secrete cytolysin/haemolysin, gelatinase and serine protease alongside cell-wall polysaccharides.

2.7.1 Surface-located proteins aggregation substance

This is a surface-located protein encoded by pheromone-responsive plasmids that may play a role in binding to human cells, in resistance to neutrophil-mediated killing and in promoting internalization by intestinal cells. It is proposed that aggregation substance, by mediating clumping and/or binding between enterococcal cells and target cells, leads to increased local density of cells, necessary to induce cytolysin/haemolysin production and protease expression via quorum-sensing mechanisms. Pulsed-field gel electrophoresis analysis of clinical isolates of *E. faecalis* showed that the gene encoding Agg was not present in *E. faecium* isolates (Hällgren *et al.*, 2009)

2.7.2 Adhesion of collagen (Ace)

This is a collagen-binding protein, belonging to the microbial surface components recognizing adhesive matrix molecules (MSCRAMM) family. Ace may play a role in the pathogenesis of endocarditis (Koch *et al.*, 2004). The ace gene is apparently well conserved and specifically present in *E. faecalis* isolates (Duh *et al*., 2001). Ace has significant similarity to the A domain of the collagen-binding protein (Cna) of *S. aureus* and the *E. faecium* adhesin Acm (Nallapareddy *et al*., 2000).

2.7.3 Extracellular surface protein (Esp)

The *esp* gene consists of 5622 bp and is found at high frequency in infection-derived isolates. It is thought to promote adhesion, colonization and evasion of the immune system, and to play some role in antibiotic resistance (Foulquie Moreno *et al*., 2006). Esp also contributes to enterococcal biofilm formation, which could lead to resistance to environmental stresses, and adhesion to eukaryotic cells such as those of the urinary tract (Borgmann *et al.*, 2004). Studies have shown that disruption of the *esp* gene impairs the ability of *E. faecalis* to form biofilms. Esp-negative *E. faecalis* strains, after receiving plasmid transfer of the *esp* gene, were able to produce biofilms (Latasa *et al.*, 2006). Twenty-one out of 28 clinical isolates of *E. faecium* were found to have sequences that were specific for the *esp* gene. This goes some way to suggesting that the *esp* gene may be associated with pathogenicity, since the *esp* gene was absent from dairy isolates (Mannu *et al.*, 2003). *E. faecium* strains that carry the gene esp_{fm} have higher conjugation rates than strains that do not possess this gene. They also demonstrate higher resistance to ampicillin, ciprofloxacin and imipenem (Billström *et*

al., 2008). The Esp has been shown to promote colonization and persistence in bladder (but not kidneys) of mice (Shankar *et al*., 2001).

2.7.4 Pili

The formation of pili by enterococci is necessary for biofilm formation, the gene cluster associated with this being *ebp* (endocarditis- and biofilm-associated pili). The *ebp* operon consists of *ebpA, ebpB ebpC* and an associated *srtC* (encoding sortase C) gene (Singh *et al.*, 2007). A non-piliated mutant of *E. faecalis* was unable to produce a biofilm (Budzik and Schneewind, 2006). Enterococcal pili are heterotrimetric and the pilus shaft contains two minor pilins. A feature of Gram-positive pili is that a specific sortase is dedicated to their assembly (Mandlik *et al.*, 2008). The pili are constructed by cross-linking of multiple classes of precursor proteins that are assigned by sortases, which covalently anchor proteins with a C-terminal pilin-associated motif to the peptidoglycan (Nallapareddy *et al.*, 2006). *E. faecalis* contains two classes of sortase: sortase A links most proteins with a C-terminal sortase motif to cell wall peptidoglycan, while sortase C is designated Bps (biofilm and pilus-associated sortase) and links the pilin subunits. Secreted virulence factors of *Enterococcus* species also have a function in pathogenesis.

2.7.5 Cytolysin

Cytolysin also known as haemolysin is a secreted bacterial toxin whose genes for the production of which are located on pheromone-responsive plasmids (Koch *et al.*, 2004). Cytolysin has *β*-haemolytic properties in humans and is bactericidal against other Gram-positive bacteria. The $cylL_s$ group of genes are the non-regulatory genes of the cytolysin operons (Hällgren *et al.*, 2009), and higher incidences of these genes

occur in clinical isolates (33 %, compared to 6 % in food isolates) (Semedo *et al.*, 2003). Cytolysin is regulated by a quorum-sensing mechanism involving a two-component system. It has α-haemolytic activity against some types of blood, including human, rabbit and horse blood. Diverse animal studies have shown that cytolysin-producing strains are associated with higher lethality rates (or increased organ destruction in the rabbit endophthalmitis model) than nonproducing isogenic strains; however, the exact mechanism by which this enzyme contributes to the pathogenesis of *E. faecalis* is still unknown.

A group of hydrolytic enzymes including hyaluronidases, gelatinase and serine protease are involved in the virulence of *Enterococcus* species, although their precise roles are yet to be clearly understood (Semedo *et al.*, 2003).

2.7.6 Hyaluronidase

This acts on hyaluronic acid and is a degradative enzyme which is associated with tissue damage. Hyaluronidase depolymerizes the mucopolysaccharide moiety of connective tissue, thus facilitating spread of enterococci as well as their toxins through host tissue (Kayaoglu and Orstavik, 2004). Hyaluronidase is encoded by the chromosomal *hyl* gene. One study showed that, out of 26 vancomycin-resistant *E. faecium* clinical isolates, seven (27 %) carried the *hyl* gene, but it was found in only 14 % of faecal isolates (Vankerckhoven *et al.*, 2004).

2.7.7 Gelatinase

The main role of both gelatinase and serine protease in enterococcal pathogenesis is thought to be in providing nutrients to the bacteria by degrading host tissue, although they also have some function in biofilm formation (Gilmore, 2002;

Mohamed and Huang, 2007). Gelatinase (GelE) is an extracellular zinc metallo-endopeptidase secreted by *E. faecalis* (Koch *et al.*, 2004). It is able to hydrolyse gelatin, casein, haemoglobin and other bioactive peptides. The gene (*gelE*) encoding GelE is located on the chromosome and is regulated in a cell-density-dependent manner. Another gene *sprE*, coding for a serine protease, is located directly downstream from and is cotranscribed with *gelE* (De Fátima Silva Lopes *et al.*, 2006). Transcription of *gelE* and *sprE* is regulated in a growth-phase-dependent fashion by the quorum-sensing system encoded by the *fsr* (faecal streptococci regulator) locus (Sifri *et al.*, 2002).

2.7.8 Quorum sensing

This occurs when a bacterial population produces a signal via an autoinducing peptide (AIP), regulated by a two-component system. AIP then accumulates in the environment by increased expression of the communication signal, or by increased numbers of cells producing the signal. Once the AIP reaches a threshold concentration, it interacts with a cell-surface receptor or re-enters the cell and causes a cascade of transcriptional regulation (Alksne and Projan, 2000; Gobbetti *et al.*, 2007). The *fsr* locus contains the *fsrA*, *fsrB* and *fsrC* genes. The *fsrA* gene is monocistronically transcribed into a response regulator, and *fsrB* and *fsrC*, encoding a processing enzyme and a sensor kinase respectively, are co-transcribed (Kreikemeyer*et al.*, 2004). FsrB liberates gelatinase biosynthesis activating pheromone (GBAP) peptide, and with the accumulation of GBAP a transition from exponential to stationary phase occurs and *gelE* and *sprE* are induced. It has been shown that in *E. faecalis* when mutations in *fsrA*, *fsrB* and *fsrC* are present, a reduction in biofilm formation of 28–32 % occurs (Mohamed and Huang, 2007). All of twelve *E. faecalis* endocarditis strains were

positive for the *fsr* locus while 10 out of 19 stool strains had the *fsr* locus, indicating the importance of *fsr* in virulence and disease (Kreikemeyer*et al.,* 2004).

2.7.9 *E. faecalis* antigen A (EFA)

Another factor that seems to be specific to *E. faecalis* isolates is the *E. faecalis* antigen A (EfaA), which encodes a putative ABC transporter that is regulated by manganese and appears to be important for full virulence (Singh *et al*., 1998; Low *et al*., 2003).

2.7.10 Cell-wall polysaccharides

Polysaccharides on the surface of enterococci may represent an effective way to prevent phagocytosis. Some of these appear to be variable capsular carbohydrates, and several have been described in *E. faecalis* and *E. faecium* (Arduino *et al*., 1994; Huebner *et al*., 1999; Rakita *et al*., 2000; Hancock and Gilmore, 2002).

2.7.11 Enterococcal polysaccharide antigen (EPA)

Thisis a common *E. faecalis* cell-wall polysaccharide, and mutants with an insertion in genes of the *epa*cluster showed attenuation in the mouse peritonitis model and reduced biofilm formation and are more susceptible to neutrophil killing (Teng *et al*., 2002).

2.8 Clinical Infections Caused by Enterococci

Historically, *E. faecalis*, *E. faecium* and other species (e.g. *Enterococcus gallinarum*, *Enterococcus avium*, *Enterococcus casseliflavus* and *Enterococcus raffinosus*) accounted for 80–90%, 5–20% and 2–4% of all enterococcal infections,

respectively (Lewis and Zervos, 1990; Patterson *et al*., 1995). However, in the 1990s, a shift to a higher incidence of *E. faecium* infections has been observed in the United States, coincident with increased antibiotic pressure and development of vancomycin resistance by this species.

2.8.1 Urinary tract infections

Since the early 1900s enterococci have been known to cause urinary tract infections (UTIs). Elderly men, the presence of an indwelling bladder catheter, structural abnormalities of the urinary tract and recent urologic instrumentation are recognized risk factors for the isolation of enterococci from UTIs, which has been reported in ~15% of nosocomial cases (Murray, 1990). In contrast, enterococci cause less than 5% of uncomplicated UTIs in young women. Despite the associated low morbidity and mortality, enterococcal UTIs have clinical importance because of additional costs of hospitalization and therapy.

2.8.2 Intra-abdominal and pelvic infections

Enterococci are frequently found in intra-abdominal and pelvic infections, including salpingitis, endometritis and abscesses after cesarean section, but very rarely as the sole agent. The role of enterococci in the early phases of these infections remains controversial, since most of these resolve with antibiotics that do not specifically target enterococci, although occasionally breakthrough enterococcal bacteraemia does occur. Animal experiments show that enterococci act synergistically with other bacteria in polymicrobial intra-abdominal infections (Montravers *et al*., 1997). Enterococci are also a cause of spontaneous bacterial peritonitis in cirrhotic and nephrotic patients and peritonitis in patients on continuous ambulatory peritoneal dialysis.

2.8.3 Endocarditis

Enterococci are the third most common cause of infective endocarditis, following viridans streptococci and *S. aureus*, and account for 5–20% of cases (Megran, 1992). *E. faecalis* is most often the species isolated, but other enterococcal species also cause endocarditis. Enterococcal endocarditis has an acute or, more frequently, subacute onset affects men (especially elderly men) more frequently than women and often has a genitourinary origin, although GI or biliary tract sources are also probable sources. A high percentage of women with enterococcal endocarditis have a recent history of abortion, cesarean section or genitourinary manipulation. Even though subjects with prior valvular heart disease are common in the series of enterococcal endocarditis, enterococci also affect normal valves (mainly aortic and mitral valves) and prosthetic valves, for which enterococci have been implicated in approximately 7% of the cases (Rice *et al*., 1991; Megran, 1992).

Enterococci are also among the organisms to consider in intravenous drug users with endocarditis, although, interestingly, they usually do not affect the tricuspid valve. As with other enterococcal infections, the mortality rate (15–20%) is probably affected by the seriousness of the underlying disease present in most of these patients (Megran, 1992).

2.8.4 Bacteraemia

Enterococcal bacteraemia without endocarditis is a much more common event than endocarditis. When there are no signs of intra-abdominal, genitourinary tract or intravascular catheter-related infections, the source of the bacteraemia is usually presumed to be translocation of enterococci from the GI tract. Enterococcal

bacteraemia is frequently observed in patients with serious underlying disease and in those who have undergone major surgery, have received antibiotic therapy or have urethral or intravascular catheters (Pallares *et al*., 1993). The severity of the underlying disease, the invasiveness of in-hospital procedures (surgeries, intravascular and bladder catheter placements, etc.), the length of intensive care unit (ICU) stay and the suppression of the normal gut flora by antibiotic pressure (especially antibiotics with antianaerobic effect) are factors that have been associated with enterococcal infections, particularly with *E. faecium* strains. When these factors are prolonged, resistant strains acquired from the hospital environment (i.e. ampicillin resistant *E. faecium*, *E. faecalis* with high-level resistance (HLR) to gentamicin, vancomycin-resistant enterococci (VRE) and, possibly, in the future, linezolid- and quinupristin–dalfopristin-resistant *E. faecium* strains) are allowed to persist and proliferate in the GI tract, predisposing to subsequent disseminated enterococcal infection.

It has been speculated that the presence of enterococcal bacteraemia by itself is a marker of a serious underlying condition. Of note, patients with bacteraemia due to *E. faecium* are more often seriously ill or are receiving immunosuppressors or had been previously treated with antibiotics than patients with *E. faecalis* bacteraemia (Noskin *et al.*, 1995; Suppola *et al*., 1998), which may explain, at least in part, the higher mortality associated with *E. faecium* observed in some series (up to 50%) (Noskin *et al.*, 1995). Thus, the difference in mortality rates observed in infections caused by these two species may be related to host factors (and perhaps, resistance) rather than to existing differences in virulence.

2.8.5 Nosocomial infections

In the United States, enterococci have been ranked as the third most common agent recovered from nosocomial bloodstream infections (Edmond *et al*., 1999). The high incidence of enterococcal infections has been attributed to the widespread use of antimicrobial agents to which enterococci are resistant, the high proportion of patients who are immuno-compromised and with invasive devices and the nosocomial spread of resistant strains. Of note, a dramatic increase in the proportion of VRE among nosocomial isolates has been observed over the past 15 years, reaching 26% of the ICU isolates collected in 2000 (NNIS, 2001). Enterococci are important pathogens in nosocomial-acquired UTIs and are the second most common agent isolated, both in Europe (Bouza *et al*., 2001) and in US hospitals (Mathai *et al*.,2001). Enterococci are also increasingly reported as a cause of catheter-related bloodstream infection (Sandoe *et al*., 2002).

2.8.6 Neonatal infections

Enterococci cause neonatal sepsis and/or meningitis, and it was reported as the second most common isolate in a point prevalence study of nosocomial infections in neonatal ICUs in the United States (Sohn *et al*., 2001). Although this may occur in normal-term infants, low-birth-weight infants and premature infants with severe underlying conditions are a high-risk group for enterococcal infections. Use of a central catheter, the time the central line was in place, GI surgery, prior antibiotic use and intubation have been identified as risk factors for neonatal enterococcal disease (Sohn *et al*., 2001). As with adults, VRE may also spread rapidly among hospitalized neonates (Malik *et al*., 1999).

2.8.7 Other less common infections

Besides neonatal meningitis, enterococci have also been cited as a cause of shunt infections and meningitis in older children and adults (Durand *et al*., 1993). In most of these patients, invasive procedures of the central nervous system, underlying diseases, previous antibiotic therapy and even disseminated strongyloidiasis were the predisposing factors for infection. Enterococci have also been found, usually with other microorganisms, in diabetic foot infections, decubitus ulcers, burns and postsurgical abdominal wounds (Lewis and Zervos, 1990).

2.9 Antibiotics Resistance of Enterococcus

Anantibioticis an agent that killsmicroorganismsor inhibits their growth.The term "antibiotic" originally described only those formulations derived from living organisms but is now also applied tosyntheticantimicrobials, such as thesulphonamides. The term also used to be restricted to antibacterials (and is often used as a synonym for them by medical professionals and in medical literature), but its context has broadened to include all antimicrobials. Enterococci show either intrinsic resistance where resistance genes are located on the chromosome, or they possess acquired resistance determinants which are located on plasmids or transposons (Salminen and Wright, 2004). Examples of the intrinsic antibiotic resistance include resistance to beta-lactams, cephalosporins, sulfonamides, and low levels of clindamycin and aminoglycosides (Murray, 1990; Salminen and Wright, 2004).

Resistance to chloramphenicol, erythromycin, high levels of clindamycin and aminoglycosides, tetracycline, high levels of beta-lactams, fluoroquinolones, and glycopeptides such as vancomycin are examples of acquired resistance (Murray, 1990).

The antibiotic resistance of *Enterococcus* is well documented. Bacteria may show resistance to glycopeptides such as vancomycin and teicoplanin, which are licensed in the UK, and to aminoglycosides (Kacmaz and Aksoy, 2005). Antibiotic resistance has been of growing concern for a number of years. Vancomycin was first used in the clinical arena in 1972 and the first vancomycin-resistant enterococci were recognized only 15 years later. NNIS reported an increase of 7.6 % in VRE between 1989 and 1993 (Metan *et al.*, 2005). It has been reported that if glycopeptide-resistant enterococci (GRE) are present in an infected patient rather than an antibiotic-susceptible strain, clinical treatment failure is increased by 20 % and mortality is increased from 27 % to 52 % (Brown *et al.*, 2008). When assessing the studies on enterococcal antibiotic resistance, the pattern that is emerging is the possible occurrence of multidrug resistant strains (Peters *et al.*, 2003).

In both the Surveillance and Control of Pathogens of Epidemiological Importance (SCOPE) and SENTRY (Antimicrobial Resistance Surveillance Program) databases, figures show that, of enterococcal isolates from the bloodstream, 2 % of *E. faecalis* and 60 % of *E. faecium* isolates are resistant to vancomycin (Bearman and Wenzel, 2005). Resistance rates of *Enterococcus* species have reached endemic or epidemic proportions in North America, with Europe having lower, but increasing, levels (Mutnick *et al.*, 2003). Enterococcal antibiotic resistance is not exclusive to the clinical arena but is also prevalent in the food industry. The presence of VRE in individuals who have been hospitalized, when they have not previously been in hospital or taken antibiotics, suggests that VRE may have been contracted through the food chain. GRE may emerge in the food chain through use of avoparcin in animal feed (Mannu *et al.*, 2003).

Glycopeptide resistance in enterococci involves a two-component system where the cell wall composition is altered from the peptidoglycan precursor d-Ala-d-Ala (vancomycin-susceptible) to d-Ala-d-lactate (d-Lac). The latter has 1000 times less affinity for vancomycin, while d-Ala-d-Ser has a sevenfold decrease in affinity for vancomycin, thus removing the susceptible target (Gilmore, 2002). The genes involved in this two-component system are *vanS*/*vanR*. The VanS sensor kinase is activated in response to vancomycin, resulting in the activation of d-Lac or d-Ser peptidoglycan precursor and the repression of d-Ala-d-Ala (Stephenson and Hoch, 2002). To date six gene clusters associated with glycopeptide resistance have been identified in *Enterococcus* species: *vanA* to *vanG*. The three main types of resistance are those encoded by the *vanC*, *vanA* and *vanB* clusters. Intrinsic *vanC* resistance is specific to *E. gallinarum, E. casseliflavus* and *E. flavescens*, and the *vanC* operon is chromosomally located and is not transferable. The *vanA* resistance operon comprises seven genes (*vanH*, *vanA*, *vanX*, *vanR*, *vanS*, *vanY* and *van*Z) and is acquired through the Tn*1546* transposon (Gilmore, 2002). Over 100 enterococcal isolates from humans, animals and food have shown *vanA* resistance residing on Tn*1546* (Williams and Hergenrother, 2008). The transfer of *vanB* (acquired) resistance occurs through the exchange of transposon Tn*1547* and/or Tn*5382*. Both *vanA* and *vanB* are present on the chromosome but can also be carried on a plasmid (Gilmore, 2002; Klare *et al.*, 2003). *Enterococcus* species do not possess cytochrome enzymes and thus cannot produce the energy required to take up antibiotics into the cell. This means they show resistance to aminoglycosides at low levels (Klare *et al.*, 2003). Antibiotic resistance in *Enterococcus* species can be transferred by pheromone-mediated conjugative plasmids or transposons. The resistance genes may be passed on not only to antibiotic-susceptible enterococci, but also to other pathogens (Giraffa, 2002).

In contrast to Gram-negative conjugation systems, conjugation of *Enterococcus* species does not require pili, and involves a pheromone-induced system (Andrup and Andersen, 1999). Bacteria containing conjugative plasmids respond to pheromones (plasmid specific) for genetic exchange; these bacteria generally have a narrow recipient range for conjugation, including only closely related species. This lateral transfer of genetic elements leads to rapid dissemination of antibiotic resistance. The plasmids occurring in *Enterococcus* species can also be transmission vehicles for transposons (Williams and Hergenrother, 2008). The most extensively investigated pheromone-inducible plasmids in *E. faecalis* are pCF10, pAD1 and pPD1. In the case of pAD1 the *trans*-acting regulatory protein encoded by the *traE* gene is expressed (Folli *et al.*, 2008). The transfer of these plasmids occurs in response to specific sex pheromone peptides secreted by plasmid-free recipient cells. Uptake of the exogenous pheromone by the donor cell causes it to express proteins involved in the conjugation process. Production of aggregation substance (Agg) on the donor cell surface facilitates contact with the recipient cell by binding to enterococcal binding substance (EBS) displayed on the surface, resulting in conjugation and the ability to pass antibiotic resistance on to the recipient cell (Clewell *et al.*, 1993). The pAD1 plasmid has also been shown to carry the Tn*917* transposon associated with *E. faecalis*; conjugal transfer of Tn*916* involves excision of a circular intermediate that is transferred via a plasmid into the recipient cell where it inserts into the recipient chromosome (Gilmore, 2002). Pheromones released for plasmids pCF10, pAD1 and pPD1 are pAD1 or cCF10, cAD1 and cPD1 respectively (Folli *et al.*, 2008). In *E. faecalis* the proteins RepA, RepB and RepC and the *par* locus are involved in the regulation of the pheromone-responding pAD1 replicon. The *repA* gene encodes a replication initiator protein, while *repB* and *repC* are involved in control of the replication frequency and stability of the plasmid

(Weaver *et al.*, 2009). *Enterococcus* plasmids can also be utilized for the genetic exchange of virulence factors.

Most importantly and a contributing factor to the pathogenesis of enterococci is their resistance to a wide range of antibiotics (Ogier and Serror, 2008). Enterococci either have intrinsic resistance traits or they possess acquired resistance factors (Salminen *et al*., 2004). Examples of intrinsic antibiotic resistance include resistance to beta-lactams, cephalsporins, sulphonamide and low levels of clindamycin and aminoglycoside (Murray, 1990); Salminen *et al*., 2004). Resistance to chloramphenicol, erythromycin, high levels of clindamycin and aminoglycosides, tetracycline, high levels of beta-lactams, fluoroquinolones, and glycopeptides such as vancomycin, are examples of acquired resistance (Murray, 1990). This suggests that treatment of enterococcal infections can be difficult as they possess intrinsic resistance to many antibiotics. However, treatment of enterococcal diseases has been successful using the combination of cell-wall active antibiotics such as penicillin or ampicillin with aminoglycosides (streptomycin and gentamicin) which act synergistically (Simjee and Gill, 1997). But in the 1970s, a high level of streptomycin and gentamicin resistance was reported. Furthermore, strains resistant to penicillin, streptomycin or penicillin-gentamicin combinations were also found (Moellering, 1992). A strain of *E. faecalis* producing a beta-lactamase identical to that produced by *S. aureus* was described in 1983, and it is believed that this strain of Enterococcus acquired the gene from *S. aureus.* This finding causes a major therapeutic problem with the treatment of enterococcal diseases (Mollering, 1999).

2.9.1 Penicillin

Enterococci are resistant to penicillin due to intrinsic differences between pbp 5 alleles, which is acquired via two mechanisms - the first mechanism is via the production of penicillin-binding proteins (PBP) with decreased affinity for the antibiotic (Rybkine *et al*., 1998). The second mechanism involves hydrolysis of the penicillin molecule by beta-lactamase. Resistance based on PBPs is more common among *E. faecium* strains. Even though beta-lactamase producing *E. faecalis* causes infections, it poses only a minor problem as there are several penicillinase-inhibitor antibiotic combinations available (e.g. Augmentin).

2.9.2 Tetracycline

Tetracycline resistance in enterococci can be conferred by two major groups of tetracycline resistance genes (Huys *et al*., 2004; Wilcks *et al*., 2005). The first group confers resistance by ribosomal protection and include the genes tet(M), tet(O), and tet(S). A second group mediates energy-dependent efflux of TC from cells and is represented in enterococci by the tet(K) and tet(L) genes. The gene, tet(U), encodes low-level resistance in E. faecium via an unknown mechanism (Chopra and Roberts, 2001).

2.9.3 Gentamicin

It is also believed that the gene encoding high level resistance (HLR) to gentamicin in *E. faecalis* is acquired from staphylococci as both HLR genes in *E. faecalis* and staphylococcus contain the same nucleotide sequence. *E. faecium* acquired HLR to gentamicin after its appearance in *E. faecalis* and it was first reported in 1998. Acquired HLR to gentamicin results from transfer of genes encoding aminoglycoside-

modifying enzymes by conjugative plasmids and transposons (Simjee and Gill, 1997). HLR to gentamicin in enterococci is due to the synthesis of one or more of a series of aminoglycoside adenyltransferases(AAD), and aminoglycoside phosphotransferases (APH) (Simjee and Gill, 1997).

2.9.4 Vancomycin

Vancomycin resistance is of particular concern because this antibiotic was considered a remedy for the treatment of multiple-resistant enterococcal infections (Cetinkaya *et al*., 2000). Three vancomycin resistance phenotypes can be distinguished on the basis of the level and inducibility of resistance. Van A type is the most frequent transferable vancomycin-resistant phenotype that is associated with a high level of inducible resistance to vancomycin. The Van B type is acquired inducible resistance to various levels of vancomycin and Van C type contributes to low level vancomycin resistance (Cetinkaya *et al*., 2000).

2.9.5 Linezolid

This is one of the oxazolidinone class of antimicrobial drugs, inhibits bacterial protein synthesis by binding to the 50s subunits of 23S rRNA (Gomez-Gil *et al*., 2009). In 2000, linezolid was approved in the United States and has been heavily marketed to treat methicillin- resistant *S. aureus* (MRSA) and vancomycin-resistant enterococcal (VRE) infections (Souli *et al*., 2009). Linezolid resistance has been reported in vitro and in clinical isolates of *E. faecalis, E. faecium* and *S. aureus* (Allen and Bierman, 2009; Wilson *et al*., 2003). Linezolid resistance in clinical isolates is normally associated with a point mutation in the central region of domain V of the 23S rRNA gene, leading to a nucleotide change from Guanine (G) to Uracil(U) at position 2576 in

the 23S subunit that is the target of the oxazolidinone class of antimicrobial drugs (Souli *et al*., 2009).

A number of studies have attempted to compare the resistance spectra of different enterococci from different origins including food, animal, human and environmental sources by using different antibiotics such as vancomycin (da Costa *et al*., 2007; Zdragas *et al*., 2008), ampicillin (da Coata *et al*., 2007; Patterson and Kelly, 1998), streptomycin (Poeta *et al*., 2005), gentamicin (Mirandaand Zemelman,2002; Poeta *et al*., 2005), chloramphenicol (Franz*et al*., 2001; Poeta *et al*., 2005) tetracycline (Gevers *et al*., 2003; Macovei and Zurek, 2006), erythromycin (Macovei and Zurek, 2006; Patterson and Kelly, 1998), ciprofloxacin (Leavis *et al*, 2006) and linezolid (Gomez_Gil *et al*., 2009; Souli *et al*., 2009). Particular attention should be given to the study of antibiotic resistance, as several recent outbreaks of antibiotic-resistant enterococci emphasize the need for laboratories to be able to detect the various types of resistance. Furthermore, a recent study by (Zdragas *et al*., 2008) found VRE in coastal waters an d highlights the necessity to investigate the occurrence and distribution of antibiotic-resistant enterococci, especially VRE, in natural waters in order to address the public health risk (Zdragas *et al*., 2008).

2.10 Use of Enterococci

Enterococci are also found routinely in sewage, and their presence has been used to monitor fecal contamination. Because of their production of lactic acid, enterococci have been used as starters in the manufacture of cheese, and they have been isolated from cheese products as well as from certain meats and other foods. But some strains of *E. faecium* such as *E. faecium* SF68 has been used to treat diarrhoea as an

alternative to antibiotic treatment (Psoni, *et al*., 2006), furthermore, some entorococci species constitute important components in cheese production and are considered to play an important role in ripening & aroma development (Domig *et al*., 2003; Psoni *et al*., 2006). Some enterococci are also used as probiotics to improve human health in the food industry. Enterococci are usually considered heavy fermentors due to their lack of Krebs cycle and respirator chain (Huycke *et al*., 1998), and because of this enterococci have been used as fermentors in the food industry.

Enterococci are present in many sorts of food, especially food that origin from animals, due to the presence of enterococci in the GI of animals. Historically isolation of *E. faecium* and *E. faecalis* from food has been an indication of faecal contamination in the production line of the food (Klein, 2003). Today enterococci are considered a natural part of the normal microbial flora of food (Klein, 2003) because of their ability to produce bacteriocins, and their organolepticproperties. Enterococci are used in the food industry as e.g. in the cheese industry were they might contribute to the ripening and aroma of the cheese (Foulquié-Moreno *et al*., 2006). The levels of enterococci in cheese curds lies between 10^4 and 10^6 cfu/g, while in the ripened cheese the cfu/g is between 10^5 to 10^7 *E. faecium* and *E. faecalis* are the dominant species (Foulquié-Moreno *et al*., 2006). In some cheeses, like Mozzarella, the enterococci are the predominant microorganism used (Foulquié-Moreno *et al*., 2006).

The levels of enterococci varies from cheese to cheese as a result from the production season, the extent of contamination of the milk and water used, the survival in the dairy environment, and survival and growth under the particular conditions of the cheese production and ripening (Foulquié-Moreno *et al*., 2006). When a bacterial strain is to be used as a probiotic, live microbial feed supplements that benefit and improve

the host intestinal microbial balance, they have to be able to adhere to cells and exclude or reduce the adherence of pathogenic adherence, they have to persist and multiply, and produce acids, hydrogen peroxide and bacteriocins antagonisticto pathogen growth. The strains have to be safe, in the meaning of being non-invasive, non-carcinogenic and non-pathogenic, and it has to contribute to the formation of a balanced normal flora (Salminen *et al*., 1996). Both *E. faecalis* and *E. faecium* have been used as probiotics. *E. faecium* SF68 has been clinically effective in the prevention of antibiotic associated diarrhoea in children, and has made the duration of diarrhoea in adults, it has also been used as feed probiotic, in dry dog food, and in milk in Denmark (Foulquié-Moreno *et al*., 2006), which shows the broad use of probiotics in society.

The uses of enterococci as probiotics are controversial. While some strains benefit the host, the emergence of antibiotic resistant strains, and the increase infections in humans caused by antibiotic resistant enterococci, especially VRE, has lead to concern regarding the use of enterococci as probiotic (Folquié-Moreno *et al*., 2006).

2.11 Water

Water is one of the most abundant and essential resources of man, and occupies about 70% of earth's surface. About 97% of this volume of earth's surface water is contained in the oceans, 21% in polar ice and glaciers, 0.3- 0.8% underground, 0.009% in inland freshwaters such as lakes, while 0.00009% is contained in rivers (Eja, 2002). According to Botkin and Keller (1998), more than 97% of earth's water is in the oceans and ice caps, and glaciers account for another 2%. Also, the ocean comprises 97%, while 3% of the earth's water is fresh (Kulshneshtha, 1998). A water molecule contains an oxygen and two hydrogen atoms that are connected by covalent

bonds. Water is a liquid at standard ambient temperature and pressure, but it often co-exists on Earth with its solid state, ice, and gaseous state, steam (water vapor).

Water in its pure state is acclaimed key to health and the general contention is that water is more basic than all other essential things to life. Man requires a regular and accessible supply of water which forms a major component of the protoplasm and provides an essential requirement for vital physiological and biochemical processes. Man can go without food for twenty eight days, but only three days without water, and two third of a person's water consumption per day is through food while one third is obtained through drinking (Muyi, 2007).

2.11.1 Ground water

This is the water beneath the surface where all the voids in the rocks and soil are filled. It is a source of water for wells, boreholes and springs. Ground water is already used extensively in Nigeria through wells and boreholes. Ground water has long been considered as one of the purest forms of water available in nature and meets the overall demand for rural and semi-rural people (Tyagi *et al*., 2002). This was considered as the major source of water for human activities (consumption inclusive) especially in the rural area (Fasunwon *et al*., 2008).

However, the large scale industrial growth has caused serious concerns regarding the susceptibility of groundwater contamination due to discharge of waste materials. Waste materials near factories are subjected to reaction with percolating rain water and therefore reach the aquifer system and as such degrade the groundwater quality (Tyagi *et al*., 2002). Contaminants also find their way into ground water through activities like seepage of municipal landfills, septic tank effluents etc.

2.11.2 Borehole water

This is the water obtained from borehole drilled into the aquifer or ground water zone, which is usually a fully saturated subterranean zone, some distance below the water table (NWRI, 1997). A borehole is an hydraulic structure which when properly designed and constructed, permits the economic withdrawal of water from an aquifer. It is a narrow well drilled with machine. Unfortunately borehole water like water from other sources is never entirely pure. It varies in purity depending on the geological conditions of the soil through which the ground water flows and some anthropogenic activities. Until very recently, ground water has been thought of as being a standardNevertheless, there are various ways borehole water may suffer pollution e.g. Land disposal of solid wastes, sewage disposal on land, agricultural activities, urban runoff and polluted surface water (Birmingham *et al*., 1997). The WHO recommends that boreholes should be located at least 30m away from latrines and 17m from septic tanks (Bonjoch *et al*., 2004) of water purity in itself, and to a certain extent, that is indeed true (Miller, 1992) needs. Households therefore seek other alternative sources which in most cases are unsafe.

2.11.3 Spring Water

A spring is a component of the hydrosphere. Specifically, it is any natural situation where water flows to the surface of the earth from underground. Thus, a spring is a site where the aquifer surface meets the ground surface. A spring may be the result of karst topography where surface water has infiltrated the Earth's surface (recharge area), becoming part of the area groundwater. The groundwater then travels through a network of cracks and fissure-openings ranging from intergranular spaces to

large caves. The water eventually emerges from below the surface, in the form of akarst spring.Spring water is groundwater that rises to the ground surface; they are often used as sources for bottled waters

2.11.4 Tap water

This is also referred to as running water, city water, municipal water etc. It is water supplied to a tap (valve) inside the household or workplace, replacing the manual carrying of water from sources outside the building. Its uses include drinking, washing, cooking, and the flushing of toilets. For these water sources to be consumed safely they must receive adequate treatment and meet drinking water regulations (Hall and Dietrich, 2000) Calling a water supply "tap water" distinguishes it from the other main types of fresh water which may be available; these include water from rainwater-collecting cisterns, water from village pumps or town pumps, or water carried from streams, rivers, or lakes (whose potability may vary).In general, only piped water is usually regarded as safe (Sullivan et al., 2003). When this factor is considered only 30% of the world population has access to safe source of water. Ironically, in Nigeria, only 6% of the population has access to such source (WHO and UNICEF, 2010). In general, most communities in the country are presently not serviced or at best under-serviced by water utilities due to the inability of the designated WSA to meet their.

2.11.5 Water vending

This is usually formal or informal. It is formal when it is undertaken by formal bodies, such as water utilities themselves or registered associations, or by small scale informal supplies. Formal vendors generally supply water in tankers and the water is obtained either from treated utility supplies or from registered sources. On the other

hand, informal vendors obtain water from many different sources, protected and unprotected and deliver small quantities of water for domestic use in a variety of ways ranging from carts and cycles to containers or wheel barrows, trolleys and animal-drawn or mechanized carts and tanker trucks.

In general, vended water is common in many parts of the world where scarcity of supplies or lack of infrastructure limits access to suitable quantities of drinking-water (WHO and UNICEF, 2000). However, the greatest challenge to water service delivery through this mode is the quantity and quality of supplies. Vended water has been associated with outbreaks of diarrhoea disease as some of the vended water is obtained directly from unprotected source such as unprotected wells and surface water including rivers/streams, ponds and canals etc. (WHO, 2006).

2.12 Water Quality Standards

The incidents of water borne disease and epidemics nationwide arising from drinking water of doubtful quality have become of great concern. The primary purpose of the guideline for drinking water quality is the protection of public health (WHO, 2006). As described by Horsefall and Spiff (1998), water quality standard is a measure, principle or rule established by authority set to protect the water resource for uses such as drinking water supply, recreational uses and aesthetics, agriculture (irrigation and livestock watering), protection of aquatic life and industrial water supplies.

In order to maintain water quality, guidelines for drinking water was set up by the World Health Organisation. A guideline value represents the level (a concentration or number) of a constituent that ensures aesthetically pleasing water and does not result in any significant risk to the health of the consumer (WHO, 2010).

2.12.1 Quality of drinking water

In general, certain requirements must be met for water to be fit for human consumption. If these requirements are met, the water can be described as 'wholesome', or simply 'potable water supply'. According to Eja (2002), the requirements are:

i. Freedom from organisms and chemical substances which might be injurious to health. This is the most important requirement.

ii. Drinking water should be of such composition that consumers do not question the safety of the water. This requirement implies that turbidity, colour, taste and odour should be low. Macro organisms (e.g. warms, aquatic and fly nymphs) should be absent.

iii. Drinking water should be suitable for housekeeping and for this reason, iron and manganese content should be low, because these substances colour laundry (like shirt) during washing. Iron causes a brown colour, while manganese causes a black colour. Hardness should be low, because water with a high hardness causes scale formation in water heaters by precipitation of calcium carbonates. Moreover, a high hardness implies that a high dosage of detergents is required for washing.

iv. Drinking water should not be aggressive to materials such as lead, copper, asbestos, cement and concrete, cast iron, galvanized steel, PVC (Polyvinylchloride) and PE (Polyethylene). This is because pipes, tubes and apparatus used in distribution systems and plumbing installations may consist of

these materials. Pipes, tubes and apparatus affected by water cause many problems.

2.13 Enterococcus and Water Quality

Water for human consumption must be free from all objectionable odour, turbidity, taste, enteric pathogenic bacteria or their indicators and must not fluctuate in its quality (Dawson and Sartory, 2000). Okafor (1985) reported that the sanitary quality of water is the relative extent of the absence of suspended matter, colour, taste, unwanted dissolved chemicals, bacteria indicative of faecal pollution and other aesthetically offensive objects. In safeguarding public water supplies therefore, public health authorities and engineers rely on information obtained from the results of frequent bacteriological test. Itah *et al.,* (1996) reported that in bacteriological water analysis, the recovery of conventional indicator bacteria such as the coliforms, *Escherichia coli* (faecal coliform), faecal enterococci and anaerobic endospore forming *Clostridium perfringens* (Welchii) provides a reliable means of assessing the extent of pollution as their presence is indicative of a possible presence of enteric pathogenic bacteria in such water sources.

Microbiological quality and safety of water is imperative and is often evaluated in order to address public health risks (Layton *et al*., 2010). Water contaminated with human faecal matter is generally regarded as a significant risk to human health, since it has a high chance of also containing human-specific enteric pathogens, including *Salmonella* spp. Hepatitis A virus and/or Norwalk group viruses. Animals and birds can also serve as reservoirs for a variety of enteric pathogens (Scott *et al*., 2002) that can be

transferred to natural water bodies by different means such as storm water runoff (Jeng *et al*., 2005).

Understanding the origin of faecal contamination plays an important role in assessing associated health risks as well as for determining actions necessary to remedy the problem. Microbial indicators are used to predict the presence of potential risk associated with pathogenic microorganisms (Stoeckel and Harwood, 2007). Indicator organisms are useful as they circumvent the need to detect every pathogen that may be present in water. Indicators should be readily detected, easily enumerated, have survival characteristics that are similar to those of the pathogens of concern and are strongly associated with presence of pathogenic microorganisms (Scott *et al*., 2002). Total and faecal coliforms have been used widely for many years as indicators for determining the sanitary quality of water. In recent years, scientists have studied more ways that coliform ecology, occurrence and resistance to stress differs from that of many of the pathogenic microorganisms for which they are a proxy (Scott *et al*., 2002). Therefore, alternative indicators to coliforms have been proposed including E. coli, Enterococcus sp and *Clostridium perfringens* (Stevens *et al*., 2003). However, several studies of both recreational and drinking water samples have suggested that enterococci are more relevant indicators of faecal contamination than faecal coliforms and *E. coli* (Grammenou *et al*., 2006; Kinzelman *et al*., 2003). The enterococci tend to persist longer in the environment than coliform and are approximately an order of magnitude less numerous than faecalis coliforms and *E. coli* in human faeces, but are still sufficiently numerous to be detected after significant dilution (Stevens *et al*., 2003). Previous epidemiological studies have demonstrated a correlation between the concentration of enterococci in surface waters and an increase in swimmer-associated

gastroenteritis (He and Jiang, 2005). Certain members of the genus, particularly *E. faecalis* (Farrell *et al*., 2003) and *E. faecium*, are becoming increasingly important as opportunistic pathogens (Low *et al.*, 2001). Environmental water quality studies may benefit from focusing therefore, on a subset of *Enterococcus* spp that are consistently associated with sources of faecal pollution including domestic sewage, that poses significant human health risks.

Among the genus *Enterococcus*, *Enterococcus faecalis* and *Enterococcus faecium* are the species most commonly found to be associated with human nosocomial infections (Witte *et al*., 1999), with *E. faecalis* accounting for approximately 75% of all enterococcal infections, and *E. faecium* accounting for the majority of the remainder (Huycke*et al*., 1998).Other enterococcal species known to cause human infections include *Enterococcus avium, Enterococcus gallinarum, Enterococcus casseliflavus, Enterococcus durans, Enterococcus raffinosus and Enterococcus mundtii* (De Perio *et al*., 2006).

Virulence traits in enterococci include adherence to host tissue, invasion and abscess formation, resistance to and modulation of host defense mechanisms, secretion of cytolysins and other toxic products and production of plasmid-encoded pheromones (Franz *et al.*,1999; Eaton and Gasson, 2001). A number of genes encoding for virulence factors (especially in *E. faecalis*) have been sequenced and characterized and their effects have been shown in human and animal studies (Franz *et al*., 2001). Recent molecular screenings of *Enterococcus* virulence determinants indicated that medical *E. faecalis* strains had more virulence determinants than did food strains, which inturn had more than starter strains. Multiple determinants, e.g those involved in adherence, cytolysin and pheromone production mechanisms, were harboured mostly by *E.*

faecalis and to a lesser extent, by *E. faecium* (Eaton and Gasson, 2001; Franz *et al.*, 2001). Many of these enterococcal virulence traits, such as cytolysin production, the adhesion ability and antibiotic resistance, have been shown to be transmissible by gene transfer mechanisms (Chow *et al.*, 1993; Wirth, 1994).

Enterococci are *non-pathogenic* organisms causing no harm in specific conditions, but can become *opportunistic* pathogens when the immune system of an individual is suppressed. Production of pathological changes directly through production of toxins or indirectly through induction of inflammation, causing infections such as urinary tract infections, bacteraemia, colonize open wounds and cause skin ulcer, endocarditis and meningitis. There are well-defined patient populations (e.g. liver-transplant patients (Papanicolaou *et al.*, 1996), neonates (Christie *et al.*, 1994), and patients with haematological malignances (Chadwick *et al.*, 1996) who would clearly benefit from improved treatment options for enterococcal infections. Enterococci now rank among the top three nosocomial bacterial pathogens (Richards *et al.*, 2000; Wisplinghoff *et al.*, 2004), and strains resistant to currently available antibiotics pose real therapeutic difficulties (Hunt, 1998).

2.14 Molecular Characterization of Enterococci– Polymerase Chain Reaction

Molecular microbiological techniques may improve the sensitivity of microbial detection compared to culturingand therefore enable the identification of enterococci with greater precision. Most Enterococcal strains produce a cell wall associated glycerol teichoic acid that is identified as Lancefield's serological group D antigen. The G+C content of the DNA ranges from 32 to 44mol%. The genome size is in the range of approximately 2000 to 3500kb (Mc Shan and Shankar, 2002; Singh andMurray, 1994).

PCR amplification of DNA has become a key protocol in many biological laboratories that allows repeated synthesis of a specific DNA sequence. A typical PCR cycle involves denaturing the double stranded DNA into single strands, annealing of two short oligonucleotide primers to two opposite single strands and extending the primer sequences using a DNA polymerase enzyme to complete the synthesis of strands complimentary to the original single strands. This cycling process is repeated to obtain an exponential increase in the copies of the original DNA strand. The oligonucleotide primers are often unique, consisting of 18-25 bases, carefully chosen to flank and allow amplification of the target DNA. Each step in the PCR cycle requires specific temperatures. Template denaturation occurs at a temperature greater than the melting temperature of the DNA (94^0C). Primer annealing occurs at a lower temperature which is normally 50-70 ^{0}C. Primer extension typically occurs at 72^0C (Pepper and Gerba, 2004).

PCR has been used frequently to confirm the presence of different enterococcal species in environmental (Zdragas *et al*., 2008), clinical (Dutka-Malen *et al*., 1995) and food samples (Macovei and Zurek, 2006) using species specific primers. Furthermore, this technique has been widely applied to screen for antibiotic resistance in enterococci for example resistance to ampicillin (Rybkine *et al*., 1998), tetracycline (Gevers *et al*., 2003; Poeta *et al*., 2005), erythromycin (Patterson and Kelly 1998; Poeta *et al*., 2005), gentamycin (Poeta *et al*., 2005) or vancomycin (Poeta *et al*., 2005; Zdragas *et al*., 2008). In addition, presence of virulence factors can also be evaluated using PCR (Eaton and Gasson, 2001; Macovei and Zurek, 2006). PCR-based methods are capable of scanning part or all of the entire microbial genome and are widely used for research purposes to obtain more accurate and specific information to characterise enterococci.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

This study was conducted in Zaria, Kaduna state. Zaria township is made up of the following wards: Zaria city, Tudun Wada, Kongo/Gyallesu, TudunJukun, TukurTukur, Wusasa, Sabon Gari, Muchiya/Chikaji, GRA, Kwangila Hanwa, Palladan, Samaru, and Zango. Zaria-Nigeria is located at 11.1113 (latitude in decimal degrees), 7.7227 (longitude in decimal degrees) at an elevation/altitude of meters. The average elevation of Zaria, Nigeria is 624 meters and has a population of 975153 according to GeoNames geographical database. (www.mongabay.com).Water samples were collected from Sabon Gari, Samaru and Zaria City, the sources included: Wells, Boreholes, Tap water, and Vended water (mairuwa).

3.2 Sample size

The sample size was determined using a reported 14% prevalence rate of *Enterococcus s*pp contamination of water samples in Chile (Juan *et al*., 2005), the sample size was determined using the formula described by (Naing *et al*., 2006).

$$n = \frac{Z^2 p (1-p)}{d^2}$$

n = Number of sample

Z = standard normal distribution at 95% confidence limit = 1.96

p = Prevalence rate of contamination of previous study = 14% = 0.14

d = Absolute desired precision of 5% = 0.05

Therefore

$$n = \frac{1.96^2 \times 0.14 \times (1-0.14)}{0.05^2}$$

$n = 185$

Therefore a total number of 240 water samples were collected and subjected to *Enterococcus* isolation. A total of 80 samples was collected from each point (Sabon gari, Samaru and Zaria city) and were screened for the presence *Enterococcus* spp.

3.3 Sample Collection

Samples were collected from various sources such as wells, boreholes, vendedwater (mairuwa) and tap water. One litre of each sample was collected in sterile bottles and was transported in a cooler on ice packs to the laboratory where they were prepared for analyses immediately upon arrival and screened within 6h of collection for presence of *Enterococci.*

3.4 Preparation of Media

The media used in this study were: Bile Esculin Agar, Nutrient Agar, Methyl Red-Voges Proskauer medium, Blood Agar Base, and Mueller-Hinton Agar. They were all prepared according to manufacturers' instructions and the prepared media were then stored at 8-15°C for use during cultivation.

3.4.1 Bile esculin AGAR

Forty four point five grams(44.5g) of the medium was weighed and suspended in 100ml of distilled water, mixed well and dissolved by heating with frequent agitation. Sterilization was done in an autoclave at 121^0C, pressure of 15psi for 15 minutes. The medium was then cooled to 45-50^0C, mixed well and 15-20ml was dispensed into each sterile Petri-dish.

3.4.2 Mueller Hinton agar

Thirty eight grams(38g) of the medium was weighed and suspended in 100ml of distilled water, mixed well and dissolved by heating with frequent agitation. Sterilization was done in an autoclave at 121^0C, pressure of 15psi for 15 minutes. The medium was then cooled to 45-50^0C, mixed well and 15-20ml dispensed into the sterile Petri-dishes.

3.4.3 Methyl Red-VogesProskauer medium

Seventeen grams (17g) of the medium was weighed and suspended in 100ml of distilled water, mixed well and dissolved by heating with frequent agitation. The medium was dispensed into tubes and sterilized in an autoclave at 121^0C for 15 minutes. Few colonies of the isolates were inoculated into Methyl red Voges-Proskauer broth (MR-VP) and incubated for 12-18hours until a good growth was observed. 0.6ml of α-naphthol solution and 0.2ml of 40% potassium hydroxide were added to 2.5ml of the culture broth and were shaken well. Formation of a pink-red product within 5-15min of dilution was recorded positive.

3.4.4 Blood agar base

Approximately 40grams of the medium was weighed and suspended in 100ml of distilled water, mixed well and dissolved by heating with frequent agitation. Sterilization was done in an autoclave at 121^0C for 15 minutes. The medium was then cooled to 45-50^0C and 5-7mls of sheep blood was added aseptically to the medium. It was well mixed and dispensed into petri-dishes.

3.5 Isolation of *Enterococcus* spp

The Membrane-filtration (MF) technique described by Itah *et al.*, (1996) was employed in this work. Briefly, 100ml of each aseptically collected water sample was millipore-filtered using 0.45µm pore size membrane filter (Millipore Corporation, England). After filtration, bacteria were retained on the membrane filter. The whole membrane filter for each sample was aseptically transferred to Bile Esculin agar, a highly selective medium for *Enterococcus* using sterile forceps. The inoculated agar plates were incubated invertedly at 37^0C for 18-24hours. The plates were examined after 24 hours and colonies with grayish colonies with black centres, yellow colonies, and brownish colonies were considered tentatively as *Enterococcus sp*. isolates pending the biochemical characterization.

Colonies presumed to be *Enterococcus* were picked and sub-cultured to get pure cultures and then streaked on nutrient agar slants, incubated for 24hours and stored at 12^0C until required.

3.6Identification of presumptive *Enterococcus* isolates.

The presumptive *Enterococcus* isolates stored on nutrient agar slants were subjected to Gram staining and microscopic examination for motility. The presumptive *Enterococcus* isolates were identified biochemically using catalase test, oxidase test, survival in 6.5%Nacl,Esculin hydrolysis test and bile solubility, which wasfurther confirmed using the Microgen kit(Uk).

3.7Gram Staining

This technique was used to stain a slide in which a smear was made, to observe the bacterial micro-flora present based on their gram stain reaction. The slide was

"Heat-fixed" with the specimen by passing it over a Bunsen burnerflame, several times. The fixed smear was flooded with crystal violet solution and allowed to remain for 1minute. This was rinsed off with flooded with crystal violet solution and allowed to remain for 1minute. This was rinsed off with distilled water. The slide was flooded with iodine solution and allowed to remain for one minute. The slide was rinsed off with distilled water. The slide was flooded with decolorizer- ethanol for one to five seconds. This was again rinsed off with distilled water. The slide was flooded with safranin and allowed to remain for 30 seconds. This was rinsed off with distilled water. The slide was then dried in an upright position. The slide was microscopically examined under a 100X objective. Deep violet to blue colouration was recorded as Gram positive isolates.

3.8Bile Esculin (BE) test

Few colonies of the *Enterococcus* spp isolates were inoculated using the streak method on Bile Esculin Medium and this was incubated at 37^0C for 24 hours. The light-dark brown colonies with a brown–black surrounding (hydrolysis of esculin) were recorded as positive.

3.9Growth in 6.5% Sodium Chloride broth

Few colonies of the isolates were picked using a sterile wire loop and inoculated into the 6.5% sodium chloride broth and incubated at 37^0C for seven days. Increase in turbidity in the broth was recorded as positive.

3.10Voges-Proskauer (VP) Test

Two to three colonies of the isolates were inoculated into Methyl red-Voges-Proskauer broth (MR-VP) and incubated for 24hours. 0.6ml of α-naphthol solution and

0.2ml of 40% potassium hydroxide were added to 2.5ml of the culture broth and were shaken well. Formation of a pink-red product within 5-15min of dilution was recorded as positive.

3.11Haemolysis Test

A colony was picked from a 24hr culture and streaked on blood agar. The plates were incubated at 37^0C for 24 hours. Colonies with α-haemolysis, β-haemolysis (colonies surrounded by a clear zone), γ-haemolysis (colonies that have no clear zone) were recorded.

3.12Biochemical Characterisation using Microgen™ Kits

The isolates were further confirmed using Microgen™ Kits which were as follows

Enterococcus spp. (Microgen® STREP-ID)

3.12.1 Inoculation and incubation

A single colony was placed on a blood plate for α or β haemolysis and the observed reactions were recorded on the report form provided. Single colonies from a 18-24 hour culture were emulsified in the suspending medium (MID-62b) supplied in the kit to produce a suspension equivalent to 2.0 MacFarland standard and was mixed thoroughly. Three drops of Hippurate solution (MID-62d) were added to empty tubes. One or two colonies were emulsified in the Hippurate solution. The tubes were capped and incubated at 35-37^0C for 18-24 hours. The adhesive tape covering the microwell test strip was carefully peeled back. Three or four drops (approximately 100µL) of the bacterial suspension was added to each of the micro-well test strips(s). After inoculation, well 12 was overlaid with three or four drops of mineral oil. The top of the

microwell test strip(s) was sealed with the adhesive tape removed earlier and was incubated at 35-37^{0}C.

3.12.2 Reading of results and addition of reagents

The adhesive tape was removed and all positive reactions were recorded with the aid of the colour chart on the forms provided. The appropriate reagents were added to the wells as follows: One drop of VP I reagent and one drop of VP II were added to well 8 and read after 15-30 mintues. The formation of the deep red or pink colour indicated a positive colour and a clear background indicated a negative VP reaction. One of pyrrolidonyl arylamidase (PYR) reagent was added to well 11 and was read after 5-10 minutes. Formation of pale pink to a very deep red colour indicated a positive result. Three drops of Ninhydrin reagent was carefully added to the Hippurate test. The reagent was not mixed into the test tubes and the reagent overlaid the inoculum. The tubes were incubated at room temperature (37^{0}C) for 10-15 minutes. The development of a purple colour in the upper reagent indicated a positive Hippurate reaction while a clear colour indicated a negative reaction.

3.12.3 Identification

On the Report Forms, the substrates have been organized into the triplets (sets of 3 reactions) with each substrate assigned a numerical value (1, 2 or 4). The sum of the positive reactions for each triplet forms single digit of the octal code that is used to determine the identity of the isolate. The Octal Code was entered into the Microgen Identification System Software (MID-60) which generated a report of the most likely organism in the selected database.

3.13 Antibiotic Susceptibility Test

The antibiotic susceptibility testing of the enterococcus strains was carried out using the disc diffusion technique to determine the minimum inhibitory concentrations (MICs) of the isolates. The purified *Enterococcus* isolates were grown in nutrient broth overnight and standardized by comparing with 0.5 MacFarland standard suspensions. Sterile cotton swab was used in transferring the culture to freshly prepared pre-dried Mueller Hinton agar plates. A forcep was used to transfer each antibiotic sensitivity disc on the plate and incubated for 24hours at 37^{o}C (Oladipo and Adejumobi, 2010). The antibiotics discs and their concentrations that were used include: ampicillin (10µg), ciprofloxacin (5µg), gentamycin (30µg), chloramphenicol(30µg), tetracycline (30µg), and vancomycin (30µg),erythromycin (15µg).*Enterococcus faecalis* from National Collection of Industrial Bacteria, Scotland (NCIB 775) obtained from the Department of Microbiology, Obafemi Awolowo University, Ile-Ife, Nigeria was used as a pathogenic strain.

3.13.1Measuring the diameter of the inhibition zone

After incubation, the test plates were examined for confluent growth and zone of inhibition. The diameter of each zone of inhibition was measured in millimetre (mm) using a ruler on the underside of the plate. The interpretation of the measurement as sensitivity, intermediate and resistant was made according to Clinical Laboratory Standards Institute (CLSI, 2013) manual. The intermediate readings were considered as sensitive for the assessment of the data (Akpan *et al*., 2011) and recorded as sensitive or resistant depending on their respective MIC (minimum inhibition concentration) breakpoint. Zones of inhibition of ≥18 mm were considered sensitive, 13 to 17mm

intermediate or marginally sensitive and < 13 mm resistant (Coyle, 2005; Cheesbrough, 2006; Tripathi *et al*., 2011).

3.14 Determination of Antibiotic Resistance of the Isolates

The multiple antibiotic resistance was determined using MAR index for each isolate by dividing the number of antibiotics to which the isolate was resistant by the total number of antibiotics tested (Olayinka *et al*., 2004).

$$\text{MAR index} = \frac{\text{Number of antibiotics to which isolate was resistant}}{\text{Total number of antibiotics tested.}}$$

3.15Statistical Analysis

Statistical analysis was carried out by calculating in percentages, means and SPSS version 19.0 from SPSS Inc., USA was used to calculate Analyses of variance.

3.16 Molecular Characterization of Enterococcal Isolates

This was done to confirm if the isolates carried genes that confers resistance to antibiotics or virulence gene. This required using primers specific for such genes. Amplification at the expected size confirms presence of the gene.

3.17 Primers

Primer sequences specific for the various genes to be targeted were gotten from literature and synthesised; this included the virulent gene- cytolysin (*cylA*) and antibiotic resistance gene i.e. vancomycin resistance (*vanA* and *vanB*).

Table 3.1: Primer sequences for *Enterococcus* spp

Gene	Sequence	Size(bp)	Accession No
vanA	*vanA*1 5'-GCT ATT CAG CTG TAC TC-3'	783	X56895
	*vanA*2 5'-CAG CGG CCA TCA TAC GG-3'		
vanB	*vanB1* 5'-CAT CGC CGT CCC CGA ATT TCA AA-3'	297	U00456
	*vanB*2 5'-GAT GCG GAA GAT ACC GTG GCT-3'		
cylA	*cylA*-F 5'-ATGGATGGGACAGATGGAAA-3'	519	Pangallo *et al.*, 2004
	cylA-R 5'-AGCTGCGCTTACTTCTGGAG-3'		

3.18 DNA Extraction

The bacteria were grown on Luria Betani (LB) medium to obtain an 18-24 broth culture. The bacterial DNA was extracted using a commercially obtained Extraction kit (Bioneer) in accordance with the manufacturer's instructions. The procedure involved transferring 200µl of the broth culture into a clean sterile micro-centrifuge tube; followed by the addition of 200µl of binding buffer and 20µl of proteinase K. This was incubated for 10minutes at 55^{o} C-60°C after which the lysis buffer was added and allowed to stand for about 45secs at room temperature. The mixture was then transferred into the spin column and centrifuged at 12,000rpm for one minute. This was followed by the addition of 500µl of wash buffer 1 and further centrifugation at 12,000rpm for one minute. After centrifugation, the flow through was discarded and the spin column was transferred into a new column and 500µl of wash buffer 2 was added, followed by centrifugation at 10,000rpm for another one minute. The spin column was transferred into a sterile micro-centrifuge tube and the DNA eluted by addition of 100µl of the elution buffer, this was spinned at 12,000rpm for one minute. The concentration of the DNA was determined by quantifying using Nano drop equipment and this was stored at 4^{o}C until required for further assay.

3.19 Determination of DNA Concentration

The Nano Drop Spectrophotometer was used to determine the concentration of the DNA and the upper and lower optical surfaces of the Nano Dropwas cleaned. 1 to 2µl of clean deionized water was pipetted on the lower optical surface. The lever arm was closed and tapped a few times to bathe the upper optical surface. The lever was lifted and both surfaces wiped off with Kim wipe. The Nano Drop software on the computerwas opened,and nucleic acid module was selected,

the spectrophotometer was initialized by placing 1µl of purified wateron the lower surface, then lowering the upper arm and then selecting initialize in the Nano Drop software. When this was completed in ~10secs, both surfaces were cleaned with Kim wipe. A blank measurement was performed by loading 1µl of deionized water and blank was selected, when it was complete in ~20secs, both surfaces were wiped with Kim wipe. Measurement of nucleic acid was done by loading 1µl of the DNA sample and selecting measure, this was completed in ~20secs. When it was done, both surfaces were wiped clean by adding 2µl of water and drying it. The result observed was recorded and the system shut down.

3.20 Primer Reconstitution

The primer which is usually in lyophilized form was reconstituted to get the master stock 100µM. This was gotten by adding nuclease free water, the amount of water (µl) added was calculated by multiplying the amount of lyophilized powder (nmol) by 10 to get 100µM primer stock. The concentration for working stock was 10µM that was 10times solution.

3.21 Polymerase Chain Reaction

The PCR kit used was Bioneer PCR kit and was carried out according to the manufacturer's instruction. The PCR tube already contained lypholized PCR master mix, to which 1µl of the constituted forward primer, 1µl of backward, 1µl of the template and 17µl of nuclease free water was added. The PCR conditions were as follows-

Pre-denaturation- 95^{o}C for 5minutes
Denaturation - 95^{o}C for 30seconds

Annealing - varying temperatures for 30seconds, depending on the primer

Extension - 72^{o}C for 30seconds

This was carried out at various cycles ranging from 30 – 45 cycles; also optimization was done to get the best band of PCR amplification product.

3.22Gel Electrophoresis

The gel was prepared by dissolving 1.2g of agarose powder in 100ml one time Tris Acetate Buffer, boiled and allowed to cool, after which 5µl of ethidium bromide was added and inturn poured into the electrophoretic tank to cast the gel. For analysis, 5 µl aliquot of the amplicon was transferred into a PCR tube, one time loading dye was added and and 5µl loaded inside each well of the agarose gel using the horizontal electrophoresis apparatus (Pharmacia, Stockholm, Sweden). This was allowed to run alongside a 100kb ladder, for about 30 - 40 minutes after which it was photographed on a UV transilluminator and documented in the Gel documentation system.

CHAPTER FOUR

4.0 RESULTS

A total of two hundred and forty water samples collected from different sources and location in Zaria were analysed. In all, four *Enterococcus* spp were identified, which included *E. gallinarum, E. mundtii, E. avium* and *E. cecorum*, among which *E. avium* had the highestoccurrence (n=20, 58.8%), this was closely followed by *E. gallinarum* with (n=8, 23.5%), *E. mundtii* was next with (n=4, 11.7%), and *E. cecorum* had the leastoccurrence (n=2, 5.88%) as shown in Fig 4.1.

Fig 4.2 shows the mean frequency distribution of Enterococci, it was observed that Zaria city had the highest occurrence of 15 while Sabon gari had the least of 7 isolates, Samaru was intermediate with 12. This gave a total of 34 *Enterococcisp* isolated in all from the 240 water samples collected giving a prevalence of 14.2%. Sabon gari was significantly different with (1.86) compared to Zaria city (4.33) and Samaru (4.17), but no significant difference was seen between Zaria city and Samaru.
On the other hand, the mean frequency of Enterococci amongst the various sources of water samples collected was calculated and presented in Fig 4.3. It was observed that well water had the highest mean of 4.33, which was significantly different ($P<0.05$) from water samples collected from vended water (mairuwa), Borehole and Tap with mean frequencies of 2.33, 3.0 and 1.67 respectively.There was no significant difference between borehole water and tap water.

Table 4.1 shows the biochemical characterization and identification of *Enterococcus* spp isolated from water samples from different locations in Zaria using the Microgen Streptococcus-ID system. All the isolates were able to hydrolyse Esculin and ferment sugars such as melibiose, lactose, ribose and β-galactosidase.

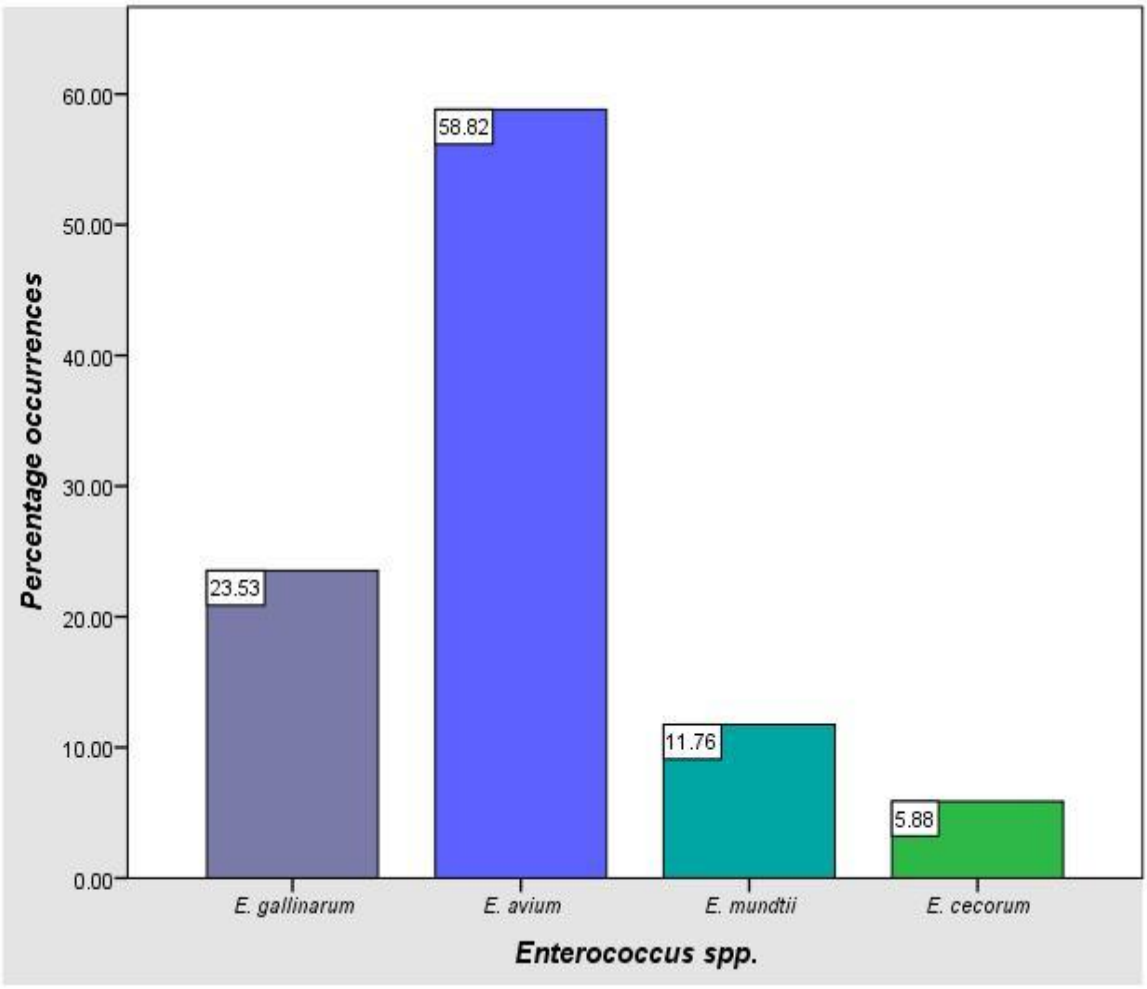

Fig 4.1: Percentage of all *Enterococcus sp* isolated from the water samples.

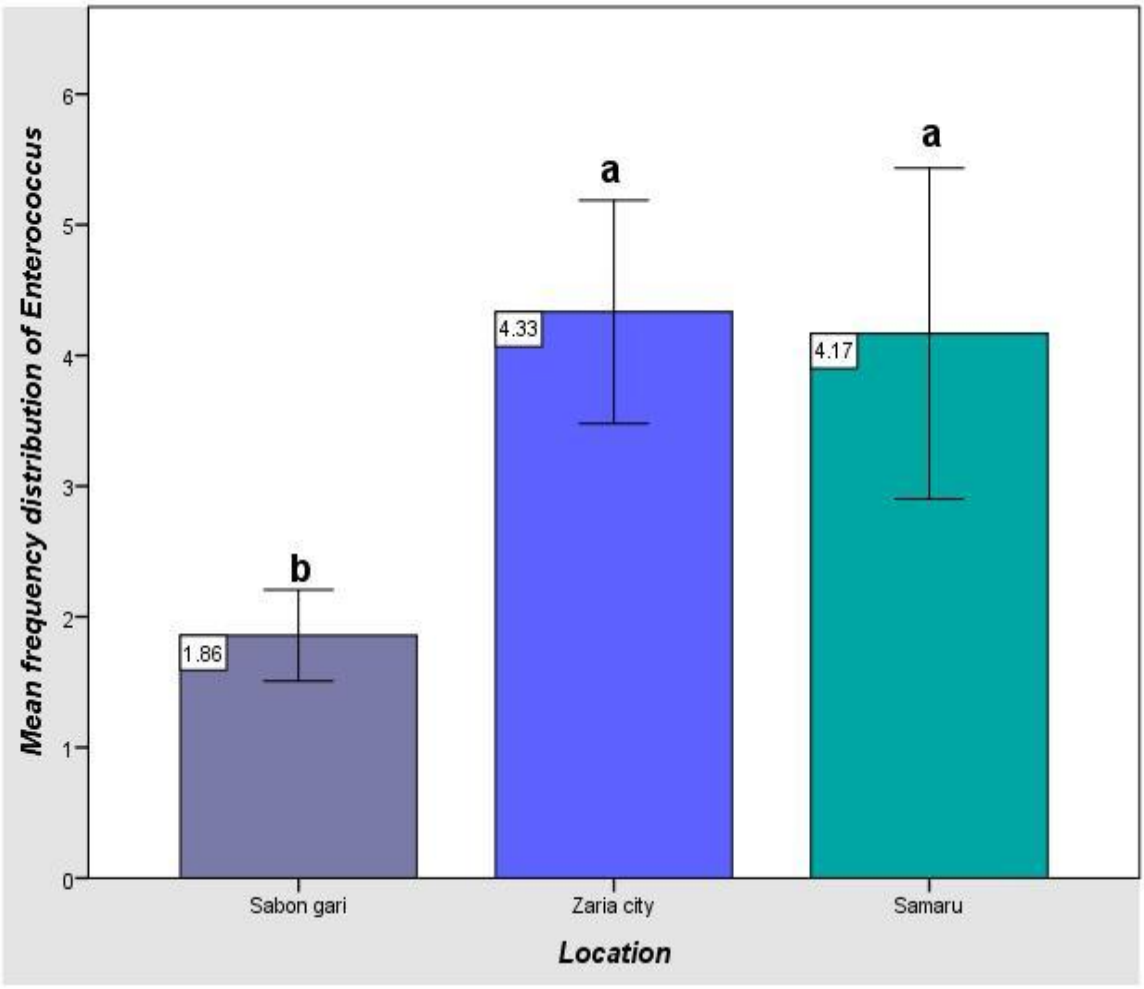

Fig 4.2: Mean frequency of Enterococci in the different locations sampled

*Bars with different top-placed alphabets are statistically different (P<0.05)

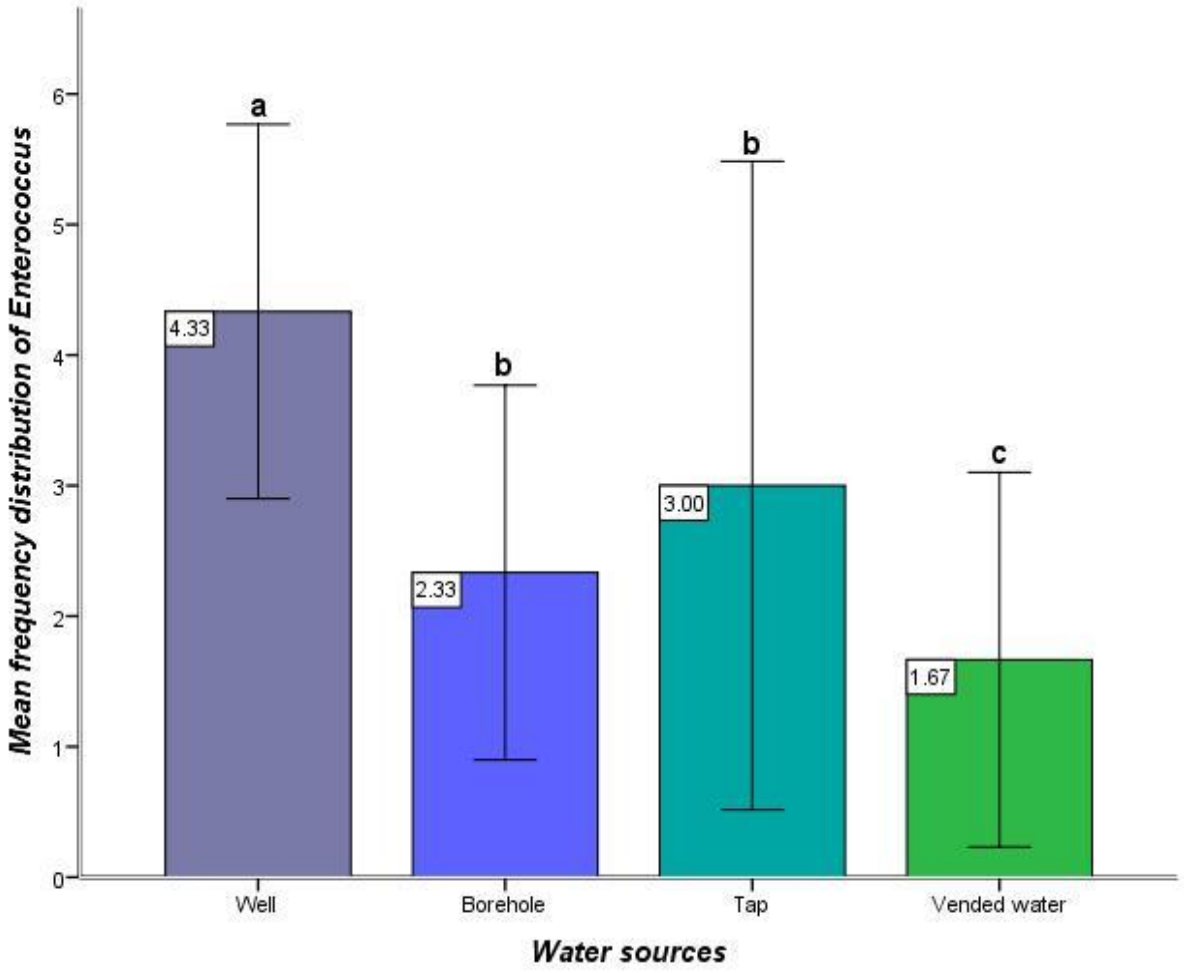

Fig 4.3: Mean frequency distribution of enterococci gotten from the different sources

*Bars with the same top-placed alphabets are not statistically different (P>0.05)

Table 4.1: Biochemical characterization and identification of *Enterococcus* spp isolated from water samples from different locations in Zaria using the Microgen Streptococcus-ID system.

HIP	AHE	BHE	MEL	SOR	INU	LAC	ARA	RIB	ESC	VP	PHS	βGA	PYR	ARG	Identity
+	-	-	+	+	-	+	+	+	+	+	+	+	+	-	*E.avium*
+	-	-	+	+	+	+	+	+	+	+	+	+	+	-	*E.gallinarum*
-	-	+	+	-	-	+	-	+	+	+	+	+	+	-	*E.cecorum*
-	-	-	+	+	-	+	+	+	+	+	+	+	+	-	*E.mundtii*

Key: HIP= Hippurate, AHE= α-haemolysis, BHE= β-haemolysis, MEL= Melibiose, SOR= Sorbitol, INU= Inulin, LAC= lactose, ARA= Arabinose, RIB= Ribose, ESC= Esculin, VP= Voges Proskauer, PHS= Phosphatase, βGA= β-galactosidase, PYR= Pyyronidonyl aramylydase, ARG= Arginine.

Table 4.2 shows the antimicrobial sensitivity of the 34 strains of Enterococciisolated from water samples in Zaria. More than 80% of the isolates were found to be susceptible to Chloramphenicol, Ciprofloxacin, Gentamicin and Tetracycline whereas More than half of the isolates were found to be resistant to Ampicillin, Erythromycin and Vancomycin thus making the percentage susceptibility to Ampicillin, Erythromycin and Vancomycin to be 21%, 16% and 13% respectively. The pathogenic strain was found to susceptible to all the antibiotics used.

Table 4.3 shows the resistant pattern of the isolated enterococci isolated. Ten resistance phenotypes were obtained, all from the multiple resistance types with varying combinations of 2, 3, 4, 6 and 7 antibiotics. Three resistance phenotype was found with the single antibiotic resistance type. Highest frequency (3) was found in combinations of two, three and four antibiotics, while combination for seven occurred just once. Multidrug resistance is regarded as resistance to four or more antibiotics. Seven (23%) of the Enterococciisolates exhibited multidrug resistance.

Multiple antibiotic resistance index (MARI) of the isolates is shown in Fig. 4.4. Multiple antibiotic resistance index (MARI) is calculated as

$$\text{MARI} = \frac{\text{Number of antibiotics to which the isolate is resistant}}{\text{Number of antibiotics tested}}$$

For the molecular studies, there was assay for antibiotic resistant gene vancomycin (*vanA* and *van B*) and virulent gene cytolysin (*cyl A*). A total of 21 isolates were used to check for presence of*van A* and *B* genes, *van A*gene wasn't detected in any of the isolates including the pathogenic strain, while the *van B* gave an amplification in 5 isolates including the pathogenic strain, but at a different product size, thus it was concluded as not present. The *cyl A* gene was assayed for in all the 34 isolates and was

found to be absent in all of them including the pathogenic strain. The faint band underneath the bands was said to be primer dimers and this was verified while running the documentation of amplicon gel.

Table 4.2: Antibiotic sensitivity of isolated *Enterococci* from water samples

Antibiotic used (Class/Structural group)	Abbreviation	Disc content	Number (%) resistant organisms	Number (%) susceptible organisms
AMPICILLIN (β-lactam penicilin)	AMP	10	27 (**79**)	7 (21)
CHLORAMPHENICOL (Chloramphenicol)	C	10	4 (12)	30 (88)
CIPROFLOXACIN (Fluoroquinolones)	CIP	30	4 (12)	30 (88)
GENTAMICIN (Aminoglycosides)	GN	10	1 (3)	33 (**97**)
ERYTHROMYCIN (Macrolides)	E	15	18 (53)	16 (47)
TETRACYCLINE (Tetracycline)	TE	30	4 (12)	30 (88)
VANCOMYCIN (Glycosides)	VA	30	21 (62)	13 (38)

Key: AMP= Ampicillin, C= Chloramphenicol, CIP= Ciprofloxacin, GN= Gentamicin, E= Erythromycin, TE= Tetracycline, VA= Vancomycin

Table 4.3: Resistant pattern of Enterococciisolated from water samples; n=31

Single antibiotic resistance		Multiple antibiotic resistance		
Number of isolates (%) in the category	Resistance phenotype	Number of antibiotic combinations	Number of isolates (%) with the pattern	Resistance phenotype
1 (3)	AMP	2	3 (10)	ERY,VAN
6 (19)	ERY		3 (10)	AMP,CIP
1 (3)	C		1 (3)	AMP,VAN
		3	7 (23)	AMP,ERY,VAN
			1 (3)	AMP,C,ERY
			1 (3)	AMP,TET,VAN
		4	4 (13)	AMP,ERY,TET,VAN
			1 (3)	AMP,C.ERY,VAN
			1 (3)	AMP,CIP,ERY,VAN
		7	1 (3)	AMP,C,CIP,GEN,ERY,TET,VAN

Key: AMP= Ampicillin, C= Chloramphenicol, CIP= Ciprofloxacin, GN= Gentamicin, E= Erythromycin, TE= Tetracycline, VA= Vancomycin

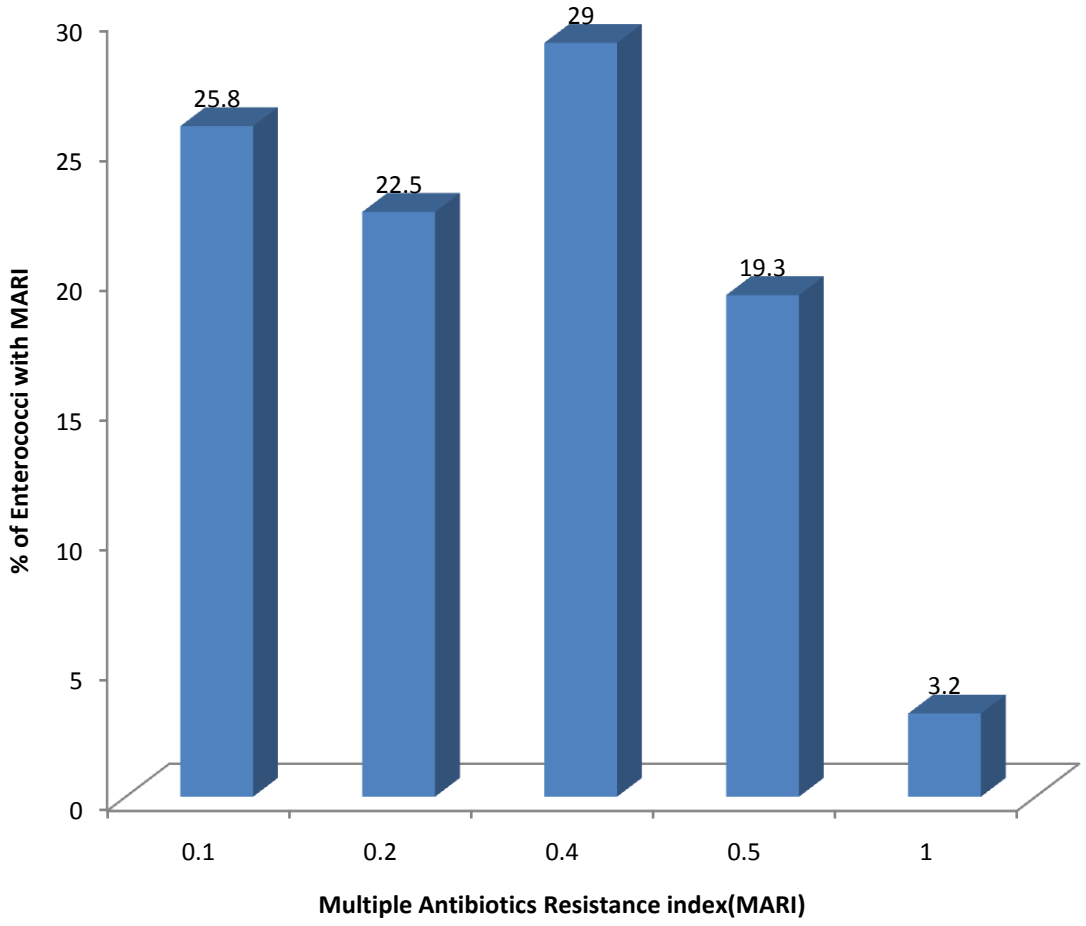

Fig. 4.4: Multiple antibiotics resistance indices of *Enterococcus* spp. isolated from water samples

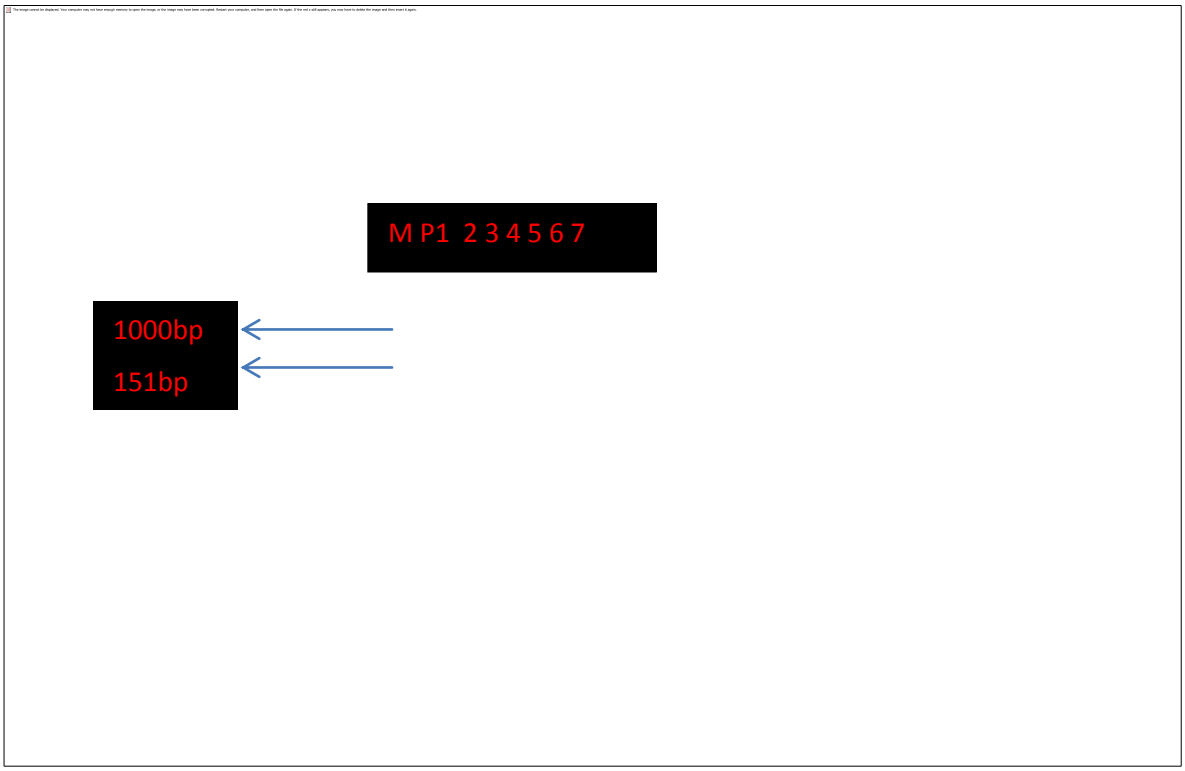

Plate 4.1: Electrophoretic gelpicture of amplification gotten from*van B* geneprimer of *Enterococcus*

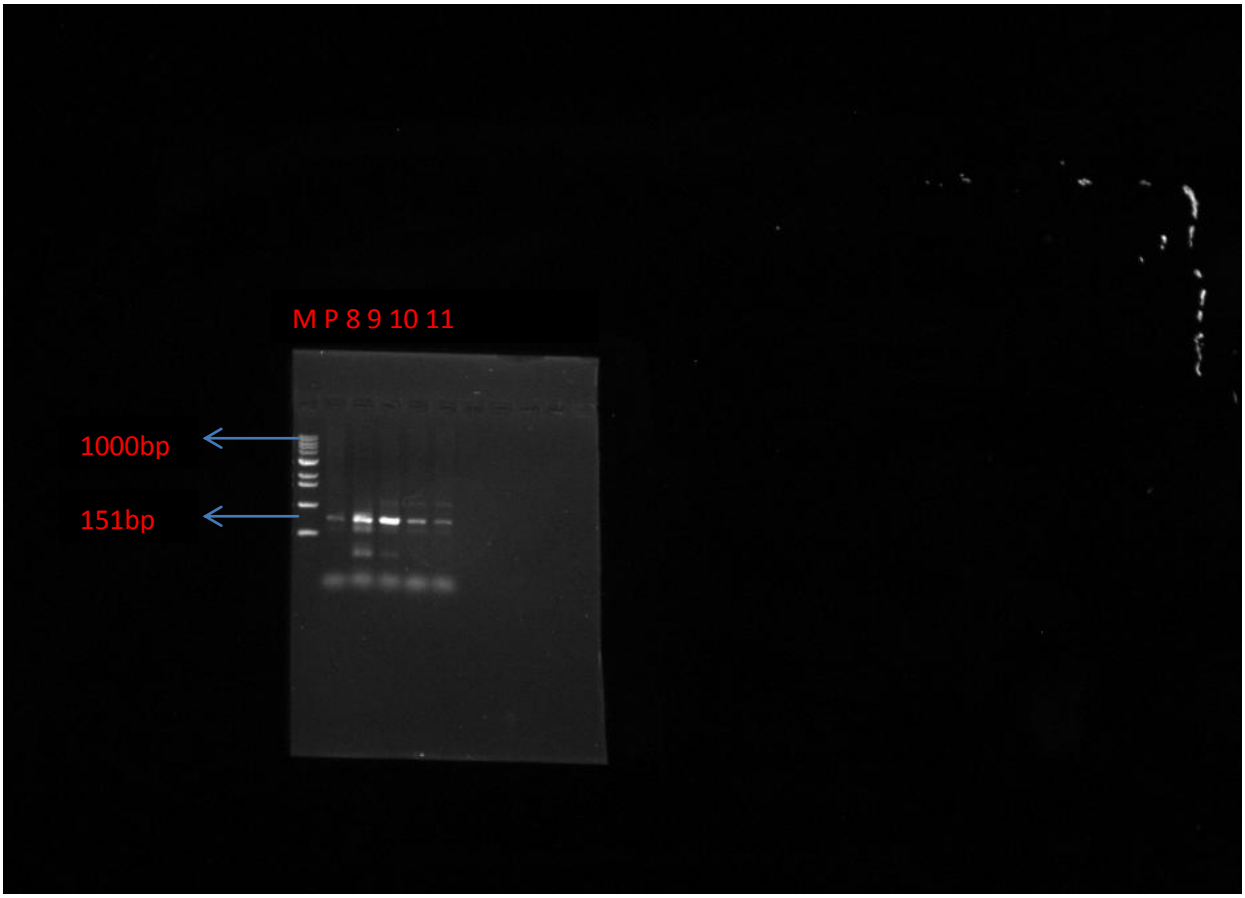

Plate 4.2: Electrophoretic gel picture of amplification gotten from*van B* gene primer of *Enterococcus*

CHAPTER FIVE

5.0 DISCUSSION

Enterococci are widely spread in nature; they are used as indicators of faecal contamination in environmental waters. This study identified four different strains of Enterococci with the prevalence of *E. avium* highest with 58.82% (Fig 4.1), this result was in contrast with work of Collins and Merapello, (2011), where *E. avium* was the least among the Enterococci isolated from ground water in Mmabato, South Africa, while *E. gallinarum* followed with 23.53% - slightly higher than the result obtained by (Olawale *et al*., 2010), *E. mundtii* had 11.76% and lastly *E. cecorum* with 5.88%. However, all the species isolated have been previously isolated from different samples as reported by other researchers (Irani, 2011,Daminabo, *et al.*, 2013 and David, *et al*., 2013). There are reports of isolation of other species such as*E. faecalis, E. faecium, E. casseliflavus,* and *E. hirae* from water samples (Moore*et al*., 2008), however these species were not isolated in the present study. This difference in results may be due to several reasons including geographical differences, types of collection method and type and source of samples. The high prevalence of *E. avium* and *E.gallinarum* could be as a result of the fact that they are known to be of animal origin (birds and chicken respectively), and it was observed during sample collection that most of the households had these animals around the sample source. *E. mundtii* isolation from water could be as a result of plants growing in some of the wells or borehole overhead tanks, because they are known to be of plant origin. This implies that people can come down with enterococcal related infections when they consume such water or when their immune system is compromised.

Zaria city had the highest number of enterococci compared to other locations. This might be due to the high population of people in the area and the fact that most of

the houses were built very close to one another and thus no enough space to maintain the rule of digging wells or borehole at least 100m away from toilets, hence the level of contamination of ground water with faeces from humans and animals is high.The mean frequency of distribution of enterococci via location pinpointed out Sabon gari with the least distribution of Enterococci (1.86) compared to Zaria city and Samaru (4.33) and (4.17) respectively.

The mean frequency distribution of Enterococci among the different sources pointed out that well water had an average frequency of 4.33, indicating it as quite unsafe for consumption, and recommends vended water (mairuwa) with an average of 1.67enterococcal frequency.Well water samples were observed to be more contaminated with Enterococci compared to other sources, this result agrees with the work of (Peter *et al*., 2012), This high level of contamination may be due to periodicity and intensity of rainfall which had been shown to impact the level of microbial contaminant in wells, and the more time between events, the more contaminants accumulate and are washed into the well (Abbott *et al*., 2006) coupled with the seepage from nearby septic tanks and feacal contamination from birds and pets. Also shallow and uncovered wells could be a contributing factor to contamination. The tap water which is always considered safe due to pre-treatment before circulation were also found to be next in contamination to the well water (9). This is in agreement with works of (Mathur *et al.,* 2003) and (Oluyege *et al*., 2006), who reported that treated water, may have a wide range of organisms which include indigenous species, saprophytic species as well as human pathogen such as*Enterococcus feacalis*, *E. feacium, E. durans, E. avium* and other species of Enterococcus. This may be as a result of cross connections or back flow of water, inadequate quantity of chemicals for treatment of pipe-borne water to kill microbes or discontinuing follow up process from source. The presence of

*Enterococci*in borehole water was not unusual as it conforms to work of (Aderiye *et al.*, 1992; Peter *et al*., 2012), who isolated Enterococci from water obtained from boreholes. This can be due to the fact that most sanitary pipelines are located in close proximity to water supply including borehole locations which traverse the septic tank absorption fields or shallow dug boreholes. The lower count in vended water (mairuwa)could be as a result of the fact that most of them get their water from boreholes which had lesser number of Enterococcal contaminations and also some of them do wash their containers occasionally, thus reducing source of contamination.

Enterococci have the ability to transfer plasmids to both closely and distantly related Gram-positive bacteria (Clewell, 1993; Bøhle *et al*., 2011),which has been an advantage in keeping their resistant plasmids in circulation over the years, therefore, the antimicrobial susceptibility tests of Enterococci sppto selected antibiotics indicated that the Enterococcus spp from the water samples collected were susceptible to Gentamicin with 97% susceptibility. Three of the isolates were susceptible to all the antibiotics used while the remaining were resistant to at least one of the antibiotics.This may due to the fact that gentamicin is expensive and therefore may not be readily abused. These findings are in agreement with work of Odeyemi *et al*. (2009); and Collins and Merapelo (2011),which also had the highest susceptibility with gentamicin. Therefore, this result implies that gentamicin is a drug of choice for the treatment of infection with enterococci.

Resistance to antibiotics commonly leads to a failure of treatment with other antimicrobials.The most frequent resistance properties of *Enterococcus* spp obtained from the water samples were resistant to tetracycline (12%), Erythromycin (53%), ampicillin (79%), ciprofloxacin (12%), Chloramphenicol (12%) and vancomycin (62%).Enterococci are intrinsincally resistant to macrolides (e.g.erythromycin),

tetracycline and often exhibit high-level resistance to glycopeptides (e.g.vancomycin).These findings are in consonance with the reports of Moore *et al*. (2008), Peter *et al*. (2012), Valerie*et al*. (2001), who also had high resistance with these antibiotics.In this study, a large proportion of the *Enterococcus* species isolated were resistant to three or more antibiotics and hence were termed multiple antibiotic resistant (MAR) isolates. The high ampicillin resistance could be as a result of the abuse of the drug, since it is relatively cheap and readily available. Previous studies have shown that an amino acid substitution in ampicillin-resistant enterococci usually occur (Mohn *et al*., 2004). Multiple antibiotic resistance index (MARI) gives an indirect suggestion of the probable source of the organism. MAR index greater than 0.2 indicates that an organism must have originated from an environment where antibiotics are often used (Olayinka *et al.*, 2004). Among the isolated *Enterococcus* spp from water, 16 (52%) of the tested bacteria had MAR index greater than 0.2.

Vancomycin resistance should be of public health concern because the Enterococci isolated in this study were mostly those known to be of animal origin, for example,*E. avium* in birds and *E. gallinarum* in chicken. Epidemiological evidence from Europe suggests that VRE are horizontally transmitted from animals to human (Bates *et al*., 1993, Bates *et al.*, 1997 and Stobberingh *et al*., 1999). Thus this indicates that great care should be taken to avoid introducing these organisms into the environment via nonnosocomial routes such as drinking water, because of the difficulties encountered in treating infections.

The distribution of selected genes conferring the virulence to enterococcal strains was studied with the aim to assess the pathogenic potential of environmental strains. Cytolysin, a virulent gene (*cyl A*) was assayed for in the isolates, *cyl A* gene was absent

in all the tested isolates including the pathogenic strain, this agrees with work of DebRoy and Maddox, 2001, who didn't get the cytolysin gene in the enterococcal strains tested. However, this can be as a result of the organisms habouring other cytolysin genes which were not tested for. The other genes in the cytolysin operon are *cyl L* which serves as a precursor, *cyl B*- for transport of cytolysin,*cyl M*- for posttranslational modification of cytolysin, *cyl A* was chosen because it is the one responsible for the activation of the cytolysin gene, therefore,other cytolysin genes can be present but not active without the *cylA* gene to activate them. Another reason could be that the organisms isolated in this study were not *E. faecium* and *E. faecalis* (Pangallo *et al*., 2004) which are known from literature mostly to harbor cytolysin genes, although the *E. faecium* which was a pathogenic strain gotten in this study didn't possess it. The absence of *cyl A* in the isolates from the water sources is an indication that the Enterococci cannot cause cytotoxin borne illnesses.

Molecular studies were carried out on the isolates and pathogenic strain, although the pathogenic strain was susceptible to all the antibiotics it was tested against using disc diffusion method, it was found to also give an amplification with the *van B* gene primer at a different product size.

Vancomycin resistant gene was also screened for in the Enterococcal isolates, these were *van A* and *van B*. *Van A* was absent in all the isolates including the pathogenic strain which confirms the result that it is often seen in *E. faecalis* and *faecium*(Pangallo *et al*., 2004, Irani, 2011) which were not isolated in the present study, though the pathogenic strain *E. faecalis* too didn't possess the *van A*gene. The absence in the pathogenic strain might be that the organism had lost the virulent gene through mutation or never possessed the gene. The *van B* gene primer gave amplification in six of the isolates which were*E. gallinarum* and*E. mundtii*, also the

pathogenic strain, though, the expected size was 297bp, PCR amplification was obtained at 151bp. This could be as a result of non-specific primer binding or failure on the part of the researcher to report the actual sequence gotten in his work; also difference in location of isolation could be a reason.*E. gallinarium* is known to possess intrinsic resistance *van C*, althougha first case was reported by (Corso *et al*., 2005). A report of *Enterococcus gallinarum* harbouring a *van B* gene was made by CDC, (2005), that wasn't the case in this study with the *E. gallinarum* isolated.*Enterococcus mundtii* which are now grouped under the faecium group have been implicated in disease in humans even though they are usually of plant or soil origin (Tomomi *et al.*, 2005), was expected to have either of the genes tested for, but was found absent in all of them.

CHAPTER SIX

6.0 SUMMARY ANDCONCLUSION

6.1 Summary and Conclusion

The result of this study shows that water samples in Zaria are predominantly contaminated with four different species of *Enterococcus* namely *Enterococcus avium, Enterococcus gallinarum*, *Enterococcus mundtii* and *Enterococcus cecorum* with *Enterococcus avium*having the highest frequency of occurrence 58.82%.

The result of the antibiotics assay revealed the efficacy of gentamicin, ciprofloxacin, chloramphenicol and tetracycline against all the enterococci with gentamicin being the most effective, while the isolates were more resistant to Ampicillin, Vancomycin and Erythromycin respectively.The highest zones of inhibition recorded in mm were Ampicillin (24), chloramphenicol (30), ciprofloxacin (30), gentamicin (27), erythromycin (28), tetracycline (26), and vancomycin (23).

The molecular studies confirmed that the organisms didn't harbour an antibiotic resistance gene and virulent gene. This study has been able to bring to limelight the extent of water contamination with Enterococci which should be viewed with serious concern because of the potential health risk that could result thereof noting that most people do not treat their water before use.

6.2 Recommendations

In view of the danger and health risks associated with Enterococci as faecal indicator in water, the following measures are therefore recommended:

1. There should be provision of proper water management systems and treated safe water supply to residents in these communities.

2. Physicochemical analysis should be carried out on the water samples in further studies to know the components of the water.
3. The results presented herein demonstrate the need for a continued surveillance of antimicrobial resistance among water and food-borne pathogens.
4. Enlightenment programs should be organized by the public health officials to educate the populace on the implication of Enterococcal infection.
5. The constant monitoring for the presence of virulent enterococci in recreational water is recommended to prevent increased risk of developing disease as a result of exposure to virulent environmental enterococci.
6. Education on siting of wells far away from toilets should be emphasized.
7. Proper covering of wells should be encouraged, to reduce contamination with faecal materials.
8. Molecular studies should be affordable to increase the amount of research that could be carried out on Enterococci to understand the pathogenicity of the organism.
9. Proper monitoring of pipelines should be done to reduce contamination.
10. Researcher should please try to report the exact results they get from their analysis, so as not to mislead other researcher citing such works.

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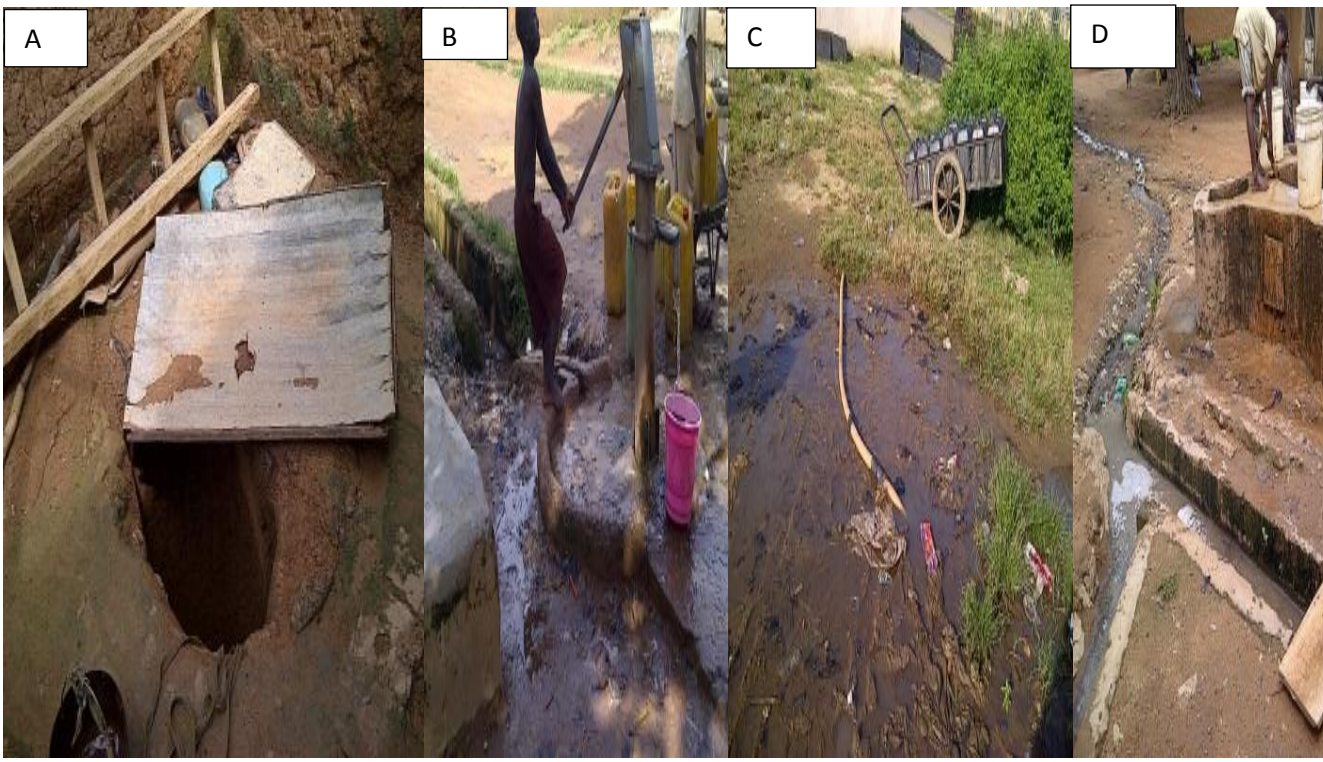
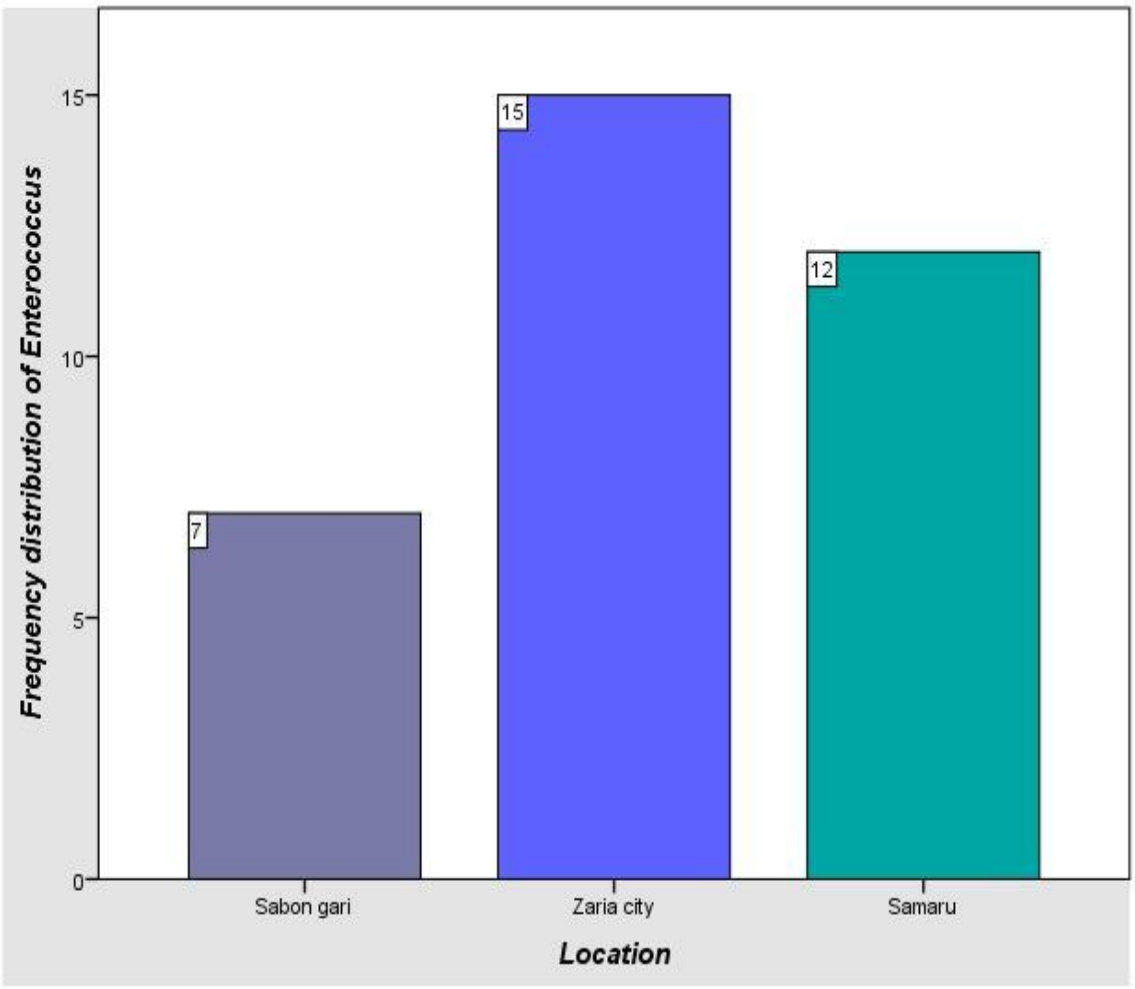
APPENDICES

Appendix I: Showing various sources from which water samples were collected in Zaria Kaduna state.

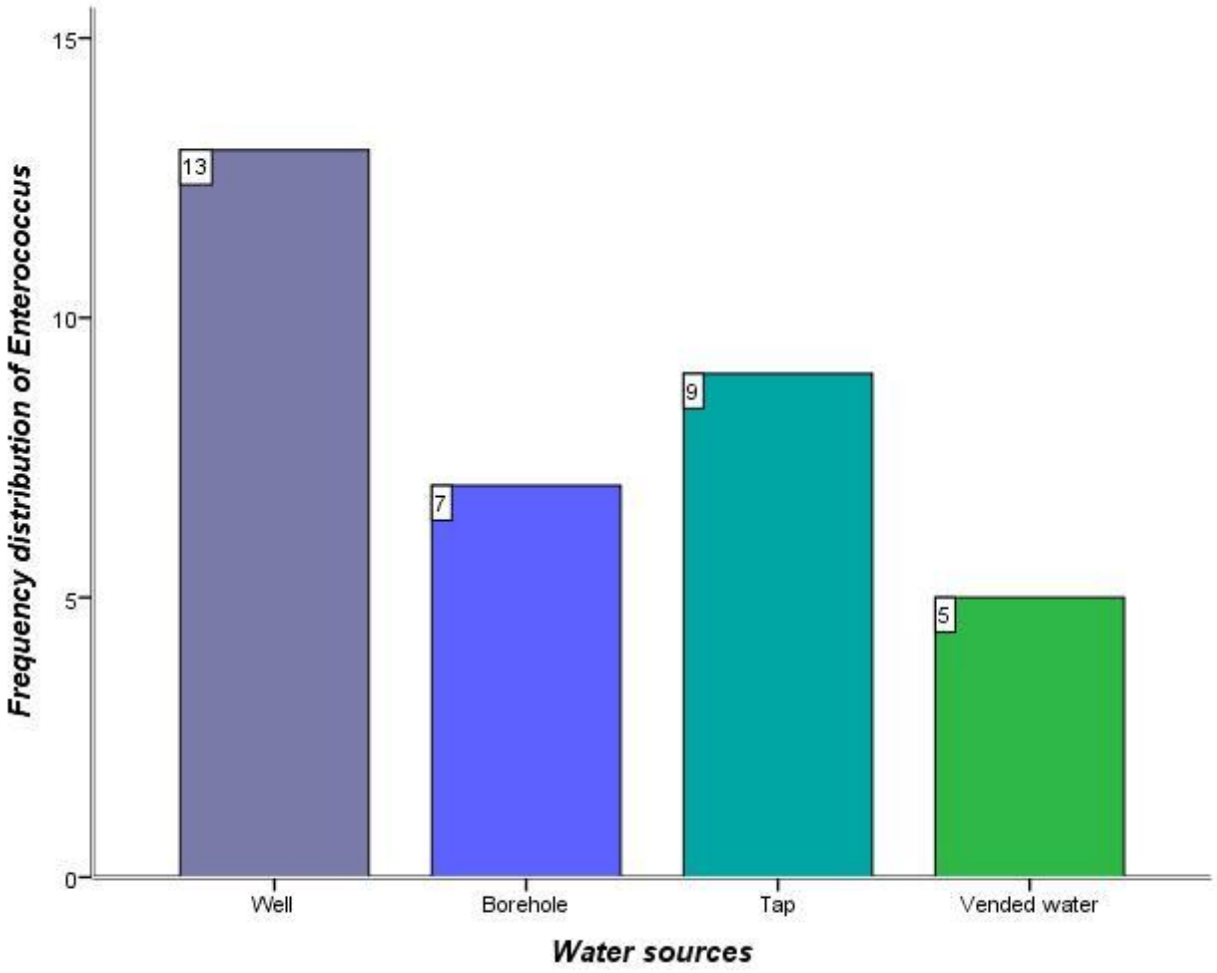


A- Well water source
B- Manual borehole water source
C- Source of vended water (mairuwa)
D- Motorised borehole source

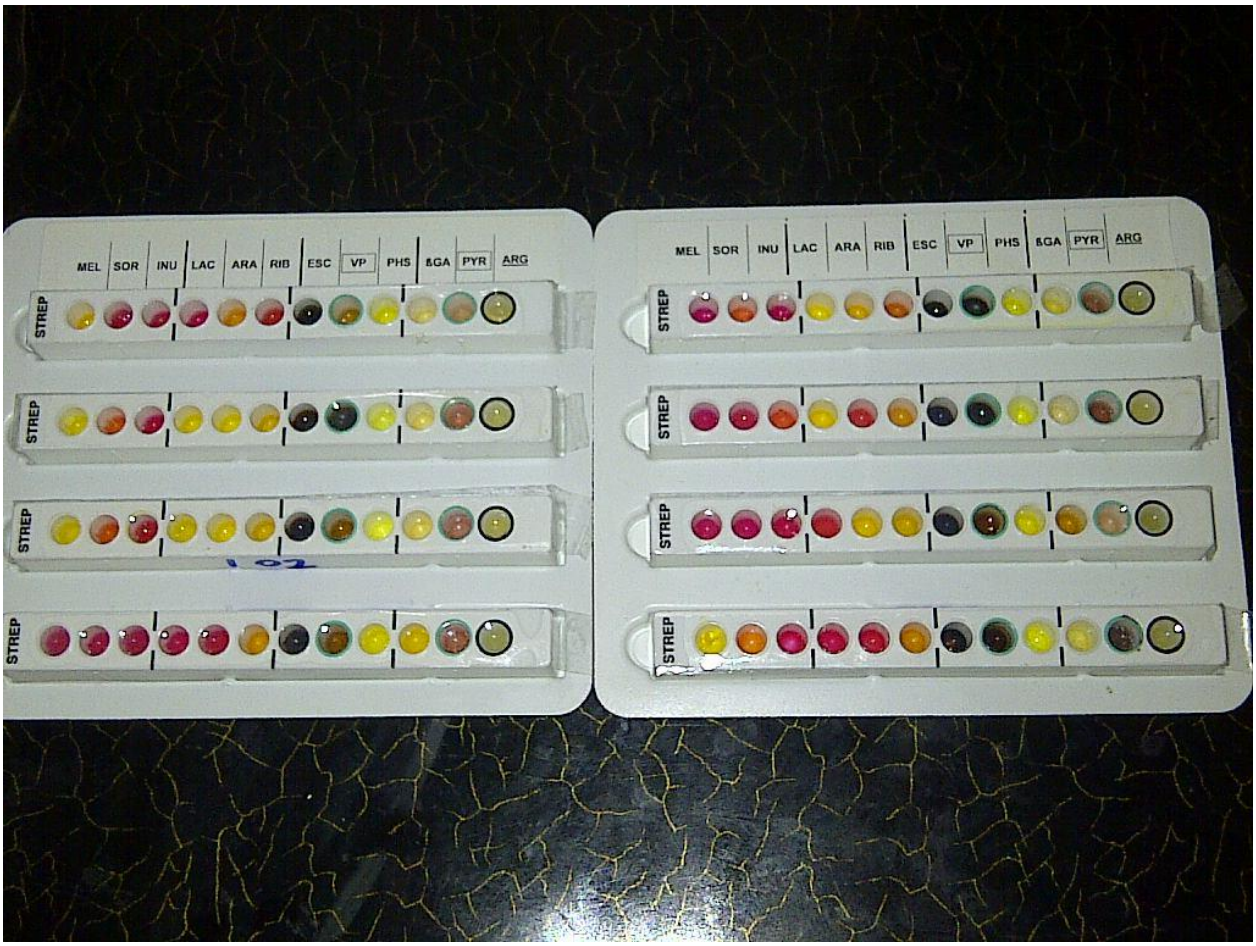
Appendix II: Showing number of *Enterococcus* spp isolated from different locations



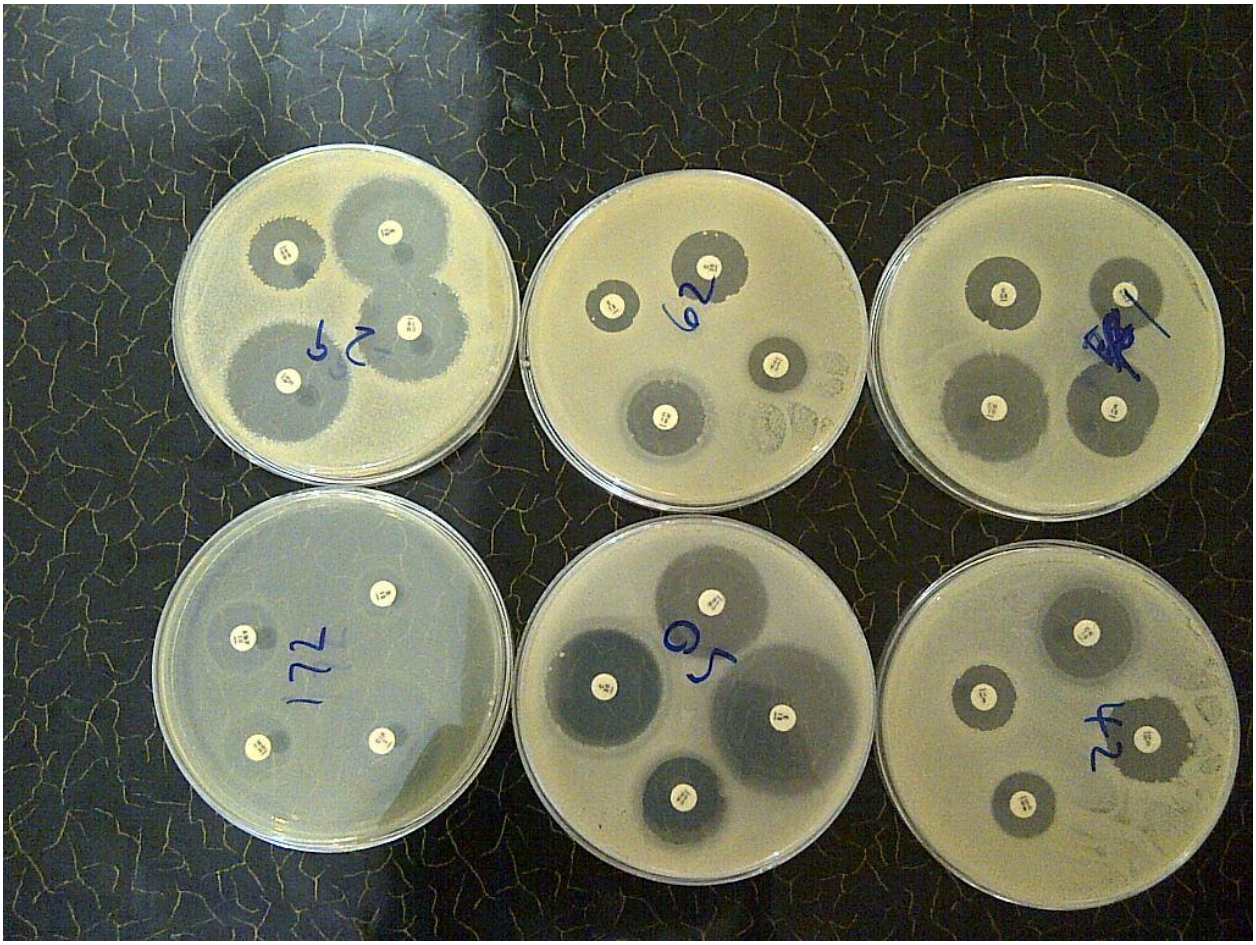
Appendix III:Showing number of Enterococcus gotten from each water source in all the locations



Appendix IV: Biochemical characterization and identification of Enterococci from water samples in Zaria, Kaduna State.



Appendix V: Zones of inhibition of isolates to selected antibiotics.



Key: Plate 29-*Enterococcus gallinarum*

Plate 42-*Enterococcus cecorum*

Plate 50-*Enterococcus avium*

Plate 62-*Enterococcus mundtii*

Plate 41-*Enterococcus gallinarum*

Plate 172-*Enterococcus avium*

Appendix VI: Antibiotic sensitivity of isolated Enterococci from water samples

Enterococcus isolates from various selling sites in Zaria	Amp (10µg)	C (30µg)	Cip (5µg)	Gen (10µg)	Ery (15µg)	Tet (30µg)	Van (30µg)
	Zone of inhibition (mm)*						
WSa200	11 (R)	19 (S)	24 (S)	17 (S)	8 (R)	16 (S)	6 (R)
WSa180	18 (S)	20 (S)	26 (S)	16 (S)	6 (R)	18 (S)	7 (R)
WSa140	9 (R)	20 (S)	24 (S)	20 (S)	8 (R)	16 (S)	6 (R)
WSa152	8 (R)	22 (S)	24 (S)	17 (S)	9 (R)	16 (S)	6 (R)
WSa188	13 (R)	21 (S)	24 (S)	19 (S)	8 (R)	18 (S)	6 (R)
WSa195	7 (R)	21 (S)	15 (R)	16 (S)	19 (S)	17 (S)	16 (S)
WSa172	17 (S)	28 (S)	16 (S)	15 (S)	8 (R)	15 (S)	6 (R)
WSa176	7 (R)	26 (S)	14 (R)	17 (S)	6 (R)	16 (S)	6 (R)
WSa169	7 (R)	30 (S)	20 (S)	15 (S)	19 (S)	15 (S)	6 (R)
WSa085	6 (R)	6 (R)	6 (R)	6 (R)	6 (R)	6 (R)	6 (R)
WSa172	13 (R)	25 (S)	21 (S)	13 (S)	8 (R)	16 (S)	6 (R)
WSa193	7 (R)	20 (S)	21 (S)	14 (S)	7 (R)	15 (S)	6 (R)
WZc010	10 (R)	20 (S)	15 (R)	15 (S)	7 (R)	16 (S)	6 (R)
WZc058	6 (R)	10 (R)	30 (S)	23 (S)	10 (R)	23 (S)	23 (S)
WZc064	7 (R)	26 (S)	17 (S)	16 (S)	19 (S)	16 (S)	14 (R)
WZc042	8 (R)	21 (S)	22 (S)	20 (S)	15 (S)	15 (S)	15 (S)
WZc029	27 (S)	29 (S)	27 (S)	27 (S)	28 (S)	26 (S)	18 (S)
WZc007	12 (R)	22 (S)	29 (S)	21 (S)	14 (S)	16 (S)	21 (S)
WZc001	7 (R)	28 (S)	16 (S)	14 (S)	6 (R)	15 (S)	6 (R)
WZc050	25 (S)	28 (S)	27 (S)	26 (S)	25 (S)	19 (S)	19 (S)
WZc071	7 (R)	24 (S)	17 (S)	15 (S)	9 (R)	9 (R)	6 (R)

WZc056	6 (R)	24 (S)	16 (S)	15 (S)	14 (S)	13 (R)	13 (R)
WZc017	8 (R)	20 (S)	28 (S)	17 (S)	25 (S)	17 (S)	14 (R)
WZc043	26 (S)	24 (S)	32 (S)	24 (S)	24 (S)	19 (S)	20 (S)
WZc022	18 (S)	9 (R)	29 (S)	27 (S)	21 (S)	23 (S)	18 (S)
WZc028	8 (R)	18 (S)	24 (S)	20 (S)	25 (S)	19 (S)	16 (S)
WZc030	7 (R)	18 (S)	20 (S)	22 (S)	19 (S)	18 (S)	15 (S)
WSg088	6 (R)	9 (R)	17 (S)	18 (S)	9 (R)	18 (S)	6 (R)
WSg117	17 (S)	14 (S)	25 (S)	18 (S)	7 (R)	17 (S)	6 (R)
Wsg133	7 (R)	14 (S)	25 (S)	20 (S)	25 (S)	18 (S)	16 (S)
WSg119	11 (R)	23 (S)	27 (S)	13 (S)	6 (R)	13 (R)	6 (R)
WSg102	6 (R)	23 (S)	16 (S)	14 (S)	7 (R)	14 (R)	6 (R)
WSg161	8 (R)	21 (S)	25 (S)	23 (S)	28 (S)	20 (S)	16 (S)
WSg165	11 (R)	30 (S)	22 (S)	19 (S)	22 (S)	16 (S)	16 (S)

Key: Amp= Ampicillin, C= Chloramphenicol, Cip= Ciprofloxacin, Gen= Gentamicin, Ery= Erythromycin, Tet= Tetracycline, Van= Vancomycin, WSa= Water Samaru, WSg= Water Sabon gari, WZc= Water Zaria city.

*Resistance/Susceptibility range (CLSI, 2013).

Appendix VII: Biochemical characterization and identification of Enterococci from water samples using the Microgen Strept-ID.

PROFILE NO	HIP	AHE	BHE	MEL	SOR	INU	LAC	ARA	RIB	ESC	VP	PHS	βGA	PYR	ARG	OCTAL CODE
WSa200	+	-	-	+	+	-	+	+	+	+	+	+	+	+	-	46776
WSa180	+	-	-	+	+	-	+	+	+	+	+	+	+	+	-	46776
WSa140	+	-	-	+	+	-	+	+	+	+	+	+	+	+	-	46776
WSa152	+	-	-	+	+	-	+	+	+	+	+	+	+	+	-	46776
WSa188	+	-	-	+	+	+	+	+	+	+	+	+	+	+	-	47776
WSa195	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	16776
WSa172	+	-	-	+	+	-	+	+	+	+	+	+	+	+	-	46776
WSa176	+	-	-	+	+	-	+	+	+	+	+	+	+	+	-	46776
WSa169	+	-	-	+	+	+	+	+	+	+	+	+	+	+	-	47776
WSa85	-	-	+	-	-	-	+	-	+	+	+	+	+	+	-	16576
WSa172	+	-	-	+	+	-	+	+	+	+	+	+	+	+	-	46776
WSa193	+	-	-	+	+	+	+	+	+	+	+	+	+	+	-	47776
WZc010	+	-	-	+	+	+	+	+	+	+	+	+	+	+	-	47776
WZc058	-	-	+	+	+	-	-	+	+	+	+	+	+	+	-	16376
WZc064	-	-	+	+	+	-	+	+	-	+	+	+	+	+	-	16676
WZc042	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	16776
WZc029	+	-	-	+	+	-	+	+	+	+	+	+	+	+	-	46776
WZc007	-	-	-	+	+	-	+	+	+	+	+	+	+	+	-	06776
WZc001	+	-	-	+	+	-	+	+	+	+	+	+	+	+	-	46776
WZc050	-	-	-	+	+	-	+	+	+	+	+	+	+	+	-	06776
WZc071	+	-	-	+	+	-	+	+	+	+	+	+	+	+	-	46776
WZc056	+	-	-	+	+	+	+	+	+	+	+	+	+	+	-	47776
WZc017	+	-	-	+	+	+	+	+	+	+	+	+	+	+	-	47776
WZc043	-	-	+	+	-	-	+	-	+	+	+	+	+	+	-	14576
WZc022	+	-	-	+	+	+	+	+	+	+	+	+	+	+	-	47776
WZc028	+	-	-	+	+	+	+	+	+	+	+	+	+	+	-	47776
WZc030	+	-	-	+	+	-	+	+	+	+	+	+	+	+	-	46776

WSg088	+	-	-	+	+	+	+	+	+	+	+	+	+	+	-	47776
WSg117	-	-	+	-	+	-	+	+	+	+	+	+	+	+	-	12776
Wsg133	-	-	+	+	-	-	+	-	+	+	+	+	+	+	-	14576
WSg119	-	-	-	+	+	-	+	+	+	+	+	+	+	+	-	06376
WSg102	+	-	-	+	+	-	+	+	+	+	+	+	+	+	-	46776
WSg161	-	-	+	-	+	-	+	+	+	+	+	+	+	+	-	12776
WSg165	-	-	-	+	+	-	+	+	+	+	+	+	+	+	-	06376

Key: WSg= Water Sabon gari, WSa= Water Samaru, WZc= Water Zaria city.