THE PUBLIC HEALTH SIGNIFICANCE OF *ENTEROCOCCI* FROM DAIRY CATTLE IN LUSAKA PROVINCE, ZAMBIA

By

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A dissertation Submitted to the University of Zambia in Partial Fulfillment of the requirements for the Degree of Master of Science in Tropical Infectious Diseases and Zoonosis

THE UNIVERSITY OF ZAMBIA

LUSAKA

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DECLARATION

I, MATENGE MUTALANGE, declare that I have written this dissertation and that the work has not been submitted to any other institution for any other degree or professional qualification. Where assistance was sought, it has been acknowledged accordingly.

Candidate Name: Matenge Mutalange

Signature …….. ..

Date……..

CERTIFICATE OF APPROVAL

The University of Zambia approves the dissertation submitted by MATENGE MUTALANGE as fulfilling the partial requirements for the award of the Master of Science Degree in Tropical Infectious Diseases and Zoonosis by the University of Zambia.

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Supervisor	Signature	Date
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ABSTRACT

*Enterococc*i are commensal Gram-positive bacteria found in the intestines of humans and animals, but can also cause infections. *Enterococcus faecalis* and *Enterococcus faecium* are a common cause of urinary tract infections, wound infections, bacteremia, infective endocarditis in humans and bovine mastitis in dairy animals. Notably, *Enterococci* infections in the nosocomial setting (hospital-acquired infections) are usually difficult to treat while Infections of animals with *Enterococci* are rarely targeted explicitly with antimicrobial agents. However, as normal inhabitants of the intestinal tract, *Enterococci* are exposed to antimicrobial selection every time animals are exposed to antimicrobials. Thus, *Enterococci* inhabiting non-human reservoirs such as poultry and cattle play a critical role in acquiring and disseminating antibiotic resistance determinants. Antibiotic-resistant bacteria associated with animals may be easily transmitted to humans via food chains such as milk, meat and eggs. These resistant bacteria are also widely distributed in the environment via animal and human waste. Currently, there is little information about *Enterococci* infections in the dairy cows in Zambia. Therefore, this study was conducted to evaluate the public health significance of *Enterococcus* species in milk and faecal matter of dairy cow in Lusaka province, Zambia.

This cross-sectional study was carried out from September 2022 to March 2023 in selected districts of Lusaka Province, including Chilanga, Chongwe, and Lusaka districts. Three cows from each farm were randomly selected and both milk and faecal matter were collected and thus ninety-nine raw milk and 99 faecal samples were collected from 33 dairy farms. Both milk and faecal samples were processed to isolate *Enterococcus species* using standard microbiology procedures. *Enterococcus species* were tentatively identified based on phenotypic characterisation and confirmed with polymerase chain reaction (PCR) utilising the *tuf* gene. PCR was also used to speciate *Enterococci* using the *SodA* gene for *E. faecalis* and *E. faecium*. In order to determine susceptibility patterns, isolates were subjected to seven different antibiotics using the Kirby-Bauer disk diffusion

method, while vancomycin-resistant isolates on disc diffusion were subjected to minimum inhibitory concentration (MIC) testing using the E-strip method. Erythromycin-resistant isolates were screened for the presence of *erm* A, *B, and C* resistance genes. *Enterococci* recovery rate was (40%) and (90%) in milk and faecal samples, respectively. Using the *tuf* gene, the 50 randomly selected isolates were confirmed as, *Enterococci* species, of which (22%) were *E. faecalis* and (8%) were *E. faecium*. Antibiotic susceptibility patterns showed the highest resistance was to ampicillin, 63% and 57% in faecal matter and raw milk, respectively. The faecal matter *Enterococci* isolates had the highest resistance patterns in almost all the antibiotics except tetracycline and chloramphenicol. The prevalence of multi-drug resistance was 15% and 34% in milk and faecal matter isolates, respectively. The prevalent erythromycin resistance gene was *erm* B. The detection of *Enterococci* spp. in milk indicates poor hygienic conditions and contamination and, thus, a public health threat as *Entrococci* may be transmitted to humans through milk consumption. These findings highlight need to formulate education programs to farmers on good hygiene practices to prevent dissemination.

ACKNOWLEDGEMENTS

This research would not have been possible without the support of many people. Firstly, I would like to thank my sponsors Africa Centre of Excellence for the Infectious Diseases Human and Animals (ACEIDHA), for the financial help rendered during the year of study.

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Last but not least, I am grateful to my family and friends for the support and encouragement I received during my study period. Above all, I thank the Almighty God for His guidance throughout my studies.

DEDICATION

This work is dedicated to my father, Boniface Mutalange, and my mother, Precious Moono Mutalange.

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LIST OF ACRONYMS AND ABBREVIATIONS

%	Percentage
℃	Degree Celsius
µl	Icroliter
ACEIDHA	Africa Centre of Excellence for the Infectious Diseases Human and Animals
AMR	Antimicrobial Resistance
AMU	Antimicrobial Use
ARGs	Antimicrobial Resistant Genes
AST	Antimicrobial Susceptibility Testing
CLSI	Clinical Laboratory Standards Institute Guidelines
DW	Distilled Water
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleotide Triphosphate
Erm A, B, C	Erythromycin *A, B,C* genes
GIT	Gastrointestinal Tract
GRZ	Government Republic of Zambia
HCAIs	Health Care Associated Infections
HGT	Horizontal Gene Transfer

MDR	Multi-Drug Resistance
MgCl	Magnesium Chloride
MIC	Minimum Inhibitory Concentration
MIN	Minute
PCR	Polymerase Chain Reaction
TBE	Tris-borate-ethylenediaminetetraacetic Acid
UNZA	University of Zambia
UTH	University Teaching Hospital
UV	Ultraviolet
VRE	Vancomycin-resistant Enterococci
SEC	Second

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Enterococci are commensal bacteria in the intestines of humans and animals, but some species have been implicated in a wide range of infections. *Enterococci* inhabiting non-human reservoirs such as poultry and cattle are a public health concern that play a critical role in acquiring and disseminating antibiotic resistance determinants (Daniel et al., 2015). *Enterococci* are Gram-positive cocci that occur in pairs or short chains, catalase and oxidase-negative, and facultative anaerobes. They are mesophilic bacteria that could grow from 10ºC to 45ºC with optimal temperatures ranging between 30ºC and 35ºC. They can also grow in a wide pH range (from 4.4 to 9.6).

Athough over 50 different species have been described, *Enterococci. faecalis* and *Enterococci. faecium* account for the majority of human. Enterococcal infections and implicated in the majority of healthcare-associated infections (HCAIs) (Bhardwaj, 2019). *Enterococci* are intrinsically resistant to some antibiotics and can also acquire a wide range of antibiotic resistance genes through the horizontal exchange of mobile genetic material (Murray, 1990). Resistant *Enterococci* are selected both in humans and animals, owing to the use of antimicrobial agents in both settings.

As a result of the increasing population and livestock farming, there is increased interaction between humans and animals in livestock-keeping communities. This enhances opportunities for pathogen transmission between humans and animals and heightens the risk of emerging zoonotic diseases (Jori et al., 2021). The development of antimicrobial resistance (AMR) is considered one of the greatest threats to public health as it becomes challenging to treat infections (Hammerum, 2012). Antibiotic-resistant commensal bacteria in animals may be pathogenic to humans as they may be easily transmitted to humans via food chains (Giraffa,

2002). Resistant *Enterococci* have also been found to be widely distributed in the environment through animal and human waste that is not managed correctly (Hammerum, 2012).

Zambia recognised the public health threat of AMR and its impact on morbidity and mortality, as well as the subsequent economic consequences; this prompted the drafting of a multisectoral national action plan (NAP) that encourages the collaboration among the human, animal, and environmental sectors and provides guidance on the One health coordination (GRZ, 2021). Thus, this study was designed to evaluate the public health significance of *Enterococcus* in milk and faeces of dairy cattle in Lusaka province of Zambia.

1.2 Statement of the Problem

Zambia has seen an increase in smallholder dairy production, however, milk hygiene still require improvement (Kunda et al., 2015) . Raw milk that is consumed by the population in Western Zambia was found to be contaminated with bacteria such as *Escherichia coli* and *Staphylococcus Aureus* (Knight-Jones et al., 2016). A study conducted in Morocco showed that raw milk was contaminated by *Enterococci* that was resistant to antibitiocs (Bouymajane et al., 2018). The growing problem of AMR in humans and animals negatively affects the treatment of Enterococcal infections in humans as managing these infections becomes complex due to the limited treatment options (García-Solache and Rice, 2019). A study conducted at the University Teaching Hospital (UTH) in 2021 isolated *Enterococcus* species from blood and recorded high levels of MDR and reduced susceptibility (intermediate) to vancomycin. Similarly, a study that evaluated 91 *Enterococcus* species from 82 cattle in Kafue revealed high levels of resistance to some antibiotics (Mutalange et al., 2021; Mubita et al., 2008). Another study investigating the prevalence and AMR patterns of *Enterococci* isolated from layer hens in Lusaka and Copperbelt Provinces of Zambia recorded a high prevalence of MDR *Enterococci*

(Mudenda et al., 2022). In Zambia high levels of inappropriate antimicrobial use have been recorded in human and animal sectors, where people in the community and poultry farmers access or purchase antibiotics without prescriptions (Mudenda et al., 2022). The source of MDR Enterococcal clinical infections and the carriage of MDR *Enterococcus* species in humans and animals is poorly understood (Weese, 2008). Although the presence of AMR commensal bacteria in food-producing animals has been found to be high, the possibility of cross-transmission between humans and animals has not been explored (Ewers et al., 2012), and there are currently no comprehensive studies on evaluating public health significance of *Enterococcus* species in milk and faeces of dairy cattle in Zambia.

1.3 Justification of the Study

Milk is widely consumed as a source of calcium in both the pediatric and adult population (Lanou et al., 2005); thus, ensuring that the milk is free of AMR commensal bacteria and is safe for human consumption is of utmost importance. Although there is a paucity of data on AMR in *Enterococcus* species from cattle in Zambia, similar resistance patterns and high levels of MDR in clinical *Enterococcus* species from humans and carriage *Enterococcus* species from cattle and poultry highlight the need for One Health-coordinated research and surveillance with a focus towards food safety (Magnusson et al., 2021). *Enterococci* can be used as bacterial markers of faecal contamination of food and water for human consumption (Roslev and Bukh, 2011), and it has been revealed that *Enterococci* from non-human sources could contaminate food intended for human consumption (Daniel et al., 2015). Science-based and epidemiologically sound research are critical in ensuring food safety and informing policy and legislation (Zaheer et al., 2020). Surveillance programs that highlight AMR trends can be used to evaluate production practices so as to inform regulators on the factors driving

contamination and spread of AMR resistance. Lastly, knowledge on AMR and ARGs can be used to formulate antibiotic stewardship programs in One Health continuum.

1.4 Signficance of the Study

This study will evaluate the presence of *Enterococci* in dairy cows ,Suscpetibility patterns and presence of resistant genes (AGRs). Thus knowledge of *Enterococci* AMR and ARGs obtained in this study can be used in surveillance and antibiotic stewardship programs in one health in Zambia among dairy farmers to prevent the emergence of zoonotic infections.

1.5 Research Question

What is the public health significance of *Enterococcus* species in milk and faeces of dairy cattle in Lusaka Province Zambia?

1.6 Objectives

1.6.1 General Objective

To evaluate the public health significance of *Enterococcus* species in milk and faecal matter of dairy cattle in Lusaka province, Zambia.

1.62 Specific Objective

1.6.2.1 To determine the prevalence of *Enterococcus in* milk and faecal matter of dairy cattle.

1.6.2.2 To determine the prevalence of *E. faecalis* and *E. Faecium* in milk and faecal matter of dairy cattle.

1.6.2.3 To determine the antibiotic susceptibility patterns of all the *Enterococci* isolates and genes in erythromycin and vancomycin resistant isolates .

1.7. Organisation of the dissertation

The dissertation is organized in six chapters. The first chapter provides the background to the study and highlights the problem statement,justification,study significance study question and

objectives. Chapter two gives a review of *Enterococci* and identifies knowledge gaps . The third chapter explains the materials used in the study and describes the methods that were used used to achieve the objectives, this chapter also described how data was analysed and ethical consideration observed in undertaking the study. Chapter four provides a detailed description of the study findings . Chapter five provides a discussion of the study findings according to study objectives and their significance to day life and compares the findings to other studies while chapter six draws conclsion from the findings and makes recommendation.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 *Enterococcus* species

Enterococci are normal flora of the intestinal tract and are classified as lactic acid bacteria (Klein, 2003).They can also grow , at high salt concentrations (up to 6.5% NaCl), and can hydrolyse esculin in the presence of 40% bile salts, a characteristic used for phenotypic identification processes (Lebreton et al., 2014). Recently, they have emerged as nosocomial pathogens largely due to their resistance to antimicrobials (Torres et al., 2018). The therapeutic failures in enterococcal infections are mainly due to intrinsic as well as transferable drug resistance (Pahadi et al., 2014). *Enterococci* exhibit a remarkable array of environments where it survives and can be found in water, soil, sewage, and food items like dairy and meat products (Torres et al., 2018).

Enterococcus species have been reported to cause UTIs, bacteraemias, and endocarditis in humans (Said et al., 2021). *Enterococcus* is among the causative agents of mastitis in dairy cattle (Różańska et al., 2019). *E. hirae, E. avium, E. durans, E. gallinarum, E. casseliflavus,* and *E. raffinosus,* are rare causes of human clinical infections and are thought to be more opportunistic in nature (Mastroianni, 2009). *E. faecalis* and *E. faecium* are also the most enterococcal species detected in the human intestine, whereas other species, such as *E. durans* and *E. avium*, are detected occasionally (Lebreton et al., 2014). In cattle and swine, the proportions of the enterococcal species vary across studies. *E. faecium, E. durans, E. hirae,* and *E. faecalis* were unanimously found in different surveys (Devriese et al., 1992). In some works, *E. faecalis* was the predominant enterococcal species in the gut of bovines and swine (Hwang et al., 2009). In others, *E. hirae* and *E. faecium* were described as the more abundant bacteria in both livestock species (Iweriebor et al., 2016).

E. faecalis and *E. faecium* have become increasingly important pathogens worldwide, especially due to life-threatening hospital-acquired infections (Bhardwaj, 2019). In cattle, udder inflammations (mastitis) are the most frequent and cost-generating illness of dairy cows worldwide and can be caused by a variety of bacterial pathogens, including *Enterococci* (Klaas and Zadoks, 2018). A study that was carried out in South Korea indicated the presence of *E. faecalis* and *E. faecium*, and these isolates exhibited high virulence properties (Kim et al., 2022). Thus, if good husbandry methods are not practiced, virulence genes can be transmitted to other animals and cause infections and humans through the milk, contributing to potential pathogenesis in humans.

2.2 Overview of Antimicrobial Resistance

Antibiotic resistance (AMR) is reported to occur when a drug loses its ability to inhibit bacterial growth effectively. Thus, bacteria continue to multiply in the presence of therapeutic levels of antibiotics (Bush, 2017). Antimicrobials are used in animal settings to treat bacterial infections, prevent disease in a herd and act as growth promoters (Magnusson et al., 2021). The use of antibiotics such as fluoroquinolones, among other antimicrobials in livestock, poultry, and aquaculture, may reduce the effectiveness of these antibiotics due to the development of resistance in zoonotic pathogens (Lekshmi et al., 2017).

Multi-drug resistance (MDR) occurs when a bacteria species become resistant to atleast one antibiotic in three or more antibiotic classes. According to Mellata 2013, MDR has resulted in the failure of clinical treatments and has caused significant economic and health issues affecting the livestock industry and human healthcare (Mellata, 2013). MDR *Enterococci* are important nosocomial pathogens and a growing clinical challenge as these organisms have developed resistance to virtually many antimicrobials using diverse genetic strategies (Eliopoulos and Gold, 2001). Thus, treatment of these infections becomes complex due to

limited drug options. Mudenda et al 2022 recorded 86% of MDR *Enterococci* in layer hens in Zambia.

AMR represents a significant threat to animal and human livelihood(Bengtsson and Greko, 2014). However, the situation in Africa is compounded by several factors that include a lack of access to appropriate antimicrobial therapy, weak regulations that forbid the irrational purchase and use of antimicrobials for humans and animals, weak surveillance systems, lack of updated antimicrobial use and treatment guidelines and lack of continuing education on antimicrobial use (Kimera et al., 2020).

AMR in animals leads to an increased frequency of treatment failures and increased severity of infections (Bengtsson and Greko, 2014). Further, limited treatment, either through the occurrence of resistance or restrictions on their use, has consequences for animal health, welfare, and productivity (Caceres et al., 2017). For livestock owners, AMR may lead to financial losses directly through higher mortality and indirectly through reduced production and growth, and thus, human livelihood may be affected due to higher prices of commodities and food from animal production (Catry et al., 2010).

2.3 Antimicrobial Use and Resistance in Animals

In dairy cattle, antibiotics are used for prophylaxis, therapeutics, and metaphylaxis and as growth promoters to keep animals healthy and ensure high production (Loo et al., 2019). AMU around the globe in livestock has been estimated to be 73% (Van Boeckel et al., 2015). The intensification of livestock farming drives the use of antibiotics to meet the growing human demand (Loo et al., 2019). Another study that estimated the AMU and AMR in food-producing animals and the environment revealed a high prevalence of farms using antimicrobials ranging between 77.6% in Nigeria and 100% in Tanzania, Cameroon, Zambia, Ghana and Egypt (Kimera et al., 2020). Mastitis infections account for the major use of

antibiotics in dairy cattle (Loo et al., 2019). In Zambia, AMU among poultry farmers was found at 86% (Mudenda et al., 2022). The irrational use of antimicrobials in food production contributes to the development of AMR.

Antibiotic resistance in dairy cattle is due to the overuse and misuse of antibiotics (Loo et al., 2019); thus, creating enormous selection pressure in animals and increasing the likelihood that bacteria will adapt and multiply to produce a more resistant population (Bush, 2017). The agricultural industry relies on antimicrobials to improve animal health and productivity, especially in intensively reared species (Manyi-Loh et al., 2018). As with humans, animals are also susceptible to diseases that necessitate treatment. However, treatment of pathogenic organisms in a farming context heighten selective antibiotic pressure that influences the overall commensal flora of the intestine (Bengtsson and Greko, 2014). Mastitis is a major condition prompting the use of antibiotics in dairy cattle **(**Cheng and Gu Han, 2020). Antibiotic resistance is complex and challenging because zoonotic bacterial pathogens are not regularly cultured, and their resistance to commonly used antibiotics still needs to be investigated (Manyi-Loh et al., 2018). The spread of AMR is facilitated by the high density of animals involved in primary production (Aminov and Mackie, 2007). Consequently, the farming industry has been established as an AMR reservoir 'hotspot' (Bester and Essack, 2010), thus creating a route for transferring resistant organisms from animals to humans. The presence of antibiotic-resistant *Enterococci* in animal food products and faecal material of animals has therefore become a major public health concern that affects both human and veterinary medicine (Daniel et al., 2015).

2.4 Mechanisms of AMR Development

There are two types in which bacteria gain resistance; this includes natural and acquired (Reygaert, 2018). Natural resistance is also known as intrinsic resistance. It is defined as a trait shared universally within a bacteria species that makes it resistant to certain

antimicrobials or family of antimicrobials, without previous antimicrobial exposure or the need for mutation or further acquisition of resistance gene. In this mechanism, resistant genes exist naturally but are only expressed to resistance levels after exposure to an antibiotic**.** Acquired resistance is achieved by transferring genetic bacteria that confers resistance; in this mechanism, genes are acquired through horizontal gene transfer (HGT). HGT is the movement of genetic information between organisms without being its offspring (Reygaert, 2018). Bacteria have acquired a variety of important traits through HGT, including antibiotic resistance genes. There are three mechanisms of horizontal gene transfer which are transformation, transduction, and conjugation (Villa et al., 2019).

Transformation is a form of genetic recombination in which a DNA fragment from a dead, degraded bacterium enters a competent recipient bacterium and is exchanged for a piece of DNA of the recipient (Sinha et al., 2013). It involves only homologous recombination of DNA regions having nearly the same nucleotide sequences and hence involves strains of the same bacteria regions.

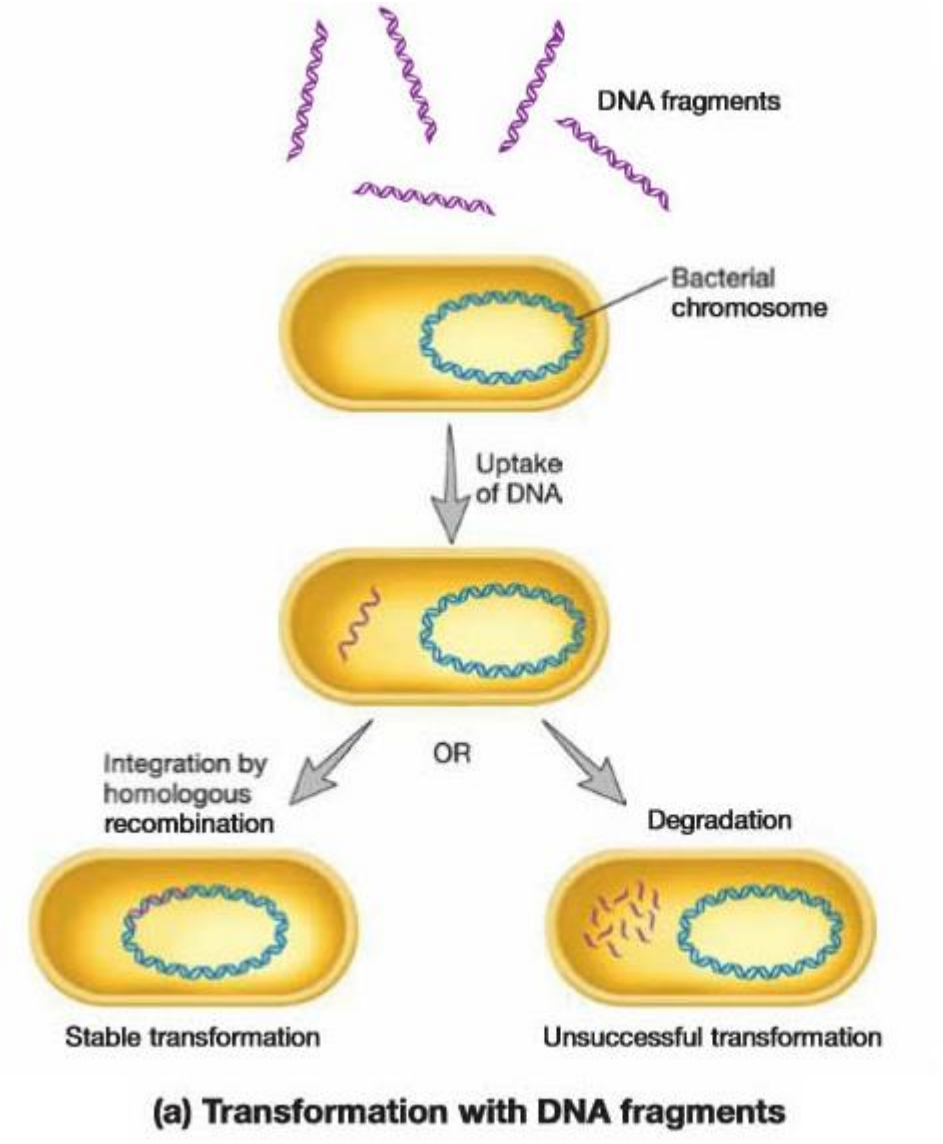

Figure 1: The diagram above shows the transfer of genetic material using transformation. (Source: https://istudy.pk/bacteria-transformation/).

Transduction involves the transfer of a DNA fragment from one bacterium to another by a bacteriophage (Schneider, 2021). During the replication of bacteriophages, the phage capsid accidentally assembles around a small fragment of bacteria DNA. When the bacteriophage, called a transducing particle, infects another bacterium, it injects the fragment of donor DNA into the recipient.

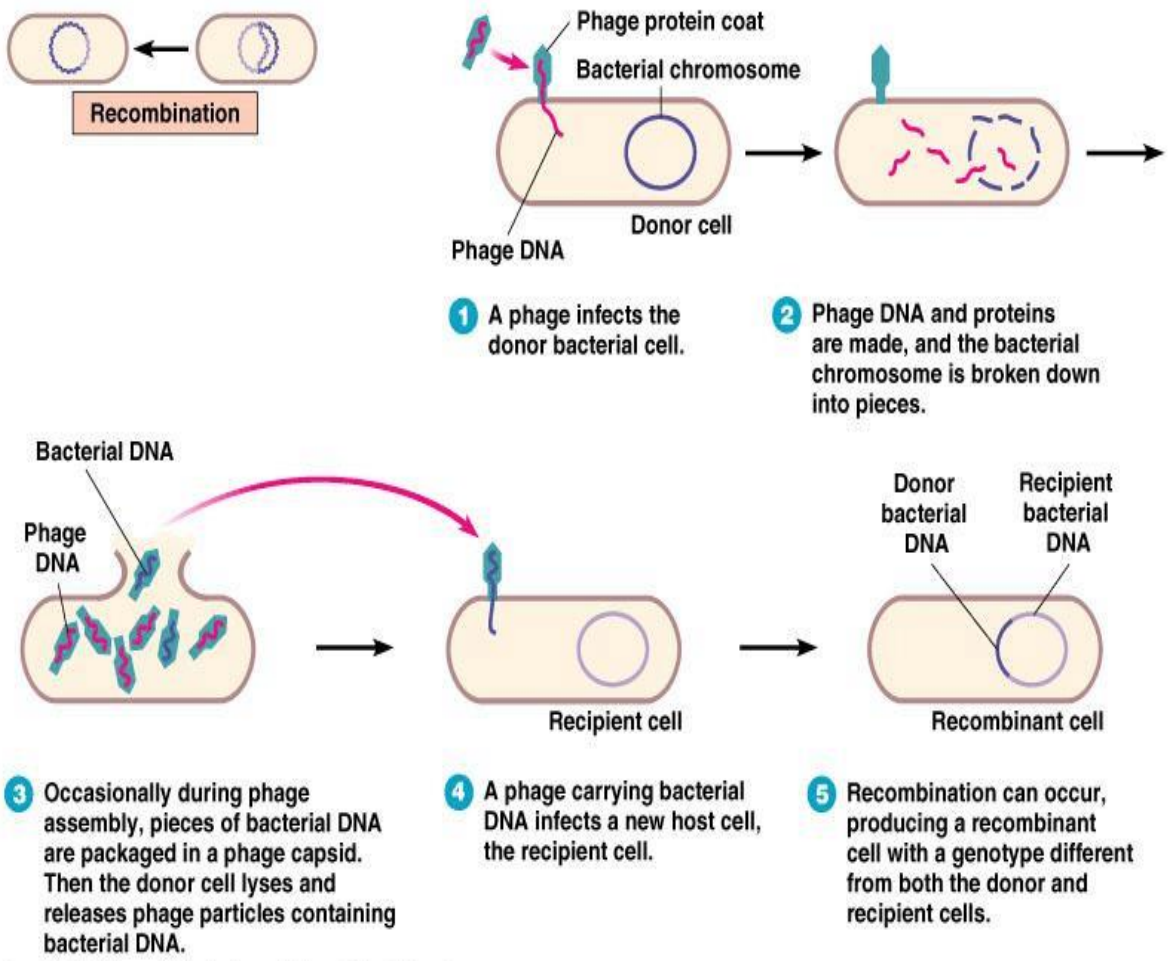

Figure 2: The diagram above shows the transfer of genetic material using transduction. (Source:https://www.onlinebiologynotes.com/transduction-generalised-and-specialised-transduction/)

Conjugation recombination is the transfer of DNA from a living donor bacterium to a living recipient bacterium by cell-to-cell contact. It is encoded by plasmids or transposons (Virolle et al., 2020).

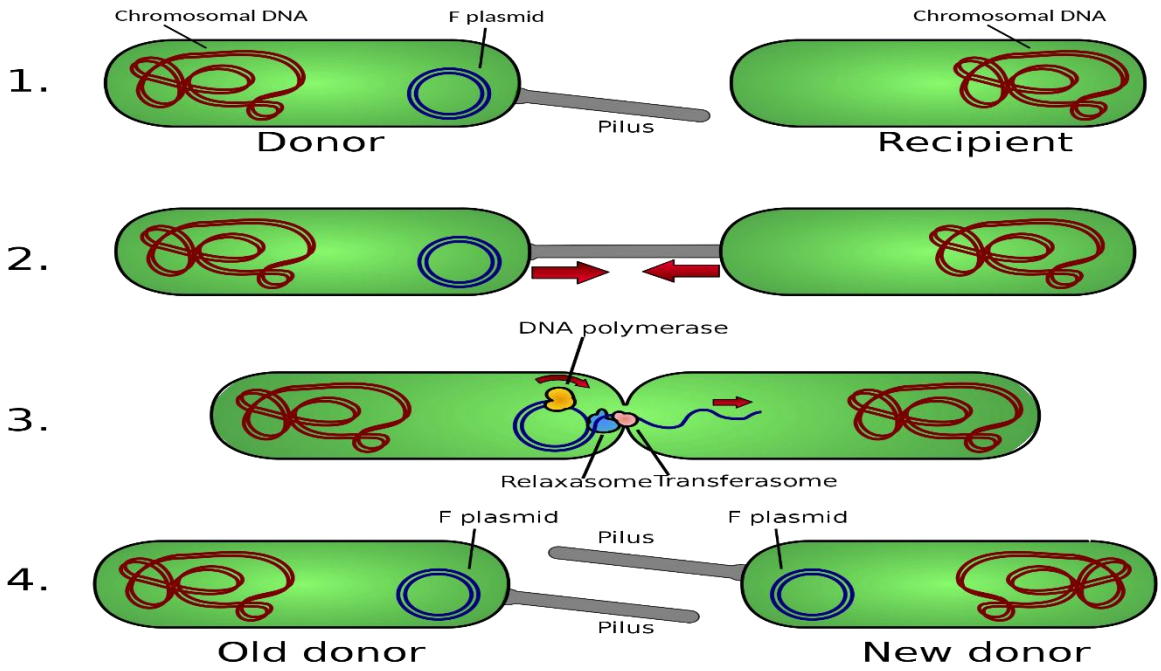

Figure 3: The diagram above shows the transfer genetic material using conjugation. (Source:https://en.m.wikepedia.org/wiki/bacteria-conjugation).

2.5 Mechanism of Antibiotics

Antibiotics can be divided into two groups based on their effect on microbial cells through two mechanisms, which are either bactericidal or bacteriostatic (Bernatová et al., 2013); bactericidal antibiotics kill the bacteria, and bacteriostatic antibiotics suppress the growth of bacteria. Five bacterial targets have been exploited in the development of antimicrobial drugs: cell wall synthesis, protein synthesis, DNA synthesis, ribonucleic acid, and intermediary metabolism (Hooper, 2001).

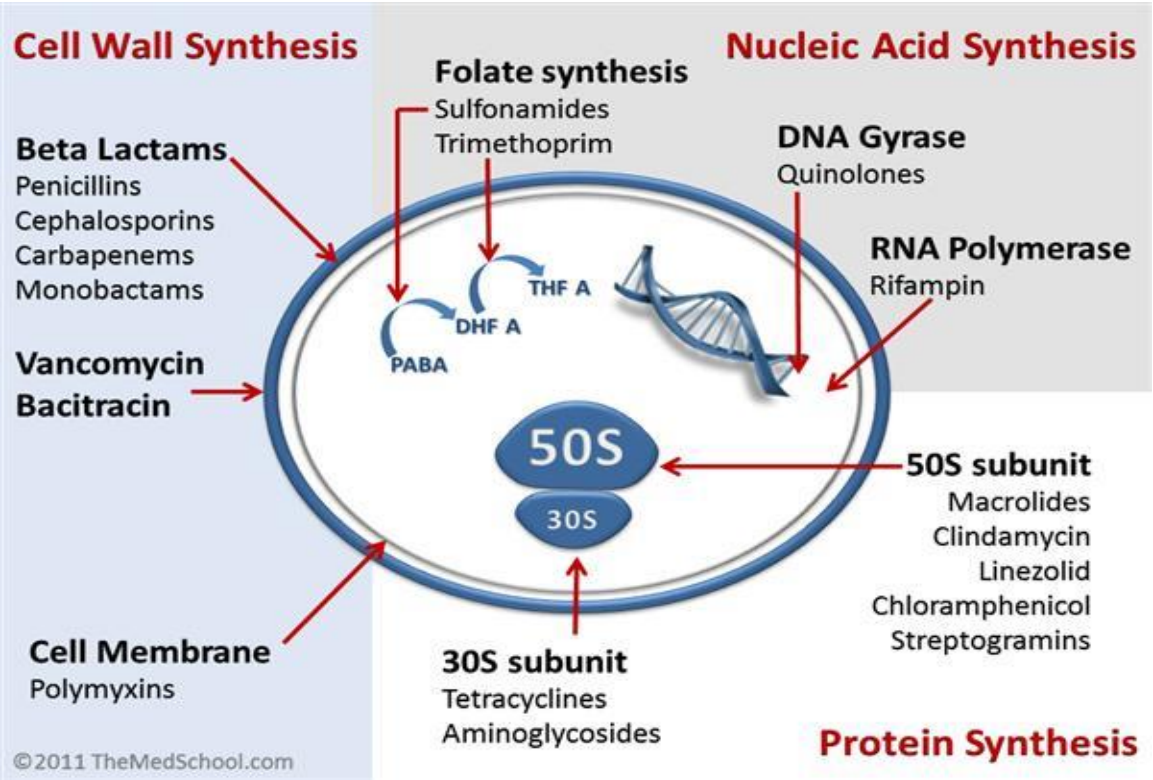

Figure 4: The diagram above shows antibiotics and their specific bacteria target (Source:https://www/researchgate.net/figure/Antibiotic-target-sites-Madigan- and –Martinko-2006-fig11-319881509).

MDR *Enterococci* display a wide repertoire of antibiotic resistance mechanisms, including modification of drug targets, inactivation of therapeutic agents, overexpression of efflux pumps, and a sophisticated cell envelope adaptive response that promotes survival in human and animal hosts (Miller et al., 2014).

Enterococci are among the best-known family of microbes equipped with intrinsic and acquired antibiotic resistance, and of particular concern are *E. faecali*s and *E. faecium* as they have multiple acquired resistance mechanisms, thereby resulting in MDR (Murray, 1990)**.** High levels of resistance may exist to β-lactams through β-lactamase enzymes and altered binding proteins, vancomycin through changes in peptidoglycan synthesis, and

aminoglycosides through enzymatic degradation, and thus, few options remain to treat systemic multi-drug-resistant *Enterococci* infections (Miller et al., 2014).

E. faecalis is intrinsically resistant to quinupristin-dalfopristin, one of the antibiotic classes reserved for multi-drug resistant (MDR) infections; similarly, *E. faecium* is intrinsically resistant to ampicillin, an antibiotic commonly used in the treatment of Enterococcal infections (Kristich et al., 2014). The other intrinsic resistance is resistance to vancomycin in the rarely isolated *E. gallinarium* and *E. casseliflavus* (Eliopoulos and Gold, 2001).

2.6 Transfer of Resistance Between Non-Human and Human reservoirs

Enterococcus species are a natural part of the intestinal flora of most animals (Getachew et al., 2013). Infections of animals with *Enterococci* are rarely targeted explicitly with antimicrobial agents. However, as normal inhabitants of the intestinal tract, *Enterococci* are exposed to antimicrobial selection every time animals are subjected to antimicrobial therapy or given antimicrobial agents for growth promotion, prophylaxis, and metaphylaxis (Hammerum et al., 2010). *Enterococci* are one of the traditional bacterial markers of faecal contamination of food and water for human consumption, and it has been accepted for several decades that *Enterococci* from non-human sources could contaminate food intended for human consumption (Daniel et al., 2015b). *Enterococci* with resistance genes may reach humans through consuming contaminated food and direct contact with infected farms (Getachew et al., 2012). *Enterococci* can cause food intoxication by producing biogenic amines and can be a reservoir for worrisome opportunistic infections and virulence traits (Gordon et al., 1992).

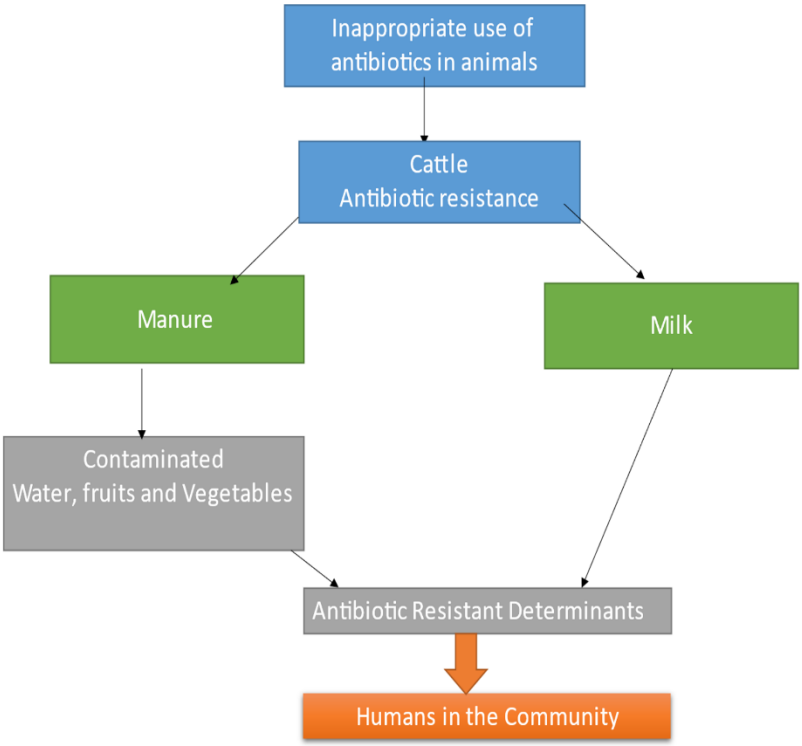

Figure 5: The diagram above shows the transfer of antibiotic determinants from animals to humans.

2.7 *Enterococci* in livestock faecal matter and susceptibility patterns to Antimicrobials

Animals excrete as much as 90% of the antibiotics administered orally or added to the feeds through faeces or urine (Wang et al., 2020). As a consequence of poor soil fertility faced by small and large-scale farming systems in sub-Sahara African countries, chemical, and organic fertilisers are frequently added to soil to improve its quality, texture, and crop yield (Materechera, 2010). Organic fertilisers consist of manure that is animal faecal matter and thus has the possibility to carry AMR *Enterococci*.

Studies have shown that *E. faecium* and *E. faecalis*, resistant to antimicrobials, are of increased public health concern worldwide as they may compromise the ability of various treatment regimens to control disease and infection in human medicine (García-Solache and Rice, 2019). A study carried out in Canada showed Enterococcus prevalence of 54% in

bovine feaces and cattle production systems, with the dominant species being *E. hirae* at 92% (Zaheer et al., 2020b). Another study in Australia evaluated 800 *Enterococcus* species from dairy cattle and observed 96 *E. faecalis* and 120 *E. faecium*, with MDR being 5% in *E. faecium* and 12% in *E. faecalis* (Barlow et al., 2017). The low level of AMR observed in this study was attributed to comprehensive controls around the use of antimicrobials in food-producing animals. Another study in Australia had a prevalence of 69.3% *Enterococcus* species from beef cattle, with *E. hirae* dominating the rectal swab samples. Resistance was observed to lincomycin (60.6%), ciprofloxacin (6.7%), and tetracycline (4.8%) (Messele et al., 2022). A study in South Africa had 71 *Enterococcus* species isolated from healthy cattle, with *E. faecium* being the dominant species. *Enterococci* isolates were susceptible to chlortetracycline and vancomycin, 38% were intermediate to erythromycin, and resistance was seen in at least one antimicrobial, with enrofloxacin resistance at 55%, followed by amoxicillin resistance at 3% (Mupfunya et al., 2021). In Tanzania, 46.7% *E. faecalis*, 39.4% *E. faecium*, 3.6% *E. avium*, and 2.4% *E. gallinarum* were isolated from cattle faecal matter (Madoshi et al., 2018**).** The only published data available from Zambia on *Enterococcus* in faeces of cattle was carried out in 2008 by Mubita et al where a total of 82 cattle faecal samples were collected from the Kafue basin; after evaluation, 62 were *E. faecalis*, and 29 were *E. faecium*, and these Enterococci isolates were resistant to gentamicin, co-trimoxazole and penicillin and moderately resistant to tetracycline at 38% in *E. faecium* isolates and 30% *E. faecalis* isolates (Mubita et al., 2008). The increasing AMR burden reported in different studies poses a danger to public health as these drugs are used in the clinical setting. Furthermore, since 2008, no other published data has been published on susceptibility patterns of *Enterococcus* species in cattle in Zambia.

2.8 *Enterococci* in Milk and susceptibility patterns

Enterococci are among the most common lactic acid bacteria in raw milk (Gao et al., 2018), and therefore raw milk may serve as a source of enterococci for dairy products. *Enterococci* may be present in large numbers in dairy products (up to 10^8 CFU g^{-1}). They gain entry into milk and milk products through the water supply, equipment, and unsanitary and unhygienic conditions of production and handling (Garg and Mital, 1991). The milking environment and improper handling of raw milk were found to be the source of vancomycin-resistant enterococci (VRE) rather than the animals (Ahmed M Hammad et al., 2022). In Australia, *Enterococci* were detected in 96% of 211 raw milk samples (McAuley et al., 2015); which is a public health concern as raw milk is used to make different dairy products. A study conducted in Morocco showed that 11.3% (17/150) of samples were positive for *Enterococcus* species in the milk. Of these, 64.7% were identified as *E. faecalis*, 17.6% as *E. faecium*, 11.8% as *E. durans*, and 5.9% as *E. hirae* and the antimicrobial susceptibility testing showed that all *Enterococcus* strains resisted ampicillin and tetracycline (Bouymajane et al., 2018). In Egypt, there was a 90% prevalence in fresh raw milk cheese; 30.8% and 10.8% were *E. faecalis* and *E. faecium*, respectively, and no VRE was detected (Ahmed M. Hammad et al., 2022). In Zambia a study by (Kunda et al., 2015) indicated that some of the raw milk On composition, was below recommended standards and minimum legal limit and thus pose a risk health to consumers. Another study carried in Western Zambia reflected high proportion of samples contaminated with *Staph. aureus* and *E. coli*, particularly after storage, suggesting poor handling and faecal contamination respectively and thus indicative of poor hygiene (Knight-Jones et al., 2016). there is no published data on *Enterococcus* in milk despite an increased raw milk intake among the general population. Raw milk consumed by the population is contaminated with strains of *Enterococcus* resistant to antibiotics used in breeding for

prophylactic purposes(Bouymajane et al., 2018). thus, this requires raising awareness of those involved in producing and marketing milk to take measures to apply good hygienic practices.

2.9 *Enterococci* resistant genes

Enterococcus species can accumulate multiple genetic elements encoding virulence traits and antibiotic resistance genes and develop a prominent biofilm in Enterococcal infections (Ch'ng et al., 2019). The biofilm of *Enterococcus* promotes tolerance to harsh environmental conditions and contributes significantly to persistence during infection and food processing environment, causing ecological contamination (Fernandes et al., 2017). Additionally, biofilms produced by *Enterococcus* species increase their inherent and acquired resistance to antibiotics (Ch'ng et al., 2019), posing a significant challenge to infection treatment, especially in virulent strains. *Enterococci* are intrinsically resistant or tolerant to many antimicrobials and easily acquire high-level drug resistance via horizontal gene transfer (Golob et al., 2019).

Erythromycin resistance among *Enterococci* is associated with the presence of erythromycin resistance methylase *(erm)* genes, such as *erm(A), erm(B),* and *erm(C)* (Tian et al., 2019). The predominant *erm* gene in erythromycin-resistant enterococci isolates is *the erm(B)* gene, which encodes the ribosomal RNA methylase (Celik et al., 2014). This *erm (B)* was also reported in a study carried out in an Iranian hospital (Emaneini et al., 2016). Similarly, another study indicated that the predominant gene conferring resistance to erythromycin in *Enterococcus* species was *erm(B)* (Portillo et al., 2000).

There are two major genotypes for acquired vancomycin resistance, *VanA*, and *VanB.* The genes encoding the VanA phenotype result in high-level resistance to vancomycin and teicoplanin and are carried on a transferable plasmid or conjugative transposon. *VanA* is mostly found *in E. faecium* and, less frequently, in *E. faecalis*. *VanB* is associated with

variable resistance to vancomycin, but isolates are usually susceptible to teicoplanin. One study conducted in Switzerland showed the occurrence of *VanA* genes in all isolates from cattle, poultry, and swine faecal matter (Wist et al., 2020).

2.10 Diagnosis of *Enterococcus* species

The diagnosis of *Enterococcus* species is made using conventional and molecular techniques (Kristich et al., 2014). Conventional techniques involve the use of culture, Gram stain, and use of biochemicals based on bacteria characteristics (Cherkaoui et al., 2010). Conventional methods have different strengths and weaknesses. The results of conventional method is that results are accurate and cost-effective, but the process is tedious and time-consuming. The culture media identification usually takes at least 3-4 days after sampling (Law et al., 2015). Molecular techniques based on specific nucleotide sequences such as Polymerase Chain reaction (PCR) with the characteristics of fast, accurate, and simple, are now widely utilised for pathogen identification. Molecular identification has been able to speciate Enterococci into species (Kristich et al., 2014).

Choosing the appropriate therapy for enterococcal infections is paramount to improving clinical outcomes; thus, accurate, precise, and reproducible antimicrobial susceptibility testing of enterococci becomes critical to guiding therapy (Khan et al., 2022). Antimicrobial testing involves conventional and automated methods (Kohner et al., 1997). Most clinical laboratories in the united states rely on commercial automated systems (cASTs), while low-resource settings primarily deploy manual disks and gradient-based methods (Humphries et al., 2018). Disk diffusion and gradient diffusion methods are feasible manual methods for assessing *Enterococci* (Khan et al., 2022). However, disk diffusion relies on phenotypic testing, and results are unavailable until the third day. Susceptibility results are affected by

certain factors such as , the size of the plate, depth of the agar, and overlapping zones of inhibition, affecting the reproducibility (Wanger, 2007). Gradient-based methods of minimum inhibitory concentration (MIC) measure the level of susceptibility or resistance of specific bacteria strains by determining the lowest concentration of an antibiotic at which bacterial growth is completely inhibited (Kowalska-Krochmal and Dudek-Wicher, 2021) . A study conducted at the UTH in Zambia indicated that 14 isolates were resistant to the vancomycin disc diffusion method; however, no VRE was recorded when gradient MIC was performed (Mutalange et al., 2021). Therefore, it is vital to check for MIC of *Enterococci* that appear resistant with vancomycin disk diffusion.

Automated AST systems include Vitek 2, Phoenix, and Microscan, of which Vitek 2 provides a relatively accurate and conservative performance for most antimicrobials (Zhou et al., 2018). The Vitek2 system was reported to have acceptable performance against multidrug-resistant enterococcal isolates with high concordance (Khan et al., 2022).

2.11 Prevention, Treatment, and Control of Enterococcal Infections in Animals

Enterococci are one of the environmental causative agents of mastitis (Różańska et al., 2019). The high tolerance of *Enterococci* to harsh conditions allows for the persistence in the environment, and thus, potential infections of the mammary glands are simple and easy. Several antimicrobial agents are used to treat clinical and subclinical mastitis. These include b-lactam, penicillin G, and macrolides erythromycin, the most commonly used drugs to treat bovine mastitis (El-Diasty et al., 2019).

Prevention of Enterococcal infections is cardinal as the bacteria are resistant, which complicates treatment options; thus, proper cleaning of animal production facilities can minimise the persistence of environmental *Enterococci* in milking facilities and slaughterhouses. A study indicated that the milking environment was the source of milk

contamination (Ahmed M Hammad et al., 2022). Good hygiene and proper cleaning are crucial both in animal production and clinical setting to minimise faecal contamination of milk and meat with *Enterococci.*

2.12 Information gap

Zambia has recognised the public health threat of AMR and its impact on morbidity and mortality. One health approach is critical to ensure morbidity and mortality rates are reduced. However, zoonotic pathogens that are commensal to animals but pathogenic to humans are rarely investigated, and studies of *Enterococci* in cattle in Zambia are scarce and, thus, if not appropriately monitored, could contribute to the emergence of zoonotic diseases. This study will evaluate the significance of *Enterococci* in dairy cattle and knowledge of *Enterococci* AMR and ARGs. The data obtained can be used in surveillance and antibiotic stewardship programs in one health in Zambia among dairy farmers to prevent the emergence of zoonotic infections.

CHAPTER THREE

3.0 MATERIAL AND METHODS

3.1 Study Design

This cross-sectional study was carried out from September 2022 to March 2023.

3.2 Study Site

The study was conducted in three districts of Lusaka Province, namely, Chilanga, Chongwe, and Lusaka. These three districts were selected because they had more dairy cattle farmers in the province.

3.3 Study Population

The study included dairy cows from Lusaka province dairy farms.

3.3.1 Inclusion Criteria

Lactating dairy cows from farms whose owners gave consent were included.

3.3.2 Exclusion Criteria

Dairy cattle farms outside Lusaka and farms in Lusaka where dairy farm owners did not consent.

3.4 Sample Size

The AusVet Epitools (https://epitools.ausvet.com.au/oneproportion) was used to estimate the number of farms to be included in the study, using sample size calculation to estimate a simple proportion (apparent prevalence). The assumptions made to determine the sample size areas shown in Table 1. Further, we assumed that there are approximately 12 dairy farmers in each of the selected study districts, bringing the study population to 36 dairy farms, and this formed the basis for adjusting the sample size for a finite population to 33 farms.

Table 1: Variable inputs in the AusVet Epitool (https://epitools.ausvet.com.au/oneproportion)

Estimated Proportion	0.5
Desired precision of the estimate	0.05
Confidence level	0.95
Population size (infinite) Estimated sample size (finite)	385 33

Sampling was stratified equally by district . This gave approximately amount of 11 farms per district as shown in table 2. We sampled three animals from each farm and collected milk and faecal matter from each animal.

Table 2: Distribution of Samples and Farms by District

District	**Number of farms**	**3 Milk samples per farm**	**Three faecal samples per farm**
Chilanga	11	33	33
Lusaka	11	33	33
Chongwe	11	33	33
Total	**33**	**99**	**99**

3.5 Experimental Approach

3.5.1 Sample Collection and Detection of *Enterococci*

Samples were collected from dairy cattle belonging to different independent owners. From each farm, three animals were sampled. Five grams (g) of Fresh faecal samples from individual cattle were collected directly from the rectum using pregnancy diagnosis gloves and put in sterile collection tubes. Similarly, milk samples were collected from three lactating cows. Firstly, the udder was cleaned using clean water, dried with blotting paper, and sanitized with alcohol. Approximately 5ml of milk was collected from the midstream into

sterile collection tubes. All samples were stored at 4°C until they were processed in the laboratory.

The detection and isolation of *Enterococci* species involved using standard microbiological methods. One ml and one gram of milk and faecal matter, respectively, were placed in nine ml of enrichment media (Axide dextrose) and incubated for 24 hours in an aerobic incubator at 37°C, after which all samples were cultured on Slanetz and Bartley using the streak method then incubated in an aerobic incubator at 37°C. After 24 hours of incubation, the plates were examined macroscopically for *Enterococcus* characteristic growth, size, shape, and texture. Gram stain was used to differentiate isolated bacteria into Gram-positive and Gram-negative, while the catalase test was used to differentiate among the Gram-positive bacteria. The presumptive diagnosis was made when typical enterococcal colonies on Bile Aesculin Azide (BEA) agar hydrolysed aesculin in the presence of bile and turned the media dark brown or black after incubation. Selection of typical enterococcal colonies, which appeared small and translucent with a zone colour of between brown-black and black, was made.

3.5.2 Confirmation and Determination of *E. faecalis* and *E. faecium* Using Molecular Analysis

Presumptive *Enterococci* isolates were subjected to the DNA extraction using the crude method; this was done by subjecting pure colonies to 100µl distilled water in Eppendorf tubes, boiled on a thermal block for 10 min at 95°C and centrifuged at 2000 revolution for 4 min. The supernatant was pipetted and used as a template DNA in a PCR reaction and molecular identification (Jackson et al., 2004) using the *tuf* gene described below. The tuf gene was used to confirm the *Enterococci* genus. The PCR reactions were carried out in a 20µl volume reaction tube containing One Taq2X Master mix with standard buffer, 2.5 mM MgCl, 0.16 Mm dNTPS premixed. 0.5µl primer, 0.75 units of Taq Polymerase, 2.0µl of

DDW and 8.0µl of DNA template. The temperature conditions were as follows initial denaturation at 94℃ for 3 minute and final denaturation at 94℃ for 1 minute followed by 35 cycles at 53 ℃ for 30sec annealing,68℃ for 1 minute initial extension and final extension at 68℃ for 5 minute and hold at 4℃. The PCR products were separated by electrophoresis in a 1% agarose gel in 0.5 x Tris-borate-ethylenediaminetetraacetic acid (TBE) buffer stained with 0.5µl ethidium bromide. Each well was loaded with 5 µl of the PCR product. The samples were separated with a DNA ladder at 100v for 30 minute and visualised under ultra-violet (UV) light.

After confirming the genus using the *tuf* gene, the DNA was then used to speciate *Enterocooci* into *E. faecalis* and *E. faecium* using species-specific PCR primers listed in Table 3. The PCR reactions were carried out in a 20µl volume reaction tube containing One Taq2X Master mix with standard buffer, 2.5 mM MgCl, 0.16 Mm dNTPS premixed. 0.5µl primer, 0.75 units of Taq Polymerase, 2.0µl of DDW and 8.0µl of DNA template. The temperature conditions were initial denaturation at 94℃ for 3 minute and final denaturation at 94℃ for 1 min, followed by 35 cycles at 55℃ and 48℃ for 30sec annealing for E. faecalis, E. faecium, respectively, 68℃ for 1 minute initial extension and final extension at 68℃ for 5 minute and hold at 4℃. Gel electrophoresis was used to determine the band size and confirm the target gene. The PCR products were separated by electrophoresis in a 1% agarose gel in 0.5 x TBE buffer stained with 0.5µl ethidium bromide. Each well was loaded with 5 µl of the PCR product. The samples were separated with a DNA ladder at 100v for 30 min and visualised under UV light.

Table 3: Primers set for the confirmation of *Enterococcus* species and confirmation of *E. faecalis* and *E. faecium*

Purpose	Target gene	Primer sequence F/R (5′-3′)	Amplicon size (bp)	Annealing temperature (°C)
Enterococcus gene	*Tuf*	F-TACTGACAAACCATTCATGATG R-AACTTCGTCACCAACGCGAAC	112	53
E. faecalis	*SodA*	*(F)* ACTTATGTGACTAACTTAACC *(R)* TAATGGTGAATCTTGGTTTGG	360	55
E. faecium	*SodA*	(F) GAAAAAACAATAGAAGAATTAT *(R)* TGCTTTTTTGAATTCTTCTTA	215	48

3.5.3 Antimicrobial Susceptibility Testing

After identifying the organisms by conventional microbiology tests, the confirmed *Enterococcus* isolates were subjected to antimicrobial susceptibility testing (AST) using the Kirby -Bauer disk diffusion method. Pure colonies were grown on nutrient agar (Oxoid) and then suspended into 2ml of normal physiological saline to make a 0.5 McFarland standard suspension. A sterile swab spread these bacteria evenly on a Mueller Hinton agar (Oxoid) plate. Oxoid antibiotic discs shown in table 4 were then gently placed on the Mueller Hinton agar plate, ensuring that the discs were not closer than 24mm from centre to centre. After 24 hours of incubation in an aerobic incubator at 37°C, the inhibition zones were measured using a vernier calliper. The interpretation of susceptible (S), intermediate (I), and resistant (R) was based on the M100 Clinical Laboratory Standards Institute guidelines (CLSI) guidelines for Enterococcus spp. (Weinstein and Lewis, 2020).

Table 4: Antimicrobial agent and disk content concentration

Antibiotics	Concentration
Penicillin	10 units
Ciprofloxacin	5µg
Tetracycline	30µg
Erythromycin	15µg
Ampicillin	30µg
Vancomycin	30µg
Chloramphenicol	30µg

In order to confirm vancomycin resistance, all *Enterococcus* isolates that were resistant to vancomycin discs were subjected to MIC testing using vancomycin E-strips. The pure colonies grown on nutrient agar were then suspended into 2ml of normal physiological saline to make a 0.5 McFarland standard suspension. The suspension was then spread evenly on the Mueller Hinton agar plate using a sterile swab; sterile forceps were used to place the E-strip on the agar plate.

3.5.4. Determination of Erythromycin Resistance Genes

Enterococcus isolates resistant to erythromycin were screened for resistance genes conferring resistance by using PCR primers listed in Table 5. The PCR reactions were carried out in a 20µl volume reaction tube containing One Taq 2X Master mix with standard buffer, 2.5 mM MgCl, 0.16 Mm dNTPS premixed. 0.5µl primer, 0.75 units of Taq Polymerase, 2.0µl of DDW and 8.0µl of DNA template. The temperature conditions were initial denaturation at 94℃ for 3 min and final denaturation at 94℃ for 1 min, followed by 35 cycles at 55℃,52℃ and 48℃ for 30sec annealing for *erm A, B, C* genes, respectively, 68℃ for 1 min initial extension and final extension at 68℃ for 5 min and hold at 4℃. Gel electrophoresis was used to determine the band size and confirm the target gene. The PCR products were

separated by electrophoresis in a 1% agarose gel in 0.5 x TBE buffer stained with 0.5µl ethidium bromide. Each well was loaded with 5 µl of the PCR product. The samples were separated along with a DNA ladder at 100v for 30 min and visualized under UV light.

Table 5: Primers set for the determination of Erythromycin resistance genes

Purpose	**Target genes**	**Primers sequence F/R (5′-3′)**	**Amplicon size (bp)**	**Annealing temperature °C**
Erythromycin resistant genes	*ErmA*	*(F)* AAGCGGTAAAACCCCTCTGAG *(R)* TCAAAGCCTGTCGGAATTGG	441	55
	ErmB	*(F)* CATTTAACGACGAAACTGGC *(R)*GGAACATCTGTGGTATGGCG	425	52
	ErmC	*(F)*CAAACCCGTATTCCACGATT *(R)*ATCTTTGAAATCGGCTCAGG	295	48

3.6 Data Analysis

Data obtained from this study were analyzed using WHONET and Excel. Descriptive data were presented in graphs and proportions.

3.7 Ethical Consideration

The ethical clearance for this study was obtained from the ethics review committee of Excellence in Research Ethics and Science approval (ERES) Converge Ethics Committee (Ref. No 2022-0ct-011). Permission to collect milk and faecal samples from dairy cattle was sought from the Ministry of Livestock. The authorisation to conduct this research was obtained from National Health Research Authority in Zambia (Ref. No

NHRA000011/21/03/2023). Consent was sought from the farm owners. The information obtained during the research was kept secure and used only for academic and research purposes. For confidentiality purposes, the names of the farmers, farms, and animals were not written in the data collection book; codes were used. Farmers were free to participate in the research willingly, and the study risk for collection of feacal matter and milk directly from the cow was minimized by letting a qualified veterinary officer collect the samples.

CHAPTER FOUR

4.0 RESULTS

4.1 Detection and Isolation of Enterococcus from Milk and Faecal Matter

A total of 40 and 89 *Enterococci* were recovered from milk and faecal samples, respectively. Of these, 50 isolates were randomly selected and confirmed with the *tuf* gene (figure 6).

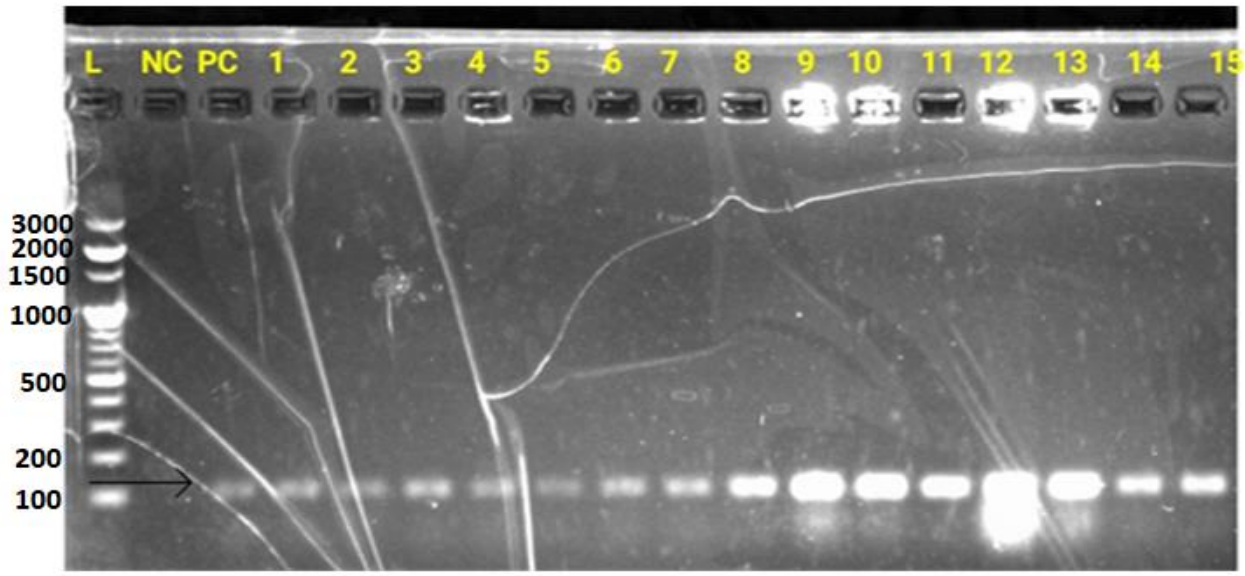

Figure 6: Image of *tuf* gene amplicons electrophoresed on Agarose gel.
Key: Lane L -100 bp Ladder; lane (1-15) *tuf* gene PCR amplicons of 112 bp; Lane NC - Negative control and Lane PC – Positive control.

4.2 Determination of *E. faecalis* and *E. faecium*

The detection rates of E. faecalis and E. faecium are shown Table 6. Among the 50 confirmed samples, a total of eleven *E. faecalis* were confirmed using the *SodA* gene, of which ten were from faecal samples and one from milk. Four *E. faecium* were detected, and all of which were from the faecal samples.

Table 6: Determination of *E. faecalis* and *E. faecium*

Sample Type	Enterococcus species	Proportion of isolates detected % (n)actual number
Faecal matter	*E. faecalis*	20 (10)
	E. faecium	8 (4)
Raw milk	*E. faecalis*	2 (1)
	E. faecium	0

4.3 Determination of Antimicrobial Susceptibility Patterns

4.3.1 Susceptibility Patterns of *Enterococcus* species in milk

The antimicrobial susceptibility patterns of *Enterococcus* species in milk were as shown in Figure 7. Most isolates were resistant to ampicillin (57%) and tetracycline (33 %). While 4% of the isolates were resistant on disc method to vancomycin, but upon using the MIC, all isolates were susceptible. The *Enterococcus* species was most susceptible to penicillin and chloramphenicol at 91% and 78%, respectively (Figure 7). A total of 5/40 (15%) isolates were MDR from 4 farms.

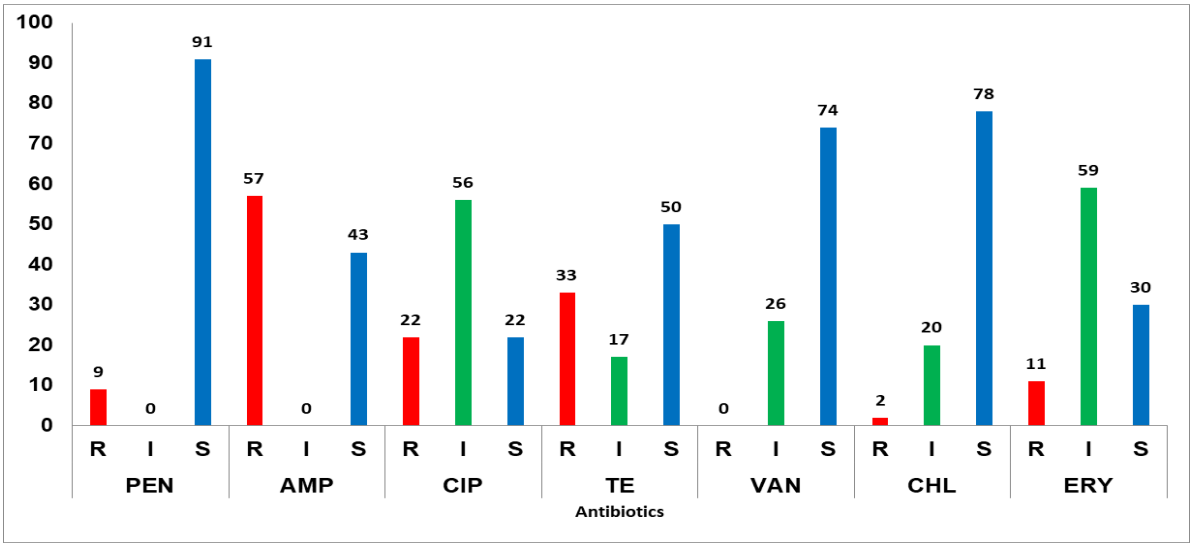

Figure 7: Antimicrobial Susceptibility Patterns of *Enterococci* isolated from Milk

Abbreviations: PEN= Penicillin, AMP= Ampicillin, CIP= Ciprofloxacin, TE= Tetracycline, VAN= Vancomycin, CHL= Chloramphenicol, ERY= Erythromycin. Key: R=Resistance, I=Intermediate and S=Susceptible.

4.3.2 Susceptibility Patterns of *Enterococcus* species in Faecal Samples

The antimicrobial susceptibility patterns of *Enterococcus* species in faecal matter were as shown in Figure 8 Most of the isolates were resistant to ampicillin (63%), ciprofloxacin (39%), and tetracycline (26%), while 10% of the isolates were resistant on disc method to vancomycin, but upon using the MIC, all isolates were susceptible. The *Enterococcus* species was most susceptible to penicillin and chloramphenicol at 82% and 67%, respectively (Figure 8). A total of 30/89 (34%) isolates were MDR from 13 farms.

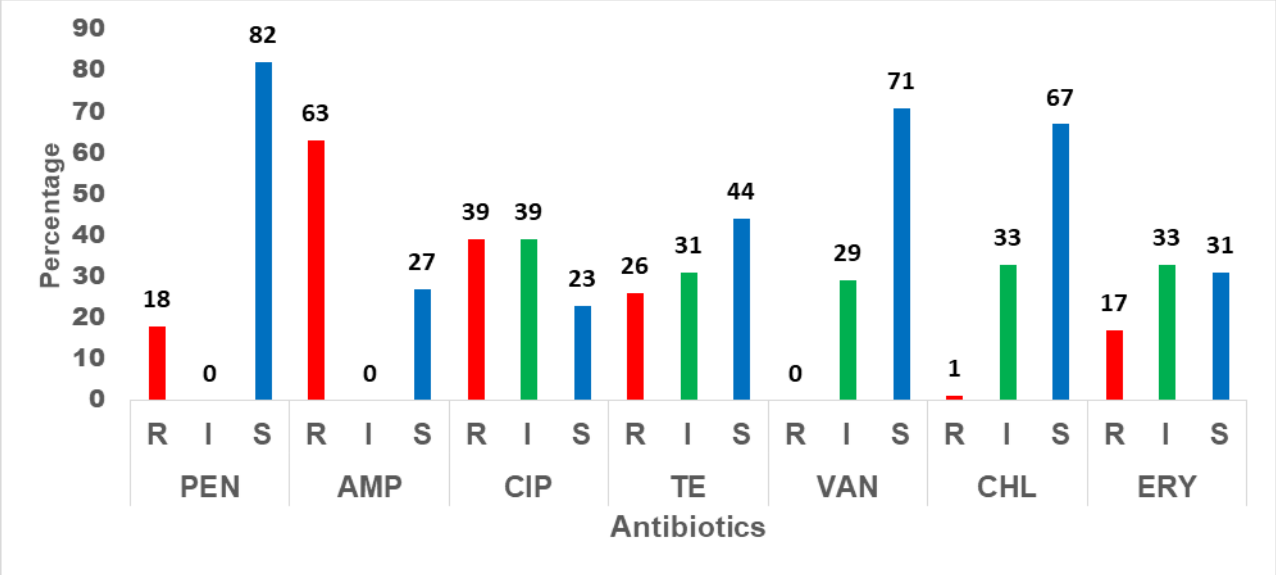

Figure 8: Antimicrobial Susceptibility Patterns of *Enterococci* isolated from faecal matter

Abbreviations: PEN= Penicillin, AMP= Ampicillin, CIP= Ciprofloxacin, TE= Tetracycline, VAN= Vancomycin, CHL= Chloramphenicol, ERY= Erythromycin.

key: R=Resistance, I=Intermediate and S=Susceptible.

4.4 Comparison of Resistance Profiles of Enterococcus in Faecal and Milk

The comparison of *Enterococci* from faecal matter and milk, as shown in (Figure 9), reveals that *Enterococcus* species in faecal had a higher percentage of resistance patterns to most antibiotics except tetracycline and chloramphenicol. The highest resistance was observed to ampicillin at 62% and 57% in stool and milk, respectively. Tetracycline resistance profiles showed 33% and 26% in milk and faecal, respectively.

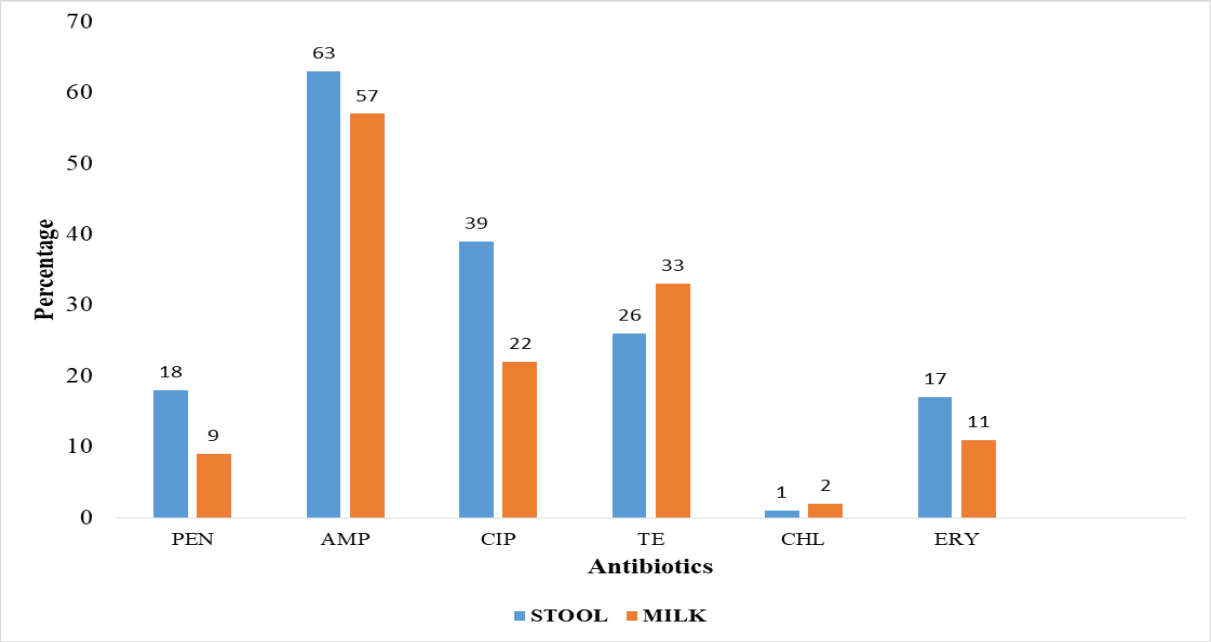

Figure 9: Comparison of Resistance Profiles of *Enterococcus* from faecal and Milk

Abbreviation: PEN= Penicillin, AMP= Ampicillin, CIP= Ciprofloxacin, TE= Tetracycline, VAN= Vancomycin, CHL= Chloramphenicol, ERY= Erythromycin

4.5 Determination of Erythromycin and Vancomycin Resistance Genes

Of the 13 isolates tested for *erm A, B,* and *C* genes, eight were positive for *erm* B as shown in figure 10, and no *erm A* and *C* were detected as shown in table 7. Since there was no VRE, no van genes were investigated.

Table 7: Determination of erythromycin resistant genes

erm genes	No of isolates
*Erm*A	0
*Erm*B	8
*Erm*C	0

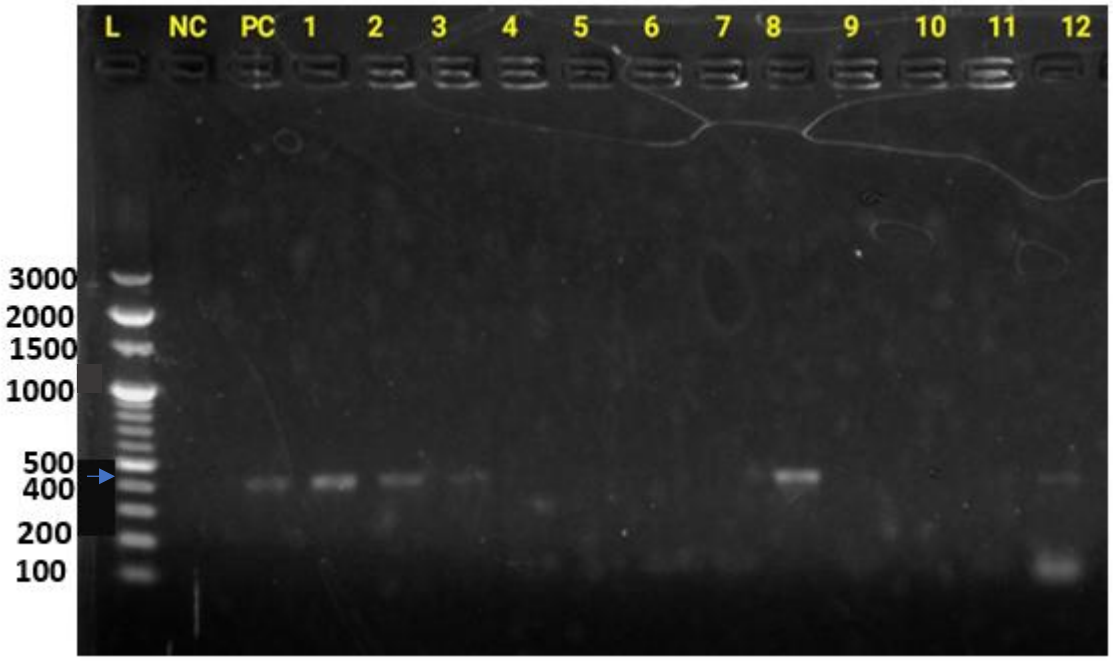

Figure 10: Image of *erm* B gene amplicon electrophoresed on Agarose gel. L-100bp, NC, and PC correspond to Ladder, Key: Lane L -100 ; Lane NC -Negative control and Lane PC – Positive control.. PCR product band size 425 base pairs obtained on samples labelled 1,2,3,8 and 12

CHAPTER FIVE

5.0 DISCUSSION

The study was carried out to evaluate the public health significance of *Enterococci* from milk and faecal matter of dairy cattle as they may serve as reservoirs and transmitters of ARGs. The prevalence of *Enterococcus* species, their antimicrobial susceptibility patterns and the presence of ARGs in milk and faecal matter from dairy cattle in three selected dairy farms in the Lusaka province of Zambia was determined.

The prevalence of *Enterococcus* species in the raw milk of dairy cattle screened in this study was 40%. This prevalence was lower than what was found in Austria (96%) (McAuley et al., 2015) and Egypt (90%) (Hammad et al., 2015) but higher than the findings recorded in France (8%) (Jamet et al., 2012) and 11.3% in Morocco (Bouymajane et al., 2018). The presence of *Enterococcus* species in raw milk indicates poor hygienic conditions and contamination and thus increases the chance for humans to acquire antibiotic-resistant strains if they consume milk that is not processed correctly (Garroni et al., 2020) . The acquisition and dissemination of antibiotic-resistant strains increase the risk burden to the most vulnerable individuals in the community, such as infants, the elderly, and those with compromised immunity, and further limits treatment options for these individuals once they acquire an infection (Prestinaci et al., 2015)**.**

The prevalence of *Enterococcus* species in the faecal matter of dairy cattle in this study was 90%. This finding was similar to a study that was carried out in Australia that isolated 96% *Enterococcus* from rectal swabs from beef cattle (Messele et al., 2022) and to another study in Australia that isolated 88.5% *Enterococcus* from beef cattle faecal matter and 84.1% from faecal matter of dairy cattle (Barlow et al., 2017). The findings in the present study were higher than those from a South African study that recorded a recovery rate of 71% (Mupfunya et al., 2021). *Enterococcus species* are commensal natural gut microflora in animals and humans; therefore, a high carriage rate has no negative implications for animals

or humans. However, they may play a role if they acquire and carry antibiotic-resistance genes, which may be transmitted to humans.

Our study detected 22% *E. faecalis* and 8% *E. faecium* among the 50 randomly selected *Enterococcus* subjected to speciation. The prevalence of *E. faecalis* and *E. faecium* vary in different environments and geographic locations, In a study done in Australia of *Enterococcus* isolates from faecal cattle showed that 6.4% were *E.faecalis* and 8.0% were *E.faecium* (Barlow et al., 2017). Similar to our findings, a study that isolated *Enterococcus* isolates from milk in Morocco found the presence of *E. faecalis* (64.7%) to be higher than *E. faecium* (17.6% (Bouymajane et al., 2018)). *E. faecalis* and *E. faecium* are implicated in most healthcare-associated Enterococcal infections in humans (Bhardwaj, 2019). *Enterococcus* species might disseminate into food products and the environment, which may be transmitted to humans; hence good husbandry practices are required to prevent zoonotic infections.

Antimicrobial resistance patterns of *Enterococcus* species in faecal matter ranged from one percent to 63%. The resistant *Enterococci* in the GIT could be reservoirs of ARGs and promote the transmission of ARGSs to other bacteria and humans. If humans become infected with resistant bacteria, it becomes difficult to treat such infections thereby leading to a public health burden. Animals excrete as much as 90% of the antibiotics administered orally or added to the feeds through faeces or urine (Wang et al., 2020b). Further, due to the poor soil fertility of small and large-scale farming systems in sub-Saharan countries, chemical and organic fertilizers derived from faecal matter are frequently added to soil to improve its quality, texture, and crop yield. This practice increases the dissemination of antibiotic resistance genes from faecal matter of cows to the environment with possible transmission to humans.

Resistance to ampicillin in *Enterococcus* species isolated from milk was high at 57%, The results were higher to a study in Morocco that revealed 100% resistance to ampicillin in all the isolated *Enterococcus* species from raw milk . Similarly, high resistance to ampicillin was observed at 63% in the faecal matter of dairy cattle; this was similar to a study that was carried out in Zambia in 2008, which recorded ampicillin resistance at 51.7% and 75.8% E. *faecium* and *E. faecalis,* respectively (Mubita et al., 2008). Our results, however, were contrary to a study conducted in Tanzania that recorded low resistance to ampicillin (1%) (Madoshi et al., 2018) and another study in Portugal that had no ampicillin-resistant isolates (Ramos et al., 2012). The difference in the results could be attributed to the levels of AMR awareness among farmers and the use of antibiotics for prophylaxis and therapeutic use (Friedman et al., 2007; Raymond et al., 2006).

A study that was carried out in 2021 at the University Teaching Hospital (UTH), a referral hospital that provides specialised care for patients from Lusaka province and all the other provinces in Zambia, recorded 79.5% resistance to ampicillin in *Enterococcus* species isolated from bloodstream infections (Mutalange et al., 2021). This was higher than the findings in milk and faecal matter isolates due to misuse and over-use of antimicrobials in humans, such as easy access to antimicrobials, self-medication and self-prescribing which sometimes leads to suboptimal dosing and non-adherence to completion of prescribed course (Dar et al., 2015**).** Other factors that have led to high use of antimicrobials in humans are Inadequate medical services and medications as well as narratives and myth about antibiotics (Planta, 2007),(Smith et al., 2015). The high resistance of *Enterococcus* species to ampicillin in dairy milk, cattle faecal, and humans warrants intensified stewardship programs that will regulate the irrational use of antibiotics in animals and humans.

The molecular basis of vancomycin resistance has been described with evidence that VREs may act as reservoirs and sources of other antimicrobial genes (Ahmed and Baptiste, 2018).

In our study, no VRE was detected from milk and faecal matter. In milk, our findings were similar to a study done in Morocco which did not detect VRE in milk (Bouymajane et al., 2018b). However, our results were in contradiction with those in Egypt, which found 91.6% of VRE in retail raw milk (Ahmed M. Hammad et al., 2022). In faecal matter, our results were lower than a study done in Iran, which found a VRE of 71.9% using disc diffusion method (Nasiri and Hanifian, 2022), and a study from Switzerland that found a VRE to be at 1.7% from faecal matter of healthy cows which used MIC gradient method (Wist et al., 2020). This difference could be attributed to the method used, as our study used the disc diffusion method and MIC. Disc diffusion method susceptibility results are affected by certain factors, and thus, the size of the plate, depth of the agar, and overlapping zones of inhibition, affecting the zone of inhibition of a particular antibiotic (Wanger, 2007) unlike the gradient-based methods. The discrepancy in the vancomycin susceptibility of the isolates by disc diffusion method and the gradient MIC E-strips highlights the need to confirm all the vancomycin disc-resistant strains with the MIC E-strips since no isolate was VRE when tested using E-strips despite being resistant using disc diffusion. The differences observed in VRE could also be attributed to the antibiotic panel used to treat infections in the respective study areas where the samples were drawn.

In this study, tetracycline-resistant isolates from milk were found to be at 33%, lower than what was found in Korea (73.4%) (Yoon and Lee, 2021) and higher than what was recorded in a study in Egypt (29.1%) (Hammad et al., 2022). The differences could be attributed to antimicrobial use and antibiotic awareness among farmers (Chanvatik et al., 2019). Mainda et al., (2015) conducted a study in dairy cattle and observed high resistance of *E. coli* to tetracycline which they attributed to high usage of tetracycline, as a drug of choice for the treatment of theileriosis (corridor disease) .

Ciprofloxacin resistance was the second-highest resistance (39%) observed in faecal matter *Enterococcus* isolates in our study. This was similar to a study done in Iran that had ciprofloxacin resistance of 39.2% (Nasiri and Hanifian, 2022). A study by Mutalange et al. in 2021 recorded 71.8% ciprofloxacin resistance in *Enterococcus* species from human bloodstream infections (Mutalange et al., 2021). The difference between the high prevalence observed in humans and animals could be that this antibiotic is not recommended for use in animals (Zimmerman et l., 2010). Ciprofloxacin is also one of the most used antibiotics to treat urinary tract infections and sexually transmitted infections in humans (Thai et al., 2022). Despite the law requiring a prescription for the drug to be accessed, it is one of the most abused antibiotics in the community, as people quickly access the drug off the counter without prescriptions (Kalungia et al., 2022).

The detection of 15% and 34% of MDR isolates in milk and faecal matter is a public health burden as this limits the treatment drug options and might increase disease burden and reduce milk productivity due to poor animal welfare. Arias et al. 2010 indicated that the management of MDR enterococcal infections was a challenge as there were limited drug options (Arias et al., 2010), and according to Mellata 2013, failure of clinical treatments has caused significant economic and health burden that have affected both the livestock industry and human healthcare (Mellata, 2013).

The comparison of resistance profiles of Enterococcus showed that *Enterococcus* isolates in the faecal matter had a higher percentage of resistance patterns. This could be attributed to the fact that *Enterococcus* species is part of the normal flora in the GIT, and every time an animal is subjected to any antibiotic for therapeutic purposes, there is selective pressure, thereby gaining resistance to antibiotics. It is possible that *Enterococcus* species in the milk could have come from the environment , the milking environment and improper handling of raw milk were found to be the source of VRE rather than the animals (Ahmed M Hammad et

al., 2022). Therefore, there is need to monitor the environment as a possible source of pathogens as this will promote good husbandry practices. In a study carried out in Zambia on antimicrobial use in poultry, the prevalence of antibiotic use was 83%, and commonly used drugs were tetracycline and gentamicin (Mudenda et al., 2022). There is a need to carry out awareness on antimicrobial use in the farming sector and increase the awareness on the negative effects of misuse and overuse of antimicrobials in both animals and humans.

A total of eight *Enterococcus* isolates showed the presence of *ermB* genes; our findings agree with a study done by Portillo et al who described *erm*B as the predominant gene conferring resistance to erythromycin in *Enterococcus species* (Portillo et al., 2000). The predominant *erm* gene in erythromycin-resistant enterococci isolates is the *ermB* gene, which encodes the ribosomal RNA methylase (Celik et al., 2014). The *ermB* was also reported in a study carried out in an Iranian hospital (Emaneini et al., 2016). A study by Mutalange et al. of *Enterococcus* species in humans detected erythromycin resistance at 97.4% (Mutalange et al., 2021); however, erythromycin-resistant genes were not investigated. Creating a reservoir of resistant *Enterococci* in dairy cattle could promote ARG ttansmission to humans. ARGs have accelerated microbial threats to human health as the genes confer resistance to antibiotics used in animal and human infections. In a study by Zhang et al. 2022, 23.78% of the ARGs pose a health risk, especially those conferring multi-drug resistance, complicating treatment options (Zhang et al., 2022). There is a need to carry out surveys on ARGs to antibiotics and the health risks of ARGs to help manage threats to human and animal health.

Study Limitation

Most farmers were skeptical about participating due to fear of finding their milk contaminated with strains of *Enterococcu*s as milk production was their source of income. The farmers were subsequently educated on the importance of this study as it would help

them practice good animal husbandry practices that would lead to the production of safe milk without contamination that negatively affects the general population, as well as reduce the occurrence of antimicrobial resistant infections as a result of consuming unsafe milk.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

This study detected a 40% prevalence of *Enterococci* in milk, which indicate poor hygienic conditions and contamination. Further, detection of *E. faecalis* and *E. faecium* pose a danger of dissemination to humans through the consumption of milk and the use of faecal matter as manure in vegetable production if good husbandry is not practiced. Detection of MDR-resistant *Enterococcus* species bacteria and ARGs in dairy cattle is a public health concern as pathogenic bacteria may be transmitted to humans

6.2 Recommendations

The following recommendations are made.

1. There is need to formulate education programs for the farmers on good hygiene practices to prevent milk contamination.
2. Establish/strengthen antimicrobial stewardship programs that would manage the irrational use of antibiotics in the animal and human sector.
3. Increase/incorporate surveillance on zoonotic pathogens that are normal flora but can also cause infections in both animals and humans.

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APPENDICES

Appendix A. Informed Consent Form

Project title: EVALUATION OF THE PUBLIC HEALTH SIGNIFICANCE OF ENTEROCOCCI FROM DAIRY CATTLE IN LUSAKA PROVINCE, ZAMBIA

This informed consent form is for farmers who keep dairy cattle in Lusaka district and whom we invite to participate in research on the evaluation of the public health significance of enterococci from dairy cattle in Lusaka province, Zambia.

Name of Principal Investigator: Matenge Mutalange

Name of Organization: University of Zambia

Name of Proposal: EVALUATION OF THE PUBLIC HEALTH SIGNIFICANCE OF ENTEROCOCCI FROM DAIRY CATTLE IN LUSAKA PROVINCE, ZAMBIA

Introduction

I am Matenge Mutalange, doing a Master of Tropical infectious diseases and Zoonoses at the University of Zambia, School of Veterinary Services. The research focuses on Enterococcus in dairy milk and faecal matter In cattle in the Lusaka district, which seems to be a public health concern. I will give you information and invite you to be part of this research. You do not have to decide today whether you will participate in the research. Before you decide, talk to anyone you feel comfortable with about the research.

There may be some words that you do not understand. Please ask me to stop as we go through the collection process, and I will take time to explain. If you have questions later, you can ask them of me or any other staff.

Purpose of the research

Enterococci inhabiting non-human reservoirs such as poultry and cattle appear to play a critical role in the acquisition and dissemination of antibiotic resistance determinants (Daniel et al., 2015), and as such, the public health concern This information will be cardinal in

coming up with appropriate interventions meant to improve our hygiene practices regarding cattle if need be.

Participant selection

We are inviting farmers who keep dairy cattle to participate in this study

Voluntary Participation

Your participation in this research is entirely voluntary. It is your choice whether to participate or not. You may change your mind later and stop participating even if you agreed earlier.

Procedures and Protocol

We will collect milk from the cattle udder and faecal matter of the same cattle. And all samples will be analysed in the public health laboratory at the University of Zambia.

Risks

This study will not pose more than minimal risk.

Benefits

If you participate in this research and are interested in the findings of Enterococcus in your cattle at your farm, that information will be shared with you regarding your cattle alone.

Reimbursements

You will not be given any money or gifts to participate in this research.

Confidentiality

We will not be sharing the identity of those participating in the research. This includes the farm owners, cattle identities and name of the farm.

The information that we collect from this research will be kept confidential. Information about you that will be collected during the research will be put away, and no one but the researchers will be able to see it. Any information about you will have a number instead of

your name. Only the researchers will know your number, and we will lock that information up with a lock and key.

Sharing the Results

The knowledge we get from this research will be shared with ERES, Veterinary services and the University before it is made widely available to the public. Confidential information will not be shared. We will also publish the results so that other interested people may learn from our research.

Right to Refuse or Withdraw

You do not have to participate in this research if you do not wish to, and refusing to participate will not affect you. You may stop participating in the research at any time that you wish.

Whom to Contact

If you have any questions, you may ask them now or later, even after the study has started. If you wish to ask questions later, you may contact the following:

1. Matenge Mutalange

   Master student in Tropical infectious diseases and zoonosis

   The University of Zambia,

   Lusaka, Zambia

   Phone number: 0978686349

   matengemutalange@gmail.com

2. ERES Converge IRB

   Ethics Research Board

0955155633/ 0955155633

eresconverge@yahoo.co.uk

3. Prof John Muma(Supervisor)

Public health lecturer and research

University of Zambia

0977912099

Jmuma@unza.zm

Appendix B: Participant consent form (ENGLISH)

Dear participant,

My name is MATENGE MUTALANGE; I am enrolled in the Master of Science in Tropical Infectious Diseases and Zoonosis programme at the School of Veterinary Sciences, University of Zambia. In partial fulfilment of my studies, I am required to undertake a research project. My research topic is "EVALUATION OF THE PUBLIC HEALTH SIGNIFICANCE OF ENTEROCOCCI FROM DAIRY CATTLE IN LUSAKA PROVINCE, ZAMBIA".

Consent

The information concerning this study has been fully explained to me, and I have been able to ask questions, all of which have been answered to my satisfaction. I understand that my participation in this study is voluntary and that I am free to withdraw at any time, without giving reason and without cost and that I am free to skip any questions I may deem personal or otherwise. I have been assured that my information will be kept private and strictly confidential. I voluntarily agree to take part in this study.

…….. or ……

…../…../……

Participant Signature Thumb print Date

I, the undersigned, have taken the time to explain to the above participant the nature and purpose of this study in a way they could understand. I have explained the possible benefits of this study and I invited the participant to ask questions and make any clarifications concerning this study.

…… ……. ……./……/…….

Name Signature Date

Appendix C: Participant consent form consent form Nyanja

Wokondewa wanga ofuna kuthengako mbali,

Dzina langa ndine Matenge Mutalanga, mwana wa sukulu wapa University of Zambia (UNZA) Ndichita masters mu Tropical infectious disease and zoonosis mu sukulu ya veterinary sciences. Iyi khani yofufudza ifunika kuti nisilize sukulu yanga. Muntu wa iyi khani yofufudza ndi "Enterococcus in daily milk and faecal matter in cattle in Lusaka, Zambia"

Kuvomekeza kuthengako mbali

Khani ya kufufudza iyi yalondoledwa kuli ine ndipo mafuso yanga yonse yayankidwa bwino bwino. Kutegako mbali muli iyi kani ya kufufudza ndikufana kwanga sono ndinga leke nthawi ili yonse kopanda kulondolola kuli konse.

_____ ____/__/____

Otengako mbali signature/kufwatika Tsiku

Ine mwine wa khani iyi yo fufudza na fotokodza bwino bwino kuli ulionse ofuna kuthengako mbali kuli iyi khani ya kufufudza zawibwino otengako mbali kuli khani yofufudza.

Appendix E: Institutional Approval, Ethics Approval, NHRA Approval And Permission From the Ministry Of Livestock

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: 29372
Vet. Clinic Telephone: 291515

P. O. Box 32379
Lusaka, Zambia

Your Ref:

Our Ref:

22nd February, 2022

Matenga Mutalange
Department of Disease Control
School of Veterinary Medicine
P. O. Box 32379
LUSAKA

Dear Mutalange

RE: APPROVAL OF RESEARCH PROPOSAL

At the meeting of the School Board of Graduate Studies held on 17th February, 2022, your research proposal entitled: ***"Evaluation of The Public Health Significance of Enterocci From Cattle and Farmworkers"*** was tabled and discussed. I am therefore, pleased to inform you that the research proposal was subsequently approved by the Board.

On behalf of the Board, I wish you success as you apply for ethical approval and carry on with your research activities.

Yours sincerely

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

Dean, School of Veterinary Medicine
Head, Disease Control
File

ERES CONVERGE

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Tel: +260 955 155 633
+260 955 155 634
Cell: +260 977 493 220
Email: eresconverge@yahoo.co.uk

I.R.B. No. 00005948
F.W.A. No. 00011697

18th January, 2023.

Ref. No. 2022-Oct-011

The Principal Investigator
Matenge Mutalange
MSc Of Science in Tropical Infectious Disease and Zoonosis
Department Of Public Health
School Veterinary Medicine
University Of Zambia

Dear Matenge Mutalange

RE: EVALAUTION OF THE PUBLIC HEALTH SIGHNIFICANCE OF ENTEROCOCCI FROM DAIRY CATTLE IN LUSAKA PROVINCE, ZAM BIA.

Reference is made to your protocol submission. The IRB resolved to approve this study and your participation as Principal Investigator for a period of one year.

Review Type	**Ordinary**	Approval No. **2022-Oct-011**
Approval and Expiry Date	Approval Date: 18th January, 2023	Expiry Date: 17th January, 2024
Protocol Version and Date	Version - Nil.	17th January, 2024
Information Sheet, Consent Forms and Dates	• English.	17th January, 2024
Consent form ID and Date	Version - Nil	17th January, 2024
Recruitment Materials	Nil	17th January, 2024
Other Study Documents	Data Collection Sheet, Focus Group Discussion.	17th January, 2024
Number of participants approved for study	-	17th January, 2024

Specific conditions will apply to this approval. As Principal Investigator it is your responsibility to ensure that the contents of this letter are adhered to. If these are not adhered

to, the approval may be suspended. Should the study be suspended, study sponsors and other regulatory authorities will be informed.

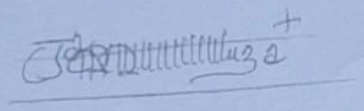
Conditions of Approval

- No participant may be involved in any study procedure prior to the study approval or after the expiration date.
- All unanticipated or Serious Adverse Events (SAEs) must be reported to the IRB within 5 days.
- All protocol modifications must be IRB approved prior to implementation unless they are intended to reduce risk (but must still be reported for approval). Modifications will include any change of investigator/s or site address.
- All protocol deviations must be reported to the IRB within 5 working days.
- All recruitment materials must be approved by the IRB prior to being used.
- Principal investigators are responsible for initiating Continuing Review proceedings. Documents must be received by the IRB at least 30 days before the expiry date. This is for the purpose of facilitating the review process. Any documents received less than 30 days before expiry will be labelled "late submissions" and will incur a penalty.
- Every 6 (six) months a progress report form supplied by ERES IRB must be filled in and submitted to us.
- A reprint of this letter shall be done at a fee.

Should you have any questions regarding anything indicated in this letter, please do not hesitate to get in touch with us at the above indicated address.

On behalf of ERES Converge IRB, we would like to wish you all the success as you carry out your study.

Yours faithfully,
ERES CONVERGE IRB



Dr. Jason Mwanza
Dip. Clin. Med. Sc., BA., M.Sc., PhD
CHAIRPERSON

NATIONAL HEALTH RESEARCH AUTHORITY
Lot No. 18961/M, off Kasama Road, Chalala, P.O. Box 30075, LUSAKA
Tell: +260211 250309 | Email: znhrasec@nhra.org.zm | www.nhra.org.zm

Ref No: NHRA000011/21/03/2023 21st March 2023

The Principal Investigator
Ms. Mutalange, Matenge
University of Zambia,
LUSAKA

Dear Ms Matenge,

RE: REQUEST FOR AUTHORITY TO CONDUCT RESEARCH

The National Health Research Authority is in receipt of your request for ethical clearance and authority to conduct research titled **"Evaluation of The Public Health Significance of Enterococcus from Diary Cattle in Lusaka Province Zambia."**

I wish to inform you that following submission of your request to the Authority, our review of the same and in view of the ethical clearance, this study has been **approved** on condition that:

1. The relevant Provincial and District Medical Officers where the study is being conducted are fully appraised;
2. Progress updates are provided to NHRA bi-annually from the date of commencement of the study;
3. The final study report is cleared by the NHRA before any publication or dissemination within or outside the country;
4. 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, University leadership, and all key respondents.

Yours sincerely,
NATIONAL HEALTH RESEARCH AUTHORITY

Prof Godfrey Biemba
DIRECTOR/CHIEF EXECUTIVE OFFICE