Determination of the Occurrence, Virulence Genes, and Antimicrobial Resistance Profiles of *Enterococcus faecalis* and *Enterococcus faecium* Isolated from Poultry of Chongwe, Lusaka, Kitwe, and Ndola Districts, Farmworkers from the Copperbelt Province Poultry Farms, and Clinical Specimens from Kitwe Teaching Hospital in Zambia

Submitted by

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A thesis submitted to the University of Zambia in fulfilment of the requirements for the degree of Doctor of Philosophy in Microbiology

THE UNIVERSITY OF ZAMBIA

LUSAKA

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This thesis of Grace Mwikuma has been approved as fulfilling the requirements for the award of Doctor of Philosophy in Microbiology by the University of Zambia

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ABSTRACT

Enterococcus has great genetic plasticity and is highly versatile, hence does not only acquire and express mobile genetic elements (MGEs) but also disseminates them to pathogenic and non-pathogenic species of the same or of different genera. As such, it has been associated with various clinical syndromes and is ranked among the major causes of nosocomial infections worldwide. The study aimed to determine the occurrence, virulence genes, and antimicrobial resistance profiles of *Enterococcus faecalis* and *Enterococcus faecium* isolated from poultry of Chongwe, Lusaka, Kitwe, and Ndola Districts, Farmworkers from the Copperbelt Province Poultry Farms, and Clinical Specimens from Kitwe Teaching Hospital in Zambia. A total of 833 samples comprising 492 poultry droppings, 108 urine, and 3 rectal swabs from farmworkers, and 138 urines, 89 stool, and 3 pus swabs from patients attending Kitwe Teaching Hospital were processed. Phenotypic methods were employed to identify *Enterococcus* while confirmation and species identity were determined by genotypic methods. Drug resistance patterns were determined using the standard disc diffusion method and interpreted according to CLSI 2020 guidelines. Polymerase chain reaction was used to identify and detect resistance and virulence genes. In this study the prevalence of *Enterococcus* was 31.1% in poultry, 30.8% in farmworkers' specimens (all being *E. faecalis*), and 29.6% in clinical samples (with *E. faecalis* making up 58.8% of these). Both *E. faecalis* and *E. faecium* showed high resistance to several antibiotics, with *E. faecium* generally more resistant than *E. faecalis*. The majority of *E. faecalis* isolates were multidrug-resistant, with 89.7% in poultry, 75% in farmworkers, and 97.4% in clinical samples. All *E. faecium* isolates in clinical samples were multi-drug resistant (MDR). The widespread MDR pattern was resistance to ampicillin, chloramphenicol, ciprofloxacin, erythromycin, nitrofurantoin, penicillin, tetracycline, vancomycin combination. The frequently detected resistance genes in *E. faecalis* were *tetK* and *tetM* from Kitwe and Ndola, respectively, and in *E. faecium*, it was *aac(6')-Ie-aph(2")-LA* and *ermB* from Kitwe, and *aac(6')-Ie-aph(2")-LA* from Lusaka. In farmworkers, the frequently detected resistance gene in *E. faecalis* was *tetL*. In clinical specimens, the commonly detected resistant gene in *E. faecalis* was *ermB*, and in *E. faecium*, it was *aac(6')-Ie-aph(2")-LA* and *tetM*. The study also investigated the presence of gelatinase, aggregation substance, enterococcal surface protein, cytolysin, pheromone cAD1 precursor lipoprotein, *E. faecalis* endocarditis antigen Regulator of *gelE* and *sprE* expression and collagen-binding cell wall protein in *E. faecalis* and *E. faecium*, and *E. faecium* specific cell wall adhesion. Overall, 95.5% of all *E. faecalis* and *E. faecium* isolates possessed one of the virulence genes tested. The most prevalent virulence gene was *ace* being found in 95.5% of all isolates, followed by *cad1*. The least common virulence gene was *esp* which was detected in 56.0% of all *E. faecalis* and *E. faecium* isolates. The findings indicate a potential for the transmission of antibiotic-resistant strains between these populations and suggest the possibility of zoonotic transmission. Poultry and its products can be contaminated thereby posing a threat to consumers. There is also the possibility of increased healthcare costs and a higher risk of treatment failure due to infection with resistance strains. The discovery highlights the need for enhanced surveillance and monitoring of antibiotic resistance and virulence genes in both human and animal populations as well as other settings.

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TABLE OF CONTENTS

Page

List of Tables

Page

List of Figures

Page

List of Abbreviations and Acronyms

ace	adhesin of collagen from *Enterococcus*
AIDS	Acquired immunodeficiency syndrome
AMGP	antimicrobial growth promoters
AMR	antimicrobial resistance
API	analytical profile index
ARE	antimicrobial resistance enterococci
asa1	aggregation substance
AST	antimicrobial susceptibility test
BEA	bile esculin azide agar
BSI	bloodstream infections
CAUTI	catheter-associated urinary tract infection
CC	Clonal complex
CLABSI	central line-associated bloodstream infection
CNS	Central nervous system
CR-BSI	catheter-related bloodstream infections
CTns	conjugative transposons
cylA	Cytolysin
CVCs	central venous catheters
ddl	d-alanine: d-alanine ligase gene
DNA	Deoxyribonucleic acid
ECC	Enterococcal clonal complex
efaA	*E. faecalis* endocarditis antigen
EFSA	European Food Standards Agency

ESKAPE	*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa,* and *Enterobacter species*
esp	Enterococcal surface protein
FIB	faecal indicator bacteria
GC	guanine and cytosine
gelE	Gelatinase
GIT	gastrointestinal tract
GRAS	generally regarded as safe
GRZ	Government of the Republic of Zambia
HAI	Hospital-acquired infections
HiRECC	High-risk Enterococcal clonal complex
HIV	Human immunodeficiency virus
hyl	Hyaluronidase
ICU	Intensive care unit
IS	Insertion sequence
LAB	lactic acid bacteria
MDR	Multidrug Resistance
MGEs	mobile genetic elements
MLST	Multilocus sequence typing
MRSA	methicillin-resistant *Staphylococcus aureus*
MSCRAMMs	microbial components recognising adhesive matrix molecules
MVLST	multi-virulence locus sequence typing
NHRA	National Health Research Authority
PAIs	pathogenicity islands
PBP	penicillin-binding protein

PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
PICCs	peripherally inserted central venous catheters
QPS	qualified presumption of safety
ROS	reactive oxygen species
rRNA	Ribosomal ribonucleic acid
SCFA	short-chain fatty acids
sodA	superoxide dismutase gene
Tn	Transposon
tRNA	transfer ribonucleic acid
tuf	elongation factor
UNZA	University of Zambia
UNZABREC	University of Zambia Biomedical Rese3arch Ethics Committee
UTI	urinary tract infections
vanA	vancomycin resistance gene
VRE	vancomycin-resistant enterococci
VREF	Vancomycin-resistant *E. faecium*
VRSA	vancomycin-resistant *Staphylococcus aureus*
WGS	Whole Genome Sequence
ZNPHI	Zambia National Public Health Institute

List of Appendices

CHAPTER ONE

INTRODUCTION

1.0 Background

Enterococcus is a genus of Gram-positive bacteria in the family of Enterococcaceae, and the order Lactobacillales of the phylum Firmicutes (Ludwig et al., 2009). *Enterococcus* is a normal flora of the gastrointestinal tract (GIT) of animals including birds (Soodmand et al., 2018). Being ubiquitous, it is found in soil, plants, sewage, and fresh and saltwater (Zaheer et al., 2020). Species of the genus *Enterococcus* have emerged as pathogens of medical and public health importance (Ahmed and Baptiste, 2018). This is due to their adaptability to selective pressure of antimicrobials and ability to acquire and transfer mobile genetic elements (MGE) to species of the same genus or other pathogenic or non-pathogenic bacteria (Sanderson et al., 2022) and subsequent development of antimicrobial resistance. MGE can move within or between deoxyribonucleic acid (DNA) molecules (Tokuda and Shintani, 2024). These include insertion sequences (IS), Transposons (Tn) gene cassettes or integrons and those that can transfer between bacterial cells for example plasmids and integrative conjugative elements (Tokuda and Shintani, 2024). These elements play an important role in facilitating horizontal genetic exchange and promoting the acquisition and transmission of resistance genes (Partridge et al., 2018; Tokuda and Shintani, 2024).

Some virulence genes are located on mobile genetic elements (MGEs), such as pathogenicity islands (PAIs) or plasmids. In the case of enterococci, MGEs are widespread and encompass conjugative transposons (CTns), PAIs, and plasmids, all contributing to resistance, virulence, and adaptation to the host (Tokuda and Shintani, 2024). Studies have demonstrated that a variety of virulence factors can exist on multiple PAIs, which are also capable of being transferred (Partridge et al., 2018). Virulence factors encoded on MGEs can spread through horizontal gene transfer, a process that can turn harmless bacteria into pathogenic threats (Partridge et al., 2018; Michaelis and Grohmann, 2023). As a result, *Enterococcus* has been associated with many clinical conditions including urinary tract

infections (UTI), endocarditis, bacteraemia, and mastitis in humans and animals (Krawczyk et al., 2021).

Enterococci have also been ranked among the major causes of nosocomial infections worldwide (Pillay et al., 2018). This is especially so with *Enterococcus faecalis* (E. *faecalis*) and *Enterococcus faecium* (*E. faecium*). The emergence of vancomycin-resistant *Enterococcus* (VRE) and multidrug-resistant (MDR) *Enterococcus* in poultry are of major public health concern. This is because of limited treatment options for infections caused by such species as well as the possibility of dispersion between poultry and humans (Rehman et al., 2018; Soodmand et al., 2018). Vancomycin-resistant *Enterococcus* is a nosocomial pathogen that exhibits multidrug resistance and virulence. *Enterococcus faecium* has transitioned from being a commensal organism to an *Enterococcus* species, *Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa,* and *Enterobacter species* (ESKAPE) pathogen. Vancomycin-resistant *Enterococcus* has been reported worldwide (Lee et al., 2019). In the United States, VRE causes more infections than other nosocomial pathogens and successfully evades the effect of antimicrobial drugs (Zhou et al., 2020).

In 2017, Zambia developed a Multi-sectoral National Action Plan in recognition of the public health threat, its impact on morbidity and mortality, and the economic outcomes of antimicrobial resistance (Republic of Zambia NAP on AMR, 2017). The plan notes that VRE has also been documented in Zambia, and routine laboratory testing at Lusaka UTH collects data on antimicrobial susceptibility for epidemic-prone bacterial pathogens such as VRE. From this it can be deduced that the VRE reported in Zambia is humans (Republic of Zambia NAP on AMR, 2017). Despite that recognition, not so much has been done in terms of surveillance and research on Enterococci particularly, *E. faecalis and E. faecium* in Zambia in comparison with other commensals of the gastrointestinal tract (GIT) such as *Staphylococci, Salmonella, Shigella, Campylobacter,* and *Escherichia coli*. This is why there is currently no information on the occurrence of virulence genes and sparse information on antimicrobial resistance genes of *E. faecalis* and *E. faecium* which exist in humans and poultry in Zambia.

1.1 Statement of the Problem

Virulence genes are prevalent in *Enterococcus* species from a wide variety of reservoirs including poultry and humans. These virulence genes can enhance the pathogenicity of *Enterococcus* and can be transmitted through horizontal gene transfer between animals and humans, and the environment. Existing research indicates that *Enterococcus* possess the capacity to acquire, express, and transmit resistance and virulence genes, contributing significantly to the spread of these traits. Notably, *Enterococcus* has been documented as having transmitted the *vanA* operon to methicillin-resistant *Staphylococcus aureus* (MRSA), resulting in vancomycin resistance *Staphylococcus aureus* (George et al., 2021). The transmission of such virulent and resistant clones is associated with heightened morbidity, posing challenges in the effective treatment of cases. Dispersion of *Enterococcus* species with such traits entails the dissemination of multidrug resistance and virulence determinants to other microbes. Most of these genetic determinants are found in clonal complexes (CCs) which have been classified as high-risk enterococcal clonal complexes (HiRECC) because of their association with the hospital environment and their role in colonization and causation of nosocomial infections. These HiRECCs have been found in humans as well as animals including poultry (Bortolaia and Guardabassi, 2015). Transmission of these clones has been postulated and poultry could be the source of these strains (Stępień-Pyśniak et al., 2019).

Poultry products rank among the most widely consumed globally, with an annual production exceeding 90 billion tons of chicken meat (http://www.fao.org/3/bi6407e). In Zambia, poultry is the most consumed meat making up about 50% of the total meat consumption, with meat trailing at 28% followed by pork, fish and other meats at 16% and 6%, respectively (https://africanfarming.net/livestock/poultry/zambian-poultry-sector-shows-significant-growth). Commercial poultry production environments emerge as significant sources of MDR microbes due to extensive antimicrobial usage during production processes (Mudenda et al., 2023). Enterococci can contaminate poultry products and pose a risk to human health if consumed (Chai et al., 2017). Given this context, understanding the occurrence of *Enterococcus* becomes imperative for effectively controlling and preventing the transmission of enterococcal infections.

In Zambia, the problem of MDR in *E. faecalis* and *E. faecium* is not well defined. To the best of available information, there is no information on the occurrence of virulence genes and there is sparse information on antimicrobial resistance profiles of *E. faecalis* and *E. faecium* isolated from poultry and humans in Zambia. Therefore, determining the occurrence of virulence genes and antimicrobial-resistance profiles of *E. faecalis* and *E. faecium* isolated from poultry and humans contributes to the broader understanding and management of antimicrobial resistance.

1.2 Study Justification

Understanding the virulence factors of *Enterococcus* can help in predicting the severity and course of infections. This can aid in developing effective treatment strategies. Investigating antimicrobial resistance genes sheds light on how these bacteria develop resistance to specific drugs. Such insights are critical for designing new medications that can effectively combat resistant strains. By studying various aspects of *Enterococcus*, we enhance our understanding of its transmission and disease-causing mechanisms. This knowledge informs prevention strategies to curb the spread of these bacteria and outbreaks. Enterococci play a significant role in healthcare-associated infections. Therefore, understanding their virulence and resistance mechanisms directly impacts public health policies and practices within healthcare settings. Because *Enterococcus* often coexists with other bacteria in infections, understanding its virulence and resistance patterns helps manage complex cases effectively. Analysing virulence and resistance genes also helps to forecast future trends in *Enterococcus* infections. This foresight aids in preparedness and prevention of future outbreaks. Enterococci are known to form biofilms. Some virulence genes are responsible for biofilm-formation, studying such genes provides insights into this process and potential strategies for disruption. Additionally, resistance genes can be transferred to other bacteria in the environment. Studying these genes can help us understand this process and its impact on environmental health.

There is a lack of comprehensive data on the virulence and antimicrobial resistance genes of *E. faecalis* and *E. faecium* that are present in poultry and humans in Zambia. A study done by Mubita and others (2008) highlighted resistance patterns of *E. faecalis* and *E.*

faecium isolated from cattle to commonly used antibiotics. However, the study did not investigate the associated resistance and virulence genes carried by the two pathogenic species of *Enterococcus*. Another Zambian study by Chisanga and others (2017) focused on other urinary tract infection pathogens which were found in significant numbers. Apart from mentioning that *E. faecalis* was detected, no further tests were carried out. The most recent study conducted by Mudenda and others (2022) was on the prevalence and antimicrobial resistance of *Enterococcus* and no speciation was done. The study did not state which species of *Enterococcus* were resistant to which specific drugs. It is therefore important to carry out this research to provide a better understanding of virulence and resistance determinants involved in the emergence of antimicrobial-resistant *Enterococcus*.

This study contributes valuable insights into the distribution of antimicrobial resistance and virulence genes among *Enterococcus faecalis* and *Enterococcus faecium* originating from both poultry and humans and provides essential knowledge for informed strategies in mitigating the transmission of enterococcal infections.

1.3 Research Questions

1.3.1 What is the occurrence of *Enterococcus faecalis* and *Enterococcus faecium* isolated from poultry of Chongwe, Lusaka, Kitwe, and Ndola Districts, Farmworkers from the Copperbelt Province Poultry Farms, and Clinical Specimens from Kitwe Teaching Hospital in Zambia?

1.3.2 What are the antimicrobial resistance patterns and associated antimicrobial-resistant genes found in *E. faecalis* and *E. faecium* from poultry of Chongwe, Lusaka, Kitwe, and Ndola Districts Farmworkers from the Copperbelt Province Poultry Farms, and Clinical Specimens from Kitwe Teaching Hospital in Zambia?

1.3.3 What virulence genes are present in *E. faecalis* and *E. faecium* from poultry of Chongwe, Lusaka, Kitwe, and Ndola Districts, Farmworkers from the Copperbelt Province Poultry Farms, and Clinical Specimens from Kitwe Teaching Hospital in Zambia?

1.4 Study Objective

1.4.1 General Objective

The study aimed to determine the occurrence, virulence genes, and antimicrobial resistance profiles of *Enterococcus faecalis* and *Enterococcus faecium* from poultry of Chongwe, Lusaka, Kitwe, and Ndola Districts, Farmworkers from the Copperbelt Province Poultry Farms, and Clinical Specimens from Kitwe Teaching Hospital in Zambia.

1.4.2 Specific Objectives

1.4.2.1 To establish the occurrence of *E. faecalis* and *E. faecium* from poultry of Lusaka, Chongwe, Kitwe, and Ndola Districts, farmworkers from the Copperbelt Province Poultry farms and Clinical Specimens from Kitwe Teaching Hospital in Zambia.

1.4.2.2 To determine antimicrobial resistance patterns and their associated antimicrobial-resistant genes in *E. faecalis* and *E. faecium* from poultry of Lusaka, Chongwe, Kitwe, and Ndola Districts, farmworkers from the Copperbelt Province Poultry farms and Clinical Specimens from Kitwe Teaching Hospital in Zambia.

1.4.2.3 To assess the presence of virulence genes in *E. faecalis* and *E. faecium* from poultry of Lusaka, Chongwe, Kitwe, and Ndola Districts, farmworkers from the Copperbelt Province Poultry farms and Clinical Specimens from Kitwe Teaching Hospital in Zambia.

CHAPTER TWO

LITERATURE REVIEW

2.0 General Overview of Enterococci

The normal habitat of enterococci is the gastrointestinal tracts of humans and animals. They have also been recovered from plants and vegetables, in soil, water, and food, both processed and unprocessed (Hamzah and Kadim, 2018; Igbinosa and Beshiru, 2019). Recovery of enterococci from the above sources is necessitated by their ability to survive at very low nutrient content, biofilm formation, growth on acidic, neutral and alkaline pHs and at low and high temperatures (García-Solache and Rice, 2019; Stępień-Pyśniak et al., 2019). For example, *Enterococcus faecalis* and *Enterococcus faecium* can survive at 60°C for 30 minutes (Zhu et al., 2022). *Enterococcus faecalis'* membrane is impermeable and durable, and this could confer the ability to grow at varying pH and temperatures (Abe and Honda, 2023).

Enterococcus is thought to possess a cation homeostasis mechanism that contributes to its resilience against variations in pH, salt, metals, and desiccation (Ali et al., 2022). Cation homeostasis is any process involved in the maintenance of an internal steady state of divalent cations within an organism or cell (Weiss and Carver, 2018). Metals are components of many proteins and are co-factors in enzymatic reactions required for proteins to perform vital functions for life (Murdoch and Skaar, 2022). Therefore, the concentration of metals is maintained at levels, which keep critical biological processes going while restricting toxicity (Monteith and Skaar, 2021).

2.1 Taxonomy, History, and Phylogenetic Status of *Enterococcus*

Enterococcus is a genus of Gram-positive bacteria in the family of Enterococcaceae and the order Lactobacillales of the phylum Firmicutes. They are catalase-negative, facultative anaerobes which appear as single cocci, in pairs and chains of different lengths (Safe and Feriala, 2022). Using physical characteristics alone, one cannot easily differentiate them from *Streptococcus*. *Enterococcus* does not form spores or capsules but grows over a wide range of environmental conditions such as extreme temperatures ranging from 10 to 45°C,

pH of 4.5 to 9.9 and high salt concentration (Gaca and Lemos, 2019). Enterococci belong to lactic acid bacteria (LAB) which produce bacteriocins (Almeida-Santos et al., 2021) which in turn assist them to survive among various microbial communities. LAB are characterized by low guanine (G) and cytosine (C) content of <50 mol% (Sharma et al., 2020).

The history of *Enterococcus* dates back to 1899 when Thiercelin described a Gram-positive saprophytic coccus, of intestinal origin, capable of causing infection (Thiercelin, 1899). In the same year, MacCallum and Hastings (1899) characterized a similar organism from a lethal case of endocarditis. This organism is now known as *Enterococcus faecalis*. In 1903, Thiercelin and Jouhaud included it in the genus *Enterococcus*. Andrewes and Horder renamed it *Streptococcus faecalis* in 1906. It was named '*Streptococcus*' because it reacted to Lancefield group D antisera which was used to group *Streptococcus* species based on carbohydrate antigens on the cell wall (Lancefield, 1933) and '*faecalis*' to stress that it was characteristic of human intestines. Schleifer and Kilpper-Balz (1984) discovered that *Streptococcus faecalis* and *Streptococcus faecium* were different from *Streptococcus* and transferred them to the genus *Enterococcus* after nucleic acid studies showed that these two species are distantly related to *Streptococcus bovis* and *Streptococcus equinus*.

New species have continued to be discovered from different reservoirs. The genus *Enterococcus* currently has more than 60 recognized species (Schwartzman et al., 2023). Of these *Enterococcus faecalis* and *Enterococcus faecium* are the most commonly isolated as well as most important species worldwide (Kim et al., 2021). *Enterococcus faecalis* is known to cause about 80 to 90% of human infections followed by *Enterococcus faecium* which accounts for about 5 to 10% of infections (Ferede et al., 2018). Other *Enterococcus* species which have been found in association with human disease include *E. casseliflavus, E. raffinosus, E. avium, E. cecorum, E. dispar, E. durans, E. gallinarum, E. gilvus, E. hirae, E. mundtii* and *E. pallens* (Teixeira et al., 2015; Toc et al., 2022).

Studies have shown that *E. gallinarum* and *E casseliflavus* can cause bacteraemia (Bilman and Çiçek, 2015; Britt and Potter, 2016). They have also been associated with cases of

endocarditis and meningitis (Monticelli et al., 2018). *E. casseliflavus* has also been involved in endophthalmitis (Berenger et al., 2015; Nguyen and Hartnett, 2017). *E. pallens* was found in association with spontaneous peritonitis in patients with liver cirrhosis and was isolated from ascites fluid (Lévesque et al., 2016). *E. gilvus* is involved in human infections (Farsi et al., 2023). *E. pallens* was implicated in cases of bacterial peritonitis (Lévesque et al., 2016).

E. raffinosus has been known to cause endocarditis (Ishida et al., 2022) it is known to cause bacteraemia in adult patients (Lee et al., 2022). *E. durans* was reported as the cause of bacteraemia over a period of 20 years in a tertiary hospital (Ryu et al., 2019). Though rarely isolated from human infections, *E. mundtii* was reported from a human urinary tract infection in Iran (Sharifi-Rad et al., 2016).

Enterococcus hirae is on record as a cause of infections in animals. It caused diarrhoea in rats, endocarditis in chickens, mastitis in cattle, cholangitis and pancreatitis in cats and septicaemia in birds (Pinkes et al., 2019; Piccinini et al., 2023). *E. hirae* is a rare zoonotic pathogen which has been reported in connection with pyelonephritis, infective endocarditis and biliary tract infections in humans (Dicpinigaitis et al., 2015; Bollam et al., 2021; Nakamura et al., 2021). *E. dispar* was isolated among others in cases of urinary tract infections in India (Goel et al., 2016).

2.2 Important Characteristics of *Enterococcus*

Species of *Enterococcus* are capable of growing over wide temperatures ranging from 10 to 45°C though some species such as *E. faecalis* and *E. faecium* can withstand the temperature of 60°C for 30 minutes (Gaca and Lemos, 2019; Zhu et al., 2022). They resist 40% bile and grow in 6.5% sodium chloride concentrations (García-Solache and Rice, 2019). They are facultative anaerobes, which produce lactic acid as the main end product of carbohydrate fermentation (Wang et al., 2021). *Enterococcus* has a low G and C content of 34–45% (Zhong et al., 2017). As a genus *Enterococcus* plays a dual role in humans; being useful and beneficial as well as being harmful as opportunistic nosocomial pathogens (Braïek and Smaoui, 2019).

2.2.1 Useful and Beneficial Roles of *Enterococcus*

Enterococcus has not been recommended for the qualified presumption of safety (QPS) list (Zommiti et al., 2022) and has not given the generally recognized as safe (GRAS) status (Zommiti et al., 2022) because of the prevalence of virulence and resistance genes and their ability to cause disease. QPS list contains biological agents which are intentionally added to food or feed based on evidence that they do not raise safety concerns (EFSA BIOHAZ Panel, 2023). A substance or microbe could attain GRAS status only if experts evaluate its safety and give it general recognition of safety. Strains of different species of *Enterococcus* are highly competitive and useful. This is due to their resistance to a wide range of pH and temperature, their proteolytic and lipolytic activities, and many other properties including their ability to produce bacteriocins (Hanchi et al., 2018; Terzić-Vidojević et al., 2021).

2.2.1.1 Proteolytic Activity

Enterococcus's ability to tolerate a high salt content of 6.5% and pH range of 4.6 to 9.9 has made it possible for it to occur at high population levels in fermented foods. It's these beneficial technological properties that make some *Enterococcus* species play an important role in food production, either as starter or non-starter lactic acid bacteria (Roopashri et al., 2023), and are also used in the fermentation of meat products such as dry sausages (Terzić-Vidojević et al., 2021). In the production of dairy products, enterococci are used to hasten the ripening of cheeses because of their proteolytic and lipolytic activities coupled with their ability to breakdown citrate and produce aromatic compounds which improve organoleptic qualities such as taste, flavour, colour and texture (Hanchi et al., 2018). Citrate metabolism contributes to the sensory traits of fermented foods by the production of acetoin, acetaldehyde, diacetyl, and 2,3-butanediol with very distinct flavours which influence the quality of cheese, fermented milk, cream and butter in a significant way (Rajendran et al., 2023; Rutkowska et al., 2022). The production of carbon dioxide enhances the texture of fermented products.

Biscola and others (2016) suggested proteolytic activity of *E. faecalis* VB63F could reduce the allergenicity of bovine protein milk in children. Breakdown of proteins (proteolysis) frequently leads to the loss of linear epitopes, destruction of the epitope and

loss of most conformational epitopes because of the changes in the structural folding of the protein (Scheiblhofer et al., 2017; Pekar et al., 2018; Bøgh and Larsen, 2021). This consequently leads to a reduction of product allergenicity (Scheiblhofer et al., 2017; Pekar et al., 2018).

2.2.1.2 Bacteriocin Production

According to Aljohani and others (2023), bacteriocins are peptides that are synthesized in the ribosome of bacteria and serve as antimicrobial agents. These peptides have the capability to either kill or inhibit the growth of other bacterial strains, irrespective of their close genetic relation to the producing bacteria. Interestingly, the producing bacteria remain unharmed due to the action of specific immunity proteins. They are used to extend food preservation time, treat pathogen disease and for cancer therapy, and to maintain human health (Aljohani et al., 2023).

Bacteriocins produced by *Enterococcus* are called enterocins. Most enterocins act by forming pores in the cell membrane of target strains thereby depleting the pH gradient and transmembrane potential resulting in the leakage of intracellular molecules (Braïek et al., 2018). Enterocin A degrades the cell wall structure which leads to the lysis of target strains (Du et al., 2022). The activity of enterocins is shown against foodborne pathogens and spoilage bacteria (Baños et al., 2016). *Enterococcus* produce enterocins which act as potential inhibitors of biofilm formation and prevent food spoilage thereby extending shelf life and enhancing the hygienic safety of food (Braïek et al., 2018). They do so by preventing the development of pathogenic bacteria such as *Staphylococcus aureus, Vibrio cholerae* and *Listeria monocytogenes* Braïek et al., 2018). Enterocins also showed exceptional antimicrobial inhibitory activities against *Bacillus cereus* and *Clostridium* species (Arbulu et al., 2015; O'Connor et al., 2015; Baños et al., 2016; Caly et al., 2017) and antagonistic activities against *Escherichia coli, vibrio cholera, Pseudomonas aeruginosa,* fungi and viruses (Braïek et al., 2018; Braïek and Smaoui, 2019; Kasimin et al., 2022).

Some scientists are considering bacteriocins as an alternative to drugs in the fight against emerging antimicrobial resistance (Pircalabioru et al., 2021)). Enterococcal bacteriocins

are useful as potential drug candidates for the treatment of multi-drug resistant (MDR) pathogens (Pircalabioru et al., 2021)) such as Vancomycin Resistance *Enterococcus* and Methicillin-resistant *Staphylococcus aureus*. Enterocins K1 and EJ97 showed some potential of being used in the Treatment of Vancomycin-Resistant Enterococcal Infections (Reinseth et al., 2020). Enterocin P was found with activity against *L. monocytogenes, S. typhi*, and *S. aureus* (Tanhaeian et al., 2019). In a study conducted by Borrero and others (2015), three bacteriocins exhibited potent antimicrobial activity against *E. faecalis.* These are enterocin A, hiracin JM79 and enterocin P. Bacteriocin production is now being considered as a probiotic trait (Romero-Luna et al., 2022).

2.2.1.3 Probiotic Uses

Probiotics are defined as live microorganisms which, when administered in adequate amounts, have beneficial effects on the host (Reid et al., 2019). According to Plaza-Diaz and others (2018), a probiotic should be preferably of human origin, safe, and free of vectors that can transfer resistance to antibiotics and of pathogenicity or toxicity factors and should be able to survive intestinal conditions, demonstrate antagonism against pathogens and stimulate immune response apart from conferring beneficial effects on the host. It should also maintain viability, growth efficacy and activity upon subjection to technological treatment (Plaza-Díaz et al., 2018). Some species and strains of *E. faecalis* and *E. faecium* are used as probiotics because they have demonstrated the above qualities. Health benefits probiotics bestow on the host include prevention and treatment of some diseases such as necrotizing enterocolitis, acute infectious diarrhoea, acute respiratory tract infections, antibiotic-associated diarrhoea, and infant colic (EFSA BIOHAZ Panel, 2023), enhancing intestinal barrier function, anti-inflammatory activity, antimutagenic, anticarcinogenic, hypocholesterolaemic, and immune regulation effects (Latif et al., 2023). They have also been used in different applications including human and veterinary medicine, and pharmaceutical and food industries (Baral et al., 2021). Probiotic strains of *Enterococcus faecium* AL41 have been used to treat infections caused by *Campylobacter jejuni* in chickens (Letnická et al., 2017; Plaza-Díaz et al., 2018).

Some studies show that *E. durans* M4-5 was found to generate butyrate, short-chain fatty acids (SCFAs), which induced significant anti-inflammatory effects and contributed to the

integrity of the intestinal epithelium (Jeevaratnam and Nallala, 2017). Li and others (2018) showed that *Enterococcus durans* KLDS6.0933 had probiotic properties of lowering human serum cholesterol levels. Whole genome sequencing was performed on *Enterococcus durans* KLDS6.0933 to understand the genetic basis of its cholesterol removal ability (Li et al., 2018). An enterocin produced by *Enterococcus faecium* T1 was observed to inhibit the growth of numerous Gram-negative and Gram-positive bacteria including *Pseudomonas putida*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Escherichia coli*, *Salmonella typhimurium*, *Shigella flexneri*, *Shigella sonnei*, *Staphylococcus aureus* and *Listeria monocytogenes* (Liu et al., 2018). The strain *E. durans* LAB18s was recommended for use as a source of dietary selenium supplementation (Pieniz et al., 2017).

The European Food Standards Agency (EFSA) authorised certain strains of enterococci for use as silage additives and dietary supplements to stabilise microbial communities of the digestive tract in both monogastric and ruminant animals (Braïek and Smaoui, 2019). For example, strains of *E. faecium* NCIMB 11181 and *E. faecium* DSM 7134 were approved as feed additives for calves and piglets. *E. faecium* SF68® and *E. faecalis* Symbioflor 1 are used either to prevent or treat diarrhoea in pigs, poultry, livestock, and pets (Zommiti et al., 2022).

2.2.1.4 Microbial indicator of faecal water contamination

Faecal indicator bacteria (FIB) are bacteria that can be used in assessing the safety of drinking water (Schneeberger et al., 2015; Baia et al., 2022). They can be included in a drinking water monitoring program to provide information on the quality of the source water, the adequacy of treatment, post-treatment ingress into the distribution system and the delivery of safe drinking water to the consumer (Wei et al., 2017; Holcomb and Stewart, 2020). Enterococci are widely used as tools for assessing water quality in many parts of the world as they are relatively abundant in human and animal faeces, ease of culture, and have correlated with human health outcomes in fresh and marine waters. Though *E. coli* is a good indicator of faecal contamination, it has limitations. *E. coli* is more sensitive to environmental stresses and disinfection than enteric viruses, protozoa and some bacterial pathogens. Enterococci have shown that they are also better able than

coliforms to survive unfavourable conditions in aquatic environments, disinfectants commonly used in drinking water such as chlorine and monochloramine, and ultraviolet (UV) light (Owoseni and Okoh, 2017). This is why they are preferentially used as FIB for recreational water, drinking water and water re-use applications (Baia et al., 2022).

In some water environments, enterococci can persist longer and can be carried further than *E. coli*. Thus, enterococci may indicate faecal contamination in water or water quality deficiencies likely not captured by traditional faecal indicator monitoring that might otherwise be missed. The attribute of reliability makes enterococci the preferred FIB for the detection of faecal contamination of fresh water and recreational water (Yuan, 2016).

Enterococci can be used as a verification indicator to provide more useful information on the quality of the drinking water source, the adequacy of drinking water treatment and the microbial condition of the distribution system (Schneeberger et al., 2015; Wei et al., 2017; Baia et al., 2022). They can also be tested routinely to provide complementally information and can be used during an audit of a drinking water system in order to gain more information about the microbiological quality in the system and detect potential vulnerabilities in the system (Fout et al., 2017; Holcomb and Stewart, 2020).

Research suggests monitoring enterococci for untreated groundwater wells and in drinking water distribution systems as enterococci can survive longer than *E. coli* in some contaminated groundwater supplies (Schneeberger et al., 2015). For surface water sources, however, research suggests the use of *E. coli* as an indicator of faecal contamination or pathogen presence as enterococci are not more sensitive than *E. coli* (Motlagh and Yang, 2019).

The presence of *Enterococcus* in untreated groundwater systems can indicate the potential vulnerability of the source water (Wei et al., 2017; Baia et al., 2022). Where drinking water disinfection is inadequate, *Enterococcus* may persist longer than *E. coli* (da Silva et al., 2020) . The presence of enterococci in the distribution system may indicate failure of the treatment barrier that is in place or point to a potential pathway for entry of faecal contaminants. Use of *Enterococcus* in verification of the effectiveness of system barriers

in place and detection of water quality deficiencies which may go undetected if only *E. coli* is used as the indicator of faecal contamination (Fout et al., 2017).

Enterococci can persist in favourable habitats such as soils, especially tropical soils (Devane et al., 2020), sand, and masses of aquatic vegetative material that provide nutrients and protection from environmental stresses (Hassard et al., 2016). For instance, Enterococci survived for more than 6 months in sun-dried algal mats which were subsequently stored at 4°C (Whitman et al., 2003).

Enterococcus is also regarded as being more resistant to environmental stresses, like increased salinity or desiccation, than *E. coli* (Perera et al., 2020). This is attributable in part to their thicker Gram-positive cell wall (Mai-Prochnow et al., 2016). Some studies have reported enterococci as being capable of surviving longer than *E. coli* in marine waters (Wang et al., 2018; Sagarduy et al., 2019).

When enterococci undergo stress in the water environment, they can enter a viable but non-culturable (VBNC) state (Hassard et al., 2016). This is the state in which they do not grow on laboratory media but are live and capable of resuscitation when conditions become favourable (Zhang et al., 2021). This is a primary survival strategy for bacteria (Pazos-Rojas et al., 2024).

The persistence of *Enterococcus* possessing multiple virulence and antimicrobial-resistance determinants can pose significant public health concerns. The presence of antibiotic-resistant genes (ARGs) and ARB in the environment can exacerbate the risk of spreading antibiotic resistance and this can lead to a situation where antibiotics become less effective against infections (Rahman et al., 2023)). Horizontal gene transfer plays a significant role in the dissemination of ARGs and can make the issue of antibiotic b resistance pollution more severe (Michaelis and Grohmann, 2023). Antibiotic resistance is a major threat to global public health and food safety. The spread of resistance in the environment has the potential to proliferate more ARB, which can then transfer to humans and other organisms (Michaelis and Grohmann, 2023). The persistence of ARBs harbouring virulence determinants can generally lead to problems in healthcare and food

processing, water distribution systems, packaging, industrial manufacturing, marine industries, and sanitation. (Shineh et al., 2023).

2.2.2 Harmful Roles of *Enterococcus*

Although enterococci have a lot of beneficial attributes, some *Enterococcus* species have however been implicated as the causative agents of a variety of infections such as endocarditis, urinary, bacteraemia, central nervous system infections, and abdominal and pelvic infections as well as nosocomial infections (Braïek and Smaoui, 2019; Schneeberger et al., 2015). The importance of enterococci as significant multidrug-resistant pathogens especially in hospital-acquired infections is facilitated by their ability to acquire and donate mobile genetic elements (MGEs) and their intrinsic resistance to antibiotics and disinfectants due to a hardened cell wall (Lebreton et al., 2017). Lebreton and others (2017) hypothesized that the hardened cell wall of *Enterococci* was a consequence of natural selection in response to environmental stresses such as starvation and desiccation which allow them to adapt. Most *Enterococcus* species especially those from clinical environments exhibit several virulence and resistance genes and have been recovered from various habitats in different parts of the world (Foka et al., 2019; Gouliouris et al., 2019). Enterococci have emerged as an important cause of multi-drug resistance (MDR) and hospital-acquired infections (HAI) due to the harmful traits they exhibit. It is in this vein that enterococci is neither generally regarded as safe nor is it on the list of qualified assumption of safety organisms (Hanchi et al., 2018).

2.2.2.1 Virulence genes

According to Braïek and Smaoui, (2019) a virulence factor (also called virulence determinant or virulence gene) is an effector molecule that enhances the capacity of a microorganism to cause illness. They play an important role in the pathogenicity of an organism. *Enterococcus* species are endowed with various virulence genes, which help them to colonize and cause infection as well as evade the host's defences. These include collagen-binding cell wall protein (*ace*), aggregation substance (*asa1*), Pheromone cAD1 precursor lipoprotein (*cad1*), *cytolysin* (*cylA*), *enterococcal surface protein* (*esp*), *E.*

faecalis endocarditis antigen Regulator of *gelE* and *sprE* expression, (*fsr*), *gelatinase* (*gelE*), and *E. faecium* specific cell wall adhesin (*efm*) (Song et al., 2019).

Collagen-binding cell wall protein or Adhesin to Collagen from *Enterococcus faecalis* (*ace*) is a protein that plays a crucial role in the pathogenesis of enterococcal infections (Hashem et al., 2021). It belongs to the family of Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs). *ace* binds specifically to collagen, which is an abundant structural protein in host tissues ((Marques et al., 2023). This interaction allows *Enterococcus* to attach to host cells. The initial attachment is a critical step in colonization and subsequent infection (Sharma et al., 2020).

Aggregation substances (*agg* and *asa1*) are pheromone-inducible surface proteins that promote aggregate formation during bacterial conjugation and mediate the specific binding to epithelial cells for colonization and also the interchange of plasmids which may carry virulence traits and antibiotic resistance genes (Chajęcka-Wierzchowska et al., 2017). The *agg* gene increases the hydrophobicity of the enterococcal surface inducing localization of cholesterol to phagosomes resulting in delayed or prevention of fusion with lysosomal vesicles (Lee et al., 2023). The *asa1* enables the conjugative transfer of sex pheromone gene-containing plasmids through the clumping of one *Enterococcus* to another (Zou et al., 2020) thereby helping in mating and conjugation which results in the accumulation of bacteria at the site of infection (Ferguson et al., 2016).

Pheromone cAD1 precursor lipoprotein (*cad1*) is a virulence gene associated with *Enterococcus.* The *cad1* gene encodes a protein known as the pheromone cAD1 precursor lipoprotein. Unlike other virulence factors, *cad1* is not directly related to bacterial pathogenicity or virulence (Iweriebor et al., 2015), but it's involved in cell-cell communication within bacterial populations (Oli et al., 2022). It produces a pheromone (cAD1) that serves as a signalling molecule. The cAD1 pheromone facilitates sexual aggregation (clumping) of bacterial cells. During aggregation, plasmids (small DNA fragments) are exchanged between cells. Through direct contact plasmids that carry antibiotic-resistant genes can be transferred and thus can lead to the spread of antibiotic resistance genes among bacteria (Iweriebor et al., 2015).

Cytolysin (*cylA*) is an exotoxin that is produced by some strains of *E. faecalis.* The operon that encodes *cytolysin* is either carried on a plasmid or integrated into the bacterial chromosome. It is conveyed on a pathogenicity island and regulated by a process called "tele sensing" (Wu et al., 2021). This enables the bacteria to detect the presence of target cells in the vicinity and respond by producing more toxins. *Cytolysin* acts as a bacteriocin as well as a *β*-haemolysin. As a *β*-haemolysin, it constitutes a peptidic toxin which lyses cells by forming pores in the cytoplasmic membrane of target bacterial cells (Teixeira et al., 2015) and eukaryotic cells in response to quorum signals (Fiore et al., 2019). As a bacteriocin, it acts against Gram-positive bacteria (Stępień-Pyśniak et al., 2019) and some Gram-negative bacteria. Studies have shown that bacteriocins-producing *Enterococcus* has bactericidal effect on other bacteria (Vimont et al., 2017; Abanoz and Kunduhoglu, 2018; Almeida-Santos et al., 2021). According to Braïek and Smaoui (2019), the frequency of death caused by infection due to a cytolysin-producing *Enterococcus* is five times higher than that observed in a noncytolysin-producing enterococcal infection.

Enterococcal surface protein (*esp*) is a surface protein encoded on a pathogenicity island in both *E. faecalis* and *E. faecium* (Goh et al., 2017). It is found at high frequency in isolates recovered from infections. The *esp* is important in host colonization (Zou and Shankar, 2016). It is associated with biofilm formation and strengthens enterococcal biofilms thereby enhancing the pathogenic potential of *Enterococcus* (Spiegelman et al., 2022). *ESP* helps with evasion of the immune response of the host (Daniel et al., 2017).

E. faecalis endocarditis antigen regulatory system (*fsr*) plays a significant role in the pathogenesis of *E. faecalis* infections, particularly in endocarditis. The fsr system regulates the expression of two proteases namely gelatinase (*gel*E) and serine protease (*sprE*) (Marques et al., 2023). These proteases are involved in controlling various virulence factors, including biofilm formation and *gelE* production (Hashem et al., 2021). Enterococcal endocarditis is a serious clinical manifestation characterized by bacterial biofilm-like vegetations on the aortic valve and adjacent areas within the heart (Nappi, 2024). The *fsr* system contributes to the formation of vegetation. Understanding its role helps researchers explore potential therapeutic targets for managing these infections.

Gelatinase (*gelE*) is an extracellular Zinc-metallo-endopeptidase involved in the hydrolysis of gelatin, collagen, β-insulin, haemoglobin, casein, and other bioactive peptides (Too and Masila, 2024). *Gelatinase* cleaves fibrin thereby damaging host tissue and enhancing the spread of bacteria to other parts of the host tissues (Too and Masila, 2024). This protease promotes the aggregation of cells into microcolonies which is the first step in biofilm formation (Too and Masila, 2024). In this way, it plays an important role in the formation of biofilms. Biofilms allow enterococci to colonise tissues and persist in the site of infection (Schulze et al., 2021). Gelatinase promotes inflammation by hydrolysing haemoglobin and other peptides, and the sex pheromones transfer plasmids which carry antibiotic-resistant genes (García-Solache and Rice, 2019).

The *E. faecium*-specific cell wall adhesin (*efm*) is a protein located on the cell surface of *E. faecium* and it plays a significant role in the pathogenesis of *E. faecium* infections. It facilitates the adhesion of the bacterium to various host surfaces, including mucosal barriers in the gastrointestinal (GI) tract. The mucosal barrier prevents adhesion and invasion by (pathogenic) bacteria. *E. faecium*-specific cell wall adhesin allows *E. faecium* to overcome this barrier and adhere to the GI tract (Fiore et al., 2019). By adhering to these surfaces, *E. faecium* can establish colonization and potentially cause infections. Additionally, it may help the bacterium evade host defence mechanisms. Understanding the role of *efm* is essential for managing infections caused by *E. faecium* and developing targeted interventions (Fiore et al., 2019).

2.2.2.2 Antimicrobial Resistance

Despite constituting approximately 1% of the total human intestinal microbe population (Tedim et al., 2015; Fiore et al., 2019), enterococci have earned recognition as pathogens due to their resistance to numerous antibiotics and possession of virulence determinants. Human activities such as urbanization, domestication of animals, introduction, and broad application of antibiotics in health care and animal husbandry have selected for drug resistance and the merging of traits in some *Enterococcus* species, especially *Enterococcus faecalis* and *E. faecium* (García-Solache and Rice, 2019). Antimicrobial resistance genes play an important role in the pathogenicity of *Enterococcus* strains (Golob et al., 2019).

There are three main reasons why Enterococci have emerged to be multidrug resistant. These are intrinsic resistance to antimicrobial agents like beta-lactams and aminoglycosides and acquired resistance through mobile genetic elements like transposon and plasmids against glycopeptides, quinolones, tetracyclines, macrolides and streptogramin or through the horizontal transfer of resistance genes (Von Wintersdorff et al., 2016). Studies have shown that resistance to aminoglycosides, beta-lactamase-induced ampicillin resistance and glycopeptides are the most significant resistance observed in *Enterococcus* (Arzanlou et al., 2020; Kakoullis et al., 2021). Resistance to high-level beta-lactams is due to mutation or overproduction of penicillin binding protein 5 (PBP5) (Hunashal et al., 2023; Sethuvel et al., 2023). Resistance to low-level aminoglycosides is due to reduced uptake (Wistrand-Yuen et al., 2018). Acquired resistance is because of the acquisition of foreign DNA or mutation in the existing DNA (Larsson and Flach, 2022). High-level aminoglycoside resistance is believed to come about by ribosomal mutation or acquisition of aminoglycoside modifying enzyme via plasmid (Doi et al., 2016; Yang and Hu, 2022; Zhang et al., 2023). Resistance to chloramphenicol is enzymatic or plasmid-borne while resistance to high-level erythromycin is due to transposon encoding macrolide resistance (Algarni et al., 2022; Manyahi et al., 2023). Resistance to glycopeptides can either be plasmid-borne or chromosomal and is due to the absence of a cluster of genes in Enterococcus (Wistrand-Yuen et al., 2018).

Moulded by the competitive dynamics of their surroundings, enterococci have undergone a variety of adaptations and genetic flexibility, equipping them to prosper in contemporary healthcare environments. (Farsi et al., 2023). Progress in contemporary healthcare has enhanced capacity to prolong the lives of severely ill individuals, which, in turn, has raised the likelihood of infections and led to a swift turnover of patients, accompanied by the extensive utilization of antibiotics (Muteeb et al., 2023). With a repertoire of various antibiotic resistance factors, enterococci seize this opportunity to thrive within their ecological habitat, namely the gastrointestinal tract of hospitalized patients, enabling them to gain a competitive edge and establish dominance in the intestinal microbiota (Hassan et al., 2018). Multidrug-resistant (MDR) Enterococci swiftly spread throughout the hospital environment originating from the gastrointestinal (GI) tract mainly through contaminated hands, clothing and hospital utensils (Ramos et al., 2020).

Multidrug resistance strains are not only of public health importance but also of major concern worldwide because of the reduced treatment options for illnesses they cause (Daniel et al., 2015) and the possibility of being reservoirs for drug resistance genes (Ahmad et al., 2021). Enterococci may readily transfer drug resistance genes to other bacteria (Conwell et al., 2017). For example, cases of vancomycin-resistant *Staphylococcus* aureus (VRSA) have been reported (Cong et al., 2020). In that case, *Staphylococcus* acquired the vancomycin resistance gene (*vanA*) from vancomycin resistance *Enterococcus* (VRE) which colonized the same *Staphylococcus* infected patient (Cong et al., 2020). This signifies the importance of drug resistance *Enterococcus* even when the species are considered non-pathogenic and shows that resistance in one species can affect the evolution of resistance in another pathogenic or non-pathogenic species. It also shows that failure to control one species can negatively affect patient outcomes in those infected with another bacterium. It is therefore important to determine virulence and resistance genes carried by *Enterococcus* isolated from poultry and humans.

2.2.2.3 Resistance to disinfectants

Disinfectants are extensively used in healthcare systems and households to comply with health standards, as well as in food processing and agricultural industries to meet quality and they are also part of pharmaceutical products and cosmetics ((Kim et al., 2018). It is therefore startling that resistance to disinfectants is emerging at a fast rate (Wassenaar et al., 2015). It is believed that a lack of understanding of the principles behind biosecurity and misuse of disinfectants are incentives behind the abrupt advent of disinfectant resistance (Roca et al., 2015).

Use of biosecurity and disinfectants is considered the only feasible way of preventing unwanted bacterial growth (Bragg et al., 2018). Biosecurity measures are meant to protect animals and humans against harmful biological or biochemical substances. Extensive use of disinfectants has proved efficacious in the prevention and or reduction of infections (Alajlan et al., 2022). It is in this vein that failure in biosecurity is life-threatening to both humans and animals. Some studies have shown an association between antibiotic and disinfectant resistance in some bacteria (Khan et al., 2016) while others show that the use of benzalkonium chloride (BC) disinfectants promote antibiotic resistance by co-selection

(Kim et al., 2018). A study conducted by Sobhanipoor and others (2021) showed a correlation between reduced susceptibility to chlorhexidine digluconate (CHX) and high-level resistance to gentamicin showing that exposure to disinfectants can promote antibiotic resistance with resultant MDR bacteria (Sobhanipoor et al., 2021). A study by Wieland and others, (2017) indicated that residual disinfectant might select antimicrobial-resistant enterococci. In 2020, Wang and colleagues. carried out a study to determine the effects of ultraviolet disinfection on vancomycin-resistant *Enterococcus faecalis*. The findings indicated that ultraviolet disinfection did not have a notable impact on the vancomycin resistance of *E. faecalis.*

2.3 Clinical Significance of *Enterococcus*

Several studies have shown that *Enterococcus* colonize human intestines and cause various diseases including urinary tract infections (UTIs) and liver abscesses in humans (Mücke et al., 2017) and locomotive disorders and septicaemia in poultry (Dolka et al., 2017). Clonal complexes of *Enterococcus* harbour mobile genetic elements that are associated with resistance and virulence genes. An instance of this is the role played by the transposon Tn1546 in spreading vancomycin resistance (specifically, vanB1 determinant) among *Enterococcus* populations globally. This transposon often coexisted with plasmids that could transfer between bacteria by conjugation (Partridge et al., 2018). Such incidences result in emergence of resistance and resultant nosocomial infections, which are difficult to treat (Buultjens et al., 2017; Dai et al., 2018).

The clinical relevance of *Enterococcus* has been intensified by the emergency of high-risk enterococcal clonal complex (HiRECC) which is associated with the hospital environment and is involved in nosocomial infections leading to increased morbidity, mortality and cost (Brinkwirth et al., 2021). This impacts negatively on the global economy by increasing expenditure associated with increased duration of hospitalization (Serra-Burriel et al., 2020). Also, not to be underestimated is the disability-adjusted life years (DALYs) due to illness caused by nosocomial infections (Mitkova et al., 2022). The morbidity of a population entails significant government expenditure on the provision of medical care and medicines to citizens. This affects the dynamics of budget expenditure

especially if the government must provide financial support to citizens during their disability (Khavjou et al., 2020).

2.4 Reservoirs of *Enterococcus*

Most *Enterococcus* species are normal flora of the gastrointestinal tracts of animals ranging from birds to humans and they are also found in a variety of habitats (Marques et al., 2023). They can endure a broad range of harsh environmental conditions for prolonged periods (García-Solache and Rice, 2019). Such conditions include high and low temperatures ranging from 10°C to 45°C and can withstand 60°C for 30 minutes (Gaca and Lemos, 2019; Zhu et al., 2022). They can grow in an environment with high salt (NaCl), concentration of 6.5% (Marques et al., 2023). They hydrolyse esculin in the presence of 40% bile salts. They also grow in acidic as well as alkaline pH (-6.9). They withstand lethal levels of sodium dodecyl sulphate, sodium hypochlorite, hyperosmolarity, ethanol, and hydrogen peroxide (Meena et al., 2020). This adaptability accounts for their wide distribution in nature.

2.4.1 Human Reservoirs

For many years, *Enterococcus* was considered commensals of the gastrointestinal tract and opportunistic pathogen because it caused infection in immune-compromised patients such as HIV/AIDS patients, the elderly, solid organ and bone marrow transplant patients, cancer patients and patients with prolonged stay in the hospital (Ramos et al., 2020). A severely weakened immunity is unable to keep commensal bacteria in check. Modern medicine has created circumstances that facilitate the pathogenic behaviour of enterococci through the overuse of antibiotics. Enterococci are good at acquiring and exchanging antibiotic resistance determinants and are naturally resistant to many antibiotics. These properties give enterococci a selective advantage in environments with heavy antibiotic usage, like the hospital, and may allow them to out-compete other species. Exposure of commensal enterococci during antibiotic treatment of infectious disease caused by other bacteria select for resistance.

Commensal enterococci become pathogens by acquiring antibiotic resistance and virulence genes from bacteria of the same or different species (Golob et al., 2019). The

ability of enterococci to survive and cause infection in a host is additionally facilitated by the possession of virulence genes. *Enterococcus* species harbour virulent genes and it is usually those isolated from patients which exhibit more virulent genes than those isolated from environmental sources (Fiore et al., 2019). A study by Ruiz-Garbajosa and colleagues (2006) showed that virulence genes (*ace*, *agg*, *cylA*, *esp* and *gelE*) were more common among *Enterococcus* strains isolated from intensive care units (ICUs) than among isolates from people in the community. Isolates from ICU harboured *gelE* and *cylA* more frequently than isolates from patients in the community (Ruiz-Garbajosa et al., 2006).

Certain strains of *E. faecalis* and *E. faecium* are primarily responsible for a variety of infections. These include endocarditis, urinary tract infections (UTIs), mastitis, bacteraemia, bloodstream infections (BSIs), particularly those related to vascular catheters, and infections in the abdomen and pelvis. Additionally, infections of the skin and soft tissues, infections in the joints and bones, infections in the central nervous system (CNS), lung infections, wound infections, and hospital-acquired outbreaks are also caused by some strains of *E. faecalis* and *E. faecium* (Pillay et al., 2018; Morgan et al., 2024).

2.4.2 Non-Human Reservoirs

Enterococcus has been isolated from reservoirs other than the human gut. They have been recovered from a variety of habitats such as poultry, insects, animals like cats, dogs, cattle, pigs, and horses; soil, plants and various other environments (Braïek and Smaoui, 2019).

2.4.2.1 *Enterococcus* in Poultry

Wild and domesticated birds including poultry are part of normal habitats of enterococci. Like in animals and humans, *Enterococcus* inhabit the gut of poultry. Antimicrobial use in animal production has been linked to the development and spread of resistant bacteria. In poultry production antibiotics are used for prevention and treatment of infections and as Antimicrobial Growth Promoters (AMGPs) (Agyare et al., 2019). This is a cause for concern especially in commercial poultry farms where the use of antimicrobials is massive. Massive use of antimicrobials provides substantial selection pressure that contributes to the spread of clones and the acquisition of resistance determinants from

resistant bacteria. For example, some species of *Enterococcus* such as *E. cecorum* which was identified as a commensal have become a significant cause of morbidity and mortality in poultry (Dolka et al., 2017; Jung et al., 2018). *Enterococcus* species has been implicated in septicaemia, endocarditis, and other diseases (Souillard et al., 2022). *E. cecorum* causes spondylitis and osteomyelitis in poultry (Dolka et al., 2017; Jung et al., 2018). MDR strains of *Enterococcus* have been recovered from poultry (Bertelloni et al., 2015; Ribeiro et al., 2023; Cagnoli et al., 2024).

Vancomycin-resistant *Enterococcus* has also been isolated from poultry. For example, Maia and others (2020) isolated Vancomycin-Resistant *Enterococcus* from raw chicken meat, raw pork, and boiled meats in Brazil. In a study carried out by (Semedo-Lemsaddek et al., 2021) to evaluate and compare the antimicrobial resistance profiles and virulence genes of enterococci isolated from Portuguese conventional and free-range broiler farms, the majority of the enterococcal isolates were resistant to more than one antibiotic. Virulence factors also have been detected in poultry. For example, between August and September 2020, Isichei-Ukah and others detected virulence genes in *Enterococcus* isolated from feed, water, and faecal samples from selected poultry farms in different commercial farming areas of Benin City in Nigeria (Isichei-Ukah et al., 2024). The virulence gene, *gelE* was detected from broiler chickens in Portugal (Semedo-Lemsaddek et al., 2021). Because of the ability of *Enterococcus* to disseminate virulence and drug resistance determinants, it is probable that the resistant strains of *Enterococcus* found in poultry may be transmitted to humans and other bacteria and hence can be a health hazard (Bortolaia and Guardabassi, 2023). It was therefore important to carry out this study to determine the zoonotic potential of *Enterococcus* inhabiting poultry.

2.4.2.2 *Enterococcus* in Other Environments

Other environments where *Enterococcus* has been detected include natural waters such as rivers, ponds, lakes and oceans (Rodrigues and Cunha, 2017; Adegoke et al., 2022), groundwater such as wells and aquifers (Naphtali et al., 2019), recreational waters like pools, beaches and water parks (Adeniji et al., 2019), and wastewater such as sewerage and treated wastewater effluents (Iweriebor et al., 2015; Adegoke et al., 2022; Gotkowska-Płachta and Gołaś, 2023). Monitoring their presence in water bodies is important for

assessing water quality and the potential risk of contamination. They are also important for detecting the efficiency of the water treatment processes that are in place (Gotkowska-Płachta and Gołaś, 2023).

Enterococcus has also been recovered from soil, especially in areas contaminated with faecal matter or where there is agricultural runoff (Cortez et al., 2023). In soil, *Enterococcus* play a role in decomposition processes and nutrient cycling (Aguilar-Paredes et al., 2023; Jangid and Dalal, 2023). They are often used as indicators of soil quality and pollution (Aguilar-Paredes et al., 2023). *Enterococcus* is commonly found in the environments where animals and livestock are raised (Marques et al., 2023) as well as in households with pets, such as dogs and cats (Stępień-Pyśniak et al., 2016; Yuan et al., 2023). *Enterococcus* species are notorious for their ability to survive in hospital environments and can be responsible for healthcare-associated infections (Ahmed and Baptiste, 2018; Marques et al., 2023). They are often found on medical equipment and surfaces (Ssekitoleko et al., 2020). They also persist in hospital environments where they contaminate utensils, equipment, gowns and hands of other hospital workers (Mitchell et al., 2015). *Enterococcus* can be found on surfaces in various indoor and outdoor environments, contributing to its role as an indicator of cleanliness and hygiene. *Enterococcus* may be detected in environmental monitoring studies, such as those assessing pollution or microbial diversity (Gotkowska-Płachta and Gołaś, 2023).

Enterococci can be found in various food products, including meat, poultry as well as processed and unprocessed foods (Igbinosa and Beshiru, 2019)). They are sometimes used as indicators of food safety and hygiene (Terzić-Vidojević et al., 2021). Some *Enterococcus* species are intentionally used in the fermentation of food products like cheese and yoghurt, so they are detected in these products. They contribute to the development of flavour and texture in these products (Nami et al., 2019; Dapkevicius et al., 2021). *Enterococcus* species have been isolated from various plant surfaces, including leaves, stems, and roots (Grudlewska-Buda et al., 2023). They can act as both plant pathogens (Pandova et al., 2024) and beneficial plant-associated microorganisms ((Hanchi et al., 2018). They have also been isolated from ready-to-eat food like dishes purchased in bars and restaurants in Olsztyn, Poland (Chajęcka-Wierzchowska et al., 2017).

Studies have demonstrated that insects such as house flies and cockroaches are potential vectors for the dissemination of antimicrobial-resistant and potentially virulent *Enterococcus* species (Onwugamba et al., 2018). The potential for transmission of MDR enterococci by animals to humans has long been exhibited by several studies (Ahmed and Baptiste, 2018; Marques et al., 2023). Some studies have demonstrated genetic relatedness of *Enterococcus* isolates from humans with those from animals suggesting that animals can be a source of human infections (Aun et al., 2021; Marques et al., 2023).

2.5 Transmission of *Enterococcus*

Some experiments which have been carried out have shown that transmission of enterococci occurs in many ways (Bhagwat and Annapure, 2019; Cimen et al., 2023). These include transmission through contact, contaminated hands, devices, and objects, foodborne, aerosols and through birth canal (Bhagwat and Annapure, 2019; Cimen et al., 2023). Studies have shown the potential of contaminated eggs to facilitate the rapid spread of *E. faecalis* to nearly all chicks during hatching (Reynolds and Loy, 2020; Karunarathna et al., 2022).

The spread of enterococcal infections from patient to patient is thought to occur through the hands of healthcare workers and that it is the primary mode of transmission (Zhou et al., 2020). Several studies have reported transient carriage of *Enterococcus* on the hands of healthcare workers (Montoya et al., 2019; Vasilakopoulou et al., 2020). In situations where decontamination is irregular, *Enterococcus* can persist for 4 months on inanimate surfaces where they can become a source of infection (Porter et al., 2024). Interhospital transmission of *Enterococcus* was investigated by Saito and others in 2022. Thirty-seven hospitals participated in the study while thirteen hospitals participated in the screening tests for vancomycin-resistant *Enterococcus faecium.* This study was carried out in Aomori prefecture, Japan and a total number of 500 cases of vancomycin-resistant *Enterococcus faecium* were obtained. PFGE and whole genome sequencing showed that the multi-jurisdictional outbreaks were caused by *vanA*-type vancomycin-resistant *Enterococcus faecium* ST1421 (Saito et al., 2022). This interhospital transmission could have been necessitated by the transfer of patients from one hospital to another.

Detection of the same variants of the *vanA* gene cluster (Tn *1546)* encoding vancomycin resistance in *Enterococcus* of both human and animal origin could indicate horizontal transfer of Tn*1546* between *Enterococcus* species of different origin (Ahmed and Baptiste, 2018). *E. faecium* isolates of animal origin in themselves may not constitute a human hazard, but they could disseminate antimicrobial resistance genes to other pathogenic *Enterococcus* species. *Enterococcus faecalis* of animal origin appears to be a human threat because the same types could be detected in *E. faecalis* from animals, food, humans in the community, and patients.

Certain research has suggested that the utilization of avoparcin in farm animals can lead to the selection of vancomycin-resistant *E. faecium*, even when vancomycin has never been used for animal treatment (Simm et al., 2019; Ramos et al., 2020; Zhou et al., 2020). Further investigations have revealed that *Enterococcus* strains present in both animal and human populations possess identical genetic lineages, highlighting the unhindered transmission of resistance genes among various *Enterococcus* species across different reservoirs (Cattoir, 2022). This finding is significant because it suggests that antimicrobial-resistant *Enterococcus* species in animals could pose a potential threat to human health. The use of antimicrobial treatments in the modern agriculture business has resulted in a reservoir of resistant bacteria in food animals that can be transmitted to people via contaminated food or the environment (Manyi-Loh et al., 2018).

Objects, such as phones, doorknobs, towels, and soaps can transmit *Enterococcus* from one person to another if contaminated (Mwamungule et al., 2015; Edi et al., 2023). Contamination takes place when a patient with enterococcal infection touches the anal area and without washing hands touches an object, phone, towel or through a handshake with another patient or health worker or by touching the clothing of a patient (Mwamungule et al., 2015; Edi et al., 2023). Inadequate hand washing can increase the risk of spreading infection, especially in hospitals. Insufficient cleaning and disinfection of tools used in healthcare facilities such as catheters and dialysis ports can increase the risk of transmitting *Enterococcus* infections (Costa et al., 2021).

Bortolaia and Guardabassi (2015) asserted that foodborne transmission is higher for poultry meat than for other food products of animal origin possibly because of lower hygiene and high risk of carcass contamination associated with poultry slaughtering. Antimicrobial-resistant *Enterococcus* in animals can serve as reservoirs from which resistance genes can be transferred to *Enterococcus* species or other bacteria in humans (Zaheer et al., 2020). In that case transmission can either be through human consumption of food of animal origin, by direct contact between animals and humans, or via the environment (Zaheer et al., 2020; Bortolaia and Guardabassi, 2023). The presence of dominant strains of *Enterococcus* in hospitals likely indicates adaptation to the hospital environment and this may be associated with factors in the environment, in the patient, or in the strain itself e.g., selective pressure from antimicrobial use, immune status of the patient, or virulence of the strain, respectively.

In a study carried out by Bhagwat and Annapure (2019) in which they compared cesarean delivered (c-section) with vaginally delivered neonates, it was found that vaginally delivered neonates had lactic acid bacteria while those delivered by c-section had no bacteria. Further analysis revealed several *Enterococcus* species including *E. canintestini*, *E. rivorum* and *E. dispar* which were transmitted from mother to infant born through vaginal delivery.

2.6 Diagnosis of *Enterococcus*

Routine diagnosis of *Enterococcus* is made by isolation of organisms through culture of various specimens on enrichment, general, and selective media (García-Solache and Rice, 2019). Depending on the protocol, this may be followed by conventional biochemical tests and then molecular techniques (Weterings et al., 2021).

2.6.1 Conventional Methods

Conventional methods employed in the identification of *Enterococcus* include phenotypic methods (such as culture), the Analytical Profile Index and MALDI-TOF Mass Spectrometry.

2.6.1.1 Phenotypic Methods

In most clinical microbiological laboratories, the primary method of identifying *Enterococcus* species is based on phenotypic characterization. However, most studies have shown the identification of *Enterococcus* by phenotypic methods to be very challenging as it can take several days to accomplish mainly because of the phenotypic and biochemical similarities between many *Enterococcus* species (Kim et al., 2023). For example, *E. faecium* cannot be differentiated from other *Enterococcus* species based on the appearance of colonies because they look alike. Pigmentation is not the definitive property of *E. casseliflavus* as some strains of *E. faecalis* produce yellow pigmentation during the day (Maraccini et al., 2012).

2.6.1.2 Analytical Profile Index

The Analytical Profile Index (API) 20 Strep test kits by BioMerieux, France are employed for identifying *Enterococcus* species. Isolates (bacterial strains) are first inoculated onto Blood agar plates. These plates are then incubated for 24 hours at 37°C to allow bacterial growth. Overnight growth colonies from the Blood agar plates are collected and a bacterial suspension is made and adjusted to match the four McFarland standard, which corresponds to a bacterial concentration of 1.2×10^9 CFU/ml (colony-forming units per millilitre). Each bacterial isolate is inoculated into a set of API 20 Strep tests. These tests consist of a panel of biochemical reactions and colour changes. The reactions are specific to various metabolic pathways and help differentiate between different bacterial species. After incubation, the tests produce specific colour changes based on the bacterial metabolic activity. The resulting code number profile is obtained from these colour changes. Each bacterial species exhibits a unique profile. The code number profile is evaluated using the API Analytic Profile Index system on the website. This evaluation involves comparing the profile to a reference database. The system assigns a code or identification based on the match with known species. The assigned code is used to identify the bacterial species.

The API web or other reference sources can be consulted to determine the species name.

The API 20 Strep test kits, along with the McFarland standard and biochemical reactions, allow the identification of *Enterococcus* species. This method is widely used in clinical microbiology laboratories.

The API20 Strep method for bacterial identification has both advantages and limitations when compared to other identification methods. API20 Strep provides relatively quick results (within hours) compared to traditional culture-based methods that may take days. The test involves inoculating bacterial isolates into a set of biochemical reactions, making it straightforward to perform. It is cost-effective and widely available in clinical laboratories (Franco-Duarte et al., 2019). Limitations of API20 Strep include 1. False Positives/Negatives: Some strains may exhibit atypical reactions, leading to incorrect identification. 2. Database Dependency: Accuracy depends on the quality and comprehensiveness of the reference database. 3. Biochemical Variability: Bacterial strains within the same species can exhibit biochemical variability, affecting test results.

2.6.1.3 MALDI-TOF Mass Spectrometry

Mass spectrometry (MS) is a versatile analytical method used in various scientific fields. In MALDI-TOF Mass Spectrometry, a laser ionizes the test sample (usually proteins or peptides) by striking it with a matrix (material Matrix-Assisted Laser Desorption/Ionization (MALDI)), ions generated are accelerated and separated based on their mass-to-charge ratio (m/z) and the time taken for ions to reach the detector is measured (Time-of-Flight (TOF)), allowing determination of their mass (Stępień-Pyśniak et al., 2017). The resulting spectrum provides information about the masses of ions present in the sample. Advantages of MALDI-TOF MS include direct detection of high-molecular-weight molecules, including proteins, rapid and accurate identification of bacterial species, allows comparison with existing databases, and aids in identification to species level as different species exhibit distinct protein profiles.

MALDI-TOF MS is a valuable tool for identifying bacterial species, including those within the genus *Enterococcus*. Its ability to directly analyse cellular proteins and create strain-specific databases enhances understanding of microbial diversity and assists in clinical diagnostics.

2.6.2 Molecular Techniques

Molecular techniques have been developed to counter the drawbacks of phenotypic methods. Molecular methods based on the amplification of target genes are particularly useful as they increase the sensitivity of the methodology and are useful in identifying target molecules present in low concentrations. Molecular methods based on target gene amplification using specific primers have been successfully used to identify enterococci at the species level (Yousefi et al., 2019; Kim et al., 2022). These techniques are more accurate because they minimize false positive test results by targeting the specific molecule of interest and results can be generated in a relatively shorter time. Molecular methodologies have broader applications such as infectious diseases, genetic testing, forensics, drug resistance, and tumour marker detection and monitoring. Molecular methods have also been used to detect genes responsible for antimicrobial resistance (Saedi et al., 2020). Infection control and epidemiological studies primarily require rapid and simple means of identifying and typing clinical isolates. It is against this background that a variety of approaches have been developed.

2.6.2.1 Pulse Field Gel Electrophoresis

Pulse Field Gel Electrophoresis (PFGE) is a technique used to separate large DNA fragments inside agarose gels by exposing them to an oscillating electric field, allowing fragment lengths to be measured. Furthermore, by comparing restriction endonuclease patterns, PFGE can be used to determine the genetic relatedness of *Enterococcus* species. Because of the DNA degradation that occurs during the operation, this approach is unsuitable for typing several strains (Tabit, 2016).

2.6.2.2 Multilocus Sequence Typing

Multilocus sequence typing (MLST), on the other hand, employs housekeeping genes, typically seven, and is a technique used to analyse several loci. The seven house-keeping genes utilized by (Ruiz-Garbajosa et al., 2006) are *glucose-6-phosphate dehydrogenase* (*gdh*), *glyceraldehyde-3-phosphate dehydrogenase* (*gyd*), *phosphate ATP binding cassette transporter* (*pstS*), *putative glucokinase* (*gki*), *shikimate 5-dehydrogenase* (*aroE*), *shikimate 5-dehydrogenase* (*xpt*), and *acetyl-coenzyme A acetyltransferase* (*yqiL*). Table 2.1 shows this.

Table 2.1 Sets of Primers for MLST used by Ruiz-Garbajosa and others (2006)

HOUSEKEEPING GENES			
Gene (locus)	Primer Name	PRIMER SEQUENCE 5'-3'	Amplicon size bp
gdh	*gdh1*	GGCGCACTAAAAGATATGGT	530
(ef1004)	*gdh2*	CCAAGATTGGGCAACTTCGTCCCA	
gyd	*gyl1*	CAAACTGCTTAGCTCCAATGGC	395
(ef1964)	*gyl2*	CATTTCGTTGTCATACCAAGC	
pstS	*pstS1*	CGGAACAGGACTTTCGC	583
(ef1705)	*pstS2*	ATTTACATCACGTTCTACTTGC	
gki	*gki1*	GATTTTGTGGGAATTGGTATGG	438
(ef2788)	*gki2*	ACCATTAAAGCAAAATGATCGC	
aroE	*aroE1*	TGGAAAACTTTACGGAGACAGC	459
(ef1561)	*aroE2*	GTCCTGTCCATTGTTCAAAAGC	
xpt	*xpt1*	AAAATGATGGCCGTGTATTAGG	456
(ef2365)	*xpt2*	AACGTCACCGTTCCTTCACTTA	
yqiL	*yqiL1*	CAGCTTAAGTCAAGTAAGTGCCG	436
(ef1364)	*yqiL2*	GAATATCCCTTCTGCTTGTGCT	

Other combinations such as *gdh*, *gyd*, *pstS*, *purK*, *ddl*, *atpAn*, and *adkn*) as described by Homan and others (2002) may be used. In their study, Horman and others used the primers shown in Table 2.2;

Table 2.2 Sets of Primers for MLST used by Homan and others (2002)

HOUSEKEEPING GENES			
Gene (locus)	Primer Name	Primer Sequence 5'-3'	Amplicon Size bp
adk	*Adk1*	TAT GAA CCT CAT TTT AAT GGG	437
	Adk2	GTT GAC TGC CAA ACG ATT TT	
atpA	*atpA1*	CGG TTC ATA CGG AAT GGC ACA	556
	atpA2	AAG TTC ACG ATA AGC CAC GG	
ddl	*ddl1*	GAG ACA TTG AAT ATG CCT TAT G	465
	ddl2	AAA AAG AAA TCG CAC CG	
gdh	*gdh1*	GGC GCA CTA AAA GAT ATG GT	530
	gdh2	CCA AGA TTG GGC AAC TTC GTC CCA	
gyd	*gyd1*	CAA ACT GCT TAG CTC CAA GG C,	395
	gyd2,	CAT TTC GTT GTC ATA CCA AGC	
purK	*purK1*	GCA GAT TGG CAC ATT GAA AGT	492
	purK2	TAC ATA AAT CCC CCT GTT TY	
pstS	*pstS1*	TTG AGC CAA GTC GAA GCT GGA G	593
	pstS2	CGT GAT CAC GTT CTA CTT CC	

Multilocus sequence typing is highly unambiguous, reproducible, and scalable. It also provides good discriminatory power to differentiate isolates. However, this method has the drawback of being expensive and sometimes lacks discriminatory power to differentiate as a result it cannot be used for epidemiological investigations (Gits-Muselli et al., 2020). A multi-virulence locus sequence typing (MVLST) technique that targets virulence genes was developed to combat the limitations of MLST. The development of advanced molecular techniques such as DNA sequencing and whole genome sequencing (WGS) has made it easier to detect and determine strains' genomic similarities.

2.6.2.3 DNA sequencing

DNA sequencing is the process of determining the order of nucleotides in DNA. It includes all methods that determine the order of the four bases: adenine, guanine, cytosine, and thymine. The resolution of Whole Genome Sequencing (WGS) exceeds the discriminatory power of PFGE and provides comprehensive and high-resolution information for various purposes such as Surveillance and Outbreak Investigations (WGS

allows precise tracking of bacterial strains during outbreaks. It helps identify the source and transmission routes of infections) Source Attribution (By analysing genomic data, WGS can attribute infections to specific sources (e.g., contaminated food, water, or healthcare settings)), Genomic Studies (WGS enables in-depth exploration of bacterial genomes. Researchers can study genetic variations, evolution, and functional elements), and Prediction of Phenotypes (WGS provides insights into various phenotypic traits like 1. Serotyping: Determining bacterial serotypes (important for vaccine development), 2. Antimicrobial Resistance: Predicting resistance patterns based on genetic markers, 3' Biofilm Formation: Understanding factors related to biofilm production. 4' Pathogenicity and Virulence: Identifying genes associated with disease severity. In essence, WGS revolutionizes understanding of bacterial genomes and their impact on health and disease.

Despite being sensitive and specific, molecular techniques are difficult to adapt for use in routine laboratories because of their high cost and the requirement for highly skilled personnel. Other drawbacks of molecular methods include an abundance of false positives and false negatives results. If DNA is present in the environment, in the laboratory, and even in the instruments used to prepare the reaction mix it will result in the false detection of a pathogen. DNA is less easily removable from surfaces and lab equipment (Tabit, 2016). The presence of inhibitors can cause false negative results. DNA-based assays detect both viable and non-viable pathogens. A false positive result can arise due to the detection of the genomic DNA of a dead pathogenic organism that is still contained in the sample.

2.7 Treatment of Enterococcal Infections

2.7.1 Treatment of Enterococcal Infections in Humans

The history of antibiotics dates back to 1928 when the Professor of Bacteriology at St Mary's Hospital in London, Alexander Fleming discovered penicillin. With this discovery, diseases were treated with remarkable results. As time passed, clinicians noticed that some microorganisms did not respond well to certain antimicrobial agents. For example, some Enterococci did not respond well to penicillin (a cell wall active agent) due to an inherent tolerance to the killing action of these compounds (Williamson et al., 1983). In 1944 they discovered that the addition of Streptomycin (aminoglycoside) to

penicillin improved outcomes (Schatz and Waksman, 1944). Using that combination, the cure rates for infective enterococcal endocarditis improved from 40% to 88% (Robbins and Tompsett, 1951). This effect was seen even though *Enterococcus* exhibits inherent resistance to aminoglycosides. The use of a cell wall-active agent in combination with an aminoglycoside became a standard of care for serious enterococcal infections and it is still useful in our time (Baddour et al., 2005).

The introduction of antimicrobials in clinical practice gave rise to modern-day antimicrobial-resistant *Enterococcus* (Miller et al., 2014). As more and more antimicrobials were being used in clinical practice more *Enterococcus* species took advantage of the situation to select for antibiotic resistance thereby emerging as multi-drug resistance (MDR) bacteria. Although the use of vancomycin was related to the emergence and spread of methicillin-resistant *Staphylococcus aureus* (MRSA), *Enterococcus* acquired resistance to vancomycin and by 1988 the first vancomycin-resistant *Enterococcus* (VRE) was identified (Uttley et al., 1988). Due to their ability to acquire, maintain and disseminate resistance genes, it is on record that *Enterococcus* donated vancomycin resistance genes to MRSA (Chang et al., 2003; Ray et al., 2003). It is these characteristics which make the treatment of enterococcal infections challenging. Treatment of enterococcal infections is achieved by medications or surgery and at times other interventions are employed.

2.7.1.1 Medical Treatment

Treatment using medications is usually based on susceptibility test results (Wang et al., 2021). In uncomplicated cases, a single drug may be used for treatment (Zaheer et al., 2020; Codelia-Anjum et al., 2023). Ampicillin is preferred when there is no antibiotic resistance (Hemapanpairoa et al., 2022; Zacharopoulos et al., 2023). Ampicillin inhibits the synthesis of Enterococcus's external cell wall, ultimately causing their death (Baran et al., 2023). In severe or serious infections such as bacteraemia, endocarditis, sepsis or meningitis, a combination of cell wall active drug and aminoglycoside such as ampicillin or vancomycin and gentamycin or streptomycin respectively may be required (Ezeh et al., 2023; Herrera-Hidalgo et al., 2023). For vancomycin-resistant enterococcal infection, Linezolid or Daptomycin is administered (Reinseth et al., 2020; Markwart et al., 2021).

For localized infections, Ampicillin or beta-lactamase inhibitors such as clavulanate/sulbactam are used (Carcione et al., 2021; Straub et al., 2022). Nitrofurantoin may alternatively be used (Salm et al., 2022). In their review, Del Turco and others (2021) recommended 2-4 weeks of treatment for enterococcal bloodstream infections related to endovascular devices (central venous catheters (CVCs) including haemodialysis catheters, peripherally inserted central venous catheters (PICCs), or midline catheters).

2.7.1.2 Surgical Treatment

In cases were medical treatment fails to treat enterococcal infections, surgery may be required (Pochhammer et al., 2021). For patients with enterococcal endocarditis, valve-replacement surgery may be considered in refractory congestive heart failure cases when medical therapy fails to clear bacteraemia, if a valve ring abscess forms, or if septic emboli develop after therapy initiation (Pettersson and Hussain, 2019; Benedetto et al., 2020; Arayakarnkul et al., 2022; Papadimitriou-Olivgeris et al., 2024). In the case of enterococcal intra-abdominal infections, surgical intervention may be necessary for conditions such as cholecystitis or the presence of intra-abdominal abscesses (Akcam et al., 2020).

2.7.1.3 Other Interventions

Other interventions used to manage catheter-related enterococcal infections include the removal of the line or intravascular device or urinary catheters (Muff et al., 2021). Indications to remove the CVC are: severe sepsis, suppurative (septic) thrombophlebitis, endocarditis, tunnel infection, port abscess, BSI which continues despite 48–72 h of adequate coverage. The CVC should be removed in most non-tunnelled CVC-related bacteraemia and fungemia cases. In tunnelled Catheters or Implantable Devices (e.g., Ports), the decision to remove the catheter or device depends on the severity of the patient's illness, documentation of vascular-access device infection, specific pathogen involved, presence of complications (e.g., endocarditis, septic thrombosis, tunnel infection, metastatic seeding) (Muff et al., 2021).

Awadh and others' (2021) study focused on patients with central line-associated bloodstream infections (CLABSI) caused by *Enterococcus*. The researchers investigated

the impact of early central venous catheter (CVC) removal on patient outcomes. The findings were that in the CLABSI group, prompt CVC removal led to favourable outcomes. The CLABSI group had significantly more CVC removals within 3 days of *Enterococcus*-related bacteraemia. Early CVC removal was associated with higher rates of microbiologic eradication. The CLABSI group without mucosal barrier injury or catheter-related bloodstream infections had fewer complications. All-cause mortality, infection-related mortality, defervescence, and relapse rates were similar in both groups. In summary, early CVC removal appears beneficial for managing enterococcal CLABSI, leading to improved outcomes and reduced complications.

Timely catheter removal is crucial in managing enterococcal infections associated with catheters (Werneburg, 2022). Early removal within three days may improve outcomes (Nguyen, 2024). However, individual patient factors and clinical judgment should guide decisions (Nguyen, 2024).

2.7.2 Treatment of Enterococcal Infections in Poultry

Enterococcosis in poultry is a significant concern in the poultry industry worldwide. (Jung et al., 2018; Laurentie et al., 2023) When poultry are affected, bloodstream infections typically occur, and sadly, birds are often found dead (Jung et al., 2018; Souillard et al., 2022). The main causes of enterococcal infections in poultry are *E. cecorum* and *E. faecalis* (Arango et al., 2023; Menck-Costa et al., 2024). To confirm the diagnosis, bacterial isolation is necessary (Yilema et al., 2017). Broilers are the mainly affected production sector (Souillard et al., 2022).

2.7.2.1 Use of Antimicrobials

To ensure that the most efficacious antimicrobial is used, culture and sensitivity are always be performed whenever possible before selecting treatment (Agyare et al., 2019; Bzdil et al., 2023). Early treatment is effective, especially during the acute stages of the disease, and is crucial, as antimicrobials tend to be ineffective if not administered promptly (Abreu et al., 2023). The efficacy of treatment deteriorates with the progression of disease within a flock (Borst et al., 2017; Ribeiro et al., 2023). Antimicrobials, including erythromycin and chlortetracycline, have been used to treat acute and subacute enterococcal infections

(Agyare et al., 2019; Mudenda et al., 2022). The current FDA-approved drugs for poultry should always be consulted when considering treatment (Wakenell, 2016). Amoxycillin appears to be the drug of first choice for most infections according to sensitivity studies in poultry (Maasjost et al., 2015).

2.7.2.2 Prevention of Enterococcal Infections in Poultry

Prevention and control of enterococcosis require preventing immunosuppressive diseases and conditions because enterococcosis often occurs secondary to another disease (García-Solache and Rice, 2019; Sangiorgio et al., 2024). Good hygiene, management, and housing strategies are imperative for controlling the disease (Oloyo and Ojerinde, 2019). In addition, ensuring proper cleaning and disinfection of facilities can decrease environmental reservoirs of the bacteria (Gržinić et al., 2023). Sanitation is particularly important in the hatchery because bacterial infections are a leading cause of embryo and neonatal chick death (Wakenell, 2016; Gržinić et al., 2023; Mertens et al., 2023). Water sanitation helps to decrease the incidence of enterococcosis (Mertens et al., 2023).

2.8 Risk Factors

2.8.1 Risk Factors of Enterococcal Infections in Humans

Risk factors of enterococcal infections include in-dwelling medical devices such as intravenous catheters and bladder catheters (Werneburg, 2022), medical procedures such as surgery and dialysis (Doganci et al., 2023), underlying disease (Luo et al., 2021), prior antibiotic treatment (Kajihara et al., 2015; MacKenzie et al., 2023), cancer treatment ((Kajihara et al., 2015; Rafey et al., 2022), prolonged hospitalization (Luo et al., 2021; Uda et al., 2021) and immunodeficiency (Giannakopoulos et al., 2019). A study carried out by Kajihara, *et al*, (2015) found abdominal surgery as the strongest risk factor for enterococcal infections, while structural abnormalities of the urinary tract, abdominal surgery and hypoalbuminemia were significant risk factors for *E. faecalis* infection. For *E. faecium* infections, immunosuppressive agent use and in situ device use were the most significant risk factors. Turjeman and others (2021) found male gender, age range of 55–75 years, catheter-associated urinary tract infection (CAUTI) and urinary retention as independent risk factors for enterococcal urinary tract infections (UTI). Treatment with

broad-spectrum antibiotics was also cited as a risk factor for enterococcal infections (Uda et al., 2021).

2.8.1.1 Indwelling Medical Devices

The use of intravascular and urinary catheter devices can contribute to the spread of enterococcal infections especially if they are not properly sterilized or are contaminated (Josephs-Spaulding and Singh, 2020). *Enterococcus* can attach to medical devices and create a biofilm that can allow them to multiply (Keogh et al., 2018). Mechanical ventilation also puts the patient at risk of infections (Basaran and Ascioglu, 2017). Transmission of enterococcal infections through contaminated medical equipment and healthcare carriers has been revealed to take place in different sections of hospitals mainly in dialysis wards (Marques et al., 2023)**.** Patients in need of haemodialysis frequently have complicated illnesses and may be given several antibiotic courses. This puts them at high risk for antimicrobial-resistant enterococcal infection. Patients undergoing haemodialysis experience frequent hospitalizations, and this coupled with cross-transmission puts those patients at risk of antimicrobial-resistant Enterococcus infections (Rodríguez-Villodres et al., 2021)**.**

2.8.1.2 Medical Procedures

Organ transplantation procedures may introduce infections through contaminated hands and devices. Patients undergoing organ transplantation are at increased risk of acquiring drug-resistant enterococcal infections (Bartoletti et al., 2018; Benamu and Deresinski, 2018). Sato and others (2020) documented *Enterococcus* as the most common causative agent of intra-abdominal infections following pancreaticoduodenectomy in their study. That showed the need to account for enterococcal infections when choosing antimicrobials to treat intra-abdominal infections after abdominal surgery. Other studies have shown liver transplantation as a risk factor for enterococcal intra-abdominal infections (Kim et al., 2019; Sato et al., 2020). The study by Kajihara and others (2015) showed cases of acquired urinary tract disorders and isolation of *E. faecalis* which coincided with the presence of artificial devices from such procedures as nephrostomies and stent placements.

2.8.1.3 Underlying Diseases

Underlying diseases such as cardiovascular abnormalities, open wounds, urinary tract infections and impaired mobility put the patient at risk of enterococcal infections. Patients with haematological malignancies are at increased risk for the acquisition of enterococcal infections (Uda et al., 2021). Dental root canal issues make patients vulnerable to infection of Enterococcus (Alghamdi and Shakir, 2020). Renal failure has also been cited as a risk factor for enterococcal infections (Sangiorgio et al., 2024).

2.8.1.4 Prolonged Hospitalization

Hospitalized patients especially those hospitalized for prolonged periods are vulnerable to enterococcal infections ((Kajihara et al., 2015; Vasilakopoulou et al., 2020). According to a study conducted by Moemen and others in 2015, risk factors for enterococcal infections which had a significant association with the ICU-acquired Vancomycin-resistant *E. faecium* (VREF) infections included transfer to the ICU from a ward and prolonged ICU stay among others. Other studies also showed that intra-hospital transfer was associated with VREF colonization or infection (Moemen et al., 2015). The patient may acquire *Enterococcus* species while in one ward which may cause infection during their stay in another ward. Prolonged hospitalization has been cited by many studies as a risk factor for enterococcal colonization and infection (Brinkwirth et al., 2021; Meschiari et al., 2023; Sangiorgio et al., 2024). The longer the patient stays in the hospital the greater the chance that they will receive antibiotics and the longer the time they will be exposed to possible pathogen transmission. Because of increased exposure to patients colonized with VRE, healthcare workers become colonized by VRE (Jackson et al., 2019) which in turn they can transmit to other patients through direct contact with them through the provision of healthcare or contaminated medical devices. Patients may get *Enterococcus* through exposure to other patients with VRE either by proximity to a VRE-colonized patient or by care from a nurse who is providing care to another VRE-colonized patient (Meschiari et al., 2023).

2.8.1.5 Prior Antibiotic Use

Risk factors for enterococcal infections include prior or prolonged antibiotic use (Uda et al., 2021). Prior use of antibiotics such as vancomycin, third-generation cephalosporins,

gentamicin, or ciprofloxacin are among the risk factors associated with VRE infections (Moemen et al., 2015). Prolonged exposure to antibiotics may increase the risk of *Enterococcus* infections due to selective pressure (Kirsch et al., 2023).

2.8.1.6 Use of Immunosuppressive Agents

The use of immunosuppressive agents such as cancer treatment weakens the immune system thereby putting the patient at risk of enterococcal infections (Kajihara et al., 2015). With weakened immunity commensal *Enterococcus* species can cause infection.

2.8.1.7 Immunodeficiency

Other people with reduced immunity such as those with HIV/AIDS are at high risk of enterococcal infections (Zike et al., 2024).

2.8.2 Risk Factors of Enterococcal Infections in Poultry

2.8.2.1 Antibiotic Use

Prior or prolonged antibiotic use is a significant risk factor. Antibiotics disrupt the normal gut microbiota, creating an opportunity for opportunistic pathogens like *Enterococcus* to thrive (Agyare et al., 2019). Overuse or misuse of antibiotics in poultry production can lead to the emergence of antibiotic-resistant strains (Abreu et al., 2023).

2.8.2.2 Contaminated Environment

Poor hygiene and contaminated litter contribute to the spread of enterococci within poultry flocks (Bzdil et al., 2023). Overcrowding and inadequate sanitation create an environment conducive to bacterial growth (Oloyo and Ojerinde, 2019).

2.8.2.3 Stress and Immunosuppression

Stressful conditions (such as transport, handling, or environmental changes) weaken the birds' immune system (Hofmann et al., 2020). Stress-induced immunosuppression makes poultry more susceptible to infections (Tian et al., 2022).

2.8.2.4 Age and Production System

Young birds are more vulnerable to enterococcal infections (Souillard et al., 2022; Bzdil et al., 2023). Intensive production systems (such as broiler farms) may increase exposure to enterococci due to close confinement and shared resources (Mudenda et al., 2022).

2.8.2.5 Biosecurity Measures

Proper biosecurity practices, including cleaning and disinfection, help prevent the introduction and spread of enterococci in poultry farms (Dhaka et al., 2023).

Worldwide, many studies have been conducted on *Enterococcus* species from various habitats. However, not so many studies have done on genetic determinants of *Enterococcus* species from poultry and humans. In Africa, few studies have determined the occurrence, virulence genes, and antimicrobial resistance profiles of *E. faecalis* and *E. faecium* isolated from poultry and humans. Most studies have mainly focused on clinical isolates and those from water samples (Ferede et al., 2018; Gotkowska-Płachta and Gołaś, 2023) or isolates from animals and not in comparison with those from humans (Pillay, et al., 2018). In Zambia, *Enterococcus* has not received the same level of attention as other gastrointestinal commensals like *Staphylococcus* and Salmonella. This explains the current lack of comprehensive data on the virulence and antimicrobial resistance genes of *E. faecalis* and *E. faecium* that are present in poultry and humans in Zambia. The aim of the study was to determine the occurrence, virulence genes, and antimicrobial resistance profiles of *Enterococcus faecalis* and *Enterococcus faecium* from poultry of Chongwe, Lusaka, Kitwe, and Ndola Districts, Farmworkers from the Copperbelt Province Poultry Farms, and Clinical Specimens from Kitwe Teaching Hospital in Zambia.

CHAPTER THREE

MATERIALS AND METHODS

3.0 Study Design

This was a cross-sectional study covering the period of December 2020 to June 2021. Phenotypic and molecular techniques were employed to determine the occurrence, virulence genes, and antimicrobial resistance profiles of *Enterococcus faecalis* and *Enterococcus faecium* from poultry, Farmworkers, and Clinical Specimens.

3.1 Study Areas

The study areas comprised of commercial farms from Chongwe, Lusaka, Kitwe and Ndola Districts, of Lusaka and Copperbelt Provinces, and Kitwe Teaching Hospital.

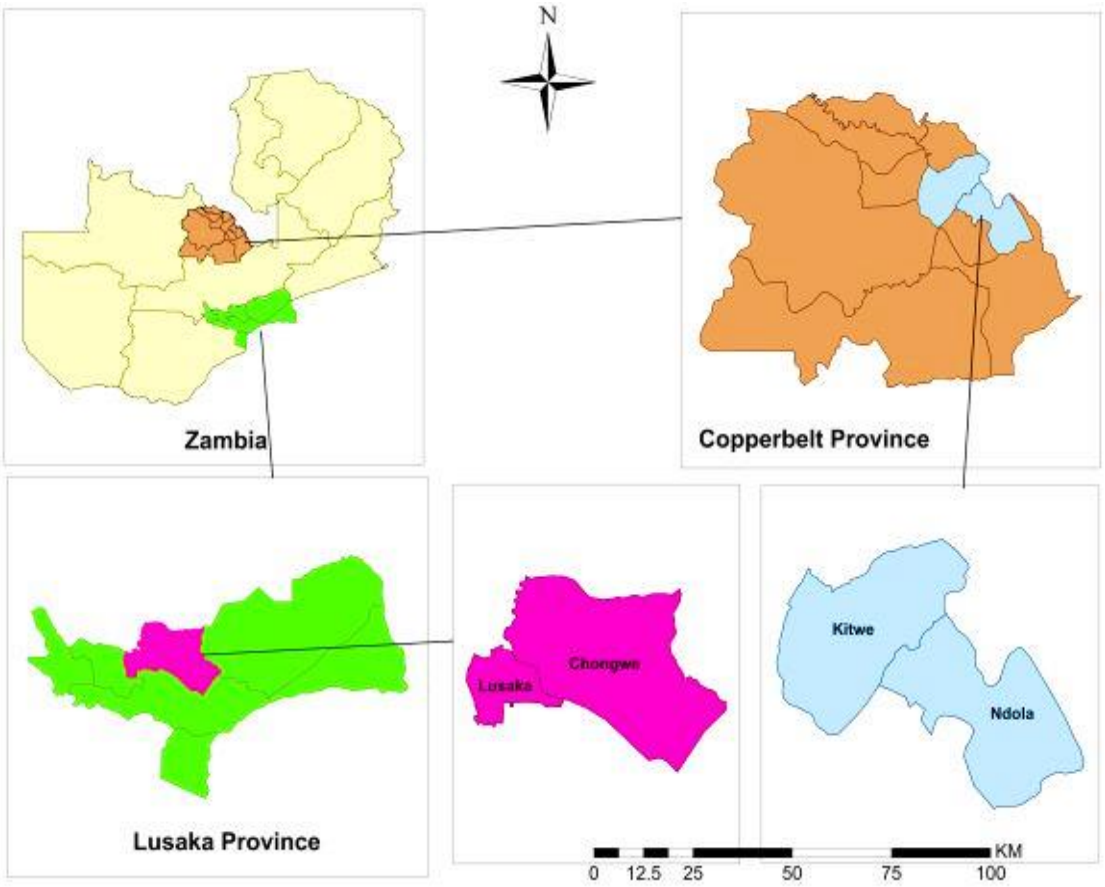

Figure 3.1 Map showing Study Areas; Chongwe and Lusaka, and Kitwe and Ndola Districts, of Lusaka and Copperbelt Provinces respectively.

3.2 Sampling Frame

The study identified *E. faecalis* and *E. faecium* from poultry droppings, farmworker and clinical specimens.

3.2.0.1 Poultry Droppings

All poultry from the commercial farms were samples originated were vaccinated, and the farmers confirmed their use of antimicrobials for growth promotion and treatment. Some farms had biosecurity measures in place. The age of chickens ranged from 10 to 100 weeks old. The poultry droppings from Lusaka Province were 107 comprising of 57 and 50 from Chongwe and Lusaka districts respectively. From Ndola and Kitwe Districts, 140 and 245 poultry droppings were collected respectively making a total of 385 poultry specimens from Copperbelt Province.

3.2.0.2 Farmworker Specimens

Farmworkers who consented to participate in the study were asked if they had any of the following; headache, general body weaknesses, fever, pain when passing urine, urinary tract infections, diarrhoea or abdominal pains. Those with none of the above were presumed healthy. All samples were from Copperbelt Province. Although 111 farmworkers consented to participate in the study, they refused to give out personal information. Of the 111 that participated in the study, 110 were males while one was female. Farmworker specimens comprised of stool, urine, and rectal swabs because some of those who consented to participate in the study were unable to submit stool specimens. They were therefore asked to submit random midstream urine or rectal swabs. Those presumably healthy individuals (farmworkers) who consented to participate in the study were from the poultry farms where poultry droppings were collected. Although the farmworkers in Lusaka province expressed interest in participating in the study and specimen containers were left with them, on the day for collection, they gave back empty contains and said they were not willing to participate in the study.

It is worth noting that collection of samples was done during the Covid-19 pandemic, during which time the government had imposed travel restrictions, and many people were sceptical about participating in studies which involved submitting specimens.

3.2.0.3 Clinical Specimens

All clinical samples were from Kitwe Teaching Hospital. The composition of clinical specimens was 138 urines, 89 stool, and 3 pus swabs and were from 105 male and 127 female patients who were suspected of or were being treatment for urinary tract infections (UTI), gastrointestinal tract (GIT) and wound infections. The age of the patients ranged from 2 to 86 years, 80 were inpatients while 150 were outpatients.

3.2.1 Sample Size Determination

To determine the sample size required to determine the presence of *Enterococcus* species in poultry, health individuals (farmworkers) and clinical samples, sampling was done at three different levels. Farm owners of 14 commercial farms in the 4 Districts consented to participate in the study.

3.2.1.1 Poultry

For poultry, the proportion "p" of 50%, the confidence level of 95%, and the margin of error of 0.05 (Pourhoseingholi et al., 2013) were used to calculate sample size. Using a web-based calculator (https://www.calculator.net/sample-size-calculator.html?) the sample size was 385.

3.2.1.2 Farmworkers

Since the prevalence of enterococci in farmworkers was unknown and no pilot study was done, a prevalence of 50% and a margin of error of 5% at a 95% confidence level were used to calculate the sample size.

The formula;

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

was used to calculate the sample size (Pourhoseingholi et al., 2013). Where n is the sample size, Z is the level of confidence, P is the expected prevalence and d is the margin of error.

Applying the formula

$n = (1.96^2 \times 0.5(1-0.5)) / 0.05^2$

$n = 385$

3.2.1.3 Clinical Specimens

For clinical samples a total of 230 urine, stool and pus swabs sent to the laboratory from hospitalized and non-hospitalized patients who were undergoing treatment for or suspected of urinary tract, gastrointestinal tract and wound infections respectively at Kitwe Teaching Hospitals in Kitwe District of Zambia were collected and analysed. For sample size calculation, a prevalence "p" of 18.03% (Mpinda-Joseph et al., 2019) was used, Z statistic at 95% confidence is 1.96 and an acceptable error of 0.05 (Pourhoseingholi et al., 2013) was used.

Formula:

$n=(z^2 \times p(1-p))/e^2$

$n=(1.96\text{^}2 \times 0.18(1-0.18))/0.05^2$

$n = 227$

Rounding up we got 230.

3.2.2 Inclusion criteria

Poultry droppings voided within 30 minutes and were not stepped on or contaminated in other ways were sampled.

For farmworker specimens, all stool specimens submitted were included. For those who did not manage to submit stool, mid-stream urine or rectal swabs were alternatively submitted.

For clinical specimens, all urine and stool specimens, and pus swabs from patients suspected of or were being treated for urinary tract infection, gastrointestinal and wound infections respectively which were received at the Kitwe Teaching Hospital Laboratory. The specimens included in the study were those received on the first day of February until the 230th sample was received on the 16th day of March 2021.

3.2.3 Exclusion criteria

All chicken droppings that were not freshly voided, those freshly voided but contaminated and those voided after 30 minutes were excluded.

All farmworkers who had signs and symptoms of illness such as headaches, general body weaknesses, fever, urinary tract infections, diarrhoea and abdominal pains were excluded from the study.

For clinical samples, all samples other than urine, stool and pus swabs that were received during the study period were excluded. All urine, stool and pus swabs that did not meet the inclusion criteria such as unlabelled or mislabelled, leaked and contaminated samples were rejected, and were therefore excluded from the study. Also excluded were specimens which were submitted by patients who were not suspected of or confirmed having urinary tract, gastrointestinal tract and wound infections. Specimens which were received after receiving the 230th sample were excluded.

3.2.4 Sampling Strategy

3.2.4.1 Chicken droppings

Chicken droppings were collected from all the four districts. Upon getting to a chicken run, the researcher had to wait and observe the chickens. Droppings were collected as soon as a chicken pooped while avoiding contamination. In case chickens stepped on the fresh droppings just after defecation, no sampling was done from those droppings. In a situation where chickens were frightened and they ran and raised dust, sample collection was discontinued. The collection of chicken droppings in each pen was done within 30 minutes. In Lusaka province, droppings collected comprised of those from layers, broilers and village chickens which were kept in chicken rans like those where broilers were kept and were raised the same way as broilers. In the Copperbelt Province, all droppings were from layers.

3.2.4.2 Farmworker Specimens

Faecal specimens from farmworkers who consented to participate in the study were collected. For those who were unable to submit faecal specimens, random midstream urine or rectal swabs were collected. Immediately after collection, rectal swabs were put in 9 ml buffered peptone water (BPW) in Falcon centrifuge tubes and kept in a cool box for transportation to the laboratory.

3.2.4.3 Clinical Specimens

Before sampling, specimens which were received in the Microbiology Section at Kitwe Teaching Hospital Laboratory were checked for acceptability. Unlabelled or mislabelled, leaked and contaminated samples were rejected. Information about age, gender, specimen type, clinical diagnosis and department or ward were noted. Urine samples of patients confirmed or suspected urinary tract infections were included in the study. Also included in the study were stool specimens from patients who had diarrhoea and those who were suspected of gastrointestinal infections. The swabs were all from wound infections. For urine samples 1 ml was sampled after mixing the specimen well and dispensed into a centrifuge tube containing 5 ml Trypticase Soy broth (TSB) using a sterile Pasteur pipette. For stool samples, about 1 g was scooped using an applicator stick and was suspended in 9 ml buffered peptone water (BPW) in Falcon centrifuge tubes. Pus swabs were put in 9 ml buffered peptone water (BPW) in Falcon centrifuge tubes. The source, types and number of all specimens processed including number of farms (for poultry and farmworkers) are shown in Table 3.1.

Table 3.1 Sources, Types and Numbers of Specimens

Area	District	Commercial Farms. n	Source of specimen		Specimen Type	Samples, n
Lusaka Province	Chongwe	4	Poultry	Layer	Droppings	57
	Lusaka	2	Poultry	Broilers (20), Layers (20), Village (10)	Droppings	50
Copperbelt Province	Kitwe	7	Poultry	Layer	Droppings	245
	Ndola	4	Poultry	Layer	Droppings	140
	Kitwe	7	Farmworkers		Urine	108
	Ndola	4	Farmworkers		Rectal swabs	3
	Kitwe (Kitwe Teaching Hospital)	N/A	Patients Suspected of or confirmed UTI		Urine	138
			Patients with GIT infections		Stool	89
			Wound Infections		Pus swabs	3
Total number of samples						833

n = number, UTI = urinary tract infections, GIT = gastrointestinal tract, N/A = not applicable. In Lusaka, one farm had broilers only while the other farm had layers and village chickens.

3.3 Diagnosis of *Enterococcus*

3.3.1 Phenotypic Methods

3.3.1.1 Culture for Presumptive Identification of *Enterococcus*

Conventional Microbiological methods employed to detect *Enterococcus* species were as described by Facklam and others (1989) with a few modifications. In brief, for stool specimens, 1g of faecal specimens from submitted specimens was scooped and suspended in 9ml buffered peptone water (BPW) (HIMEDIA, India). Swabs were placed in 5ml of BPW and incubated at 37^0c for 24 hours. In the case of urine, 1ml of urine sample was put into 5ml Trypticase Soy broth (TSB) (HIMEDIA, India), mixed and incubated at 37^0c for 24 hours. A loopful of the TSB suspension was streaked on Bile Esculin Agar (BEA) (HIMEDIA, India) and incubated at 37^0c for 24 hours. Preliminary identification was based on phenotypic characteristics including cultural traits (such as growth on Bile Esculin Agar where suspected *Enterococcus* colonies appeared black, growth at 10^oC and 45^oC), Gram Stain (Gainland Chemicals Company, United Kingdom) reaction (purple/blue colonies showed positive results), catalase and oxidase tests (where bubbles and no colour change indicated negative results, respectively).

3.3.1.2 Identification of *Enterococcus* using Analytical Profile Index (API)

Analytical Profile Index (API) 20 Strep test kits (BioMérieux, France) were used to identify 61 isolates. The procedure was performed according to the manufacturer's instructions. Briefly, a concentrated microbial suspension (Mac Farland 4 turbidity) of the suspected *Enterococcus* colonies was used to fill the tubes, then some wells were covered with mineral oil, and incubated for 24 hours at 37°C. The results were recorded on slips provided by the manufacturer and each of them produced a code that, when inputted into the database on the website after logging in (http://apiweb.biomerieux.com), displayed the identity of the target *Enterococcus* species. The website generates the identity of *Enterococcus* species by comparing the generated codes with the codes of species of *Enterococcus* in the database.

3.3.1.3 Antimicrobial Susceptibility Testing

Susceptibility to vancomycin (30 μg), erythromycin (15 μg), ampicillin (10 μg), penicillin (10U), tetracycline (30 μg), nitrofurantoin (300 μg), ciprofloxacin (5 μg) and chloramphenicol (30 μg) was determined by Kirby-Bauer disk diffusion method and interpreted according to the Clinical and Laboratory Standards Institute guidelines of 2020 (CLSI, 2020). Briefly, Mueller Hinton (MH) agar plates were brought to room temperature and the surface of the media was dried before use. Then the MH agar plates were labelled. The pure bacterial culture was suspended in normal saline and adjusted for turbidity equivalent to a 0.5 McFarland standard. A sterile swab was dipped into the suspension and pressed against the inside wall of the tube to remove excess fluid. Then the inoculum was streaked across the entire surface of the MH agar plates. Using an aseptic technique, antimicrobial disks were dispensed and pressed onto the surface of the inoculate MH agar plates and incubated at 35^{o}C for 18 to 24 hours. Using a ruler, the zones of inhibition were measured in millimetres and recorded. The interpretation was done using the CLSI, M100, zone interpretive guide, as susceptible, intermediate, or resistant. Intermediate results fell within the defined range, any result that was greater than or equal to (≥) the stipulated threshold was Susceptible and a result less than or equal to (≤) the stated value was resistant (Table 3.2). In this study, all intermediate results were taken as resistant. A reference strain, *Enterococcus faecalis* 29212 was used as the control strain.

Table 3.2 Interpretation of Zones of Inhibition

Antimicrobial susceptibility Disk	AMP	CHL	CIP	ERY	NIT	PEN	TET	VAN
Thresholds in millimetres	≥17 - ≤16	≥18 - ≤12	≥21 - ≤15	≥23 - ≤13	≥17 - ≤14	≥15 - ≤14	≥19 - ≤14	≥17 - ≤14

AMP = ampicillin, CHL = chloramphenicol, CIP = ciprofloxacin, ERY = erythromycin, NIT = nitrofurantoin, PEN = penicillin, TET = tetracycline, VAN = vancomycin

3.3.2 DNA Extraction

Colonies of overnight growth on a blood agar plate were put in an Eppendorf tube containing 0.5ml of molecular grade water, vortexed, and boiled at 95°C for 10 minutes, and then centrifuged for 5 minutes at 1500xg. The supernatant was pipetted into cryo-vials and stored at -20°C for further analysis.

3.3.3 Molecular Methods

3.3.3.1 Molecular Identification of *Enterococcus*

Molecular identification of the genus *Enterococcus* was done by PCR using genus-specific primers as described previously by (Li et al., 2012). DNA amplification of the elongation factor (*tuf*) and D -Ala- D -Ala ligase (*ddl*) was done using Phusion Flash High-Fidelity PCR Master Mix (Thermofisher Scientific, US) in the thermal cycler (Applied Biosystems, Chiba, Japan). The PCR was run under the following conditions: initial denaturation at 98°C for 2 minutes followed by 30 cycles of denaturation at 98°C for 5 seconds, annealing at 56°C for 5 seconds, extension at 72°C for 30 seconds, and final extension at 72°C for 1 minute. PCR amplicons were run on 1.5% agarose gels to which approximately 0.2-0.5µg, i.e., about 2 µl of Ethidium Bromide stock solution per 100ml was added. The expected bandwidths for *tuf* and *ddl* PCR products were 112bp and 475bp, respectively. Polymerase Chain Reaction using Genus-specific primers was done on a total number of 473 samples, segregated as 343 poultry isolates, 15 farmworker isolates and 124 clinical isolates.

For species identification, species-specific primers targeting the *superoxide dismutase* (*sodA*) gene of *E. faecalis* and *E. faecium* were used. The PCR conditions were like the conditions described above for genus identification except the annealing temperature for which was 52°C. Polymerase Chain Reaction using species-specific primers was done on 153 poultry, 13 Farmworker, and 68 clinical isolates. Table 3.3 shows the genus-specific and species-specific primers used to identify *Enterococcus*.

Table 3.3 Primers for Genus and Species Identification of Enterococci

IDENTIFICATION PRIMERS				
Target gene	Primer name	Primer sequence 5'-3'	Amplicon Size bp*	References
Tuf	*tuf-F*	TAC TGA CAA ACC ATT CAT GAT G	112	(Ke et al., 1999)
	tuf-R	AAC TTC GTC ACC AAC GCG AAC		
Ddl	*ddlF*	CAC CTG AAG AAA CAG GC	475	(Vilela et al., 2006)
	ddlR	ATG GCT ACT TCA ATT TCA CG		
SodAEfm	*sodAEfm1*	CAG CAA TTG AGA AAT AC	190	(Bensalah et al., 2006)
	sodAEfm2	CTT CTTTTATTTCTCCTGTA		
SodAEfs	*sodAEfs1*	CTGTAG AAG ACC TAA TTT CA	209	(Bensalah et al., 2006)
	sodAEfs2	CAG CTG TTT TGA AAG CAG		

*bp = base pair

3.3.3.2 Confirmation of species identity of *Enterococcus*

A total of nine PCR species-specific identified *E. faecalis* DNA samples were randomly selected, amplified, purified, and sequenced to confirm the PCR species identification results. The distribution of the nine selected isolates was such that three were from poultry, the other three were from clinical specimens, and the last three were from farmworkers. For more information, please refer to Appendix 2 on pages 129 to 131.

3.3.3.3 Detection of Antimicrobial Resistant Genes (ARG)

Detection of genes conferring resistance to glycopeptides (*vanA*), tetracyclines [*tet(M), tet(L), tet(K),* and *tet(X)*], macrolides [*erm(A)* and *erm(B)*] and aminoglycosides [*aac(6')-Ie-aph(2'')-Ia*] was performed using PCR with gene-specific primers (Table 3.2). One Taq Quick-load 2X Master Mix (Biolabs, Durham, North Carolina, USA) was used for amplification using a thermal cycler (Applied Biosystems, Chiba, Japan). The following PCR conditions were employed; Initial denaturation at 93°C for 3 minutes. The amplification cycles consisted of denaturation at 93°C for 60 seconds, and annealing at 52°C for 60 seconds, elongation at 72°C for 60 seconds. After 35 cycles amplification cycles, the final elongation step was performed at 72°C for 5 minutes. PCR amplicons were run on 1.5% agarose gel containing about 0.2-0.5µg/ml Ethidium Bromide. Expected bands for PCR products were different for each gene (Table 3.4).

Table 3.4 Primers Used for Detection of Antimicrobial Resistance Genes

Target gene	Primer name	Primer sequence 5'-3'	Amplicon size bp*	References
aac(6′)-Ie-aph(2″)-LA	*aacF*	CAG GAA TTT ATC GAA AAT GGT AGA AAA G	369	(Sabouni et al., 2016)
	aacR	CAC AAT CGA CTA AAG AGT ACC AAT C		
*Erm*A	*ermAF*	TAT CTT ATC GTT GAG AAG GGA TT	139	(Goudarzi et al., 2018)
	ermAR	CTA CAC TTG GCT TAG GAT GAA A		
*Erm*B	*ermB-1*	GAA AAG TAC TCA ACC AAA TA	639	(Sutcliffe et al., 1996)
	ermB-2	AGT AAC GGT ACT TAA ATT GTT TA		
*Tet*K	*tetK-1*	TTA GGT GAA GGG TTA GGT CC	697	(Aarestrup et al., 2000)
	tetK-2	GCA AAC TCA TTC CAG AAG CA		
*Tet*M	*tetM-1*	GTT AAA TAG TGT TCT TGG AG	576	(Aarestrup et al., 2000)
	tetM-2	CTA AGA TAT GGC TCT AAC AA		
*Tet*L	*tetL-1*	CAT TTG GTC TTA TTG GAT CG	456	(Aarestrup et al., 2000)
	tetL-2	ATT ACA CTT CCG ATT TCG G		
*Tet*X	*tet*XF	CAA TAA TTG GTG GAC CC	468	(Ng et al., 2001)
	tetXR	TTC TTA CCT TGG ACA TCC CG		
*Van*A	*vanAF*	CTG CAA TAG AGA TAG CCG CTA ACA	751	(Sting et al., 2013)
	vanAR	TGT ATC CGT CCT CGC TCC TC		

*bp = base pair

3.3.3.4 Determining the Presence of Virulence Genes in *Enterococcus*

Virulence genes *gelatinase* (*gelE*), *aggregation substance* (*asa1*), *Pheromone cAD1 precursor lipoprotein* (*cad1*), enterococcal surface protein *(esp*), *cytolysin* (*cylA*), *E. faecalis* endocarditis antigen Regulator of *gelE* and *sprE* expression, (*fsr*), *collagen-binding cell wall protein* (*ace*), and *E. faecium* specific cell wall adhesin (*efm*) were detected by PCR amplification with gene-specific primers (Table 3.7) as described previously (Song et al., 2019). DNA amplification was done using Phusion Flash High-Fidelity PCR Master Mix (Thermofisher Scientific, USA) in the thermal cycler (Applied Biosystems, Chiba, Japan). The following PCR conditions were used; initial denaturation at 98°C for 2 minutes followed by 30 cycles of denaturation at 98°C for 5 seconds, annealing at 56°C for 5 seconds, extension at 72°C for 30 seconds, and final extension at 72°C for 1 minute. PCR amplicons were run on 1.5% agarose gels suspended with 0.2-0.5µg/ml Ethidium Bromide and visualized under UV light. Each virulence gene had a different expected bandwidth (Table 3.5).

Table 3.5 Primers used for Determining Presence of Virulence Genes

Target gene	Primer name	Primer Sequence 5'-3'	Amplicon size bp	References
ace	*aceF*	ATA GAA ACG GAT TTC GGA ACA G	298	(Song et al., 2019)
	aceR	TCA AAC TCG GCA AGT GAA ATA T		
asal	*asa1F*	AAC AAG CTT GGT CTG TGT ATC	168	(Song et al., 2019)
	asa1R	TCT TCC CCT TTC TTG TTA TGA AC		
cad1	*cad1*	TTC CAA AAC TAC GCA CAA CA	423	(Song et al., 2019)
	cad1	CTT TTT CAG CAT TCA CTA ATT		
cylA	*cylAF*	GAG TTA GAT GAA TAT GGT CAT GGT	521	(Song et al., 2019)
	cylAR	AGA AAC TAG CGA TGT AGG GTA ATA		
ef1841/ fsrC	*Forward*	CAA GGC ACT ATT TCT TAC TTA GG	1,016	(Song et al., 2019)
	Reverse	AGC GCA TAA ATC AAC CAA G		
esp	*espF*	CAT CTT TGA TTC TTG GTT GTC G	695	(Song et al., 2019)
	espR	GTT ATA GGT ACG TAT GTT GCA TCA		
gelE	*gelEF*	TAT GAC AAT GCT TTT TGG GAT G	208	(Song et al., 2019)
	gelER	GCA CCC GAA ATA TAA CCC		
Efm	*efmF*	GAA AAG TTG TCA GTC GTG G	818	(Song et al., 2019)
	efmR	TGT TTG TGA CAA ACC TTC ATG		

bp = base pair

3.4 Data Analysis

Qualitative data was entered, cleaned, and validated in a Microsoft™ Excel spreadsheet. PCR results (positive or negative) were reference variables for descriptive analyses. The data was then exported to SPSS software ver. 21 (IBM Corp, Armonk, NY, USA). Univariate analysis was done for descriptive results and data was presented as frequencies, percentages, tables, and figures. Differences in antibiotic resistance phenotype and genotype were analysed using Pearson's Chi-Square test ($a = 0.05$). Odds ratios with their respective 95% confidence interval (CI) were calculated to measure the strength of associations. A probability value (p) of <0.05 was considered statistically significant.

Sequencing data were edited using BioEdit 7.2 Software. Identification was done using Basic Local Alignment Search Tool (BLAST) on the National Centre for Biotechnology Information (NCBI) website. All specimens were collected and processed by the researcher.

CHAPTER FOUR

RESULTS

4.1 Occurrence of *Enterococcus*

4.1.1 Occurrence of *Enterococcus* using Analytical Profile Index

Using the API system of identification the occurrence of *Enterococcus* selected poultry, farmworker, and clinical specimens was as depicted in Figure 4.1.

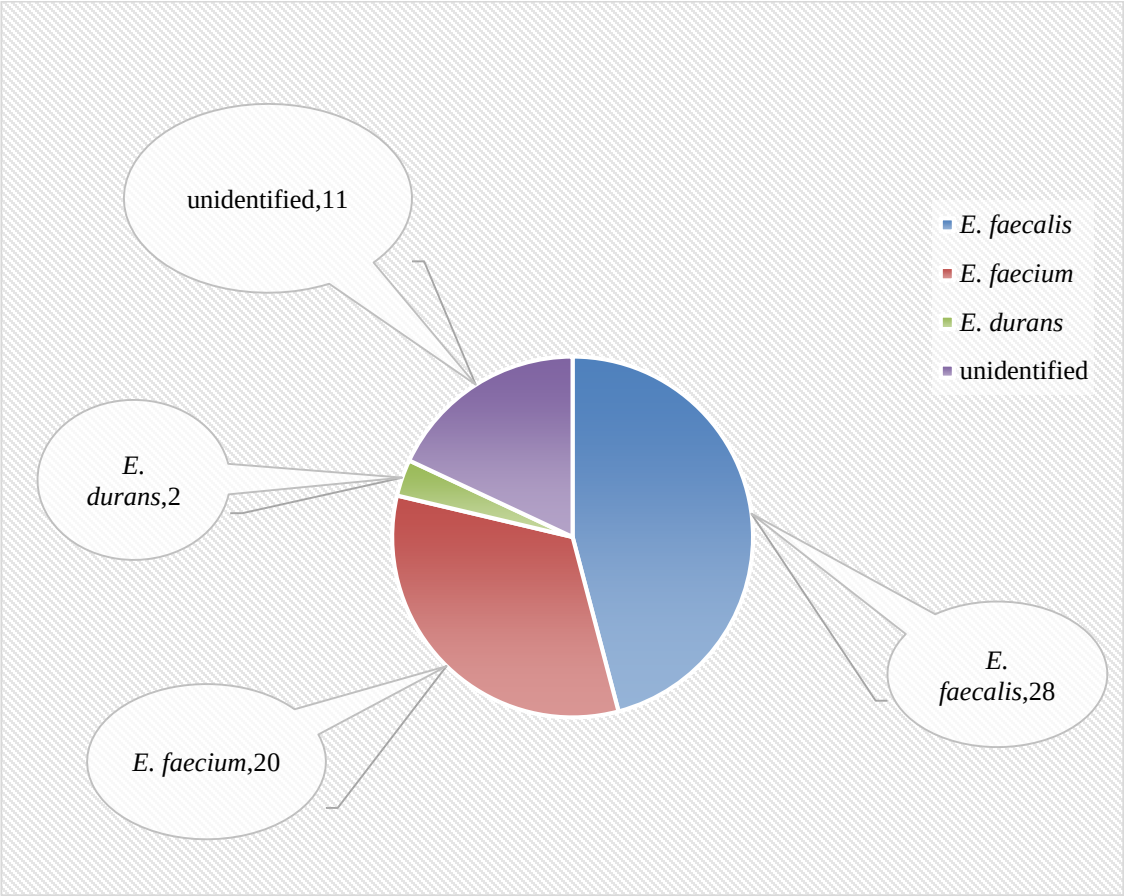

Figure 4.1 Chart showing the 61 *Enterococcus* isolates identified using API 20 Strep

4.1.2 Occurrence of *Enterococcus* using Polymerase Chain Reaction

Figures 4.2a and 4.2b are representative images of amplified DNA samples identified using genus-specific *tuf* and *ddl* primers respectively.

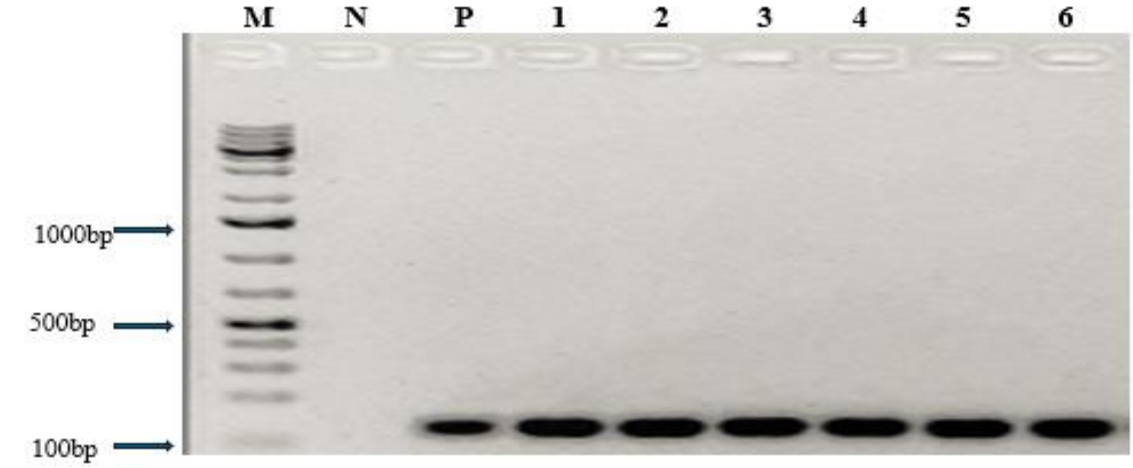

Figure 4.2a Representative agarose gel image showing the amplified 112bp *tuf* bands of the Genus *Enterococcus*. M = 100bp molecular marker, N = negative control, P = positive control (*Enterococcus faecalis* ATCC 29212 strain), 1,2,3,4,5,6 = samples

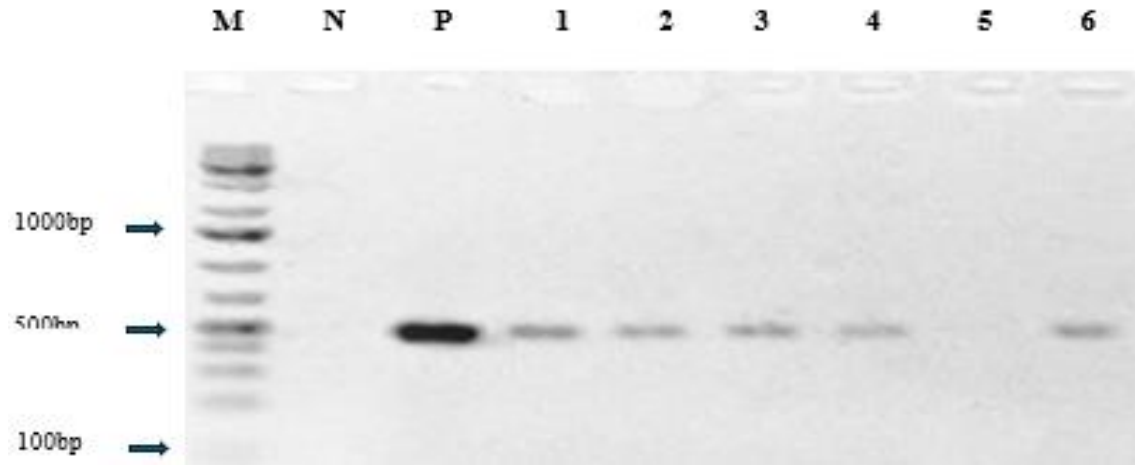

Figure 4.2b Representative agarose gel image showing the amplified 475bp *ddl* bands of the Genus *Enterococcus*. M = 100bp molecular marker, N = negative control, P = positive control (*Enterococcus faecalis* ATCC 29212 strain), 1, 2, 3,4,5,6 = samples

Using *superoxide dismutase* (*sodA*) species-specific primers for *Enterococcus faecalis* (*sodAEfs*) and *Enterococcus faecium* (*sodAEfm*), the identified *Enterococcus faecalis* and *Enterococcus faecium*, showed expected bands of 209bp and 90bp when visualized under ultraviolet light in 1.5% agarose gels as shown in figures 4.3a and 4.3b respectively.

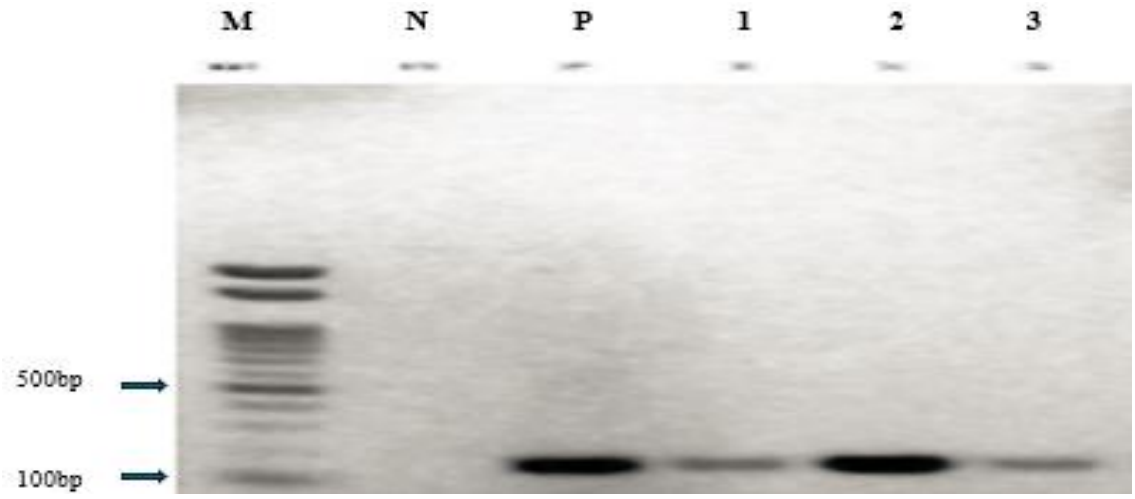

Figure 4.3a Representative agarose gel image showing the amplified 209 bp *sodAEfs* bands of *Enterococcus faecalis* species. M = 100bp molecular marker, N = negative control, P = *Enterococcus faecalis* ATCC 29212 control strain, 1,2,3 = samples

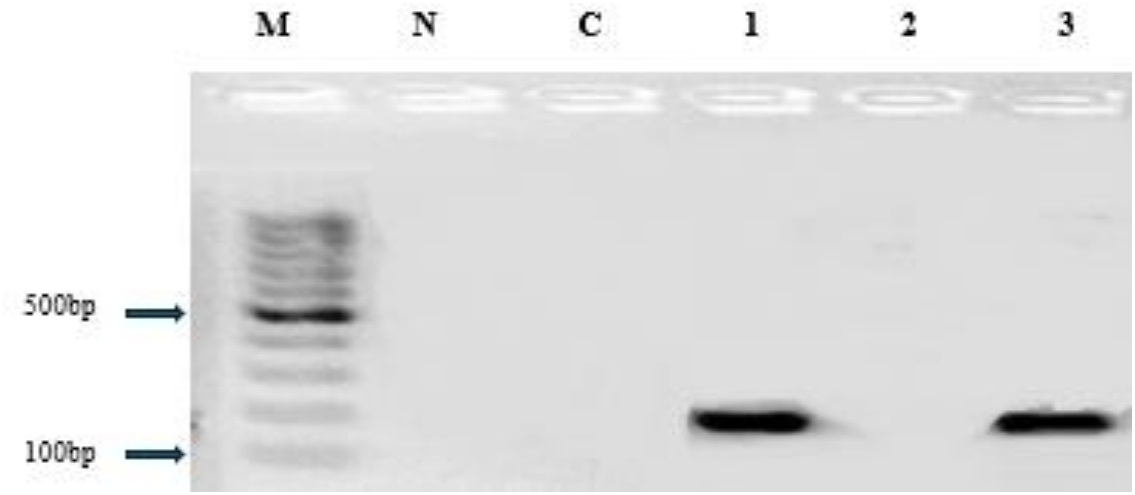

Figure 4.3b. Representative agarose gel image showing the amplified 190 bp *sodAEfm* bands of *Enterococcus faecium* species. M = 100bp molecular marker, N = negative control, C = *Enterococcus faecalis* ATCC 29212 control strain, 1,2,3 = samples.

4.1.2.1 Occurrence of *Enterococcus* in Poultry

Out of the total number of 492 specimens processed, 153 were *Enterococcus* representing a proportion of 31.1%, (153/492, CI: 27.1-35.4), while the proportion for Lusaka Province was 30.8% (33/107, CI: 22.5-40.6) and proportion for Copperbelt province was 31.2% (120/385, 26.6-36.1). Table 4.1 shows a summary of the proportion of *E. faecalis* and *E. faecium* in poultry from each of the districts in the Copperbelt and Lusaka Provinces.

Table 4.1. Proportion of *Enterococcus* in Poultry from the four Districts

Province	District	n Tested	n Positive	Proportion (%)	95% CI
Lusaka	Chongwe	57	11	19.3	10.5-32.3
	Lusaka	50	22	44.0	30.3-58.7
	Chongwe and Lusaka	107	33	30.8	22.5-40.6
Copperbelt	Kitwe	245	78	31.8	26.1-38.1
	Ndola	140	42	30.0	22.7-38.4
	Kitwe and Ndola	385	120	31.2	26.6-36.1
Both Provinces	All the 4 Districts	492	153	31.1	27.1-35.4
Area	*Enterococcus* species	n Tested	n Positive	Proportion (%)	95% CI
Four districts of Lusaka and Copperbelt Provinces	*E. faecalis*	153	58	37.9	30.3-46.1
	E. faecium	153	16	10.5	6.3-16.7
	All other *Enterococcus* species	153	79	51.6	43.5-59.7

n = number, % = percent, CI = confidence interval

4.1.2.1.1 Species-specific Proportion of *Enterococcus* Species in Poultry

The proportion of *Enterococcus* varied significantly across the districts. Lusaka district had a higher proportion of 44.0%, (22/50, CI: 30.3-58.7) than the other three districts of Kitwe, Ndola, and Chongwe of 31.8% (78/245, CI: 26.1-38.1), 30% (42/140, CI: 22.7-38.4) and 19.3% (11/57, CI: 10.5-32.3 respectively). The proportion of *E. faecalis* was higher compared to that of *E. faecium* in all districts (p=0.012), despite having a higher proportion e of *E. faecalis* + *E. faecium* in the Kitwe district (p=0.044) (Table 4.2).

Table 4.2 Species-specific Proportion of *Enterococcus* species across the study area

Province	District	Species	n Tested	n Positive	Proportion (%)	95% CI
Lusaka	Chongwe	*E. faecalis*	57	5	8.8	3.3-20.0
		E. faecium	57	1	1.8	0.1-10.6
		E. faecalis +E. faecium	57	1	1.8	0.1-10.6
		E. faecalis + other	57	2	3.5	0.6-13.2
		Other	57	2	3.5	0.6-13.2
		Total	57	11	19.3	10.5-32.3
	Lusaka	*E. faecalis*	50	8	16.0	7.6-29.7
		E. faecium	50	5	10.0	3.7-22.6
		E. faecalis +E. faecium	50	4	8.0	2.6-20.1
		E. faecalis + other	50	1	2.0	0.1-12.0
		E. faecium + other	50	1	2.0	0.1-12.0
		Other	50	3	6.0	1.6-17.5
		Total	50	22	44.0	30.3-58.7
Copperbelt	Kitwe	*E. faecalis*	245	26	10.6	7.2-15.3
		E. faecium	245	8	3.3	1.5-6.6
		E. faecalis +E. faecium	245	39	15.9	11.7-21.2
		Other	245	5	2.0	0.8-5.0
		Total	245	78	31.8	26.1-38.1
	Ndola	*E. faecalis*	140	19	13.6	8.6-20.6
		E. faecium	140	2	1.4	0.2-5.6
		E. faecalis +E. faecium	140	8	5.7	2.7-11.3
		E. faecalis +E. faecium + other	140	1	0.7	0.0-4.5
		E. faecalis + other	140	2	1.4	0.2-5.6
		Other	140	10	7.1	3.7-13.1
		Total	140	42	30.0	22.7-38.4

n = number, % = percent, CI = confidence interval, other = unidentified *Enterococcus* species

4.1.2.2 Proportion of *Enterococcus* in Farmworkers

Out of 13 *Enterococcus* species identified by PCR using genus-specific primers, 4 were *E. faecalis* (Table 4.3). The proportion of *E. faecalis* in specimens from farmworkers was 30.8% (CI: 10.36-61.13). Most of the farmworkers, i.e., 53.8% (CI: 26.13-79.59) had mixed infection of *E. faecalis*, *E. faecium* and other unidentified *Enterococcus* species.

Table 4.3 Proportion of *Enterococcus* in Farmworkers

Species	*n* Tested	*n* Positive	Proportion (%)	95% CI
Total specimens processed	111	13	11.7	6.63-19.54
E. faecalis	13	4	30.8	10.36-61.13
E. faecium	13	0	0	0
E. faecalis + *E. faecium*	13	7	53.8	26.13-79.59
Other *Enterococcus* species	13	2	15.4	2.71-46.33

n = number, % = percent, CI = confidence interval

4.1.2.3 Proportion of *Enterococcus* in Clinical Samples

The proportion of *Enterococcus* in clinical samples was 29.6% (CI: 23.84-35.98). Of these, *E. faecalis* made up 58.8%, while 14.7% (CI: 7.65-25.84) was *E. faecium.* Five isolates (7.4%) had *E. faecalis* and *E. faecium* species in them (Table 4.4). The remaining 19.1% were not identified at the species level.

Table 4.4 Proportion of *Enterococcus* in Clinical Samples

Categories	*n* Tested	*n* Positive	Proportion (%)	95% CI
Total specimens processed	230	68	29.6	23.84-35.98
E. faecalis	68	40	58.8	46.24-70.41
E. faecium	68	10	14.7	7.65-25.84
E. faecalis + *E. faecium*	68	5	7.4	2.74-17.02
Other *Enterococcus* species	68	13	19.1	10.96-30.83

n = number, % = percent, CI = confidence interval

4.1.3 Comparison of API and PCR Identification

A comparison between API and PCR identification results for select isolates from poultry, farmworker and clinical specimens was done to ascertain the agreement between the two methods. API correctly identified 24 (39.3%) of the 61 isolates (Table 4.5).

Table 4.5 Comparison between API and PCR Identities

Study ID	PCR ID	API ID	Study ID	PCR ID	API ID
Poultry (Lusaka Province)			Poultry (Copperbelt Province)		
80	*E. faecalis*	*E. faecalis*	361	*E. faecalis*	*E. faecalis*
82	*E. faecium*	*E. faecium*	399	*E. faecalis*	*E. faecalis*
84	*Efs + Efm*	*E. faecalis*	454	*Efs + Efm*	*E. faecium*
87	*E. faecalis*	*E. faecalis*	455	*Efs + Efm*	*E. faecalis*
88	*E. faecalis* + Other	*E. faecium*	476	*Efs + Efm*	*E. faecium*
89	Other *Enterococcus*	Not identified	477	*Efs + Efm*	*E. faecalis*
90	*E. faecium*	*E. faecium*	501	Other *Enterococcus*	Not identified
92	Other *Enterococcus*	*E. durans1*	506	*Efs + Efm*	Not identified
93	*E. faecalis*	*E. faecalis*	552	*Efs + Efm*	*E. faecalis*
94	*E. faecium*	*E. faecium*	555	*E. faecium*	*E. faecium*
96	*E. faecalis*	*E. faecalis*	576	*Efs + Efm*	*E. faecalis*
99	*Efs + Efm*	*E. faecium*	585	*Efs + Efm*	*E. faecium*
100	*Efs + Efm*	*E. faecium*	599	*E. faecalis*	Not identified
101	*Efs + Efm*	*E. faecium*	619	*E. faecalis*	*E. faecalis*
102	*E. faecalis*	*E. faecalis*	627	*E. faecium*	*E. faecium*
106	*E. faecalis*	*E. faecalis*	630	*Efs + Efm*	*E. faecalis*
107	*E. faecium*	*E. faecium*	702	*Efs + Efm*	*E. faecium*
Clinical Specimens (Copperbelt Province)			704	*Efs + Efm*	*E. faecalis*
149	*E. faecalis*	*E. faecalis*	714	*Efs + Efm*	*E. faecalis*
152	*E. faecalis*	*E. faecalis*	718	*Efs + Efm*	*E. faecium*
154	*E. faecalis*	*E. faecalis*	725	*E. faecalis*	*E. faecalis*
168	*Efs + Efm*	*E. faecalis*	734	*E. faecalis*	*E. faecalis*
179	Other *Enterococcus*	*E. durans1*	Farmworker (Copperbelt Province)		
252	Other *Enterococcus*	Not identified	748	*E. faecalis*	*E. faecalis*
291	*E. faecalis*	*E. faecalis*	755	Other *Enterococcus*	Not identified
305	*E. faecalis*	*E. faecalis*	763	*Efs + Efm*	*E. faecium*
315	*E. faecalis*	Not identified	765	*Efs + Efm*	*E. faecium*
317	*E. faecalis*	Not identified	767	*Efs + Efm*	*E. faecium*
320	*E. faecalis*	Not identified	770	*Efs + Efm*	*E. faecium*
326	*Efs + Efm*	Not identified	778	*E. faecalis*	*E. faecalis*
348	*E. faecalis*	Not identified	780	*Efs + Efm*	*E. faecium*
			830	Other *Enterococcus*	*E. faecalis*

PCR=Polymerase Chain Reaction, API = Analytical Profile Index, ID = Identity, *Efs = E. faecalis*, *Efm = E. faecium*

4.2 Antimicrobial Resistance

Interpretation of Antimicrobial susceptibility test results was done according to (Clinical and Laboratory Standards Institute of 2020. All intermediate test results were considered resistant, and they were indicated as such. Polymerase Chain Reaction to detect the presence of antimicrobial resistance genes was done on all *E. faecalis* and *E. faecium* DNA samples.

4.2.1 Antimicrobial Susceptibility of *E. faecalis* and *E. faecium*

In poultry isolates, both *Enterococcus faecalis* and *Enterococcus faecium* (97.3%) showed high resistance to tetracycline. Remarkably, 64.9% of both *E. faecalis* and *E. faecium* were susceptible to vancomycin. In farmworker isolates, only one *E. faecalis* isolate was susceptible to all tested antimicrobials, while one was resistant to all. All *E. faecalis* isolates from clinical specimens were resistant to ciprofloxacin and erythromycin. All *E. faecium* isolates showed phenotypic resistance to ampicillin, ciprofloxacin, erythromycin, nitrofurantoin, and penicillin. Generally, *E. faecium* isolates exhibited more resistance to all tested antimicrobials than *E. faecalis* isolates. The susceptibility profiles of *E. faecalis* and *E. faecium* to the eight antimicrobials tested are displayed in Table 4.6.

Table 4.6 Antimicrobial Susceptibility of *Enterococcus faecalis* and *Enterococcus faecium* in poultry, farmworker and clinical specimens

Species	Susceptibility Test Result	AMP n (%)	CHL n (%)	CIP n (%)	ERY n (%)	NIT n (%)	PEN n (%)	TET n (%)	VAN n (%)
Poultry									
E. faecalis (58)	Resistant	37 (63.8)	35 (60.3)	44 (75.9)	54 (93.1)	37 (63.8)	31 (53.4)	58 (100)	19 (32.8)
	Susceptible	21 (36.2)	23 (39.7)	14 (24.1)	4 (6.9)	21 (36.2)	27 (46.6)	0	39 (67.2)
E. faecium (16)	Resistant	14 (87.5)	6 (37.5)	13 (81.3)	16 (100)	8 (50)	8 (50)	14 (87.5)	7 (43.7)
	Susceptible	2 (12.5)	10 (62.5)	3 (18.7)	0	8 (50)	8 (50)	2 (12.5)	9 (56.3)
Farmworker									
E. faecalis (40	Resistant	3 (75)	1 (25)	2 (50)	3 (75)	2 (50)	3 (75)	3 (75)	1 (25)
	Susceptible	1 (25)	3 (75)	2 (50)	1 (25)	2 (50)	1 (25)	1 (25)	3 (75)
Clinical									
E. faecalis (40)	Resistant	27 (67.5)	39 (97.5)	40 (100)	40 (100)	30 (75.0)	30 (75.0)	39 (97.5)	36 (90.0)
	Susceptible	13 (32.5)	1 (2.5)	0	0	10 (25.0)	10 (25.0)	1 (2.5)	4 (10)
E. faecium (10)	Resistant	10 (100)	10 (100)	10 (100)	10 (100)	10 (100)	10 (100)	8 (80)	8 (80)
	Susceptible	0	0	0	0	0	0	2 (20)	2 (20)

n = number, AMP = ampicillin, CHL = chloramphenicol, CIP = ciprofloxacin, ERY = erythromycin, NIT = nitrofurantoin, PEN = penicillin, TET = tetracycline, VAN = vancomycin

4.2.1.1 Number of *E. faecalis* and *E. faecium* which were resistant to one or more classes of antimicrobials and their MDR Patterns

The *Enterococcus* isolates with multidrug resistance (MDR) were those resistant to three or more antimicrobial classes.

For poultry isolates, a total number of 74 *E. faecalis* (n = 58) and *E. faecium* (n = 16) were tested against eight antimicrobials. The majority of 52 (89.7%) of *E. faecalis* isolates were MDR. For *E. faecium*, only one isolate was resistant to two antibiotic classes. The rest were multidrug-resistant. For *E. faecalis* from farmworker specimens, only one isolate was susceptible to all tested antibiotics. The majority (75%) were MDR. The majority

(97.4%) of *E. faecalis* isolates of clinical origin were MDR, while 2.6% were resistant to only one class of antibiotics. All *E. faecium* isolates of clinical origin were MDR (Table 4.7).

Table 4.7 Number of isolates that were resistant to one, two, and more than two Antimicrobial Classes and MDR Patterns

Isolate (n)	All susceptible n (%)	Resistant to one antibiotic class, n (%)	Resistant to two antibiotic classes, n (%)	Resistant to more than two antibiotic classes, n (%)
Poultry				
E. faecalis (58)	0 (0)	2 (3.5)	4 (6.9)	52 (89.7)
E. faecium (16)	0 (0)	0 (0)	1 (6.3)	15 (93.7)
Farmworker				
E. faecalis (4)	1 (25)	0 (0)	0 (0)	3 (75)
Clinical				
E. faecalis (40)	0 (0)	0 (0)	1 (2.5)	39 (97.5)
E. faecium (10)	0 (0)	0 (0)	0 (0)	10 (100)
Total				
E. faecalis (102)	1 (1.0)	2 (2.0)	5 (4.9)	94 (92.1)
E. faecium (26)	0 (0)	0 (0)	1 (3.8)	25 (96.2)

n = number, % = percent

The MDR pattern exhibited by the majority of *E. faecalis* (23) and *E. faecium* (9) isolates was ampicillin, chloramphenicol, ciprofloxacin, erythromycin, nitrofurantoin, penicillin, tetracycline, vancomycin combination. Table 4.8 shows the most common combinations observed.

Table 4.8 Most common MDR patterns of *E. faecalis* and *E. faecium*

MDR Pattern	No. of Isolates	No. of Antibiotic Classes
E. faecalis		
AMP, CHL, CIP, ERY, NIT PEN, TET, VAN	23	7
AMP, CHL, CIP, ERY, NIT, TET, VAN	6	7
AMP, CHL, CIP, ERY, PEN, TET, VAN	4	6
AMP, CHL, CIP, ERY, NIT, PEN, TET	7	6
AMP, CIP, ERY, NIT, PEN, TET	5	5
CHL, CIP, ERY, NIT PEN, TET, VAN	5	6
CHL, CIP, ERY, NIT, TET	4	5
E. faecium		
AMP, CHL, CIP, ERY, NIT, PEN, TET, VAN	9	7

AMP = ampicillin, CHL = chloramphenicol, CIP = ciprofloxacin, ERY = erythromycin, NIT = nitrofurantoin, PEN = penicillin, TET = tetracycline, VAN = vancomycin

4.2.2 Presence of Antimicrobial Resistance Genes in *E. faecalis* and *E. faecium*

Polymerase Chain Reaction (PCR) using gene-specific primers was used to detect resistance genes present in *Enterococcus faecalis* and *Enterococcus faecium* DNA samples of poultry, farmworkers, and clinical origin. Figures 4.4a-d show representative images of selected resistance gene amplicons visualized on 1.5% agarose gels stained with Ethidium Bromide. A 100bp ladder was used as a gene ruler. The analysis detected the presence of some antimicrobial resistance genes in *Enterococcus faecalis* and *Enterococcus faecium* species.

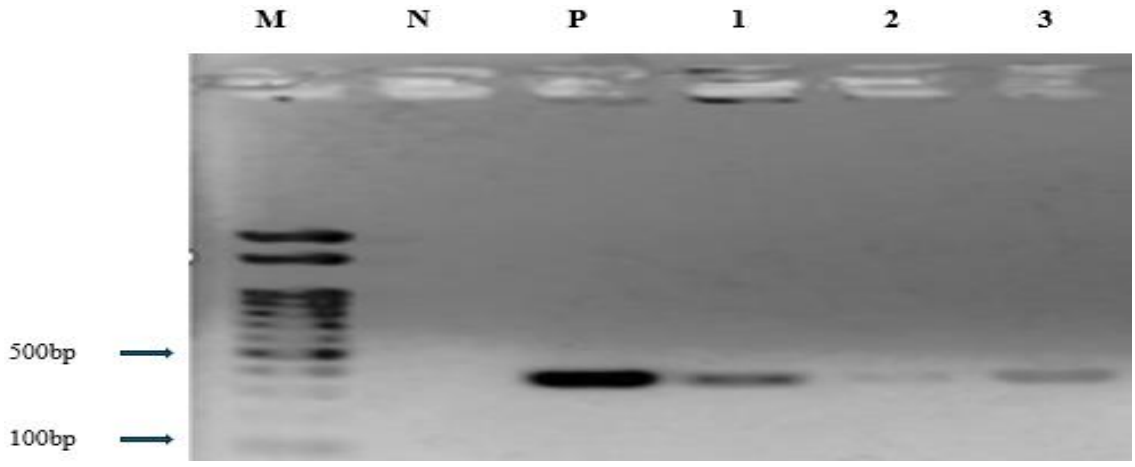

Figure 4.4a Representative agarose gel image showing the amplified 369bp *aac(6')-Ie-aph(2")-LA* resistance gene bands of *Enterococcus faecalis* and *Enterococcus faecium*. M = 100bp molecular marker, N = negative control, P = positive control (*Enterococcus faecalis* ATCC 29212 strain), 1,2,3 = samples

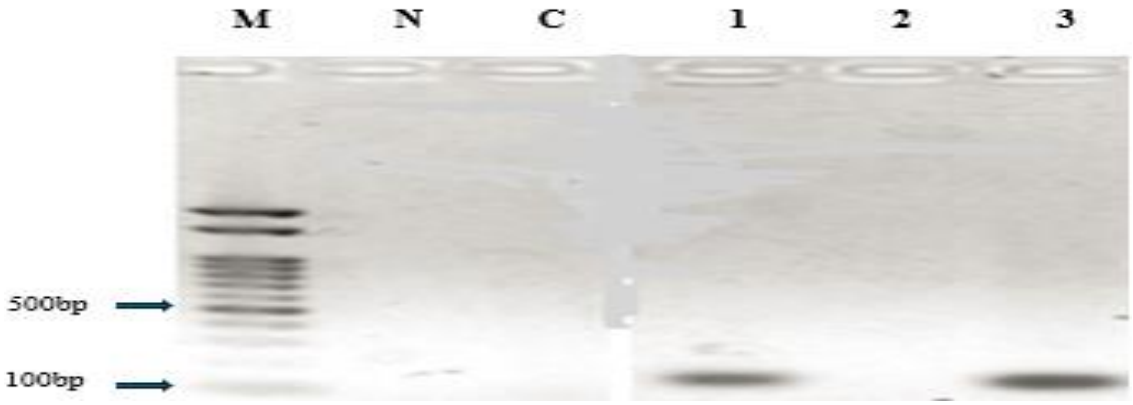

Figure 4.4b Representative Agarose gel image showing the amplified 139bp *ermA* resistance gene bands of *Enterococcus faecalis.* M = 100bp molecular marker, N = negative control, P = *Enterococcus faecalis* ATCC 29212 control strain), 1,2,3 = samples.

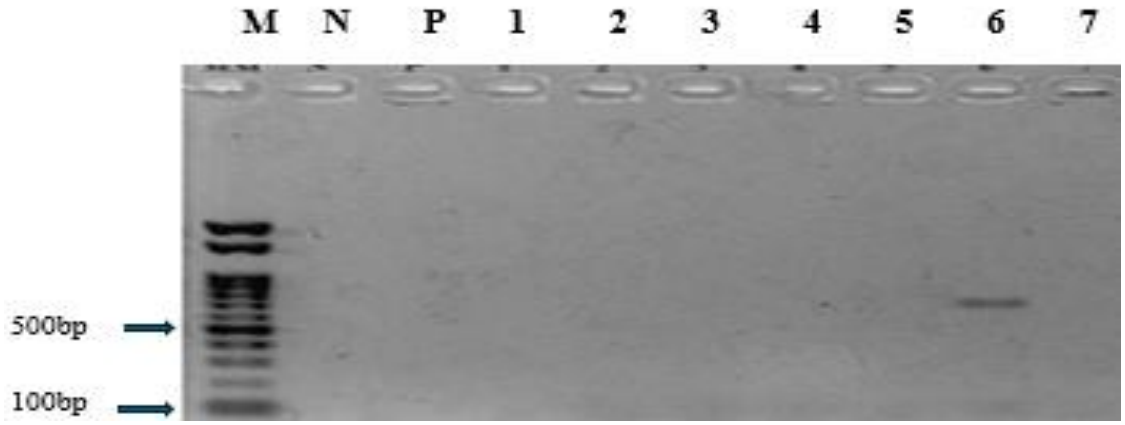

Figure 4.4c. Representative agarose gel image showing the amplified 751bp *vanA* resistance gene band of *Enterococcus faecalis.* M = 100bp molecular marker, N = negative control, P = *Enterococcus faecalis* ATCC 29212 control strain), 1, 2,3,4,5,6,7 = samples

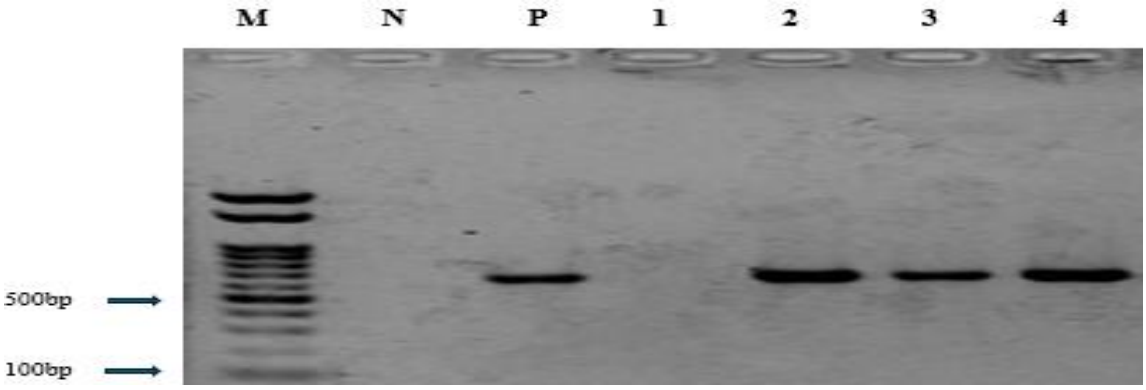

Figure 4.4d. Representative agarose gel image showing the amplified 657bp *tetM* resistance gene bands of *Enterococcus faecalis* and *Enterococcus faecium*. M = 100bp molecular marker, N = negative control, P = positive control (*Enterococcus faecalis* ATCC 29212 strain), 1,2,3,4 =6 samples

4.2.2.1 Antimicrobial Resistance Genes in *E. faecalis* and *E. faecium* from Poultry

The *aac (6')-Ie-aph (2")-LA* resistance gene encoding resistance to gentamycin was detected in E. *faecalis* (n = 33) and *E. faecium* (n = 12) isolates, representing 56.9% and 75.0% respectively. The most detected resistance gene in *E. faecalis* was *tetM* (77.6%), followed by *tetL* (70.7%), *tetK* (69.0%), *ermB* (60.3%) and *aac (6')-Ie-aph (2")-LA* (56.9%). In *E. faecium*, the *aac (6')-Ie-aph (2")-LA* resistance gene was the most frequently detected, followed by *ermB* (62.5%) and *tetM* (62.5%), then *tetK* (50.0%) and *tetL* (50.0). The *ermB* resistance gene was more common in both *E. faecalis* and *E. faecium* compared with the *ermA* gene. The *vanA* resistance gene was detected in only two *E. faecalis* isolates and none of the *E. faecium* isolates. Table 4.9 shows the number of different resistance genes detected in the *E. faecalis* and *E. faecium* isolates.

Table 4.9 Antimicrobial Resistance Genes Detected in *E. faecalis* and *E. faecium* of Poultry Origin

Resistance	*E. faecalis* (58)			*E. faecium* (16)		
	Detected, n	Proportion	Undetected, n	Detected, n	Proportion	Undetected, n
aac(6')-Ie-aph(2")-LA	33	56.9%	25	12	75.0%	4
ermA	1	1.7%	57	1	6.3%	15
ermB	35	60.3%	23	10	62.5%	6
tetK	40	69.0%	18	8	50.0%	8
tetM	45	77.6%	13	10	62.5%	6
tetL	41	70.7%	19	8	50.0%	8
tetX	3	5.2%	55	2	12.5%	14
vanA	*2*	*3.4%*	*56*	*0*	*0.0%*	*16*

n = number

4.2.2.1.1 Presence of Antimicrobial Resistance Genes in *E. faecalis* and *E. faecium* from Poultry across the Study Area

The *E. faecalis* species which harboured most of the resistance genes (80%) were from Ndola followed by Kitwe district with 68%. The *tetK* resistance gene was the most frequently detected antimicrobial resistance gene occurring in 68% of *E. faecalis* isolates from the Kitwe district, while the *tetM* antimicrobial resistance gene was most detected in 80% of *E. faecalis* isolates of the Ndola District. All *E. faecalis* isolates from the Lusaka district harboured *tetK* and *tetM* antimicrobial resistance genes. In the Chongwe district, however, it was the *tetL* antimicrobial resistance genes that were detected in all *E. faecalis*

isolates. The *E. faecium* species that harboured the majority of the antimicrobial resistance genes were from the Kitwe and Lusaka districts and ranged from 60 to 100% (3/5-5/5). The most frequently encountered antimicrobial resistance genes in *E. faecium* from the Kitwe district were *aac(6')-Ie-aph(2")-LA* and *ermB* genes followed by the *tetM* gene. In the Lusaka district, all (5/5) *E. faecium* isolates possessed *aac(6')-Ie-aph(2")-LA* gene, while 80% (4/5) possessed *ermB, tetL* and *tetX* antimicrobial resistance genes. Table 4.10 shows the distribution of resistance genes across the study area.

Table 4.10 Distribution of Antimicrobial Resistance Genes in *E. faecalis* and *E. faecium* of Poultry Origin across the study area

Province	District	Species	#RG	Resistance Genes in Poultry																TRG
				aac		*ermA*		*ermB*		*tetK*		*tetL*		*tetM*		*tetX*		*vanA*		
				Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	
Lusaka Province	Chongwe District	*E. faecalis*	5	4	1	0	5	3	2	4	1	5	0	5	0	0	5	1	4	5
		E. faecium	1	1	0	0	1	1	0	1	0	1	0	1	0	0	1	0	1	1
	Lusaka District	*E. faecalis*	8	6	2	0	8	5	3	8	0	7	1	8	0	7	1	0	8	8
		E. faecium	5	5	0	0	5	4	1	3	2	4	1	3	2	4	1	0	5	4
	Total	*E. faecalis*	13	10	3	0	13	8	5	12	1	13	0	13	0	1	12	1	12	13
		E. faecium	6	6	0	0	6	5	1	4	2	5	1	4	2	1	5	0	6	6
Copperbelt Province	Ndola District	*E. faecalis*	20	13	7	0	20	12	8	11	9	15	5	16	4	0	20	0	20	16
		E. faecium	2	1	1	0	2	2	0	1	1	2	0	2	0	0	2	0	2	2
	Kitwe District	*E. faecalis*	25	10	15	1	24	15	10	17	8	14	11	16	9	2	23	1	24	17
		E. faecium	8	5	3	1	7	5	5	3	5	1	7	4	4	1	7	0	5	5
	Total	*E. faecalis*	45	23	22	1	44	27	18	28	17	29	16	32	13	2	43	1	44	32
		E. faecium	10	6	4	1	9	5	5	4	6	3	7	6	4	1	9	0	10	6

#RG = number of isolates subjected to resistance gene detection, Pos = detected, Neg = undetected, TRG = total number of isolates which had resistance genes

4.2.2.2 Presence of Antimicrobial Resistance Genes in *E. faecalis* from Farmworker

The most frequently detected resistance gene in *E. faecalis* from farmworkers was *tetL*, followed by *tetX* being 100% and 50%, respectively. *aac(6')-Ie-aph(2")-LA*, *tetM* and *vanA* were detected in 75%, while ermA, tetM and vanA were not detected.

4.2.2.3 Presence of Antimicrobial Resistance Genes in *E. faecalis* and *E. faecium* from Clinical Specimens

The most detected resistant gene in *E. faecalis* from clinical specimens was *ermB* (97.5%). This was followed by *tetL* (95.0%) and *aac(6')-Ie-aph(2")-LA* (82.5%). *tetX* was absent in these isolates, while the occurrence of the *vanA* (2.5%) gene was infrequent. In *E. faecium*, the most commonly detected resistance gene was *aac(6')-Ie-aph(2")-LA* (90.0%) and *tetM* (80.0%), while *ermA* (10.0%) was infrequently detected and *tetX* and *vanA* were undetected (Table 4.11).

Table 4.11 Number of different Antimicrobial Resistance Genes Detected in *E. faecalis* and *E. faecium* from Clinical Specimens

Resistance gene	*E. faecalis* (40)			*E. faecium* (10)		
	Detected (n)	Proportion	Undetected	Detected (n)	Proportion	Undetected
aac(6')-Ie-aph(2")-LA	33	82.50%	7	9	90.00%	1
ermA	2	5.00%	38	1	10.00%	9
ermB	39	97.50%	1	5	50.00%	5
tetK	19	47.50%	21	4	40.00%	6
tetM	31	77.50%	9	8	80.00%	2
tetL	38	95.00%	2	5	50.00%	5
tetX	0	0	40	0	0	10
vanA	1	2.50%	39	0	0	10

N = number

4.3 Presence of Virulence Genes in *E. faecalis* and *E. faecium*

Virulence genes detected in *E. faecalis* and *E. faecium* were *ace*, *asa1*, *cad1*, *cylA*, *efm*, *esp*, *fsr* and *gelE*, and the expected bands for the PCR products were 298bp, 168bp,

423bp, 521bp, 818bp, 695bp, 1, 016bp and 208bp respectively. Figure 4.5a-g shows representative images of virulence gene amplicons visualized on 1.5% agarose gels embedded with approximately 0.2-0.5µg/ml of Ethidium Bromide. A 100bp ladder was used as a gene ruler.

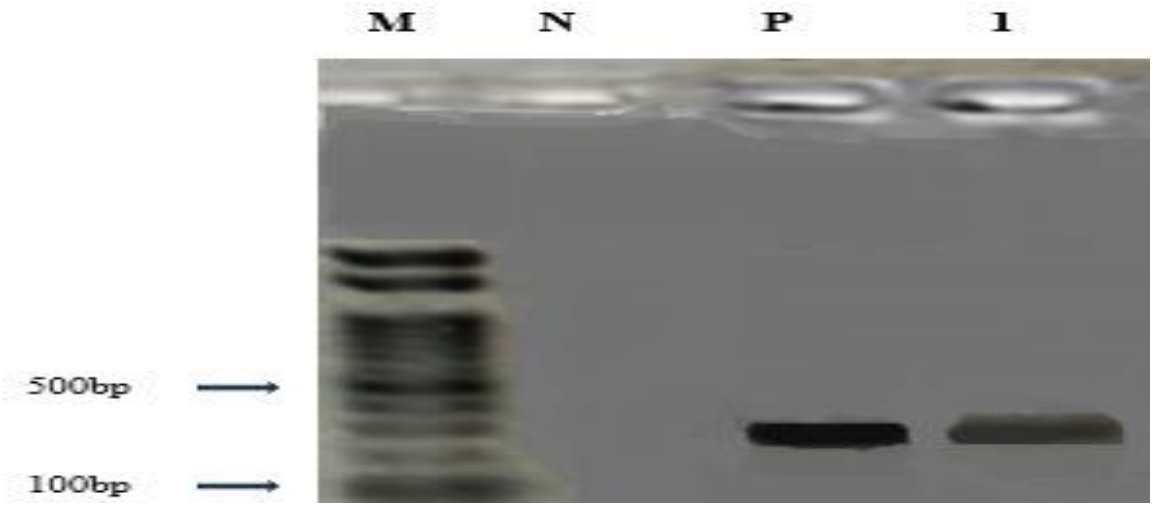

Figure 4.5a Representative agarose gel image showing the amplified 298bp *ace virule*nce gene bands of *Enterococcus faecalis* and *Enterococcus faecium*. M = 100bp molecular marker, N = negative control, P = positive control (*Enterococcus faecalis* ATCC 29212 strain), 1 = sample

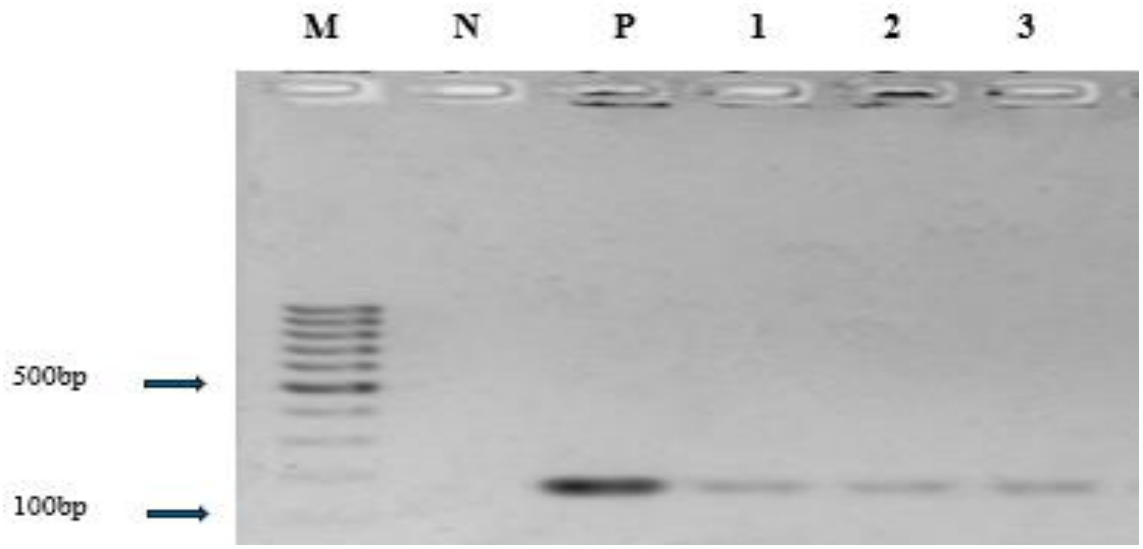

Figure 4.5b Representative Agarose gel image showing the amplified 168bp *asa1 virule*nce gene bands of *Enterococcus faecalis* and *Enterococcus faecium*. M = 100bp molecular marker, N = negative control, P = positive control (*Enterococcus faecalis* ATCC 29212 strain), 1,2,3 = 168bp samples

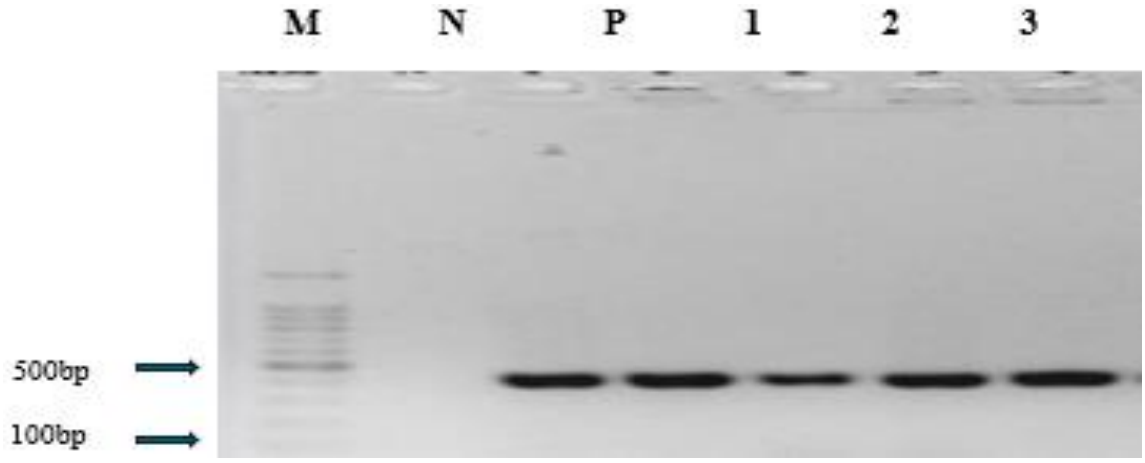

Figure 4.5c Representative agarose gel image showing the amplified 423bp *cad1 virule*nce gene bands of *Enterococcus faecalis* and *Enterococcus faecium*. M = 100bp molecular marker, N = negative control, P = positive control (*Enterococcus faecalis* ATCC 29212 strain), 1,2,3 = samples

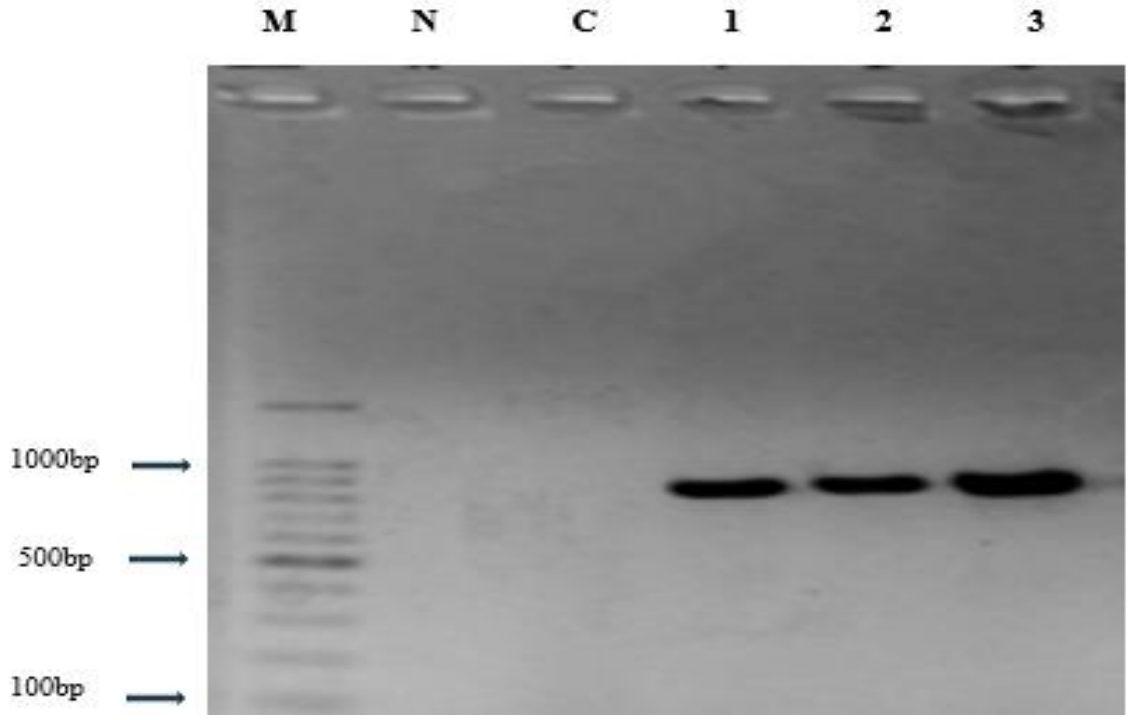

Figure 4.5d Representative agarose gel image showing the amplified 818bp *efm virule*nce gene bands of *Enterococcus faecium*. M = 100bp molecular marker, N = negative control (nuclease free water), C = Negative control (*Enterococcus faecalis* ATCC 29212 strain), 1,2,3 = samples

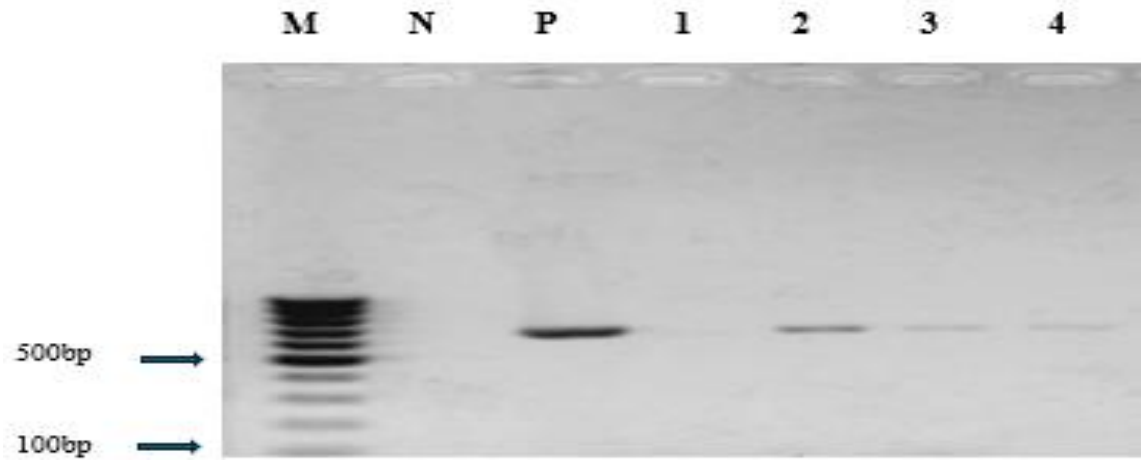

Figure 4.5e Representative agarose gel image showing the amplified 695bp *esp virule*nce gene bands of *Enterococcus faecalis* and *Enterococcus faecium*. M = 100bp molecular marker, N = negative control, P = positive control (*Enterococcus faecalis* ATCC 29212 strain), 1,2,3,4 = 695bp samples

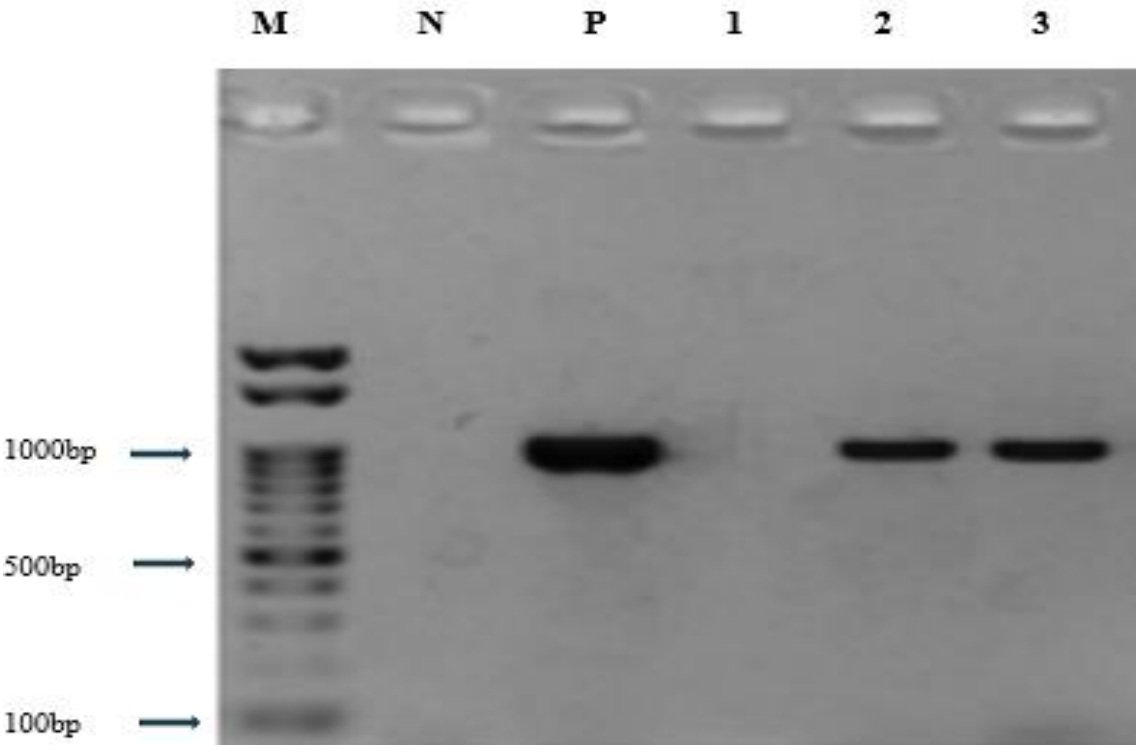

Figure 4.5f Representative agarose gel image showing the amplified 1016bp *fsr virule*nce gene bands of *Enterococcus faecalis* and *Enterococcus faecium*. M = 100bp molecular marker, N = negative control, P = positive control (*Enterococcus faecalis* ATCC 29212 strain), 1,2,3,4 = samples

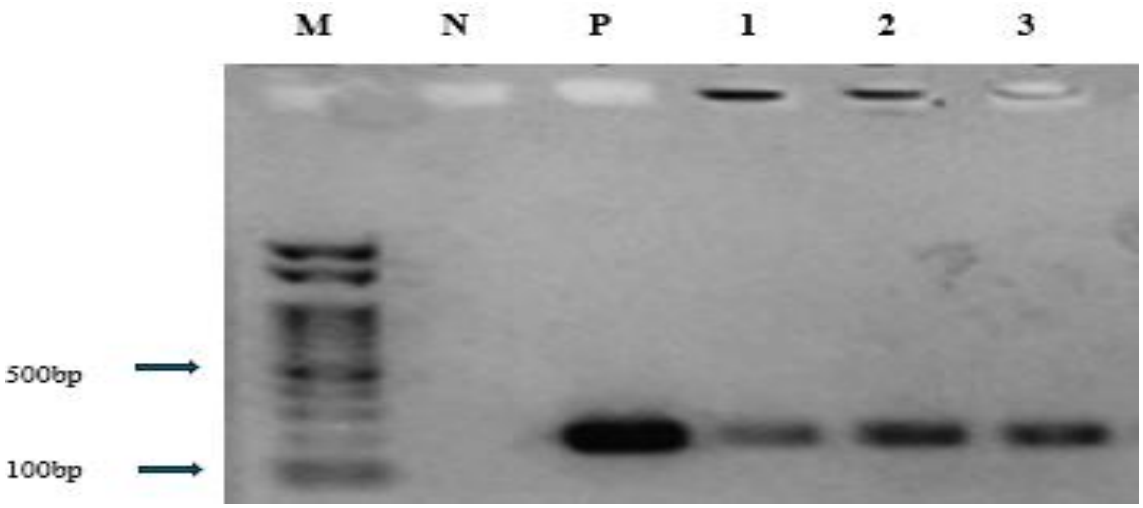

Figure 4.5g Representative agarose gel image showing the amplified 208bp *gelE virule*nce gene bands of *Enterococcus faecalis* and *Enterococcus faecium*. M = 100bp molecular marker, N = negative control, P = positive control (*Enterococcus faecalis* ATCC 29212 strain), 1,2,3 = samples

4.3.1 Presence of Virulence Genes in *E. faecalis* and *E. faecium* from Poultry

The *efm* virulence gene in addition to the seven were tested for in *E. faecium* isolates. Seven out of the 58 *E. faecalis* isolates harboured all seven virulence genes, 18 isolates had six genes, 19 had five, 13 had 4 genes and only one had 3 genes. For *E. faecium*, only one isolate out of the 16 had all virulence genes tested, two had three and the rest of the isolates had more than three genes. The adhesion of collagen from the *Enterococcus* (*ace*) gene, which is also known as collagen-binding cell wall protein, was the most frequently detected virulence gene in both *E. faecalis* (96.6%) and *E. faecium* (93.8%). The second most detected virulence gene in both *E. faecalis* (94.8%) and *E. faecium* (87.5%) species was pheromone cAD1 precursor lipoprotein (*cad1*) (Table 4.12)

Table 4.12 Virulence Genes Detected in *E. faecalis* and *E. faecium* from Poultry

Species	*ace* (%)	*asa1* (%)	*cad1* (%)	*cylA* (%)	*esp* (%)	*fsr* (%)	*gelE* (%)	*efm* (%)
E. faecalis (58)	56 (96.6)	51 (87.9)	55 (94.8)	29 (50)	26 (44.8)	43 (74.1)	47 (81.0)	-
E. faecium (16)	15 (93.8)	11 (68.8)	14 (87.5)	7 (43.8)	8 (50)	11 (68.8)	10 (62.5)	9 (56.3)

% = detection rate

4.3.1.1 Presence of virulence Genes in *E. faecalis* and *E. faecium* from Poultry across the Study Area

Most virulence genes were detected from *E. faecalis* species isolated from poultry droppings sampled from poultry farms of the Kitwe district in the Copperbelt Province. The *E. faecium* isolates, which harboured most virulence genes, were from the Lusaka District of Lusaka Province. Table 4.13 shows the distribution of virulence genes in *E. faecalis* and *E. faecium* across the study area

Table 4.13 Virulence genes detected in *E. faecalis* and *E. faecium* from Poultry across the study Area

Province	District	Species		*ace*	*asa1*	*cad1*	*cylA*	*esp*	*fsr*	*gelE*	*efm*
Lusaka Province	Chongwe	*E. faecalis*	POS	4	4	4	4	2	5	1	
			NEG	1	1	1	1	3	0	4	
		E. faecium	POS	1	0	1	0	1	1	0	1
			NEG	0	1	0	1	0	0	1	0
	Lusaka	*E. faecalis*	POS	8	5	8	3	2	8	8	
			NEG	0	3	0	5	6	0	0	
		E. faecium	POS	5	2	4	2	2	4	0	5
			NEG	0	3	1	3	3	1	5	0
Copperbelt Province	Kitwe	*E. faecalis*	POS	25	23	23	9	10	16	19	
			NEG	0	2	2	16	15	9	6	
		E. faecium	POS	7	7	7	3	3	4	8	2
			NEG	1	1	1	5	5	4	0	6
	Ndola	*E. faecalis*	POS	19	19	20	13	12	14	19	
			NEG	1	1	0	7	8	6	1	
		E. faecium	POS	2	2	2	2	2	2	2	1
			NEG	0	0	0	0	0	0	0	1

Pos = detected, Neg = undetected

4.3.2 Presence of Virulence Genes in *E. faecalis* from Farmworker Specimens

None of the *E. faecalis* isolates harboured *fsr* gene. Two of the isolates possessed 5, i.e., *asa1*, *cad1*, *cylA*, *esp*, and *gelE* while the other two had 4 virulence genes in two different combinations; one was *asa1*, *cad1*, *cylA* and *gelE* and the other being *asa1*, *cad1*, *esp*

and *gelE*. The adhesion of collagen from *Enterococcus* (*ace*), pheromone, aggregate substance (*asa1*), *cAD1* precursor lipoprotein (*cad1*), and gelatinase (*gelE*) was detected in all (100%), while *cylA* and *esp* were found in 75% of *E. faecalis*. The regulator of *gelE* and *sprE* expression (*fsr*) was the only virulence gene that was not detected in any of the *E. faecalis* isolates from farm workers.

4.3.3 Presence of virulence genes in *E. faecalis* and *E. faecium* from Clinical Specimens

Sixteen *E. faecalis* from clinical specimens had all the tested virulence genes, while the other 16 harboured 6, 6 possessed 5, and 2 carried 4 virulence genes. For *E. faecium*, 3 isolates possessed 7 genes, 2 harboured 6 genes, 3 had 5 and the remaining 2 carried 4 virulence genes. None of the *E. faecium* clinical isolates possessed all virulence genes. More than 70% of *E. faecalis* species possessed all virulence genes tested for, with *ace*, 97.5%,. being the most frequently detected. The most frequently detected virulence gene in *E. faecalis* was *collagen-binding cell wall protein* (*ace*), 97.5%. The *E. faecium-specific cell wall adhesin* (*efm*) was the most detected virulence gene in *E. faecium*. The detection rates for all virulence genes in *E. faecalis* and *E. faecium* are exhibited in Figure 4.6

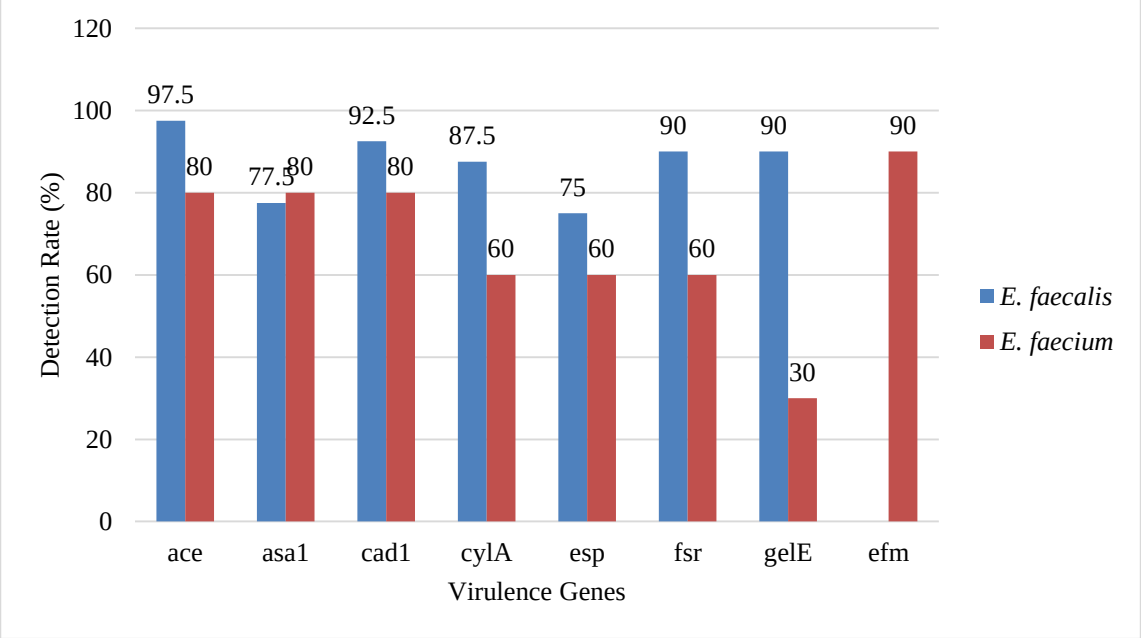

Figure 4.6 Virulence genes detected in *E. faecalis* and *E. faecium* from clinical specimens

CHAPTER FIVE

DISCUSSION

The preponderance of enterococcal infections in humans is attributable to *Enterococcus faecalis* and *Enterococcus faecium*. Their clinical significance has increased due to their resistance to a majority of commonly used medications, and they are now a leading cause of human infections (Ramos et al., 2020; Zhou et al., 2020). The study aimed to determine the occurrence, virulence genes, and antimicrobial resistance profiles of *E. faecalis* and *E. faecium* isolated from poultry droppings from the four districts, farmworkers from the Copperbelt Province poultry farms, and clinical specimens from Kitwe Teaching Hospital in Zambia.

5.1 Occurrence of *E. faecalis* and *E. faecium*

5.1.1 Occurrence of *E. faecalis* and *E. faecium* in Poultry Droppings

The overall occurrence of enterococci was 31.1%. This agrees with other studies that reported similar results in Poland (Dolka et al., 2017), and Nigeria (Ayeni et al., 2016). However, it was lower than that reported in a similar study conducted in Zambia, where the prevalence was 88.4% in laying hens ((Mudenda et al., 2022). This could be due to differences in sampling methods, the locality of farms sampled, and the number of farms sampled. Conversely, our prevalence rate was higher than reported in Ethiopia (Ferede et al., 2018), and Thailand (Noenchat et al., 2022). The differences in the isolation rates of *Enterococcus* can be attributed to antibiotic use, environmental factors, and methodology. The widespread use of antibiotics has led to the selection and dissemination of antibiotic-resistant enterococci. The environment plays a crucial role in the prevalence of *Enterococcus*. Different locations have varying environmental conditions, which can influence the growth and spread of these bacteria (Ferede et al., 2018). Enterococci are found in soil and water and can persist in the environment for long periods, making them more difficult to control and leading to increased isolation rates. The type of culture method used can influence the isolation rate and the presence of selective media may preferentially isolate enterococci (Suyemoto et al., 2017).

Among the *Enterococcus* species isolated in this study, *E. faecalis* was the most prevalent (37.9%), followed by *E. faecium* (10.5%). This agreed with the study by Stępień-Pyśniak (2016) which found *E. faecalis* to be the most prevalent species in poultry. However, our study differed slightly from another study that found species other than *E. faecalis* to be the most predominant (Rehman et al., 2018). The variations in species levels between studies might be due to differences in the type of poultry, source of chicks, sampling methods, geographical disparities, time of study, and isolation and identification procedures (Kwit et al., 2023).

5.1.2 Occurrence of *E. faecalis* and *E. faecium* in Farmworker Specimens

The overall occurrence of *Enterococcus* species from farmworkers was 11.7%. The occurrence of *E. faecalis* isolates among *Enterococcus* species was 30.8%. *E. faecium* was only detected in combination with *E. faecalis* and two species were not identified. For farmworker specimens, most *Enterococcus* isolates (83.1%, 59/71) were obtained from urine specimens. This is consistent with findings from similar studies conducted in India, Egypt and France (Said and Abdelmegeed, 2019; Sumangala et al., 2020; Mohanty and Behera, 2022) which reported that most *Enterococcus* species were from urine samples; 80.1%, 89/111; 54.4%, 56/103 and 59.5%, 25/42; 54.4% respectively. Possibly because most samples from farmworkers were urine specimens.

5.1.3 Occurrence of *E. faecalis* and *E. faecium* in Clinical Specimens

Of the 230 clinical specimens, 30.9% had *Enterococcus*. This finding aligns with a previous report by Iancu and others (2023) in Romania, which indicated the prevalence of *Enterococcus* to be 32.6%. However, our findings did not agree with an earlier study which reported a higher prevalence of 73.3% in Germany (Anderson et al., 2016). The detection rate in our study was higher than in reports from studies conducted in Ethiopia (2.1%) and Asia Pacific (3.6%) (Abera et al., 2021; Paul et al., 2021).

Enterococcus faecalis among all *Enterococcus* species accounted for 56.3% making it the most prevalent species while *Enterococcus faecium* accounted for 14.1%. This distribution agrees with other studies which found *E. faecalis* to be higher than *E. faecium*; 78.1% and 21.9% respectively in Northwest Iran (Jahansepas et al., 2018) and

82.2% and 17.8% respectively in Italy (Boccella et al., 2021). However, a study done by Horner and others (2021) shows a higher distribution of *E. faecium* (51%) than that of *E. faecalis* (43%). The differences in the distribution of *E. faecalis* or *E. faecium* may be due adaptation to environment, nutritional availability, competition and antimicrobial resistance. The two species thrive in specific niches within the gut or other environments (Cattoir, 2022). They both have different preferences for nutrient sources, which may contribute to their distribution, and as they compete for resources and space, they may outcompete each other based on their metabolic capabilities. Additionally, resistance to treatment of a species can impact its survival and prevalence.

The majority of *E. faecalis* and *E. faecium* isolates were from urine specimens. In agreement with this, the study by Boccella and others (2021) indicated that *E. faecalis* and *E. faecium* were isolated at a higher frequency from urine cultures. The majority of *E. faecalis* were from Urine specimens. *E. faecium* was only recovered from males and mainly from the urine of non-hospitalized male patients. Some factors may contribute to higher rates of *Enterococcus*-related UTIs in males. The prostate gland surrounds the male urethra. Infections in this area can lead to UTIs (John et al., 2016). Males often require urinary catheters more frequently than females due to various medical conditions (e.g., prostate enlargement, and surgery). Having a urinary catheter can increase the risk of UTI (Shuai and Li, 2021). Enlarged prostate (benign prostatic hyperplasia) or prostate infections can increase the risk of UTIs in males (Anggi et al., 2019). Bladder or kidney problems such as kidney stones or conditions that cause urine to pool and not drain properly and poor immune system may increase the risk of UTI in males (Smithson et al., 2019).

5.1.4 Comparison of API with PCR

Some researchers consider API 20 strep as the best identification system for bacteria (Rakotovao-Ravahatra, et al., 2022). It however does not accurately identify some *Enterococcus* species (Loong et al., 2020). API 20 strep results were compared with results PCR results. API 20 strep accurately identified 39.3% of *Enterococcus* species but identified only one species in isolates containing more than one species. It also misidentified 1.6% of the *Enterococcus* species. Our findings agreed with the results of

a previous study by Loong and others (2020) where nine *E. faecium* isolates were misidentified as *E. gallinarum* (n = 4), *E. durans*, (n = 3) and *leuconostoc* (n = 1). This shows the unreliability of biochemical methods for the correct identification of *Enterococcus*.

5.2 Antimicrobial Resistance Patterns and their associated antimicrobial-resistance genes of *E. faecalis* and *E*. faecium

5.2.1 Antimicrobial Resistance Patterns and their Associated Antimicrobial-Resistance Genes of *E. faecalis* and *E. faecium* from Poultry

Phenotypic resistance to critically important antimicrobials, as defined by the world Health Organization (Scott et al., 2019), was observed and 89.7% of *E. faecalis* and 93.8% of *E. faecium* isolates of poultry origin were multidrug resistant (MDR) (Table 11). Notably, all *E. faecalis* and *E. faecium* isolates of poultry origin were resistant to one or more of the tested antimicrobial classes (Table 11). These findings were similar to those of a study done earlier (Kim et al., 2021) in which the majority of *E. faecalis* and *E. faecium* isolates were resistant to one or more of the tested antimicrobials. Resistance to all tested antimicrobials was also observed in both *E. faecalis* and *E. faecium* isolates.

The distribution of resistance genes in *E. faecalis* and *E. faecium* of poultry origin differed according to the study site. The *tetK* resistance gene was the most frequently detected antimicrobial resistance gene in *E. faecalis* isolates from Kitwe District; while in Ndola District it was the *tetM* antimicrobial resistance gene that was mostly found. In *E. faecalis* from Lusaka district *tetK* and *tetM* were the mostly encountered antimicrobial resistance genes. In Chongwe district, however, it was the *tetL* antimicrobial resistance gene that was commonly detected. The majority of the antimicrobial resistance genes were found in *E. faecium* species from Kitwe and Lusaka districts. In the Kitwe district, the *aac (6')-Ie-aph (2")-LA* gene was the antimicrobial resistance gene most found. The ermB gene was the next most frequently detected. In the Lusaka district, all *E. faecium* isolates possessed the *aac (6')-Ie-aph (2")-LA* gene, while 80% possessed *ermB* and *tetL* antimicrobial resistance genes. The variation in predominant resistance genes detected

in poultry from different study sites may indicate differences in antibiotics used for growth promotion and treatment of poultry in different farms in those sites.

5.2.2 Antimicrobial Resistance Patterns and their associated antimicrobial-resistant genes of *E. faecalis* from Farmworkers

Twenty-five per cent of *E. faecalis* isolates from farmworkers were susceptible to all tested antimicrobials while 75% were MDR. Interestingly, the highest resistance in 75% of *E. faecalis* was expressed towards erythromycin, tetracycline, ampicillin and penicillin, while the least resistance was to chloramphenicol and vancomycin. This conformed with the study by Almeida-Villegas and others (2020) where erythromycin and tetracycline had the highest resistance, and with Ahmadpoor and others (2021) in which resistance to erythromycin was more common. This observation is not consistent with a report in which resistance to ampicillin and penicillin was observed in *E. faecium* of clinical origin and rarely in *E. faecalis* (Gagetti et al., 2019). Resistance to penicillin in *E. faecalis* is said to predominantly develop within high-risk enterococcal clonal complexes (HiRECCs) because of increased production of penicillin-binding protein 4 (PBP4) or genetic alterations (Gawryszewska et al., 2021; Timmler et al., 2022). This acquisition of penicillin resistance by HiRECCs could potentially signify a crucial step in the evolutionary path of *E. faecalis* as a nosocomial human pathogen (Gawryszewska et al., 2021). Penicillin resistance is said to be uncommon in non-clinical settings (Correa et al., 2022).

E. faecalis isolated from farmworker specimens was unlike *E. faecalis* isolates from Poultry and clinical specimens in that all isolates, i. e., 100% possessed *tetM* antimicrobial resistance gene, while 50% of the isolates possessed *tetX* and either *aac(6')-Ie-aph(2")-LA, ermB,* and *tetK* gene were detected in 25%. None of these isolates possessed *ermA, tetL* and *vanA* genes. This agreed with findings by Adeniji and others (2019) and Tian and others (2022) where the *tetM* resistance gene was found to be the most prevalent among *Enterococcus* species. Our findings were in contrast with the study by Safe and Feriala in which the most detected resistance gene was *tetK* (Safe and Feriala, 2022). The differences in the distribution of resistance genes may be due to

several factors, such as environmental conditions, human and animal activities, antibiotic use and misuse, horizontal gene transfer, and evolutionary processes.

5.2.3 Antimicrobial Resistance Patterns and their Associated Antimicrobial-Resistance Genes of *E. faecalis* and *E.* faecium from Clinical Specimens

More than 50.0% of *E. faecalis* isolates were resistant to all tested antimicrobials, while 100.0% of the isolates were resistant to tetracycline. On average, *E. faecium* exhibited increased resistance to antimicrobials in comparison with *E. faecalis*. Our findings are in agreement with a recent study conducted in Zambia (Mudenda et al., 2022). Our study also has some similarities with a study conducted in the Italy (Cagnoli et al., 2024), in which increased resistance of enterococci to tetracycline was observed. Furthermore, our results are consistent with findings from previous studies (Maasjost et al., 2015; Kim et al., 2018; Noh et al., 2020) where high tetracycline resistance was reported. Our results were also comparable with those of the study by (Ayeni et al., 2016), which recorded the resistance of Enterococci to erythromycin to be at 81.7% when intermediate results were included. Nevertheless, that study noted reduced resistance to tetracycline (38.3%) and chloramphenicol (5.7%) compared with our study. The observed increase in resistance to all the antimicrobials tested indicates that poultry from these four districts in Zambia can be a source of MDR enterococci. However, our study contrasted with other studies (Alduhaidhawi et al., 2022; Osman et al., 2023), which reported lower levels of resistance to antimicrobials.

In the present study, the highest levels of resistance observed in both *E. faecalis* and *E. faecium* were ciprofloxacin (100%) and erythromycin (100%). Our findings revealed that *E. faecium* exhibited higher resistance rates to most antimicrobial agents used in clinical treatment compared to *E. faecalis*. For example, all *Enterococcus faecium* isolates were resistant to ampicillin, chloramphenicol, ciprofloxacin, erythromycin, nitrofurantoin and penicillin whereas all *E. faecalis* isolates were resistant to just two drugs namely ciprofloxacin and erythromycin. These results align with previous reports (Golob et al., 2019; Tollu and Ekin, 2020; Horner et al., 2021) which supports the notion that *E. faecium* is generally more prone to develop resistance than *E. faecalis*. The infections caused by *E. faecium* pose significant clinical challenges for physicians due

to their higher resistance to drugs commonly used in clinical practice thereby limiting treatment options (Zhou et al., 2020). All (100%) *E. faecium* and 97.5% *E. faecalis* isolates were multidrug-resistant (MDR). This is concurrent with studies by (Esmail et al., 2019) in which 100% of all *E. faecalis* and *E. faecium* isolates respectively were MDR. The selective pressure exerted by the broad application of antibiotics in health care and animal husbandry enhances the development of MDR. This leads to an increase in the prevalence of resistance and the creation of reservoirs of resistance genes in some *Enterococcus* species, especially *E. faecalis* and *E. faecium* (Gousia et al., 2015).

Enterococci exhibiting resistance to erythromycin possess erythromycin resistance methylase (*erm*) genes, namely *erm(A), erm(B),* and *erm(C)*. These genes express a ribosomal RNA methylase that alters the ribosomal RNA and renders it inaccessible to erythromycin (Celik et al., 2014). The *erm (B)* gene is the most frequently observed among erythromycin-resistant enterococci. The most prevalent resistance gene in *E. faecalis* of clinical origin was *ermB* (97.5%), followed by *tetM* (95.0%) and *aac (6')-Ie-aph (2")-LA* (82.5%). These findings were in tandem with findings in a study by (Marghmalek et al., 2021) in which *ermB, tetM* and *aac (6')-Ie-aph (2")-LA* pattern of distribution was the same but with lower percentages. In that study resistance genes were distributed in the following manner: *ermB* (66.6%), *tetM* (60%), and *aac (6')-Ie-aph (2")-Ia* (28.8%).

In *E. faecium* of clinical origin, the most frequently detected resistance genes were *aac (6')-Ie-aph (2")-LA* (90.0%) and tetL (80.0%). The detection of *aac (6')-Ie-aph (2")-LA* gene in 82.5% of *E. faecalis* and 90.0% of *E. faecium* was in line with Niu and others (2016) who found that 89.3% of *Enterococcus* isolates in their study carried the *aac (6')-Ie-aph (2")-LA* gene. Other studies reported lower detection rates of the *aac (6')-Ie-aph (2")-LA* gene. These include Khani and others (2016) who conducted research in which 32.8% of *E. faecium* isolates and 67.2% of *E. faecalis* isolates carried *aac(6')-Ie-aph(2")-LA* gene and Moussa and others (2019) where the *aac(6')-Ie-aph(2")-Ia* resistance gene was detected in 40% of *E. faecalis* and *E. faecium* isolates.

Concerning *Enterococcus faecalis* strains of clinical origin, which were resistant to erythromycin, among the detected *erm* genes, *ermB* took precedence as the most prevalent. This discovery echoed the outcomes of prior studies (Tian et al., 2019; Ahmadpoor et al., 2021; Marghmalek et al., 2021). In a similar vein, the gene *tetM* emerged as the frequently identified element among *Enterococcus faecalis* isolates displaying resistance to tetracycline, in alignment with the conclusions drawn by Tian and Others (2019).

In clinical isolates, the *vanA* gene was only detected in one *E. faecalis* isolate and was not detected in *E. faecium* isolates. The phenotypic resistance exhibited by both *E. faecalis* and *E. faecium* could be due to other *van* genes that were not tested. This shows that *vanA* was not common in *Enterococcus* species in our study and is in contrast with what other studies asserted. For example, Haghi and others (2019) detected the *vanA* gene in all *E. faecium* isolates. Jahansepas and others (2018) showed that the *vanA* gene was more predominant than other *van* genes.

5.2.4 Association of Resistance Phenotypes and Genotypes in *E. faecalis* and *E. faecium*

The associations between antimicrobial resistance phenotypes and genotypes in *E. faecalis* and *E. faecium* isolates were analysed. Associations were found between genotypes of tetracycline and erythromycin-resistant phenotypes. However, genotypes showed no relationship with vancomycin-resistant phenotypes. The disparity observed between the phenotypes and genotypes in the case of vancomycin could be because vancomycin resistance in *Enterococcus* can be conferred by different gene clusters (Das et al., 2022). It is therefore likely that the observed phenotypic resistance was due to van genes other than *vanA.*

Despite identifying the gene *aac(6')-Ie-aph(2")-LA*, which confers resistance to gentamicin, in both species of *Enterococcus* under investigation, it remained challenging to establish a direct link between the observed resistance traits and the specific genetic makeup. This challenge stemmed from the unavailability of gentamicin discs containing

higher concentrations (e.g., 120 µg or 500 µg), which are typically employed to pinpoint substantial levels of aminoglycoside resistance.

5.3 Presence of Virulence Genes in *E. faecalis* and *E. faecium*

5.3.1 Presence of Virulence Genes in *E. faecalis* and *E. faecium* from Poultry

In poultry, the *ace* gene emerged as the most commonly identified virulence gene in both *E. faecalis and E. faecium*, with detection rates of 96.6% and 93.8%, respectively. Following closely were the *cad1* gene at 94.8% and 87.5%, ranking as the second most prevalent in both species. In *E. faecalis*, the third most prevalent gene was *asa1* (87.9%), succeeded by *gelE* (81%), *fsr* (74.1%), *cylA* (50%), with the *esp* gene being the least detected (44.8%). For *E. faecium*, the third most prevalent genes were *asa1* and *fsr*, both at 68.8%, followed by *gelE* (62.5%), *efm* (56.3%), with *esp* (50%) and *cylA* (43.8%) being the least detected. These findings align with a study conducted in Northern Japan, which reported higher prevalence rates of *asa1*, *efaA*, and *ace* in *E. faecalis* (46–59%) compared to *E. faecium* (Aung et al., 2023). They also agreed with the study by Shokoohizadeh and others (2018) in which *gelE,* and *asa1* genes were the most prevalent virulence genes in *E. faecalis (48.5%),* and *E. faecium* (43%) isolates. The high prevalence of *ace* and *cad1* virulence genes in poultry-associated *E. faecalis* and *E. faecium* highlights their potential role in poultry colonization and potential transmission to humans through the food chain. A study in Kenya reported virulence gene prevalence rates among 44 *Enterococcus* isolates, with 61.4% for *gelE*, 59.1% for *asa1*, 36.3% for *esp*, 25.0% for *cylA*, and 2.3% for *hyl* (Georges et al., 2022). This partially aligns with our findings, as both *asa1* and *gelE* were among the most prevalent genes in both *E. faecalis and E. faecium* species. This suggests that *E. faecalis* tends to carry more virulence genes than *E. faecium*. The differences in geographical location and the source of the isolates could explain the variation in the prevalence of virulence genes in *E. faecalis* and *E. faecium*.

5.3.2 Presence of Virulence Genes in *E. faecalis* from Farmworkers

The *ace, asa1*, *cad1,* and *gelE* genes were detected in all (100%) of the *E. faecalis* isolates from farmworker specimens. This indicated that these isolates could potentially colonize and cause infections. The *cylA* and *es*p genes were found in 75% of these

isolates, while the *fsr* gene was not detected. This aligns with a study by Nasaj and others that investigated clinical specimens in Hamadan hospitals, western Iran in 2012–14 where the gene *asa1* was 100% and 97% in *E. faecium* and *E. faecalis* respectively (Nasaj et al., 2016), and also the study by Lins and others (2019) where *gelE* was found in 100% of isolates.

5.3.3 Presence of Virulence Genes in *E. faecalis* and *E. faecium* from Clinical Specimens

Enterococccus faecalis tends to attach to non-living surfaces (abiotic surfaces) within the hospital environment. This attachment behaviour is associated with a higher occurrence of genes that encode virulence factors involved in biofilm formation (Too and Masila, 2024). These virulence factors include enterococcal surface protein, aggregation substance, and gelatinase. Biofilms formed by *E. faecalis* can be particularly resilient and contribute to persistent infections. Our study revealed that 40% of *E. faecalis* species from clinical samples possessed all virulence genes tested for, with *ace* being the most frequently detected at 97.5%. This was followed by *cad1* (92.5%), then, *fsr* and *gelE* both at 90.0%, *cylA* at 87.5%, *asa1* at 77.5% and *esp* was the least detected at 75.0%. A study conducted in Slovenia indicated that each clinical isolate of *E. faecalis* and *E. faecium* harboured two or more virulence genes, with *gelE*, *efaA*, and *asa1* being the most frequently detected ones (Golob et al., 2019). These findings similar to the present study where *gelE* and *asa1* were the commonly detected, though in higher rates. Our findings also agree with a study (Ullah et al., 2023) in which *ace* was detected at 92.7%. A study by Heidari and others (2017) found the prevalence of *ace*, *gelE* and *cylA* genes to be 90.2%, 80.4% and 64.7%, respectively. This agreed with our findings of the *gelE* gene in 90% of *E. faecalis* isolates. In contrast to our findings, Strateva and others (2016) reported lower prevalence of *asa1*(38.4%), *ace* (64.3%), *gelE* (64.3%) and *cylA* (47.1%). *E. faecalis* had higher frequencies of *esp*, *asa1* and *gelE* in comparison to *E. faecium*. Our findings revealed that *E. faecalis* isolates possessed a greater number of virulence genes compared to other studies (Niu et al., 2016; Strateva et al., 2016). These differences probably reflect variability in the types of samples or geographic regions.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

1. The study demonstrated a higher isolation rate of *E. faecalis* in both poultry and human specimens than other *Enterococcus* species.
2. All *E. faecalis* and *E. faecium* from clinical specimens showed more resistance than those from poultry and farmworker specimens and exhibited ciprofloxacin and erythromycin resistance.
3. *E. faecium* showed more resistance to antimicrobials than *E. faecalis.*
4. A high frequency of genotypic aminoglycoside resistance was detected among *E. faecium* isolates.
5. The distribution of virulence genes among *E. faecalis* isolates was higher than in *E. faecium* showing a higher virulence potential of *E. faecalis* than *E. faecium.*
6. Erythromycin and tetracycline resistance genes were more prevalent in *E. faecalis*.
7. Genes that encode virulence factors were more prevalent in *E. faecalis* strains isolated from clinical samples.
8. Nearly all *E. faecalis* and *E. faecium* isolates carried the *ace* gene signifying their ability to colonize and cause infection.
9. Many resistance and virulence genes have been detected, indicating that future infections caused by these strains could potentially lead to serious illnesses that are difficult to treat.

6.2 Limitations

1. The samples may not represent the population as they were conveniently sampled. Therefore, the findings are not generalizable.
2. Farmworkers and clinical samples were not randomly selected hence the possibility of sampling bias.
3. Some *Enterococcus* could not be identified at the species level due to a lack of additional species-specific primers and limited DNA Sequencing reagents.

4. This study lacks an analysis of the phenotypic expression of *Enterococcus*'s virulence factors, which would have contributed to the significance of the detected virulence genes.

6.3 Recommendations

1. Use of media such as Slanetz and Bartley Agar that differentiates species of *Enterococcus* by colony appearance could help detect poly infections or multiple colonization which could further aid phenotypic identification.
2. Random as opposed to convenient sampling of clinical samples for purposes of generalization of results is recommended.
3. Determination of phenotypic expression of virulence genes is highly recommended.

6.3.1 Future Research

1. Further studies should be carried out for a better understanding of the association between the presence of virulence determinants and the emergence of multidrug-resistant enterococci
2. Further research is needed to explore potential reasons behind the variations in the virulence genes present in *E. faecalis* and *E. faecium*.

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APPENDICES

Appendix 1: Demographic Information

Appendix 1.1 Demographic Information of Poultry with *Enterococcus* Species

The demographic distribution of *Enterococcus* species isolated from poultry in Lusaka Province reveals notable patterns based on age and poultry type. In the age-based analysis, poultry aged between 10 and 30 weeks old yielded the highest number of *Enterococcus* species, with a subsequent decline in isolates among those aged 31 to 50 weeks. Interestingly, the age group from 51 to 70 weeks old presented the second-highest number of isolates. When examining the distribution by poultry type, layers emerged as the predominant source of *Enterococcus* species in Lusaka Province. Layers yielded more species than both broilers and village chickens. Within this analysis, *E. faecalis* was particularly prevalent among layers, followed by broilers and village chickens. In the Copperbelt Province, all samples came from layers. The age-based analysis indicates that the age group of 31 to 50 weeks old had the highest number of *Enterococcus* species, followed by 51 to 70 weeks old and 71 to 90 weeks old. Notably, the age group of 91 to 100 weeks old yielded the least number of *Enterococcus* species. Table A1 exhibits the distribution of *Enterococcus* species isolated from poultry in Lusaka and on the Copperbelt Provinces.

Table A1 Distribution of *Enterococcus* species from poultry

Province	Age group (Weeks)	Total Number	*Efs*	*Efm*	*Efs* + *Efm*	*Efs* + *Efm* + Other	*Efs* + Other	*Efm* + Other	Other
Lusaka	13-22	15	7	3	1	0	1	0	3
	23-62	11	5	1	1	0	2	0	2
	63-92	7	2	2	3	0	0	0	0
	Total	33	14	6	5	0	3	0	5
Copperbelt	13-22	13	6	0	3	0	0	0	4
	23-62	93	37	6	32	1	1	0	16
	63-98	30	10	4	16	0	0	0	0
	Total	136	53	10	51	1	1	0	20

Efs = *E. faecalis*, *Efm* = *E. faecium*, other = Unidentified *Enterococcus* species

The age categorization of poultry in Table 8.1 was based of egg production. The earliest age at which a layer can produce an egg depending on bleed is believed to be about 14 weeks (https://blog.meyerhatchery.com/2020/06/signs-that-a-pullet-will-begin-laying-soon/) and the oldest age at which a layer can start producing eggs is about 22 weeks (https://grubblyfarms.com/blogs/the-flyer/when-do-chickens-start-laying-eggs?srsltid=AfmBOoqgmnHhN33pv-Jkka5s-c7GG6P7WZbxDJKjIY1KiyhZoWaYRgNg). The peak production age of layers ranges from 23 to 63 weeks, thereafter the production starts reducing (Yakubu and Aguda, 2020).

Appendix 1.2 Demographic Information of Farmworkers with *Enterococcus* species

Limited demographic information was available because participants declined to give personal information. However, a gender-based observation revealed that out of 111 farmworkers who submitted specimens, only one was female. Among the 110 male farmworkers, 12 specimens yielded *Enterococcus* species. As shown in Table A2, the only sample from the female farmworker yielded more than two *Enterococcus species.*

Table A2 Distribution of *Enterococcus* species from farmworkers

Gender	**Total No. of *Enterococcus* isolates**	***E. faecalis***	***E. faecalis* + *E. faecium* + Other**	**Other**
Female	1	0	1	0
Male	12	4	6	2

Appendix 1.3 Demographic Information of Clinical Specimens with *Enterococcus* species

In the analysis of clinical specimens, male patients were found to be more commonly associated with *Enterococcus* species. The age group of 31 to 45 years old yielded the highest number of isolates, followed by the 16 to 30 years old category. Furthermore, urine specimens exhibited a higher number of isolates compared to stool specimens. Notably, patients from the Outpatient Department (OPD) contributed the highest number of *Enterococcus* species (Table A3).

Table A3 Distribution of *Enterococcus* species from clinical specimens

Age range	Total n of specimens processed (230)	*Total n of Enterococcus* isolates (71)	*E. faecalis* (40)	*E. faecium* (10)	*E. faecalis* + *E. faecium* (5)	Other *Enterococcus* species (16)
2-15	51	9	4	1	0	5
16-30	70	19	9	3	1	6
31-45	62	20	12	4	2	2
46-60	14	6	5	0	1	0
61-75	23	12	8	1	0	3
76-86	7	2	1	1	0	0
Not indicated	3	2	1	0	1	0
Gender						
Male	103	39	21	10	1	7
Female	127	32	19	0	4	9
Specimen type						
Urine	138	59	37	7	3	12
Stool	89	11	3	2	2	4
Pus swab	3	1	0	1	0	0
Department						
Out-patient	150	51	30	9	1	11
In-patient	80	20	10	1	4	5

Appendix 2: Species confirmed by DNA Sequencing

Appendix 2.1 Confirmation of species identity

A total of nine PCR species-specific identified *E. faecalis* DNA samples were randomly selected, amplified, purified and sequenced to confirm the PCR species identification results. After sequencing, the sequences were edited using BioEdit software. Using the BLAST search on the National Centre for Biotechnology Information (NCBI) website, the sequences were compared with reference sequences in the GenBank. The nine DNA samples confirmed by DNA Sequencing are shown in Table A4 with their study IDs, Gene Accession numbers, Sequence identity percentage and species identity obtained after comparing with those available in the GenBank using BLAST search.

Table A4. Selected DNA samples confirmed by DNA Sequencing

Serial No.	Study ID	Source	Gene Accession No.	Percent Sequence ID (%)	Species Identity
1	263	Poultry Lusaka	CP051005.1	100	*Enterococcus faecalis* strain TH4125 chromosome
2	271	Poultry Lusaka	CP051005.1	100	*Enterococcus faecalis* strain TH4125 chromosome
3	361	Clinical	LR607329.1	99.77	*Enterococcus faecalis* strain 4928STDY7071325 genome assembly, chromosome
4	385	Clinical	CP051005.1	99.39	*Enterococcus faecalis* strain TH4125 chromosome
5	531	Poultry Coppebelt	CP051005.1	100	*Enterococcus faecalis* strain TH4125 chromosome
6	593	Poultry Coppebelt	CP030042.1	99.97	*Enterococcus faecalis* strain C25 chromosome
7	748	Farmworker	CP051005.1	100	*Enterococcus faecalis* strain TH4125 chromosome
8	778	Farmworker	LR607371.1	99.68	*Enterococcus faecalis* strain 4928STDY7387718 genome assembly
9	804	Farmworker	CP136353.1	99.56	*Enterococcus faecalis* strain ES-1390 chromosome

ID = Identity, % = percent

The *Enterococcus faecalis* isolated from Lusaka, Study IDs 263 and 271 were 100% similar to a clinical isolate 385 from Kitwe Teaching Hospital, Poultry isolate 531 and farmworker isolate 748 from Copperbelt farms. Despite being from the Copperbelt Province, isolates 531 and 748 were not from the same farm.

Appendix 2.2 Representative sequence compared with Reference Sequence in the GenBank using BLAST search.

***Enterococcus faecalis* strain TH4125 chromosome, complete genome**

Sequence ID: CP051005.1 Length: 2993403 Number of Matches: 1

Range 1: 1464348 to 1464818 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
870 bits(471)	0.0	471/471(100%)	0/471(0%)	Plus/Plus

```
Query  1        GTGGAGTTTGAAAGAGAAAAAGGTTGACGTTTTTCAGAATAAAATGACCATCTTAGGAAC  60
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1464348  GTGGAGTTTGAAAGAGAAAAAGGTTGACGTTTTTCAGAATAAAATGACCATCTTAGGAAC  1464407

Query  61       AGGTGGTGCAGCCTTATCAATCATTGCCCAAGCTGCTTTAGATGGCGTGAAAGAAATCGC  120
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1464408  AGGTGGTGCAGCCTTATCAATCATTGCCCAAGCTGCTTTAGATGGCGTGAAAGAAATCGC  1464467

Query  121      CGTTTACAACAGGAAAAGCGCGGGCTTTAACGACAGTCaaaaaaaaCTGGCAAATTTCAC  180
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1464468  CGTTTACAACAGGAAAAGCGCGGGCTTTAACGACAGTCAAAAAAAACTGGCAAATTTCAC  1464527

Query  181      TGAACGAACCAACTGTGTAATCCATTTAAATGATTTAGCGGATACTGAAAAACTAGCAAA  240
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1464528  TGAACGAACCAACTGTGTAATCCATTTAAATGATTTAGCGGATACTGAAAAACTAGCAAA  1464587

Query  241      AGATGTTGCTGAAAGCGTCTTGTTAGTTAATGCAACGAGTGTGGGTATGCATCCACATGC  300
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1464588  AGATGTTGCTGAAAGCGTCTTGTTAGTTAATGCAACGAGTGTGGGTATGCATCCACATGC  1464647

Query  301      GCATAGTAGTCCTATAGAAAATTATGCAATGATTCAACCGAAGTTATTTGTGTATGATGC  360
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1464648  GCATAGTAGTCCTATAGAAAATTATGCAATGATTCAACCGAAGTTATTTGTGTATGATGC  1464707

Query  361      TATTTATAATCCCAGAGAAACACAGTTATTAAAAGAAGCCCGTTTACGTGGCGCAGAAAC  420
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1464708  TATTTATAATCCCAGAGAAACACAGTTATTAAAAGAAGCCCGTTTACGTGGCGCAGAAAC  1464767

Query  421      AAGCAACGGCTTGGACATGCTACTTTATCAAGGCGCTGTTGCTTTTGAACA  471
                ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1464768  AAGCAACGGCTTGGACATGCTACTTTATCAAGGCGCTGTTGCTTTTGAACA  1464818
```

Appendix 3: UNZABREC Approval

UNIVERSITY OF ZAMBIA
BIOMEDICAL RESEARCH ETHICS COMMITTEE

Telephone: 260-1-256067
Telegrams: UNZA, LUSAKA
Telex: UNZALU ZA 44370
Fax: + 260-1-250753
Federal Assurance No. FWA00000338

Ridgeway Campus
P.O. Box 50110
Lusaka, Zambia
E-mail: unzarec@unza.zm
IRB00001131 of IORG0000774

16th July, 2020.

Your REF. No. 797-2020.

Ms. Grace Mwikuma,
University of Zambia,
School of Veterinary Studies,
Department of Paraclinical studies,
P.O Box 32379,
Lusaka.

Dear Ms. Mwikuma,

RE: "IDENTIFICATION OF CLONAL COMPLEXES, DISPERSION AND ANTIMICROBIAL RESISTANCE OF ENTEROCOCCI FROM POULTRY AND HUMANS IN LUSAKA DISTRICT OF ZAMBIA" (REF NO. 797-2020)

The above-mentioned research proposal was presented to the Biomedical Research Ethics Committee on 15th July, 2020. The proposal is **approved**. The approval is based on the following documents that were submitted for review:

a) **Study proposal**
b) **Questionnaires**
c) **Participant Consent Form**

APPROVAL NUMBER : REF. 797-2020

This number should be used on all correspondence, consent forms and documents as appropriate.

- **APPROVAL DATE : 15th July 2020**
- **TYPE OF APPROVAL : Standard**
- **EXPIRATION DATE OF APPROVAL : 14th July 2021**
  After this date, this project may only continue upon renewal. For purposes of renewal, a progress report on a standard form obtainable from the UNZABREC Offices should be submitted one month before the expiration date for continuing review.
- **SERIOUS ADVERSE EVENT REPORTING**: All SAEs and any other serious challenges/problems having to do with participant welfare, participant safety and study integrity must be reported to UNZABREC within 3 working days using standard forms obtainable from UNZABREC.
- **MODIFICATIONS**: Prior UNZABREC approval using standard forms obtainable from the UNZABREC Offices is required before implementing any changes in the Protocol (including changes in the consent documents).

- **TERMINATION OF STUDY**: On termination of a study, a report must be submitted to the UNZABREC using standard forms obtainable from the UNZABREC Offices.
- **NHRA:** You are advised to obtain final study clearance and approval to conduct research in Zambia from the National Health Research Authority (NHRA) before commencing the research project.
- **QUESTIONS**: Please contact the UNZABREC on Telephone No.256067 or by e-mail on unzarec@unza.zm.
- **OTHER**: Please be reminded to send in copies of your research findings/results for our records. You're also required to submit electronic copies of your publications in peer-reviewed journals that may emanate from this study. Use the online portal: unza.rhinno.net for further submissions.

Yours sincerely,

Sody Mweetwa Munsaka, BSc., MSc., PhD
CHAIRPERSON
Tel: +260977925304
E-mail: s.munsaka@unza.zm

Appendix 4: NHRA Approval

NATIONAL HEALTH RESEARCH AUTHORITY
Paediatric Centre of Excellence, University Teaching Hospital, P.O. Box 30075, LUSAKA
Tell: +260211 250309 | Email: znhrasec@gmail.com | www.nhra.org.zm

Ref No:…… **Date: 5th August, 2020**

The Principal Investigator
Ms. Grace Mwikuma,
University of Zambia,
School of Veterinary Studies,
Department of Paraclinical studies,
P.O Box 32379,
Lusaka.

Dear Ms. Mwikuma,

Re: Request for Authority to Conduct Research

The National Health Research Ethics Board (NHREB) is in receipt of your request for authority to conduct research titled **"Identification of Clonal complexes, dispersion and antimicrobial resistance of Enterococci from poultry and humans in Lusaka District of Zambia."**
I wish to inform you that following submission of your request to the Board, its review of the same and in view of the ethical clearance, this study has been **approved** on condition that:

1. **A Material Transfer Agreement is obtained and cleared by the National Health Research Ethics Board should there be any need for samples to be sent outside the country for analysis.**
2. The relevant Provincial and District Medical Officers where the study is being conducted are fully appraised;
3. Progress updates are provided to NHRA quarterly from the date of commencement of the study;
4. The final study report is cleared by the NHRA before any publication or dissemination within or outside the country;
5. After clearance for publication or dissemination by the NHRA, the final study report is shared with all relevant Provincial and District Directors of Health where the study was being conducted, and all key respondents.

Yours sincerely,

Prof. Patrick Musonda
Chairperson
National Health Research Ethics Board

Appendix 5: UNZA Approval

THE UNIVERSITY OF ZAMBIA
SCHOOL OF VETERINARY MEDICINE
OFFICE OF THE ASSISTANT DEAN (POSTGRADUATE)

Telephone: 293727
Telegrams: UNZA LUSAKA
Telex: UNZALU ZA 44370
Fax: 293727/253952
School Fax: 293727
Vet. Clinic Telephone: 291515

P.O. Box 32379
Lusaka, Zambia

Your Ref:

Our Ref:

15th July, 2020

Mwikuma Grace
C/o Paraclinical Studies
School of Veterinary Medicine
University of Zambia
P.O. Box 32379
LUSAKA

Dear Mwikuma Grace

RE: APPROVAL OF RESEARCH PROPOSAL

On behalf of the Board of Graduate Studies, I am pleased to inform you that your proposed research project entitled ***"Identification of Clonal Complexes, Dispersion and Antimicrobial Resistance of Enterococci isolated from Humans and Poultry in Lusaka Province of Zambia."*** has been approved.

I wish you success as you apply for ethical approval and carry on with your research activities.

Yours sincerely

Dr Chisoni Mumba
ACTING ASSISTANT DEAN (PG), SCHOOL OF VETERINARY MEDICINE

Cc *Director, DRGS*
Dean, School of Veterinary Medicine
Head, Paraclinical Studies
Prof. B. Hang'ombe

Appendix 6: Participant (Farmworker) information Sheet

Title of Study: Determination of the Occurrence, Virulence Genes, and Antimicrobial Resistance Profiles of *Enterococcus faecalis* and *Enterococcus faecium* Isolated from Poultry of Chongwe, Lusaka, Kitwe, and Ndola Districts, Farmworkers from the Copperbelt Province Poultry Farms, and Clinical Specimens from Kitwe Teaching Hospital in Zambia

Principal Investigator: Ms Grace Mwikuma
Co-Principal Investigator: Prof Bernard Hang'ombe,

We are carrying out a study on bacteria known as *Enterococci* from Poultry and Humans in Lusaka District of Zambia. I kindly request that you give me a bit of your time to explain what we are doing so that you can make an informed decision to participate in this study. As we discuss the content of this information sheet, please feel free to ask any questions.

Brief description of the Study
Very small living things called germs live in different reservoirs including the guts of poultry and humans. *Enterococcus* species are among those germs. These are germs which are intrinsically resistant to some antimicrobials and acquired resistance genes and pathogenic determinants which they are able to transfer to other germs. They select for antimicrobial pressure and cause infections which are not easy to treat in poultry as well as humans.

This study focuses on *Enterococci* from poultry and humans who work or are found on poultry farms. Clinical samples from select hospitals will be included to compare the germs from these with those from farmer and farmworkers and poultry. The information gained from this study will be used develop intervention strategies aimed at controlling and preventing outbreaks of *Enterococcal* disease in poultry and humans, reducing the advent of antimicrobial resistance and its effects on human and animal health.

Voluntary Participation and withdrawal
You are free to either participate in this study or not. If you agree to take part in this study, we will get faecal samples from you, your family members, and farm workers and from poultry to test for the germs which cause infections. You are free to withdraw from the study at any time and if you choose to do so there are no repercussions whatsoever.

Risks
Please note that there is no risk involved in participation in this study.

Payments and Costs
You will not be paid for participating in this study and neither will you be asked to pay.

Benefits
You will not directly benefit from this study whether you choose to participate or not.

Confidentiality
The information that will be collected in this study will be strictly confidential and will not be linked to particular farms or individuals. Study numbers will be used and not names. On completion of the study, the samples will not be stored for future use.

Contact Details
In case you have questions, complaints, or problems as a result of participating in this study, You may get in touch with Grace Mwikuma on +260 977494310 or email: mwikumag@gmail.com of Flat 7, Anmercosa Court, Harrow Crescent, Parklands, Kitwe. All research on human volunteers is reviewed by a committee that works to protect your rights and welfare. If you have questions or concerns about your rights as a research subject you may contact, anonymously if you wish, UNZABREC office calling Telephone: +260 211 256067 or E-mail: unzarec@unza.zm or visit the office at The University of Zambia Ridgeway campus, Nationalist Road, Lusaka.

Appendix 7: Informed Consent Form

Consent to participate in Research

What does your signature (or thumbprint/mark) on this consent form mean?

Your signature (**or thumbprint/mark)** on this form means:

- You have been informed about this study's purpose, procedures, possible benefits and risks.
- You have been given the chance to ask questions before you sign.
- You have voluntarily agreed to participate in this study.

Please indicate Yes or No

- I agree to allow my faecal sample and that of poultry to be used in this study ☐Yes ☐No
- I agree to allow my farm to be included in the study . ☐Yes ☐No

Name of participant: _____

_____ _____

Signatu re of participant or Thumb print Date

In signing here or sticking my thumb print, I agree that I have read and understood or the information has been explained to me and I have understood the agreement/consent form and agree to participate in the study.

Name of Witness: _____

_____ _____

Signature of Witness Date

_____ _____

Signature of recipient of form Date

_____ _____

Signature of Witness Date

The signature of the witness above means that another person has observed the consenting of the participant. The witness must be impartial and not part of the study staff.

Appendix 8: Translated Participant (Farmer/Farmworker) Information Sheet

Mulimi/ Cikumbe/ Osewenza Mupulanzi Odzipeleka Kapena Kugwapo Pa Uthenga Uyu

Mutu Wakafukufuku: Determination of the Occurrence, Virulence Genes, and Antimicrobial Resistance Profiles of *Enterococcus faecalis* and *Enterococcus faecium* Isolated from Poultry of Chongwe, Lusaka, Kitwe, and Ndola Districts, Farmworkers from the Copperbelt Province Poultry Farms, and Clinical Specimens from Kitwe Teaching Hospital in Zambia

Oyanganila Ndi: Ms Grace Mwikuma

Oyanganila Enaomuleali Akulu Ndi: Aprofesa Bernard Hang'ombe

Tili kufufuzafufuza pakafufuku wa *Enterococci* kucokera mu nkhuku ndi anthu muno mulusaka. Nimpemphako kuti munganipaseko kanthawi kotinifotokoze nchito yomweticita, ndiposo ndikupemphani kuti imwe mukhala amodzi mwaiwo otengako mbala pakafufuku omwetilinawo ise. Pomwe tikuffotokoza pa zauthenga womwe walembedwa pansi apa nkhalani omasuka kunfunsa mafuso yali onse yomwe mufuna imwe.

Kufotokoza Mwadule Pankhani Yanthu Yakafukufuku

Mungakhale odziwa kuti tuzilombo twina tumakhala mbali zanthupi zosiyana-siyana mukati mwanthupi, mwacizitsanzo mukamwa ndi mumimba ya ndzinyama ndi anthu omwe. Ngati takhala okhudzana ndi nyama kapena anthu omwe ali ndi tulombo utu tingatengeko ngati tilipafupi nao. Kalumbo *kaEnterococci* kapena kanthata aka ndikamodzi komuse kangapitendipo kangabweretse matenda yomwe yangakange kucilitsidwa nthawi zina.

Kukafukufuku uyu takhala cabe ndimbaliyaka nthata ka *Enterococci* kucokera Kunkhuku ndi anthu omwe aweta ziweto izi. Tinga tengatenge kalombo aka ku anthu amene akhala mumapulazi yankhuku. Uthenga uyu omwe udza tengedwa apa ndiofuna kudziwa ngati kalinganandi komwe kapezeka mu anthu amene amakhudzana ndi nkhuku, ndiponso ndi kuipa kwake komwe kumabwera kucokera kwa kanthata kapena kalombo aka. Tifunanso kudziwa ngati kalombo aka kapezekanso ku anthu odwala amene ali muzipatala.

Kukwapo Kwakutengako Mbali Ndikwa Ulere Ndi Kucokamo

Kugwapo kwa kafukufuku uyu ndi kwaulere kopanda malipiro yaliyonse, panthawi iliyonse mungaleke kutengako mbali kapena kusintha manganizo yanu kusatengako mbali, kopanda ukulipilitsani ciliconse kapena cina ciliconse cabwino. Mungaleke kutengako mbali panthawi ili yonse mungafune kwa imwe ene ake. Ngati simunatengeko mbali pakafukufuku uyu kulibe cobvuta ciliconse comwe mungakherenaco ai.

Kuipa Kwake

Kuikilapo /kuonjezera, mudzafunsidwa ngati munga cilole kutengako chibudzi (matuvi) yanu kuti tipime kalombo kamene kabweletsa matenda kulinga ndi kafukufuku uyu. Njira zotengelamo zomwe tifuna palibe zina zilizonse zomwe munza mvera kuwawa ai kwa inu.

Ubwino

Simudzakhala ukhudzidwa ku ubwino wakafukufuku uyu. Uthenga womwe tidzakambapo udzatengedwa ndikusewenzesedwa kaleketsa kalombo aka kanthata kupita kumunthu kapena kunyama.

Mutengo Ndi Kalipilidwe

Simudzatenga ciliconse pakugwapokwa kafukufuku uyu kapena kulipila ciliconse ai. Kungwapo ndikwa ulele.

Mwakudzipeleka Mwacinsinsi

Kafukufuku uyu udzatengadwa mwaceru ndipo simulodwa kutipatsa zamaina yanu ai. Uthenga wamene udzatengedwa pakafukufuku uyu mwawakudzipile kakudzasewenzedwa mwanjirayo funikira ndiponso osati kudwiwika kwamunthu wina wace mwayekha. Mwacitsanzo, dzina, kapena maina siyasewenzesedwa, tizasewetsanamba la yanu yakafukufuku. Ngati tatsiliza kupima zomwetiza tenga kuli imwe sitidzakhalanso ndimpata wakuti tingansewenzetso kutsongolo ai.

Momwemungagwilizane Nao

Ngati mulindifunso iliyonse, zobvuta kapena zonunzitsa kapena zotuluka lake lakafukufuku wakugwapo mungatume kwa Ms Grace Mwikuma oyanganila kwanambala iyi +260 977494310 kapena ku emelo: mwikumag@gmail.com kapena kuyumba yao pa Flat 7, Anmercosa Court, Harrow Crescent, Parklands, ku Kitwe. Zonse zofunika kapena zofuna kucokerakwa azoyangila odzipeleka ndi kabungwe kanchito yoteteza zamaufulu ndiumbwinowake. Ngati mulindifunso kapena mandaulo pa ufulu, kukhala ngati otengako mbali pakafukufuku umenewu mungagwilizane, pazambili ngati mufuna, UNZABREC Ofisi pa nambala +260-1-256067 kapena emelo: unzarec@unza.zm kapena kufi ku ofisi yamwe ipezeka pa sukulu yapamwamba ya Zambia, Ridgeway Campus, Nationalist Road, muno mulusaka.

Appendix 9: Translated Informed Consent Form

Mwine Wapulazi/ Anchito Mupulazi Kubvumekesa

Kodi kusaina kwanu papepalayi kapena kuika cala kutanthauza ciani?

Kusaina kwanu kutanthauza:

- Mwauzidwa colingo ca kafukufuku, mundandanda, ubwino wake ndi kuipa kwake.
- Mwapatsidwa danga la kunfunsa mafunso musana saine pepalayi.
- Mwadzipereka pakubvomera kutengako mbali pakafukufuku umeneyu.
- Ndinu ololedwa kucokamo kapena kuleka kafukufuku umeneyu nthawi iliyonse.

Conde ikani Inde kapena Ayi

- Ndalola chibudzi kapena matuvi anga kutiapime kalombo kamene kabweletsa matenda kulinga ndi kafukufuku uyu ☐inde ☐ayi
- Ndabvomera pulazi langa kuikidwa kukafukufuku umeneyu ☐inde ☐ayi

Dzina la otengaka mbali: _____

_____		_____
Kusaina kwa otengaka mbali	kapena kudinda cala canu	Tsiku

Pakusaina, ndabvomera ndawerenga ndi kumvetsetsa pangano yacipepala ndi kutengako mbali kukafukufuku umeneyu.

Dzina la umboni/ oimilira: _____

_____	_____
Kusaina mboni/ oimilira	Tsiku

_____	_____
Kusaina kwa olandila pepalayi	Tsiku
_____	_____
Kusaina mboni/ oimilira	Tsiku

Kusaina kwa mboni/oimilira pamwambapa kutanthauza kuti munthu wina wayendera zokhuza otengako mbali. Mboni kukhala munthu amene Sali m'modzi mwa akulu a kafukufuku umeneyu.

Appendix 10a Published Research Article 1

antibiotics

MDPI

Article

Determination of the Prevalence and Antimicrobial Resistance of *Enterococcus faecalis* and *Enterococcus faecium* Associated with Poultry in Four Districts in Zambia

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Abstract: The presence of antimicrobial-resistant *Enterococci* in poultry is a growing public health concern worldwide due to its potential for transmission to humans. The aim of this study was to determine the prevalence and patterns of antimicrobial resistance and to detect drug-resistant genes in *Enterococcus faecalis* and *E. faecium* in poultry from four districts in Zambia. Identification of *Enterococci* was conducted using phenotypic methods. Antimicrobial resistance was determined using the disc diffusion method and antimicrobial resistance genes were detected using polymerase chain reaction and gene-specific primers. The overall prevalence of *Enterococci* was 31.1% (153/492, 95% CI: 27.1–35.4). *Enterococcus faecalis* had a significantly higher prevalence at 37.9% (58/153, 95% CI: 30.3–46.1) compared with *E. faecium*, which had a prevalence of 10.5% (16/153, 95% CI: 6.3–16.7). Most of the *E. faecalis* and *E. faecium* isolates were resistant to tetracycline (66/74, 89.2%) and ampicillin and erythromycin (51/74, 68.9%). The majority of isolates were susceptible to vancomycin (72/74, 97.3%). The results show that poultry are a potential source of multidrug-resistant *E. faecalis* and *E. faecium* strains, which can be transmitted to humans. Resistance genes in the *Enterococcus* species can also be transmitted to pathogenic bacteria if they colonize the same poultry, thus threatening the safety of poultry production, leading to significant public health concerns.

Keywords: antimicrobial resistance; antimicrobial resistance genes; *Enterococcus faecalis*; *Enterococcus faecium*; prevalence; poultry; Zambia

1. Introduction

Enterococcus is a genus of Gram-positive bacteria in the family Enterococcaceae, the order Lactobacillales and the phylum Firmicutes [1]. *Enterococcus* is part of the normal flora in the gastrointestinal tract (GIT) of mammals, fish, reptiles, insects, and birds [2,3]. Being ubiquitous in nature, it is also found in soil, plants, sewage, and fresh and salt water [4,5]. Species in the genus *Enterococcus* (E) have emerged as pathogens of medical and public health importance [6]. This is partly due to their adaptability to the selective pressures of antimicrobials. They also can acquire, express, and transmit mobile genetic elements (MGEs) from/to pathogenic as well as non-pathogenic species in the same or different genus [7,8], leading to the development of antimicrobial resistance. MGEs play an important role in facilitating horizontal genetic exchange and promoting the acquisition and transmission of resistance genes [9]. These properties have made *Enterococcus* an important human pathogen responsible for several clinical conditions, including urinary tract infections (UTI), endocarditis, bacteremia and mastitis in humans and animals [10,11]. *Enterococcus* species also cause locomotive disorders and septicemia in broilers [12]. *Enterococci* is ranked among the major causes of nosocomial infections worldwide [13]. This is especially true for *Enterococcus (E) faecalis* and *E. faecium*. The emergence of

multidrug-resistant (MDR) *Enterococci* such as vancomycin-resistant *Enterococci* (VRE) and drug-resistant *Enterococci* in poultry are of major public health concern because of the limited treatment options available for infections caused by such species, as well as the possibility of dispersion between poultry and humans [3,14–16] and the transfer of resistance genes to other bacteria (9). This has led to an increase in infections caused by multidrug-resistant *Enterococci*, which can not only be very difficult to treat but can also lead to increased mortality rates [17].

Enterococcal infections can be serious and are associated with increased healthcare costs, including the cost of hospitalization, laboratory testing and antibiotic treatment [18]. Enterococcal infections can also lead to lost productivity due to missed work or school.

Although *Enterococcus faecalis* and *Enterococcus faecium* are commonly found in the guts of poultry, they can cause infections in poultry that can lead to significant economic losses for the industry. Enterococcal infections in poultry can result in decreased growth rates, reduced feed efficiency and increased mortality rates [19]. Poultry and food products of poultry origin are the most consumed worldwide [20]. *Enterococci* can contaminate poultry products and pose a risk to human health if consumed [21]. Antibiotic resistance in *Enterococci* is also a concern for the poultry industry, as the use of antibiotics in poultry production can contribute to the development and spread of antibiotic-resistant strains [22]. Therefore, the presence of antimicrobial-resistant *Enterococci*, especially multidrug resistance *Enterococcus* species, in poultry is of public health concern as it may serve as a pool from which antimicrobial resistance genes are disseminated. VRE is a nosocomial pathogen that exhibits multidrug resistance (MDR) and virulence.

Enterococcus faecium has transitioned from a commensal organism to an ESKAPE (*E. faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter species*) pathogen. ESKAPE is an acronym for a group of life-threatening nosocomial pathogens that successfully evade the effect of antimicrobial drugs and represent a model for pathogenesis, transmission, and resistance [23]. VRE cause a greater number of infections than other nosocomial pathogens in hospitals in the United States [23].

Vancomycin-resistant *Enterococci* have been reported worldwide [24], including in Zambia [25]. However, they have not been given the same attention as other commensals of the GIT such as *Staphylococci*, *Salmonella*, *Shigella*, *Campylobacter* and *Escherichia coli*. Zambia developed a multi-sectoral national action plan in recognition of the public health threat of morbidity, mortality, and economic outcomes of antimicrobial resistance. However, minimal surveillance and research have been conducted on MDR *Enterococci* in Zambia. This study aimed to determine the prevalence of antimicrobial resistance and the presence of antimicrobial-resistant genes in *Enterococcus faecalis* and

Enterococcus faecium isolates from poultry in four districts in Zambia.

2. Results

2.1. Identification

2.1.1. Identification of Enterococci using Analytical Profile Index (API)

Of the 37 poultry isolates subjected to API identification using BioMérieux's Analytical Profile Index (API) 20 Strep test kits, 19 were identified as *Enterococcus faecalis*, 15 as *Enterococcus faecium* and one as *Enterococcus durans*. Two were not identified. The reason for performing API tests on only 37 isolates was due to insufficient reagents. Particularly, the NIN, VP 1 + VP 2, ZYM A and ZYM B were enough for only 38 samples (one control *Enterococcus faecalis* ATCC 29212 strain and the 37 isolates).

2.1.2. Identification of Enterococci Using Polymerase Chain Reaction (PCR)

PCR was run on 343 suspected *Enterococcus* DNA samples extracted from poultry droppings using genus-specific primers for elongation factor (*tuf*) and d-alanine-d-alanine ligase (*ddl*) genes. PCR was subsequently run on 153 positive DNA samples using species-specific primers for *Enterococcus faecalis* and *Enterococcus faecium*. The most common *Enterococcus* species was *E. faecalis* (37.9%), followed by *E. faecium* (10.5%). Remarkably, 38.6% of the isolates contained more than one species, with 34.6% of the total *Enterococcus* isolates containing both *E. faecalis* and *E. faecium*. Adding the latter to *E. faecalis* and *E. faecium*, *E. faecalis* would still be the most predominant species, followed by *E. faecium* (Fig 1). The word "Other" represents *Enterococcus* species—identified by PCR using genus-specific *ddl* and *tuf* gene primers—which could not be identified through PCR due to lack of additional species-specific primers, or DNA sequencing due to the unavailability of reagents.

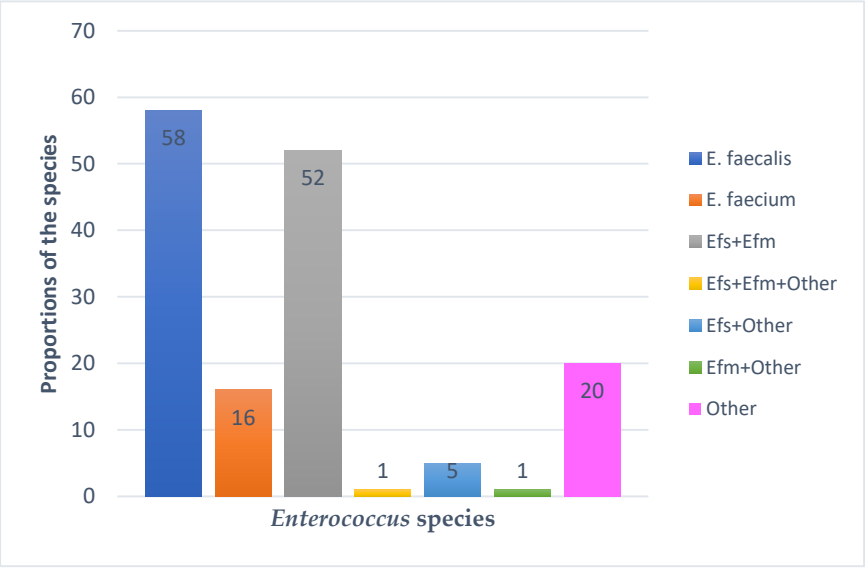

Figure 1. *Enterococcus* species identified using species-specific *E. faecalis* and *E. faecium* primers.

2.1.3. Comparing API and PCR Identification

API and PCR results were compared to ascertain the agreement between the two methods. API correctly identified 17 (45.9%) of the 37 isolates. API could not identify isolates with more than one species and only picked one of the species in samples with two or more species (16, 43.2%). It also misidentified an isolate that contained *E. faecalis* and another species as *E. faecium*, and it was not able to identify two isolates. Additionally, API identified one isolate as *E. durans1*, but this could not be confirmed as the corresponding species-specific primers were not available (Table 1).

Table 1. API and PCR Identities.

Study ID	PCR ID	API ID	Study ID	PCR ID	API ID
80	*E. faecalis*	*E. faecalis*	454	*E. faecalis* + *E. faecium*	*E. faecium*
82	*E. faecium*	*E. faecium*	455	*E. faecalis* + *E. faecium*	*E. faecalis*
84	*E. faecalis* + *E. faecium*	*E. faecalis*	476	*E. faecalis* + *E. faecium*	*E. faecium*
87	*E. faecalis*	*E. faecalis*	477	*E. faecalis* + *E. faecium*	*E. faecalis*
88	*E. faecalis* + Other	*E. faecium*	501	Other enterococci	Not identified
89	Other enterococci	Not identified	552	*E. faecalis* + *E. faecium*	*E. faecalis*
90	*E. faecium*	*E. faecium*	555	*E. faecium*	*E. faecium*
92	Other enterococci	*E. durans1*	576	*E. faecalis* + *E. faecium*	*E. faecalis*
93	*E. faecalis*	*E. faecalis*	585	*E. faecalis* + *E. faecium*	*E. faecium*
94	*E. faecium*	*E. faecium*	619	*E. faecalis*	*E. faecalis*
96	*E. faecalis*	*E. faecalis*	627	*E. faecium*	*E. faecium*
99	*E. faecalis* + *E. faecium*	*E. faecium*	630	*E. faecalis* + *E. faecium*	*E. faecalis*
100	*E. faecalis* + *E. faecium*	*E. faecium*	702	*E. faecalis* + *E. faecium*	*E. faecium*
101	*E. faecalis* + *E. faecium*	*E. faecium*	704	*E. faecalis* + *E. faecium*	*E. faecalis*
102	*E. faecalis*	*E. faecalis*	714	*E. faecalis* + *E. faecium*	*E. faecalis*
106	*E. faecalis*	*E. faecalis*	718	*E. faecalis* + *E. faecium*	*E. faecium*
107	*E. faecium*	*E. faecium*	725	*E. faecalis*	*E. faecalis*
361	*E. faecalis*	*E. faecalis*	734	*E. faecalis*	*E. faecalis*
399	*E. faecalis*	*E. faecalis*			

- PCR = Polymerase Chain Reaction, API = Analytical Profile Index, ID = Identity.

2.2. Prevalence of *Enterococci*

2.2.1. Overall Prevalence

The overall prevalence of *Enterococci* was 31.1% (153/492, CI: 27.1–35.4), while the prevalence in Lusaka Province was 30.8% (33/107, CI: 22.5–40.6) and the prevalence in Copperbelt Province was 31.2% (120/385, 26.6–36.1). Table 2 contains summaries of the prevalence of *E. faecalis* and *E. faecium* (combined and separate) in poultry from districts in the Copperbelt and Lusaka Provinces.

Table 2. Prevalence of *Enterococcus* in Poultry from the four Districts.

Factor	Categories	n Tested	n Positive	Prevalence (%)	95% CI
Overall	Positivity	492	153	31.1	27.1–35.4
Province	Lusaka	107	33	30.8	22.5–40.6
	Copperbelt	385	120	31.2	26.6–36.1
District	Lusaka	50	22	44.0	30.3–58.7
	Chongwe	57	11	19.3	10.5–32.3
	Kitwe	245	78	31.8	26.1–38.1
	Ndola	140	42	30.0	22.7–38.4
Enterococci isolates	*E. faecium*	153	16	10.5	6.3–16.7
	E. faecalis	153	58	37.9	30.3–46.1
	All other *Enterococcus* species	153	79	51.6	43.5–59.7

- n = number, % = percent, CI = confidence interval.

2.2.2. Species-Specific Prevalence of Isolates

The prevalence of *Enterococci* varied significantly across the districts. Lusaka district had the highest prevalence at 44.0% (22/50, CI: 30.3–58.7) compared with the other three districts of Kitwe, Ndola, and Chongwe (p = 0.038). The prevalence of *E. faecalis* was higher than that of *E. faecium* in all districts (p = 0.012) except Kitwe district (p = 0.044) (Table 3).

Table 3. Prevalence of specific species across the study area.

Factors	Categories	Species	n Tested	n Positive	Prevalence (%)	95% CI
Districts	Chongwe	*E. faecium*	57	1	1.8	0.1–10.6
		E. faecalis	57	5	8.8	3.3–20.0
		E. faecalis + *E. faecium*	57	1	1.8	0.1–10.6
		E. faecalis + other	57	2	3.5	0.6–13.2
		Other	57	2	3.5	0.6–13.2
		Total	57	11	19.3	10.5–32.3
	Lusaka	*E. faecium*	50	5	10.0	3.7–22.6
		E. faecalis	50	8	16.0	7.6–29.7
		E. faecalis + *E. faecium*	50	4	8.0	2.6–20.1
		E. faecium + other	50	1	2.0	0.1–12.0
		E. faecalis + other	50	1	2.0	0.1–12.0
		Other	50	3	6.0	1.6–17.5
		Total	50	22	44.0	30.3–58.7
	Kitwe	*E. faecium*	245	8	3.3	1.5–6.6
		E. faecalis	245	26	10.6	7.2–15.3
		E. faecalis + *E. faecium*	245	39	15.9	11.7–21.2
		Other	245	5	2.0	0.8–5.0
		Total	245	78	31.8	26.1–38.1
	Ndola	*E. faecium*	140	2	1.4	0.2–5.6
		E. faecalis	140	19	13.6	8.6–20.6
		E. faecalis + *E. faecium*	140	8	5.7	2.7–11.3
		E. faecalis + *E. faecium* + other	140	1	0.7	0.0–4.5
		E. faecalis + other	140	2	1.4	0.2–5.6
		Other	140	10	7.1	3.7–13.1
		Total	140	42	30.0	22.7–38.4
Species	Overall	*E. faecium*	492	16	3.3	1.9–5.3
		E. faecalis	492	58	11.8	9.1–15.1
		E. faecalis + *E. faecium*	492	52	10.6	8.1–13.7
		E. faecalis + *E. faecium* + other	492	1	0.2	0.0–1.3
		E. faecium + other	492	1	0.2	0.0–1.3
		E. faecalis + other	492	5	1.0	0.4–2.5
		Other	492	20	4.1	2.6–6.3
		Total Isolates	492	153	31.1	27.1–35.4

- n = number, % = percent, CI = confidence interval, other = unidentified enterococcus species.

2.3. Antimicrobial Susceptibility Test Results

2.3.1. Antimicrobial Susceptibility of *Enterococci*

All intermediate test results were considered resistant. Both *Enterococcus* species showed very high (97.3%) resistance to tetracycline, while 94.6% were resistant to erythromycin and 77.0% were resistant to ciprofloxacin. Remarkably, 64.9% of both *Enterococcus* species were susceptible to vancomycin. More than 90.0% of *E. faecalis* isolates were resistant to erythromycin and tetracycline and more than 50.0% were resistant to ampicillin, chloramphenicol, and ciprofloxacin. Less than 20.0% of the *E. faecalis* isolates were resistant to vancomycin. All *E. faecium* isolates in this study were resistant to erythromycin. More than 80.0% of *E. faecium* isolates exhibited phenotypic resistance to ampicillin, ciprofloxacin, and tetracycline, while less than 40.0% showed resistance to chloramphenicol and vancomycin. Susceptibility profiles of *E. faecalis* and *E. faecium* to the eight antimicrobials tested are provided in Table 4.

Table 4. Antimicrobial Susceptibility Profiles of *Enterococcus faecalis* and *Enterococcus faecium.*

Species	Susceptibility Test Result	AMP n (%)	CHL n (%)	CIP n (%)	ERY n (%)	NIT n (%)	PEN n (%)	TET n (%)	VAN n (%)
E. faecalis	Resistant	37 (63.8)	35 (60.3)	44 (75.9)	54 (93.1)	37 (63.8)	31 (53.4)	58 (100)	19 (32.8)
	Susceptible	21 (36.2)	23 (39.7)	14 (24.1)	4 (6.9)	21 (36.2)	27 (46.6)	0	39 (67.2)
E. faecium	Resistant	14 (87.5)	6 (37.5)	13 (81.3)	16 (100)	8 (50)	8 (50)	14 (87.5)	7 (43.7)
	Susceptible	2 (12.5)	10 (62.5)	3 (18.7)	0	8 (50)	8 (50)	2 (12.5)	9 (56.3)
Total	*E. faecalis*	51 (68.9)	41 (55.4)	57 (77.0)	70 (94.6)	45 (60.8)	40 (54.1)	72 (97.3)	26 (35.1)
	E. faecium	23 (31.1)	33 (44.6)	17 (23.0)	4 (5.4)	29 (39.2)	34 (46.0)	2 (2.7)	48 (64.9)

- n = number, % = percent, AMP = ampicillin, CHL = chloramphenicol, CIP = ciprofloxacin, ERY = erythromycin, NIT = nitrofurantoin, PEN = penicillin, TET = tetracycline, VAN = vancomycin.

2.3.2. Number of Enterococcus Isolates Resistant to One, Two, Three or More Antimicrobial Classes.

Multidrug resistance (MDR) is defined as resistance to three or more classes of antimicrobials. The results of our study show that none of the isolates were susceptible to all antimicrobial classes tested. Of the 74 *E. faecalis* and *E. faecium* isolates tested against eight antimicrobials, only two (2.7%) were resistant to one class of antimicrobials. A total of 5 (6.8%) isolates were resistant to two classes of antimicrobials. The majority of *E. faecalis* and *E. faecium* isolates (67, 90.5%) were MDR (Table 5).

Table 5. Number of Isolates Resistant against one, two, three or more Antimicrobial Classes.

Isolate (Total Number)	All Susceptible n (%)	Resistant to One Class of Antibiotic, n (%)	Resistant to Two Classes of Antibiotics, n (%)	Resistant to Three or More Classes of Antibiotics, n (%)
All *Efs* and *Efm* (74)	0 (0)	2 (2.7%)	5 (6.8%)	67 (90.5%)

E. faecalis (58)	0 (0)	2 (3.5%)	4 (6.9%)	52 (89.7%)
E. faecium (16)	0 (0)	0 (0)	1 (6.3%)	15 (93.8%)

- n = number, % = percent, *Efs* = *E. faecalis*, *Efm* = *E. faecium*.

2.4. Detected Antimicrobial Resistance Genes

2.4.1. Presence of Antimicrobial Resistance Genes in *E. faecalis* and *E. faecium*

The *aac(6')-Ie-aph(2")-LA* resistance gene encoding resistance to gentamycin was detected in 33 and 12 *E. faecalis* and *E. faecium* isolates, respectively, representing 60.8% of the isolates. The *ermB* resistance gene was more common in both *E. faecalis* and *E. faecium* compared with the *ermA* gene. The *vanA* resistance gene was detected in only two *E. faecalis* isolates and in none of the *E. faecium* isolates. Table 6 shows the number of different resistance genes detected in the *E. faecalis* and *E. faecium* isolates.

Table 6. The number of different resistance genes detected in *E. faecalis* and *E. faecium* isolates.

Resistance Gene	Total isolates Tested	Detected				Undetected
		E. faecalis (58)	Proportion (*E. faecalis*)	*E. faecium* (16)	Proportion (*E. faecium*)	
aac(6')-Ie-aph(2")-LA	74	33	44.6%	12	16.2%	29
ermA	74	1	0.01%	1	0.01%	72
ermB	74	35	47.3%	10	13.5%	29
tetK	74	40	54.1%	8	10.8%	26
tetM	74	45	60.8%	10	13.5%	19
tetL	74	41	55.4%	8	10.8%	25
tetX	74	3	0.04%	2	0.03%	69
vanA	74	2	0.03%	0	0	72

2.4.2. Resistance Genes in *E. faecalis* and *E. faecium* isolates across the Study Area

The most common resistance genes in *E. faecalis* isolates from Chongwe district in Lusaka Province were *tetL* and *tetM*, as they were found in all five isolates. These were followed by *aac* and *tetK*, which were detected in four of the five isolates, and *ermB*, which was detected in three isolates. The most common resistance genes in isolates from Lusaka district were *tetK* and *tetM*, which were found in all 8 *E. faecalis* isolates. These were followed by *tetL* (7/8), *aac* (6/8) and *ermB* (5/8). Resistant genes commonly detected in *E. Faecalis* from Ndola district were *tetM* (16/20), *tetL* (15/20), *aac* (13/20), *ermB* (12/20) and *tetK* (11/20). In *E. faecalis* isolated from Kitwe, *tetK* (17/25) was the most prevalent resistance gene, followed by *tetM* (16/25), *ermB* (15/25), *tetL* (14/25) and *aac* (10/25). The most detected resistance genes in *E. faecium* isolates from Kitwe district were *aac* (5/8) and *ermB* (5/8), followed by *tetM* (4/8) and *tetK* (3/8). In Lusaka district, the most common genes were *aac* (5/5) followed by *ermB* (4/5), *tetL* (4/5), *tetK* (3/5) and *tetM* (3/5).

Table 7 shows all resistance genes detected in *E. faecalis* and *E. faecium* isolates from the four districts in Zambia.

Table 7. Resistance genes detected in *E. faecalis* and *E. faecium* isolates from poultry in Copperbelt and Lusaka Provinces.

Area	Species	#RG	Resistance Genes																TRG
			aac		*ermA*		*ermB*		*tetK*		*tetL*		*tetM*		*tetX*		*vanA*		
			Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	
Lusaka Province	*E. faecalis*	13	10	3	0	15	8	5	12	1	13	0	13	0	1	12	1	12	13
	E. faecium	6	6	0	0	6	5	1	4	2	5	1	4	2	1	5	0	6	6
Copperbelt Province	*E. faecalis*	45	23	22	1	44	27	18	28	17	29	16	32	13	2	43	1	44	32
	E. faecium	10	6	4	1	9	5	5	4	6	3	7	6	4	1	9	0	10	6
Chongwe	*E. faecalis*	5	4	1	0	5	3	2	4	1	5	0	5	0	0	5	0	4	5
	E. faecium	1	1	0	0	1	1	0	1	0	1	0	1	0	0	1	0	1	1
Lusaka	*E. faecalis*	8	6	2	0	8	5	3	8	0	7	1	8	0	1	7	0	8	8
	E. faecium	5	5	0	0	5	4	1	3	2	4	1	3	2	1	4	0	5	4
Ndola	*E. faecalis*	20	13	7	0	20	12	8	11	9	15	5	16	4	0	20	0	20	16
	E. faecium	2	1	1	0	2	2	0	1	1	2	0	2	0	0	2	0	2	2
Kitwe	*E. faecalis*	25	10	15	1	24	15	10	17	8	14	11	16	9	2	23	1	24	17
	E. faecium	8	5	3	1	7	5	5	3	5	1	7	4	4	1	7	0	5	5

- #RG = number of isolates in which resistance-gene detection was conducted; Pos = detected, Neg = undetected, TRG = total number of isolates containing resistance genes.

2.5. Association between antimicrobials and resistance genes

Differences in antimicrobial resistance patterns and resistance genes in both enterococcus species were analyzed to assess possible associations between resistance phenotypes and their corresponding genotypes. A positive association between phenotype and genotype was found for tetracycline ($p = 0.047$) and erythromycin ($p = 0.008$), but there was no association between genotype and the vancomycin resistance phenotype ($p = 0.051$) (Table 8).

Table 8. Association between antimicrobial results and their corresponding resistance genes.

Antibiotic	Genes	X^2—Value	*p*-Value
TET	*tet*	3.945	0.047 ***
ERY	*erm*	6.947	0.008 ***
VAN	*vanA*	3.795	0.051

- X^2 = Chi-square value; *p*-Value = significant at <0.05; TET = Tetracycline; ERY = Erythromycin; VAN = Vancomycin; *tet* = all tetracycline genes (*tetM*, *tetL*, *tetK* and *tetX*); *erm* = both *ermA* and *ermB* genes.

3. Discussion

The prevalence, antimicrobial susceptibility patterns and presence of resistance genes in poultry droppings from four districts in Zambia were determined. The overall prevalence of *Enterococci* was 31.1%. This is in agreement with other studies that reported similar results in Poland [26], Malaysia [27] and Nigeria [28]. However it was lower than that reported in a similar study

conducted in Zambia, where the prevalence was 88.4% in laying hens [29]. This could be due to differences in sampling methods, farms sampled and the number of farms sampled. Another previous study [30] also reported a higher prevalence than that reported in the present study. Conversely, the prevalence rate in our study was higher than the rates reported in Ethiopia [31], Pakistan [32] and Thailand [33]. The differences in the isolation rates of *Enterococci* can be attributed to several factors, including antibiotic use, environmental factors and methodology. The widespread use of antibiotics has led to the selection and dissemination of antibiotic-resistant *Enterococci*. *Enterococci* are found in soil and water and can persist in the environment for long periods of time, making them more difficult to control and leading to increased isolation rates. The isolation rate can also be influenced by the type of culture method used and the presence of selective media that may preferentially isolate *Enterococci* [34].

Among the *Enterococcus* species isolated in this study, *E. faecalis* was the most prevalent (37.9%), followed by *E. faecium* (10.5%). This was in agreement with other studies [35–38] which found *E. faecalis* to be the most prevalent species in poultry. However, our study differed slightly from some studies that found species other than *E. faecalis* to be the most predominant [16, 39, 40]. The variations in species levels between studies might be due to differences in the type of poultry, source of chicks, sampling methods, geographical disparities, the time of study and isolation and identification procedures [40].

Although API 20 strep is considered the best identification system for bacteria [41], it does not accurately identify some species of *Enterococci* [42]. In the present study, we validated API 20 strep results using PCR. PCR conducted using species-specific primers identified 91.9% of samples containing both single and multiple species. API 20 strep accurately identified 45.3% of *Enterococcus* species, but identified only one species in isolates containing more than one species. It also misidentified 2.7% of the *Enterococcus* species. Our findings were in agreement with the results of previous studies [42–45].

In the present study, phenotypic resistance to critically important antimicrobials, as defined by the WHO [46], was observed and 90.5% of *E. faecalis* and *E. faecium* isolates were multidrug resistant (MDR) (Table 5). Notably, all *E. faecalis* and *E. faecium* isolates were resistant to one or more of the tested antimicrobials (Table 5). These findings were similar to those of a study done earlier [47] in which the majority of *E. faecalis* and *E. faecium* isolates were resistant to one or more of the tested antimicrobials. Resistance to all tested antimicrobials was also observed in both *E. faecalis* and *E. faecium* isolates.

More than 50.0% of *E. faecalis* isolates were resistant to all tested antimicrobials, while 100.0% of the isolates were resistant to tetracycline. On average, *E. faecium* exhibited increased resistance to antimicrobials in comparison with *E. faecalis*. Our findings are in agreement with a recent study conducted in Zambia [29]. Our

study also has some similarities with a study conducted in the Czech Republic [48], in which increased resistance of *Enterococci* to tetracycline, erythromycin and nitrofurantoin were observed, as well as a study from USA [49], in which *Enterococci* resistance to tetracycline, penicillin and ciprofloxacin was documented. Furthermore, our results are consistent with findings from previous studies [50–56] where high tetracycline resistance was reported. Our results were also comparable with those of the study by Fracalanzza et al. [57], which recorded the resistance of *Enterococci* to erythromycin to be at 82.0% when intermediate results were included. Nevertheless, that study noted reduced resistance to tetracycline (38.3%) and chloramphenicol (5.7%) compared with our study. The observed increase in resistance to all the antimicrobials tested indicate that poultry from these four districts in Zambia can be a source of MDR *Enterococci*. However, our study contrasted with other studies [58,59], which reported lower levels of resistance to antimicrobials.

Although the gene *aac(6′)-Ie-aph(2″)-LA*, which encodes resistance to gentamicin, was detected in 60.8% of both *Enterococci* species tested, an association with susceptibility could not be determined, as discs containing high concentrations of gentamicin (for example 120 μg or 500 μg), which are used to detect high-level aminoglycoside resistance, were not available.

The associations between antimicrobial resistance phenotypes and genotypes in *E. faecalis* and *E. faecium* isolates were analyzed. Associations were found between genotypes and tetracycline and erythromycin resistant phenotypes. However, genotypes showed no relationship with vancomycin resistant phenotypes. The disparity observed between the phenotypes and genotypes in the case of vancomycin could be due to the fact that vancomycin resistance in *Enterococci* can be conferred by different gene clusters [60–62].

4. Materials and Methods

4.1. Study Design and Sites

A cross-sectional study was conducted in selected farms in Chongwe and Lusaka (Lusaka Province) and Ndola and Kitwe (Copperbelt Province) districts in Zambia (Figure 2). The two provinces are among those that harbor most of the commercial poultry farms in Zambia.

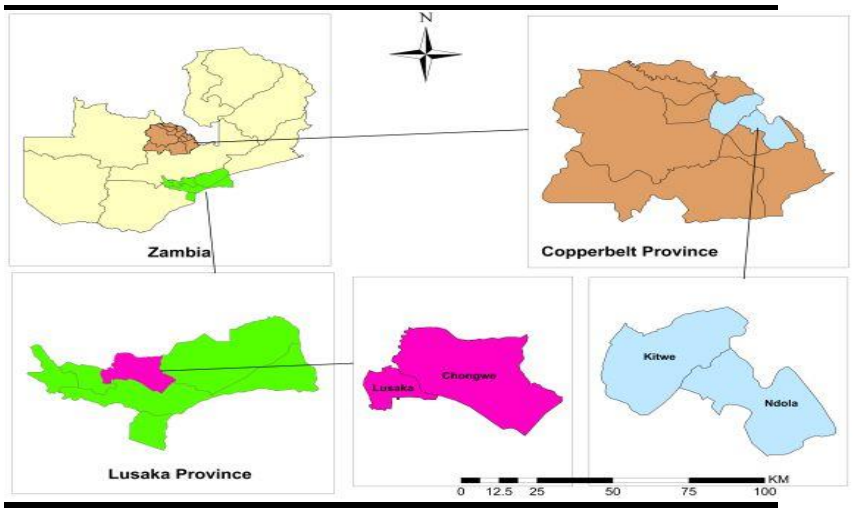

Figure 2. Map of the study areas.

4.2. Sample Collection

A total of 492 freshly voided poultry droppings were collected from layers, broilers and village chickens. Five different visits were made to selected poultry farms in four districts in the Copperbelt and Lusaka Provinces in Zambia (Figure 2). Of the total samples collected, 57 were from farms in Chongwe, while 50 were from Lusaka district in Lusaka Province. Of the 385 samples from Copperbelt Province, 140 and 245 came from Ndola and Kitwe districts, respectively.

4.3. Laboratory Investigations

4.3.1. Isolation of *Enterococci*

Conventional microbiological assays were performed to detect and identify *Enterococcus* species as described by Facklam and Collins [63]. Briefly, 1 g of poultry droppings was suspended in 9 mL buffered peptone water (BPW) (HIMEDIA, India), mixed, and incubated at 37 °C for 24 h. A loopful of the BPW suspension was streaked on Bile Esculin Agar (BEA) (HIMEDIA, India) and incubated at 37 °C for 24 h. Following this, colonial traits were noted and smears of suspect colonies (small black shiny colonies on BEA) were made and stained using Central Drug House's (CDH) Gram's color staining kit from India. Gram-positive cocci appearing in chains, doubles or singles were characteristic of enterococci. A total of 343 suspected *Enterococcus* isolates were recovered from the 492 samples tested. These were stored in 20% glycerol at −20 °C for subsequent experiments.

4.3.2. Identification of Enterococci Using Analytical Profile Index (API)

Species identification based on phenotypic characteristics and biochemical tests was conducted using BioMérieux's Analytical Profile Index (API) 20 Strep test kits. A total of 37

isolates were identified using the API 20 Strep test kits. The reasons for this are stated in Section 2.1.1.

4.3.3. DNA Extraction

Colonies grown overnight on a blood agar plate were placed in a test tube containing 0.5 mL of molecular grade water, vortexed and boiled at 95 °C for 10 min and then centrifuged for 5 min at 1500× *g*. The supernatant was pipetted into cryo-vials and stored at -20 °C for further analysis.

4.3.4. Molecular Identification of Enterococci

Molecular identification of the *Enterococcus* species was conducted using single PCR and the genus-specific and species-specific primers shown in Table 9, following the procedure described by Li et al. (2012) [64]. PCR amplification of *elongation factor* (*tuf*) and *D -Ala- D -Ala ligase* (*ddl*) in the extracted DNA was conducted using Phusion Flash High-Fidelity PCR Master Mix (Thermofisher Scientific, USA) in a thermal cycler (Applied Biosystems, Chiba, Japan) under the following PCR conditions: initial denaturation at 98 °C for 2 min followed by 30 cycles of denaturation at 98 °C for 5 s, annealing at 56 °C for 5 s, and extension at 72 °C for 30 s. A final extension was performed at 72 °C for 1 min. PCR amplicons were run on 1.5% agarose gels. The expected bandwidths for *tuf* and *ddl* PCR products were 112 bp and 475 bp, respectively. For species identification, species-specific primers (Table 1) targeting the superoxide dismutase (*sodA*) gene in *E. faecalis* and *E. faecium* were used. No primers were available for other species. The PCR conditions were similar to those used for genus amplification, except for the annealing temperature, which was set to 52 °C for both species.

Table 9. Primers for Enterococcus Genus and Species identification.

IDENTIFICATION PRIMERS				
Target Gene	**Primer Name**	**Primer Sequence 5′-3′**	**Amplicon Size bp**	**References**
tuf	*tuf-F*	TACTGACAAACCATTCATGATG	112	[65]
	tuf-R	AACTTCGTCACCAACGCGAAC		
ddl	*ddlF*	CACCTGAAGAAACAGGC	475	[66]
	ddlR	ATGGCTACTTCAATTTCACG		
sodAEfm	*sodAEfm1*	CAGCAATTGAGAAATAC	190	[67]
	sodAEfm2	CTTCTTTTATTTCTCCTGTA		
sodAEfs	*sodAEfs1*	CTGTAGAAGACCTAATTTCA	209	[67]
	sodAEfs2	CAGCTGTTTTGAAAGCAG		

- bp = base pairs.

4.3.5. Determination of Antimicrobial Resistance Levels

Susceptibility to vancomycin (30 μg), erythromycin (15 μg), ampicillin (10 μg), penicillin (10 U), tetracycline (30 μg), nitrofurantoin (300 μg), ciprofloxacin (5 μg) and chloramphenicol (30 μg) was determined using the disk diffusion method according

to the Clinical and Laboratory Standards Institute guidelines [68]. The disks used for susceptibility testing were manufactured by HIMEDIA, India. Diameters of the zones of inhibition were recorded in millimeters (mm) and interpreted as susceptible, intermediate or resistant. In this study, intermediate results were taken as resistant. A reference strain, *Enterococcus faecalis* 29,212, was used as a control strain.

4.3.6. Detection of Antimicrobial Resistant Genes (ARG)

Genes conferring resistance to aminoglycosides [*aac(6′)-Ie-aph(2″)-LA*], which in this study was abbreviated as "*aac*", macrolides (*ermA* and *ermB*), tetracyclines (*tetM*, *tetL*, *tetK*, and *tetX*) and glycopeptides (*vanA*) were detected in a single PCR using the gene-specific primers shown in Table 10. One Taq Quick-load 2X Master Mix (Biolabs, Durham, North Carolina, USA) was used for amplification in a thermal cycler (Applied Biosystems, Chiba, Japan). The following PCR conditions were employed: initial denaturation at 93 °C for 3 min followed by 35 cycles of denaturation at 93 °C for 60 s, annealing at 52 °C for 60 s and elongation at 72 °C for 60 s. The final elongation step was performed at 72 °C for 5 min. PCR amplicons were run on 1.5% agarose gels. The expected sizes of the PCR products differed for each gene (Table 10).

Table 10. Primers used for the Detection of Resistance Genes.

PRIMERS FOR RESISTANCE GENES				
Target Gene	**Primer Name**	**Primer Sequence 5′-3′**	**Amplicon Size (bp)**	**References**
aac(6′)-Ie-aph(2″)-LA	*aacF*	CAGGAATTTATCGAAAATGGTAGAAAAG	369	[69]
	aacR	CACAATCGACTAAAGAGTACCAATC		
*erm*A	*ermAF*	TATCTTATCGTTGAGAAGGGATT	139	[70]
	ermAR	CTACACTTGGCTTAGGATGAAA		
*erm*B	*ermB-1*	GAAAAGTACTCAACCAAATA	639	[71]
	ermB-2	AGTAACGGTACTTAAATTGTTTA		
*tet*K	*tetK-1*	TTAGGTGAAGGGTTAGGTCC	697	[72]
	tetK-2	GCAAACTCATTCCAGAAGCA		
*tet*M	*tetM-1*	GTTAAATAGTGTTCTTGGAG	576	[72]
	tetM-2	CTAAGATATGGCTCTAACAA		
*tet*L	*tetL-1*	CATTTGGTCTTATTGGATCG	456	[72]
	tetL-2	ATTACACTTCCGATTTCGG		
*tet*X	*tetXF*	CAATAATTGGTGGTGGACCC	468	[73]
	tetXR	TTCTTACCTTGGACATCCCG		
*van*A	*vanAF*	CTGCAATAGAGATAGCCGCTAACA	751	[74]
	vanAR	TGTATCCGTCCTCGCTCCTC		

- bp = base pairs.

4.4. Data Analysis

Data were entered, cleaned and validated in a Microsoft™ excel spreadsheet (MS Office Excel® 2016). The data were then exported to SPSS software ver. 21 (IBM Corp, Armonk, NY, USA). PCR results (positive or negative) were reference variables for descriptive analyses. Univariate analyses were conducted for descriptive statistics and data are presented as frequencies, percentages and prevalence.

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Appendix 10b Published Research Article 2

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Antimicrobial Resistance Profiles of Enterococcus Faecalis and Enterococcus Faecium Isolated from Clinical Specimens at Kitwe Teaching Hospital in Zambia

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Abstract

This research examined the occurrence of *Enterococcus faecalis* and *Enterococcus faecium* in clinical samples processed at Kitwe Teaching Hospital Laboratory, along with their resistance to antimicrobials and presence of virulence determinants. A total of 230 clinical specimens were analyzed to identify suspected *Enterococcus* strains. Standard laboratory techniques were employed to isolate, characterize, and confirm the species of *Enterococcus*, followed by testing their susceptibility to antimicrobials and detection of resistance and virulence genes using polymerase chain reaction. Among the 230 cultured specimens, 89 were stool samples, 3 were swabs, and 138 were urine specimens. Out of these, 71 isolates were confirmed *Enterococcus* by PCR using genus specific primers. The resulting prevalence rate of 30.9% was thus obtained. The most prevalent species was *Enterococcus faecalis*, accounting for 56.3% of the 71 *Enterococcus* isolates. The prevalence of *E. faecium* was 14.1%. All tested *E. faecalis* isolates displayed resistance to ciprofloxacin and erythromycin. Furthermore, high rates of resistance were observed among *E. faecalis* isolates for chloramphenicol and tetracycline (97.5%), vancomycin (90.0%),

nitrofurantoin (75.0%), penicillin (75.0%) and ampicillin (67.5%). Only two *E. faecium* isolates exhibited susceptibility to tetracycline and vancomycin, while the remaining isolates were 100% resistant to all other tested antimicrobials. Multidrug resistance was detected in all *E. faecium* isolates. In addition, various antibiotic resistance genes (*aac(6')-Ie-aph(2")-LA*, *ermA*, *ermB*, *tetK*, *tetL*, *tetM*, *tetX*, *vanA*) were identified in both *E. faecalis* and *E. faecium* isolates. A positive association between phenotype and genotype was found for tetracycline and erythromycin.

Keywords: antimicrobial susceptibility, antimicrobial resistance genes, *Enterococcus faecalis*, *Enterococcus faecium*, prevalence, clinical specimens, Zambia

1. Introduction

Enterococci are bacteria commonly found as normal flora in the gastrointestinal tracts of animals and humans (Lebreton, Willems, and Gilmore, 2014, Soodmand, *et al*, 2018). *Enterococcus* has spread widely as a hospital and a community acquired pathogen on a global scale (Zhou, *et al.*, 2020; Guzman Prieto, *et al.*, 2016). It has gained clinical relevance due to its implication in many clinical syndromes including urinary tract infections, bacteraemia, endocarditis, wound infections, endophthalmitis and root canal (NI and Huycke, 2014; Abat, *et al.*, 2016; Todokoro, *et al.*, 2017). *Enterococcus faecium* and *Enterococcus faecalis* are responsible for the majority of human enterococcal infections (Georges, *et al.*, 2022; Horner, *et al.*, 2021). *Enterococci* have also earned recognition due to their ability to acquire and transfer virulence and antimicrobial resistance determinants, from and to other commensal and pathogenic bacteria in animals and humans (Krawczyk, *et al.*, 2021; Ramos, *et al.*, 2020). There have been reports of genetic similarities between animal strains and those causing infections in humans (Lee, et al., 2021; Ahmed, *et al.*, 2018; Zischka, *et al.*, 2015). Cases of human infections caused by animal strains, as well as the transfer of virulence and resistance traits from animals to humans, have been documented (Ngbede, *et al.*, 2017; Iseppi, *et al.*, 2020; Miranda, *et al.*, 2021). Such reports and cases are of significant concern for public health. Studies conducted in other parts of the world have demonstrated that the prevalence of antimicrobial resistance and virulence traits among *Enterococcus* species varies depending on geographical location and antimicrobial usage (Barbosa-Ribeiro, *et al.*, 2016; Shridhar, and Dhanashree, 2019). Few studies have investigated the prevalence, characteristics and antimicrobial resistance of *Enterococci*. These include studies on *Enterococci* from poultry in some districts on the Copperbelt and Lusaka Provinces (Mwikuma, *et al.*, 2023; Mudenda, *et al*, 2022), from clinical samples at the University Teaching Hospital (Mutalange, *et al.*, 2021) and at cattle interface areas of Kafue basin in Zambia (Mubita, *et al.*, 2008). However, there is a lack of information on the occurrence and antimicrobial resistance of *Enterococcus faecalis* and *Enterococcus faecium* isolated from clinical specimens at Kitwe Teaching Hospital, Copperbelt Province, Zambia. Considering the potential risk of harmful enterococcal strains; *Enterococcus faecalis* and *Enterococcus faecium* possibly being transmitted in the hospital environment and the role of *Enterococcus* in the spread of antimicrobial resistance genes, it is crucial to assess the prevalence and antibiotic resistance in clinical *Enterococcus faecalis* and *Enterococcus faecium*, as they may contribute to outbreaks of hospital acquired infections. It is therefore important to monitor the occurrence of resistant *Enterococcus* species in clinical specimens to prevent nosocomial infections. The current study aims to provide insight into the occurrence, species diversity and antibiotic resistance potential of *Enterococcus faecalis* and *Enterococcus faecium* isolated from clinical specimens at Kitwe Teaching Hospital in Zambia.

2.0 Materials and Methods

2.1 Study design, site and period

A cross-sectional study was conducted at Kitwe Teaching Hospital on the Copperbelt Province in Zambia from February to March, 2021.

2.2 Study Population

All pus swabs, urine and stool samples which were received in the laboratory from 1st February to 16th March, 2021 i.e., from first sample until the 230th specimen was reached were conveniently included in the study. The samples included in this study were from both hospitalized and non-hospitalized male and female patients ranging from 2 to 86 years old. Only 2 patients had their age not indicated on the request forms.

2.3 Sample Size and Sampling Frame

For determination of sample size prevalence "p" of 18.03% (Mpinda, *et al.*, 2019), Z statistic of 1.96 at 95% confidence and acceptable error of 0.05 (Pourhoseingholi, *et al.*, 2013) were employed.

Formula:

$$n = \frac{z^2 \text{ x } p(1-p)}{e^2}$$

$$n = \frac{1.96^2 \text{x } 0.18(1-0.18)}{0.05^2}$$

n = 227

Rounding up it gives us 230.

2.4 Sample Collection

From 1st February to 16th March, 2021 all stool and urine samples which were sent to laboratory were included in study. This was done from starting with first sample until the number 230 specimens was reached. After which sampling was discontinued.

2.5 Laboratory Investigations

2.5.1 Culture of *Enterococcus*

To detect and characterize *Enterococcus* species, standard Microbiological methods were employed to as described by Facklam and Collins (1989) with a few modifications. In brief, 1g of faecal specimens was suspended in 9ml buffered peptone water (BPW) (HIMEDIA, India), while swabs were placed in 5ml of BPW and incubated at 37°C for 24hrs. 1ml of the overnight suspension was put into 5ml Trypticase Soy broth (TSB) (HIMEDIA, India), mixed and incubated at 37°C for 24 hours. For urine, 1ml of urine samples was dispensed into 5ml Trypticase Soy broth (TSB), mixed and incubated at 37°C for 24 hours. A loopful of the TSB suspension was streaked on Bile Esculin Agar (BEA) (HIMEDIA, India) and incubated at 37^0c for 24 hours. A total of 230 clinical specimens comprising of swabs, urine and stool were collected and processed at Kitwe Teaching Hospital Laboratory.

2.5.2 Phenotypic Characterization of *Enterococcus* Species

Species identification was based on phenotypic characteristics including colonial morphology, Gram Stain (Gainland Chemicals Company, United Kingdom), catalase test and biochemical tests. A total of 124 suspect bacterial colonies (small black shiny) were stored in 20% glycerol at -20℃ pending subsequent

experiments.

2.5.3 DNA Extraction

Colonies of overnight growth on a blood agar plate were put in a test tube containing 0.5ml of molecular grade water, vortexed and boiled at 95°C for 10 minutes, and then centrifuged for 5 minutes at 1500*xg*. The supernatant was pipetted into cryo-vials and stored at -20°C for further analysis

2.5.4 Molecular Identification of *Enterococci*

Confirmation of the genus *Enterococcus* was done by PCR using genus-specific primers (table 1) as described previously by Li and colleagues (2012). Extracted DNA PCR amplification of *elongation factor* (*tuf*) and *D -Ala- D -Ala ligase* (*ddl*) was done using Phusion Flash High-Fidelity PCR Master Mix (Thermofisher Scientific, US) in the thermal cycler (Applied Biosystems, Chiba, Japan) under the following PCR conditions; initial denaturation at 98°C for 2 minutes followed by 30 cycles of denaturation at 98°C for 5 seconds, annealing at 56°C for 5 seconds, extension at 72°C for 30 seconds, and final extension at 72°C for 1 minute. PCR amplicons were run on 1.5% agarose gels. The expected band width for *tuf* and *ddl* PCR products was 112bp and 475bp, respectively. For species identification, species-specific primers (Table 1) targeting the *superoxide dismutase* (*sodA*) gene of *E. faecalis* and *E. faecium* were used. No other primers for species were available. The PCR conditions were as described above for genus except the annealing temperature which was 52°C for both.

Table 1. Primers for Genus and Species identification of *Enterococci*

IDENTIFICATION PRIMERS				
Target gene	**Primer name**	**Primer sequence 5'-3'**	**Amplicon Size bp**	**References**
Tuf	*tuf-F*	TAC TGA CAA ACC ATT CAT GAT G	112	Ke, *et al*, 1999
	tuf-R	AAC TTC GTC ACC AAC GCG AAC		
Ddl	*ddlF*	CAC CTG AAG AAA CAG GC	475	Vilela, *et al*, 2006
	ddlR	ATG GCT ACT TCA ATT TCA CG		
sodAEfm	*sodAEfm1*	CAG CAA TTG AGA AAT AC	190	Bensalah, Flores and Mouats, 2006
	sodAEfm2	CTT CTTTTATTTCTCCTGTA		
sodAEfs	*sodAEfs1*	CTGTAG AAG ACC TAA TTT CA	209	Bensalah, Flores and Mouats, 2006
	sodAEfs2	CAG CTG TTT TGA AAG CAG		

bp = base pair

2.5.5 Determination of Levels of Antimicrobial Resistance

Susceptibility to vancomycin (30 μg), erythromycin (15 μg μg), ampicillin (10 μg), penicillin (10U), tetracycline (30 μg), nitrofurantoin (300 μg), ciprofloxacin (5 μg), chloramphenicol (30 μg), and gentamicin (120 μg) was determined by disk diffusion method according to the Clinical and Laboratory Standards Institute guidelines (2012). Diameters of zones of inhibition were recorded in millimeters and interpreted according to Clinical and Laboratory Standards Institute (2012) as susceptible or intermediate or resistant. All intermediate results were taken as resistant. A reference strain, *Enterococcus faecalis* 29212 was used as control strain.

2.5.6 Detection of Antimicrobial Resistant Genes (ARG)

Detection of genes conferring resistance to glycopeptides (*vanA*), tetracyclines [*tet(M), tet(L), tet(K),* and *tet(X)*], macrolides [*erm(A)* and *erm(B)*] and aminoglycosides [*aac(6')-Ie-aph(2'')-Ia*] was performed using PCR with gene-specific primers (Table 2). One Taq Quick-load 2X Master Mix (Biolabs, Durham, North Carolina, USA) was used for amplification using a thermal cycler (Applied Biosystems, Chiba, Japan). The following PCR conditions were employed; Initial denaturation at 93°C for 3 minutes. The amplification cycles consisted of denaturation at 93°C for 60 seconds, and annealing at 52°C for 60 seconds, elongation at 72°C for 60 seconds. After 35 cycles amplification cycles, the final elongation step was performed at 72°C for 5 minutes. PCR amplicons were run on 1.5% agarose gels. Expected PCR products were different for each gene (Table 2).

Table 2 Primers used for Detection of Resistance Genes

PRIMERS FOR RESISTANCE GENES				
Target gene	**Primer name**	**Primer sequence 5'-3'**	**Amplicon size bp**	**References**
aac(6′)-Ie-aph(2′′)-LA	*aacF*	CAG GAA TTT ATC GAA AAT GGT AGA AAA G	369	Sabouni, *et al.*, 2016
	aacR	CAC AAT CGA CTA AAG AGT ACC AAT C		
***erm*A**	ermAF	TAT CTT ATC GTT GAG AAG GGA TT	139	Goudarzi, *et al.*, 2016
	ermAR	CTA CAC TTG GCT TAG GAT GAA A		
***erm*B**	ermB-1	GAA AAG TAC TCA ACC AAA TA	639	Sutcliffe, *et al.*, 1996
	ermB-2	AGT AAC GGT ACT TAA ATT GTT TA		
***tet*K**	tetK-1	TTA GGT GAA GGG TTA GGT CC	697	Aarestrup, *et al.*, 2000
	tetK-2	GCA AAC TCA TTC CAG AAG CA		
***tet*M**	tetM-1	GTT AAA TAG TGT TCT TGG AG	576	Aarestrup, *et al.*, 2000
	tetM-2	CTA AGA TAT GGC TCT AAC AA		
***tet*L**	tetL-1	CAT TTG GTC TTA TTG GAT CG	456	Aarestrup, *et al.*, 2000
	tetL-2	ATT ACA CTT CCG ATT TCG G		
tetX	*tetXF*	CAA TAA TTG GTG GAC CC	468	Ng, et al., 2001
	tetXR	TTC TTA CCT TGG ACA TCC CG		
***van*A**	*vanAF*	CTG CAA TAG AGA TAG CCG CTA ACA	751	Sting, *et al.*, 2013
	vanAR	TGT ATC CGT CCT CGC TCC TC		

bp = base pair

3.0 RESULTS

3.1 Patient Demographic Characteristics and Isolate Identification

Specimens from 230 patients; 150 from out-patients (non-hospitalized) and 80 from in-patients (hospitalized) were included in this study. More than half of the specimens 55.2% (127/230) were from female patients. Specimens from male patients accounted for 44.8% (103/230). The age of patients ranged from 2 to 86 years old. Two had no age indicated on their request forms. The 230 specimens comprised

of 138 urine, 89 stool and 3 pus swabs. During the study period, a total of 71 *Enterococcus* species were isolated, 59 from urine, 11 from stool and 1 from pus swab. Table 4 shows the species identities in relation to age range and gender, along with specific sources of the isolates, which consisted of *E. faecalis* (40, 56.3%), *E. faecium* (10, 14.1%), a combination of *E. faecalis* and *E. faecium* (5, 7.0%) and other *Enterococcus* species (16, 22.5%) which were not identified to species level due to unavailability of other species-specific primers other than those for *E. faecalis* and *E. faecium* as well as inadequate DNA sequencing reagents.

Table 4. Patient Demographic Characteristics and Isolate Identification

Age range	Total n of specimens processed (230)	*Total n of Enterococcus* isolates (71)	*E. faecalis* (40)	*E. faecium* (10)	*E. faecalis* + *E. faecium* (5)	Other *Enterococcus* species (16)
2-15	51	9	4	1	0	5
16-30	70	19	9	3	1	6
31-45	62	20	12	4	2	2
46-60	14	6	5	0	1	0
61-75	23	12	8	1	0	3
76-86	7	2	1	1	0	0
Not indicated	3	2	1	0	1	0
Gender						
Male	103	39	21	10	1	7
Female	127	32	19	0	4	9
Specimen type						
urine	138	59	37	7	3	12
Stool	89	11	3	2	2	4
Pus swab	3	1	0	1	0	0
Department						
Out-patient	150	51	30	9	1	11
In-patient	80	20	10	1	4	5

n = number

3.2 Prevalence of *Enterococcus faecalis* and *Enterococcus faecium*

The prevalence of *Enterococcus* was 30.9% (71/230, CI: 25.15-37.3). The prevalence of *Enterococcus faecalis* among all *Enterococcus* species was 56.3% (40/71, CI: 44.1-67.9). Table 5 shows summary of the prevalence of *E. faecalis* and *E. faecium* (occurring as single isolates as well as in combination) in clinical specimens at Kitwe Teaching Hospital in the Copperbelt Province.

Table 5. Prevalence of *E. faecalis and E. faecium*

Factor	Categories	*n* Tested	*n* Positive	Prevalence (%)	95% CI
Overall	Positivity	230	71	30.9	25.15-37.3
Enterococci isolates	*E. faecalis*	71	40	56.3	44.1-67.9
	E. faecium	71	10	14.1	7.3-24.8
	E. faecalis + *E. faecium*	71	5	7.0	2.6-16.3
	Other *Enterococcus* species	71	16	22.5	13.8-34.3

n = number, % = percent, CI = confidence interval

3.3 Antimicrobial Susceptibility of *E. faecalis and E. faecium*

All *E. faecalis* isolates were resistant to ciprofloxacin and erythromycin. Most of the *E. faecalis* isolates were resistant to chloramphenicol and tetracycline (97.5%), while 90.0% were resistant to vancomycin. Eighty percent of *E. faecium* isolates were resistant to vancomycin and tetracycline. All *E. faecium* isolates showed phenotypic resistance to ampicillin, ciprofloxacin, erythromycin, nitrofurantoin and penicillin. Generally, *E. faecium* isolates exhibited more resistance to the eight antimicrobials tested than *E. faecalis* isolates (Table 6).

Table 6. Antimicrobial Susceptibility of *E. faecalis and E. faecium*

Species (Total)	Susceptibility Test Result	AMP n (%)	CHL n (%)	CIP n (%)	ERY n (%)	NIT n (%)	PEN n (%)	TET n (%)	VAN n (%)
E. faecalis (40)	Resistant	27 (67.5)	39 (97.5)	40 (100)	40 (100)	30 (75.0)	30 (75.0)	39 (97.5)	36 (90.0)
	Susceptible	13 (32.5)	1 (2.5)	0	0	10 (25.0)	10 (25.0)	1 (2.5)	4 (10)
E. faecium (10)	Resistant	10 (100)	10 (100)	10 (100)	10 (100)	10 (100)	10 (100)	8 (80)	8 (80)
	Susceptible	0	0	0	0	0	0	2 (20)	2 (20)

n = number, AMP = ampicillin, CHL = chloramphenicol, CIP = ciprofloxacin, ERY = erythromycin, NIT = nitrofurantoin, PEN = penicillin, TET = tetracycline, VAN = vancomycin

3.4 Number of *E. faecalis* and *E. faecium* which were resistant to one, two and more than two antimicrobial classes

The majority (97.4%) of *E. faecalis* isolate were multidrug resistant, while 2.6% were resistant only to one class of antibiotics (Table 7). All *E. faecium* isolates were MDR.

Table 7. Number of *E. faecalis* and *E. faecium* which were resistant to one, two and more than two antimicrobial classes

Isolate (Total	All susceptible	Resistant to one class of antibiotic,	Resistant to two classes	Resistant to more than two classes of

number)	n (%)	n (%)	of antibiotic, n (%)	antibiotic, n (%)
E. faecalis (102)	0 (0)	0 (0)	1 (2.5)	39 (97.5)
E. faecium (6)	0 (0)	0 (0)	0 (0)	10 (100)

n = number, % = percent

3.5 MDR patterns

3.5.1 *E. faecalis* MDR Patterns

Only one *E. faecalis* isolate was resistant to two tested antibiotics. Thirty nine isolates were resistant to more than two antibiotic classes. (Table 8). The MDR pattern exhibited by the majority of *E. faecalis* isolates (18, 45%) was AMP, CHL, CIP, ERY, NIT, PEN, TET, VAN combination.

Table 8. MDR patterns of *E. faecalis*

MDR PATTERNS	No. of isolates	No. of antibiotic classes
CIP, ERY	1	2
CHL, CIP, ERY, PEN, TET	1	5
CHL, CIP, ERY, NIT, TET, VAN	3	6
AMP, CHL, CIP, ERY, TET, VAN	2	6
CHL, CIP, ERY, PEN, TET, VAN	3	6
AMP, CHL, CIP, ERY, NIT, PEN, TET	3	6
AMP, CHL, CIP, ERY, NIT, TET, VAN	3	7
AMP, CHL, CIP, ERY, PEN, TET, VAN	2	6
CHL, CIP, ERY, NIT, PEN, TET, VAN	4	7
AMP, CHL, CIP, ERY, NIT, PEN, TET, VAN	18	7

AMP = ampicillin, CHL = chloramphenicol, CIP = ciprofloxacin, ERY = erythromycin, NIT = nitrofurantoin, PEN = penicillin, TET = tetracycline, VAN = vancomycin

3.5.2 *E. faecium* MDR patterns

None of the *E. faecium* isolates was susceptible to all tested antibiotics. Two isolates were resistant to two antibiotics and the rest were resistant to three or more antibiotics (Table 9).

Table 9. MDR patterns of *E. faecium*

MDR patterns	*No. of isolates*	*No. of antibiotic classes*
AMP, CHL, CIP, ERY, NIT, PEN	2	6
AMP, CHL, CIP, ERY, NIT, PEN, TET, VAN	*8*	*7*

AMP = ampicillin, CHL = chloramphenicol, CIP = ciprofloxacin, ERY = erythromycin, NIT = nitrofurantoin, PEN = penicillin, TET = tetracycline, VAN = vancomycin

3.6 Presence of Antimicrobial Resistance Genes in *E. faecalis* and *E. faecium*

The most commonly detected resistant gene in *E. faecalis* was *ermB*. This was followed by *tetL* and *aac(6')-Ie-aph(2")-LA*. TetX was absent in these isolates, while the occurrence of vanA gene was infrequent. In *E. faecium*, the most commonly detected resistance gene was *aac(6')-Ie-aph(2")-LA* and *tetM*, while *ermA* was seldomly detected and *tetX* and *vanA* were undetected (Table 10).

Table 10. The number of different resistance genes detected in *E. faecalis* and *E. faecium* isolates

Resistance gene	*E. faecalis* (40)			*E. faecium* (10)		
	Detected (n)	Proportion (%)	Undetected	Detected (n)	Proportion (%)	Undetected
aac(6')-Ie-aph(2")-LA	33	82.50%	7	9	90.00%	1
ermA	2	5.00%	38	1	10.00%	9
ermB	39	97.50%	1	5	50.00%	5
tetK	19	47.50%	21	4	40.00%	6
tetM	31	77.50%	9	8	80.00%	2
tetL	38	95.00%	2	5	50.00%	5
tetX	0	0	40	0	0	10
vanA	**1**	2.50%	39	0	0	10

n = number

3.7 Association between antimicrobials and resistance genes

Differences in antimicrobial resistance patterns and resistance genes in both *Enterococcus* species were analyzed to assess possible associations between resistance phenotypes and their corresponding genotypes. A positive association between phenotype and genotype was found for tetracycline ($p = 0.047$) and erythromycin ($p = 0.008$), but there was no association between genotype and the vancomycin resistance phenotype ($p = 0.051$) (Table 11).

Table 11. Association between antimicrobial results and their corresponding resistance genes.

Antibiotic	Genes	X^2—Value	*p*-Value
TET	*tet*	3.945	0.047 ***
ERY	*erm*	6.947	0.008 ***
VAN	*vanA*	3.795	0.051

X^2 = Chi-square value; *p*-Value = significant at <0.05; TET = Tetracycline; ERY = Erythromycin; VAN = Vancomycin; *tet* = all tetracycline genes (*tetM*, *tetL*, *tetK* and *tetX*); *erm* = both *ermA* and *ermB* genes

4.0 Discussion

Detection of *E. faecalis* and *E. faecium* in this study is remarkable because these two species are the leading causes of enterococcal infections world-wide (Zhou, *et al.*, 2020; García-Solache and Rice, 2019). Of interest too is the detection of antimicrobial resistance genes as well as virulence genes in these *Enterococcus* species in clinical specimens. It is important to note that these findings are of public health importance whether they have caused infection in the host or are just colonizing. Patients harboring such

Enterococcus species have the potential to act as reservoirs from which the organisms can be transmitted to healthcare workers, the surrounding environment and other patients (Jackson, *et al.*, 2019; Cassone, *et al.*, 2020). The current research presents an assessment of the current trends in species distribution, antimicrobial susceptibility, and virulence trait profiles of clinical enterococcal isolates at Kitwe Teaching hospital in Zambia. The majority of *Enterococcus* isolates (59/71) were obtained from urine specimens. This is consistent with findings from similar studies conducted in India, Egypt and France (Mohanty and Behera, 2022; Sumangala, Sharlee and Sahana Shetty, 2020; Said and Abdelmegeed, 2019) which reported 80.1%, 89/111; 54.4%, 56/103 and 59.5%, 25/42; 54.4% respectively.

In this study prevalence of *Enterococcus* was 30.9%. This finding align with previous report which indicated prevalence of *Enterococcus* to be 32.6% by Lancu and others in Romania (2023) and also agreed with a study done earlier whose prevalence was 31.1% in poultry droppings (Mwikuma, *et al.*, 2023). However our findings did not agree with an earlier study which reported higher prevalence of 43.42% in India (Sreeja, *et al.*, 2012). The present study's prevalence was higher than reports from studies conducted in Ethiopia (2.1%), Asian pacific (3.6%) and in USA and Canada (18.0% and 21.2%, respectively) (Abera, *et al.*, 2021; Paul, Nirwan and Srivastava, 2017; Low, *et al.*, 2001).

The prevalence of *Enterococcus faecalis* among all *Enterococcus* species was 56.3% (40/71, CI: 46.24-70.41) making it the most prevalent species. *Enterococcus faecium* accounted for 14.1%. This distribution agrees with other studies which found *E. faecalis* to be higher than *E. faecium*; 69.2% and 11.3% respectively in Saudi Arabia (Salem-Bekhit, *et al.*, 2012) and in Kuwait with 85.3% and 7.7% respectively (Udo *et al.*, 2003). However, a study done by Jia, Li and Wang (2014) shows a higher distribution of *E. faecium* (58.7%) than that of *E. faecalis* (33.0%). The majority of *E. faecalis* and *E. faecium* isolates were from urine specimens and were from age ranging from 16 to 45 years old. In agreement with this, studies by Boccella and others (2021) and Salem-Bekhit and others (2012) indicated that *E. faecalis* and *E. faecium* were isolated at higher frequency from urine cultures (32.5% and 46.6% respectively). *E. faecium* was only recovered from males and mainly from urine of non-hospitalized patients.

In the present study, the highest levels of resistance observed in both *E. faecalis* and *E. faecium* was to ciprofloxacin (100%) and erythromycin (100%). Our findings revealed that *E. faecium* exhibited higher resistance rates to most antimicrobial agents used in clinical treatment compared to *E. faecalis*. For example; all *Enterococcus faecium* isolates were resistant to ampicillin, chloramphenicol, ciprofloxacin, erythromycin, nitrofurantoin and penicillin whereas all *E. faecalis* isolates were resistant to just two drugs namely ciprofloxacin and erythromycin. These results align with previous reports (Horner, *et al.*, 2021; Tollu and Ekin, 2020; Cui, *et al.*, 2020; Golob, *et al.*, 2019) which supports the notion that *E. faecium* is generally more prone to develop resistance than *E. faecalis*. The infections caused by *E. faecium* pose significant clinical challenges for physicians due to their higher resistance to drugs commonly used in clinical practice thereby limiting treatment options (Zhou, *et al.*, 2020). All (100%) *E. faecium* and 97.5% *E. faecalis* isolates were multidrug resistant (MDR). This is concurrent with a studies by Esmail, Abdulghany and Khairy (2019), and Tremblay and colleagues (2011) in which 100% of all *E. faecalis* and *E. faecium* isolates respectively were MDR. Selective pressure exerted by broad application of antibiotics in health care and animal husbandry enhance development of MDR. This leads to increase in prevalence of resistance and creation of reservoirs resistance genes in some *Enterococcus* species, especially *Enterococcus faecalis* and *E. faecium* (Ahmad, *et al.*, 2011).

The most prevalent resistance gene in *E. faecalis* was *ermB* (97.5%), followed by *tetM* (95.0%) and *aac(6')-Ie-aph(2")-LA* (82.5%). In *E. faecium*, the most frequently detected resistance genes were *aac(6')-*

Ie-aph(2")-LA (90.0%) and tetL (80.0%). The detection of *aac(6')-Ie-aph(2")-LA* gene in 82.5% of *E. faecalis* and in 90.0% of *E. faecium* was in line with Niu and others (2016) who found that 89.3% of *Enterococcus* isolates in their study carried the *aac(6')-Ie-aph(2")-LA* gene. Although the gene *aac(6')-Ie-aph(2")-LA*, which encodes resistance to gentamicin was detected in both *Enterococci* species tested, an association between the phenotype and genotype could not be established as gentamycin discs containing high concentrations of gentamicin such as 120 µg or 500 µg, which are used for detection of high-level aminoglycoside resistance, were not available. Amongst the *erm* genes detected, *ermB* was the most prevalent gene in erythromycin resistant *Enterococcus faecalis* isolates. This finding concurred with the findings of earlier studies (Ahmadpoor , et al., 2021; Marghmalek, *et al.*, 2021; Kim, *et al.*, 2019; Tian, *et al.*, 2019). *tetM* was the most frequently detected gene among the tetracycline resistant *Enterococcus faecalis* isolates. Our findings collaborated with the findings of Tian and colleagues (2019).

The *vanA* gene was only detected in one *E. faecalis* isolate and was not detected in *E. faecium* isolates. The phenotypic resistance exhibited by both *E. faecalis* and *E. faecium* were due to other *van* genes which were not tested. This shows that *vanA* was not common in *Enterococcus* species in our study and is in contrast with what others studies assert. For example, Daghighi and others (2014) shows that 89.3% samples had *vanA* gene. Haghi, Lohrasbi, and Zeighami (2019) detected *vanA* gene in all *E. faecium* isolates. Others studies which shows predominance of *vanA* over other *van* genes include Jahansepas and others (2018) and Kristich, Rice and Arias (2014).

5.0 Conclusion

In essence, this study underscores the clinical significance of detecting and understanding the prevalence, distribution, and resistance patterns of *E. faecalis* and *E. faecium*. The findings contribute to the broader understanding of antimicrobial resistance and the potential impact on patient care, healthcare practices, and public health strategies. The study further shed light on the challenges posed by multidrug resistance and genetic factors affecting resistance. Addressing the challenges posed by these organisms requires a multi-faceted approach, encompassing appropriate antibiotic stewardship, infection control measures, and further research into novel treatment strategies to mitigate the impact of antimicrobial resistance.

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