OCCURRENCE AND CHARACTERIZATION OF *ENTEROCOCCUS* SPECIES ISOLATED FROM LIVESTOCK AND VEGETABLE FARMS IN ZARIA, NIGERIA

BY

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Declaration

I declare that the work in this thesis entitled "**Occurrence and characterization of *Enterococcus* species isolated from livestock and vegetable farms in Zaria, Nigeria"** has been carried out by me in the Department of Veterinary Microbiology. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other Institution.

Emmanuel Ochefije NGBEDE ....
Signature Date

Certification

This thesis entitled "**OCCURRENCE AND CHARACTERIZATION OF *ENTEROCOCCUS* SPECIES ISOLATED FROM LIVESTOCK AND VEGETABLE FARMS IN ZARIA, NIGERIA" by** Emmanuel Ochefije NGBEDE meets the regulations governing the award of the degree of Doctor of Philosophy in Veterinary Microbiology of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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Dedication

I dedicate this work to my parents, teachers and in the loving memory of Late Rev. Fr. Dr. Martin I. Debaleh for their selfless sacrifices towards making my future great. This dedication is but small and humble in view of your commitments.

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Abstract

This study was carried out to determine the occurrence of *Enterococcus* species and characterize *Enterococcus faecalis* and *E. faecium* isolates from poultry, cattle and vegetable farms in Zaria, Nigeria. A total of 670 samples consisting of cattle rectal swabs and manure (n=130 and 65 respectively), poultry cloacal swabs and manure (n=130 and 65 respectively), irrigation water (n=40), vegetables (52 lettuce, 20 cabbage and 48 carrot) and soil (n=120). The samples were collected between January, and July, 2014 in Zaria, Nigeria. Isolation and speciation was carried out using standard microbiological techniques and further confirmation of *E. faecium* isolates using polymerase chain reaction (PCR). Further characterization of the isolates identified as *E. faecium* and *E. faecalis* was carried out by detection of phenotypic expression of virulence and resistance traits, detection of some of the corresponding genes by PCR and transferability of some resistance traits. Sequencing of the *pbp*5 gene was also carried out to determine the amino acid substitutions responsible for high level ampicillin resistance among the isolates. *Enterococcus* was isolated from 200 (29.9%) out of the 670 samples collected. Two hundred and sixty seven enterococcal isolates were recovered from the 200 samples. Except for irrigation water, *Enterococcus* was isolated from all sample types. Nine species; *E. faecium* (n=130), *E. faecalis* (n=5), *E. raffinosus* (n=6), *E. avium* (n=1), *E. gallinarum* (n=48), *E. casseliflavus* (n=3), *E. mundtii* (n=4), *E. durans* (n=1) and *E. hirae* (n=5) were differentiated from the 267 enterococcal isolates, while 64 were unidentifiable. The isolation rates were *Enterococcus faecium* (48.7%) followed by *E. gallinarum* (17.9%) and were the predominant species isolated from all sample types (except for lettuce). Virulence factors observed were haemolysin (20.7%), gelatinase (9.6%), aggregation substance (1.48%) and

biofilm (83.7%) which were expressed in addition to carriage of their corresponding genes; *cyl*A (1/19), *gel*E (10/19) and *asa*1 (14/20) except for enterococcal suface protein (*esp*) gene. However, a silent *gel*E gene was detected in 2 isolates. High percentages of resistance were detected for erythromycin (54.1%), tetracycline (52.6%) and ampicillin (46.7%) and lower values were observed for quinupristin/dalfoprsitin (3.70%), chloramphenicol (6.7%) and ciprofloxacin (8.1%). All the isolates were susceptible to vancomycin. MIC of gentamicin and ampicillin above resistance breakpoints (≥500µg/ml and ≥16µgml, respectively) were observed among the high level gentamicin (35/37) and ampicillin (7/63) resistant isolates. Multiple drug resistance (MDR) to three to six different antibiotics was observed (45.9%) among the isolates tested, with majority of the MDR originating from poultry sources. Twenty four different MDR patterns were identified with the CN-E-S-TE phenotype (11 isolates) followed by AM-CN-E-S-TE (seven isolates) being the predominant MDR pattern. Resistance to AM, E and TE frequently occurred among the 24 different MDR phenotypes. Genes responsible for resistance to gentamicin: *aac(6')-Ie-aph(2'')* (19/19), *aph(2')*-Ic (8/9), *aph(3')*-IIIa (9/10) and *ant(4')* (4/10), ampicillin; *pbp5*, erythromycin; *erm*(B) (19/19) and tetracycline: *tet*(K) (9/10) *tet*(L) (20/20) *tet*(M) (18/19) and *tet*(O) (10/10) were detected among the *E. faecium* and *E. faecalis* isolates tested. In addition to co-carriage of more than one gene (aminoglycoside modifying genes and tetracycline resistant genes) mediating resistance to the same antibiotic, co-carriage of the *erm*(B)+*tet* gene in a single strain was observed among most of the isolates. Five of the seven *E. faecium* isolates presented a total of nine amino acid substitutions in PBP5 protein. Four new amino acid substitution; 500Glu→Leu, 502Asp→Arg, 612IIe→X and 614IIe→Phe in addition to known polymorphism 470His→Gln, 496Asn→Lys, 497Phe→IIe, 499Ala→Thr, 525Glu→Asp resulted from mutations of the *pbp*5 gene. *In*

vitro intra and inter species transfer of determinants coding for either or both of erythromycin and tetracycline resistance to recipient cells was observed in six (75%) out of the eight isolates tested. In conclusion, enterococci from livestock and vegetable farms in Zaria harbour a variety of virulence and antimicrobial resistance determinants that give them the potential to cause antimicrobial resistant nosocomial infections. They may also serve as reservoirs of virulence and antimicrobial resistance genes of bacteria that could eventually be transmitted either to other bacteria, animals or even to humans.

Table of Contents

	Page
Cover Page - - - - - - - - -	i
Fly page - - - - - - - - -	ii
Title Page - - - - - - - - -	iii
Declaration - - - - - - - - -	iv
Certification - - - - - - - - -	v
Dedication - - - - - - - - -	vi
Acknowledgments - - - - - - - - -	vii
Abstract - - - - - - - - -	viii
Table of Contents - - - - - - - - -	xi
List of Tables - - - - - - - - -	xvi
List of Figures - - - - - - - - -	xvii
List of Plates - - - - - - - - -	xviii
List of Appendices - - - - - - - - -	xx
List of Abbreviations - - - - - - - - -	xxi

1.0 INTRODUCTION - - - - - - - - 1

1.1 Background of the Study - - - - - - - 1

1.2 Statement of the Research Problem - - - - - 3

1.3 Justification of the Study - - - - - - - 6

1.4 Research Questions - - - - - - - - 7

1.5 Aim of the Study - - - - - - - 8

1.6 Objectives of the Study - - - - - - - 8

2.0 LITERATURE REVIEW - - - - - - - 9

2.1. The Organism *Enterococcus* - - - - - - 9

2.2 Taxonomy of *Enterococcus* Species - - - - - - 10

2.3 Characteristics of *Enterococcus* species - - - - - 12

2.4 Genome Structure and Characteristics of *Enterococcus* Species - 13

2.5 Isolation Methods for Enterococci - - - - - - 14

2.6 Phenotypic Identification of *Enterococcus* species - - - - 15

2.7 Genotypic Identification and Typing of *Enterococcus* Species - 20

2.8 Ecology and Epidemiology *Enterococcus* Species - - - - 23

2.9 Clinical Significance of *Enterococcus*: Virulence and Antimicrobial Resistance - - - - - - - - - 24

2.10 Virulence Factors in *Enterococcus* Infection - - - - 27

2.10.1 Aggregation substance (AS) - - - - - - - 28

2.10.2 Enterococcal surface protein - - - - - - - 29

2.10.3 Enterococcal cytolysin (haemolysin) - - - - - - 29

2.10.4 *Enterococcus* gelatinase - - - - - - - 31

2.10.5 *Enterococcus* hyaluronidase - - - - - - - 32

2.10.6 Microbial surface components recognizing adhesive matrix molecules (MSCRAMM) - - - - - - - - 32

2.10.7 Collagen binding adhesin of *Enterococcus faecium* (Acm) - - - 33

2.11 Antimicrobial Use and Evolution of Resistance in Livestock Production 33

2.12 Antimicrobial Resistance in *Enterococcus* - - - - - 34

2.12.1 Resistance to beta (β)-lactam antibiotics - - - - 36

2.12.2 Resistance to the aminoglycoside antibiotics - - - - 38

2.12.3 Resistance to the glycopeptide antibiotics - - - 42

2.12.4 Resistance to the tetracyclines - - - - - 45

2.12.5 Macrolide-lincosamide-streptogramin resistance (MLS) - - 46

2.12.6 Resistance to the fluoroquinolones - - - - - 47

2.12.7 Resistance to streptogramin antibiotics - - - - 48

2.13 Treatment and Control of Enterococcal Infections - - 49

2.13 Transfer of antimicrobial resistance determinants - - 50

3.0 MATERIALS AND METHODS - - - - - 52

3.1 Study Area - - - - - - - - 52

3.2 Study Design - - - - - - - - 52

3.3 Sampling - - - - - - - - 53

3.3.1 Sample site and sample collection - - - - - 53

3.3.2 Number of samples collected - - - - - - 54

3.3.3 Processing of samples- - - - - - - 55

3.4 Isolation and Identification of Enterococci- - - - 56

3.4.1 Isolation of enterococci - - - - - - 56

3.4.2 Biochemical identification/speciation- - - - - 56

3.4.3 Molecular Identification of *Enterococcus* species - - - 57

3.4.4 Analysis of PCR products - - - - - - 59

3.5 Phenotypic Screening of the *Enterococcus faecium* and *E. faecalis* for Expression of Virulence Traits - - - - - 59

3.5.1 Assay for haemolysin (cytolysin) production - - - - 59

3.5.2 Assay for gelatinase production - - - - - 60

3.5.3 Assay for biofilm production - - - - - - 60

3.5.4 Assay for aggregation substance production - - - - 61

3.6. Determination of the Antibiotic Susceptibility Profile of the *Enterococcus faecium* and *E. faecalis* Isolated - - - - - 61

3.6.1 Disc diffusion test - - - - - - 62

3.6.2 Minimum inhibitory concentrations (MICs) - - - - 62

3.7 Molecular Detection of Virulence and Resistance Genes-- - 63

3.7.1 Detection of virulence genes - - - - - - 63

3.7.2 Detection of antibiotic resistance genes - - - - 64

3.7.3 Analyses of PCR products - - - - - - 67

3.7.4 Sequencing of the C-terminal region of the *pbp*5 gene - - 67

3.8 Conjugal Transfer of Antibiotic Resistance Phenotypes - - 69

3.9 Data Analysis - - - - - - - - - 70

4.0 RESULTS - - - - - - - - 71

4.1 Occurrence and Distribution of *Enterococcus* Species Isolated from Poultry, cattle and Vegetables Farms - - - - 71

4.1.1 Isolation rate of *Enterococcus* species from all sample types- - 71

4.1.2 Distribution and diversity of *Enterococcus* species - - - 71

4.2 Phenotypic Expression of Virulence Factors among *Enterococcus* Isolated from Poultry, cattle and Vegetables Farms - - - 75

4.3 Virulence Encoding Genes among *Enterococcus* Isolated from Poultry, cattle and Vegetables Farms - - - - - 77

4.3.1 Detection of *asa*1, *esp and cyl*A *and gel*E - - - - 77

4.4 Antimicrobial Susceptibility Profile of *Enterococcus* Isolated from Poultry, cattle and Vegetables Farms - - - - - 81

4.4.1 Antimicrobial susceptibility profiles - - - - - 81

4.4.2 Minimum Inhibitory Concentration (MIC) of the ampicillin and gentamicin against *Enterococcus* isolates - - - - - - 81

4.4.3 Frequency of Multidrug resistance and resistance phenotypes among *Enterococcus* isolates - - - - - - - 82

4.5 Antimicrobial Reistance Genes among *Enterococcus* Isolated from Poultry, cattle and Vegetables Farms - - - - - 88

4.5.1 Genes encoding aminoglycoside resistances - - - - 88

4.5.2 Detection of the *pbp*5 gene among the ampicillin resistant isolates - 93

4.5.3 Detection of the *erm*(B) and *tet* genes among the erythromycin and tetracycline resistant isolates - - - - - - - 96

4.6 Transferability of Antimicrobial Resistance Genes - - 102

5.0 DISCUSSION - - - - - - - 104

6.0 CONCLUSION AND RECOMMENDATIONS - - - - 126

6.1 Conclusion - - - - - - - - 126

6.2 Recommendations - - - - - - - 128

REFERENCES - - - - - - - - 129

APPENDICES - - - - - - - - 179

List of Tables

Tables	Titles	Page
Table 2.1	Phenotypic characteristics used for the identification of *Enterococcus* species	18
Table 2.2	Genes encoding resistance to aminoglycoside modifying enzymes among enterococci.	40
Table 2.3	Clinically important vancomycin resistance enterococci phenotypes and genotypes	43
Table 3.1	List of primer and sequences used for the amplification of some virulence and resistance genes from *Enterococcus* isolated from poultry, cattle and vegetable farms in Zaria, Nigeria	65
Table 3.2	List of primers and sequences used for the amplification of antibiotic resistance genes from *Enterococcus* isolated from vegetable and livestock farms in Zaria, Nigeria	68
Table 4.1	Positive samples and isolation rate of *Enterococcus* from poultry, cattle and vegetable farms in Zaria, Nigeria	72
Table 4.2	Occurrence and diversity of *Enterococcus* species isolated from poultry, cattle and vegetable farms in Zaria, Nigeria	73
Table 4.3	Phenotypic expression of virulence factors by the *E. faecium* and *E. faecalis* isolated from poultry, cattle and vegetable farms in Zaria, Nigeria	76
Table 4.4	Antimicrobial resistance profile of *E. faecium* and *E. faecalis* isolates from poultry, cattle and vegetable farms in Zaria, Nigeria	84
Table 4.5	Minimum inhibitory concentration of ampicillin and gentamicin on the *E. faecium* and *E. faecalis* isolated poultry, cattle and vegetable farms in Zaria, Nigeria	85
Table 4.6	Occurrence of multidrug resistant (MDR) phenotype by the *Enterococcus faecalis* and *E. faecium* isolated from poultry, cattle and vegetable farms in Zaria, Nigeria	86
Table 4.7	Distribution of multidrug resistance phenotypes expressed by the *E. faecium* and *E. faecalis* isolates from poultry, cattle and vegetable farms in Zaria, Nigeria	87
Table 4.8	Amino acid polymorphism detected in the C-terminal regions of *pbp5* of 5 *Enterococcus faecium* isolated from livestock and vegetable farms in Zaria, Nigeria	95
Table 4.9	Resistance profile of donor and transconjugants from the mating experiment of Enterococcus isolated from livestock and vegetable farms in Zaria.	103

List of Figures

Figures	Titles	Page
Figure 2.1	16S rRNA dendrogram of showing the phylogenetic position of *Enterococcus* species	11
Figure 2.2	Identification key for *Enterococcus* species	19
Figure 2.3	Major mechanisms of antibiotic resistance in enterococci	35

List of Plates

Plates	Titles	Page
Plate 4.1	Gel electrophoresis image of the PCR detection of *ddl* gene (550bp) among *Enterococcus* isolated from livestock and vegetable farms in Zaria, Nigeria.	74
Plate 4.2	Gel electrophoresis image of the PCR detection of *asa*1 (375bp) among enterococci isolated from livestock and vegetable farms in Zaria, Nigeria	78
Plate 4.3	Gel electrophoresis image of the PCR detection of *cyl*A gene (500bp) among enterococci isolated from livestock and vegetable farms in Zaria, Nigeria	79
Plate 4.4	Gel electrophoresis image of the PCR detection of *gel*E gene (213bp) among enterococci isolated from livestock and vegetable farms in Zaria, Nigeria	80
Plate 4.5	Gel electrophoresis image of the PCR detection of *aac(6´)*-Ie-*aph(2´´)*-Ia gene (348bp) among enterococci isolated from livestock farms in Zaria, Nigeria	89
Plate 4.6	Gel electrophoresis image of the PCR detection of *aph(2´´)*-Ic gene (444bp) among enterococci isolated from livestock farms in Zaria, Nigeria	90
Plate 4.7	Gel electrophoresis image for the PCR detection of *aph(3´)*-III*a* gene (523bp) among enterococci isolated from livestock farms in Zaria, Nigeria	91
Plate 4.8	Gel electrophoresis image of the PCR detection of *ant(4´)*-Ia gene (294bp) among enterococci isolated from livestock farms in Zaria, Nigeria	92
Plate 4.9	Gel electrophoresis image of the PCR detection of *pbp*5 gene (659bp) among ampicillin resistant *Enterococcus* isolated from livestock and vegetable farms in Zaria, Nigeria	94
Plate 4.10	Gel electrophoresis image of PCR detection of the *erm*(B) gene (539bp) among enterococci isolated from livestock farms in Zaria, Nigeria	97
Plate 4.11	Gel electrophoresis image of the PCR detection of *tet*(L) gene (267bp) among enterococci isolated from livestock farms in Zaria, Nigeria	98
Plate 4.12	Gel electrophoresis image of the PCR detection of *tet*(M) gene (406bp) among enterococci isolated from livestock farms in Zaria, Nigeria	99

Plate 4.13 Gel electrophoresis image of the PCR detection of *tet*(K) gene (169bp) among enterococci isolated from livestock farms in Zaria, Nigeria 100

Plate 4.14 Gel electrophoresis image of the PCR detection of *tet*(O) gene (515bp) among enterococci isolated from livestock farms in Zaria, Nigeria 101

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List of Appendices

Appendix	Titles	Page
Appendix I	Isolation of *Enterococcus* from poultry, cattle and vegetable farms in Zaria, Nigeria	179
Appendix II	Phenotypic resistance pattern (resistant to ≤ antimicrobial agent) of the *E. faecium* and *E. faecalis* isolated from poultry, cattle and vegetable farms in Zaria, Nigeria	180
Appendix III	Minimum inhibitory concentration of ampicillin and gentamicin on *E. faecium* and *E. faecalis* isolated from poultry, cattle and vegetable farms in Zaria, Nigeria	181
Appendix IV	Translated nucleotide sequence of sample 17 with amino acid substitutions at position 470.	182
Appendix V	Translated nucleotide sequence of sample 22 with amino acid substitutions at positions 470.	183
Appendix VI	Translated nucleotide sequence of sample 23 with amino acid substitutions at positions 470, 499, 525, 612 and 614.	184
Appendix VII	Translated nucleotide sequence of sample 41 with amino acid substitutions at positions 470, 497, 499, 500 and 502.	185
Appendix VIII	Translated nucleotide sequence of sample 45 with amino acid substitutions at position 470.	186
Appendix IX	Translated nucleotide sequence of sample 51 (control strain) with amino acid substitutions at position 470	187
Appendix X	Positive samples (1 and 4) and positive control (3) indicated by the liquefaction of the gelatin medium and uninoculated/negative control indicated by gelling of the medium after incubation for 10 minutes at 4°C.	188
Appendix XI	Point of eclipse (A) indicating minimum inhibitory concentration of ampicillin for an *Enterococcus* isolate	189
Appendix XII	0.45µm Milipore filter paper and disc inoculated with a mixture of donor and recipient enterococci	190

List of Abbreviations

AFLP	Amplified fragment length polymorphism
AME	Aminoglycoside modifying enzyme
API	Analytical Profile Index
AS	Aggregation substance
CLSI	Clinical Laboratory Standard Institute
DNA	Deoxyribonucleic Acid
GRE	Glycopeptide resistant enterococci
HAI	Hospital Associated Infection
MIC	Minimum inhibitory concentration
MLST	Multi-locus sequence typing
MLVA	Multi-locus variable number of tandem repeats analysis
MSCRAMM	Microbial surface components recognizing adhesive matrix molecules
NaCl	Sodium Chloride
PCR	Polymerase Chain Reaction
PFGE	Pulsed-field gel electrophoresis
PYRase	Pyrrolidonylaryamidase
Q/D	Quinupristin Dalfopristin
RAPD	Random amplified polymorphic DNA analysis
RFLP	Restriction fragment length polymorphism
SB	Slanetz Bartley
SSR	Single sequence repeats
TE	Tris-EDTA buffer

TTC	2,3,5-triphenyl tetrazolium chloride
VRE	Vancomycin-resistant enterococci
WHO	World Health Organization

CHAPTER ONE

INTRODUCTION

1.1 Background

The Genus *Enterococcus* is comprised of Gram positive cocci related to the lactic acid bacteria (Castillo-Rojas *et al.,* 2013; Jahan *et al.,* 2015). They colonize the gastrointestinal tract of animals and humans as commensals and some species are reported to be adapted to soil, water and vegetation (Byappanahalli *et al.,* 2012; Klibi *et al.,* 2013a; Jahan *et al.,* 2015). Some members of the genus are used as probiotics, starter cultures (Jurkovič *et al.,* 2007; Barbosa *et al.,* 2014; Hammad *et al.,* 2015) and as "indicator bacteria" for tracking the evolution and dissemination of virulence and antibiotic resistance traits in different ecosystems (Kasimoglu-Dogru *et al.,* 2010; Silva *et al.,* 2010).

This organism once regarded as a commensal and unassociated with infectious disease has however, evolved in recent times into one of the most important nosocomial pathogen worldwide (Leite-Martins *et al.,* 2015; Yang *et al.,* 2015). The organism has gained notoriety in recent times an important nosocomial pathogen and trafficker of virulence and antibiotic resistance determinants (Hegstad *et al.,* 2010; Wang *et al.,* 2014a, b). Two species; *E. faecium* and *E. faecalis* are responsible for more than 95% of infections caused by this organism (Lindenstrauß *et al.,* 2011; Hayakawa *et al.,* 2013). They are a cause of endocarditis, urinary and genital tract infections, meningitis, wound infections and septicaemia in humans (Murray, 1990; Poulsen *et al.,* 2012). Although, enterococci are rarely reported as a cause of diseases in animals, they have been implicated in cases of diarrhoea and mastitis in cattle (Butaye *et al.,* 2001; Kuyucuoglu, 2011). As intestinal inhabitants, enterococci are under selective pressure from most antibiotics administered

making them prone to developing resistance to commonly used antibiotics (Butaye *et al.*, 2001). Their intrinsic ruggedness allows them to persist in the environment and survive the host defense system via production of virulence traits (van Tyne and Gilmore, 2014).

Enterococci have been implicated in food-borne infections and the spread of antibiotic resistance through the food chain (Trivedi *et al.,* 2011; Chajęcka-Wierzchowska *et al.,* 2012). Although reservoirs for antibiotic-resistant enterococci have not been completely determined, animals, humans, food, and the inanimate environment have been suspected as sources for some resistant clinical isolates (Thal *et al.,* 1995). Recent studies have provided evidence for spread of multiresistant as well as vancomycin and gentamicin resistant *Enterococci faecalis* and *E. faecium* in the US and Europe through the food chain from animals especially cattle, poultry, pigs and vegetable consumption to humans (Hershberger *et al.,* 2005). Studies from some parts of the world have established that livestock production environment, through the abuse of antibiotics, selects for *Enterococcus* clones that harbour clinically important virulence and resistance traits (Nilsson, 2012; Andersson and Hughes, 2014; Radhouani *et al.,* 2014). Entry of resistant and virulent enterococci into the food chain and subsequent colonization of human populations or dissemination of genes encoding these traits via food produce and water contaminated with animal and human faeces raises serious health concern (Ahmad *et al.,* 2011; Jamet *et al.,* 2012). Factors that facilitate the emergence and spread of virulent and resistant enterococci and the corresponding genes include: abuse of antibiotics in livestock production, use of untreated livestock manure for fertilization of vegetable beds and consumption of raw vegetables especially salad vegetables which are consumed without prior heat treatment (Abougrain *et al.,* 2010; Seow *et al.,* 2012; Eraky *et al.,* 2014).

1.2 Statement of the Research Problem

Nosocomial infections are a major cause of morbidity and mortality, particularly in developing countries like Nigeria (Ige *et al.,* 2011). The global emergence of increased virulence and antimicrobial resistance among commensals such as *Enterococcus* and pathogenic bacteria undermines the management of infectious diseases (Perez-Fontain *et al.,* 2011; Yusuf *et al.,* 2014). Members of the genus *Enterococcus* have received increasing attention as opportunistic as well as primary pathogens in humans and sometimes animals (Semedo-Lemsaddek *et al.,* 2013). *Enterococcus* is currently ranked as the third leading cause of nosocomial infections worldwide being implicated as a major cause of endocarditis, wound infections and urinary tract infections (Huycke *et al.,* 1998; Sydnor and Perl, 2011; Rajkumari *et al.,* 2014; Sidler *et al.,* 2014; Khan *et al.,* 2015).

The significance of *Enterococcus* as a nosocomial pathogen is associated with selection of specific clones that evolve through acquisition of antibiotic resistance and virulence determinants by horizontal gene transfer (Wardal *et al.,* 2013). They are known for their notorious capacity to acquire and disseminate resistance genes to other commensals and pathogenic bacteria (Jamet *et al.,* 2012; Santestevan *et al.,* 2015). Notable is the transfer of resistance genes to clinically important bacteria such a methicillin resistance *Staphylococcus aureus* (MRSA), *Lactococcus, Listeria* spp, *Clostridium difficile, E. coli* and streptococci (Doucet-Populaire *et al.,* 1991; Noble *et al.,* 1992; Biavasco *et al.,* 1996; Jasni *et al.,* 2010; Jurado-Rabadan *et al.,* 2014). Therefore, they may contribute significantly to the increasing report of virulence and antimicrobial resistance among commensals and pathogenic organisms in the community and hospital settings.

Reports from different parts of the world have also indicated that enterococci from livestock, manure and vegetables harbor clinically important virulence and resistance genes (Mangalappalli-IIIathu *et al.,* 2010; Micallef *et al.,* 2013; Novais *et al.,* 2013; Garder *et al.,* 2014). However, the emergence and spread of virulent and antibiotic-resistant enterococci by livestock are not fully understood, because of the striking differences observed in different geographical areas (Li *et al.,* 2014a). Abuse of antibiotic in livestock production has been reported to select for resistant and virulent enterococci (Jiménez *et al.,* 2013; Novais *et al.,* 2013). Therefore, the spread of infectious and multidrug resistant enterococci from the livestock environment to food especially vegetables and water through use of livestock manure for fertilizing vegetables and agricultural runoff from farm lands could increase the prevalence of these strains in the human population and become a potential risk to human health (Mishra *et al.,* 2008; Ben Said *et al.,* 2015). These findings raise a major health concern in Nigeria where misuse of antibiotics in animal production, practice of using untreated animal manure for fertilization of soil and consumption of some vegetables without prior heat treatment is relatively common.

Although only rarely have enterococci originating from animals caused human infection (Larsen *et al.,* 2010), the occurrence of the virulent hospital adapted human CC17 clone among animals (Damborg *et al.,* 2009; Freitas *et al.,* 2011; Kwon *et al.,* 2012) and the transfer of virulence and resistance determinants from animal strains to human strains have been widely reported (Hammerum, 2012). Isolates from nosocomial infections in humans have also been reported to be indistinguishable from isolates from animals suggesting that animals could pass virulent and resistant strains to humans (Kwon *et al.,* 2012; Poulsen *et al.,* 2012; Getachew *et al.,* 2013). Livestock therefore, pose potential

sources for antimicrobial resistant and virulent enterococci or their associated genes to enter the food chain and subsequent colonization of human populations via food produce and water contaminated with animal and human wastes (Hammad *et al.,* 2015).

In spite of the above stated threats, the contribution of livestock sources as reservoirs and to the emergence of virulence and antimicrobial resistant enterococci as well as their role in the dissemination of virulent and resistance determinants in Nigeria remain to be evaluated. Data on the occurrence of virulence and antimicrobial resistance among enterococci from livestock sources and vegetables in Nigeria to the best of our knowledge after extensive literature search are scarce. Few studies have investigated the occurrence and characteristics of antimicrobial resistance among enterococci in some parts of Nigeria (Iregbu *et al.,* 2002; Ndubuisi *et al.,* 2011; Olawale *et al.,* 2011; Ogunbanwo *et al.,* 2012; Oladipo *et al.,* 2013; Oladipo *et al.,* 2014; Amaechi and Nwankwo, 2015). However, most of these studies except for that by Amaechi and Nwankwo (2015) are typically targeted and limited to human populations in the hospital environments, vancomycin-resistant enterococci and ready-to-eat foods and such results cannot be readily extrapolated to animals. It is therefore, important to provide information on the ecology and characteristics of *Enterococcus* especially *E. faecalis* and *E. faecium* harboured by livestock in Nigeria. This thesis was therefore initiated, in light of concerns about the increasing prevalence of enterococci and their role in human nosocomial infections, cross-transmission of hospital-adapted enterococci between animals and humans, and a paucity of information on the role livestock may play in the spread of virulent and antibiotic resistant enterococci and encoding genes through the food chain.

1.3 Justification of the Study

The World Health Organization in its 2014 Report entitled "Antimicrobial Resistance: Global Report on Surveillance", identified key gaps in antimicrobial resistance surveillance which include a lack of understanding of the movement of antimicrobial resistant organisms or resistance genes into people from the food chain, and the role of the global food systems in the dissemination of resistance (WHO, 2014). Of recent there has been an increased interest in the study of enterococci due to their ability to cause life threatening nosocomial infection, increased resistance to many antimicrobial agents, emergence and spread of vancomycin resistant enterococci (VRE) and their ability to spread resistance and virulence traits to other bacteria. Enterococci are considered important "indicator bacteria" that could be used to track the evolution of antibiotic resistance in different ecosystems because of their status as reservoir and disseminators of virulence and resistant determinants (Rathnayake *et al.,* 2011; Byappanahalli *et al.,* 2012; Alipour *et al.,* 2014; Ben Said *et al.,* 2016).

Reports of incidence and outbreaks of disease associated with close contact between livestock and humans, contact with livestock wastes, consumption of vegetables and foods of animal origin is increasing (Johnston *et al.,* 2006; Oliveira *et al.,* 2011; Seow *et al.,* 2012). The sources of the organisms involved have in some cases been traced back to contamination by livestock faeces and use of livestock manure for fertilization of soil or irrigation water (Ibekwe *et al.,* 2011) and enterococci have been implicated in some of these outbreaks (Poulsen *et al.,* 2012).

There is increasing number of reports on virulent and antibiotic resistant enterococci from different parts of the world (Jombo *et al.,* 2011; Khan *et al.,* 2015; Ben Said *et al.,* 2016).

However, despite this information and the increased virulence and resistance among pathogens and commensals reported across Nigeria (Tambuwal *et al.,* 2009; Ige *et al.,* 2011; Jombo *et al.,* 2011; Olawale *et al.,* 2011; Ojo-Bola and Oluyege, 2014; Iduh *et al.,* 2015), information on occurrence of enterococci and their role as reservoir and disseminators of virulence and resistance determinants is scarce in Nigeria. This study will help provide information on the ecology and characteristics of *Enterococcus* from livestock and vegetables in Nigeria. It will also help improve clinical decision-making and policies on the use of antimicrobial drugs to avoid treatment failures.

1.4 Research Questions

1. What is the rate of occurrence and species distribution of *Enterococcus* from poultry, cattle, manure, vegetables, soil and irrigation water in Zaria?
2. Do the *E. faecalis* and *E. faecium* if isolated express already established virulence traits (haemolysin, gelatinase, aggregation substance and biofilm production)?
3. Do the *E. faecalis* and *E. faecium* if isolated possess already established virulence associated genes (*cylA, gelE, asa1* and *esp*)?
4. What are the antibiotic susceptibility profiles of the *E. faecalis* and *E. faecium* if isolated from from poultry, cattle, manure, vegetables, soil and irrigation water in Zaria?
5. What is the minimum inhibitory concentration (MIC) of ampicillin and gentamicin against *E. faecalis* and *E. faecium* are resistant to these antibiotics if isolated?
6. Which resistance genes and mechanisms are involved in the resistance of the isolates to some of the antibiotics?

7. Can the isolates transfer the resistance determinants by horizontal gene transfer (conjugation) to susceptible recipients?

1.5 Aim of the Study

The aim of this study is to determine the occurrence of *Enterococcus* species and characterize *Enterococcus faecalis* and *E. faecium* isolates from livestock and vegetable farms in Zaria.

1.6 Objectives of the Study

The objectives of this study were;

1. To culture and isolate *Enterococcus* species from from poultry, cattle, manure, vegetables, soil and irrigation water in Zaria
2. To investigate the phenotypic expression of some virulence traits (production of haemolysin, gelatinase, aggregation substance and biofilm) by *E. faecalis* and *E. faecium* if isolated.
3. To characterize *E. faecalis* and *E. faecium* if isolated for carriage of some virulence genes (*asa1*, *cylA*, *esp*, *gelE*).
4. To determine the antimicrobial susceptibility profiles of *E. faecalis* and *E. faecium* if isolated.
5. To determine the minimum inhibitory concentration of antimicrobial susceptibility profiles of *E. faecalis* and *E. faecium* if isolated.
6. To determine the genes responsible for resistance to some of the antibiotics and the mechanism of resistance among the ampicillin resistant isolates.
7. To investigate the transferability of resistance determinants by the isolates.

CHAPTER TWO

LITERATURE REVIEW

2.1 The Organism *Enterococcus*

Enterococci are natural members of the digestive microbiota in animals and humans, but may be present in soil, surface water and plants (Castillo-Rojas *et al.,* 2013; Ferguson *et al.,* 2013). They have also been detected in some food items such as meat and cheese where their presence is regarded as an indicator of faecal contamination (Martin *et al.,* 2009; Zago *et al.,* 2009; Riboldi *et al.,* 2009). They are also commonly used as probiotics or starter cultures in the food industry due to their ability to produce bacteriocins (Franz *et al.,* 2011; Banwo *et al.,* 2013). *Enterococcus* species have evolved in recent times into one of the most important multidrug resistant nosocomial pathogen worldwide (Sood *et al.,* 2008; Sydnor and Perl, 2011; Guzman Prieto *et al.,* 2016). They are opportunistic pathogens which are harmless in healthy individuals but cause infections in immunocompromised patients, very young and elderly and patients in intensive care units (Garsin and Lorenz, 2013). Enterococci are ranked third among the most prevalent bacterial organisms encountered in nosocomial infections and implicated as an important cause of bacteraemia, endocarditis, meningitis, wound and urinary tract infections (Swaminathan and Alangaden, 2010; Kenzaka *et al.,* 2013).

Enterococcus faecium and *Enterococcus faecalis* are the two species predominantly responsible for enterococcal infections (Billington *et al.,* 2014; Yu *et al.,* 2015). In addition to nosocomial infections caused by its arsenal of virulence factors, enteroccci are reported to easily develop resistance to most antimicrobial agents resulting in treatment failures (Peterson *et al.,* 2002; Hollenbeck and Rice, 2012).

2.2 Taxonomy of the Genus *Enterococcus*

The Genus *Enterococcus* can be classified as a heterogenous group of bacteria as they share many characteristics with the genera *Aerococcus, Carnobacterium, Globicatella, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus*, and *Weissella* (Franz *et al.,* 1999; Holzapfel *et al.,* 2006). This heterogeneous nature has resulted in problems with taxonomy of species belonging to the genus (Stiles and Holzapfel, 1997; Lebreton *et al.,* 2014). The genus was initially classified under faecal streptococci as *S. faecium* and *S. faecalis*. However, in 1984, 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). The 16S rRNA sequencing data enabled the construction of a 16S rRNA-dendrogram showing the relationship between *Streptococcus, Enterococcus* and *Lactococcus* species (Figure 2.1).

The number of *Enterococcus* species fluctuates from time to time as new species are discovered and the most recent inclusions are *E. xiangfangesis* (Li *et al.,* 2014b) and *E. olivae* (Lucena-Padros *et al.,* 2014) which were added in 2014. To date the genus is comprised of 54 species and 2 subspecies namely: *E. alcedinis, E. aquimarinus, E. asini, E. avium, E. caccae, E. camelliae, E. canintestini, E. canis, E. casseliflavus, E. cecorum, E. columbae, E. devriesei, E. diestrammenae, E. dispar, E. durans, E. eurekensis, E. faecalis, E. faecium, E. flavascens, E. gallinarum, E. gilvus, E. haemoperoxidus, E. haemanniensis, E. hirae, E. italicus, E. lemanii, E. malodoratus, E. moraviensis, E. mundtii, E. olivae,*

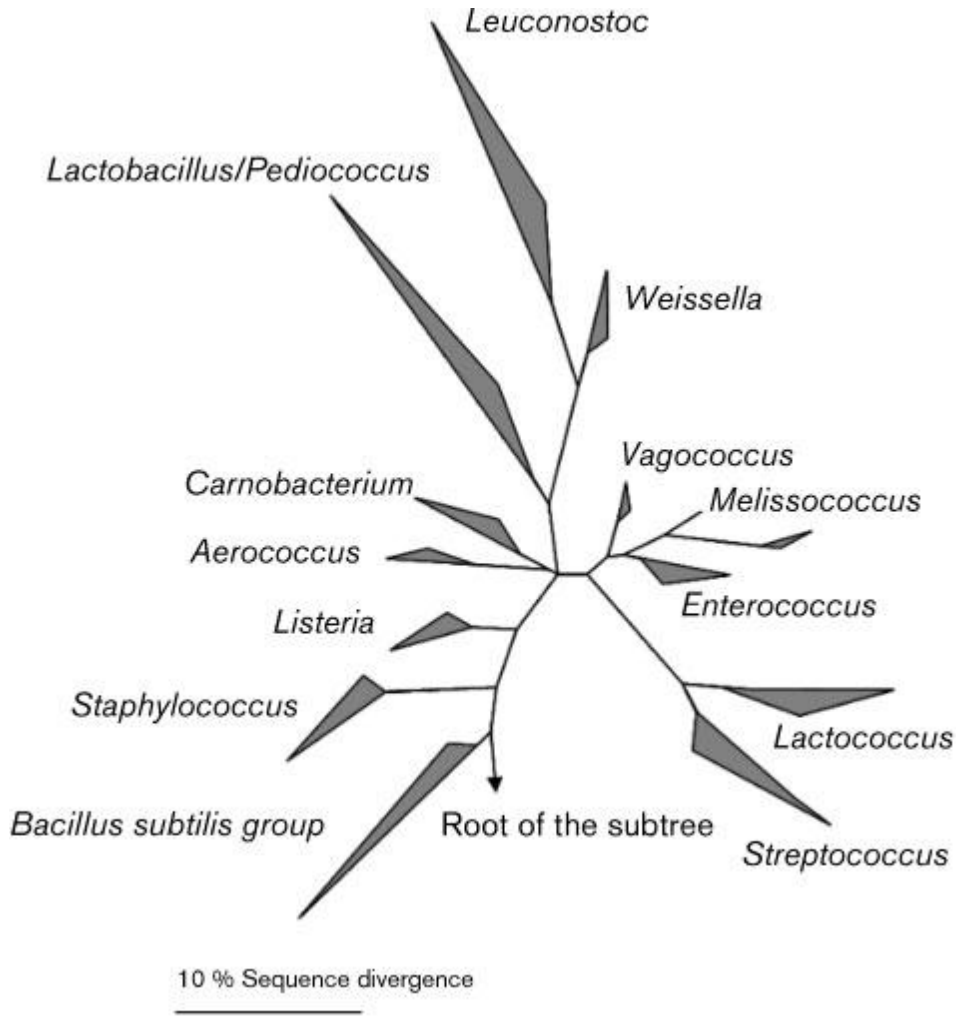


Figure 2.1: 16S rRNA dendrogram showing the phylogenetic position of *Enterococcus* species (Source: Klein, 2003).

E. pallens, E. termitis, E. phoeniculicola, E. plantarum, E. porcinus, E. pseudoavium, E. quebecensis, E. raffinosus, E. ratti, E. rivorum, E. rotai, E. saccharolyticus, E. saccharolyticus subsp. saccharolyticus, E. saccharolyticus subsp. taiwanensis, E. saccharominimus, E. seriolicida, E. solitaries, E. sulfureus, E. thailandicus, E. ureilyticus, E. ureasiticus, E. viikkiensis, E. villorum and *E. xiangfangesis* (accessed on the 16th of October 2015 at 5:29pm from http://www.bacterio.net/enterococcus.html).

2.3 Characteristics of *Enterococcus* Species

The Genus *Enterococcus* consists of Gram positive lactic acid bacteria, facultatively anaerobic, non-sporing and catalase negative cocci occurring either singly, in pairs, or short chain (Facklam and Elliott, 1995; Kajihara *et al.,* 2015). They differ from other Gram positive catalase negative cocci by their ability to grow at temperature of between 10 - 45°C (optimum 35°C), survive 6.5% NaCl concentrations, pH 4.0 - 9.6, 40% bile concentration and hydrolyze esculin (Qadri *et al.,* 1980; Chuard and Reller, 1998). They also survive heating at 60°C for 30 minutes, 71°C for 10 min, and 80°C for 1 min (Giraffa, 2002; McAuley *et al.,* 2012). These make them able to survive some food processing conditions and have been implicated in food spoilage and spread of resistance and virulence determinants through the food chain (Oprea and Zervos, 2007). As facultative organisms, enterococci grow under reduced or oxygenated conditions. They lack a Kreb's cycle and respiratory chain and thus are strict fermenters (Huycke *et al.,* 1998). However, *E. faecalis* is an exception as it can use exogenous hemin to produce d, b, and o type cytochromes (Baureder and Hederstedt, 2012). These cytochromes are only expressed under aerobic conditions and in the presence of exogenous hemin and provide a growth advantage to *E. faecalis* during aerobic growth and colonization of unsuitable sites (Winstedt *et al.,* 2000).

Motility is observed among few species such as *E. casseliflavus, E. gallinarium* in addition to pigment production by *E. casseliflavus, E. sulfureus* and *E. mundtii.*

Some exceptions to the general characteristic include the inability of *E. cecorum, E. columbae, E. avium* to grow in the presence of 6.5% NaCl, absence of Lancefield group D antigen among the "*E. avium* species group" (*E. cecorum, E. columbae, E. dispar* and *E. saccharolyticus*) and the absence of pyroglutamyl aminopeptidase (Pyrase) activity an important enzyme that aids in the identification of members of the genus *Enterococcus* including *E. cecorum, E. columbae, and E. saccharolyticus* (Domig *et al.*, 2003a).

2.4 Genome Structure and Characteristics of *Enterococcus* Species

The genus is characterized by a low guanine plus cytosine (G+C) content of 30 - 38 mol % (Lam *et al.,* 2012; Shiwa *et al.,* 2014). Data on the enterococcal genome generated to date has been targeted towards the two enterococcal species; *E. faecalis* and *E. faecium* that are most associated with clinical disease (Qin *et al.,* 2012). The first enterococcal genome sequence (*Enterococcus faecalis* V583) was first reported in 2003 (Paulsen *et al.,* 2003). However, in recent times complete or draft genome sequences of various enterococcal strains and species numbering up to hundreds have been carried out and reported (**Bourgogne *et al.,* 2008;** Shiwa *et al.,* 2014).

The genome size of the *Enterococcus* is about 2.7MB (2.50 Mb - 3.14 Mb) with about 2,703 ORFs (2,587 - 3,118), 6 copies of ribosomal rRNA, 62 tRNAs, and 32 other non-coding RNAs (Bourgogne *et al.,* 2008; Kim *et al.,* 2012a; Qin *et al.,* 2012; Tytgat *et al.,* 2016; Quintana *et al.,* 2016). *E. faecalis* genome is made up of about 3093 genes (van Schaik and Willem, 2010) compared to the 3043 genes of *E. faecium* (Lam *et al.,* 2012) and

a core genome content of 2057 - 2092 genes (McBride *et al.,* 2007; Sohlheim *et al.,* 2009) and 2172 - 2192 genes (van Schaik and Willem, 2010) respectively. About 30% of the content of both genomes is not core but made up of accessory genes, thus the enormous genome plasticity of the genus (van Schaik *et al.,* 2010; Palmer *et al.,* 2010a). This plasticity or flexibility of the genome is believed to significantly contribute to its adaptation to the hospital environment and uptake of virulence and resistance genes. Mobile genetic elements (MGE), particularly IS elements are believed to increase the genome plasticity and facilitate adaptive changes, thus enhancing the genetic variability in the hospital adapted strains (van Schaik *et al.,* 2010). Hospital isolates have been proposed to be enriched with about 150 genes, transposases and IS elements, specifically IS*16*, which possibly confers genomic plasticity to these strains (Leavis *et al.,* 2007). The IS*16* is reported to be exclusively found among the hospital-associated isolates of *E. faecium*; specifically CC17-related strains (Werner *et al.,* 2011).

2.5 Isolation Methods for Enterococci

Enterococci are facultative anaerobes and grow best at an optimum temperature of between 35 - 37°C. It can grow on any blood agar base supplemented with 5% animal blood, although horse blood is preferred when testing for haemolytic capability (Domig *et al.,* 2003a). Agars and broths used in selective isolation of *Enterococcus* are available from different companies and trade names. However, they all contain similar basic ingredients which selectively inhibit the growth of other organisms while supporting the growth of *Enterococcus* (Domig *et al.,* 2003b). Elevation of the incubation temperatures to between 42 and 44 °C and the duration of incubation (18 - 48h) may influence the selectivity of some enterococci media and reduce the growth of the background microflora. Some

additives are also added to reduce the growth of background microflora some of which include, sodium azide at a concentration of 0.2 - 0.5% which is the most widely used selective additive in *Enterococcus* media (Domig *et al.,* 2003b). It functions by inhibition of the enzyme systems (catalase, cytochrome *c* oxidase) involved in the electron transport of gram negative and other catalase positive organisms. Others are thallium acetate, potassium tellurite, potassium thiocyanate, dyes, e.g., ethyl violet, crystal violet, 2,3,5-triphenyl tetrazolium chloride (TTC) or antibiotics, e.g., kanamycin, gentamicin, nalidixic acid, oxolinic acid, polymyxin, or colistin. Tetrazolium is also added as an indicator to some agar such as Slanetz Bartley (SB) medium and this causes *E. faecalis* colonies to become bright red, whereas *E. faecium* bacterial colonies show only a partial reaction, producing a colony with a red center and a pale halo (Domig *et al.,* 2003b; Fisher and Philip, 2009).

2.6 Phenotypic Identification of *Enterococcus* Species

The enterococci share similar phenotypic and biochemical characteristics with the streptococci. Both are Gram-positive cocci, facultative anaerobes, catalase and oxidase negative. However, *Enterococcus* possesses in addition the ability to produce pyrrolidonylaryamidase (PYRase), hydrolyze bile esculin, growth under high (45°C) and low temperature extremes, growth in 6.5% NaCl and growth on MacConkey agar (Facklam and Elliott, 1995; Devriese *et al.*, 1987; Domig *et al.*, 2003a; b).

The conventional biochemical test-tube approach, although time-consuming and labour-intensive is the most popular method for the phenotypic identification of species. Popular methods are described by Facklam and Collins (1989) and Manero and Blanch (1999). Results from this method are comparable with those from molecular techniques like

polymerase chain reaction (Cartwright *et al.,* 1995; Moore *et al.,* 2006). Facklam and Collins (1989) separated *Enterococcus* species into five groups on the basis of acid formation in mannitol and sorbose broths, and arginine hydroysis. Members of Group I form acid in both mannitol and sorbose broths but do not hydrolyze arginine and the species consists of *Enterococcus avium*, *Enterococcus malodoratus*, *Enterococcus raffinosus*, *Enterococcus pseudoavium*, *Enterococcus saccharolyticus*, *Enterococcus pallens*, and *Enterococcus gilvus*. Members of Group II form acid in mannitol but not sorbose broth and hydrolyze arginine. Five species belong to this group and consists of *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus casseliflavus*, *Enterococcus mundtii*, and *Enterococcus gallinarum*. Members of Group III hydrolyze arginine but do not form acid in mannitol, sorbitol or sorbose broths. The group includes; *Enterococcus durans*, *Enterococcus porcinus*, *Enterococcus ratti*, *Enterococcus hirae*, *Enterococcus dispar*, and mannitol-negative variants of *E. faecalis* and *E. faecium*. The enterococci in Group IV do not produce acid in mannitol or sorbose broths and do not hydrolyze arginine. They are *Enterococcus asini*, *Enterococcus sulfureus*, and *Enterococcus cecorum*. Group V members consist of the variant strains of *Enterococcus casseliflavus*, *Enterococcus gallinarum*, and *Enterococcus faecalis* that fail to hydrolyze arginine.

Other useful tests include: the pigmentation test, pyruvate utilization test and tolerance of tellurite (Murray *et al.,* 2003). The pigmentation test aids in the identification of *E. casseliflavus*, *E. mundtii*, *E. pullins*, *E. gilvus* and *E. sulfureus*. These enterococci produce a yellow pigment that can be detected on several different media. The pyruvate utilization test aids in the differentiation of *E. faecalis* and *E. faecium*. This test is also used to help differentiate between *E. faecalis* variant strains and *E. hirae.* The tellurite tolerance

test aids in the differentiation of *E. faecalis* and *E. faecium*. *E. haemoperoxidus* is variable in the mannitol reaction and may be in group II or III. Murray *et al.* (2003) compiled a scheme based on these tests above for the identification of *Enterococcus* species (Table 2.1) The scheme of Manero and Blanch (1999) is based on the use of 12 biochemical tests for the rapid identification of clinical and environmental species of *Enterococcus* and the threshold of identification is over 99%, with some exceptions of 87, 91.5, and 97% for *E. avium*, *E. hirae*, and *E. durans*, respectively (Figure 2.2). Numerous commercial biochemical identification kits developed over the years for the identification of *Streptococcus* have been adapted for *Enterococcus* species identification. Commercial identification kits, such as the API Rapid ID 32 Strep and BBL Crystal identification gram-positive ID kits, API 20 STREP, API 50CH, API *zym* and automated identification systems, such as the VITEK gram-positive identification and Phene Plate PhP Plate systems have been developed to provide for rapid identification of enterococci based on reaction to a panel of biochemicals (Brtkova *et al.,* 2010). Results using these kits can be obtained within 4 - 24h, they are easier to use, less expensive and standardized (Hudson *et al.*, 2003). However, these kits suffer from some limitations such as the identification of newer species or atypical species and its inability to identify isolates from animal and the environment and a limited database (Ruoff *et al.,* 1990; Morrison *et al.,* 1997; Jackson *et al.,* 2004a; Hudson *et al.,* 2003), because they were developed for identification of human clinical isolates (Ruoff *et al.,* 1990). Also agreement of between 61 – 90% in comparison to PCR has been reported (Jackson *et al.,* 2004a; Hudson *et al.,* 2003; Gomes *et al.,* 2007; Furlaneto-Maia *et al.,* 2014).

Table 2.1: Phenotypic characteristics used for the identification of *Enterococcus* species adapted from Murray *et al.* (2003).

Species	Phenotypic characteristic[a]											
	MAN	SOR	ARG	ARA	SBL	RAF	TEL	MOT	PIG	SUC	PYU	MGP
Group I												
E. avium	+	+	-	+	+	-	-	-	-	+	+	V
E. raffinosus	+	+	-	+	+	+	-	-	-	+	+	V
E. gilvus	+	+	-	-	+	+	-	-	+	+	+	-
E. pallens	+	+	-	-	+	+	-	-	+	+	-	+
E. saccharolyticus[b]	+	+	-	-	+	+	-	-	-	+	-	+
E. malodoratus	+	+	-	-	+	+	-	-	-	+	+	V
E. pseudoavium	+	+	-	-	+	-	-	-	-	+	+	+
E. devriesei[b]	+	+	-	-	+	-	-	-	-	+	-	-
E. hawaiiensis	+	+	-	-	+	-	-	-	-	-	+	-
Group II												
E. faecium	+[d]	-	+	+	V	V	-	-	-	+[d]	-	-
E. casseliflavus	+	-	+[d]	+	V	+	-[d]	+[d]	+[d]	+	V	+
E. gallinarum	+	-	+[d]	+	-	+	-	+[d]	-	+	-	+
E. mundtii	+	-	+	+	V	+	-	-	+	+	-	-
E. faecalis	+[d]	-	+[d]	-	+	-	+	-	-	+[d]	+	-
E. haemoperoxidus[b]	+[c]	-	+[c]	-	-	-	-	-	-	+	-	+
E. sanguinicola	+	-	+	-	-	-	+[a]	-	-	+	-	-
Lactococcus sp.	+	-	+	-	-	-	-	-	-	V	-	-
E. silesiacus	+	-	+	-	-	-	-	-	-	+	-	-
Group III												
E. dispar	-	-	+	-	-	+	-	-	-	+	+	+
E. canintestini[b]	-	-	+	-	-	+	-	-	-	+	+	+
E. hirae	-	-	+	-	-	+	-	-	-	+	-	-
E. durans	-	-	+	-	-	-	-	-	-	-	-	-
E. ratti	-	-	+	-	-	-	-	-	-	-	-	-
E. villorum	-	-	+	-	-	-	-	-	-	-	-	-
Group IV												
E. cecorum[b]	-	-	-	-	-	+	-	-	-	+	+	-
E. phoeniculicola[b]	-	-	-	+	-	+	-	-	-	+	-	+
E. aquimarinus[b]	-	-	-	+	-	+	-	-	-	+	-	+
E. sulfureus	-	-	-	-	-	+	-	-	+	+	-	+
E. asini[b]	-	-	-	-	-	-	-	-	-	+	-	-
E. caccae	-	-	-	-	-	-	-	-	-	+	+	+[c]
E. termitis[b]	-	-	-	-	-	-	-	-	-	+	+	+
Group V												
E. canis[b]	+	-	-	+	-	-	-	-	-	+	+	+
E. columbae[b]	+	-	-	+	+	+	-	-	-	+	+	-
E. moraviensis[b]	+	-	-	+	-	-	-	-	-	+	+	+
E. hermanniensis	+	-	-	-	-	-	-	-	-	-	-	-
E. italicus	V	-	-	-	V	-	-	-	-	+	+	+
Vagococcus fluvialis	+	-	-	-	+	-	-	+	-	+	-	+

Man: Mannitol, SOR: Sorbose, ARG: Arginine, ARA: Arabinose, SBL: Sorbitol, RAF: Raffinose, TEL: Tellurite, MOT: Motility, PIG: Pigmentation, SUC: Sucrose, PYU: Pyruvate, MGP: Methylglucopyranoside

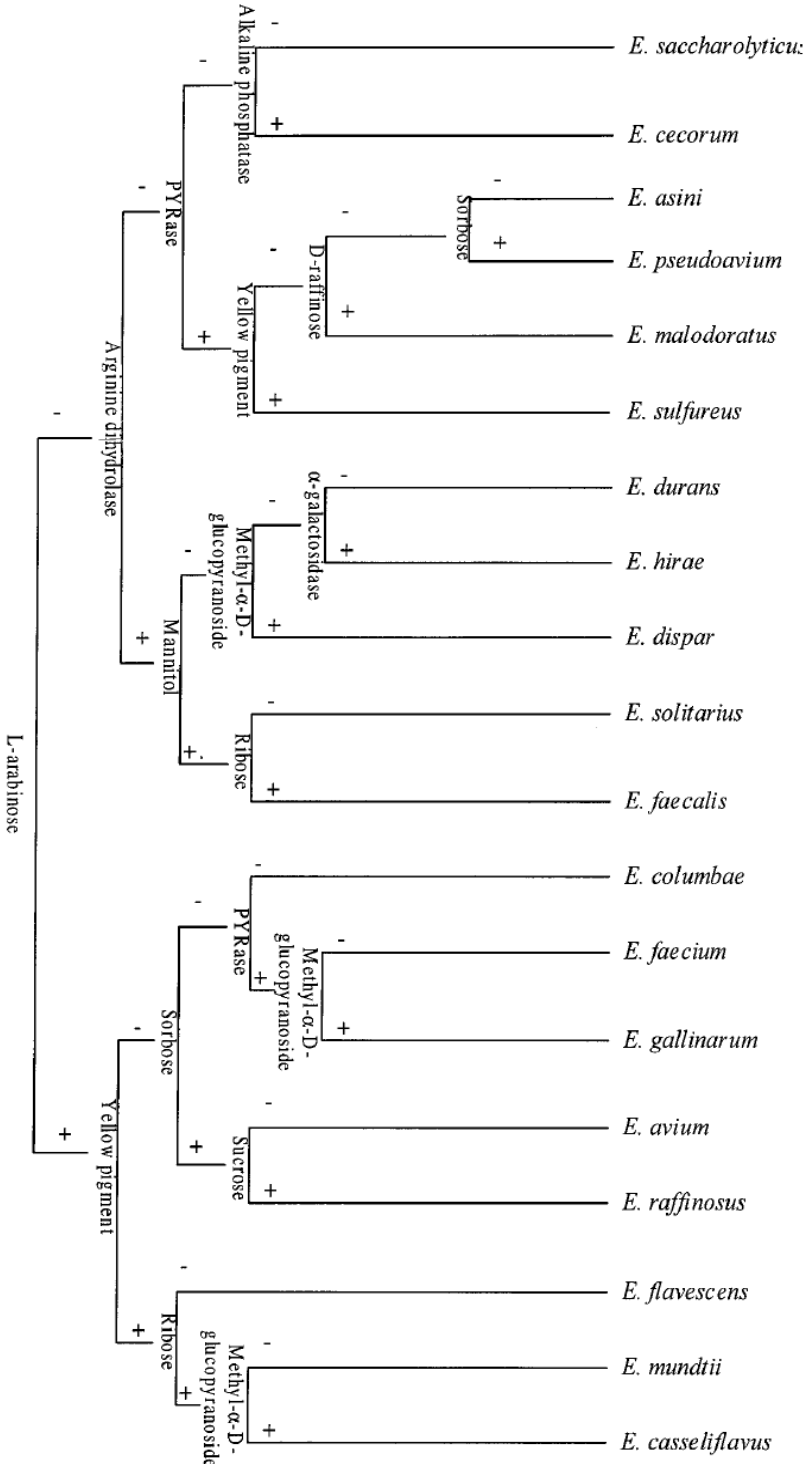


Figure 2.2: Identification key for *Enterococcus* spp
Adapted from Manero and Blanch (1999). PYRase; pyrrolidonyl aminopeptidase.

2.7 Genotypic Identification and Typing of *Enterococcus* Species

In recent years, in an effort to improve the specificity and increase the rapidity with which clinical infections are diagnosed, molecular methods utilizing the polymerase chain reaction have been used for genotypic identification of enterococci to the species level. However, these methods are not a common finding in clinical settings in developing countries except in research facilities where they are used for identification, strain typing and in the epidemiological study of outbreaks.

A number of PCR amplification, sequencing and sequence comparison of target genes have also allowed differentiation of species of enterococci (Jackson *et al.,* 2004a). The most common one are those targeting the 16S rRNA, *sodA* (manganese-dependent superoxide dismutase) and *ddl* (d-alanine-d-alanine ligase) (Jackson *et al.,* 2004a). These genes are essential constituents of the bacteria genome and are therefore preferred for diagnostic purposes (Jackson *et al.,* 2004a). Other techniques include the Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) which is based on analysis of the protein composition of the bacteria resulting in production of a species-specific spectra of peptides and protein masses considered as fingerprints of the bacteria and identified by mass spectrometry (Raemy *et al.,* 2013). Microarrays (Lehner *et al.,* 2005; Hampagne *et al.,* 2011), PCR-restriction fragment length polymorphisms (PCR-RFLP) (Scheidegger *et al.,* 2009), Intergenic transcribed spacer (ITS) –PCR of the 16S – 23S rRNA intergenic region (Tyrrell *et al.,* 1997; Nowakiewicz *et al.,* 2015), DNA-DNA hybridization, Fluorescence *in situ* hybridization using fluorescence labeled oloigonucleotide probes targeting the rRNA (Waar *et al.,* 2005; Wellinghausen *et al.,* 2007).

Molecular typing of enterococci during outbreak is commonly performed by pulsed-field gel electrophoresis (PFGE). This technique involves macrorestriction of the genomic DNA using restriction enzymes followed by a subsequent separation of the restriction fragments by pulse field electrophoresis. This technique offers a high reproducibility and has a high discriminatory power, however it suffers from some drawbacks such as its laborious nature, low inter-laboratory reproducibility resulting in unsuitability for global, long term epidemiologic studies and phylogenic or population structure studies (Ruiz-Garbajosa *et al.,* 2006). Although, this technique is still currently the gold standard for investigation of the clonal relationships and genetic diversity among enterococci, other molecular techniques have emerged in the last decade (Jureen *et al.*, 2004; Werner, 2013). These techniques are majorly based on polymorphism in DNA patterns, partial or whole genome analysis. Examples include amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA analysis (RAPD), multi-locus variable number of tandem repeats analysis (MLVA) and multi-locus sequence typing (MLST) (Chiew and Hall, 1998; Homan *et al.,* 2002; Burtscher *et al.,* 2006; Al-Badah *et al.,* 2015).

MLST identifies alleles from DNA sequences of the internal fragments of house keeping genes (Homan *et al.,* 2002). It involves the sequencing of seven enterococcal house keeping genes; *gyd* (glyceradehyde-3-phosphate dehydrogenase), *pstS* (phosphate-ATP-binding cassette transporter*), gki* (glucokinase), *aro* (shikimate-5-dehydrogenase), *gdh* (glucose-6-phosphate dehydrogenase), *xpt* (xanthine phosphoribosyltransferase) *and yiq* (acetyl-CoA acetyltransferase*)* for *E. faecalis* (Homan *et al.,* 2002) and *adk* (adenylate

kinase), *atpA* (ATP synthase), *ddl* (D-alanine: D-alanine ligase), *gyd* (glyceradehyde-3-phosphate dehydrogenase), *gdh* (glucose-6-phosphate dehydrogenase), *purK* (phosphoribosyl-aminoimidazolcarboxylase) *and pstS* (phosphate-ATP-binding cassette transporter) for *E. faecium* (Ruiz-Garbajosa *et al.,* 2006). MLST is highly reproducible, provides an unambigous nomenclature for genotypes and clones in addition to easy storage of these data in a form that can be exchanged via the internet (www.mlst.net) between laboratories (Top *et al.,* 2004; Homan *et al.,* 2002; Ruiz-Garbajosa *et al.,* 2006). However, it is labour intensive, time consuming, expensive and suffers from impracticability and application outside major research facilities (Top *et al.,* 2004). MLVA is based on the detection of differences/genetic variations in the variable number of tandem repeats (also referred to as single sequence repeats, SSR) present at multiple loci on the chromosome of enterococci (Buhnik-Rosenblau *et al.,* 2013). These genetic variations are detected by PCR using primers targeting the flanking regions of the tandem repeats (Top *et al.,* 2008). The MLVA types are discriminated by the gains or loss of discrete repeats which yield unambiguous numerical data. Thus the target repeats used are usually those not subject or resulting from selective pressure. The discriminatory power of MLVA is comparable to that of PFGE (Top *et al.,* 2008). AFLP involves the detection of polymorphism among small restriction fragments originating from both variable and conserved DNA sequences in the enterococcal genome (Routsi *et al.,* 2003). This method combines restriction enzyme analysis of the genomic DNA with PCR amplification (Willem *et al.,* 2000; Jureen *et al.,* 2004). A variation of the AFLP is the fluorescence based analysis of the fragment (fbAFLP) (Antonishyn *et al.,* 2000). Although rapid, the AFLP technique suffers from poor reproducibility and impossible inter-laboratory data exchange (Top *et al.,* 2004). The RAPD analysis involves amplification of discrete regions in the enterococcal genome using

short oligonucleotide primers of abirtary sequences (Barbier *et al.,* 1996). It has been extensively used in genotyping of enterococcus from different sources (Banerjee, 2013; Al-Badah *et al.,* 2015; Nieto-Arribas *et al.,* 2011; Martins *et al.,* 2009; Barbosa *et al.,* 2009).

2.8 Ecology and Epidemiology of *Enterococcus* Species

The origin of *Enterococcus* species vary from animal or human to environmental sources (Fisher and Philip, 2009; Boehm and Sassoube, 2014). They are an integral part of the gastrointestinal microflora of both animals and humans and their distribution is very similar in these sources (Byappanahalli *et al.,* 2012). *E. faecium* and *E. faecalis* are the most common in the human gastrointestinal tract, *E. faecium* in production animals while *E. mundtii, E. casseliflavus* and *E. gallinarum* are adapted to plant sources (Lebreton *et al.,* 2014). *E. cecorum* is a member of the enterococcal flora of pigs and poultry; *E. hirae* is a frequent inhabitant of the porcine gut and may occur in poultry, cattle, dogs, and cats, *E. asini* is specific for donkeys (Aarestrup *et al.,* 2002). *E. faecalis* and *E. faecium* are also regularly isolated from cheese, fish, sausages, minced beef, and pork due to contamination and the ability of *Enterococcus* species to survive the heating process (Franz *et al.,* 1999; Giraffa, 2002; Fontana *et al.,* 2009; Nieto-Arribas *et al.,* 2011). Urban sewage and farmland using pig manure and crops produced from such land have been reported to be 100% positive for *Enterococcus* in comparison to 33% on lands to which animal fertilizers were not applied (Kuhn *et al.,* 2003). The distribution of *Enterococcus* species has also been reported to vary with geographical location (Diarra *et al.,* 2010; Layton *et al.,* 2010). In Europe the most common species isolated from both clinical and environmental sources are *E. faecalis* and *E. faecium* (Goosens *et al.,* 2003). Also between 2003 and 2005 in some parts of Europe such as Denmark and Sweden, *E. hirae* was the predominant species

isolated from slaughtered animals as reported by Kuhn *et al.,* 2003; 2005), a situation which is gradually changing with the increasing isolation of *E. faecium* instead of *E. hirae* (Hammerum *et al.,* 2007).

2.9 Clinical Significance of *Enterococcus*: Virulence and Antimicrobial Resistance

Virulence and antimicrobial resistance of *Enterococcus* spp. is linked to particular species, with *E. faecium* and *E. faecalis* being the most relevant members of this genus with regard to clinical significance (Billington *et al.,* 2014; Yu *et al.,* 2015). The possession of virulence factors and resistance to antibiotics by *Enterococcus* are considered as a major concern (Nami *et al.,* 2015). They are also important for the assessment of safety or categorization of enterococci as safe or unsafe especially in the food industry (Araujo and Ferreira, 2013). *Enterococcus* may harbour antibiotic resistance or virulence genes that can be transferred to pathogenic or other commensal bacteria causing nosocomial infection that are difficult to treat (Yuste *et al.,* 2014). One of the most important resistance is that to vancomycin, a very potent antibiotic and used as the last line of defense in treatment of clinical infections by multidrug-resistant pathogens (Praharaj *et al.,* 2013).

Nococomial infections, also referred to as "Hospital-acquired infections (HAI)" or "Health care associated infections," are defined by the World Health Organization as an infection that was not present or incubating at the time of admission but present after 48 hours of hospitalization or stay at a healthcare facility. It also includes infections that occurred within 4 weeks following discharge from a health facility or occupational infections among staff of the facility (WHO, 2002; Hensley and Monson, 2015). Hospital-

acquired infections (HAIs) have been recognized as the most frequent adverse consequences of healthcare delivery worldwide, threatening the safety of both patients and healthcare workers (Tao *et al.,* 2014). Reports have shown that 7 and 10 of every 100 hospitalized patients in developed and developing countries respectively acquire at least one nosocomial infection during hospitalization (WHO, 2015).

Enterococcus has over the last two decades emerged as an important nosocomial pathogen (von Gottberg *et al.,* 2000; van Schaik *et al.,* 2010; Sujatha and Parga, 2012). Nosocomial infection caused by this organism have been associated with increased patient morbidity, mortality, and health care costs especially among the very young, elderly and immunocompromised (Argemi *et al.,* 2013; Kenzaka *et al.,* 2013). Although infections in veterinary settings are far less reported, various studies have also identified *Enterococcus* as a major nosocomial pathogen in veterinary settings (Ghosh *et al.,* 2012; Chung *et al.,* 2014; Milton *et al.,* 2015). The emergence of *Enterococcus* as a leading nosocomial pathogen paralleled the emergence of strains within the species that are resistant to most antimicrobial drugs used to treat human infections (Shepard and Gilmore, 2002). Infections caused by *Enterococcus* present a treatment challenge to clinicians because this organism has a natural (intrinsic) resistance to a large number of antimicrobial agents and can acquire high level resistance traits (Bethea *et al.,* 2004; Rossi, 2011; Ramaswamy *et al.,* 2013). Notable are vancomycin-resistant strains (VRE) and the emerging gentamicin and ampicillin resistant strains all of which display resistance to almost all clinically available antibiotics used for their treatment and control (Arias *et al.,* 2010; Sierr-Hoffman *et al.,* 2012; Sakoulas *et al.,* 2013; Wang *et al.,* 2014a, b; O'Driscoll and Crank, 2015).

As opportunistic pathogens, enterococci are currently ranked third as a leading cause of nosocomial infections (Ramaswamy *et al.,* 2013; Cornejo-Juarez *et al.,* 2015). *E. faecium* and *E. faecalis* are the most important causes of nosocomial infections associated with *Enterococcus* species (Billington *et al.,* 2014; Yu *et al.,* 2015). They are responsible for about 95% of all enterococcal infections (Salem-Bekhit *et al.,* 2012; Aamodt *et al.,* 2015). They have been implicated in numerous outbreaks of nosocomial infections in both veterinary and human facilities (Sagel *et al.,* 2008; Johnson *et al.,* 2010; Tuon *et al.,* 2011; Escaut *et al.,* 2013). HAIs, such as bacteremia, endocarditis, wound, urinary tract and skin or soft tissue infections, are among the most frequent infections caused by *Enterococcus* (Rajkumari *et al.,* 2014; Sandoval *et al.,* 2015). Other species that have been reported although rarely to cause clinical infections include *E. mundtii*, *E. hirae, E. gallinarum* and *E. casseliflavus* (Reid *et al.,* 2001; Anghinah *et al.,* 2013; Bourafa *et al.,* 2015). Unique characteristic such as; the ability to survive the harsh hospital environment, possession of virulence traits and the ability to acquire and transfer virulence and resistance determinants to other bacteria gives *Enterococcus* the ability to successfully colonize, persist, spread and cause infection in the hospital environment (Zoletti *et al.,* 2011; Biswas *et al.,* 2014) .

The clinical manifestion of enterococcal infection in animals is well characterized in poultry. The acute form of enterococcosis in poultry present clinical signs related to septicemia and include depression, lethargy, ruffled feathers, diarrhea, and a decrease in egg production. The subacute/chronic form presents with depression, lameness, and head tremors. If untreated, most affected birds die (Chadfield et al., 2004). Egg transmission or fecal contamination of hatching eggs can often lead to late embryo mortality, and an

increased number of hatchlings are unable to "pip" through the shell at hatch (Oktay et al., 2009; Stalker *et al.,* 2010). Pericarditis, hepatitis, femoral head necrosis and vertebral osteomyelitis causing lameness and hind-limb paresis are common pathologies associated with enterococcosis in poultry (Oktay et al., 2009; Jung and Rautenschlein, 2014). Enterococci have also been implicated as a cause of mastitis in cattle (Kateete *et al.*, 2013), however in dogs, cats and humans it does not present specific signs but is associated with wound infections, urinary tract infection, hepato-biliary sepsis, bacteremia, endocarditis and periodontal disease (Poulsen *et al.,* 2012; Oliveira *et al.,* 2016; Semedo-Lemsaddek *et al.,* 2016).

The reported incidence of nosocomial infections caused by enterococci in Nigerian hospitals vary between 1.8% to 22.5% and antimicrobial resistant strains have been implicated in most of these infections (Olawale *et al.,* 2011; Ndubuisi *et al.,* 2011; Olajubu *et al.,* 2012).

2.10 Virulence Factors in *Enterococcus* Infection

In producing infection, enterococci must be able to colonize host tissues, invade and evade the host immune defense mechanisms and produce pathological changes (Banerjee and Anupurba, 2015). Virulence of enterococcal species is mediated by many factors which may act synergistically to produce disease (Nami *et al.,* 2015). Most prominent among these virulence determinants are the cytolysins (hemolysin), enterococcal surface protein, aggregation substance (AS), hyaluronidase, gelatinase, microbial surface components recognizing adhesive matrix molecules (MSCRAMM), enterococcal capsule, extra cellular

superoxide and cell wall polysaccharides (Beceiro *et al.,* 2013; Banerjee and Anupurba, 2015).

2.10.1 Aggregation substance (AS)

AS is a pheromone-inducible surface glycoprotein that mediates aggregate formation during conjugation, thus aiding in plasmid transfer as well as adhesion to eukaryotic cell surfaces (Schlievert *et al.,* 1998; Sava *et al.,* 2010). It is an *E. faecalis* surface-anchored protein and facilitates the transfer of plasmids between cells by allowing the adherence of the donor bacterium cells to recipient cells (Dunny, 1990). The presence of AS increases the hydrophobicity of the cell surface resulting in the localization of cholesterol to the phagosomes of the macrophages. The localization of cholesterol to the phagosomes promotes the delay or prevent fusion with lysosomal vesicles (Hirt *et al.,* 2000; Waters and Dunny, 2001). AS is encoded by the *asa1* gene (Sava *et al.,* 2010) and its secretion can also be induced by host factor during *in vivo* growth (Chandler *et al.,* 2005). It promotes the internalization and intracellular survival of enterococci in polymorphonuclear leukocytes (PMNs) (Submuth *et al.,* 2000; Golinska *et al.,* 2013) and has also been shown to promote translocation of enterococci through the intestinal epithelia cells into the systemic circulation (Sava *et al.,* 2010; Waters *et al.,* 2003). It is also involved in adherence to several extracellular matrix proteins especially epithelial cells of the endocardium and urinary tract resulting in endocarditis and increased adherence to renal tubular cells (Chuang-Smith *et al.,* 2010; Rathnayake *et al.,* 2012).

2.10.2 Enterococcal surface protein

Enterococcocal surface protein (*esp*) is associated with the bacterial cell wall that promotes adhesion, colonization and evasion of the immune system (Shankar *et al.,* 2001; Jarzembowski *et al.,* 2015). It has also been reported to play some role in antibiotic resistance (Tendolkar *et al.,* 2004; Kafil and Mobarez, 2015). The *esp* gene encodes an MSCRAMM-like protein and is located on a putative pathogenicity island. Expression of *esp* has been associated with initial adherence and biofilm formation on abiotic surfaces (Heikens *et al.,* 2009). The close proximity of *esp* to cytolysin operon which is regulated by a quorum sensing mechanism, helps in the biofilm formation, inducing resistance to environmental stresses, and adhesion to eukaryotic cells such as those of the urinary tract (Upadhyaya *et al.,* 2011).

2.10.3 Enterococcal cytolysin (haemolysin)

Cytolysin also referred to as haemolysin is a bacterial toxin, possesses β-haemolytic properties on horse, rabbit and humans erythrocytes (Upadhyaya *et al.,* 2010; Tsikrikonis *et al.,* 2012) and has bactericidal or bacteriocin activity against other Gram-positive bacteria (Cotter *et al.,* 2005). The molecular characterization of the cytolysin structure indicates it contains lanthionine residues, which resulted in its classification as a class I bacteriocin i.e the lantobiotic a class of bacteriocins (Haas *et al.,* 2002). The enterococcal haemolysin is related to the streptolysin produced by *Streptococcus pyogenes* (Cox *et al.,* 2005).

Expression of cytolysin is known to require the presence and functionality of eight genes: *cylL*L and *cylL*S, which encode the cytolysin structural peptides, *cyl*M, *cyl*B, *cyl*A, and *cyl*I, which encode proteins enrolled in the post-translational modifications into

peptides (secretion, extracellular activation, and immunity) and the *cyl*R1 and *cyl*R2, which are regulators of cytolysin expression (Cox *et al.,* 2005). Enterococcal cytolysin is a secreted and posttranslationally modified protein (Shankar *et al.,* 2004; Coburn and Gilmore, 2003). The gene encoding the cytolysin production is usually located on pheromone-responsive plasmids or integrated into the chromosome within a pathogenicity island and is regulated by a two-component regulatory system via a quorum sensing mechanism (Haas *et al.,* 2002; Upadhyaya *et al.,* 2009). Because the cytolysin operon is located on transmissible plasmids and a mobile pathogenicity island, its transference between strains can easily occur (van Tyne *et al.,* 2013).

Cytolysin is expressed as two peptide subunits, CylLL (the long subunit) and CylLS (the small subunit) and its production is regulated by CylLs, which can interact with the regulatory protein CylR1 on the cell membrane (Haas *et al.,* 2002; Jett *et al.,* 1994). Cytolysin production and activation involves several stages which include synthesis, modification and transportation. The two subunits LL and LS are ribosomally synthesized and post-translationally modified by the protein CylM. The modified peptides are proteolytically cleaved and secreted from the cell by an ABC transporter; CylB. The secreted peptides are then activated by further cleavage by a serine protease; CylA. Cells producing cytolysin usually protect themselves from the destructive action of the toxin using CylI which is located in the cell wall (Coburn and Gilmore, 2003). Transcription of the cytolysin operon is highly influenced by environmental conditions of culturing and thus could be transcribed at low levels in cases of an uninduced state, as in the case of *in vitro* tests (van Tyne *et al.,* 2013). Enterococcal cytolysin has been implicated in the

pathogenesis of infection mediated through lysis of erythrocytes, polymorphonuclear cells (PMN), macrophages or the destruction of other host cells (Garsin *et al.,* 2014).

2.10.4 *Enterococcus* gelatinase

Gelatinase (GelE) is an extracellular zinc metallo-endopeptidase secreted by enterococci (Teixeira *et al.,* 2012). It is able to hydrolyse gelatin, casein, haemoglobin, fibrinogen, collagen, insulin, immune peptides and other bioactive peptides and shares similarity to the elastase and aurolysin produced by *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Sifri *et al.,* 2002; Sieprawska-Lupa *et al.,* 2004; Fraga *et al.,* 2014). Gelatinase contributes to virulence by degradation of specific host proteins and damaging host tissue to provide means of invasion and provision of nutrients to the bacteria, in addition to their function in biofilm formation (Teixeira *et al.,* 2012).

Gelatinase production is encoded by the g*elE* gene which is an operon with the serine protease *sprE* with the latter located immediately downstream from *gelE* (Gaspar *et al.,* 2009). GelE and SprE expression is regulated by a quorum sensing two -component system located on the *fsr* (faecal streptococci regulator) locus which has sequence similarity with the *Staphylococcus* accessory gene regulator (AGR) (Qin *et al.,* 2000). The *fsr* operon comprises four genes: *fsrA, fsrB, fsrC* and *fsrD* which regulate the expression of gelatinase (Teixeira *et al.,* 2012). *fsrD* encodes an autoinducing cyclic peptide: gelatinase biosynthesis activating pheromone (GBAP) while the *fsrB* is responsible of processing, exporting and cyclization of the GBAP. Accumulation of GBAP in the extracellular space is sensed by the *fsrC* histidine kinase sensor leading to activation of the response regulator *fsrA* and

therefore expression of GelE and SprE, which are located downstream from *fsr* locus (Sava *et al.,* 2010; Thomas *et al.,* 2008).

GelE has been shown to have a role in development of biofilms of *E. faecalis* (Hancock and Perego, 2004). Although, the mechanism is not fully known GelE has been reported to promote biofilm formation by increasing cell surface hydrophobicity. It cleaves cell surface proteins at hydrophobic residues and therefore, enhanced cell attachment to the surface (Carniol and Gilmore, 2004). Results from studies using mouse models have reported that it has a role in the development of peritonitis, endocarditis and endophthalmitis (Upadhyaya *et al.,* 2010).

2.10.5 *Enterococcus* hyaluronidase

Hyaluronidase is a degradative enzyme that acts on hyaluronic acid resulting in tissue damage (Rice *et al.,* 2003). It depolymerizes the mucopolysaccharide moiety of connective tissue, thus facilitating spread of enterococci as well as their toxins through host tissue. The hyaluronidase is encoded by the chromosomal *hyl* gene (Rice *et al.,* 2003; Fisher and Phillips, 2009).

2.10.6 Microbial surface components recognizing adhesive matrix molecules (MSCRAMM)

MSCRAMM - like proteins include a secretion signal sequence, a region with immunoglobulin like folds that is usually the ligand-binding domain, and a C-terminal LPXTG-like motif (Leu-Pro-any amino acid -Thr-Gly is the recognition signal for sortase enzyme) that is recognized and cleaved by sortases (transpeptidase enzymes). These MSCRAMMs covalently attach the protein to the cell-wall peptidoglycan (Nadapareddy *et*

al., 2003; Hendrickx *et al.,* 2009). These proteins contribute to virulence by promoting attachment to host tissues and thus increasing the colonizing ability of the strains that harbour them (Hendrickx *et al.,* 2009; Sillanpaa *et al.,* 2009).

2.10.7 Collagen binding adhesin of *Enterococcus faecium* (Acm)

Acm is a MSCRAMMs, and they are typically adhesins and pili (Sillanpaa *et al.,* 2008; Sillanpaa *et al.,* 2009). The *acm* gene is reportedly present in almost all enterococci although functional Acm is expressed exclusively by clinical isolates (Nadapareddy *et al.,* 2008). Acm primarily interacts with collagen type I, and secondarily with collagen type IV (Paganelli *et al.,* 2013). Acm plays a role in the pathogenesis of enterococcal endocarditis, and elicits an anti-Acm IgG antibodies commonly detected in patients diagnosed of enterococcal endocarditis (Nadapareddy *et al.,* 2008).

2.11 Antimicrobial Use and Evolution of Resistance in Livestock Production

In spite of the indispensible role played by antimicrobial agents in decreasing illness and death associated with infectious diseases in animals and humans (Tadesse *et al.,* 2012), selective pressure exerted by their use has been the major driving force behind the emergence and spread of drug-resistance traits among pathogenic and commensal bacteria (Aarestrup *et al.,* 2008).

Antimicrobial use in animal production as growth promoters, for prophylaxis or chemotherapy is a common practice worldwide (Phillips *et al.,* 2004; Chattopadhyay, 2014). The most commonly used antimicrobials are similar or related to those administered

to humans, including penicillins, tetracyclines, cephalosporins (including ceftiofur, a third-generation cephalosporin), fluoroquinolones, avoparcin (a glycopeptide that is related to vancomycin), and virginiamycin (a streptogramin that is related to quinupristin-dalfopristin). Since the introduction of these drugs, varying levels of resistance to them have developed, rendering some of them therapeutically useless (Mathew *et al.,* 2007; Marshall and Levy, 2011). This is in addition to the emergence of multidrug resistant bacteria in the animal production settings, the community and hospital environment (Marshall and Levy, 2011). Concerns regarding the increasing incidence of multidrug resistant bacteria (MDR) in livestock production and risk of these MDR strains entering the food chain has been reported (Doyle, 2015; Marshall and Levy, 2011).

2.12 Mechanism of Antimicrobial Resistance in *Enterococcus* Species

The most pressing issues currently facing clinicians in hospitals worldwide regarding the treatment of enterococcal infection is the unreliability of the currently available therapeutic options resulting from the organism's ease of developing resistance (Tarai *et al.,* 2013). Some of the major mechanisms of antibiotic resistance that adversely affect the treatment of enterococcal infections are as schematically presented by Arias and Murray (2012) (Figure 2.3).

The reported increase in the antimicrobial resistance in *Enterococcus* isolated from humans and animals, associated with the widespread use of antimicrobials has become a significant public health issue worldwide (Marshall and Levy, 2011; Wegener, 2012;

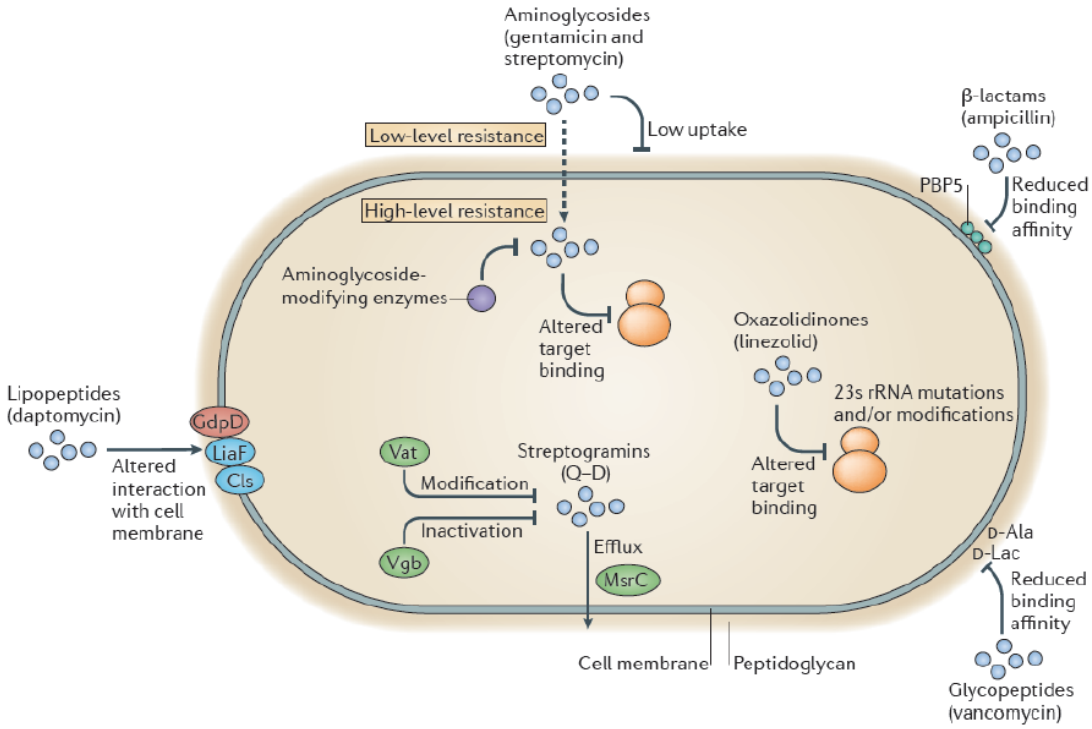


Figure 2.3: Major mechanisms of antibiotic resistance in enterococci. Adapted from Arias and Murray (2012)

Chang *et al.,* 2015). Antibiotic resistance in *Enterococcus* occur through several mechanisms, including alteration by mutations of the antibiotic target, changes in cell permeability and efflux, and horizontal transfer of resistance genes (Rodriguez-Rojas *et al.,* 2013). The three types of resistance of importance in *Enterococcus* because of their implication in clinical therapy are: high level resistance to aminoglycosides, ampicillin and vancomycin (Wang *et al.,* 2013; Sadowy and Luczkiewicz, 2014).

2.12.1 Resistance to beta (β) -lactam antibiotics

Beta (β) - lactam antibiotics exert bactericidal activity by covalently binding and inactivating penicillin-binding proteins (PBPs), resulting in interference with the synthesis and remodeling of the bacterial peptidoglycan cell wall (Hollenbeck and Rice, 2012; Zhang *et al.,* 2012). Three different resistance mechanisms mainly account for enterococcal resistance to the β-lactams: overproduction of penicillin-binding proteins (PBP4 or PBP5), decreased affinity for PBP4 or PBP5 by the occurrence of point mutations at the penicillin binding domain and production of a β - lactamase (Hiraga *et al.,* 2008; Hollenbeck and Rice, 2012; Conceicao *et al.,* 2014). Alterations in PBPs account majorly for resistance to the β-lactams among enterococci (Hsieh *et al.,* 2006) while enzymatic inactivation via production of β-lactamases has only been reported in few clinical isolates (Chambers, 1999; Ono *et al.,* 2005; Sarti *et al.,* 2012).

Enterococci possess an intrinsic and acquired resistance to low level and high level β-lactam respectively. The intrinsic resistance of enterococci to β-lactam antibiotics stems from the production of low-affinity PBP5 that is able to function in place of other PBPs that

are already saturated by β - lactam antibiotics (Hollenbeck and Rice, 2012). This low-level resistance renders penicillins bacteriostatic and cephalosporins practically ineffective (Rice *et al.,* 2009). Minimum inhibitory concentrations (MICs) of ampicillin, penicillin, piperacillin and imipenem have been reported to be approximately 100-fold higher than that for streptococci, with MICs of penicillin against *Enterococcus faecalis* and *Enterococcus faecium* typically in the ranges of 2 – 8 μg/ml and 8 – 16 μg/ml respectively (Rice *et al.,* 2004; Miller *et al.,* 2014).

High level resistance to β-lactams is as a result of acquisition of β- lactamases or mutations in PBP5 (and PBP4 in *E. faecalis*) (Ono *et al.,* 2005). β - lactamases production in enterococci is encoded by plasmid-mediated *bla* genes, which are highly homologous to the *bla*Z genes of *S. aureus,* except for its constitutive expression presumably due to a lack of a repressor protein (Leclercq, 1997). Mutations of the PBP4 or 5 cause a reduction in binding affinity to β-lactams and/or its overproduction. Reduction in binding affinity has been implicated in the development of highly resistant strains that have MICs of ampicillin up to 512 μg/ml (Ono *et al.,* 2005). Mutation of the PBP is usually associated with an 800-fold reduction in MIC of ampicillin (Sifaoui *et al.,* 2001).

Until recently ampicillin resistant enterococci were rarely recovered from animals and humans in the community, they were restricted to the hospital environment. More than 90% of *E. faecium* recovered from health care associated infections in the U.S. are ampicillin resistant (de Regt *et al.,* 2012). As high as 48.8% prevalence of ampicillin resistant enterococci have also been reported in hospital environments in Europe (Simonsen *et al.,* 2003), 36 – 89% in Africa (Abamecha *et al.,* 2015; Aamodt *et al.,* 2015; Martins *et*

al., 2015). However, recent reports have shown healthy individuals in the community and animals harbor ampicillin resistant isolates with a prevalence of 0 – 6% among healthy individuals (Duh *et al.*, 2001; Novais *et al.*, 2006), 23 – 76% among dogs (Damborg *et al.*, 2009; de Regt *et al.*, 2012), 59.1% in farm animals and their environment (Novais *et al.*, 2013).

2.12.2 Resistance to the aminoglycoside antibiotics

Aminoglycosides act by binding specifically to the aminoacyl site (A-site) of the 16S rRNA within the 30S ribosomal subunit of prokaryotic and interfering with protein synthesis (Doi and Arakawa, 2007). Enterococci generally exhibit a moderate level of intrinsic resistance to clinical concentrations of aminoglycoside that has been attributed to poor uptake of antibiotics i.e. its inability to enter the cell wall (Chow, 2000; Hollenbeck and Rice, 2012). The intrinsic resistance of enterococci to aminoglycosides has also been suggested to be mediated by proteins involved in electron transport associated with facultative anaerobic metabolism of enterococci (Chow, 2000). Costa *et al.* (1993) also reported a chromosomally encoded 6′- N-aminoglycoside acetyltransferase (*aac(6′)*-Ii) to confer low-level intrinsic resistance to aminoglycosides. A moderate species-specific intrinsic resistance to aminoglycosides has been observed in *E. faecium*. This intrinsic resistance is enhanced by a chromosomally encoded rRNA methyltransferase, *EfmM* that uses S-adenosyl methionine as a methyl donor to methylate a specific residue on 16S rRNA has been reported (Galloway-Pena *et al.*, 2011).

Acquisition of mobile genetic elements is responsible for high-level aminoglycoside resistance in enterococci i.e ≥ 500µg/ml (Hollenbeck and Rice, 2012; Behnood *et al.*,

2013). Acquired resistance results in high level aminoglycoside resistance. High-level aminoglycoside resistance in enterococci is encoded by aminoglycoside modifying enzymes (AME) that inactivate or chemically modify these drugs (Labby and Garneau-Tsodikova, 2013). Three major groups of this enzyme include aminoglycoside phosphotransferase (APH), aminoglycoside acetyltransferases (AAC), and aminoglycoside adenyltransferases (AAD) (Table 2.2) which function by using ATP to phosphorylate a hydroxyl group, using an acetyl-CoA to acetylate an amino group and using ATP to adenylate a hydroxyl group respectively, on the antibiotic (Shaw *et al.,* 1993; Ramirez and Tolmasky, 2010; Frye and Jackson, 2013). Modification of the aminoglycosides by these enzymes results in their inability to bind to the target site on the 30S ribosomal subunit and thereby looses antibacterial activity (Doi and Arakawa, 2007). The bifunctional enzyme APH(2″)-Ia-AAC(6′)-Ie is the most wide-spread and also clinically important AME in enterococci (Vakulenko and Mobashery, 2003; Padmasini *et al.,* 2014; Li *et al.,* 2015). It mediates high level resistance to all aminoglycosides except streptomycin (Choi and Woo, 2013). It is coded for by the *aph(2")-Ia-aac(6')-Ie* gene a member of the APH(2″)-I phosphotransferase family. Others aminoglycoside modifying enzyme genes (*aph*(2″)-Ib, Ic and Id) in the APH(2″)-I phosphotransferase family are located on various transposons or conjugative plasmids (Chen *et al.,* 2010; Choi and Woo, 2013; Gold *et al.,* 2014). The members of the family *aph(2")-I; aph(2")-Ib, aph(2")-Ic and aph(2")-Id* have been identified to confer aminoglycoside resistance although their MICs may be as low as 256 µg/ml, an MIC which would be interpreted as aminoglycoside susceptible by some laboratories that use an MIC of 500 µg/ml as a cut-off (Hollenbeck and Rice, 2012).

Table 2.2: Genes encoding different aminoglycoside modifying enzymes among enterococci.

Resistance genes	**Aminoglycoside**				
	Gentamicin	**Amikacin**	**Tobramycin**	**Kanamycin**	**Streptomycin**
aac(6')-Ie-aph(2")-Ia	R	R	R	R	S
aph(2")-Ib	R	S	R	R	S
aph(2")-Ic	R	S	R	R	S
aph(2")-Id	R	S	R	R	S
aph(3')-IIIa	S	R	S	R	S
aac(6')-Ii	S	S	R	R	S
ant(3')-Ia	S	S	S	S	R
ant(4")-Id	S	R	R	R	S
ant(6')-Ia	S	S	S	S	R

Adapted from Chow (2000).

High-level resistance to streptomycin occurs most commonly through enzymatic modification of the antibiotic or by single point mutations to the ribosome. Two nucleotidyltransferases, Ant(6′)-Ia and Ant(3″)-Ia, have been reported to be capable of inactivating streptomycin (and structurally related aminoglycosides) (Chow, 2000). The gene *aadE* also known as *ant(6)*-Ia encodes a 302-aa adenyltransferase, which confers streptomycin resistance and together with *aph*A3, are carried on transposon *Tn*5405. Whereas resistance caused by aminoglycoside-modifying enzymes (AME) will typically have MICs in the 4,000 to 16,000 µg/ml range, ribosomal mutations result in MICs of 128,000 µg/ml (Hollenbeck and Rice, 2012).

Other acquired AMEs that occur among enterococci but are of less clinical significance because they do not confer gentamicin or streptomycin resistance are Aph(3′)-IIIa, an aminoglycoside phosphotransferase that confers resistance to kanamycin and Ant(4″)-Ia, a nucleotidyltransferase that confers resistance to tobramycin, amikacin, neomycin and kanamycin (Vakulenko *et al.,* 2003). Genes encoding the AMEs are often located on plasmids promoting the rapid dissemination of the aminoglycoside resistance trait from *Enterococcus* to other bacteria. The bifunctional aminoglycoside resistance gene *aac(6')-Ie-aph(2")-Ia* gene for example is usually flanked by inverted repeats of IS256 resulting in a composite transposons such as the Tn*5281* reported in *E. faecalis* (*as against the* Tn*4001* in *S. aureus)* which also promotes the rapid dissemination of this gene (Wendlandt *et al.,* 2013).

High level aminoglycoside resistance was first described in *Enterococcus* in 1979 in France (Murray, 1990; Dadfarma *et al.,* 2013). The emergence and spread of high-level resistance to aminoglycosides, in particular to gentamicin, is diffused worldwide. They

have been identified with increasing frequencies from clinical samples of hospitalized patients, and in the intestinal tract of humans (del Campo *et al.,* 2004) and animals (Klibi *et al.,* 2015a; Jaimee and Halami, 2016) and vegetables. High level aminoglycoside resistance has been more frequently found in *E. faecium* than in *E. faecalis* in Europe, USA, Asia (Kwon *et al.,* 2012; Choi and Woo, 2013), Africa (Klibi *et al.,* 2015a; Ben Sallem *et al.,* 2016). Frequencies of resistance ranging from 0 – 60% have been reported from humans and animals (Fracalanzza *et al.,* 2007; Han *et al.,* 2011; Kwon *et al.,* 2012; El-Ghazawy *et al.,* 2015). Reports of high level aminoglycoside resistance among enterococci from animals and humans in Nigeria are however scarce.

2.12.3 Resistance to the glycopeptide antibiotics

Vancomycin acts by binding to the D-ala-D-ala terminus of the pentapeptide precursor, thereby inhibiting cell wall synthesis. Glycopeptide-resistant organisms modify these pentapeptide precursors, replacing the terminal D-ala with D-lac or D-ser (Hollenbeck and Rice, 2012) (Table 2.3). These modified cell wall precursors bind glycopeptides with 1,000-fold lower affinity than do normal precursors (Reynolds and Courvalin, 2005; Schaberle *et al.,* 2011). First described in 1986, glycopeptide-resistant enterococci (GRE) have since emerged as a major cause of nosocomial infections and an antimicrobial dilemma (Woodford, 1998). Although glycopeptide resistance occurs in *E. faecalis*, majority of GRE (VRE) infections are attributed to *E. faecium* (Hollenbeck and Rice, 2012).

VanA and VanB operons encoded by the *vanA* and *vanB* genes are the most prevalent VRE in nosocomial infections (Leclercq, 2009; Zheng *et al.,* 2009). In the VanA

Table 2.3: Clinically important vancomycin resistance phenotypes and genotypes haboured by enterococci

Phenotypes	Genotypes (gene cluster)	Gene Location	VAN MIC	Teico MIC	Expression	Transferable by conjugation	Termination of peptidoglycan precursor	Species
VanA	*vanA* gene cluster	Chromosome and plasmid (Tn*1546*)	>128	>64	Inducible	+	D-Ala-D-Lac	*E. faecium* *E. faecalis*
VanB	*vanB* gene cluster	Chromosome and plasmid (Tn*1547*)	16 – 1024	<2	Inducible	+	D-Ala-D-Lac	*E. faecium* *E. faecalis*
VanC	*vanC1, vanC2, vanC3* gene cluster	Chromosome	4 – 16	0.5 – 1	Constitutive	-	D-Ala-D-Ser	*E. gallinarum, E. casseliflavus, E. flavescens*
VanD	*vanD* gene cluster	Chromosome	64 – 128	4	Inducible or constitutive	-	D-Ala-D-Lac	*E. faecium* *E. faecalis*
VanE	*vanE* gene cluster	Chromosome	16	0.5	Inducible	-	D-Ala-D-Ser	*E. faecalis*

VAN: Vancomycin , Teico: Teicoplanin, MIC: Minimum inhibitory concentration. Table adapted from Sujatha and Praharaj (2012) and Mendez-Alvarez *et al.* (2000)

phenotype, the *Enterococcus* expresses a high resistance to both vancomycin and teicoplanin while in the VanB phenotype, resistance is expressed to vancomycin but not teicoplanin, although a constitutive expression (which may be selected by teicoplanin exposure) may result in resistance to both compounds (Hollenbeck and Rice, 2012). *vanA* is usually mobilized on Tn*3*-family transposon Tn*1546* found on both non-conjugative and conjugative plasmids while *vanB* is most often carried on a Tn*5382*/*1549* or related conjugative transposons (Hegstad *et al.,* 2010; Launay *et al.,* 2006; Miller *et al.,* 2014). VanB carrying transposons have been identified in pheromone-sensitive and conjugative plasmids (Miller *et al.,* 2014). The *vanC* resistance is intrinsic to *E. gallinarum* and *E. casseliflavus*. These species carry the *vanC* gene which is chromosomally encoded and confers low-level resistance to vancomycin through the change of the terminal dipeptide from D-Ala-D-Ala to D-Ala-D-Ser (Arias *et al.,* 2000) in addition to production of an altered D-Ala-D-Ser dipeptide via VanT, a serine racemase that catalyzes the change from L-Ser to D-Ser (Arias *et al.,* 1999). These species also harbour a bifunctional D,D-pepti-dase/DdcY designated $VanXY_C$ that hydrolyzes the typical D-Ala-D-Ala precussor in the cytoplasm (Fines *et al.,* 1999). The *vanE* gene is also chromosomally encoded and expresses low-level resistance to vancomycin (Hollenbeck and Rice, 2012).

Vancomycin resistant *Enterococcus* was first reported in France in 1986 (Leclercq *et al.,* 1988). About 35.5% of enterococcal hospital associated infections in the US are reported to be resistant to vancomycin (Sievert *et al.,* 2013). This is in contrast to the report from other parts of the world such as Canada which has a lower (6%) prevalence rate (Zhanel *et al.,* 2013) and Europe which has a variable prevalence ranging from less than 1% in France, Spain and Sweden to greater than 20% in Greece, Ireland, Portugal and the

United Kingdom and an overall prevalence of 4% (EARS, 2015). The few studies on VRE in from some countries in Africa, indicates the prevalence ranges between 0% - 35% (Ndubuisi *et al.,* 2014; Olawale *et al.,* 2011; Abebe *et al.,* 2014; Ben Sallem *et al.,* 2015) from clinical samples of human and 0% in food producing animals (Klibi *et al.,* 2015a).

2.12.4 Resistance to the tetracyclines

Resistance to tetracycline by bacteria is mediated by three mechanisms; ribosomal protection, efflux pump and the recently identified enzymatic inactivation mechanism encoded by the rare *tet*(X) gene (Chopra and Roberts, 2001; Volkers *et al.,* 2011; Woodford, 2005). Only two of these mechanisms encoded by six different genes have been reported in enterococci (Huys *et al.,* 2004; Frye and Jackson, 2013). The ribosomal protection in *Enterococcus* is mediated by the *tet*(M), *tet*(O), *tet*(S) genes while the energy dependent efflux mechanisms is mediated by the *tet*(K) and *tet*(L) genes (Huys *et al.,* 2004; Rathnayake *et al.,* 2012; Frye and Jackson, 2013). The sixth gene *tet*(U) was initially detected in *E. faecium* but its mechanism of action is unknown (Ridenhour *et al.,* 1996). However, a recent report by Caryl *et al.* (2012) suggests that it does not confer resistance to tetracycline and is not a *tet* resistance determinant but rather a misannotated 3´ end of a gene encoding a rolling-circle replication initiator protein. *tet*(L), *tet*(M) and *tet*(O) are the predominant tetracycline resistance determinants in enterococcal isolates in contrast to the *tet*(K) that is more common among staphylococci (Klare *et al.,* 2003). The *tet*(M) gene is often associated with the Tn916/1545 family of conjugative transposons which have a broad host range, while the other determinants may be located on the chromosome [*tet*(L)] or on conjugative plasmids [*tet*(L), tet(O), *tet*(S)] (Klare *et al.,* 2003).

Tetracycline is one of the most widely used and abused antibiotic in animal production and human medicine (Marshall and Levy, 2011; Hao *et al.,* 2014; Adenipekun *et al.,* 2015). The routine application of these agents has result in the development of resistance against them among many bacteria (de Vries *et al.,* 2011). Worldwide resistance rates of close to 100% have been reported against tetracyclines among enterococci from animals and humans (Aarestrup *et al.,* 2000; Lin *et al.,* 2013) therefore, limiting their effectiveness in the treatment of many bacteria infections.

2.12.5 Macrolide-lincosamide-streptogramin resistance (MLS_B)

Acquired resistance to MLS antibiotics among enterococci is associated with three resistance mechanisms: modification of the drug target due to a single alteration of the 23S rRNA and confers cross-resistance to macrolide-lincosamide-streptogramin B (MLS_B) antibiotics, inactivation of the drug which confers resistance to only the structurally related MLS antibiotics, and the active efflux pump mechanism encoded by the *mefA* and *mreA genes (both associated with macrolide resistance), msrA (associated with macrolide and streptogramin resistance and mefE* genes.

Like tetracycline, the macrolides are extensively used especially in poultry production resulting in the detection of a high rate of resistance to this class of drug (Lin *et al.,* 2007; EMA, 2011; Marshall and Levy, 2011; Hao *et al.,* 2014; Oluwasile *et al.,* 2014). The most studied among the macrolide are tylosin and erythromycin. High rate of use of these agents in livestock production has been associated with the development of resistance among enterococci with prevalence rates reaching 90% in isolates from human and animals (Aarestrup *et al.,* 2000; Jackson *et al.,* 2004b; de Vries *et al.,* 2011; Zou *et al.,* 2011; Kim *et*

al., 2012b; Ben Said *et al.,* 2015). However, national surveys in the EU show that proportion of erythromycin-resistant *E. faecalis* and *E. faecium* isolated from broiler was decreasing as a result of a reduction in use in livestock production (EFSA, 2010).

2.12.6 Resistance to the fluoroquinolones

Resistance to clinically important fluoroquinolones is usually attributed to mutations within the drug's targets: DNA gyrase and topoisomerase IV two important enzymes required for DNA replication (Gruger *et al.,* 2004; Drlica *et al.,* 2009). Both enzymes are comprised of two subunits: GyrA and GyrB for DNA gyrase and ParC/GrlA and ParE/GrlB for the topoisomerase IV (Bebear *et al.,* 1998; Ince *et al.,* 2002). GyrA and ParC/GrlA are the major targets of the fluoroquinolones and mutations predominantly at the quinolone-resistance-determining-region (QRDR) of these targets result in decreased binding affinity of the drug (Ruiz, 2003; Fabrega *et al.,* 2009). Mutations in the target genes, specifically *gyrA* and *parC,* have been described in *E. faecium* and *E. faecalis* (el Amin *et al.,* 1999; Petersen and Jensen, 2004). Efflux pump is also an important resistance mechanism of *Enterococcus* to the quinolones and is mediated through the NorA and *qnr* in *E. faecium* and *E. faecalis* respectively (Hawkey, 2003; Arsene and Leclercq, 2007).

Reports from different parts of the world indicates the resistance to fluroquinolones among enterococci is on a steady increase especially among human isolates with a prevalence of about 14.4% in 1993 (Tankovic *et al.,* 1996) to 20.4 in 2007 (Yasufuku *et al.,* 2011) and 58.1% in 2012 (Jai *et al.,* 2014). High rates of up to 55%, 47% and 23% associated with the use of enrofloxacin have been reported in cattle, pigs and poultry (Hershberger *et al.,* 2005). Kwon *et al.* 2012) also reported a high prevalence of 66.7%

among human enterococcal isolates compared with 10.0%, 0% and 31.8% in chicken, pig and companion animals. In Africa prevalence of ciprofloxacin resistant enterococci of 6 - 22.2% was reported among food animals (Klibi *et al.,* 2013b; Klibi *et al.,* 2015a)

2.12.7 Resistance to streptogramin antibiotics

E. faecalis have an intrinsic resistance to streptogramins and therefore this drug acts as a bacteriostatic agent while *E. faecium* is highly susceptible to the streptogramins (Werner *et al.,* 2002). Thus the approval by the FDA of the streptogramin B/A combination quinupristin-dalfopristin as one of two antibiotics for the treatment of infections caused by vancomycin-resistant *E. faecium* (Hollenbeck and Rice, 2012). Three mechanisms are involved in the acquired resistance to the streptogramins by *Enterococcus*: acetylation of the antibiotic, efflux of the antibiotic, and dimethylation of the 23S rRNA target site. The best understood mechanism of streptogramin resistance is dimethylation of the 23S rRNA which confers the MLS_A or MLS_B resistance phenotype to *Enterococcus*. This resistance phenotype usually occurs through acquisition of either the *ermA* or *ermB* genes on broad host range plasmids such as pAMβ1. If these plasmids also contain *vatE* or *vatD* genes, then they will confer in addition to MLS_B resistance phenotype, resistance to quinupristin-dalfopristin which is a combination of streptogramina A and B (Werner *et al.,* 2002; Christensen *et al.,* 2008). Virginiamycin acetyltransferase genes *vatD, vatE* and *vatH* are among the streptogramin resistance genes with probable veterinary origins. *vatD* and *vatE* (formerly called *satG*) have been isolated from plasmids alongside *erm* and *vgbA* genes that reduce susceptibility to streptogramin B - thus providing full resistance to quinupristin-dalfopristin. VgbA is the only known acetyltransferase with activity against streptogramin

B in enterococci. The ABC-efflux channel VgaD also plays a role in acquired streptogramin resistance, independent of the intrinsic ABC-efflux channels encoded by *lsa* genes in *E. faecalis* and *msrC* gene in *E. faecium* (Hollenbeck and Rice, 2012).

Resistance to streptogramin by *Enterococcus* was first reported in humans in 1997 (Woodford *et al.,* 1997). Worldwide only a small proportion of quinupristin/dalfopristin resistance has been reported from human sources compared to animals sources. High rate of up to 63% have been reported among farm animals especially poultry (Hayes *et al.,* 2005). Resistance levels of as high as 67%, was reported three decades ago from broilers (Aarestrup *et al.,* 2001; Jensen *et al.,* 2002; Hershberger *et al.,* 2005), however, with the ban on the use of virginiamycin across the globe a steady decrease in quinupristin/dalfopristin resistant *Enterococcus* is been reported from animals; 20%, 0.3% and 5% from chicken, cattle and food produce respsectively (Jackson *et al.,* 2007).

2.13 Treatment and Control of Enterococcal Infections

Several antimicrobial agents with significant effect on enterococci have emerged within the last two decades, however, less than 100% clinical and microbiological success have been achieved with their use (Arias *et al.,* 2010; Kristich *et al.,* 2014). In order to improve the efficacy of the antibiotics and reduce the development of resistance, the use of these agents as a combination to achieve a synergistic effect has been advocated (Arias *et al.,* 2010). Traditionally, treatment of enterococci infection is achieved with a combination of cell wall active antimicrobials such as β-lactams or glycopeptides, and an aminoglycosides (Linden, 2007; Arias *et al.,* 2010). The commonly used β-lactam, glycopeptides (cell wall inhibitors) and aminoglycoside are ampicillin, vancomycin and

gentamicin respectively (Linden, 2007; Houlihan *et al.,* 2000). The synergism is achieved via enhanced intracellular uptake of the aminoglycosides caused by the increased permeability of bacteria after the inhibition of cell wall synthesis by the β-lactams or glycopeptides.

Other antimicrobials include linezolid which displays a bacteriostatic activity on *Enterococcus* by inhibiting protein synthesis, daptomycin which interfers with the cytoplasmic membrane causing depolarization and cessation of protein, DNA and RNA-synthesis (Canton *et al.,* 2010; Casapao *et al.,* 2013), quinupristin-dalfopristin (Q/D) and Tigecycline that act by inhibition of protein synthesis (Wang *et al.,* 2014a) (Pontikis *et al.,* 2013) are used for the treatment of enterococcal infection.

2.14 Transfer of Antimicrobial Resistance Determinants

It is well recognized that virulence and antimicrobial resistance genes can be transferred among bacterial populations (Beceiro *et al.,* 2013). *Enterococcus* has been known for a long time as a genus with a high number of mobile genetic elements resulting from a frequent exchange of genetic material across species, genus and family barriers (Werner *et al.,* 2013). Bacteria can acquire antibiotic resistance either through *de novo* gene mutation(s) or by horizontal/lateral transfer (conjugation, transduction or transformation) of mobile genetic elements (Tang *et al.,* 2014). Two major mechanisms; transformation and conjugation are the means by which enterococci establish themselves in the game of uptake and dissemination of resistant determinants (Davies and Davies, 2010). Conjugation involves the transfer of genetic material between bacteria through direct cell-to-cell contact

or via the sex pilli while transformation is the process in which a recipient cell takes up bacterial free DNA molecules from the environment (Filloux, 2010).

The roles of mobile genetic elements such as plasmids and transposons in the dissemination of resistant determinants among Gram positive and negative bacteria have been established (Ploy *et al.,* 2000; Bennett, 2008; Berglund, 2015). Virulence and antibiotic resistance genes are carried on mobile genetic elements such as transposons, integrons and plasmids (extrachromosomal genetic material that may be large and can contain a variety of resistance genes which serve as vectors for transfer within the same species or between different species) (Stokes and Gillings, 2011; Skippington and Regan, 2011; Baquero *et al.,* 2013). The capability of *Enterococcus* species originating from clinical, food and commensal sources to transfer virulence and resistance genes *in vitro* (filter or broth mating) and *in vivo* (within the gastrointestinal tract environment) have been well documented (Moubareck *et al.,* 2003; Lester *et al.,* 2006; Hummel *et al.,* 2007). Plasmids and transposons have also been characterized as the mobile elements responsible for intra- and interspecies transfer of these virulence and resistance traits (Palmer *et al.,* 2010b; Mason *et al.,* 2010; Jasni *et al.,* 2010).

Enterococcus have been shown to horizontally transfer resistance traits to other bacteria that cause infection in animals and humans such as; *Staphylococcus aureus* (Noble *et al.,* 1992; Clewell and Dunny, 2002; Weigel *et al.,* 2003), *E. coli* (Jurado-Rabadan *et al.,* 2014), *Clostridium difficile* (Jasni *et al.,* 2010), and *Listeria* spp (Doucet-Populaire *et al.,* 1991; Biavasco *et al.,* 1996).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

This study was conducted in Zaria, Kaduna State, Nigeria located between latitude 11°7´N 11°12´N and longitudes 07°41´E. The study sites included vegetable farms located along the banks and tributaries of the ABU dam, Kubani river, Galma river and Basawa River where vegetable food produce are grown and some cattle and poultry farms within Zaria which are the sources of manure for vegetable farms. Effluents from sewage treatment plants, abattoir, domestic sewage and wastes, agricultural run-offs such as manures are discharged into these rivers (Agbogu *et al.,* 2006; Chigor *et al.,* 2010; Yebpella *et al.,* 2011). Farms in these areas have been used for cultivation of food produce for many years. They grow mainly vegetables (cabbage, spinach, onion, okra, lettuce, cucumber, tomatoes, carrot, pepper), maize and sugar cane destined for open markets in the immediate Zaria environment, other parts of the state as well as some neighboring states (Katsina, Kano, Jos).

3.2 Study Design

A cross-sectional design was used for this study. Vegetables (carrot, cabbage, lettuce) and soil were collected from vegetable farms while rectal/cloacal swabs and manure were collected from poultry and cattle farms. The farms where samples were collected were selected based on the willingness of the farmers and owners to allow their farms to be included in the study and the presence of the vegetables, animal or manure on the farm (convenience/non probability sampling technique). Sampling for this study was carried out from January to July, 2014.

3.3 Sampling

Sampling and sample processing were carried out as described by Kuhn *et al.* (2003) and Schwaiger *et al.* (2010)

3.3.1 Sample site and sample collection

Sampling from livestock farms: Livestock samples were collected from 13 cattle farms and and 13 poultry farms. The farms are located in Shika, Tudun Wada, Wusasa, Zango and Samaru, all in Zaria. In each of the farm visited, 15 samples comprising of 10 cloacal/rectal swabs from 10 of the birds/cattle and five manure samples were collected. The cloacal and rectal swabs were collected directly from the cloaca and rectum of the birds and cattle, respectively, using sterile swab sticks. In the case of manure, five different composite manure (a composite manure sample comprise five different manure samples (10g each) collected from different sites of the manure heaps/bags). Samples of about 50g each were collected into a sterile polythene bag.

Sampling from vegetable farms: The vegetables were collected between January and April, 2014, a period characterized by intense vegetable farming in the study area. Fourteen farms from five vegetable farming sites located along banks of the rivers or their tributaries were used for the study viz: PZ (four farms), Galma (four farms), Zango (four farms), ABU dam (two farms) and Basawa (two farms). Four different samples of each of the vegetable type available in the farm were collected.

Vegetable samples: The vegetable types sampled include the following salad vegetables: lettuce, cabbages and carrots which were collected directly from the farms. Each farm selected for sampling was divided into four quadrants and one sample (total of four) was collected from each quadrant. For each produce type in each farm, two to three of the

respective vegetables collected three meters apart in each quadrant of the field of cultivation, constituted a sample i.e. for cabbage and lettuce, two to three heads of lettuce or cabbage constituted a sample while for carrots two to three complete bunches constituted a sample.

Soil samples: Four different composite soil samples were collected from each of the vegetable farms from where vegetables were sampled. About 50g of soil sample each were collected into sterile polythene bags from the four different quadrants of the farm. A composite soil sample comprised of three different soil samples from a single quadrant mixed together to obtain a composite (50g) soil sample.

Water sample: Water sample was collected directly from the source of irrigation water (tip of the outlet of the irrigation pump machine) in each of the farms where vegetable was collected. A volume of 100ml of each water sample was collected into a sterile bottle. The bottle was then corked, appropriately labeled and kept on ice for onward transportation to the Laboratory.

3.3.2 Number of samples collected

A total of 670 samples comprising 130 cloacal/rectal swabs and 65 manure samples were collected from each of the poultry and cattle farms while 48 carrot, 20 cabbage, 52 lettuces and 120 soil samples were collected from the vegetable farms. All the samples collected were placed in sterile polythene bags or bottles, properly marked by recording sample type, source/farm identity, sample number, and date of collection as appropriate. The polythene bag or bottle containing the sample was kept in insulated coolers containing ice-packs and transported to the Microbiology Laboratory of the Department of Veterinary

Microbiology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria for processing. All samples were processed and analyzed within 6 hours of collection.

3.3.3 Processing of samples

Cloacal/rectal swab: Each cloacal/rectal swab was inoculated directly into nine mililiters of bile esculin azide broth (Hardy Diagnostic, USA).

Manure: Ten grams of the soil or manure was homogenized in 90ml of 0.1% peptone water. One milliliter of the homogenized sample was then inoculated into nine mililiters of the bile esculin azide broth (Hardy Diagnostic, USA) for selective enrichment.

Vegetables: For leafy greens such as lettuce and cabbage, leaves from the outside and inside sections of the produce were used to make up 25g of sample while for carrots two to three of the different roots were chopped into smaller pieces. The sample was then packed in a stomacher bag containing 225ml of 0.1% peptone water and homogenized using a stomacher (Stomacher Lab Blender 400) for three to four minutes. To avoid possible cross-contamination, the knives used were flamed after processing each sample while disposable gloves were changed after processing of each sample.

Irrigation water: The irrigation water was vigorously shaken to mix and one milliliter of the water inoculated directly into nine milliliters of bile esculin azide broth (Hardy Diagnostic, USA).

Soil: In the case of soil samples; 25g of each composite soil sample was weighed and mixed with 225ml of 0.1% peptone water. The mixture was manually shaken for 20 seconds and then allowed to sit for about two minutes. The liquid supernatant was then collected and 1ml inoculated into the enrichment broth (bile esculin azide broth).

3.4 Isolation and Identification of Enterococci

3.4.1 Isolation of enterococci

The selective enrichment broth (bile esculin azide broth) was incubated at 45°C and the broth evaluated after 48 h for blackening/browning (esculin hydrolysis), indicative esculin hydrolysis. A loopful of the broth showing presumptive enterococcal growth (browning/blackening of the broth) was streaked onto the surface of bile esculin azide agar (Hardy Diagnostic, USA) plate and incubated at 37°C for 24h. Enrichment broth showing no growth or blackening/browning after 48h of incubation, were considered negative for enterococci and discarded. From each of the streaked bile esculin azide agar plates, three to three colonies with different morphology showing esculin hydrolysis (small, translucent with brownish-black or black coloration) were individually picked and subcultured on tryptone soya agar (TSA) (Oxoid, USA) plates and incubated at 37°C for 24h to obtain pure colonies. The suspected colonies were then subjected to Gram staining, catalase test, test for production of L-pyrrolidonyl-*β*-naphthylamide-PYR using the PYRase strips (Oxoid, UK) and growth in tryptone soya broth (TSB) supplemented with 6.5% NaCl. Gram positive, catalase negative cocci occurring singly, in pairs or chains that are PYRase positive and grew in the TSB containing 6.5% NaCl were identified presumptively as *Enterococcus* species (Facklam and Collins, 1989). The presumptive *Enterococcus* species were inoculated onto brain heart infusion agar (BHI) slants and incubated overnight at 37°C followed by storage at 4°C until required for further characterization.

3.4.2 Biochemical identification/speciation

The presumptive enterococci were further characterized to species level based on the following biochemical tests: arginine decarboxylation using Moeller decarboxylase

medium supplemented with arginine (Hardy Diagnostic, USA), fermentation of carbohydrates: mannitol, ribose, sorbitol, L-arabinose, D-xylose, sucrose, D-raffinose, methyl-α-D-glucopyranoside (MDG) (Sigma, USA) and pigment production as described by Facklam and Collins (1989), Manero and Blanch (1999) Schwaiger *et al.* (2010).

The carbohydrate fermentation test was carried out using phenol red broth (Hardy Diagnostic, USA) as the basal medium. The sugars were added at 1% concentrations to the phenol red broth for the corresponding tests. The uninoculated sugar was incubated for 24h at 37°C prior to inoculation to test for sterility. Following inoculation the sugars were incubated for 24h at 37°C and then observed for colour change. A change in colour of the broth from pink to yellow was considered positive for fermentation. The isolates were then assigned to different species based on the result of the biochemical tests.

3.4.3 Molecular identification of *Enterococcus* species

DNA extraction: Genomic DNA extraction was carried out as described by Laird *et al.* (1991) with slight modifications which included overnight incubation in lysis buffer and ethanol washing of the DNA pellets. Briefly, the isolates were grown overnight in five milliliters of brain heart infusion broth (Oxoid, UK) overnight followed by centrifugation at 11200*g* for 10 minutes to pellet the cells. Cell pellets were then suspended in the lysis buffer (100mM Tris HCl pH 8.5, 5mM EDTA, 0.2% sodium dodecyl sulfate (SDS), 200mM NaCl) and incubated for 2hr at 55°C followed by an overnight incubation at 37°C. The mixture was then centrifuged at 11200*g* for 10 min to pellet the cellular debris after which the supernatant (lysate) was harvested. Isopropanol (600µl) was then added to the lysate and the mixture votexed for one minute to precipitate the DNA. The samples were

then left on the bench for 30 min and then the mixture centrifuged at 11200*g* for 2 minutes to pellet the DNA followed by decanting of the isopropanol. The pelleted DNA was then washed twice by addition of 500µl of 70% ethanol and centrifugation at 11200*g* for 2mins. The ethanol was then decanted carefully so as not to dislodge the DNA pellet and the tubes left open in a sterile chamber for 30 minutes to allow for evaporation of any residual ethanol. After the evaporation the pellet was resuspended in 150µl of TE buffer (10mM Tris HCl, 0.9mM EDTA pH 8.0) and left overnight at 37°C to dissolve the DNA pellet. After the complete dissolution of the DNA, 5µl of RNase was added to the extracted DNA. The success of the DNA extraction and the DNA integrity were then assessed by electrophoresis of 10µl of the extracted DNA on 0.7% agarose gel stained with ethidium bromide in Tris Borate EDTA. The DNA concentration and purity were then determined spectrophotometrically using NanoDrop 2000 spectrophotometer (ThermoScientific, UK) at an absorbance of 260nm and A_{260}/A_{280} respectively. The DNA was then stored at 4°C until required for further analyses.

Polymerase chain reaction: The polymerase chain reaction (PCR) was performed using a GeneAmp PCR System 9700 (Applied Biosystem, USA). Polymerase chain reaction targeting the D-alanine: D-alanine ligase (*ddl*) gene of *E. faecium* was carried out on the *E. faecium* isolates using primers (F1: GCAAGGCTTCTTAGAGA and F2: CATCGTGTAAGCTAACTTC) and conditions. Briefly, the reaction was carried out in a total reaction volume of 10µl containing 3µl of DNA template, 0.5 µl 50mM $MgCl_2$, 0.8µl 2.5mM dNTPs, 0.8µl DMSO, 2.9µl H_2O, 1.0µl of 10x PCR buffer, 0.1µl (2.5U) of *Taq* DNA polymerase (Qiagen), 0.5µM each of forward primer and reverse primers. Amplification of the DNA was carried out using the following thermal cycling conditions: an initial denaturation for 5 min at 94°C; 30 cycles of 30 s at 94°C (denaturation), 90 s at

50°C (annealing), and 60 s at 72°C (extension); and a final extension step of 10 min at 72°C.

3.4.4 Analysis of PCR products

After amplification, 5µl of the amplicons was mixed with gel loading buffer and electrophoresed in a 2% agarose gel for 45 minutes at 100 V in 0.5x TBE (Tris borate-EDTA) containing 0.05 mg/L of ethidium bromide. A 100-bp Plus DNA ladder (Invitrogen, Belgium) was used as a molecular size marker. The gel image was photographed using a UV light source ENDURO^{TM} GDS Gel Documentation System (Labnet International, Inc, USA).

3.5 Phenotypic Screening of the *E. faecium* and *E. faecalis* for Expression of Virulence Traits

3.5.1 Assay for haemolysin (cytolysin) production

The isolates stored on BHI slants were streaked on tryptone soya agar (TSA) and incubated overnight at 37°C. Production of cytolysin was then assessed by picking a colony of the *E. faecalis* or *E. faecium* from the TSA and inoculating onto TSA agar (Oxoid, UK) supplemented with 5% horse blood as described by Semedo *et al.* (2003). Plates were then incubated at 37 °C for 48h after which they were examined for β- haemolysis observed as complete zones of clearing around the colonies. *E. faecalis* MMH594 was used a positive control.

3.5.2 Assay for gelatinase production

Production of gelatinase was detected by observation for gelatin hydrolysis using a nutrient gelatin broth (Oxoid, UK) supplemented with 13% gelatin (Sigma, UK). A heavy inoculum of an overnight culture of each test isolates was stab-inoculated into tubes containing nutrient gelatin. The inoculated tubes, an uninoculated and inoculated control tubes were incubated at 37°C for 24 - 72h followed by observation for gelatin liquefaction. Gelatin normally liquefies at 28°C and above so to confirm that liquefaction was due to gelatinase activity, the tubes were removed from the incubator after the various incubation periods and kept at 4°C for 15 - 20 minutes. Afterwards, the tubes were tilted to observe if gelatin has been hydrolyzed. Hydrolyzed gelatin will result in a liquid medium even after exposure to the low temperature while the uninoculated control medium will remain solid. *E. faecalis* MMH594 was used as positive control.

3.5.3 Assay for biofilm production

Congo red agar (CRA) was prepared using brain heart infusion agar, containing 5% sucrose, 0.08% Congo red, 1.5% NaCl and 2% glucose as described by Kaiser *et al.* (2013). Briefly, four microliter of overnight growth in TSB of each of the test isolates were spoted on the CRA plates and incubated at 37 °C for 24 h. After the 24h incubation period, the colonies of the test isolates were observed for production of rough black or red colonies. Biofilm producing isolates formed black colonies, while the non-biofilm producing isolates formed red colonies. *E. faecalis* MMH594 was used as positive control.

3.5.4 Assay for aggregation substance production

E. faecalis JH2-2 was grown for 6 h at 37°C in brain heart infusion broth (Oxoid, USA) and centrifuged afterwards at 4032*g* for 10 minutes. The supernatant containing the pheromone (a protein that promotes donor and recipient cell aggregation leading to conjugative transfer of plasmids) that induces pheromone-responsive plasmids was harvested and autoclaved for 15 min (Macovei and Zurek, 2006; 2007). Two mililiter of the pheromone containing supernatant was mixed with two milliliters of fresh brain heart infusion broth (Oxoid, USA) followed by addition of 50µl of each test isolates. The mixture was then incubated for 4 h and 24h at 37°C and examined for the formation of aggregates (clumping) (Waar *et al.*, 2002). Tubes showing clumping (as determined by the naked eye) were considered positive for aggregation substance expression. *E. faecalis* OG1RF (pCF10) and *E. faecalis* FA2-2 pMV158 were used as positive and as negative controls, respectively.

3.6. Determination of the Antibiotic Susceptibility Profiles of the *Enterococcus faecium* and *E. faecalis* Isolated

The susceptibility profile of each isolate to a panel of 10 antimicrobial agents was determined using the disc diffusion while the minimum inhibitory concentration of gentamicin and ampcillin was determined using the E-test strip. Both techniques were carried out and interpreted as recommended by the Clinical and Laboratory Standard Institute (CLSI, 2011). Briefly, colonies (four to five) of the test isolates from overnight cultures on TSA plates were picked and emulsified in one milliliter of sterile normal saline contained in a test tube. The turbidity of the suspension was adjusted to match a 0.5 MacFarland's standard with normal saline. A sterile swab stick soaked in the suspension of

the organism was spread on Mueller-Hinton agar plates (Oxoid, UK) to create a uniform lawn. The pre-inoculated plates were used for the disc diffusion test and determination of the minimum inhibitory concentrations.

3.6.1 Disc diffusion test

The susceptibility of the isolates to a panel of 10 antimicrobial agents (belonging to nine different antimicrobial classes) was determined. Antibiotic discs (Oxoid, UK) impregnated with the following antimicrobials (and concentrations) were used: Ampicillin (10µg), ciprofloxacin (5µg), erythromycin (15µg), quinupristin-dalfopristin (15µg), tetracycline (30µg), chloramphenicol (30µg), rifampin (5µg), gentamicin (120µg), streptomycin (300µg) and vancomycin (30µg). The antimicrobial discs were placed on the surface of each of the pre-inoculated Mueller-Hinton plates using disk dispenser (Oxoid, USA) (two plates were used per isolate and five different antibiotic discs were dispensed on each plate) and the plate incubated aerobically at 37°C for 24h. After the period of incubation, the diameters of the antibiotic inhibition zones were measured to the nearest millimetre (mm) and recorded as susceptible (S), intermediate (I) or resistant (R) (CLSI, 2011).

3.6.2 Minimum inhibitory concentrations (MICs)

MICs of gentamicin (1024-0.06µg/ml) and ampicillin (256 - 0.016µg/ml) against *E. faecalis* and *E. faecium* (resistant to gentamicin or ampicillin from the disc diffusion test) was determined using the E-test strip (Biomerieux, France). The MIC determination was carried out as described by the manufacturer and the values interpreted using the CLSI guidelines (CLSI, 2011). Briefly, E-test strips impregnated with varying concentrations of

the antibiotics were carefully placed onto the pre-inoculated Mueller Hinton agar plates. The plates were then incubated for 24h at 37°C. After the period of incubation an inhibition ellipse was observed around the strip. The point where the eclipse intersected the strip corresponds to the antimicrobial concentration no longer inhibitory to the growth of the organism. The corresponding concentration of antibiotic at the point of intersection on the strip was read as the MIC in µg/ml. Where the growth intersects on the line between two sections, the MIC was read as the value in the lower section. In cases where growth of the test organism was observed along the entire length of the strip, the MIC was read as greater than the highest value on the E-test strip. On the other hand, where the growth of the test organism was inhibited around the strip, the MIC was read as less than the lowest value on the E-test strip. The MIC values was interpreted as susceptible, intermediate and resistant and the MIC_{50} and MIC_{90} values defined as the MICs at which 50% and 90% of the investigated isolates were inhibited, respectively, were also calculated. The MIC_{50} and MIC_{90} were calculated from the 50^{th} and 90^{th} percentile of the MIC values of all the isolates.

3.7 Molecular Detection of Virulence and Reistance Genes

Molecular characterization was carried out using facilities in the Bioscience Laboratory of the International Institute for Tropical Agriculture, Ibadan, Nigeria.

DNA extraction was carried out as previously described under section 3.4.3

3.7.1 Detection of virulence genes

The isolates were screened for carriage of some virulence genes; *asa1* (aggregation substance), *cylA (*cytolysin), *gelE* (gelatinase) and *esp* (enterococcal surface protein). PCR to amplify each of the virulence genes was carried using a monoplex PCR, previously

published primers (Table 3.1) and conditions described by Vankerckhoven *et al.* (2004) and Eaton and Gasson (2001). Briefly, PCR for detection of each gene was carried out in a 10 µl PCR mixture containing; 3µl of DNA template, 0.5 µl 50mM $MgCl_2$, 0.8µl 2.5mM dNTPs, 0.8µl DMSO, 2.9µl H_2O, 1.0µl of 10x PCR buffer, 0.1µl (2.5U) of *Taq* DNA polymerase (Qiagen), 0.5µM each of forward and reverse primers. Amplification was carried out using an initial denaturation step of 95°C for 5 min followed by 30 cycles of denaturation (94°C for 1 min), annealing (56°C for 1 min), and extension (72°C for 1 min), followed by a final extension of 5 min at 72°C . *E. faecalis* MMH594 was used as the positive control for the detection of *cyl*A, *esp* and *gel*E genes.

3.7.2 Detection of antibiotic resistance genes

Isolates that were resistant to ampicillin, gentamicin, erythromycin and tetracycline were screened for carriage of some genes responsible for the corresponding resistance.

Aminoglycoside resistance genes: PCR to amplify genes encoding four aminoglycoside modifying enzymes; *aac(6')*-Ie-*aph(2")*-Ia, *aph(2")*-Ib, *aph(3´)*-III*a*, and *ant(4´)*-Ia genes was carried using a monoplex PCR, previously published primers (Table 3.1) and conditions described by Vakulenko *et al*. (2003). Briefly, PCR for each gene was carried out in a 10µl total reaction volume containing 3µl of DNA template, 0.5 µl 50mM $MgCl_2$, 0.8µl 2.5mM dNTPs, 0.8µl DMSO, 2.9µl H_2O, 1.0µl of 10x PCR buffer, 0.1µl (2.5U) of *Taq* DNA polymerase (Qiagen), 0.5µM each of forward and reverse primers. Amplification was carried out using the following thermal cycling conditions: an initial denaturation for 3 min at 94°C; 35 cycles of 40 s at 94°C (denaturation), 40 s at 55°C (annealing), and 40 s at 72°C (extension); and a final extension step of 2 min at 72°C. *E. faecalis* ATCC 49532 and

Table 3.1: List of primers and sequences used for the amplification of some virulence and aminoglycoside reisstance genes from *Enterococcus* isolated from vegetable and livestock farms in Zaria, Nigeria

Genes	Primer Sequence 5′ → 3′	Product size	Reference
***asa*1**	ASA1F:GCACGCTATTACGAACTATGA ASA2R:TAAGAAAGAACATCACCACGA	**357**	Vankerckhoven *et al.*, 2004
***gel*E**	GEL1F: TATGACAATGCTTTTTGGGAT GEL2R: AGATGCACCCGAAATAATATA	**213**	Vankerckhoven *et al.*, 2004
***cyl*A**	CYT IAF: CTCGGGGATTGATAGGC CYT IIbR: GCTGCTAAAGCTGCGCTT	**517**	Eaton and Gasson, 2001
Esp	**F** AGATTTCATCTTTGATTCTTGG **R** AATTGATTCTTTAGCATCTGG	**510**	Vankerckhoven *et al.*, 2004
***aac(6´)*-Ie-*aph(2´´)*-Ia**	F:CAGAGCCTTGGGAAGATGAAG R:CCTCGTGTAATTCATGTTCTGGC	**348**	Vakulenko *et al*., 2003
***aph(2´´)*-Ic**	F:CCACAATGATAATGACTCAGTTCCC R:CCACAGCTTCCGATAGCAAGAG	**444**	Vakulenko *et al*., 2003
***aph(3´)*-IIIa**	F:GGCTAAAATGAGAATATCACCGG R: CTTTAAAAAATCATACAGCTCGCG	**523**	Vakulenko *et al*., 2003
***ant(6´)*-Ia**	F:CAAACTGCTAAATCGGTAGAAGCC R:GGAAAGTTGACCAGACATTACGAACT	**294**	Vakulenko *et al*., 2003

E. faecium SF11770 Ib (chow) were used as positive control for detection of *aac(6')-Ie-aph(2")-Ia* and *aph(2")-Ib* genes respectively.

Erythromycin resistance gene: PCR to amplify the erythromycin resistance methylase gene (*erm*B) encoding ErmB responsible for dimethylation of the adenine residue at position 2085 in 23S rRNA was carried using a monoplex PCR, previously published primers (Table 3.2) and conditions described by Sutcliffe *et al.* (1996). Briefly, PCR for detection of the *erm*(B) gene was carried out in a 10µl total reaction volume containing 3µl of DNA template, 0.5µl 50mM $MgCl_2$, 0.8µl 2.5mM dNTPs, 0.8µl DMSO, 2.9µl H_2O, 1.0µl of 10x PCR buffer, 0.1µl (2.5U) of *Taq* DNA polymerase (Qiagen), 0.5µM each of forward and reverse primers. Amplification was carried out using the following thermal cycling conditions: an initial denaturation step at 94°C for 3 min, 94°C for 3 min, 55°C for 3 min, 72°C for 30 min, and 72°C for 4 min (final extension). *E. faecalis* JH2-2:*Tn1545* was used as the positive control.

Tetracycline resistance genes: PCR to amplify genes encoding active efflux (*tetK*, *tetL*) and ribosomal protection (*tetM and tetO*) was carried out using a monoplex PCR, previously published primers (Table 3.2) and conditions described by Ng *et al*. (2001). Briefly, PCR for each gene was carried out in a 10µl total reaction volume containing 3µl of DNA template, 0.5 µl 50mM $MgCl_2$, 0.8µl 2.5mM dNTPs, 0.8µl DMSO, 2.9µl H_2O, 1.0µl of 10x PCR buffer, 0.1µl (2.5U) of *Taq* DNA polymerase (Qiagen), 0.5µM each of forward and reverse primers. Amplification was carried out using the following thermal cycling conditions: an initial denaturation for 5 min at 94°C followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1·5 min. *E. faecalis* CG110: *Tn916* was used as the positive control for the *tet*M gene.

Ampicillin resistance gene: PCR to amplify the *pbp5* gene encoding the production of low affinity penicillin binding protein 5 (PBP5) was carried using a monoplex PCR, previously published primers (Table 3.2) and conditions described by Jureen e*t al*. (2003). Briefly, PCR for detection of the *pbp*5 gene was carried out in a 10µl total reaction volume containing; (PBP5F-1 and PBP5R). Amplification was carried out using the following thermal cycling conditions: an initial denaturation step of 95°C for 5 min, followed by 30 cycles of denaturation (94°C for 30s), annealing (54°C for 30 s), and extension (72°C for 2 min), followed by one cycle consisting of 7 min at 72°C. *E. faecium* 9730357-1 was used a positive control.

3.7.3 Analysis of PCR products: Refer to page section 3.4.4

3.7.4 Sequencing of the C-terminal region of the *pbp*5 gene

The products from the PCR amplification of *pbp*5 were purified by addition of two volume of absolute ethanol followed by incubation at room temperature and centrifugation at 11200*g* for 15minutes. The purified product was then sequenced on both strands (forward and reverse) using primers PBP5F-2 and PBP5R using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit and analysed on an ABI PRISM 3130xl, genetic analyzer (Applied Biosystems, USA). The sequences obtained were analyzed using BioEdit version 7.2 software to generate a consensus sequence which was compared with that of the *pbp*5 gene reference sequence X84860 in the GeneBank and then translated into amino acid sequences. The nucleotide sequences were deposited in the GenBank database under accession number KX377704, KX377705, KX377706, KX377707.

Table 3.2: List of primers and sequences used for the amplification of erythromycin, tetracycline and ampicillin resistance genes from *Enterococcus* isolated from vegetable and livestock farms in Zaria, Nigeria.

Genes	Primer Sequence 5′ → 3′	Product size	Reference
***erm*B**	F: GAAAAGGTACTCAACCAAATA R: AGTAACGGTACTTAAATTGTTTAC	539	Sutcliffe *et al..*, 1996
***tet*L**	F: TCG TTA GCG TGC TGT CAT TC R: GTA TCC CAC CAA TGT AGC CG	267	Ng *et al.*, 2001
***tet*M**	F: GTG GAC AAA GGT ACA ACG AG R: CGG TAA AGT TCG TCA CAC AC	406	Ng *et al.*, 2001
***tet*O**	F: AAC TTA GGC ATT CTG GCT CAC R: TCC CAC TGT TCC ATA TCG TCA	515	Ng *et al.*, 2001
***pbp*5**	PBP5F-1: AACAAAATGACAAACGGG PBP5R : TATCCTTGGTTATCAGGG PBP5F-2:GACAAACGGGATCTCACAAG	659	Jureen *et al.*, 2003

3.8 Conjugal Transfer of Antibiotic Resistance Phenotypes

Conjugal transfer experiments were carried out to determine the transferability of tetracycline and erythromycin resistance phenotypes from the donor to the recipient strain. *E. faecalis* JH2-2 which is resistant to rifampin and susceptible to tetracycline and erythromycin was used as the recipient strain while the donor strains were eight multidrug resistant isolates displaying resistance to erythromycin and tetracycline.

Filter mating experiment was carried out as described by Lester *et al.* (2004). Briefly, an equivalent of 1.0 x 10^9 bacterial cells of the overnight cultures of donor and recipient strains in brain heart infusion broth (Oxoid, UK) were mixed in a 1:1 ratio (100µl of each). One hundred microliters of the mixed culture was then dispensed onto a 47-mm sterile filter (0.2µm, Millipore, USA) placed on a 5% brain heart infusion plate. The mixed culture was allowed to soak into the filter and the plates incubated at 37°C for 24h for mating to occur. After the period of incubation the cells were washed from the filter paper using five mililiters of normal saline. Serial dilution of the rinsate was carried out in normal saline and dilutions of 10^{-3} and 10^{-5} were spread on antibiotic supplemented plates to determine the transferability and the number of transconjugants. Plates containing 15µg/ml erythromycin and 30µg/ml rifampin (ER) or 10µg/ml tetracycline and 30µg/ml rifampin (TR) were used for detecting transconjugants; 15 µg/ml erythromycin or 10µg/ml tetracycline for detecting donors; and transconjugants and plates containing 30µg/ml rifampin for detecting recipients and transconjugants. The plates were incubated at 37°C for 24 - 72h and checked for the absence (donor and recipient plates) or presence (mating mixture plate) of growth. The transconjugants grown on selective media were purified and

their antimicrobial suceptibility tested using the disk diffusion tests as recommended by CLSI (2011).

3.9 Data Analysis

Data obtained were expressed as percentages and presented in tabular forms or as plates. Fisher's exact test was used to determine association between categorical variables (isolation rate vs type of sample and occurrence of multidrug resistance vs type of sample) and values of $p \leq 0.05$ were considered significant. Isolation rates were calculated by dividing the total number of samples positive by total number of samples collected and multiplied by 100.

CHAPTER FOUR

RESULTS

4.1 Occurrence and Distribution of *Enterococcus* Species Isolated from Poultry, Cattle and Vegetables Farms in Zaria, Nigeria

4.1.1 Isolation rate of *Enterococcus* species from all sample types

Out of the 670 samples collected and examined for the presence of *Enterococcus*, *Enterococcus* species were isolated from 200 (29.9%) samples (Table 4.1). *Enterococcus* was recovered from 58 (44.6%) cloacal swabs and 29 (44.6%) manure samples in 10 and eight poultry farms respectively, while from 10 cattle farms enterococci were recovered from 45 (34.6%) and 26 (40.0%) of rectal swabs and manure samples, respectively (Table 4.1; Appendix 1). In the vegetable farms *Enterococcus* was isolated from 1 (2.01%) carrot, 8 (40.0%) cabbage and 15 (28.8%) lettuce samples. In the case of soil from the vegetable farms, enterococci were isolated from 6 (12.8%), 2 (10.0%) and 10 (19.2%) soil sample from carrot, cabbage and lettuce farms respectively (Table 1).

4.1.2 Distribution and diversity of *Enterococcus* species

Of the 267 enterococcal isolates recovered from the 200 *Enterococcus* positive samples, the species identity of 64 (24%) of the isolates could not be ascertained and were classified as *Enterococcus* spp. The remaining 203 isolates were distributed across 9 different species: *E. faecium* (n=130), *E. faecalis* (n=5), *E. raffinosus* (n=6), *E. avium* (n=1), *E. gallinarum* (n=48), *E. casseliflavus* (n=3), *E. mundtii* (n=4), *E. durans* (n=1) and *E. hirae* (n=5) (Table 4.2).

Table 4.1: Positive samples and isolation rates of *Enterococcus* from poultry, cattle and vegetable farms in Zaria, Nigeria.

Sample Type	**Total samples collected**	**[a]No of Samples Positive (%)**	**No. of *Enterococcus* Isolates (% isolation rate)**
Poultry Cloacal Swab	130	58 (44.6)	72 (55.4)
Poultry Manure	65	29 (44.6)	33 (50.8)
Cattle Rectal Swab	130	45 (34.6)	60 (46.2)
Cattle Manure	65	26 (40.0)	41 (63.1)
Carrot	48	1 (2.08)	1 (2.0)
Cabbage	20	8 (40.0)	10 (50.0)
Lettuce	52	15 (28.8)	27 (51.9)
Soil (Carrot farm)	48	6 (12.8)	7 (14.6)
Soil (Cabbage farm)	20	2 (10.0)	3 (15.0)
Soil (lettuce farm)	52	10 (19.2)	13 (25.0)
Water	40	0	0
Total	**670**	200 (29.9)	267

[a]*p value for association between sample type and isolation of Enterococcus = p<0.0001*

Table 4.2: Occurrence and diversity of *Enterococcus* species isolated from poultry, cattle and vegetable farms in Zaria, Nigeria

Sample Type	**Total No. of Isolates**	**No. (%) of Individual *Enterococcus* species**									
		faecium	*faecalis*	*raff*	*avium*	*Gall*	*cass*	*mund*	*hirae*	*dur*	Unident
Poultry Cloacal Swab	72	49(68.1)	4 (5.6)	1(1.4)	0	6(8.3)	0	1(1.4)	1(1.4)	1(1.4)	9(12.5)
Poultry Manure	33	27(81.8)	0	1(3.0)	1(3.0)	3(12.1)	1(3.0)	0	0	0	0
Cattle Rectal Swab	60	20(33.3)	1(1.7)	0	0	14(23.3)	1(1.7)	2(3.3)	2(3.3)	0	20(33.3)
Cattle Manure	41	12(29.3)	0	0	0	7(17.1)	1(2.4)	0	2(4.9)	0	19(46.3)
Carrot	1	1(100)	0	0	0		0	0	0	0	0
Cabbage	10	5(50.0)	0	0	0	1 (10.0)	0	0	0	0	4(40)
Lettuce	27	5(18.5)	0	1(3.7)	0	11(40.7)	0	0	0	0	10(37)
Carrot (soil)	7	5(71.4)	0	0	0	1(14.3)	0	0	0	0	1 (14.3)
Cabbage(soil)	3	2(66.7)	0	0	0	0	0	0	0	0	1(33.3)
Lettuce (soil)	13	4(30.8)	0	3(23)	0	5	0	1(7.7)	0	0	0
Total	**267**	**130(48.7)**	**5 (1.9)**	**6 (2.2)**	**1(0.4)**	**48(18)**	**3(1.1)**	**4(1.5)**	**5(1.8)**	**1(1.4)**	**64(24)**

raff: E. raffinosus, gall: E. gallinarum, cass: E. casseliflavus mund: E. mundtii, dur: E. durans, Unident: Unidentified

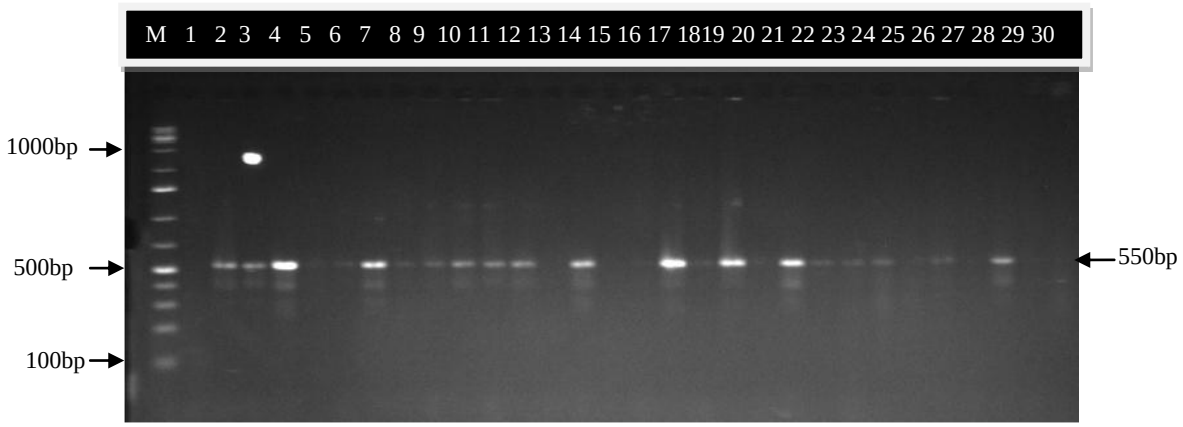


Plate 4.1: Electrophoresis image of the PCR detection of the *ddl* gene (550bp) among enterococci isolated from livestock and vegetable farms in Zaria, Nigeria. Lane M: 100bp Plus DNA ladder and Lanes 1 – 30 isolates. Positive isolates: Lanes 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27

4.2 Phenotypic Expression of Virulence Factors among *Enterococcus* Species Isolated from Poultry, Cattle and Vegetable Farms

Production of β - haemolysin, gelatinase, aggregation substance and ability to form biofilm were detected in 28 (20.7%), 13 (9.62%), 2 (1.48%) and 113 (83.7%) of the 135 isolates tested, respectively The virulence traits were expressed more by isolates from cloacal swabs of poultry followed by isolates from rectal swabs of cattle. Aggregation substance was the virulence trait least expressed by the isolates (Table 4.3). None of the *E. faecalis* produced haemolysin or aggregation substance but three produced both gelatinase and biofilm. Also isolates obtained from carrot, lettuce and soil from lettuce farms expressed only one (biofilm production) of the virulence traits evaluated (Table 4.3).

Table 4.3: Phenotypic expression of virulence factors by the *E. faecium* and *E. faecalis* isolated from poultry, cattle and vegetable farms in Zaria, Nigeria

Sample Type	Total No. of Isolates	No. (%) of Isolates Producing			
		Hemolysin	Gelatinase	Aggregation Substance	Biofilm
Poultry Cloacal Swab	53	15(28.3)	6[a] (11.3)	1 (1.9)	47[c] (88.7)
Poultry Manure	27	4 (14.8)	1 (3.7)	0	21 (77.8)
Cattle Rectal Swab	21	4 (19.0)	5[b] (23.8)	1 (4.8)	16 (76.2)
Cattle Manure	12	1 (8.33)	0	0	10 (83.3)
Carrot	1	0	0	0	1 (100)
Cabbage	5	1 (20)	0	0	3 (60.0)
Lettuce	5	0	0	0	5 (100)
Carrot (soil)	5	2 (40.0)	1 (20.0)	0	5 (100)
Cabbage (soil)	2	1 (50.0)	0	0	2 (100)
Lettuce (soil)	4	0	0	0	3 (75.0)
Total	**135**	**28 (20.7)**	**13 (9.62)**	**2 (1.48)**	**113 (83.7)**

[a]one, [b]two and [c]three *E. faecalis* isolates

4.3 Virulence Encoding Genes among the *Enterococcus* Isolated from Poultry, Cattle and Vegetable Farms

4.3.1 Detection of *asa*1, *esp and cyl*A *and gel*E genes

The PCR amplification of enterococcal virulence genes revealed a high incidence of genes encoding for aggregation substance (*asa*1) which was found among 14 out of 20 isolates tested (Plate 4.2). *cyl*A encoding hemolysin/cytolysin production was detected in only one of the 19 isolates (Plate 4.3), gelatinase production (*gel*E) among 10 of the 19 isolates tested with the gelatinase gene *gelE* absent in 5 of the 13 isolates that phenotypically expressed its production, but was present in two isolates that did not phenotypically express it (Plate 4.4), and none of the isolates tested harbored the *esp* gene encoding the enterococcal surface protein.

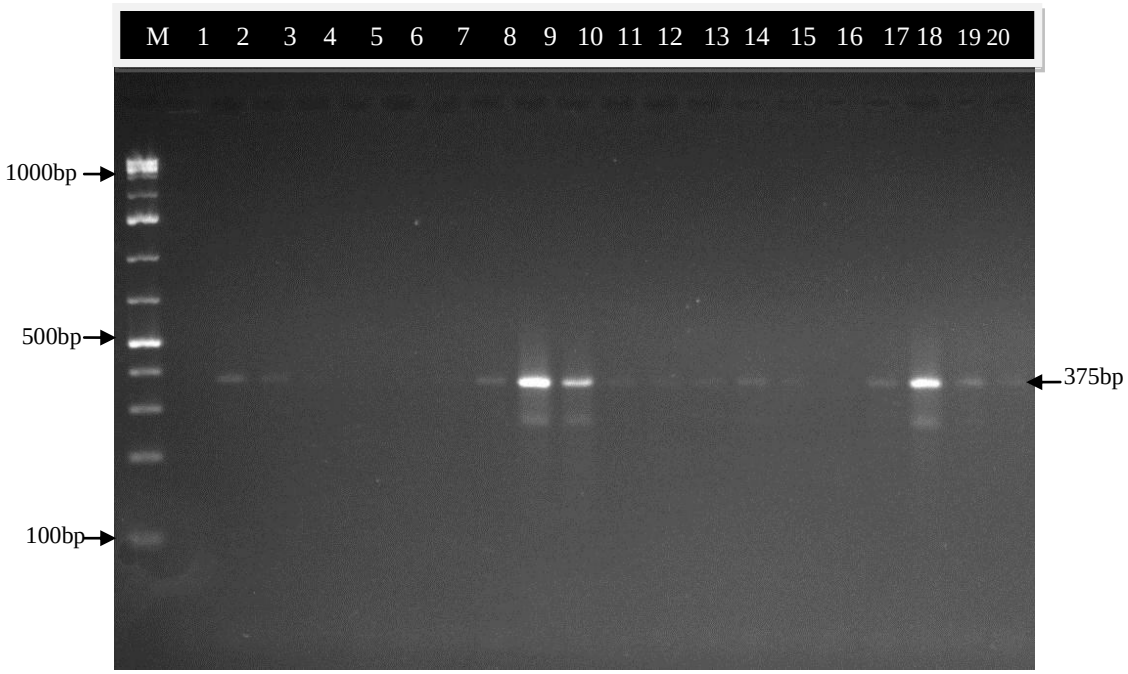


Plate 4.2: Gel Electrophoresis image of the PCR detection of the *asa*1 (375bp) gene among enterococci isolated from livestock and vegetable farms in Zaria, Nigeria. Lane M: 100bp Plus ladder, Lanes 1 – 20 isolates (Lanes 1, 6, 8, 15 are isolates from rectal swab of cattle, lane 2 is an isolate from soil in a carrot farm, lanes 3, 4, 9, 10, 11, 12, 14, 17, 19 are isolates from cloacal swab of poultry, lanes 5, 13, 16, 18 are isolates from poultry manure while lane 7 is an isolate from soil in a cabbage farm). Positive isolates: Lanes 2, 3, 7, 8, 9, 11, 12, 13, 14, 15, 17, 18, 19, 20

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Plate 4.3: Gel Electrophoresis image of the PCR detection of the *cyl*A (500bp) gene among enterococci isolated from livestock and vegetable sources in Zaria, Nigeria. Lane M: 100bp Plus ladder, Lane PC: Positive control *E. faecalis* MMH594 Lane 1 – 19 isolates (Lanes 1, 6, 9, 16, are isolates from rectal swab of cattle, lanes 2, 7 are isolates from soil in a carrot farm, lanes 3, 5, 10, 12, 13, 14, 18, 19 are isolates from cloacal swab of poultry, lanes 4, 15, 17 are isolates from poultry manure while lane 8 is an isolate from soil in a cabbage farm). Positive isolate: Lane 14

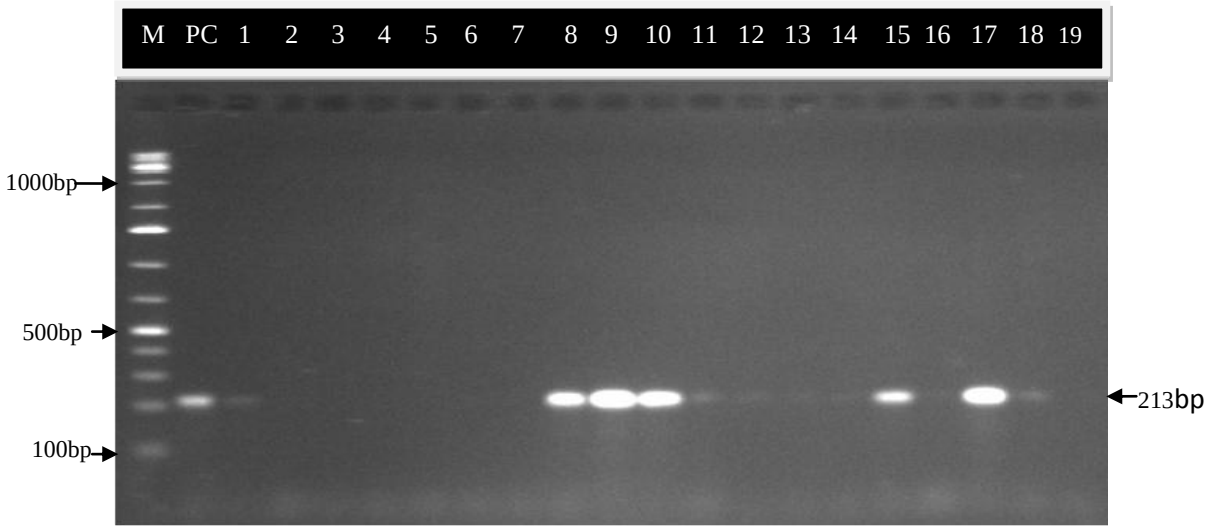


Plate 4.4: Gel Electrophoresis image of the PCR detection of *gel*E (213bp) gene among enterococci isolated from livestock and vegetable farms in Zaria, Nigeria. Lane M: 100bp Plus DNA ladder, Lane PC: Positive control *E. faecalis* MMH594 and Lane 1 - 19 isolates (Lanes 1, 6, 8, 15 are isolates from rectal swab of cattle, lane 2 is an isolate from soil in a carrot farm, lanes 3, 4, 9, 10, 11, 12, 14, 17, 19 are isolates from cloacal swab of poultry, lanes 5, 13, 16, 18 are isolates from poultry manure while lane 7 is an isolate from soil in a cabbage farm). Positive isolates: Lanes 1, 8, 9, 10, 11, 12, 14, 15, 17, 18

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4.4 Antimicrobial Susceptibility of *Enterococcus* Species Isolated from Cattle, Poultry and Vegetable Farms

4.4.1 Antimicrobial susceptibility profiles

Out of the 135 isolates tested only 14 (10.4%) were susceptible to all 10 antimicrobial agents while a high proportion (89.6%) of the isolates were resistant to at least one (≥1) antimicrobial agent (Table 4.4). Vancomycin was the only antimicrobial agent to which all of the 135 isolates were susceptible. Resistance among the isolates was observed more to erythromycin (54.1%) followed by tetracycline (52.6%), ampicillin (46.7%) and rifampicin (39.3%) (Table 4.4). The agents to which low frequencies of resistance was observed other than vancomycin were quinupristin-dalfopristin; (3.70%), chloramphenicol (6.67%) and ciprofloxacin (8.14%). Also high level resistance to the aminoglycosides (gentamicin and streptomycin) were detected in 37 (25.9%) and 32 (23.7%) of the *Enterococcus* isolates, respectively (Table 4.4).

4.4.2 Minimum Inhibitory Concentration (MIC) of ampicillin and gentamicin against *Enterococcus* isolates

The minimum inhibitory concentration (MIC) of ampicillin and gentamicin for the 63 and 37 isolates phenotypically resistant to ampicillin and high level gentamicin (120µg) respectively, from the disc diffusion was determine by E-test. Seven (11.5%) out of the 63 ampicillin resistant isolates had MIC equal to or greater than the breakpoint value (≥ 16µg/ml). Six out of the seven isolates expressed MIC values > 265, while one expressed MIC value ≥ 16µg/ml (Table 4.5 and Appendix 3). The MIC values of ampicillin that inhibited the growth of 50% (MIC_{50}) and 90% (MIC_{90}) of the tested isolates were ≤ 4µg/ml and ≤ 16µg/ml respectively (Table 4.5) while the modal MIC of ampicillin was 1 µg/ml and displayed by 11 isolates (Appendix 3).

Thirty five (94.6%) out of the 37 gentamicin resistant (from the disc diffusin test) isolates expressed resistance to MICs of ≥ 500 µg/ml which is beyond the established breakpoints. The MIC of gentamicin that inhibited the growth of 50% (MIC_{50}) and 90% (MIC_{90}) of the isolates was ≥ 1024 µg/ml (Table 4.5).

4.4.3 Frequency of Multidrug resistance and resistance phenotypes among *Enterococcus* isolates

Multiple drug resistance (MDR), defined as resistance to three or more antibiotics of different classes in this study, was observed in 62 (45.9%) of the 135 isolates tested. MDR was observed more frequently in isolates from poultry sources (66.7% and 55.6% for poultry cloacal swabs and manure, respectively). There was a significant association (*p=0.0035*) between source of the isolates from rectal swab (i.e. poultry or cattle) and occurrence of MDR but no significant association (*p=0.200*) was observed between source of the manure (i.e. poultry or cattle) and occurrence of MDR. MDR isolates were resistant to as few as three antibiotics and, as many as seven different antibiotics and up to six different antibiotic classes (Table 4.6). A total of 24 different phenotypic patterns were identified among the 62 MDR isolates. The predominant MDR pattern was the CN-E-S-TE phenotype which was expressed by 11 (18.3%) of the 62 MDR isolates, followed by the AM-CN-E-S-TE (7 isolates) and CN-E-TE (6 isolates) patterns. Five resistance patterns (AM-E-TE, CN-E-TE, AM-CN-E-TE, CN-E-S-TE and AM-CN-E-S-TE) were common among isolates from cloacal swabs and poultry manure while only one pattern (AM-E-R) was common to isolates from cattle and cattle manure. Also, co-resistance to AM, E and TE frequently occurred among the 24 different phenotypes i.e resistance to the three antibiotics occurred in 12 (50%) out of the 24 MDR phenotypes (Table 4.7).

Table 4.4: Antimicrobial resistance profile of *E. faecium* and *E. faecalis* isolated from poultry, cattle and vegetable farms in Zaria, Nigeria

Sample Type	**Total No. Tested**	**No. (%) of isolates resistance to antibiotics**									
		QD	**CN**	**VAN**	**RIF**	**ERY**	**CIP**	**STR**	**CHL**	**AMP**	**TET**
Poultry Cloacal Swab	53	5[a] (9.4)	25[b] (47.1)	0	8[b] (15.1)	39[c] (73.5)	6 (11.3)	25[c] (47.1)	5[c] (9.4)	23[a] (43.3)	43[c] (81.1)
Poultry Manure	27	0	10 (35.7)	0	11 (39.2)	20 (71.4)	4 (14.3)	4 (14.3)	2 (7.1)	12 (42.9)	17 (60.7)
Cattle Rectal Swab	21	0	2 (7.1)	0	10 (35.7)	7[b] (35.0)	1 (5.0)	3 (15.0)	2[b] (7.1)	10 (35.7)	5[b] (25.0)
Cattle Manure	12	0	0	0	9 (75.0)	3 (25.0)	0	0	0	6 (50.0)	4 (33.3)
Carrot	1	0	0	0	1 (100)	0	0	0	0	1 (100)	0
Cabbage	5	0	0	0	3 (60.0)	0	0	0	0	2 (40.0)	0
Lettuce	5	0	0	0	4 (80.0)	1 (20.0)	0	0	0	3 (60.0)	1 (20.0)
Carrot (soil)	5	0	0	0	2 (40.0)	1 (20.0)	0	0	0	2 (40.0)	0
Cabbage (soil)	2	0	0	0	1 (50.0)	-	0	0	0	2 (100)	0
Lettuce (soil)	4	0	0	0	4 (100)	2 (50.0)	0	0	0	2 (50.0)	1 (25.0)
Total (%)	**135**	**5 (3.70)**	**37 (25.9)**	**0**	**53 (39.3)**	**73 (54.1)**	**11 (8.14)**	**32 (23.7)**	**9 (6.67)**	**63 (46.7)**	**71 (52.6)**

[a]Four isolates are *E. faecalis,* [b]one isolate is *E. faecalis,* [c] three isolates are *E. faecalis* QD: Quinupristin-Dalfopristin, CN: Gentamicin,VAN: Vancomycin, RIF: Rifampicin, ERY: Erythromycin, CIP: Ciprofloxacin, STR: Streptomycin, CHL: Chloramphenicol, AMP: Ampicillin, TET: Tetracycline

Table 4.5: Minimum inhibitory concentration of ampicillin and gentamicin against *E. faecium* and *E. faecalis* isolated from poultry, cattle and vegetable farms in Zaria, Nigeria

Antibiotics	**Minimum Inhibitory Concentration (µg/ml)**					**No. of Isolates Tested**	**No. (%) of Isolates Resistant at breakpoints**
	$MIC_{Test\ Range}$	**$MIC_{Breakpoint}$**	**MIC_{50}**	**MIC_{90}**	**MIC_{Mode}**		
Ampicillin	0.016 – 256	≥ 16	≤ 4	≤ 16	≤ 1	63	7 (11.1)
Gentamicin	0.164 – 1024	≥ 500	≥1024	≥1024	>1024	37	35 (94.6)

MIC_{50}: Lowest concentration of gentamicin/ampicillin that inhibits growth of 50% of the isolates
MIC_{90}: Lowest concentration of gentamicin/ampicillin that inhibits growth of 90% of the isolates

Table 4.6: Occurrence of multidrug resistant (MDR) phenotype by the *Enterococcus faecalis* and *E. faecium* isolated from poultry, cattle and vegetable farms in Zaria, Nigeria

Sample Type	**Total No. Tested**	**No. (%) of isolates resistant ≥ 3 (MDR) antibiotic**	***p value*[c]**
Poultry Cloacal Swab	53	35[a] (66.0)	0.0035[*]
Cattle Rectal Swab	21	6[b] (28.6)	
Poultry Manure	27	15 (55.6)	0.2000[**]
Cattle Manure	12	4 (33.3)	
Cabbage	5	0	ND
Carrot	1	0	
Lettuce	5	1 (20.0)	
Cabbage (soil)	2	0	ND
Carrot (Soil)	5	0	
Lettuce (Soil)	4	1 (25.0)	
Total	**135**	**62 (45.9)**	

[a]Four isolates are *E. faecalis*, [b] one isolates is *E. faecalis* MDR: Multidrug resistance, [c]*p value* for association between sample type and MDR, ND: Not determined [*] *p <0.05*, [**] *p>0.05*

Table 4.7: Distribution of multidrug resistance phenotypes expressed by the *E. faecium* and *E. faecalis* isolates from poultry, cattle and vegetable farms in Zaria, Nigeria

Multidrug Resistance Phenotypes	No. of Isolates with Resistance Phenotype						Total
	Cloacal of Poultry	Poultry Manure	Rectal Swab of Cattle	Cattle Manure	Lettuce	Soil of Lettuce	
AM-E-R	0	2	2	2	0	0	6
AM-E-TE	1	1	0	0	0	0	2
AM-R-TE	0	0	0	1	0	0	1
AM-QD-R	1	0	0	0	0	0	1
CHL-E-TE	0	1	0	0	0	0	1
CIP-R-TE	2	0	0	0	0	0	2
CN-E-TE	4	1	1	0	0	0	6
E-S-TE	3	0	0	0	0	0	3
E-R-TE	0	0	0	0	0	1	1
AM-E-S-TE	1	0	0	0	0	0	1
AM-E-R-TE	0	1	0	1	1	0	3
AM-CN-E-TE	1	1	0	0	0	0	2
CHL-E-R-S-TE	1	0	0	0	0	0	1
CIP-CN-R-TE	0	1	0	0	0	0	1
CN-E-S-TE	8	2	1	0	0	0	11
AM-CIP-E-S-TE	0	0	1	0	0	0	1
AM-CN-E-R-TE	1	0	0	0	0	0	1
AM-CN-E-S-TE	4	2	1	0	0	0	7
AM-CN-R-S-TE	0	1	0	0	0	0	1
AM-CHL-E-QD-S-TE	3[b]	0	0	0	0	0	3
AM-CIP-CN-E-S-TE	3	0	0	0	0	0	3
AM-CIP-CN-E-R-TE	1	0	0	0	0	0	1
AM-CIP-CN-E-R-TE	0	2	0	0	0	0	2
AM-CHL-CN-E-QD-S-TE	1	0	0	0	0	0	1
Total	**35**	**15**	**6**	**4**	**1**	**1**	**62**

Isolate is *E. faecalis*, [b]Two of the isolates are *E. faecalis*

4.5 Antimicrobial Resistance Genes among *Enterococcus* Species Isolated from Cattle, Poultry and Vegetable Farms

4.5.1 Genes encoding aminoglycoside resistance

All the aminoglycoside-resistant *Enterococcus* species tested harboured the *aac(6')-Ie-aph(2'')* gene (Plate 4.5). *aph(2')*-Ic was detected in eight out of nine isolates tested (Plate 4.6) while *aph(3')*-IIIa and *ant(4')* were detected in nine and four respectively out of the 10 isolates tested (Plates 4.7 and 4.8). Three of the isolates harboured all the four assayed genes that code for aminoglycoside resistance while eight harboured the trio of *aac(6')-Ie-aph(2''), aph(2')*-Ic and *aph(3')*-IIIa genes and two harboured the duo of *aac(6')-Ie-aph(2'')* and *aph(3')*-IIIa genes.

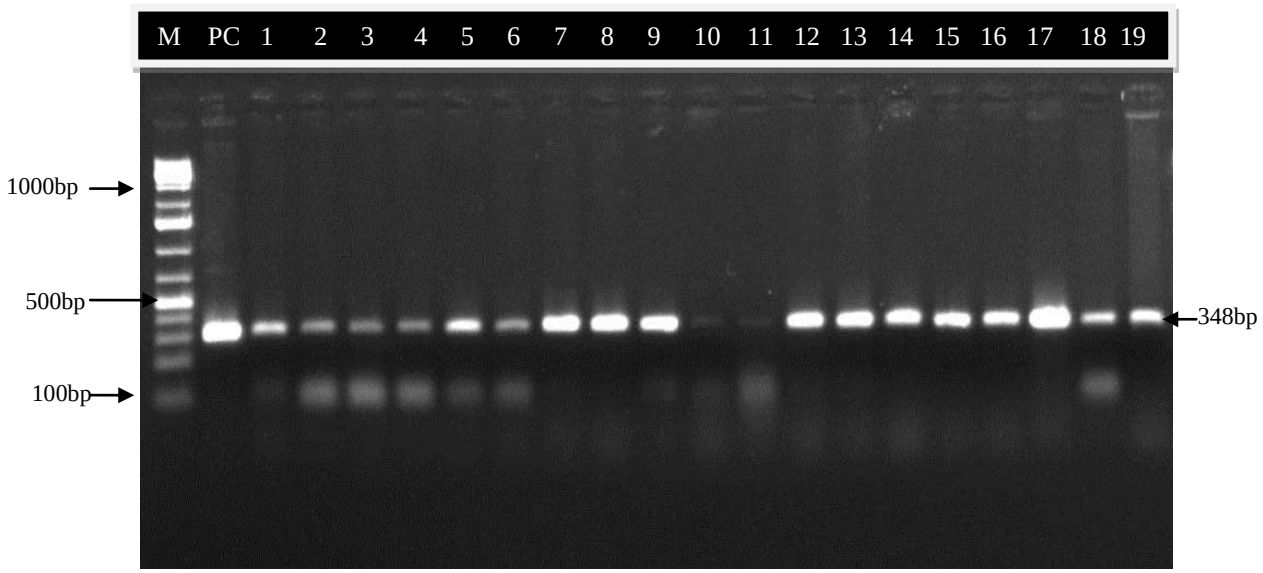


Plate 4.5: Gel Electrophoresis image of the PCR detection of the *aac(6´)*-Ie-*aph(2´´)*-Ia gene (348bp) among *Enterococcus* isolated from livestock farms in Zaria, Nigeria. Lane M: 100bp Plus DNA ladder, Lane PC: Positive control *E. faecium* ATCC 7889 and Lanes 1 – 19: test isolates (Lanes 1, 3, 5, 6, 9, 10, 11, 12, 13, 15, 16, 17, 19 are isolates from cloacal swabs of poultry, lanes 2, 4, 7, 8, 18 are isolates from poultry manure, while lane 14 is an isolate from rectal swab of cattle). Positive isolates: All lanes (1 – 19)

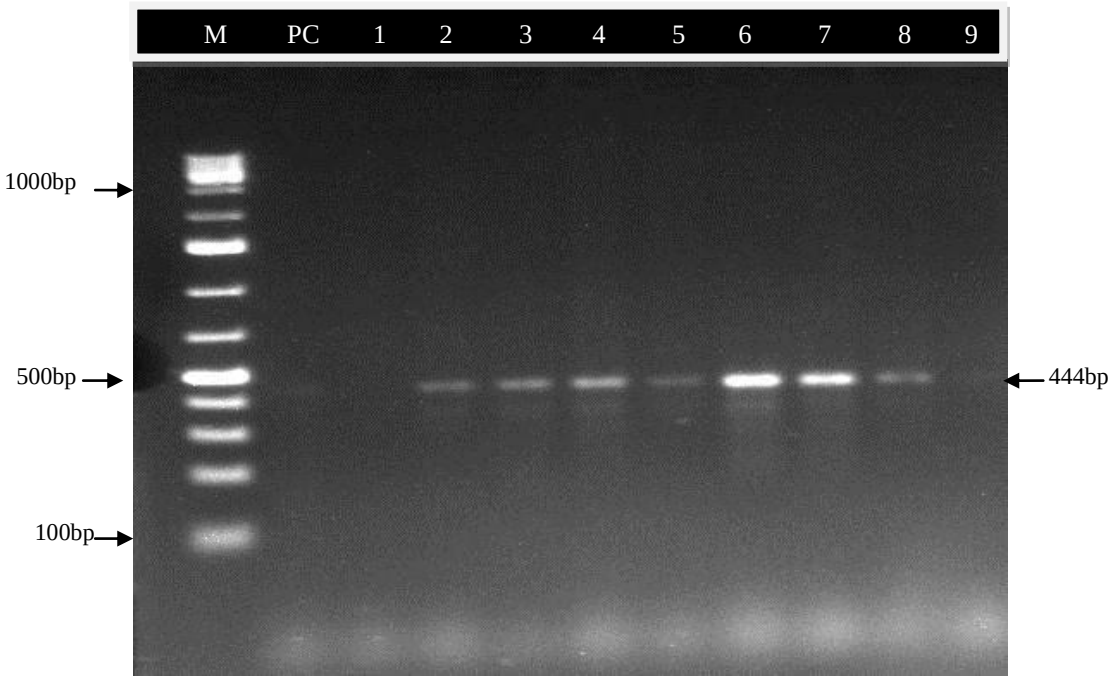


Plate 4.6: Gel image representing PCR detection of the *aph(2´´)*-Ic gene (444bp) among *Enterococcus* isolated from livestock farms in Zaria, Nigeria. Lane M: 100bp Plus DNA ladder, Lane PC: positive control *E. faecium* SF11770 Ib (chow) and Lanes 1 - 9 are the test isolates (Lanes 1, 3, 5, 8, 9 are isolates from cloacal swabs of poultry while lanes 2, 4, 6, 7 are isolates from poultry manure). Positive isolates: Lanes 2, 3, 4, 5, 6, 7, 8, 9

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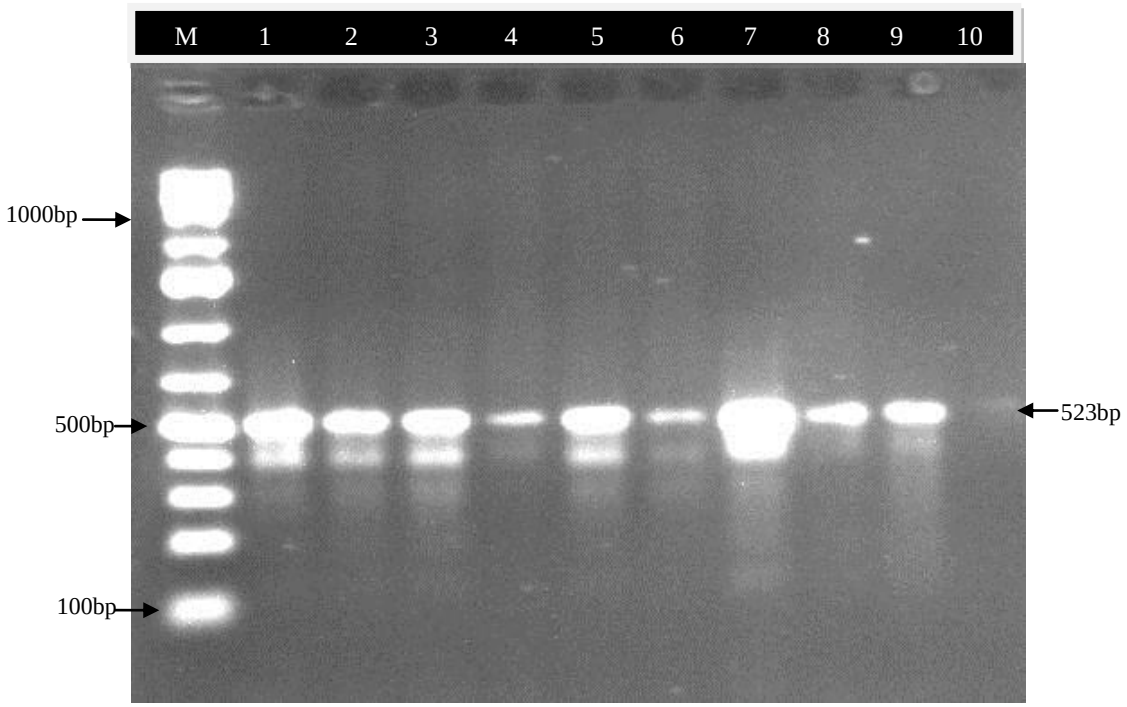


Plate 4.7: Gel Electrophoresis image for the PCR detection of the *aph(3´)*-III*a* gene (523bp) among *Enterococcus* isolated from livestock farms in Zaria, Nigeria. Lane M: 100bp Plus DNA ladder and Lanes 1 – 10: Test isolates (Lanes 1, 3, 5, 8, 9, 10 are isolates from cloacal swab of poultry, lanes 2, 4, 6, 7 are isolates from poultry manure). Positive isolates: Lanes 1 - 9

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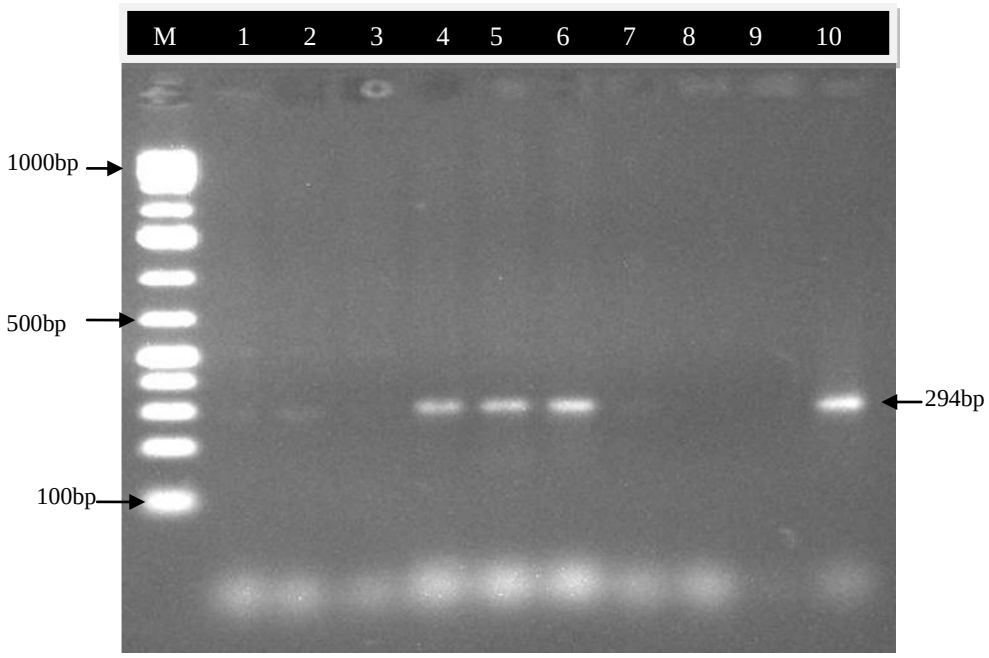


Plate 4.8: Gel image representing PCR detection of the *ant(4´)*-Ia gene (294bp) among *Enterococcus* isolated from livestock farms in Zaria, Nigeria. Lane M: 100bp Plus DNA ladder and Lanes 1 – 10: Test isolates (Lanes 1, 2, 4, 7, 8, 9, 10 are isolates from cloacal swab of poultry, lanes 3, 5, 6 are isolates from poultry manure). Positive isolates: Lanes 4, 5, 6, 10

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4.5.2 Detection of the *pbp*5 Gene among the Ampicillin Resistant Isolates

Amplification of the *pbp*5 gene: The *pbp*5 gene was amplified from six of the seven isolates displaying high level ampicillin resistance (Plate 4.10). The test isolates possessing the *pbp*5 gene in lanes 1 - 4, 6 and 7 were all *E. faecium* while the gene was not amplified in the *E. faecalis* isolate on lane 5 (Plate 4.9).

Sequence Analysis of *pbp5*: The C - terminal region of the six isolates that showed high-level ampicillin resistance (MICs of ≥ 256 μg/ml) were sequenced and the nucleotide sequences obtained were analyzed. Only six isolates including the control isolate (*E. faecium* 9730357-1) produced analyzable sequences and the sequences had 96% - 99% identity with the reference sequence X84860 (Table 4.8). Analysis of the C-terminal region revealed a total of 9 different amino acid changes. Insertion of glutamine instead of histidine at position 470 (His470 → Gln) was a common substitution observed among the isolates. In addition substitutions; His470 → Gln, 496Asn→Lys, 497Phe→IIe, 499Ala→Thr, 500Glu→Leu, 502Asp→Arg, 525Glu→Asp, 612IIe→X and 614IIe→Phe were also detected. Three alleles (A - C) based on the pattern of amino acid substitutions in the C-terminal region were detected (Table 4.8). The sequences were submitted to the GeneBank and accession numbers (KX377704, KX377705, KX377706 KX377707) were assigned to them (Table 4.8).

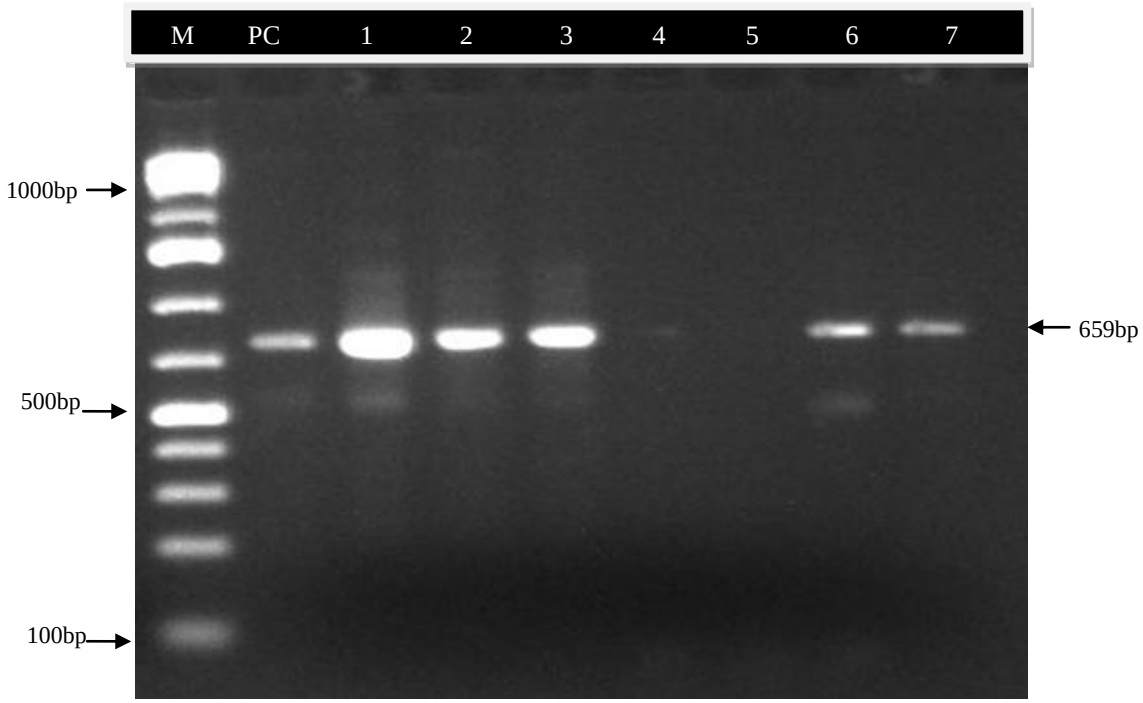


Plate 4.9: Gel image representing PCR detection of the *pbp*5 gene (659bp) among ampicillin resistant *Enterococcus* isolated from livestock and vegetable farms in Zaria, Nigeria. Lane M: 100bp Plus DNA ladder, Lane PC: Positive control *E. faecium* 9730357-1 and Lanes 1 – 7 are the test isolates (Lanes 1, 2 are isolates from cattle manure, lane 3 is an isolate from lettuce while lane 4, 5, 6, 7 are isolates from cloacal swab of poultry). Positive isolates: 1, 2, 3, 6, 7

Table 4.8: Amino acid polymorphism detected in the C-terminal region of *pbp5* gene of five ampicillin resistant *Enterococcus faecium* isolates recovered from livestock and vegetable farms in Zaria, Nigeria.

Sample ID	Species	Source of isolate	Amino Acid Changes at Different Positions of the C-terminal region									Alleles	% similarity to X84860	MIC
			470	496	497	499	500	502	525	612	614			
X84860	*E. faecium*	NK	H	N	F	A	G	D	E	I	I			ND
(51) 9730357-1	*E. faecium*	NK	**Q**	N	F	A	G	D	E	I	I		99	ND
41	*E. faecium*	Cloacal swab	**Q**	N	**I**	**Y**	**L**	**R**	D	I	I	A	96	≥ 265
23 **KX377704**	*E. faecium*	Lettuce	**Q**	**K**	F	**T**	G	D	**D**	**X**	**F**	B	96	≥ 265
45 **KX377706**	*E. faecium*	Cloacal swab	**Q**	N	F	A	G	D	E	I	I	C	99	16
17 **KX377705**	*E. faecium*	Cattle manure	**Q**	N	F	A	G	D	E	I	I	C	99	≥ 265
22 **KX377707**	*E. faecium*	Cattle manure	**Q**	N	F	A	G	D	E	I	I	C	99	≥ 265

H: Histidine, Q: Glutamine, N: Asparagine, K: Lysine, A: Alanine, T: Threonine, E: Glutamic acid, D: Aspartic acid, I: Isoleucine, F: Phenylalanine, Y: Tyrosine, X: undetermined or nonstandard aminoacid, ND: not determined MIC: minimum inhibitory concentration, NK: Not known. Amino acids substitutions in green and red indicates previously reported and novel subsititutions respectively.

4.5.3 Detection of the *erm*(B) and *tet* Genes among the Erythromycin and Tetracycline Resistant Isolaes

Amplification of the *erm*(B) gene: the result of the PCR for detection of the erythromycin methylase (*erm*B) gene showed that all the 19 isolates tested possessed the *erm*(B) gene (Plate 4.10).

Amplification of the *tet* gene: *tet*(L) gene was detected in all the 20 isolates tested (Plate 4.11) while *tet*(M) gene was detected in 18 out of the 19 isolates tested (Plate 4.12). Out of the 10 isolates tested for carriage of the *tet*(K) and *tet*(O) genes encoding efflux and ribosomal protection, nine (Plate 4.13) and ten (Plate 4.14) isolates were identified to harbour the respective genes. Furthermore 15 isolates carried the duo of *tet*(L) and *tet*(M) genes, 10 harboured the trio of *tet*(L), *tet*(M) and *tet*(O) genes while 5 isolates harboured all four [(*tet*(K), *tet*(L), *tet*(M), *tet*(O)] genes.

Co-carraige of *erm*(B) and *tet* genes: cocarraige of *erm*(B) and *tet*(L) genes was observed among all the isolates tested while 13 of the isolates harboured *erm*(B), *tet*(L) and *tet*(M). Also five of the isolates harboured the *erm*(B) and all the four *tet*[(K), (L), (M) (O)] genes tested.

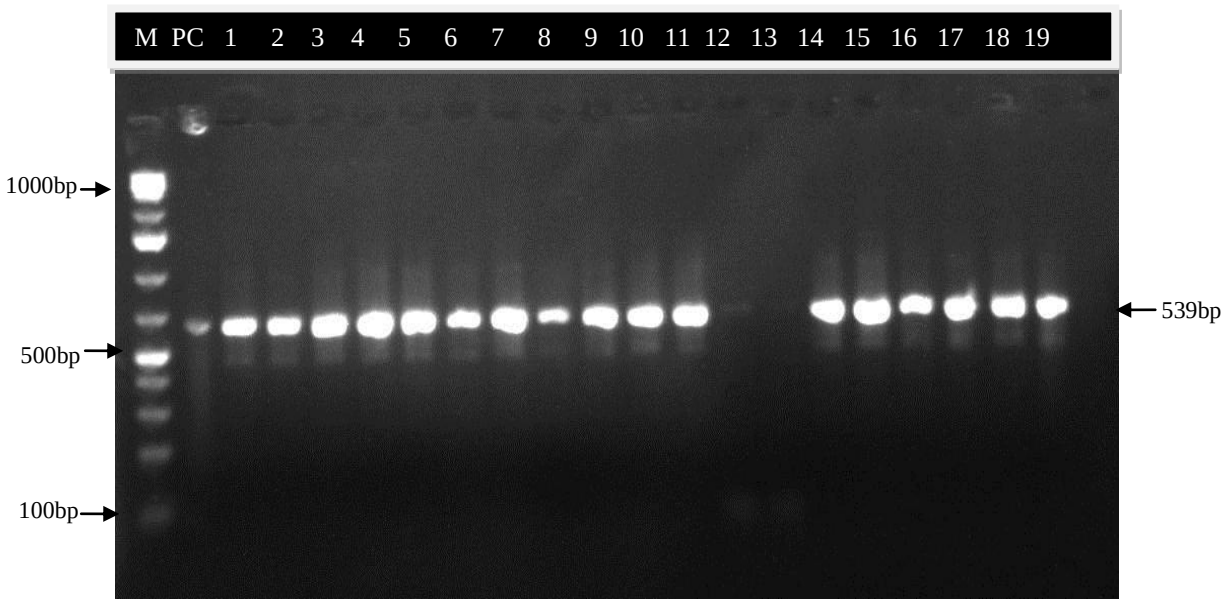


Plate 4.10: Gel image representing PCR detection of the *erm*(B) gene (539bp) among *Enterococcus* species isolated from livestock farms in Zaria, Nigeria. Lane M: 100bp Plus DNA ladder, Lane PC: Positive control *E. faecalis* JH2-2:*Tn1545* and Lanes 1 – 19: test isolates (Lanes 1, 3, 5, 6, 10, 11, 12, 13, 14, 15, 17, 18, 19 are isolates from cloacal swab of poultry, lanes 2, 4, 7, 9 are isolates from poultry manure while lanes 8 and 16 are isolates from rectal swab of cattle). Positive isolates: All lanes (1-19)

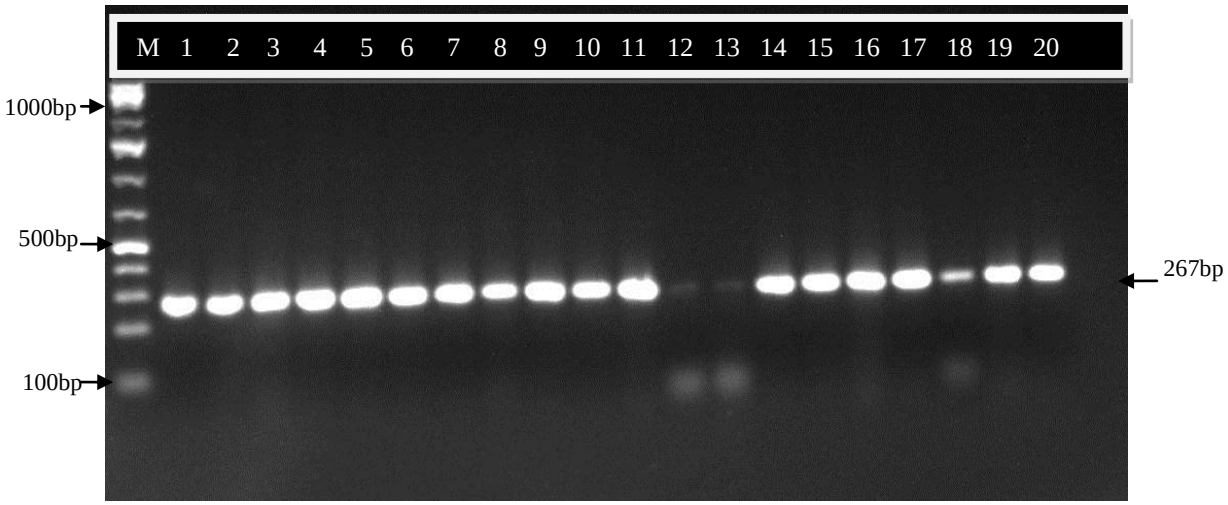


Plate 4.11: Gel image representing PCR detection of the *tet*(L) gene (267bp) among *Enterococcus* species isolated from livestock farms in Zaria, Nigeria. Lane M: 100bp Plus DNA ladder and Lane 1 – 20: test isolates (Lanes 1, 3, 5, 6, 10, 11, 12, 13, 14, 15, 17, 18, 19 are isolates from cloacal swab of poultry, lanes 2, 4, 7, 9 are isolates from poultry manure while lanes 8 and 16 are isolates from rectal swab of cattle). Positive isolates: All lanes (1 – 20)

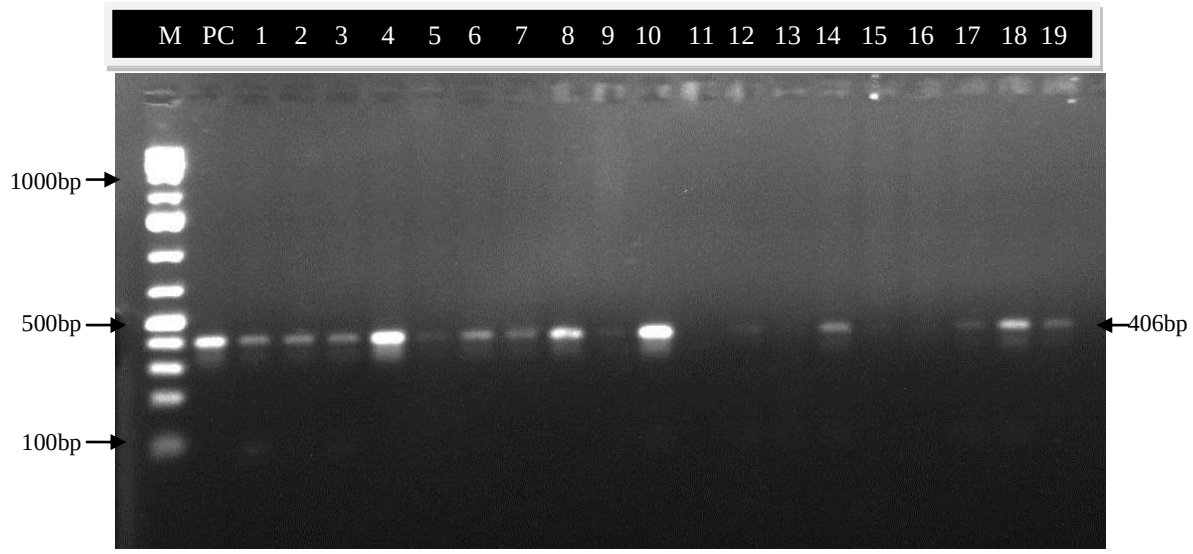


Plate 4.12: Gel image representing PCR detection of the *tet*(M) gene (406bp) among *Enterococcus* species isolated from livestock farms in Zaria, Nigeria. Lane M: 100bp Plus DNA ladder, Lane PC: Positive control *E. faecalis* CG110:Tn916 and Lanes 1 – 19 are the test isolates (Lanes 1, 3, 5, 6, 10, 11, 12, 13, 14, 15, 17, 18, 19 are isolates from cloacal swab of poultry, lanes 2, 4, 7, 9 are isolates from poultry manure while lanes 8 and 16 are isolates from rectal swab of cattle). Positive isolates: Lanes 1-10 and 12 - 19

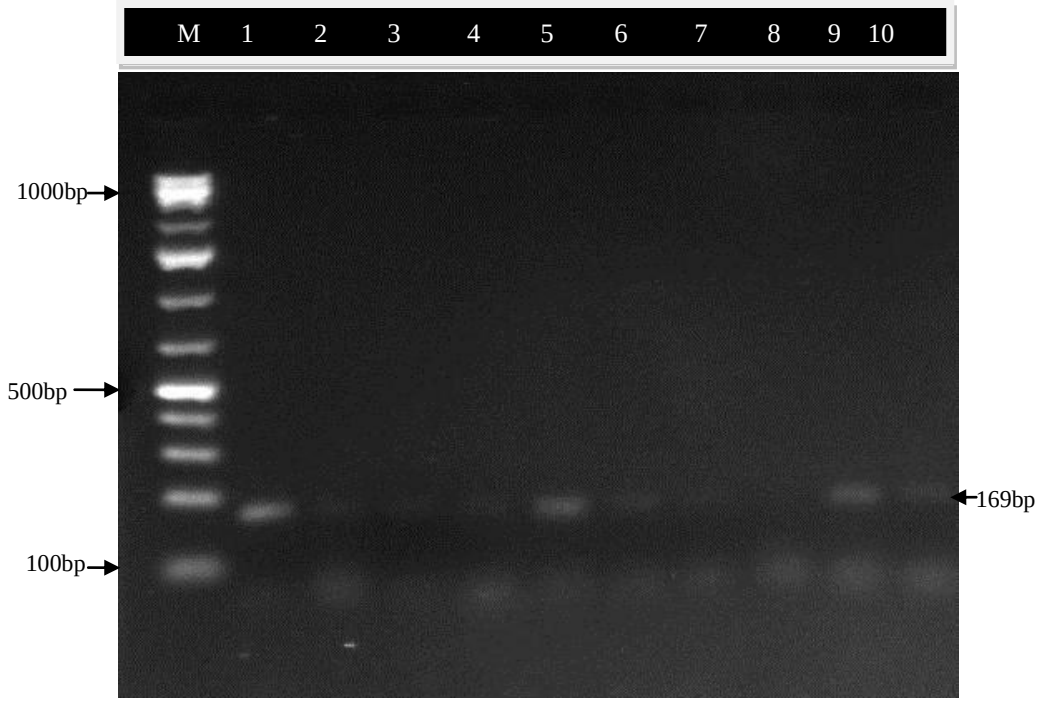


Plate 4.13: Gel image representing PCR detection of the *tet*(K) gene (169bp) among *Enterococcus* species isolated from livestock farms in Zaria, Nigeria. Lane M: 100bp Plus DNA ladder and Lanes 1 - 10: test isolates (Lanes 1, 3, 6, 8 are isolates from poultry manure, lanes 2, 4, 5, 9, 10 are isolates from cloacal swabs of poultry, while lane 7 is an isolate from rectal swab of cattle). Positive isolates: lanes 1 – 7, 9, 10

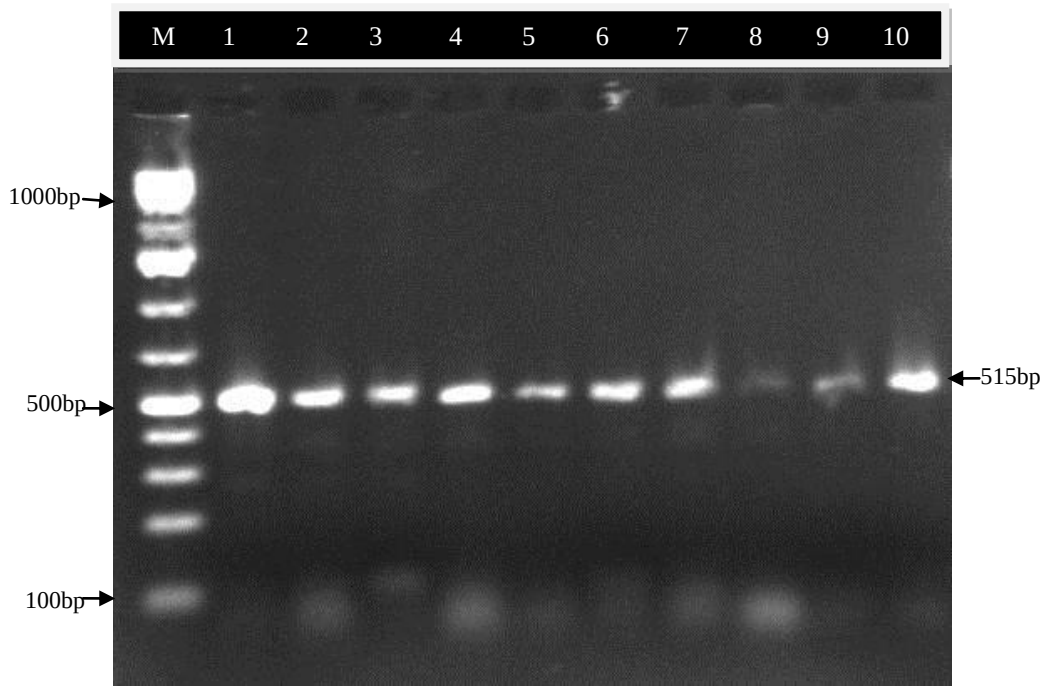


Plate 4.14: Gel image representing PCR detection of the *tet*(O) gene (515bp) among *Enterococcus* species isolated from livestock farms in Zaria, Nigeria. Lane M: 100bp Plus DNA ladder and Lane 1 - 10: test isolates (Lanes 1, 3, 5, 6, 10 are isolates from cloacal swabs of poultry, lanes 2, 4, 7, 9 are isolates from poultry manure, while lane 8 is an isolate from rectal swab of cattle). Positive isolates: All lanes (1 – 10)

4.6 Transferability of Antimicrobial Resistance Genes

Eight (three *E. faecalis* and five *E. faecium*) multi-drug resistant *Enterococcus* strains were selected and examined for the potential of horizontal gene transfer by filter conjugation assays. Only 6 (three *E. faecalis* and three *E. faecium*) of the isolates transferred traits conferring resistance to tetracycline and or erythromycin to recipient *E. faecalis* JH2-2 (Table 4.9). Three isolates transferred both tetracycline and erythromycin resistance traits while two and one isolate transferred only the tetracycline and erythromycin resistance traits respectively to the recipient strain. When the new antibiotic susceptibility profiles of the transconjugants were determined it was discovered that in addition to the erythromycin and tetracycline tested for, other resistance phenotypes (gentamicin, chloramphenicol and streptomycin) were also transferred (Table 4.9).

Table 4.9: Resistance profiles of donors and transconjugants from the mating experiment of *Enterococcus* isolated from livestock and vegetable sources in Zaria, Nigeria.

Isolate No. (Species)	**Antibiotic Resistance Profile**	
	Test Isolates	**Transconjugants (*E. faecalis* JH2-2)**
29 *E. faecium*	AMP-CN-E-S-TE	CN-E-RIF
49 *E. faecium*	AMP-CN-E-S-TE	CN-RIF-S-TE
54 *E. faecium*	AMP-CIP-CN-E-S-TE	RIF-TE
114 *E. faecalis*	AMP-C-CN-E-QD-S-TE	C-CN-E-QD-RIF-TE
118 *E. faecalis*	AMP-C-E-QD-S-TE	E-QD-RIF-S-TE
165 *E. faecalis*	AMP-C-E-QD-S-TE	C-E-QD-RIF-S-TE

TE: Tetracycline, AMP: Ampicillin, E: Erythromycin, S: Streptomycin, CN: Gentamicin, C: Chloramphenicol, RIF: Rifampicin, FA: Fusidic acid, Van: Vancomycin, CIP: Ciprofloxacin. *E. faecalis* JH-2 is susceptible to E, S, TE, CIP, AMP, C, Van, CN but resistant to RIF

CHAPTER FIVE

DISCUSSION

Enterococcus has emerged in recent times as an important nosocomial pathogen and trafficker of virulence and antimicrobial resistance genes (Diarra *et al.,* 2010; Leite-Martins *et al.,* 2014; Yang *et al.,* 2015). In this study *Enterococcus* was isolated from 29.9% of the total samples collected and distributed across all sample types with the exception of irrigation water. This finding underscores the status of *Enterococcus* as a known commensal of the gastrointestinal tract of animals and environmental contaminant. It also supports its reported ubiquitous nature and hardiness/ruggedness which gives them the capability to survive harsh conditions and spread in the environment resulting in contamination of soil and vegetables (Klibi *et al.,* 2015a). This finding agrees with those of other studies that have detected *Enterococcus* from cattle (Jackson *et al.,* 2011; Klibi *et al.,* 2015a), poultry (Yoshimura *et al.,* 2000; Diarra *et al.,* 2010; Klibi *et al.,* 2015a; b; Amaechi and Nwankwo, 2015) and vegetable farms (Abriouel *et al.,* 2008; Micallef *et al.,* 2013; Ben Said *et al.,* 2015) in different parts of the world.

In contrast to the findings of other studies (Abriouel *et al.,* 2008; Micallef *et al.,* 2013; Ben Said *et al.,* 2015) *Enterococcus* was not isolated from irrigation water in vegetable farms. Our inability to isolate enterococci from the water samples may likely be attributed to the isolation methodology used in our study and the likelihood that the number of *Enterococcus* in the irrigation water at the time of sampling was low and below the detectable level of our isolation methodology. Other studies utilized the membrane filtration which concentrates the organism followed by inoculation onto a selective media with enrichment in some cases (Castillo-Rojas *et al.,* 2013) while we inoculated an aliquot

of the water sample directly into the enrichment broth (bile esculin azide broth). Another reason for our inability to isolate *Enterococcus* from the water samples may have been due the ability of *Enterococcus* to activate the viable but non culturable state (VBNC) to conserve their viability as a survival strategies in the presence of reduced nutrient as found in water bodies (Iweriebor *et al.,* 2015).

The high isolation rate of enterococci (28.8%) from lettuce compared to the other vegetables may be because lettuce are leafy vegetables with large surface areas and folds which makes them susceptible to contamination and adhesion by bacteria. Leaf shapes have been reported to contribute to the differences in the contamination levels of different varieties of vegetables (Maffei *et al.,* 2013). Open leaves may trap dirt and pathogen in their folds because they are always in contact with soil, manure and irrigation water (Abadias *et al.,* 2008; Mritunjay and Kumar, 2015).

Currently, 54 species have been identified in the Genus *Enterococcus* (http://www.bacterio.net/enterococcus.html), however, only nine of these were identified in this study. In this study two to three different colonies showing different morphologies on the primary isolation plate were processed for species identificataion, since it is practically impossible to process all colonies with similar morphologic characteristic on each primary isolation plate. We therefore, assume that some species may have been missed or even underestimated in that process. Also the likelihood that colonies with similar morphologies maybe different species or strains may result in us missing some species or strains.

Isolation methodology has been reported to influences the detection of some *Enterococcus* species (Butaye *et al.,* 1999; Barbosa *et al.,* 2009). The incubation temperature has also been reported to influence the isolation of some species (Haye *et al.,* 2004; Jackson *et al.,* 2005). Incubation temperature of 43 - 45°C routinely used for selective enrichment (which was also used in this study) has been reported to inhibit the growth of some strains of *E. dispar*, *E. malodoratus* and *E. sulfureus* (Devriese *et al.*, 1993; Barbosa *et al.*, 2009) while the use 6.5% NaCl (which is recommended for identification of *Enterococcus* species) for supplementation inhibit the growth of *E. cecorum* and *E. columbae* (Devriese *et al.*, 1993; Jackson *et al.*, 2005).

Similar species to the ones detected among all sample types in this study have also been reported (Ghidan *et al.,* 2008; Abriouel *et al.,* 2008; Kuhn *et al.,* 2003; Ben Said *et al.*, 2015). *E. faceium* was the most common species isolated from all sample types which is in agreement with the report of other workers (Kuhn *et al.,* 2003; Abriouel *et al.,* 2008; Ben Said *et al.,* 2015). The low prevalence of *E. faecium* from cattle sources is consistent with its reportedly low abundance in cattle (Gelsimino *et al.*, 2002; Jackson *et al.*, 2011) while the detection of *E. faceium* on vegetables and soil may have resulted from contamination from animal or human faeces, fertilization of the soil with animal manure or amending of the soil with wastes from refuse dumps since this species is adapted to animals and humans. *E. faecium* and *E. faecalis* are the most frequently and clinically important species involved in human infections (Werner *et al.,* 2013; Ben Sallem *et al.,* 2015). The isolation of the clinically important *E. faecium* from all sample types raises concern of whether potentially pathogenic strains can also be found in these environments. Of importance also is whether they can contribute to the spread of antibiotic resistance and virulence capabilities developed in clinical settings. *E. casseliflavus, E. munditt* and *E. sulferus* are plant adapted

Enterococcus species (Svec *et al.,* 2002; Byappanahalli *et al.,* 2012). However, *E. casseliflavus* and *E. mundtti* were detected in this study from all the livestock sources which include cattle rectal swab, poultry cloacal swab and manure. This finding suggests that feeding regimes or habitats may be major factors influencing the distribution of *Enterococcus* species especially among food animals (Devriese *et al.,* 2006). The detection of these species from manure, cloaca of poultry and rectal swabs of cattle may have resulted from consumption of pasture or pecking of soil by chicken, where these species are abundantly present. This is in addition to likely contamination of the manure from environmental sources i.e. vegetations and soil where the manure was kept. *E. gallinarum* was the second most predominant species detected especially from cattle. This finding was not in agreement with other studies which reported *E. faecalis* and *E. hirae* as the next dominant species in cattle (Devriese *et al.,* 1992; Kuhn *et al.,* 2003; Jackson *et al.*, 2011; Klibi *et al.*, 2015a) and thus may be a further reflection of the influence of geographical location on the species diversity of *Enterococcus* (Hudson *et al.,* 2003; Jackson *et al.,* 2005).

Sixty four (24%) of the *Enterococcus* isolates in this study were unidentified to species level. Our inability to identify these isolates tagged "unidentified" to species level was due to the similarities in their biochemical characteristics, a limitation that has also been reported (Haye *et al.*, 2004; Facklam *et al.*, 2007).

Despite being known as commensal organisms, enterococci can act as opportunistic pathogens, especially in the very young, elderly and immunocompromised patients who are under hospitalization for long periods (Hammad *et al.,* 2015). For enterococci to cause infection in any host it must be able to colonize, invade host tissue, translocate through

epithileial tissue, evade the immune response and cause pathological changes (Hammad *et al.,* 2014). Virulence factors contribute to the ability of enterococci to cause infection (Mundy *et al.,* 2000). It is of interest to note that most of the isolates encountered in this study harboured and expressed at least one gene coding for a virulence factor. Production of aggregation substance (AS) which is an important component of the bacterial pheromone-responsive genetic exchange system was the least detected virulence trait expressed phenotypically among the isolates. However, in contrast to reports (Vankerckhoven *et al.,* 2004; Sienko *et al.,* 2015) that the *asa1* gene encoding this virulence trait was detected more among *E. faecalis* isolates it was found more common among *E. faecium* in this study. *asa1* is known to mediates the adherence of enterococci to epithelial cells of the intestinal tract and renal tubules and endocardium (Sava *et al.,* 2010; Rathnayake *et al.,* 2012). It also promotes enterococci survival in the polymorphonuclear leukocytes and macrophages by increasing the hydrophobicity of the enterococcal surface, which may prevent or delay phagocytosis and the subsequent destruction of the organism (Submuth *et al.,* 2000; Klibi *et al.,* 2013b; Pieniz *et al.,* 2015). In addition, the aggregation protein is reported to be involved in adherence to eukaryotic cells as well as invasion and translocation of *Enterococcus* through intact colonic mucosa (Valenzuela *et al.,* 2008). This protein has also been recognized to play an important role in the ability of enterococci to cause endocarditis (Sava *et al.,* 2010; Rathnayake *et al.,* 2012). It is responsible for transfer of mobile genetic elements (MGE) especially conjugative plasmids in the pathogenicity island (PAI) on which they are located by promoting increase tight contact between the recipient and donor strains (Dunny, 1990). Also worthy of note is that because the gene encoding aggregation factor is located within the pathogenicity island, it is susceptible to excision, circularization, and horizontal intra- and interspecies transfer to other

enterococcal species and bacteria (Hammad *et al.,* 2014). The finding of this gene among some of the isolates suggests in addition to potential pathogenicity, an increased capability of most of the isolates to transfer virulence or resistance genes by conjugation. This is also supported by the result of the detection of isolates that transferred erythromycin and or tetracycline resistance determinants to the recipient strain in this study.

The *gelE* gene that encodes the production of gelatinase enzyme involved in the hydrolysis of gelatin, collagen, heme and other proteins was detected among some of the gelatinase positive and negative isolates. Gelatinase is also reportedly important for the degredation of misfolded proteins and cleavage of pheromones responsible for the induction of conjugal plasmid transfer (Waters *et al.,* 2003; Strzelecki *et al.,* 2011). Gelatinase has also been shown to cleave and degrade fibrin layer surrounding bacteria and allows further dissemination of the organism; a serious concern in the pathogenicity of *Enterococcus*. This is in addition to its ability to degrade antimicrobial peptides, which are part of the innate immune system, rendering the host immunocompromised (Schmidtchen *et al.,* 2002). Although, *gelE* gene has been more frequently reported among *E. faecalis*, and rarely in *E. faecium* (Coque *et al.,* 1995; Vankerckhoven et al., 2004; Sienko *et al.,* 2015), in the present study, the *gelE* gene was detected among both *E. faecalis* and *E. faecium* strains. The detection of *gelE* genes among isolates that do not phenotypically express gelatinase activity indicates the occurrence of silent *gelE* genes among the isolates. This phenomenon has also been reported by other authors (Eaton and Gasson, 2001; Creti *et al.,* 2004; Han *et al.,* 2011; Trivedi *et al.,* 2011; Strzelecki *et al.,* 2011; Comerlato *et al.,* 2013; Klibi *et al.,* 2015b). It is reported that the presence of *gelE* gene is not solely enough for expression of gelatinase activity, since possession of a complete *fsr* operon is essential for its expression (Hancock and Perego, 2004; Lopes *et al.,* 2006). The inability to

phenotypically express gelatinase activity has also been attributed to a loss of one or more genes such as deletion of a 23.9-kb involving the *fsr* locus that regulates *gelE* expression (Nakayama *et al.,* 2002; Singh *et al.,* 2005). The *fsr* operon has been reported to be easily damaged, lost or deleted especially during cold storage (freezing) of cells in the laboratory (Perin *et al.,* 2014). Eaton and Gasson (2001) reported that culture conditions, and laboratory manipulation of the strains can result in the loss of the structural genes resulting in the loss of gelatinase activity during *in vitro* tests. Interestingly, some isolates positive for gelatinase activity/production were negative for carriage of the *gel*E gene. Other authors (Trivedi *et al.,* 2011; Klibi *et al.,* 2015b; Strzelecki *et al.,* 2011) have reported similar findings and this may likely be as a result of the carriage of a divergent gelatinase gene or presence of other protease that cleave gelatin and display similar behavior like gelatinase (Camargo *et al.,* 2006).

The results of this study demonstrate that the carriage of *asa1* or *gelE* gene does not necessarily result in phenotypic expression since lack of promoter or other sequences required for their expression could result in an absence of gene expression. Also the detection of *gel*E and *asa*1 genes among the isolates have important significance in the epidemiology of dissemination of virulent enterococci as these putative virulence genes are reported to occur frequently among isolates of the ST6 (CC2) and CC17 clones causing hospital infections in parts of the world (Getachew *et al.,* 2013; Yang *et al.,* 2015).

Although haemolytic activity was observed among the isolates only one of them was positive for *cyl*A gene. This finding suggests the existence of other possible haemolytic determinants among the isolates expressing β-haemolytic activity (Channaiah *et al.,* 2010).

Although isolates possessing the *esp* gene were not detected in this study the *esp* gene is known to code for enterococcal surface protein and reside on a large pathogenicity island (PAI) in *E. faecium* and *E. faecalis* (McBride *et al.,* 2009). The gene has been proposed as an important marker of the presence for the PAI and identification of hospital acquired clones (Shankar *et al.,* 2006). The Esp protein promotes the initial adherence to surfaces during biofilm formation and urinary tract colonization (Shankar *et al.,* 1999; Shankar *et al.,* 2006).

Antimicrobial use for growth promotion, prophylaxis and chemotherapy is a common practice in animal production (Allen *et al.,* 2013). The use of these agents for such purposes have been linked to the rising cases of drug resistance among commensals and pathogens in animal production, community and hospital environments (Ahmad *et al.,* 2011; Marosevic *et al.,* 2014). Antimicrobial resistance of commensals such as *Enterococcus* has been used as indicators to estimate the resistance level in the environment (Silva *et al.,* 2010; Werner *et al.,* 2013).

Only 14 (10.4%) isolates (belonging to the *E. faecium* species) out of the 135 in this study were susceptible to all the antibiotic agents tested. The detection of antibiotic resistant strains among the isolates in the present study is a matter of concern. This is because the faeces from these animals become manure, and the manure from these animals are used to fertilize agricultural soil while the vegetables are consumed without prior heat treatment (McEwen and Fedorka-Cray, 2002). This promotes the introduction of these resistant strains or their corresponding genes into the food chain.

The frequency of use of a particular antimicrobial agent directly influences the selection of resistant strains and frequency of occurrence of resistance to that agent (Levy, 2002; Wales and Davies, 2015). Varying rates of resistance against the antimicrobial agents tested in this study were observed among the isolates suggesting the role of antibiotic use in the resistance detected. The differences and similarities in the observed resistance patterns in this study in comparison with the findings of other workers may have been influenced by the protocol of administration of the individual antimicrobials in the areas where the studies were carried out. Geographical location (Houndt and Ochman, 2000; Pickering, 2004), management system (Jackson *et al.,* 2004b; Akwar *et al.,* 2008; Pyorala *et al.,* 2014; Beukers *et al.,* 2015), laws on judicious antimicrobial use (McEwen and Fedorka-Cray, 2002; Marshall and Levy, 2011) have been reported to influence the emergence of antimicrobial resistance in bacteria. Although to the best our knowledge official data on antimicrobial consumption in animal production in Nigeria is not available. The antibiotics commonly used are penicillin (amoxicillin), tetracycline (oxytetracycline, doxycycline), macrolides (tylosin, erythromycin) aminoglycosides (gentamicin, streptomycin), a combination of sulphonamides and trimethoprim, quinolones (enrofloxacin and to a lesser extent ciprofloxacin), bacitracin, polymixins, colistins and nalidixic acid. These drugs are used in subtherapeutic doses and in some cases as single agents for growth promotion, prevention and treatment of diseases. However antimicrobial use in animal production may not be the only reason for selection of resistance clones. The high prevalence of antibiotics use by farmers in different parts of Nigeria is reflected in the higher resistance expressed by the *Enterococcus* isolates especially those originating from poultry farms in the present study.

Although, resistance of enterococci to other drugs is also of importance because of its potential of transfer of resistance genes to other bacteria (Huddleston, 2014), the three most important types of resistance in enterococci from clinical point of view are high-level ampicillin, aminoglycoside and glycopeptide resistance (Wang *et al.,* 2013; Sadowy and Luczkiewicz, 2014). This is because these antibiotics are the mainstay for the treatment of enterococcal infections.

Noteworthy in this study however is the fact that although the isolates were resistant to other antibiotics tested, none of the isolate was resistant to vancomycin. This finding is comforting as vancomycin is considered the drug of last resort in the treatment of enterococcal infection and those from multidrug resistant bacteria (Adegoke and Okoh, 2011; Hasan and Jarhult, 2015). The use of vancomycin or it analogue; avoparcin (a glycopeptides growth promoting agent with similar chemical structure to vancomycin) have been reported to select for vancomycin resistant enterococci (VRE) (Eisner *et al.,* 2005; Hammerum, 2012; Nilsson, 2012). Resistance of enterococci to both avoparcin and vancomycin is mediated by the *van*A gene thus resulting in a cross-resistance between the two antimicrobials (Harada *et al.,* 2012). These agents are not reported to be commonly used in animal production in the Nigeria. Therefore, the absence of VRE strains in this study is not surprising. Studies from some parts of Europe and Africa where this agent was used in the past have reported the carriage of VRE among livestock and vegetables (Kruse *et al.*, 1999; van den Bogaard *et al.,* 2000; Mason *et al.*, 2004; Hammerum, 2012; Klibi *et al.,* 2015b). The absence of VRE isolates in the present study agrees with reports from other parts of the world like USA and Canada where the agent avoparcin is commonly used in livestock production (Hammerum, 2012; Nilsson, 2012). This finding may also infer that even if VRE are present, they may occur in a low proportion compared with vancomycin

susceptible enterococci and may have being missed during our selection of colonies since non-vancomycin supplemented plates were used for the isolation. Also, in contrast to the findings of this study, Inabo (2014) reported a vancomycin resistance of up to 50% in a study of *Enterococcus gallinarum* and *E. faecium* isolated from faecal droppings of poultry in Zaria.

It is important to highlight the detection of ampicillin resistant *Enterococcus faecium* in this study. Until recently ampicillin resistant enterococci (ARE) were rarely isolated from animals and humans in the community (Tremblay *et al.,* 2013; Conceicao *et al.,* 2014). They occur only in the hospital environment and are used as markers for the identification of hospital acquired resistant *E. faecium* clones (de Regt *et al.,* 2012). The detection of ARE from animal sources in this study therefore supports the assertion that other sources are emerging as ARE reservoirs and not only the hospital environment maybe reservoir for dissemination of ARE clones. The detection of ARE from animals in this study is in agreement with the findings of other workers around the world who detected ARE in animals (Poeta *et al.,* 2006; Damborg *et al.,* 2009; de Regt *et al.,* 2012; Known *et al.,* 2012; Novais *et al.,* 2013).

Although 63 ampicillin resistant isolates were detected from the disc diffusion test, only seven had MIC values beyond the resistance breakpoint recommended by the CLSI (2011). The discrepancy may have resulted from the expression of intrinsic resistance to ampicillin (Marothi *et al.,* 2005). The most common mechanisms of resistance to the β-lactams among enterococci are overproduction of low affinity PBPs and point mutations in the genes encoding these PBPs resulting in production of a lower affinity PBP (Poeta *et al.,* 2006; Conceicao *et al.,* 2014). Decreased affinity due to point mutations in the gene encoding low-affinity PBP5, the most common mechanism of ampicillin resistance in

enterococci, was also assessed in this study. PBP5 has 2778 nucleotides and 680 amino acids distributed in four domains; the first comprises amino acids 1 to 45, which is the N membrane anchoring region in the cell wall; the second domain; N-terminal extension contains amino acids 46 - 162, the third N-terminal domain contains amino acids 163 - 345 and the fourth one, which is the penicillin-binding domain located at the C-terminal portion of the protein, involves amino acids 346 to 680 (Zorzi *et al.,* 1996; Galloway-Pena *et al.,* 2011). The C-terminal domain harbours three specific conserved motifs; $_{422}$STFK$_{425}$, $_{480}$SDN$_{482}$ and $_{617}$KTG$_{619}$) that have been identified as the main targets of β-lactams (Zorzi *et al.,* 1996). Penicillin binding occurs at the C-terminal transpeptidase domain and substitutions of amino acids in the vicinity of conserved regions in the C-terminal part have been proposed to decrease the affinity for β –lactams resulting in high-level ampicillin resistance (Hsieh *et al.,* 2006). Sequencing of the *pbp*5 gene in the present study revealed that amino acid substitution (470His→Gln) located around two active-site-defining motifs (STFK and SDN) was significantly common in all the high level ampicillin resistant isolates. Also in addition to substitutions 496Asn→Lys, 497Phe→IIe, 499Ala→Thr, 525Glu→Asp previously described (Jureen *et al.,* 2004; Poeta *et al.,* 2007a, b; Belhaj *et al.,* 2008), substitution 500Glu→Leu, 502Asp→Arg, 612IIe→X and 614IIe→Phe were detected in some of our strains. Although amino acid polymorphism at the C-terminal region of the PBP is implicated in ampicillin resistance, other factors such as mutations at the N region in addition to those of the C-region have been suggested to likely play a role in the decreased affinity to β-lactams (Jurren *et al.,* 2004; Poeta *et al.,* 2007b).

Gentamicin is the preferred aminoglycoside for the treatment of enterococcal infections (Malinverni, 1996). It is reported to be a good predictor of resistance to other

aminoglycosides except streptomycin because resistance to gentamicin and streptomycin occurs by different mechanisms (Abamecha *et al.,* 2015). The occurrence of gentamicin (aminoglycoside) resistance among the *Enterococcus* isolates (25.9%) is in agreement with the findings of other authors (Han *et al.,* 2011; Klibi *et al.,* 2015a; b; Ben Said *et al.,* 2015; Ben Said *et al.,* 2016). High-level aminoglycoside resistance (HLAR) in enterococci is conferred by production of aminoglycoside modifying enzymes (AMEs) or mutation in the 30S ribosomal subunit (Chow, 2000; Hollenbeck and Rice, 2012). Aminoglycoside modifying enzyme genes; *aac(6')-Ie-aph(2'')- Ia* (19/19), *aph(2')*-Ic (8/9), *ant(4')* (4/10) and *aph(3')*-IIIa (9/10) were detected in this study. Majority of the high level gentamicin resistant (HLGR) isolates contained the *aac(6′)-Ie-aph(2″)-Ia* gene that encodes the bifunctional aminoglycoside modifying enzyme AAC(6)-Ie-APH(2)-Ia supporting the assertion by other authors that this gene may be the major gene responsible for HLGR in *Enterococcus* (Donabedian *et al.,* 2003; Klibi *et al.,* 2015a; Ben Said *et al.,* 2015; Ben Said *et al.,* 2016). The bifunctional enzyme causes *Enterococcus*, to be resistant (MIC ≥ 500 μg/ml) to all the clinically important aminoglycosides with the exception of streptomycin (Sadowy and Luczkiewicz, 2014; Osuka *et al.,* 2016). Possession of this gene eliminates the synergism between aminoglycosides and a cell-wall-active agent like ampicillin and vancomycin (Chen *et al.,* 2006). The *aac(6')-Ie–aph(2")-Ia* gene is commonly harboured on the composite transposon Tn5281 (IS256-related) or its variants as part of conjugative enterococcal plasmids or sometimes on the chromosome (Watanabe *et al.,* 2009; Behnood *et al.,* 2013).

The significance and clinical importance of resistance to these classes (ampicillin and gentamicin) of antibiotics relates to the inability to attain synergistic bactericidal activity (Hammad *et al.,* 2014). The detection of isolates resistant to these antibiotics in this study is therefore a source of worry as these two agents in combination are the drugs of choice for the treatment of enterococcal infections (Arias *et al.,* 2010; Fernandez-Hidalgo *et al.,* 2013). Resistance to one or both of these agents as observed in this study therefore results in a loss of this synergistic bactericidal effect and narrows the spectrum of the therapeutic armamentarium available to clinicians for the treatment of enterococcal infections.

Neither chloramphenicol nor ciprofloxacin is an antimicrobial approved for use in livestock in Nigeria. However, a relatively low resistance to chloramphenicol (6.7%) and ciprofloxacin (8.1%) was observed in this study. This finding is consistent with the use of these agents for livestock production in Nigeria (Nsofor *et al.,* 2013; Oluwasile *et al.,* 2014) and other parts of the world (Kasimoglu-Dogru *et al.,* 2010).

Chloramphenicol has a broad spectrum of activity against both Gram positive and negative bacteria, thus its effectiveness in the control of bacterial infection (Ng *et al.,* 2014). Its use has however, been prohibited due to its reported ability to induce bone marrow aplasia, aplastic anaemia, agranulocytosis and leukemia (Young and Kaufman, 2008; Yuan and Shi, 2008; Mintzer *et al.,* 2009). Despite this ban however, chloramphenicol is widely used in poultry production in Nigeria for the prevention and control of infection (Olatoye *et al.,* 2012) especially during brooding. This may be the likely reason for the occurrence of seven out of the nine chloramphenicol resistant isolates in samples from poultry sources. The observed levels of chloramphenicol resistance may

also be partly explained by the common use of florfenicol, a fluorinated derivative that shows some cross resistance with chloramphenicol (Schwarz *et al.,* 2004). The low occurrence of chloramphenicol resistance detected in this study agrees with the findings reported by Poeta *et al.* (2006) and Masjost *et al.* (2015); however it is in contrast to the report of Kasimoglu-Dogru *et al.* (2010) who detected a prevalence of 53% in Turkey.

The observed resistance against ciprofloxacin (also a quinolone antimicrobial not licensed for veterinary use) may also be explained by cross-resistance with other quinolones licensed for veterinary use, such as enrofloxacin and norfloxacin (Literak *et al.,* 2013). The fluoroquinolones especially enrofloxacin an analogue of ciprofloxacin is currently emerging with increasing popularity as a common drug in poultry production due to its broad spectrum activity and the emergence of multidrug resistant *Salmonella* (Alo and Ojo, 2007; Oluwasile *et al.,* 2014; Adesokan *et al.,* 2015). Fluoroquinolones are classified as "critically important in human medicine" by the WHO resulting in the prohibition of its use in food animals in some countries (WHO, 2011). The detection of isolates resistant to ciprofloxacin in this study is therefore a source of concern since there is also convincing evidence that its use in poultry production results in selection for resistant *Campylobacter* and *Salmonella* which can in addition to chloramphenicol resistance genes from enterococci be transferred to humans (McDermott *et al.,* 2002; McEwen and Fedorka, 2002; Price *et al.,* 2007).

A high rate of resistance to the two most common classes of drugs used in animal production, the macrolide- erythromycin (54.1%) and tetracycline (52.6%) was observed especially among the livestock isolates in this study. Although the high rate of resistance to

tetracycline and erythromycin among the isolates from the livestock sources is worrisome, the result is predictable. This is because these agents or their analogues (oxytetracycline, chlotertracycline and tylosin in the case of erythromycin) are the most widely used group of antibiotics in livestock production worldwide (Marshall and Levy, 2011; Hao *et al.,* 2014). The resistance rates to tetracycline or erythromycin varied between the sample types with higher rates encountered among isolates originating from rectal and cloacal swabs compared to manure samples. This finding is in agreement with numerous studies reporting the widespread occurrence of tetracycline and erythromycin resistance genes among livestock and in the environment (Aarestrup *et al.,* 2000; Jackson *et al.,* 2004b; Jackson *et al.,* 2011; Klibi *et al.,* 2015b; Beukers *et al.,* 2015; Ben Said *et al.,* 2015; Ben Said *et al.,* 2016).

Macrolides such as erythromycin are not used in the treatment of enterococcal infections, but they are used to treat other bacterial diseases in animals and humans (Jackson *et al.,* 2004b). They are common substitutes for treatment of individuals with penicillin allergy and thus resistance to erythromycin, a representative of the macrolide would be a matter of concern (Betriu *et al.,* 2003). Macrolides such as erythromycin and tylosin are commonly used in the treatment of respiratory diseases among livestock. Although tylosin is most commonly used for treatment and growth promotion in the animal production in Nigeria (Oluwasile *et al.,* 2014; Adesokan *et al.,* 2015) compared to erythromycin, its use can select for cross resistance to other macrolides (Boerlin *et al.,* 2001). Tylosin use has been reported in many studies from different parts of the world to select for resistance to erythromycin among enterococci (Jackson *et al.,* 2004b; da Costa *et al.,* 2010; Beukers *et al.,* 2015).

Macrolide resistance is usually mediated by three mechanisms; target site modification (mutations or methylation of the 23S rRNA), efflux pump or inactiviation of the antibiotic (Leclercq, 2002). The *erm*(B) (erythromycin ribosome methylase), a gene responsible for modification of the 23SrRNA target site resulting in resistance of the organism to the effect of erythromycin was detected in majority (18/19) of the erythromycin resistant isolates in this study. Although other mechanisms of resistance to erythromycin were not studied, the detection of the *erm*(B) gene in majority of the isolates tested supports previous reports that methylation maybe the most common mechanism *Enterococcus* uses to resist the bactericidal effect of erythromycin (Leclercq, 2002; Zou *et al.,* 2011; Klibi *et al.,* 2015a; Jia *et al.,* 2014). It is therefore also possible that the isolates may habour other macrolide resistance genes not screened for in this study. This gene is reported to be located on conjugative plasmids or transposons such as Tn*917*, Tn*1545*, Tn*5384*, and Tn*5385* (Okitsu *et al.,* 2005) although some authors have also reported its occurrence on chromosome (Jackson *et al.,* 2004b). It is reported to confer cross resistance to the macrolide, lincosamide and streptogramin B antibiotics resulting in the MLS_B phenotype and its acquisition could play an important role in the evolution of erythromycin resistance in *Enterococcus* (Leclercq, 2002; Jackson *et al.*, 2004b).

The wide popularity of tetracycline as a constituent of most drugs used in livestock production, availability over the counter, less expensive and long term usage in animal production in Nigeria may have accounted for the high tetracycline resistance rate observed in this study (Adenipekun *et al.,* 2015). The routine application of these agents has result in the development of resistance against them by most bacteria (de Vries *et al.,* 2011). Tetracycline is not a drug of choice for the treatment of enterococcal infection but they are

used parentally to treat a range of infections including bacteria, mycoplasma, and some protozoa (Domenech *et al.,* 2015).

The mechanism of enterococci resistance to the tetracyclines is usually by ribosomal protection mediated by the *tet*(M), *tet*(O), *tet*(S) genes and the energy dependent efflux mechanisms mediated by the *tet*(K) and *tet*(L) genes (Huys *et al.,* 2004; Rathnayake *et al.,* 2012; Frye and Jackson, 2013). Four (*tet*(K), *tet*(L) *tet*(M) and *tet*(O)) of these genes were detected among the tetracycline resistant isolates in this study. In agreement with the findings of other authors (Aarestrup *et al.,* 2000; Del campo *et al.,* 2003; Klibi *et al.,* 2015a) the *tet*(L) (20/20) and *tet*(M) (18/19) were the most common tetracycline resistance genes detected in this study. These genes are usually located on conjugative transposons (Tn916, Tn*5397* and Tn*5801*) and on plasmids facilitating their transfer to other bacteria (Jurado-Rabadan *et al.,* 2014). Co-carriage of genes encoding both active efflux (*tet*(K) or *tet*(L)) and ribosomal protection protein (*tet*(M) or *tet*(O)) mechanisms was also observed in this study, a phenomenon that may result in higher resistance levels to tetracycline among the enterococci isolates (Schwaiger *et al.,* 2009).

Also most of the isolates showed carriage of both the *erm*(B)+ *tet*(L) (18/19) and *erm*(B)+*tet*(M) (14/19) genes. The *erm*(B) gene is reported to be carried on the same mobile genetic element (co-linked) with *tet* genes, thus explaining why in this study co-carriage of the two genes was frequently detected in the same strain (Leclercq *et al.,* 2013). Both genes are reported to be associated with the transposon integrase gene (*int* gene) of Tn*916*/Tn*1545* family of transposons, a mobile genetic element reported to be common among enterococci (De Leener *et al.,* 2005). Therefore the co-carriage of the genes in the same isolate in this study suggests the presence of mobile genetic elements and the possibility of the genes to be transferred together (Beuker *et al.,* 2015). This linkage is

potentially problematic as administrating macrolides (such as tylosin) or tetracycline which is a common practice in livestock production in Nigeria may not only select for macrolide resistance, but also for resistance to antibiotics such as tetracycline.

Quinupristin-dalfopristin (QD) is reported to have bacteriocidal and bacteriostatic effects on *E. faecium* and *E. faecalis* respectively (Arais *et al.*, 2010). The use of virginiamycin an analogue of QD is reported to select for resistance to QD among enterococci (Thal and Zervos, 1999; Werner *et al.,* 2000). Although QD and virginiamycin use have not been reported in animal production in Nigeria, an overall resistance of 3.70% was observed among the isolates against (QD). Four out of the five isolates resistant to QD were *E. faecalis,* a species reported to possess an intrinsic resistance to QD. Since the MIC of the QD was not determined, the resistance of the *E. faecalis* to QD may likely be due to this species intrinsic resistance. Carriage of the *erm*(B) gene has also been reported to mediate resistance to QD and lincosamides; the MLS_B resistance phenotype (Thal and Zervos, 1999). High resistance to QD has been reported in areas where virginiamycin is routinely applied to animal feed as growth promoters (Hwang *et al.,* 2010).

Interestingly however, about 39% of the isolates were resistant to rifampicin with individual sources especially those having direct contact with the environment (cattle and poultry manure) recording between 35% - 100% resistance rate. Resistance to rifampin has also been reported among *E. faecium* in other parts of the world, occurring in 65.9% and 67.6% of isolates from the USA and Europe, respectively (Deshpande *et al.*, 2007). Rifampicin is a drug exclusively used in human medicine for the treatment of tuberculosis

(Asif, 2013). The exact mechanism of resistance to rifampicin among animal enteococci is still unclear as rifimpicin resistant *E. faecium* do not possess any evidence of mutations in the *rpoB* gene, enzymatic inactivation or efflux pumps traits associated with rifampin resistance (Rand *et al.,* 2007). A strong selective pressure for the selection of rifampicin has been reported to already exist in the environment (Miller *et al.,* 2014). Thus, the detection of 39.2% resistance among isolates from rectal swabs of cattle may be attributed to the likely uptake of rifampin resistant enterococci from the environment during grazing.

Somewhat worrisome also is the occurrence of multidrug resistance among 45.9% of the *E. faecium* and *E. faecalis* isolates tested in this study. The emergence of multidrug resistance among bacterial strains is a major cause of treatment failure worldwide and a serious concern to animal and public health (da Costa *et al.,* 2013; Tarai *et al.,* 2013). One of the isolates was resistant to as high as six out of the 9 antibiotics of different classes. Occurrence of MDR was observed to be higher among isolates from poultry (66%) compared to other sources, suggesting the effect of selective pressure from antibiotic use. A similar finding on the increasing occurrence of MDR among commensal organisms including enterococci has been made (Amosun *et al.,* 2012). MDR is often mediated by mobile genetic elements such as plasmids, transposons and intergrons that carry multiple resistance gene cassettes (da Costa *et al.,* 2013). The high level of multi-drug resistance observed among the enterococci in this study poses serious clinical threats to both animal and public health as these strains have the potential to cause nosocomial infection and can transfer the genetic determinants of resistance with a lot of ease.

While enterococci are rarely associated with infections in animals, their ability to horizontally transfer resistance traits to other bacteria that cause infection in animals and humans such as *Staphylococcus aureus* (Noble *et al.,* 1992; Clewell and Dunny, 2002; Weigel *et al.,* 2003), *E. coli* (Jurado-Rabadan *et al.,* 2014), *Clostridium difficile* (Jasni *et al.,* 2010), and *Listeria* spp (Doucet-Populaire *et al.,* 1991; Biavasco *et al.,* 1996) are of concern. This report gives importance to resistance profiles observed in this study especially those to ampicillin, gentamicin, tetracycline, erythromycin and ciprofloxacin. This is because tetracyclines, aminoglycosides, macrolides, fluroqinolones and β-lactams are commonly used in veterinary and human medicine to treat various bacterial infections (Marshall and Levy, 2011). The close association between livestock and humans, the use of livestock manure for fertilization of soil and the potential for consumption of vegetables contaminated with enterococci from manure increases the likelihood of transfer of virulent and resistant enterococci that can cause infections or transfer of these traits to other bacteria in the environment or gastrointestinal tract (Simjee *et al.,* 2002; Chomel and Sun, 2011).

This study demonstrated the potential of the *E. faecium* and *E. faecalis* to transfer resistance genes to a recipient (*E. faecalis* JH2-2) strain through the filter mating conjugation experiment. Six (75.5%) out of the eight isolates tested transferred at least one of either tetracycline or erythromycin resistance genes to the recipient strain. This result suggests that the elements encoding resistance to tetracycline and erythromycin in the *E. faecalis* and *E. faecium* are linked to a mobile genetic element that could be transferred *in vitro* between enterococci. The ability of the multidrug resistant isolates used in the transferability experiments to transfer most of their antibiotic resistance pattern to the

recipient strain also suggests that mobile genetic elements, such as conjugative plasmids may be mediating the resistance. This finding is in agreement with those of other researchers (Huys *et al.,* 2004; Oancea *et al.,* 2004; Silveira *et al.,* 2014) and further confirms the role livestock may play as reservoirs and disseminators of *Enterococcus* harbouring antibiotic resistance genes to other *Enterococcus* species and bacteria.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

In conclusion the findings of this study has,

- Established a 29.9% overall detection of *Enterococcus* species from livestock (40.5%) and vegetable (16.2%) farms in Zaria, Nigeria. Of the 267 enterococci isolated, the species identity of 64 (24%) of the isolates could not be ascertained and were classified as *Enterococcus* spp while the remaining were distributed across 9 different species; *E. faecium* (48.7%), *E. faecalis* (2.2%), *E. raffinosus* (2.2%), *E. avium* (0.37%), *E. gallinarum* (18.4%), *E. casseliflavus* (1.1%), *E. mundtii* (1.5%), *E. durans* (0.37%) and *E. hirae* (1.9%). *E. faecium* (48.7%) was the predominant species isolated from all sample types (except for lettuce) followed by *E. gallinarum* (18.4%).

- Demonstrated that livestock and vegetables harbour potentially virulent *E. faecalis* and *E. faecium*. The isolates were found to express some potential virulence traits such as hemolysin (20.7%), gelatinase (9.6%), aggregation substance (1.48%) and biofilm production (83.7%) in addition to carriage of the corresponding genes; *cyl*A (1/19), *gel*E (15/19) and *asa*1 (14/20). The *esp* gene was not detected in any of the isolates tested, but however silent *gel*E genes was detected in 2 isolates. This is an indication that they can constitute a reservoir for the dissemination of virulent enterococci and genes which may pose a potential risk to animal and human health due to their capability to cause nosocomial infections.

- Demonstrated that *E. faecium* and *E. faecalis* isolates from poultry, cattle and vegetable farms in Zaria were resistant to multiple antimicrobials with resistance patterns that reflect the abuse of antimicrobials in livestock environments. Low and moderate resistance rates to ampicillin (5.1%) and gentamicin (25.9%) respectively, two of the most important drugs used in the treatment of enterococcal infection were observed among the isolates. In addition, 49.1% of the isolates displayed multidrug resistance (MDR) with CN-E-S-TE resistance phenotype being the most frequently (18.3%) occurring MDR phenotype.

- Identified genes responsible for resistance to gentamicin were; *aac(6')-Ie-aph(2'')* (19/19), *aph(2')*-Ic (8/9), *aph(3')*-IIIa (9/10) and *ant(4')* (4/10), ampicillin; *pbp5*, erythromycin; *erm*(B) (19/19) and tetracycline; *tet*(K) (9/10) *tet*(L) (20/20) *tet*(M) (18/19), *tet*(O) (10/10 among the *E. faecium* and *E. faecalis* isolates tested. It also Identified in addition to previously report amino acid polymorphisms (496Asn→Lys, 497Phe→IIe, 499Ala→Thr, 525Glu→Asp) responsible for *Enterococcus faecium* resistance to ampicillin, four unreported amino acid substitutions; 500Glu→Leu, 502Asp→Arg, 612IIe→X and 614IIe→Phe were also detected in this study. This finding suggests that mutations in the *pbp*5 gene may account for the ampicillin resistance observed among the isolates.

- Demonstrated the potential of intra species transfer of some resistance determinants by six (75.5%) of the *Enterococcus* isolates tested suggesting the role of mobile elements in mediating the resistance phenotype observed.

6.2 Recommendations

Based on the findings of this study, we recommend:

1. Further studies to evaluate the risk for human health of potential transmission of enterococci linked to the consumption of food vegetables. This is in addition to public enlightenment on the importance of proper disposal and handling of faecal material from livestock, good hygiene, and treatment of manure before disposal or use to fertilize agricultural lands.

2. The need for an increased surveillance and constant monitoring of enterococci in animals for their ability to harbour and spread potential virulence and antibiotic resistance determinants. This will also provide information and knowledge that will aid in understanding the role of livestock environment in the evolution and spread of resistance among commensal and pathogenic bacteria.

3. Further research to determine the role enterococci play in nosocomial infections in both veterinary and human hospitals and the genetic relationship between human and livestock enterococci strains in Nigeria.

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Appendix I

Appendix I: Isolation of *Enterococcus* from from poultry, cattle and vegetable farms in Zaria, Nigeriaw

	No. of Samples (isolates) Positive for *Enterococcus*													Total
	Farm 1	Farm 2	Farm 3	Farm 4	Farm 5	Farm 6	Farm 7	Farm 8	Farm 9	Farm 10	Farm 11	Farm 12	Farm 13	
Cloacal swab	7 (11)	6 (7)	2 (2)	9 (11)	10 (11)	7 (9)	-	-	4 (6)	2 (2)	4 (6)	7 (8)	-	58 (73)
Manure	5 (7)	4 (4)	5 (6)	5 (5)	5 (6)	4 (4)	-	-	-	1 (1)	-	-	-	29(33)
Rectal swab	0	0	0	9 (15)	6 (7)	3 (5)	5 (6)	6 (9)	5 (6)	2 (2)	3 (3)	6 (6)	1 (1)	46 (60)
Manure	0	1 (2)	5 (6)	4 (8)	4 (6)	4 (7)	-	3 (3)	3 (3)	2 (3)	2 (2)	-	2 (2)	30 (42)
Cabbage	2 (3)	1 (1)	3 (4)	1 (1)	1 (1)	-	-	-	-	-	-	-	-	8 (10)
Cabbage soil	-	-	1 (2)	1 (1)	-	-	-	-	-	-	-	-	-	2 (3)
Lettuce	4 (7)	3 (4)	4 (6)	4 (7)	1 (1)	-	1(1)	-	-	-	-	-	-	17 (21)
Lettuce soil	4 (5)	1 (1)	3 (4)	2 (2)	-	-	-	-	-	-	-	-	-	10 (12)
Carrot	-	1 (1)	-	-	-	-	-	-	-	-	-	-	-	1 (1)
Carrot soil	-	1 (1)	4 (5)	1 (1)	-	-	-	-	-	-	-	-	-	6 (7)
Total	**22(33)**	**18(2)**	**27(35)**	**36(51)**	**27(32)**	**18(25)**	**6(7)**	**9(11)**	**12(1)**	**7(8)**	**9(11)**	**13(14)**	**3(3)**	**200**

Appendix II

Appendix II: Phenotypic resistance pattern (resistant to ≤ antimicrobial agent) of the *E. faecium* and *E. faecalis* isolated from poultry, cattle and vegetable farms in Zaria, Nigeria

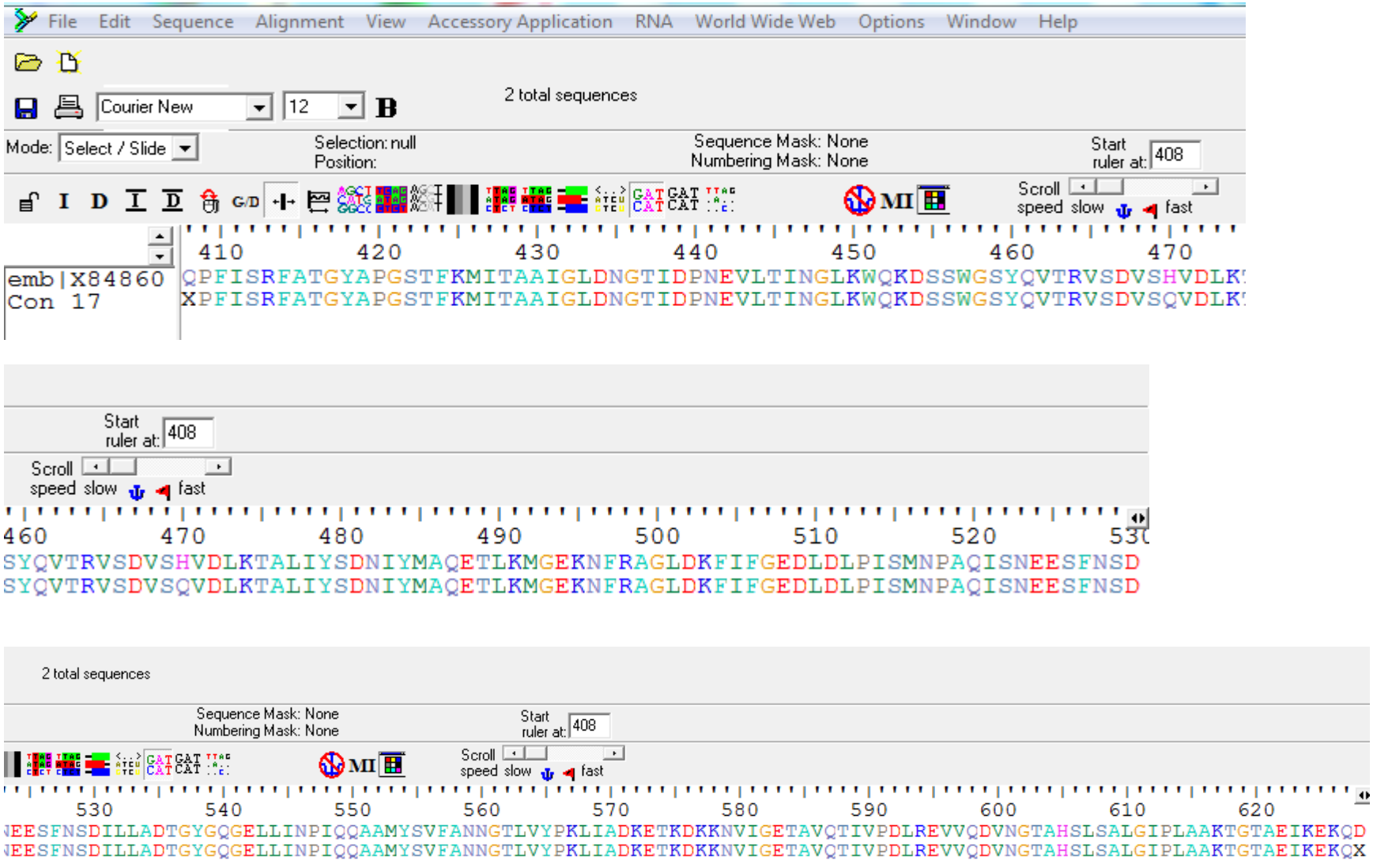
	Resistance Pattern	**Frequency**
1.	AM	5
2.	AM-C-CN-E-QD-S-TE	1
3.	AM-C-E-QD-S-TE	3
4.	AM-CIP-CN-E-R-TE	3
5.	AM-CIP-CN-E-S-TE	3
6.	AM-CIP-E-S-TE	1
7.	AM-CN-E-R-TE	1
8.	AM-CN-E-S-TE	7
9.	AM-CN-E-TE	2
10.	AM-E	2
11.	AM-CN-R-S-TE	1
12.	AM-E-R	6
13.	AM-E-R-TE	3
14.	AM-E-S-TE	1
15.	AM-E-TE	2
16.	AM-QD-R	1
17.	AM-R	18
18.	AM-R-TE	1
19.	AM-TE	2
20.	C-E	2
21.	C-E-R-S-TE	1
22.	C-E-TE	2
23.	CIP-CN-R-TE	1
24.	CIP-R	1
25.	CIP-R-TE	2
26.	CN-E-S-TE	11
27.	CN-TE	1
28.	CN-E-TE	6
29.	E	2
30.	E-R	3
31.	E-R-TE	1
32.	E-S-TE	3
33.	E-TE	7
34.	R	10
35.	R-TE	1
36.	TE	4
Total		**121**

APPENDIX III

Appendix III: Minimum inhibitory concentration of ampicillin and gentamicin on *E. faecium* and *E. faecalis* isolated from poultry, cattle and vegetable farms in Zaria, Nigeria

Antibiotics (No. of Isolates)	No. of isolates with minimum inhibitory concentration (µg/ml) of															
	0.125	0.19	0.25	0.75	0.5	1	1.5	2	3	4	6	12	16	> 256	>500	> 1024
Ampicillin (n=61)	1	1	3	4	3	11	7	6	5	8	1	-	1	6	-	-
Gentamicin (n=37)	-	-	-	1	-	-	-	-	-	-	-	1	-	-	-	35

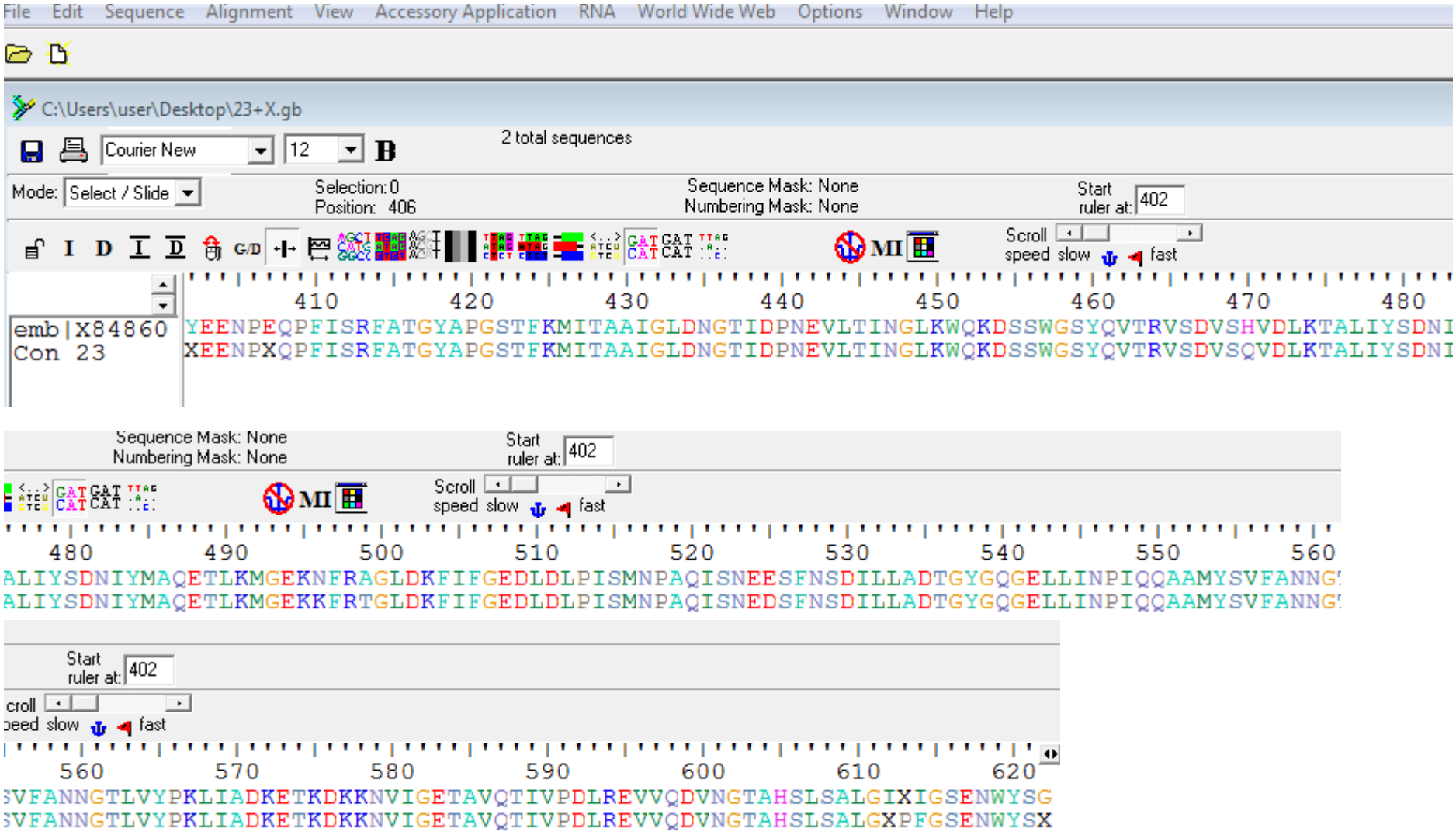
Ampicillin breakpoints = ≥ 16 µg/ml Gentamicin breakpoints = ≥ 500µg/ml



Appendix IV: Translated nucleotide sequence of sample 17 with amino acid substitutions at position 470.



Appendix V: Translated nucleotide sequence of sample 22 with amino acid substitutions at position 470.



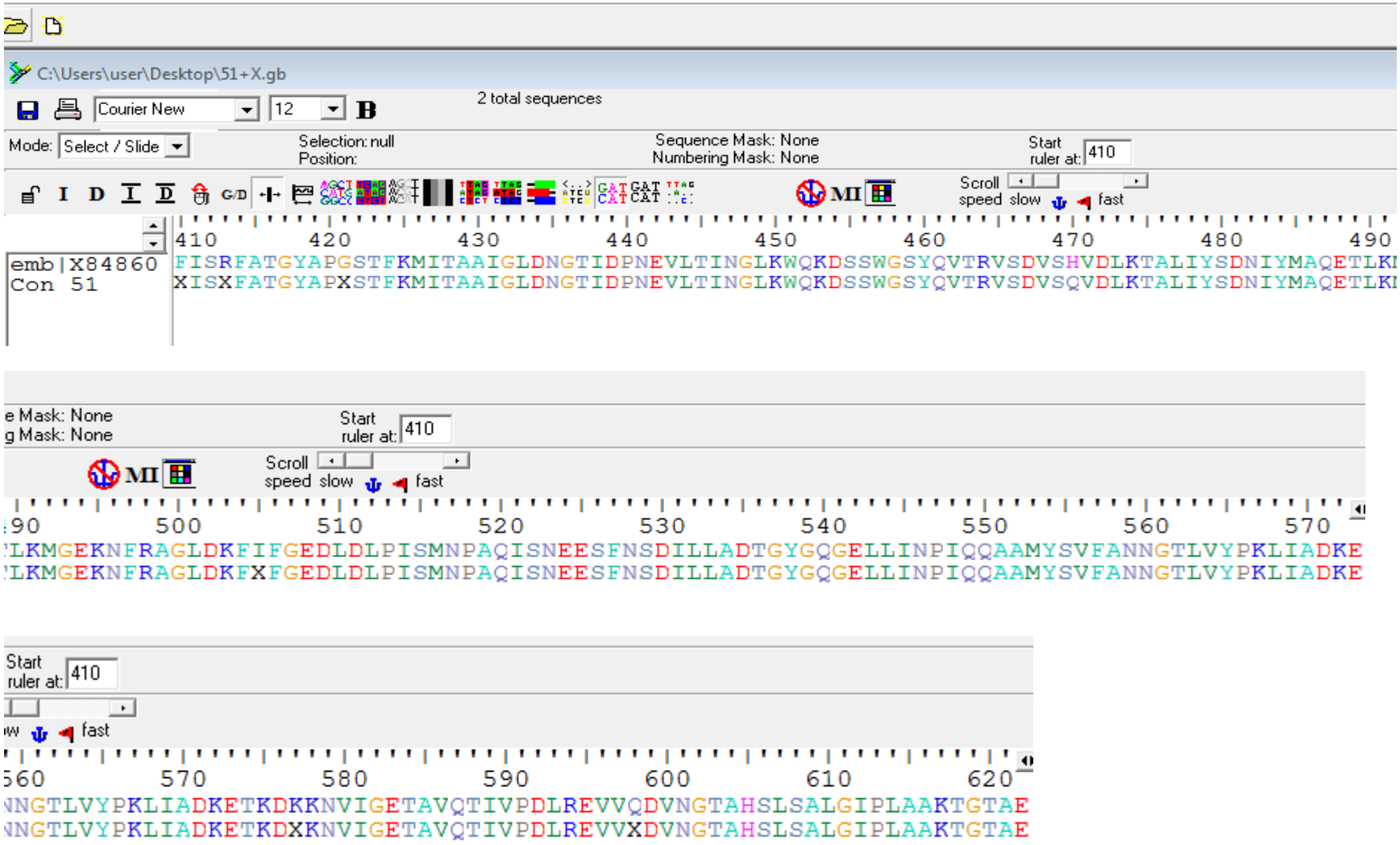
Appendix VI: Translated nucleotide sequence of sample 23 with amino acid substitutions at positions 470, 499, 525, 612 and 614



Appendix VII: Translated nucleotide sequence of sample 41 with amino acid substitutions at positions 470, 497, 499, 500 and 502



Appendix VIII: Translated nucleotide sequence of sample 45 with amino acid substitutions at position 470.



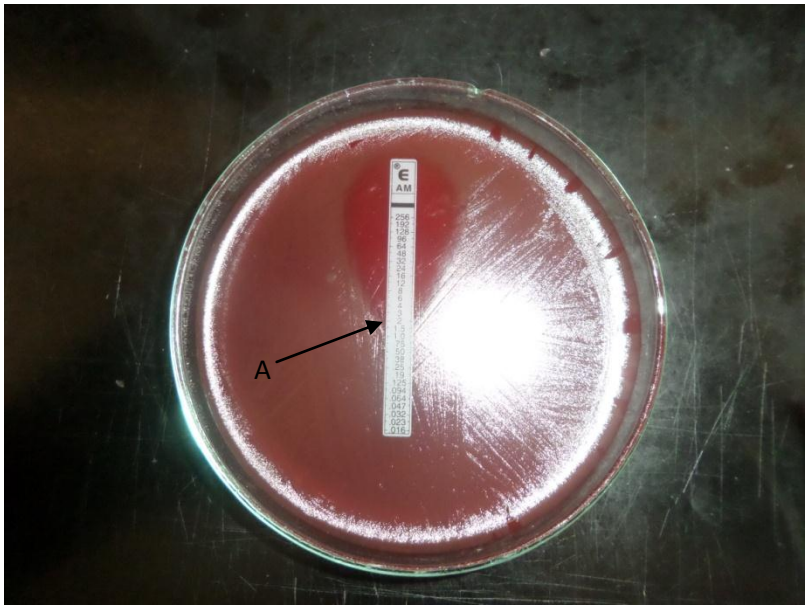
Appendix IX: Translated nucleotide sequence of sample 51 (Control strain) with amino acid substitutions at position 470.

Appendix IX



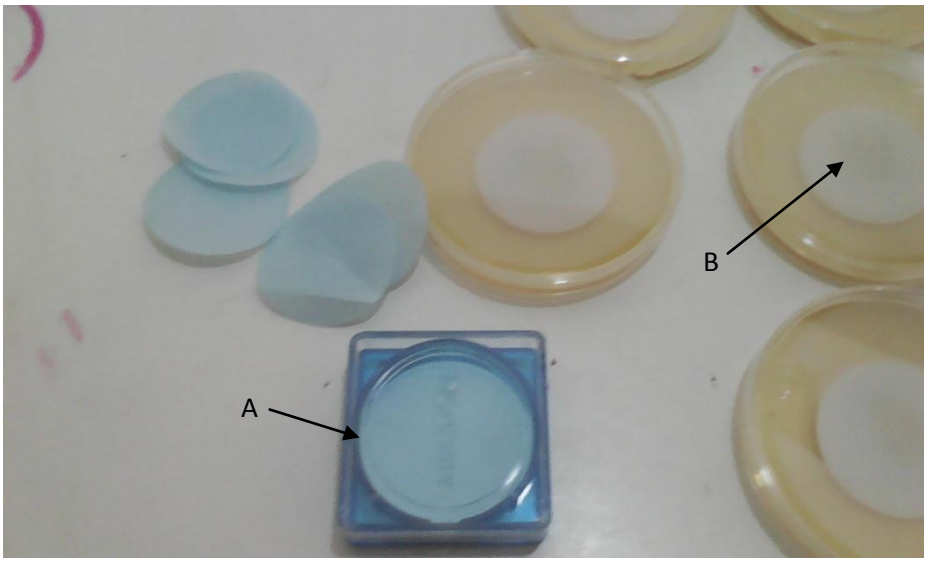
Appendix X: Positive samples (1 and 4) and positive control (3) indicated by the liquefaction of the gelatin medium and uninoculated/negative control indicated by gelling of the medium after incubation for 10 minutes at 4°C.

Appendix XI



Appendix XI: Point of eclipse (A) indicating minimum inhibitory concentration of ampicillin for an *Enterococcus* isolate

Appendix XII



Appendix XII: 0.45µm Milipore filter paper (A) and disc inoculated (B) with mixture of donor and recipient enterococci