Molecular Epidemiology of Antibiotic-Resistant *Enterococcus* spp. and *Escherichia coli* from Agricultural Soil Fertilized with Chicken Litter in uMgungundlovu District, KwaZulu-Natal Province, South Africa.

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By

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A thesis submitted to the School of Laboratory Medicine & Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Medical School, for the degree of Doctor of Philosophy in Medicine (Medical Microbiology).

This is a thesis in which the chapters are written as a set of discrete manuscripts submitted or intended for submission to internationally recognized peer-reviewed journals with an overall introduction and final summary.

This is to certify that the content of this thesis is the original research work of **Dorcas Oladayo Fatoba**, carried out under our supervision at the Antimicrobial Research Unit (ARU), Discipline of Pharmaceutical Sciences, School of Health Sciences, College of Health Sciences, Westville Campus, University of KwaZulu-Natal (UKZN), Durban, South Africa.

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DECLARATION

I, **Dorcas Oladayo Fatoba** declare that:

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Signed: Dorcas Oladayo Fatoba

Date: 27th July, 2021

DEDICATION

This thesis is dedicated to my Lord Jesus Christ, the giver of wisdom, strength, and ideas at the most critical juncture of this research.

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LIST OF ABBREVIATIONS

AMC	Amoxicillin-clavulanic acid
AMK	Amikacin
AMP	Ampicillin
AMR	Antimicrobial resistance
ARB	Antibiotic-resistant bacteria
AST	Antimicrobial susceptibility testing
CARD	Comprehensive Antibiotic Resistance Database
CAZ	Ceftazidime
CC	Clonal Complex
CHL	Chloramphenicol
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
CRISPR	Clustered regularly interspaced short palindromic repeats
CRO	Ceftriaxone
CTX	Cefotaxime
CTX-M	Cefotaximase-München
DNA	Deoxyribonucleic acid
ERY	Erythromycin
ESBL	Extended-spectrum β-lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FEP	Cefepime
FOX	Cefoxitin
GEN	Gentamicin
IPM	Imipenem
IS	Insertion sequence
LEV	Levofloxacin
LEX	Cephalexin
LZD	Linezolid
MARI	Multiple antibiotic resistance index
MDR	Multidrug resistant
MEM	Meropenem
MGE	Mobile genetic element
MLSB	Macrolide-Lincosamide-Streptogramin B

MLST	Multi-locus sequence typing
MPN	Most probable number
NIT	Nitrofurantoin
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
PCU	Population correction unit
PGAP	Prokaryotic Genome Annotation Pipeline
QD	Quinupristin-dalfopristin
RAST	Rapid Annotation using Subsystem Technology
SAPA	South African Poultry Association
SNP	Single nucleotide polymorphism
STR	Streptomycin
SXT	Sulfamethoxazole-trimethoprim
TEC	Teicoplanin
TET	Tetracycline
TGC	Tigecycline
VAN	Vancomycin
VRE	Vancomycin-resistant *Enterococci*
WGS	Whole-genome sequencing
WHO	World Health Organization

Abstract

The application of animal manure contaminated with antibiotic-resistant bacteria (ARB) and antibiotic-resistance genes (ARGs) represents a major route by which antibiotic resistance is transmitted into the soil environment. The introduction and persistence of ARB in agricultural soil may pose a risk to public health via the consumption or handling of contaminated farm produce. Understanding the impact of animal manure application on the agricultural soil resistome and the risk it poses on public health is critical. However, such information is limited in South Africa as most antibiotic resistance research focuses on humans and food animals. This study, therefore, describes the prevalence and the genomic profiles of antibiotic-resistant *Escherichia coli* and *Enterococcus* spp. isolated from agricultural soil fertilized with chicken litter and the chicken litter.

A total of 237 samples were examined and included soil before litter application, the litter-amended soil, and the chicken litter. Isolation and quantification of *Escherichia coli* and *Enterococci* were carried out using the Colilert® -18 / Quantiti-Tray® 2000 system and the Enterolert® -18® Quanti-Tray®/2000 system, respectively. The antibiotic susceptibility profiles of the isolates was determined using the Kirby-Bauer disk diffusion method. Whole-genome sequencing (WGS) and bioinformatics tools were used to determine the resistome, virulome, mobilome, clonal lineages, and phylogenies of the isolates circulating between the soil and the chicken litter.

The application of chicken litter to the soil statistically significantly increased *Enterococci* count and the number of antibiotic-resistant enterococci in the litter-amended soil. A total of 835 enterococci (680 from soil and 155 from litter) isolates recovered from the samples was dominated by *E. casseliflavus* (56%), followed by *E. faecalis* (22%), *E. faecium* (8%), *E. gallinarum* (2%) and other *Enterococcus* spp 102 (12%). Overall, 55.8% (466/835) of the enterococci isolates were resistant to one or more antibiotics with the highest rate in the litter-amended soil (68.9%, 321/466), followed by chicken litter (19.9%, 93/466) and the least in the soil samples collected before the litter amendment (11.2%; 52/466). The enterococci isolates were mostly resistant to tetracycline (33%), erythromycin (25%), and trimethoprim-sulfamethoxazole (23%), among others, intimating the high usage of these antibiotics in poultry farms in South Africa. Additionally, multidrug resistance (MDR) was recorded in 27.8% (130/466) of the enterococci isolates with MAR indices ranging from 0.13 (resistance to two antibiotics) to 0.44 (resistance to seven antibiotics). A total of 63 different resistance patterns

were recorded in the MDR enterococci isolates. Notably, enterococci count and the number of antibiotic-resistant enterococci in the litter-amended soil were reduced to levels comparable to the unamended soil at 50 and 28 days after soil amendment respectively.

The whole-genome analysis of the few selected enterococci isolates revealed eight novel sequence types (STs) (**ST1700, ST1752, ST1753, ST1754, ST1755, ST1756, ST1004, and ST1006**). Several resistance genes that confer resistance to aminoglycosides (*aac(6')-Ii*, *aac(6')-Ii*h, *ant(6)-Ia*, *aph(3')-III*, ant(9)-Ia), macrolide-lincosamide-streptogramin AB (MLS_{AB}) [*erm(B)*, *lnu*(*B*), *lnu(G)*, *lsaA*, *lsaE*, *eat(A)*, *msr(C)*], trimethoprim-sulfamethoxazole (*dfrE*, and *dfrG*), tetracycline (*tet(M)*, *tet(L)*, and *tet(S)*), fluoroquinolones (*efmA*, and *emeA*), vancomycin (*VanC {VanC-2*, *VanXY*, *VanXY*C-3, *VanXY*C-4, *VanRC*}), and chloramphenicol (*cat*) were detected in the isolates. The bioinformatics analysis further revealed that the chicken litter amendment increased the number and diversity of ARGs in the soil, resulting in increased detection of tetracycline resistance genes (*tet(M)*, *tet(L)*), and the macrolide resistance gene *erm(B)* and appearance of some ARGs (*ant(6)-Ia*, *aph(3')-III*, *lnu(G)*, *dfrG*)) that were not detected in the unamended soil. ARGs were mostly associated with diverse insertion sequences (ISs) (IS982, ISL3, IS6, IS5, IS3, IS256, IS30) and/or transposons (Tn*3*, Tn*916*, Tn*6009*) on plasmids or chromosome. The *tet(M)* and *erm(B)* were also co-located on *Tn916*-like transposons (Tn*644*, Tn*645*, and Tn*659*) in the three sample groups. Some of the isolates also harboured virulence genes that encoded adherence/biofilm formation (*ebpA*, *ebpB*, *ebpC*), anti-phagocytosis (*elrA*), and bacterial sex pheromones (*Ccf10*, *cOB1*, *cad*, and *camE*). Phylogenomic analysis showed that few isolates from litter-amended soil clustered with the chicken litter isolates. The isolates from this study also clustered with clinical and animal isolates from South Africa (Pretoria, Pietermaritzburg), Angola, and Tunisia.

There was also an increase (albeit statistically insignificant) in *E. coli* count and the number of antibiotic-resistant *E. coli* in the soil following chicken litter amendment. A total of 126 *E. coli* was recovered from the soil and chicken litter samples. In total, 76% (96/126) of the *E. coli* isolates displayed resistance to at least one antibiotic, with the highest prevalence in the litter-amended soil (71.9%, 69/96) and the least (1%, 1/96) in soil samples collected before the litter amendment.

The *E. coli* isolates displayed a high percentage resistance to tetracycline (78.1%), chloramphenicol (63.5%), ampicillin (58.3%), trimethoprim-sulfamethoxazole (39.6%), cefotaxime (30.2%), ceftriaxone (26.0%), and cephalexin (20.8%). Lower percentages of

resistance to cefepime (11.5%), amoxicillin-clavulanic acid (11.5%), cefoxitin (10.4%), nalidixic acid (9.4%), amikacin (6.3%), ciprofloxacin (4.2%), imipenem (3.1%), tigecycline (3.1%), and gentamicin (3.1%) were also recorded in the isolates. All the isolates were completely susceptible to meropenem and ceftazidime. Approximately 54% (52/96) of the resistant isolates were MDR, and the MAR indices of the isolates ranged between 0.11 (resistance to two antibiotics) and 0.56 (resistance to ten antibiotics). Overall, 38.5% (37/96) of all the resistant isolates had a MARI > 0.2, with the highest rate (51.4%) in the litter-amended soil and the least in the soil before litter amendment (2.7%). Twenty-one multidrug resistance patterns were observed among the isolates.

These results show that the soil resistome was augmented by chicken litter application. Agricultural soil and chicken litter are rich reservoirs of multidrug-resistant *E. coli* and *Enterococcus* spp. that could threaten public health through contamination of food products and the surrounding water bodies. There is therefore a need for urgent and stringent measures to mitigate the spread of antibiotic resistance in the environment via prudent use of antibiotics in food animal production and treatment of animal manure before its application onto agricultural soil.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.0 INTRODUCTION

Antimicrobial resistance (AMR) is widely recognized as a major global health challenge of the 21st century (Hernando-Amado et al., 2019). The increasing number of antibiotic-resistant bacteria (ARB) in the environment is a global threat. However, the degree of environmental contamination varies by geographical location. Human activities can lead to the contamination of the environment with antibiotics, ARB, and antibiotic resistance genes (ARGs), which can accelerate the development and spread of antibiotic resistance in the environment. Contamination can occur through land application of animal and human waste, use of antimicrobials and pesticides for crops and untreated effluent discharge from pharmaceutical industries. However, the connection between the released waste, antibiotics, and ARB in the environment as well as the public health risk associated with this contamination is not fully understood (Wellcome Trust, 2018).

The poultry industry is the biggest sector of South African agriculture, accounting for 19.8% of all agricultural production and 40% of all animal products in the country (South African Poultry Association, 2017). The South African poultry industry also dominates the agricultural sector, providing 64.3% of the animal food consumed, reaching 2.649 million tonnes per annum compared to 1.462 million tonnes of beef, mutton, goat, and pork consumption (South African Poultry Association, 2017).

The high demand for animal protein has triggered concentrated animal feeding operations (CAFOs) on a large scale, requiring veterinary antibiotics for growth promotion, prophylaxis, metaphylaxis, and treatment of diseases in livestock (Jechalke et al., 2014b; Van Boeckel et al., 2015). This practice has led to extensive use of several antimicrobial agents such as tetracycline, penicillin, sulphonamides, erythromycin, among others (Henton et al., 2011; Eagar et al., 2012). Eagar et al. (2012), in a survey of antimicrobial usage in animal production systems in South Africa, reported that 68.5% of the antimicrobials surveyed were administered as in-feed medications. The use of antibiotics in livestock farming has proven to be beneficial for economic reasons. However, the administration of sub-therapeutic antibiotics to livestock feed and water creates a suitable environment for the development and persistence of ARB and

ARGs in the gastrointestinal tract of the animals and their faecal waste (Zhu et al., 2013; Johnson et al., 2016).

The fast-growing poultry industry also generates the largest quantities of waste compared to other animal wastes. An estimation of the total animal waste generated in the Western Cape for 2015 and 2016 showed that the poultry industry generated the largest amount (32,767.69/76,102.65 tonnes) compared to other animal (sheep, cattle, pig, ostrich) wastes (Department of Environmental Affairs and Development Planning, 2017). When managed correctly, land application is the cheapest and viable way to dispose of or recycle the large volume of chicken litter waste (Bolan et al., 2010; Kyakuwaire et al., 2019). Although this waste is potentially important for soil fertility because of its relatively high nutrient content, there are safety concerns related to the application of chicken waste contaminated with antibiotic residues, ARB, and ARGs to agricultural soil (Kyakuwaire et al., 2019).

Another problem with using antibiotics in food animals is the fact that most of these antibiotics are poorly metabolized and absorbed by the animals, and approximately 75% of antibiotics are excreted via urine and faeces in an unmetabolized form or as active metabolites resulting in antibiotic residues in animal manure (Sarmah et al., 2006; Zhao et al., 2010; Qiao et al., 2012). These residues exert selection pressure for the development of resistance in the receiving environments (Heuer et al., 2011b; Zhu et al., 2013). The development and dissemination of antibiotic resistance in the soil microbial community may thus be attributed to the introduction of appreciable amounts of antibiotic residues and ARB by the application of both fresh and composted manure (Qiao et al., 2012; Zhu et al., 2013). There is a growing understanding that the environment plays a key role in the evolution and transmission of antibiotic-resistant pathogens (Larsson et al., 2018).

The role of soil environment, containing different microbial communities, in the emergence and spread of antibiotic resistance, especially with the constant influx of antibiotic–resistance related contaminants such as mobile genetic elements (MGEs), ARGs, ARB, antibiotic residues, and heavy metals, have been associated with faecal contamination of the soil via organic fertilizer and insufficient sewage infrastructure (Topp et al., 2013; Peng et al., 2015; Cheng et al., 2016; Larsson et al., 2018). In addition, antibiotic resistance is easily propagated among microorganisms via horizontal gene transfer (HGT). Therefore, soil fertilized with

animal manure such as chicken litter may be considered a rich reservoir of ARB and ARGs, which pose the danger of being transferred to other soil bacteria via the MGEs or even enter the food chain, thus posing a significant threat to human health (Jechalke et al., 2014b).

Despite the apparent dangers associated with the presence of antibiotic residues, ARB, and ARGs in untreated animal manure, no study has addressed the impact of chicken litter on soil resistome, particularly in relation to surveillance of antibiotic-resistant *Escherichia coli* and *Enterococcus* spp. in South Africa. This study therefore aimed to investigate the impact of chicken litter application on the resistome, virulome, mobilome, clonality, and phylogenies of *Escherichia coli* and *Enterococcus* spp. in agricultural soil in order to inform public health interventions.

2.0 LITERATURE REVIEW

2.1 The 'One Health' concept

Antimicrobial resistance (AMR) is recognised as a "One Health" issue which recognises that the health of humans, animals, and ecosystems are interconnected. It is considered a One Health challenge due to the rapid emergence, spread and persistence of antibiotic-resistant bacteria and antimicrobial resistance genes within and between the human, animal, and the environmental domains with evidence of interlinks across the triad (Davies and Davies, 2010; Aslam et al., 2018). Over the years, the emergence and spread of certain zoonotic disease (severe acute respiratory syndrome (SARS), Ebola, Zika virus disease, and avian influenza) outbreaks have illustrated the interconnectivity of the health of humans, animals, and the ecosystem (Destoumieux-Garzón et al., 2018).

Overuse and misuse of antibiotics in food animal production and human medicine, poor hygiene and sanitation infrastructure in developing nations, discharge of antibiotics residues into the environment from pharmacrutical industries and via animal manure or human faeces, have been suggested as the probable cause of "the global resistome" (Aslam et al., 2018).

Even though AMR exists in the three domains of One health, human health has gained more attention, and numerous studies on the global burden of AMR in humans (Robinson et al., 2016b; Rousham et al., 2018). Studies in the animal domain are on the increase, and published estimates of global consumption of veterinary antimicrobials in 2017 rated China as the highest consumer, accounting for 45% of global use, followed by Brazil (7.9%), the United States

(7.0%), Thailand (4.2%) with the least consumer being Argentina (1.5%) (Tiseo et al., 2020). Although a large percentage of antibiotic use may be justified on veterinary grounds, widespread misuse contributes significantly to the emergence of AMR (Robinson et al., 2016a, 2016b). There is growing evidence connecting the use of antibiotics in animal production to AMR in humans (Robinson et al., 2016a, 2016b).

The least understood and most under-researched domain of the One Health triad is the environment (Robinson et al., 2016a). Naturally, there is a high prevalence of different ARB in the environment that could serve as source of antibiotic resistance gene (ARG) that can be transferred to human and animal pathogens over time. This naturally occurring situation is aggravated by the influx of antibiotic resistant bacteria (ARB) and ARGs from animal and human waste together with vast quantities of antibiotic residues from the pharmaceutical industry, hospitals (Diwan et al., 2010), and intensive livestock farms. These anthropogenic activities have advanced the development and spread of ARB and ARGs (Martinez, 2009; Robinson et al., 2016a). The impact of such anthropogenic activities may be more prominent in developing countries negligent of environmental law (Robinson et al., 2016a). A critical gap in knowledge is the relative role of the environment in the development, dissemination, and persistence of ARGs. An accurate One Health strategy in combating AMR, involving all the three triads, requires a good understanding of each domain in the evolution of ARB and ARGs, their interaction, the mechanism of resistance, and the transmission routes.

2.2 Antibiotics and antibiotic resistance

2.2.1 Antibiotics

Antibiotics are chemical agents that can kill bacteria or inhibit their growth (Munita and Arias, 2016). Antibiotics are divided into several classes depending on their mechanisms of action (i.e., inhibition of nucleic acids synthesis, inhibition of cell wall synthesis, inhibition of protein synthesis, alteration of the cell membrane, and anti-metabolite activity) (Kapoor et al., 2017).

β-lactams and the glycopeptipes inhibit bacterial cell wall synthesis (Davies and Davies, 2010). β-lactams carry out their antibacterial activity by inhibiting the synthesis of the peptidoglycan layer of bacterial cell walls (ur Rahman et al., 2018). β-lactams are structural analogs of acyl-D-alanyl-D-alanine that normally binds to the active site of penicillin-binding proteins (PBPs) as a substrate of PBP during transpeptidation reaction. The transpeptidation

reaction is disrupted when *β*-lactams inactivate transpeptidase domain of PBPs. The disruption of peptidoglycan layer synthesis leads to lysis of bacterium (Soares et al., 2012; Miller et al., 2014; ur Rahman et al., 2018). Glycopeptides (vancomycin and teicoplanin) act by binding to the D-alanine-D-alanine terminus of the pentapeptide precursor, thereby inhibiting the synthesis of the cell wall. The large vancomycin molecule prevents the binding of this D-alanyl subunit with the PBP, thereby inhibiting the cell wall synthesis (Hollenbeck and Rice, 2012; Kapoor et al., 2017).

Protein synthesis is catalysed by ribosomes and cytoplasmic factors. The 70S ribosome consist of two ribonucleoprotein subunits including the 30S and 50S subunits (Yoneyama and Katsumata, 2006). Antibiotics that inhibit protein synthesis target the 30 S or 50S subunit (Figure 1a)of bacterial ribosome (Kapoor et al., 2017). Inhibitors of 30S ribosomal subunit include the aminoglycosides and tetracyclines. To reach the ribosomal target, aminoglycosides being positively-charged molecules bind to the negatively-charged outer membrane to form large pores through which antibiotic penetrate into the bacterial cell. Aminoglycosides interact with the 16S r-RNA of the 30S subunit near the A site via hydrogen bonds. Aminoglycosides cause misreading and untimely termination of translation of mRNA. Tetracyclines function as bactericidal agents by binding to the 30S ribosomal subunit and blocking the access of aminoacyl-tRNA to the acceptor (A) site of the bacteria (Hollenbeck and Rice, 2012; Miller et al., 2014).

Chloramphenicol inhibit protein synthesis by interacting with conserved sequences of the peptidyl-transferase cavity of the 23 r-RNA of the 50S ribosomal subunit. This prevents the binding of t-RNA to the A site of the ribosome (Yoneyama and Katsumata, 2006; Kapoor et al., 2017). Macrolides affect the early stage of protein synthesis i.e translocation by targeting the conserved sequences of the peptidyl-transferase center of the 23 r-RNA of the 50S ribosomal subunit (Hollenbeck and Rice, 2012; Soares et al., 2012). Macrolides, lincosamides, and streptogramin B have a similar mechanism of action. Oxazolidinones obstruct protein synthesis at different stages by binding to 23Sr RNA of the 50S subunit, and disrupt the docking of the aminoacyl-tRNA in the A site of the ribsome, thus inhibiting the delivery of peptides and the subsequent elongation of the polypeptide chain (Miller et al., 2014; Kapoor et al., 2017).

Trimethoprim and sulfamethoxazole inhibit bacterial enzymes involved in folate synthesis (Hollenbeck and Rice, 2012; Miller et al., 2014). Most bacteria do not have the ability to absorb

folate from their environment and as such need to synthesize it from the p-amino benzoic acid precusor. Trimethoprim and sulfamethoxazole inhibit enzmyes in this pathway, thereby limiting the production of dihydrofolate and tetrahydrofolate. Sulfonamides hinder dihydropteroate synthase while trimethoprim obstruct the enzyme dihydrofolate reductase at a later phase of folate synthesis (Miller et al., 2014)

Fluoroquinolones destroy bacterial cells by inhibiting two important enzymes (DNA topoisomerase IV and DNA gyrase), which are crucial for DNA replication (López et al., 2011). The two enzymes are hetero-tetramers with DNA topoisomerase IV consisting of *parC* and *parE* subunit, while DNA gyrase comprises the *gyrA* and *gyrB* subunit. Fluoroquinolones prevent the formation of replication fork by inhibiting DNA gyrase via binding to *gyrA* or *gyrB* subunit, thereby inhibiting DNA synthesis and replication (López et al., 2011).

2.2.2 Antibiotic resistance and its growing threat

The ability of bacteria to divide and multiply in the presence of therapeutic antibiotic concentrations that they were initially susceptible to, is called antibiotic resistance (Franco et al., 2009; Davies and Davies, 2010). This occurs when bacteria develop mechanisms that protect them from the effects of antibiotics or render antibiotics ineffective. The transfer of resistance genes from resistant bacteria to susceptible ones also contributes to the spread of antimicrobial resistance in a given niche. To date, the evolution and transmission of antibiotic-resistant pathogenic bacteria have become the biggest threat to the global public health care system (Davies et al., 2013; CDC, 2013). The damaging impacts of AMR are evident across the world. It has been reported that minimum of 50,000 people die each year across Europe and the US alone, with many hundreds of thousands more dying in other part of the world due to antibiotic-resistant infections. At least 700,000 people die annually due to drug–resistant infections, including 230,000 people who die from multidrug-resistant tuberculosis (World Health Organization, 2019). It has also been estimated that deaths attributable to AMR will reach 10 million by the year 2050 if the current trend of AMR is curtailed (O' Neill, 2014). Besides a high mortality rate, antibiotic resistance also imposes a tremendous financial burden on the world economies (CDC, 2013). AMR costs the European Union an estimate of 1.5 billion euros per year in healthcare and productivity losses (WHO, 2019). The World Bank in a high AMR-impact scenario study, estimated that the world will lose 3.8% of its annual gross domestic product (GDP) by 2050, with an annual shortfall of $3.4 trillion by 2030 (World

Bank, 2017). The annual reduction in global GDP due to AMR would be comparable to the losses provoked by the 2008-2009 global financial crisis. The economies of low-income countries will be more impacted than wealthy countries, resulting from higher infectious disease prevalence and greater dependence on labour incomes (World Bank, 2017). Unfortunately, the rate at which bacteria develop resistance to newly developed antibiotics after their introduction for use outweighs the rate at which new antibiotics are developed over the past decades, exacerbating the antibiotic resistance public health challenge globally (Livermore, 2011; Prestinaci et al., 2015).

2.2.3 Mechanisms of antibiotic resistance

Antibiotic resistance in bacteria can be categorised into three groups: intrinsic, mutational, and acquired resistance. Mutational resistance occurs due to spontaneous change or alteration of a single nucleotide base in bacterial chromosomes, resulting in a genetically altered bacterial population (daughter cells) that are resistant to antibiotics (Soares et al., 2012). Such mutation may confer antibiotic resistance when the daughter cells express proteins that can modify the antibiotic binding site (Munita and Arias, 2016). Intrinsic resistance refers to the natural abilities of bacteria to resist the effects of an antimicrobial agent through its innate structural or functional, characteristics without the need of mutation or acquisition of extra genes (Davies and Davies, 2010; Pikkemaat et al., 2016). This inherent characteristic is common to all members of a species and is not associated with the misuse of antibiotics. The majority of the bacteria that have this property are known antibiotic producers that are intrinsically resistant to the antibiotics they synthesize and those with similar mechanisms of action produced by other organisms (Davies and Davies, 2010; Fair and Tor, 2014). Soil bacteria are known to produce antibiotics to repel natural competition from other bacterial species within their environment (Pehrsson et al., 2013). These antibiotic producing bacteria also develop self-resistance mechanisms to protect them against their own antibiotics (Peterson and Kaur, 2018; Asante and Osei Sekyere, 2019). Additionally, the co-existence of antibiotic-producing and non-producing bacteria is also believed to have resulted in development of resistance mechanisms in non-producing environmental bacteria in order to escape the effects of naturally produced antibiotics (Peterson and Kaur, 2018). This selection pressure allows the survival and dominance of bacteria with intrinsic or acquired resistance within a given niche (Holmes et al., 2016). The developed resistance mechanisms can also be horizontally transferred to other non-

resistant bacteria through MGEs, such as transposons, plasmids, integrative conjugative elements, insertion sequences and integrons. Horizontal gene transfer (HGT) of resistance determinants helps the spread of ARGs in bacteria (Stokes and Gillings, 2011). Even though acquisition of ARGs through these MGEs accounts for more resistance mechanisms in bacteria than mutations or natural selection (von Wintersdorff et al., 2016), little is known about their role in agricultural soil fertilised with poultry manure.

Generally, bacteria resist the action and effect of antibiotics through three primary mechanisms that works concurrently with each other (Cag et al., 2016). These include target sites modification, inactivation of antibiotics by enzymes such as β-lactamase and aminoglycosides acetytransferase, decreased uptake of antimicrobial agents through efflux upregulation and porin downregulation (Cag et al., 2016).

2.2.3.1 Modification/inactivation of antibiotics agents

Modification/inactivation of antibiotics agents most commonly involves bacterial production of enzymes that inactivate antibiotics by adding specific chemical moieties to the compound or destroying the antibiotic molecules, thereby rendering the antibiotic ineffective againt its target (Munita and Arias, 2016). The production of enzymes capable of modifying antibiotics molecules is a common acquired resistance mechanism in Gram-positive and Gram-negative bacteria. The most frequent biochemical reactions by which the enzymes modify antibiotics are acetylation (aminoglycosides, chloramphenicol, and streptogramins), hydrolysis (β-lactams), phosphorylation (aminoglycosides, chloramphenicol), adenylation (aminoglycosides, lincosamides), and methylation (macrolides-lincosamides-streptogramin) (Munita and Arias, 2016).

A good example of resistance through modification of antibiotics is aminoglycosides resistance, which involves modifying the hydroxyl or amino groups of the antibiotics molecule by the aminoglycosides modifying enzymes (AMEs) (Hollenbeck and Rice, 2012). Many AMEs have been identified in both Gram-positive and Gram-negative bacteria (Ramirez and Tolmasky, 2010). These enzymes are named and classified according to their biochemical activities, which include adenyltransferase (ANT) , acetyltransferase (ACC) , and phosphotransferase (APH) . While the genes encoding these enzymes are usually located on MGEs, chromosomally encoded AMEs have also been detected in some bacterial species like *E. faecium*, *S. marcescens,* and *Providencia stuartii* (Ramirez and Tolmasky, 2010; Yoon et al., 2014). High-level resistance to aminoglycosides such as gentamycin occurs by the

acquisition of a bifunctional enzyme like *aph(2'')-Ia-aac(6')-Ie* formed from the fusion of two AME encoding genes (Hollenbeck and Rice, 2012). These enzymes inactivate gentamicin and make it unable to bind to its 30S ribosomal subunit target, thereby rendering the antibiotic ineffective. In addition to gentamicin, this protein confers high-level resistance to all aminoglycosides except streptomycin and is located on *Tn4001*-like transposon, widely distributed among enterococci and staphylococci (Munita and Arias, 2016). Other genes that confer resistance to gentamycin include; *aph(2'')-Ic*, *aph(2'')-Id* and *aph(2'')-Ib* (Hollenbeck and Rice, 2012). Resistance to other aminoglycosides has also been reported by the acquisition of adenylyltransferases (Miller et al., 2014). These include (*Ant(6')-Ia* and *Ant(3'')-Ia*) which confer resistance to streptomycin, aminoglycoside phosphotransferase (*Aph(3')-IIIa*) conferring resistance to kanamycin, and adenylyltransferase (*Ant(4'')-Ia*) conferring resistance to tobramycin, amikacin, and neomycin. Furthermore, the expression of chromosomally-encoded AMEs such as aminoglycoside 6' acetyltransferase [AAC(6')-Ii] creates steric hindrance and decreases the binding of antibiotics to the ribosomal target (Miller et al., 2014). AAC(6')-Ii is ubiquitous in *E. faecium* , where it confers resistance to tobramycin and kanamycin (Miller et al., 2014).

Modification of phenicol antibiotics is another good example of enzymatic inactivation through the acetylation of antibiotics. The chemical modification of chloramphenicol is through the enzymatic action of chloramphenicol acetyltransferase (CATs). Several CAT genes have been identified in Gram-negative and Gram-positive bacteria, and are grouped into two main types (type A and type B) (Schwarz et al., 2004; Munita and Arias, 2016). The expression of the type A genes (e.g. *cat, catl, catB, catC*) usually results in high-level resistance, while type B (e.g., *catB1, catB2, catB3*) confers low-level resistance to chloramphenicol (Schwarz et al., 2004; Munita and Arias, 2016). While most of these genes are usually found on transposons (*Tn9, Tn2424*) and plasmids (pRE25, pRUM, pHSH2), a number of them have been found located in the chromosome of some bacteria (Schwarz et al., 2004; Munita and Arias, 2016).

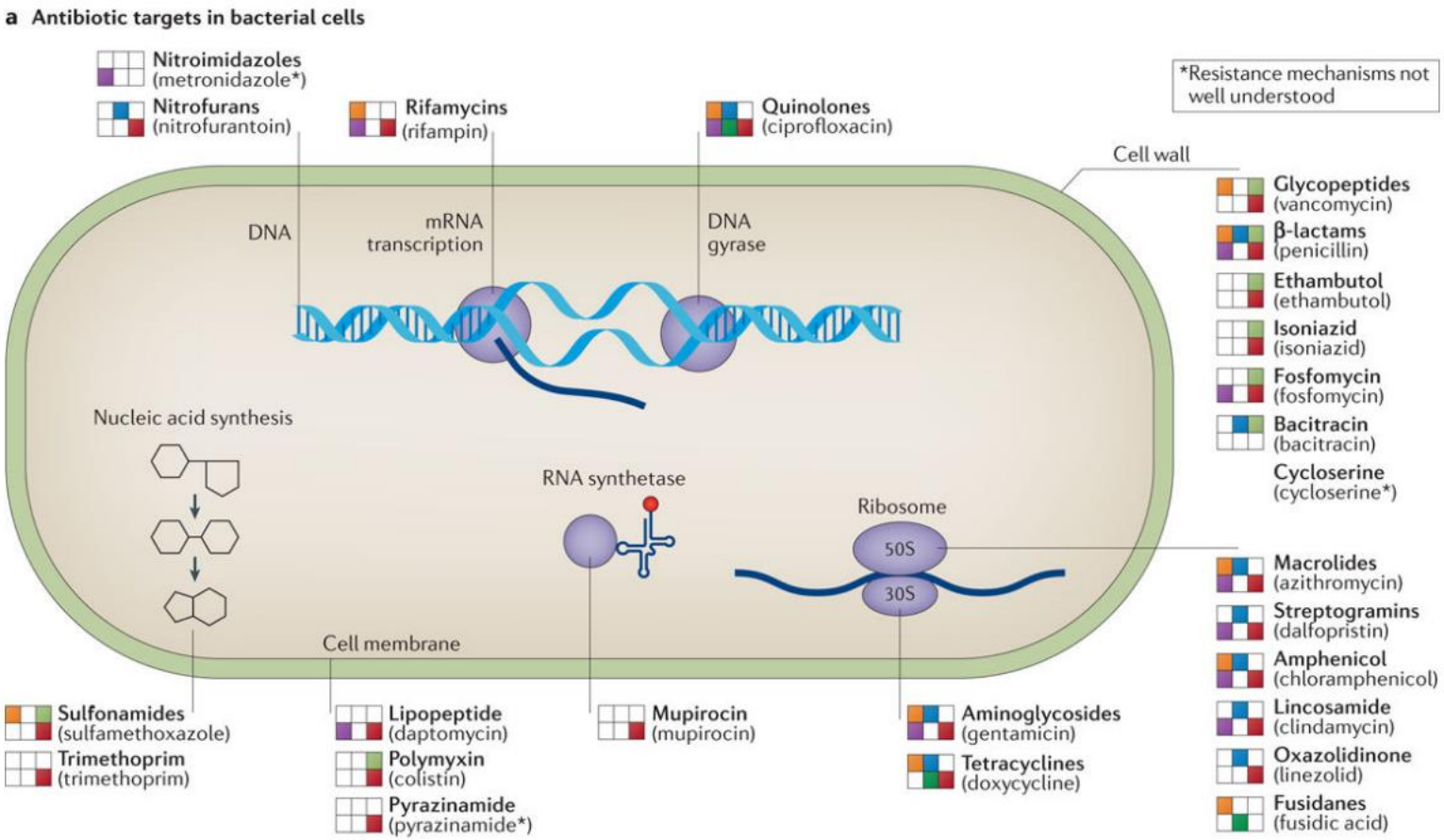

Figure 1a: Antibiotic targets and resistance mechanisms. Antibiotics are grouped by target site. Image from Boolchandani et al. (2019).

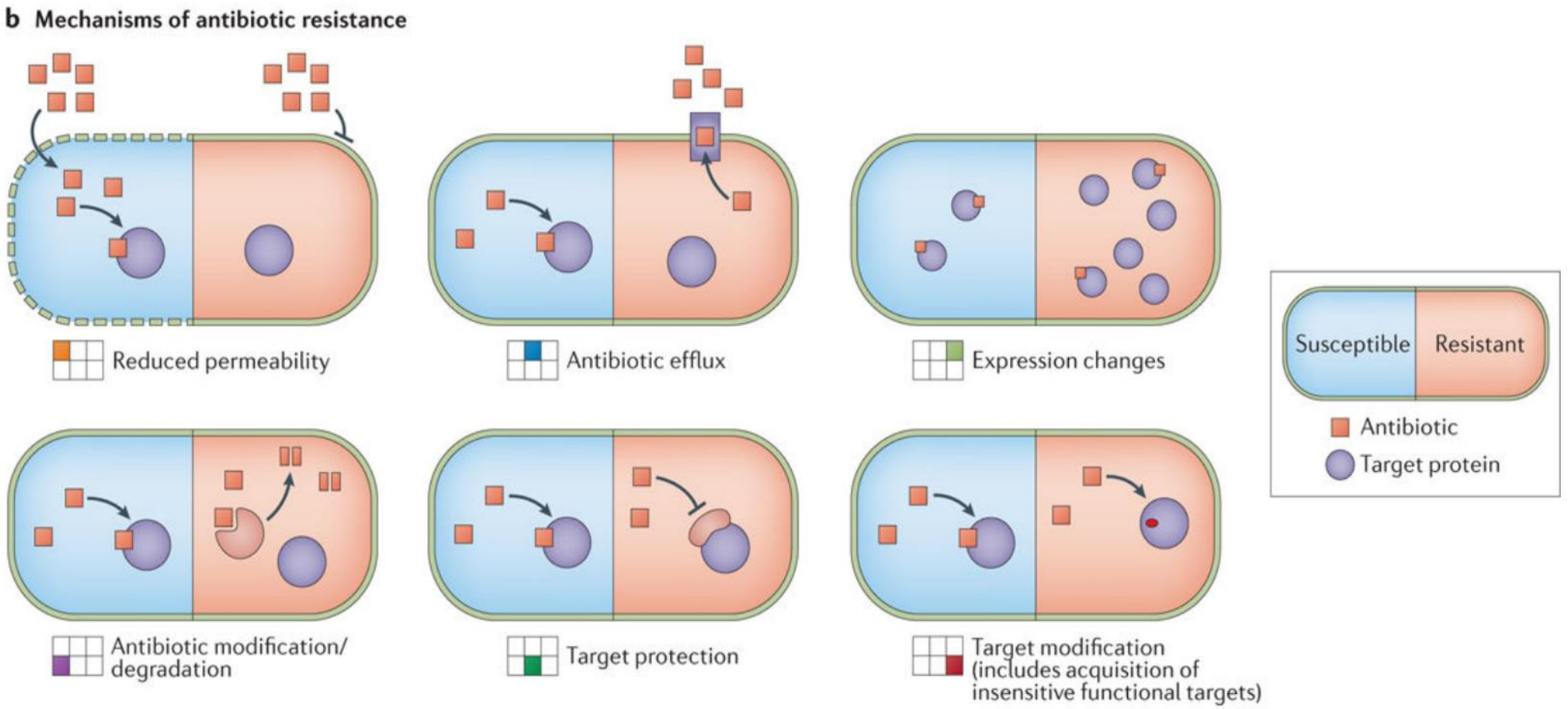

Figure 1b: Antibiotic resistance mechanisms depicted with susceptible (left side) and resistant bacteria (right side). The legend annotation position used in figure 1a is shown on the left side of each labelled mechanism. Image from Boolchandani et al. (2019).

The main mechanism of bacterial resistance to *β*-lactam antibiotics is through the destruction/degradation of the *β*-lactam ring by the action of *β*-lactamases. These enzymes destroy the amide bond of the *β*-lactam ring and render the antibiotics inactive before it reaches the PBP target (Munita and Arias, 2016). This mechanism of resistance is widespread among the *Enterobacterales* like *Klebsiella* and *E. coli*. The genes coding for *β*-lactamases (called *bla*, followed by the name of a specific enzyme, e.g., $bla_{\text{CTM-X}}$) are either expressed chromosomally or plasmid-mediated, which is often linked to the widespread dissemination of *β*-lactamases (Munita and Arias, 2016). The Ambler classification uses amino acid sequence identity and categorises the *β*-lactamases into four groups (A, B, C, and D) (Munita and Arias, 2016; ur Rahman et al., 2018). The class C, A, and D enzymes use serine for *β*-lactam hydrolysis, while class B metallo-enzymes require divalent zinc ions for substrate hydrolysis (Munita and Arias, 2016). The enzymes belonging to class A, including the penicillinases (TEM-1 and SHV-1) that hydrolyse only penicillin, ESBLs like CTX-M that hydrolyse the broad spectrum 3rd and 4th generation cephalosporins and carbapenemases like KPC (*Klebsiella pneumonia* carbapenemase), which hydrolyse the carbapenems and are commonly found in Gram-negative bacteria (Munita and Arias, 2016).

Ambler class A *β*-lactamases are generally susceptible to *β*-lactamases inhibitors such as tazobactam, clavulanic acid, and sulbactam (ur Rahman et al., 2018). This property differentiates the ESBLs from AmpC enzymes (class C *β*-lactamases) which hydrolse third-generation cephalosporins but are not inhibited by tazobactam or clavulanic acid. Also found within the ESBL group is a class D subgroup OXA enzymes that hydrolyse third-generation cephalosporins. Several ESBLs have been identified in various pathogens, but TEM, CTX-M and SHV are the most disseminated ESBLs across different epidemiological niches (ur Rahman et al., 2018). The TEM-type ESBLs evolved from mutations in the original TEM-1 penicillinase after acquiring the ability to hydrolyse third-generation cephalosporins and aztreonam due to two amino acid substitution around the active site (Sirot et al., 1987). The TEM-type ESBLs are often plasmid-mediated and are responsible for 90% of ampicillin resistance in Gram-negative bacteria (Ur Rahman et al., 2018). The SHV ESBLs are mostly found in *K. pneumoniae* and usually harboured on a plasmid. The genes encoding CTX-M enzymes have been associated with ISEcp1 insertion sequence and transposable elements such as Tn402-like transposons and are present on a broad range of conjugative plasmids or phage-like sequences (Poirel et al., 2005; Munita and Arias, 2016). Consequently, CTX-M enzymes are now the most prevalent ESBL around the world, and are responsible for most of the

cephalosporin resistance in *K. pneumonia*, *E. coli,* and other *Enterobacterales* (Riwu et al., 2020).

Unlike the Ambler class A (SHV and TEM) ESBLs, the emergence of CTX-M enzymes was not a result of alterations of existing enzymes (like SHV or TEM) but rather acquired through HGT from *Kluyvera* spp., an environmental bacterium with no major human pathogenic significance (D'Andrea et al., 2013; Munita and Arias, 2016; ur Rahman et al., 2018).

2.2.3.2 **Decreased permeability of antimicrobial agents through efflux upregulation and porin downregulation**

Bacteria can prevent accumulation of antibiotic molecules on targets by reducing it absorption into the cell or increasing its expulsion out of the cell or utilise both mechanisms simultaneously (Cag et al., 2016). The target of many clinically relevant antibiotics is located within bacterial cell, as such, antibiotic compounds need to penetrate the outer membrane to reach their targets. The outer membrane of Gram-negative bacteria consist of a lipid bilayer and porins via which antibiotics enter bacterial cells (Ude et al., 2021). Moreover, the outer membrane may be modified via substitution of one or two amino acids, thereby transforming it to a permeability barrier for antibiotics (Cag et al., 2016). This resistance mechanism is fundamental in Gram-negative bacteria with an outer membrane, which serves as a permeability barrier and provides an intrinsic resistance against hydrophilic antibiotics and other antibiotics like vancomycin (Nikaido, 2003; Peterson and Kaur, 2018). Hydrophilic antibiotics such as tetracyclines, β-lactams, and some fluoroquinolones are particularly affected by this mechanism of resistance because they often cross the outer membrane barrier through water-filled diffusion channels called porins (Pagès et al., 2008).

Additionally, upregulated efflux molecules may work singly or simultaneously with porin modifications for an efficient expulsion of antibiotics, thereby preventing build-up on targets (Poole, 2005). Different classes of efflux pumps have been characterised in both Gram-positive and Gram-negative bacteria. Efflux pump systems can be substrate-specific (specific exporters) for a particular antibiotic as observed in *mef* and *tet* genes for macrolides and tetracyclines, respectively, in pneumococci or may be broad substrate-specific (polyspecific exporters) commonly found in MDR bacteria (Schindler and Kaatz, 2016). The genes encoding efflux pumps are either located in the chromosome leading to intrinsic resistance of some bacterial species to certain antibiotics as observed in *E. faecalis* to streptogramin A or located on MGEs

(*tet* gene) (Soares et al., 2012; Munita and Arias, 2016). There are five major efflux pumps families, including (i) the small multidrug resistance family, (ii) the major facilitator superfamily (MFS), (iii) the multidrug and toxic compound extrusion family (MATE), (iv) the resistance-nodulation-cell-division family (RND), and (v) the ATP-binding cassette family (ABC) (Schindler and Kaatz, 2016). The efflux families vary from each other in terms of energy source, structure, distribution in bacterial species, and the range of substrate they can extrude. Many classes of antibiotics are affected by this mechanism of resistance, including protein synthesis inhibitors, *β*-lactams, fluoroquinolones, and polymyxins (Piddock, 2006; Munita and Arias, 2016). Tetracycline efflux is the best-studied mechanism of resistance, and various types have been identified, including *Tet(A)* to *Tet(E)*, which are found among *Enterobacterales* family members and the genera *Aeromonas*, *Moraxella*, and *vibrio*. The plasmid-borne *Tet(K)* and *tet(L)* have also been identified in Gram-positive bacteria and *Tet*(*P*) in *Clostridium* spp. Others tetracycline efflux include *Tet*(G), (H), (V), and *otrA* (Soares et al., 2012). Another class of tetracycline resistance and repressor proteins referred to as *TetA*(Z) and *TetR*(Z) was proposed in the year 2000 (Tauch et al., 2000). It is important to note that many of these efflux pumps do not affect tigecycline which are last-resort antibiotics (Grossman et al., 2012). Decreased susceptibility to tigecyline has been linked to overexpression of efflux pumps of the resistance-nodulation-cell division protein family like AcrAB-TolC, OqxAB, and AdeABC (Ruzin et al., 2007; Zhong et al., 2014)

Another clinically important phenotype mediated by the efflux mechanism is resistance to macrolides. The well-characterised efflux pumps involved in the extrusion of macrolides (e.g., erythromycin) in bacteria include the macrolide efflux (*mef*) genes (*mefA* and *mefE*), *MsrA*, and *MsrC*. The *MsrA* is a plasmid-borne resistance determinant initially described in *Staphylococcus epidermidis*, while *MsrC* is chromosomally encoded and commonly found in *E. faecalis*, where it confers low-level resistance to streptogramin B and macrolides (Miller et al., 2014). The *mef* pumps are mainly found in Streptococci as well as other Gram-positive bacteria. The *mefA* pump is usually borne on a transposon (*Tn1207*) located in the chromosome, while *mefE* is carried in the ***m***acrolide ***e***fflux ***g***enetic ***a***ssembly (MEGA) element inserted into different regions of the bacterial chromosome (Munita and Arias, 2016). Notably, macrolides resistance mediated by these pumps does not result in cross-resistance to lincosamides and streptogramins of the MLS_B group (Ross et al., 1990; Chancey et al., 2015). Moreover, Lsa efflux pumps chromosomally encoded by the *lsa* gene have been linked to the intrinsic

resistance of *E. faecalis* to lincosamides and streptogramins A (LS_A) (Poole, 2005; Miller et al., 2014)

2.2.3.3 Modification/protection of the target binding sites

Another common mechanism via which bacteria evade the actions of antibiotics is by interfering with the drug target sites (modify or protect), thereby reducing their affinity for antibiotic molecules (Munita and Arias, 2016). The genes coding for proteins responsible for target site protection and modification have been found in association with MGEs (clinically relevant ones) or bacterial chromosomes. Examples of classes of antibiotics affected by this mechanism include fluoroquinolones (*qnr*) and tetracyclines (*Tet* (*M*), *Tet*(*O*), *Tet*(*Q*), *Tet*(*S*)) among others (Soares et al., 2012; Munita and Arias, 2016). A well-known example of a target protection mechanism is the tetracycline resistance determinant *Tet* (*M*), initially detected in *Streptococcus* spp. and *Tet*(*O*) in *Campylobacter jejuni*. The binding of *Tet* (*M*) and *Tet*(*O*) to the ribosome facilitates the displacement of tetracycline from its binding site in a GTP-ase activity-dependent manner, thereby allowing protein synthesis to resume (Munita and Arias, 2016; Peterson and Kaur, 2018). These ribosome protection resistance genes are widely distributed among different bacterial species because of their association with broad-host-range conjugative plasmids and several plasmids (Munita and Arias, 2016). Chromosomal resistance determinants such as *tetM*, *tetO*, and *tetS*, which the Tn*916* transposon can transfer, have been shown to confer resistance to minocycline, doxycycline, and tetracycline (Miller et al., 2014).

The quinolone resistance protein qnr belongs to the pentapeptide repeat protein family and behaves as a DNA homologue that competes for the DNA gyrase and topoisomerase IV DNA binding site (Munita and Arias, 2016). This interaction protects DNA gyrase by reducing the opportunities of quinolone molecules to bind to DNA and the subsequent formation of quinolone-DNA gyrase complex that is lethal for bacterial cells (Miller et al., 2014; Munita and Arias, 2016). Different alleles of qnr, which have similar mechanisms, have been characterised, including *qnrS*, *qnrA*, *qnrB*, *qnrC*, *qnrVC*, and *qnrD* (Munita and Arias, 2016). It is important to note that *qnr* only confers low-level quinolone resistance; however, the presence of *qnr*-encoding genes in bacteria promote the selection of mutants strains with point mutations in genes encoding the DNA gyrase and/or topoisomerase IV, resulting in the emergence of highly resistant bacterial isolates (Aldred et al., 2014).

Bacteria may also avoid the action of antibiotics by introducing changes/modifications to the target sites of antibiotics through (i) enzymatic alterations of the binding site (e.g., the addition of methyl groups), (ii) point mutations in the genes encoding the target site and/or (iii) a replacement or bypass of the original target. All of these modifications result in reduced affinity of the antibiotics for the target sites. The enzymatic modification of the target site is best understood in the case of macrolide resistance caused by the methylation of the ribosome through the action of rRNA methylases encoded by the erythromycin ribosomal methylation (*erm*) genes. These enzymes can catalyse mono-methylation or di-methylation of an adenine residue in position A2058 of the 23S rRNA, thus preventing macrolides from binding to the 50S ribosomal subunit. Notably, the expression of *erm* genes also confers cross-resistance to macrolides, lincosamides, and streptogramin B (MLS_B) antibiotics because of their overlapping binding sites in the 23S rRNA (Miller et al., 2014; Munita and Arias, 2016). The *erm* genes are located on MGEs, which may account for their abundance among different genera, including anaerobic and aerobic Gram-positive and Gram-negative bacteria. The most frequently reported *erm* gene in enterococci and pneumococci is the *erm*(*B*) primarily located on transposons such as *Tn917* and *Tn551* and plasmids. The *erm*(*A*) and *erm*(*C*) genes are mainly distributed on transposon and plasmids, respectively, and are commonly found in Staphylococci (Munita and Arias, 2016).

Another well-characterised example of resistance due to mutations in the genes at the target site is quinolone resistance. The occurrence of chromosomal mutations at DNA topoisomerase IV and DNA gyrase regions decreases the binding affinity of quinolones with the two genes. Chromosomal mutations in the target genes, particularly *gryA* and *parC* have been reported in *E. faecium* and *E. faecalis*, butare absent in *E. casseliflavus* and *E. gallinarum* (Werner et al., 2010; López et al., 2011; Yasufuku et al., 2011).

A good example of replacement or bypass of the original target mechanism is related to vancomycin resistance. Vancomycin resistance is relevant in enterococci, particularly in *E. faecium* which harbours other resistance genes that make the treatment of diseases caused by it a serious clinical challenge (Arias and Murray, 2012). Enterococci resist the action of vancomycin by acquiring a group of genes called *van* gene clusters that code for a biochemical mechanism that reconditions peptidoglycan synthesis. This is done by replacing the last $_D$-Ala of the pentapeptide precursor with either $_D$-lactate or $_D$-serine for high- ($_D$-alanine-$_D$-lactate) and low-level ($_D$-alanine-$_D$-serine) resistance, respectively, thereby preventing vancomycin from binding to the cell wall precursor. There are nine distinct vancomycin resistance gene

clusters in *Enterococcus* species, including *vanA, vanB, vanC, vanD, vanE, vanG, vanL, vanM,* and *vanN* (Munita and Arias, 2016; Ahmed and Baptiste, 2018). These genes are distinguished from each other by their degree of resistance to glycopeptides, inducibility, and transferability (Ahmed and Baptiste, 2018). Among these vancomycin genes, *vanA* and *vanB* are most common in enterococci, particularly among the clinical VRE isolates, as they are usually found on MGEs or inserted in the chromosome (Munita and Arias, 2016). The *vanA* gene cluster confers resistance to vancomycin and teicoplanin and is usually found on a *Tn3*-family transposon (*Tn1546*), which has been detected on both conjugative and non-conjugative plasmids (Munita and Arias, 2016). The *vanB* gene cluster only confers resistance to vancomycin and is found on *Tn1547* or related conjugative transposons as well as pheromone-responsive plasmids. *Enterococcus gallinarum* and *E. casseliflavus* are intrinsically resistant to vancomycin carrying *vanC*, which modifies the pentapeptide precursor from $_{D}$-Ala-$_{D}$-Ala to $_{D}$-Ala-$_{D}$-Ser (Hollenbeck and Rice, 2012).

Genes similar (79-100% identity) to the *vanA* gene cluster (common in clinical enterococcal strains) have been found in agricultural soil organisms like *Paenibacillus thiaminoluticus, P. popilliae* and *P. apiaries* (Patel et al., 2000; Guardabassi and Agersø, 2006; Ahmed and Baptiste, 2018). Agricultural soil has been indicated as a rich reservoir of genes closely related to those conferring glycopeptide resistance in clinical bacteria and soil *Paenibacillus* has been suggested as a possible origin of vancomycin resistance in enterococci (Guardabassi and Agersø, 2006).

2.3 Use of antibiotics in animal production

The increasing demand for animal-derived protein has led to a shift to integrated intensive and large-scale livestock production systems, which require antibiotics to keep animals healthy and maintain productivity. In order to meet demand, several countries, including Brazil, Russia, India, China, and South Africa (BRICS), have moved to this cost-effective system (Van Boeckel et al., 2015). In this animal production system, antibiotics are widely used for prophylaxis, metaphylaxis, therapeutic, and growth promotion purposes (Jechalke et al., 2014a; Van Boeckel et al., 2015). To treat infections in animals, antimicrobial agents are administered at concentrations that exceed the pathogen's minimal inhibitory concentration (MIC) for an effective period (Pikkemaat et al., 2016). Prophylactic or preventive treatment involves administration of antibiotics to animals that are at risk before the expected onset of the clinical

disease, but in situations where individual treatment is impracticable, farmers engage in mass medication of the entire group of animals before the expected outbreak of disease through feed and water (Economou and Gousia, 2015; Pikkemaat et al., 2016). Growth promotion is the repeated exposure of animals to a sub-therapeutic concentration of antibiotics via feed additives to promote animal growth and feed efficiency (Van Boeckel et al., 2015).

Several antibiotics are used as feed additives to promote the growth of food animals. This accounts for more than 70% of antibiotic usage globally (Jechalke et al., 2014a; You and Silbergeld, 2014). The estimated global consumption of antibiotics in food animal production in 2010 was 63,151 (± 1,560) tonnes and is projected to increase by 67% to 105,596 (±3,605) tonnes by 2030 (Van Boeckel et al., 2015). A third of the projected future rise in global consumption of antibiotics has been attributed to the middle-income countries like South Africa, where high demand for animal protein products will trigger a further shift to large-scale intensive livestock farming in which antibiotics are routinely used at sub-therapeutic doses (Van Boeckel et al., 2015). Hence, a better understanding of the effect of unregulated use of antibiotic in livestock production is needed to assess its potential consequences on the environment that receive animal manure as organic fertiliser.

China is the highest end-user of antimicrobials in animal production (Larson, 2015; Van Boeckel et al., 2015; Zhang et al., 2015). Zhang et al. (2015) reported that the two major groups of livestock that exhaust veterinary antibiotics in China are pigs and chickens In the United States, the estimated amount of antibiotics used in food animal production as growth promoters account for 70% of all the antibiotics consumed annually in the nation (Martin et al., 2015), and a significant part of the antibiotics includes the clinically important ones needed for major surgeries and organ transplantation (Laxminarayan et al., 2013).

In contrast to China and USA reports, where the consumption of antibiotics in food animals is higher compared to humans, the estimated usage of antibiotics for animals and humans in South Africa between 2014 and 2015 ranged between 23% -26% and 77%-74%, respectively (National Department of Health, 2018). South Africa imported 4,35 tons of antimicrobials into the country in 2014, of which 23% was estimated for animal use, and the remainder (77%) for human use (National Department of Health, 2018). Between 2014 and 2015, there was an increase of 58% in estimated animal imports and 38% in human imports, resulting in a total estimated import of 6,3 tons (National Department of Health, 2018). The report of South African Animal Health Association (SAAHA) from 2014 and 2015 show that the predominant

antibiotic group used in animal health are growth promoters. Growth promoters made up more than 55% (in 2014) and 62% (in 2015) of all antimicrobials sold in animal health. These growth promoters contain antibiotics not used in human health such as ionophores (monensin sodium and salinomycin), flavophospholipol (flavomycin), olaquindox, zinc bacitracin and tylosin. The estimated consumption of tetracyclines by animals makes up about 27% of total antimicrobial sales, compared to the World Organization for Animal Health (OIE) that reported 63% for most African countries over the same period (OIE, 2016 and 2017; National Department of Health, 2018). This possibly demonstrates that South Africa's farming practices vary from those of other African countries (National Department of Health, 2018). Of concern is the increase in certain antibiotics between 2014 and 2015 especially "growth promoters" (78%), sulfonamides (398%), macrolides (120%). However, there has been a significant decrease in the use of penicillins (-49%) and fluoroquinolones (- 26%) (National Department of Health, 2018). Aside from China and India, the South Africa estimate on antibiotics consumption is similar to the reports in other low-middle income countries (O'Neill, 2016). Globally, different antibiotics are being used in animal production, depending on the purpose for which the antibiotics are given (prophylaxis, therapeutics, growth promotion), the kind of animal, and country policy rules. In the Netherlands, the commonly used antibiotics in food animal production are tetracyclines, penicillins, and trimethoprim/sulphonamides combinations, and to lesser degree aminoglycosides and macrolides (Pikkemaat et al., 2016). Austria uses tetracyclines, amphenicol, penicillins, cephalosporins (3rd generation), sulphonamides, fluoroquinolones, and aminoglycosides (Chantziaras et al., 2014).

Generally, the use of antibiotics in food animal production in developing countries, including the African continent, is to a greater extent unregulated (Maron et al., 2013). Most developing countries do not yet have relevant legislation guiding the appropriate use of veterinary antibiotics, and where it does exist, it is not strictly applied (Alonso et al., 2017; Manyi-Loh et al., 2018). Two survey studies conducted in Southwestern Nigeria and Zambia on antibiotics in the food animal industry indicate an irrational use of antimicrobial agents due to uncontrolled access and administration of veterinary antibiotics (Adesokan et al., 2015; Mainda et al., 2015). In the survey, the authors reported that farmers often purchase and administer antibiotics without veterinary prescription and supervision, even when such an act is illegal (Adesokan et al., 2015; Mainda et al., 2015; Alonso et al., 2017).

In South Africa, the use of antibiotics in animals is regulated by two Acts: Fertilisers, Farm Feeds, Agricultural Remedies and Stock Remedies Act (Act 36 of 1947), controlled by the

Department of Agriculture, Forestry and Fisheries (DAFF); and the Medicines and Related substances Control Act (Act 101 of 1965), managed by the National Department of Health (NDoH) (Henton et al., 2011; Eagar et al., 2012). Antibiotics registered under Act 36 as stock remedies are those permitted for use by the lay public (untrained consumers/ farmers), and they are available over the counter. Moreover, the administration of antibiotics registered under Act 101 for animal production may only be carried out by veterinarians (Henton et al., 2011). Eagar et al. (2012), in a survey on antimicrobial usage in food animals stated that almost all the main classes and types of antibiotics are authorised for use in food animal production under Act 36 of 1947 and Act 101 of 1965 in South Africa. Furthermore, the survey showed that the highly consumed antimicrobials were from the macrolide (tylosin) and pleuromutilin classes, followed by tetracyclines, the sulphonamide class, and lastly, the penicillins. The study also highlighted that macrolides, streptogramins, ionophores, quinoxalines, glycolipids, oligosaccharides, phosphonic acids, polipeptides and polymeric compounds, banned in the European Union as feed additives were still used as growth promoters in South Africa (Eagar et al., 2012).

Additionally, in partnership with the South African Animal Health Association (SAAHA), DAFF also reported the use of antibiotics in food animal production, to the World Organization for Animal Health (National Department of Health, 2018). The report of the South Africa National Department of Health showed that 62% of antibiotics used in food animals were used as growth promoters. The growth promoters group consist of antibiotics not used in human medicine such as tylosin, olaquindox, ionophores, zinc bacitracin and flavophospholipol (flavomycin). The most commonly used antibiotics were tetracyclines (17%) and macrolides (11%) (National Department of Health, 2018). These reports, therefore, show that South Africa presently has not aligned with the European Union policies on the use of feed premixes for growth promotion. However, chloramphenicol and nitrofurans are not permitted for use in food animal production in South Africa (Henton et al., 2011).

The use of antibiotics in agriculture and aquaculture to prevent and treat diseases and as growth promoters has been reported to establish a reservoir of ARB and ARGs in the gastrointestinal tract of livestock (Chantziaras et al., 2014; You and Silbergeld, 2014). The extensive use of antibiotics as feed additives modifies the intestinal microbiome and creates a selective advantage for the emergence of resistant bacteria first in commensals and subsequently in pathogens (Kim et al., 2012; Looft et al., 2012). A shift in the gut microbiota of pigs was reported in the USA, after 14 days of exposure to feeds containing sub-therapeutic doses of

performance-enhancing antibiotics (penicillin, chlortetracycline, and sulfamethazine) using phylogenetic, metagenomic, and quantitative PCR-based approaches (Looft et al., 2012). The exposure led to an increase in *E. coli* populations, abundance, and diversity of ARGs specific to the antibiotics used compared to the non-medicated pigs (Looft et al., 2012). Similarly, Kim et al. (2012) reported a microbial population shift representing the succession and changes in the gut microbiota of pigs in response to the growth-promoting tylosin antibiotic treatment.

Chantziaras et al. (2014), in a study from seven European countries (Austria, Belgium, Denmark, Norway, Netherlands, Sweden, and Switzerland), showed a strong correlation between the quantities of eight classes of antibiotics administered to food animals and the prevalence of antibiotic-resistant commensal *E. coli* in poultry, cattle, and pigs. Of the seven countries, Belgium ranked first (146.9 mg/PCU (population correction unit) as the highest consumer for six out of the seven classes of antibiotics included in the study, while Norway ranked lowest (10.22 mg/PCU) for the consumption of the antibiotic classes used. A strong correlation was observed between the antibiotics used and the prevalence of resistance for each antibiotics class, i.e., amphenicols, sulphonamides, streptomycin and tetracycline, aminopenicillins, fluoroquinolones, gentamicin, and third-generation cephalosporins (Chantziaras et al., 2014).

Additionally, MDR bacteria have been isolated in food animals and their wastes from developing countries, including South Africa (Adelowo et al., 2014a; Molechan et al., 2019; McIver et al., 2020), where the use of antibiotics for growth promotion remains unregulated (Maron et al., 2013). For example, *E. coli* isolates from poultry and swine wastes in a study conducted in Nigeria were resistant to trimethoprim, tetracycline, ciprofloxacin, sulphamethoxazole, streptomycin, ampicillin, spectinomycin, gentamicin, chloramphenicol, and nalidixic acid (Adelowo et al., 2014). A 'farm-to-fork study carried out in a poultry farm in uMgungundlovu District of South Africa, also reported the prevalence of relatively high levels of non-susceptibility to ampicillin, tetracycline, nalidixic acid, trimethoprim-sulphamethoxazole and chloramphenicol with the lower levels of non-susceptibility to ceftriaxone and azithromycin in *E. coli* isolates (McIver et al., 2020). Similarly, another 'farm-to-fork study conducted in the same district in South Africa isolated a significant number of *Enterococcus* spp. resistant to tetracycline, erythromycin, nitrofurantoin, streptomycin, ampicillin, chloramphenicol, ciprofloxacin, gentamicin, tigecycline, and teicoplanin (Molechan et al., 2019). In the three studies above, the authors also reported that the prevalence of resistance corresponded to the types of antibiotics used in the poultry farms.

All the above studies evaluated the impact of antibiotics on different bacteria species, but more work still needs to be done to examine the impact of antibiotic- and ARB-contaminated manure on soil bacteria and resistome. This is important as literature has shown that the application of manure to the soil can increase the abundance and diversity of ARB.

2.4 Impact of animal manure on soil resistome

2.4.1 Antibiotic residues and antibiotic resistance genes in animal manure

Active veterinary antibiotics are often found in animal manure because most of the antibiotics used in food animal production are poorly absorbed in the animal gut, resulting in the excretion of the metabolites and parent compound through urine or faeces (Lima et al., 2020). A study from China reported that an animal's average rate of antibiotic excretion is 58% (Zhang et al., 2015). Xie et al. (2018), in a review, stated that antibiotic residue concentrations in animal manure range from several nanograms per kilogram (ng/kg) to hundreds mg/kg depending on animal species and different sample locations (country). Hao et al. (2015) reported that the annual antibiotic residues in animal manure from China are between 29,000 and 87,000 tonnes. Different studies have reported different classes of antibiotics in animal manure (Conde-Cid et al., 2018; Rasschaert et al., 2020). Rasschaert et al. (2020) detected 23 different antibiotic residues belonging to 12 classes in 85 of 89 pig manure samples collected from an intensive pig farm in the northern part of Belgium. One of the samples contained 12 different antibiotics residue, while most of the samples (76) had between 1 and 6. The most frequently detected antibiotic residues were doxycycline (82%), sulfadiazine (70.8%), and lincomycin (69.7%). Doxycycline was also found in the highest concentration, with a mean of 1475.8µg/kg manure, while tylosin, which was detected in 11.2% of the samples, was the second-highest concentration (784.3 µg/kg), followed by oxytetracycline with the third-highest mean concentration of 481.9 µg/kg manure. Lincomycin, the third most frequently detected residue, had a mean concentration of 176.7 µg/kg manure, and colistin was detected in only one sample at a concentration of 116 µg/kg manure. The other 18 antibiotic residues were detected with average concentrations of less than 100 µg/kg manure (Rasschaert et al., 2020).

Another study in Spain reported a prevalence and concentrations of tetracycline (tetracycline, oxytetracycline, chlortetracycline) and sulphonamide (sulfadiazine, sulfamethazine, sulfachlorpyridazine, sulfamethoxypyridazine) residues in three different types of animal manure (pig, cattle, poultry) (Conde-Cid et al., 2018). In the study, only 42% of the manure

samples contained antibiotic residues with maximum concentrations of 106.0 mg/kg for the individual antibiotics. Tetracyclines were detected in a higher number of the samples and at higher concentrations compared to sulphonamides and the highest concentration and number of different antibiotic residues was detected in the pig manure, followed by chicken litter and the least in cattle manure (Conde-Cid et al., 2018). The high prevalence of tetracyclines and sulphonamide residues from the above studies are not unexpected, as these two antibiotic classes are commonly used in veterinary medicine due to their broad-spectrum activity, low cost, and relatively low toxicity (Leclercq et al., 2016).

2.4.2 Occurrence and variability of antibiotic resistance genes in animal manure

Antibiotic use in food animal production varies considerably from farm to farm, region to region, and is animal species-based, resulting in substantial differences in the selective pressure for antibiotic resistance in animal manure (Van Boeckel et al., 2015; He et al., 2020). ARGs are frequently detected at a higher concentration in antibiotic-contaminated manure than antibiotic-free manure (Zhu et al., 2013). Zhu et al. (2013), in a study on manure samples obtained from three large-scale commercial swine farms in China quantified ARGs with high-throughput quantitative PCR (HT-qPCR), and reported that the swine manure from animals exposed to antibiotics contained several to tens of thousands-fold more ARGs than those without antibiotic exposure. The diversity of ARGs in animal manure may vary depending on the animal species (Qian et al., 2017; He et al., 2020). A study conducted on fresh manure and compost (chicken, pig, bovine) from 12 large scale farms in China detected 109 ARGs that confer resistance to major classes of antibiotics (aminoglycosides, tetracyclines, and MLS_B) administered to the animals (Qian et al., 2017). The authors showed that chicken and pig manure had a significantly higher ARGs diversity than bovine manure, reflecting a higher use of antibiotics in the two animals due to their higher breeding density and shorter marketing period (Qian et al., 2017).

Studies have detected different ARGs in animal manure, including *tet, sul, erm, bla*, and *aadA*, which correlate with the major classes of antibiotics used in food animal production (Cheng et al., 2013; Marti et al., 2013; Gao et al., 2015)**.** The *tet* gene family involved in ribosomal protection (*tetQ, tetM, tetW, tetO*), active efflux (*tetA, tetB, tetC, tetG, tetL*), and enzymatic modification (*tetX*) of tetracyclines have been reported in different animal manure (chicken, pig, duck, sheep) collected from eight livestock farms in eastern China (Cheng et al., 2013).

Sulphonamides have been widely used for treating bacterial infections in veterinary and clinical medicine, and as such, *sul1* and *sul2* genes, which confer resistance to sulphonamides, were also detected in all the animal manure (Cheng et al., 2013). Additionally, ARGs *blaOXA*, *blaPSE*, *blaVIMgen-2*, *aad(A)*, *str(AB)*, *erm*(*ABCF*), *erm* (*E*), *tet* (*MQST*), *tet* (*ABW*), *sul1* that confer resistance to different classes of antibiotics, have also been reported in dairy and swine manure obtained from two local commercial farms in Canada (Marti et al., 2013). Gao et al. (2015) detected 25 *E. coli* isolates which harboured $bla_{\text{CTX-M +TEM}}$ genes among 32 *E. coli* isolated from pig manure samples from a pig farm in Tai'an, China.

Studies from Africa, including South Africa, have also detected multiple ARGs in poultry manure (Adelowo et al., 2014; Molechan et al., 2019; McIver et al., 2020). ARGs corroborated the the phenotypic resistance in 36 isolates *E. coli* isolates recovered from poultry manure and soil included bla_{TEM} (85%), *sul2* (67%), *sul3* (17%), *aadA* (65%), *strA* (70%), *strB* (61%), *catA1* (25%), *cmlA1* (13%), *tetA* (21%), *tetB* (17%) and *qnrS* (23%) (Adelowo et al., 2014). McIver et al. (2020) reported *blaCTX-M* (100%), *sul1*(80%), *tetA* (77%), and *tetB* (71%) genes as the most prevalent ARGs in *E. coli* isolated from the poultry manure collected in uMgungundlovu poultry farm, South Africa. Molechan et al. (2019), in a study of ARGs profiles in *Enterococcus* spp. along the farm-to-fork production chain of an intensive poultry system in uMgungundlovu Districts, South Africa reported *tetM* (76%) and *ermB* (66%) as the predominant ARGs with smaller percentages of *aph(3')-IIIa* (12%), and *vanC1* (1%). One major gap in these African studies is that the impact of the animal manure on the receiving agricultural soil was not examined. Additionally, the two South African studies did not investigate the the mobilization of the ARGs detected in the poultry manure on MGEs.

Several studies have reported on the potential mobility of the ARGs in manure, and MGEs like plasmids, transposons, and integrons have been shown to co-occur with ARGs in animal manure (Adelowo et al., 2014; Fang et al., 2016; Johnson et al., 2016). Adelowo et al. (2014) observed that all the *sul1* genes detected in the poultry manure were located on the class1 integrons. Notably, some ARGs co-occurred with integrase genes and insertion sequences (IS) as gene cassettes in animal manure, facilitating the transfer of multiple ARGs via HGT in the receiving environments (Xie et al., 2018). Johnson et al. (2016) observed that ARGs like *sul2*, *cmlA1*, *dfrA1*, *aadA*, and *qacEΔ1* clustered with class 1 integron integrase (intl1) and IS6100, in swine manure samples from Chinese farms.

Additionally, IncH12 plasmids have been implicated in co-carriage and co-transmission of several classes of resistance genes, including ESBLs (*blaCTX-M, blaCMY, blaVIM, blaSHV, blaIMP,*), quinolones (*qnrS1, qnrA1, qnrB2, oqxAB*), aminoglycosides (*aac-Ib-cr*/*aac-Ib*), fosfomycin (*fosA3*) and amphenicols (*floR*) genes in animal manure (Yang et al., 2014; Fang et al., 2016). Fang et al. (2016), in a study conducted on 739 *E. coli* isolated from the faeces of diseased pig, duck, chicken on 80 livestock farms in Guangdong Province China, detected 25 IncH12 plasmids carrying *blaCTX-M* and *oqxAB*. *aac (6')-Ib-cr* and *floR* genes, were also detected on the same IncH12 plasmids. The conjugation experiment conducted in the study revealed that *aac (6')-Ib-cr* and *floR* genes were often co-transferred with *blaCTX-M* and *oqxAB* in four transconjugants. Yang et al. (2014) also reported the co-carriage of *fosA3*, *blaCTX-M,* and *floR* genes on diverse plasmids (Incl1, IncH12, and IncN-F33:A-:B) in 58 *E. coli* isolates recovered from chicken faecal samples collected from 57 farms in China. The genetic structure, *blaCTX-M-14-fosA3-IS26*, was frequently detected on IncH12 plasmids (Yang et al., 2014).

Besides the localisation of ESBL genes on plasmids, they are often bracketed by insertion sequences, like ISEcp1 and ISCR1, which facilitate their dissemination (Lee et al., 2020).

Macrolide-resistant enterococci, harbouring *mef* and *erm* genes have been detected in swine manure examined in two studies conducted in Iowa State University, USA (Garder et al., 2014; Luby et al., 2016) and in chicken litter from broilers in Canada (Rehman et al., 2018). Several *erm* genes (*ermA, ermB, ermF*, *ermT*, and *ermC*) have been detected in animal manure, with the *erm*(*B*) gene being the most prevalent (Garder et al., 2014; Luby et al., 2016; Rehman et al., 2018).

Qian et al. (2017) reported the co-occurrence of *dfrA1, catB3, blaOXA-1,* and *TetT* genes with transposons such as *TnpA* in the three types of animal manure, i.e., chicken, cattle and, pigs. A major concern about ARGs in animal manure is the possibility of transfer to resident soil bacteria through HGT mechanisms, which can encourage the dissemination of ARGs among different microbial communities (Heuer et al., 2011a). Hence, it is important to understand the changes in the soil's ARB and ARGs content following animal manure application.

2.4.3 Effect of manure application on soil bacteria and resistance genes

Soil bacteria are important sources of antibiotics and a reservoir of resistance genes. Antibiotic resistance is a primaeval phenomenon that precedes the modern use of antibiotics in medicine, evidenced by the discovery of ARBs and ARGs in pristine environments (D'Costa et al., 2011;

Chen et al., 2016a). This finding suggests that there is intrinsic resistance in the indigenous bacterial population of soil that is not impacted by human activities (Chen et al., 2016a).

The growing intensive and large-scale livestock industry generates enormous amounts of animal manure, which are directly applied to agricultural soil as organic fertiliser (Zhao et al., 2010). Animal manure as an alternative to chemical fertilisers for soil enrichment and fertility has become common practice in many countries globally, including South Africa, due to its nutrient and cost-effectiveness (Materechera, 2010; Zhao et al., 2010). China, the largest producer and consumer of antibiotics produces over 3 billion metric tonnes of animal manure which is applied to farms with little pretreatment every year (You and Silbergeld, 2014). Land application of untreated animal manure can introduce new ARB, ARGs, MGEs, and antibiotic residues of animal manure origin to the soil. It can also increase the diversity of already existing soil ARB and ARGs, which may subsequently enter other compartments of the soil environment, the food chain, and the human population (Su et al., 2014; Udikovic-Kolic et al., 2014; Chen et al., 2016b; Cheng et al., 2019). This means that animal manure application can enhance the abundance and diversity of antimicrobial resistance determinants in soil (Zhu et al., 2013; Su et al., 2014).

Literature has shown that animal manure could increase the abundance of ARGs in the soil through various means, including enriching ARGs in soil resident bacteria by the organic nutrients in manure (Udikovic-Kolic et al., 2014). Udikovic-Kolic et al. (2014), in field experiments conducted at the Yale Farm, USA, showed that cow manure amendment increased certain ARB in soil, with a higher prevalence of β-lactam-resistant bacteria in soil amended with manure compared to soil amended with inorganic fertiliser. The authors attributed the higher frequency of ARB that harbour β-lactamases in the manure-amended soil to the enrichment of indigenous soil bacteria because the functional metagenomics detected β-lactam-resistance genes in the soil before and after the manure treatment. Similarly, a study conducted in China on swine manure samples from three large-scale farms from different Provinces and manure-amended soil samples from an agronomic field reported the enrichment of 63 unique ARGs in the manure-amended soil compared to controls, at an overall median enrichment of 192-fold for all the samples (Zhu et al., 2013). A total of 149 unique ARGs were detected in all the samples, and the ARG content of the manure-amended soil was significantly higher (three times more) than those found in the control samples. The ARGs detected in the study were those that potentially confer resistance to major classes of clinically important antibiotics

such as cephalosporins (*blaTEM*, *blaCTX-M*), macrolides (*mphA*, and *erm* genes), aminoglycosides (*aph* and *aad* genes), and tetracycline (*tet* genes) (Zhu et al., 2013).

Some studies have shown that manure amendment could also increase ARB and ARGs' diversity by importing ARB, and HGT transfer of ARGs of manure origin to indigenous soil bacteria. Chen et al. (2019) conducted a 120-day soil microcosm study in Virginia, United States, on three types of soils (sand, silt, clay) amended with raw or composted dairy manure generated from cows treated with antibiotics (pirlimycin and cephapirin) and cows with no history of antibiotics treatment (control). The study showed that the day 1 manure amendment significantly increased the ARG relative abundance and diversity in the soil (2.21 x controls), resulting in the abundance of individual ARG types compared to the unamended soils (controls). Additionally, the ARG profiles of the samples on day 1 indicated that the soils amended with manure collected during antibiotic administration to the cows and the soils amended with composted manure contained higher diversity of ARGs that conferring resistance to β-lactams, aminoglycosides, tetracyclines, MLS, and chloramphenicol compared to the unamended soils. The ARG content of the soils amended with composted manure was not statistically different from the control. Notably, the authors observed a decrease in the relative abundance of ARGs in the manure-amended soils to a level equivalents to the unamended soils after 120 days of incubation, but the diversity of ARGs was still significantly higher compared to the unamended soil (Chen et al., 2019).

Gao et al. (2015) tracked *E. coli* harbouring ESBL genes from pig manure to agricultural soil fertilised with pig manure for three years in Tai'an, China. The study indicated the possible transfer of ARB and ARGs of animal origins to the soil using enterobacterial repetitive intergenic consensus (ERIC)- polymerase chain reaction (PCR). The ESBL-producing *E. coli* isolates from the manure-amended soil and the pig manure were MDR with overlapping phenotypes, and three isolates from the soil had over 90% genetic similarity with strains from pig manure samples. Detected in the study were bla_{TEM}, and $bla_{\text{CTX-M}}$ ($bla_{\text{CTX-M-13}}$, $bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-27}}$, $bla_{\text{CTX-M-65}}$) genes with $bla_{\text{CTX-M}}$ being the most prevalent ESBL gene in the isolates from both the manured soil (7/10, 70%) and pig manure (30/32; 93.8%) samples. ESBL-producing *E. coli* was not detected in the control (soil amended with chemical fertiliser). It was further reported that eighteen isolates from the pig farm (18/32, 56.3%) and seven from

soil (7/10, 70.0%) samples harboured the IncF-type replicon plasmids, which suggest possible HGT in soil (Gao et al., 2015).

Zhu et al. (2013) detected a high level of transposase enrichment and good correlation (0.970) with ARG abundance in all the swine manure amended soil samples (1000-fold), suggesting that HGT could have facilitated the enrichment of ARGs in the soil. This is because ARGs can be easily be transmitted within and between speciesvia HGT. The most frequently detected transposable element flanking an array of ARGs in the study was the IS26 of IS6 family of insertion sequences, isolated with integrons in MDR plasmids in enterobacteria (Zhu et al., 2013).

Additionally, a field experiment set up in a long-term experiment station of the Chinese Academy in Shangdong Province, that used a high-throughput sequencing and high-throughput quantitative PCR, showed that long-term application of chicken manure substantially increased the abundance and diversity of ARGs and MGEs in soil. The enrichment of MGEs suggested that chicken manure application could accelerate ARG dissemination in soil environments since ARGs can be easily transmitted between members of the same species and the distantly related bacteria via HGT (Chen et al., 2016b). Furthermore, Chen and colleagues (2016b) observed a significant increase in the bacterial diversity in manure-amended soil following the amendment. The manure amendment increased the abundance of tetracyclines genes and caused over 100-fold enrichment of *aadE, ermB, ermD,* and *floR* genes in the soil. A total of 130 unique ARGs and five MGEs (including class 1 integrase gene) were detected, and genes conferring resistance to tetracyclines, β-lactams, $MLS_{B,}$ aminoglycosides, and multiple drugs were dominant in the samples compared to other ARGs (chloramphenicol, sulfonamide, vancomycin). The frequency of ARG detection in soil amended with chemical fertiliser was significantly lower than in soils amended with chicken manure, ranging from 42 to 100, but was similar to ARG detection in the control plot (Chen et al., 2016b).

Although the above-stated studies have indicated an increased diversity of ARGs in the soil following manure application, different studies have also reported that this increase is only for a short while because ARB of manure origin hardly survive for a long time (Marti et al., 2014). The decrease may also be due to soil matrix dilution, dissemination to other compartments of soil environment, breakdown of resistant DNA, and death of some bacteria harbouring the ARGs in the soil (Marti et al., 2014; Muurinen et al., 2017).

A field study in Southern Finland showed decreased abundance and number of manure-associated ARGs and MGEs detected in soil fertilised with manure (dairy and swine) at two and six weeks after manure application. Interestingly, six manure-associated ARGs and MGEs not found in the ditch water samples before the manure application were detected after the amendment, indicating possible dissemination to leachate water (Muurinen et al., 2017). The authors suggested that increases in ARG abundance in the soil after fertilisation are temporary and only occur annually during manure application (Muurinen et al., 2017).

A better insight was provided by Marti et al. (2014b) in a study that evaluated the fate of ARGs in manured soil over two growing seasons in Canada. Two distinct patterns of gene dynamics following manure application were observed and it was inferred that warmer and drier conditions shorten the persistence of ARB carrying ARGs in soil, whereas cooler and moist conditions promote their increase. The study showed that eventhough swine and dairy manure increased the relative abundance of *sul1, ermB, strB, intl* and *IncWrepA* genes in manure-amended farm plots, there was an exponential decomposition of the ARGs such that the ARGs content of the manured soil returned to baseline level in two months (8 weeks). The decrease was related to the warm and dry weather conditions that occurred after the amendment in 2012. However, the abundance of all the targeted ARGs increased significantly in the weeks after manure application in the spring of 2013, albeit it later declined. Overall, the authors suggested short-term period of the increase could be a crucial window when exogenous ARGs from manure are transferred to nearby microbial niches or environments (Marti et al., 2014)

A major concern with this agricultural practice is the prolonged exposure of soil to unamended animal manure, which maintains high levels of ARGs and antibiotic resistance in soil (Xie et al., 2018). Accumulation of antibiotic residues in soil due to long-term manure applications has been shown to create a selective advantage for the emergence and proliferation pre-existing ARGs in soil (Heuer et al., 2011b; Tang et al., 2015). Tang et al. (2015) demonstrated that long-term (≥ 9 years) application of pig manure significantly increased the concentration of tetracyclines residues and the abundance of several *tet* genes in paddy soils at three (Nanchang, Yingtan, and Jiaxing) experimental sites for decades in Southern China. However, only one *tet* gene increased in the fourth (Changsha) site, which suggests that significant ARGs increase may not be observed in all sites. It was further reported that the abundances of *tetA*, *tetM*, *tetQ*, and *tetW* genes statistically correlated with the concentrations of tetracycline residues and soil properties like pH and soil organic matter, indicating their importance in the resistance selection detected in the soil (Tang et al., 2015).

A study in Germany reported a significant increase in the abundance of *sul1* and *sul2* genes in soil amended with sulfadiazine (SDZ)-contaminated pig manure after two months of manure application compared to treatments with antibiotic-free manure (Heuer et al. 2011b). The authors indicated that the increase was not much of the addition of *sul* genes originating from manure but the selective pressure exerted by bioavailable SDZ in the manured soil (Heuer et al., 2011b).

Similarly, another study in Germany on soil fertilised with manure either from SDZ-treated pigs or from untreated pigs (control) reported a relative increase in the abundance of *sul1* and *sul2* genes with three orders of magnitude on day 14 after manure application than before (Kopmann et al., 2013).and was higher than the control, as revealed by quantitative PCR on days 14 and 63. The accumulated exposure of the soil bacteria to SDZ up to 63 days significantly correlated with the relative abundance of *sul1* and *sul2* in the bulk soil (Kopmann et al., 2013). The increase of *sul1* and *sul2* after applying manure from SDZ-treated pigs might be due to the increase in nutrients and selective pressure created by the SDZ in the manure. Hence fertilisation of soil with manure contaminated with antibiotics may pose a serious threat to public health because the *sul1* gene, which is usually connected with class 1-integrons, can be picked up, interchanged and lodged within gene cassettes, which may eventually spread out among Gram-negative bacteria of clinical importance (Stalder et al., 2012).

2.5 Antibiotics and the soil environment

2.5.1 Entry of antibiotics into the soil

Despite the benefits of antibiotics in treating infectious diseases in humans and animals worldwide, their continuous release into the environment and the potential adverse effects on soil microorganisms are of great concern (Larsson, 2014; Cycoń et al., 2019). A large proportion of the administered antibiotics ends up in the environment (soil and water) because they are not well metabolised. The release of antibiotics via anthropogenic activities such as land application of animal manure, sewage sludge, biosolids, aquaculture wastewater**,** disposal of unused therapeutic drugs, discharge of pharmaceutical effluents, and municipal wastewater containing disinfectants and antibiotics also contribute to the AMR burden in the environment (Kraemer et al., 2019; Manyi-Loh et al., 2018). Antibiotics released into the environment can bio-accumulate to concentrations that can exert selective pressure for ARGs in various ecosystems and alter the microbiota of plants, wild animals, and their environment (Kraemer

et al., 2019; Manyi-Loh et al., 2018). Thus, understanding the occurrence, impact and persistence of antibiotics in the environment is important in mitigating the potential risk these emerging pollutants may pose on public health.

2.5.2 Fate of antibiotics in the soil environment

Once antibiotic residues enter the soil environment, they are subject to several biotic and abiotic processes like sorption (Martínez-Hernández et al., 2016), degradation/transformation (Pan and Chu, 2016). These residues can also be absorbed by plants (Bassil et al., 2013; Carter et al., 2014) and washed off to surface and groundwater (Pan and Chu, 2017a). These processes are interrelated and categorised into transport/transfer and degradation (transformation) processes (Conde-Cid et al., 2020). The retention/transport processes such as leaching, sorption (adsorption/desorption), run-off, uptake by plant, volatilisation, and diffusion involve the movement of antibiotics from one phase to another in the soil, or within the same phase (Conde-Cid et al., 2020). Degradation processes, including biodegradation, hydrolysis, photodegradation, oxidation, and reduction, involve a structural change of antibiotics. Sorption (adsorption/desorption) and degradation are the most relevant processes among the above-stated processes with regards to determining the persistence of antibiotics in soils and their transfer from the soil to other compartments of the environment such as plants or surface water (Pikkemaat et al., 2016; Conde-Cid et al., 2020). The two processes are affected by different factors, including physical-chemical properties of the antibiotic residue, soil properties (organic carbon content, pH, ionic strength, texture), and climatic factors (rainfall, humidity, temperature) (Wegst-Uhrich et al., 2014a)

2.5.2.1 Sorption (Adsorption/desorption)

Adsorption is defined as the adherence of molecules, atoms, or ions from a liquid, dissolved solid, or gas to the surface of a solid phase without any changes in the composition of the latter, while desorption is the reverse process, i.e., release of an adsorbed substance from a surface (Conde-Cid et al., 2020). This process produces a film of the adsorbate on the surface of the adsorbent. Sorption (adsorption/desorption) determines the mobility/retention of antibiotics in soil and their potential transport to surface water (run-off), groundwater (leaching), and crops (plant uptake) (Pikkemaat et al., 2016). The persistence and transport of antibiotics in the environment depend on the sorption properties of the antibiotics. Additionally, adsorption can also affect the bioavailability of antibiotics towards soil microorganisms, thereby constraining microbial degradation (Wegst-Uhrich et al., 2014; Pikkemaat et al., 2016). However, an

increase in adsorption does not directly mean a proportional reduction in degradation. Adsorption reduces the antimicrobial potency of antibiotics, while desorption produces a reactivation of the antimicrobial potency (Conde-Cid et al., 2020). The most significant factors that affect the sorption of antibiotics in the soil are soil pH, organic matter content, soil texture, clay content, and cationic exchange capacity (Wegst-Uhrich et al., 2014; Ahmed, 2017). The potential variations in the above-stated factors suggest that the degree to which antibiotics adsorb to soil may vary widely. Generally, decreases in pH have been reported to result in increased sorption of cationic forms of antibiotics (Wegst-Uhrich et al., 2014; Park and Huwe, 2016). A sorption test and column experiment in Germany on agricultural soil showed that sulfadimethoxine, sulfamethoxazole, and sulfamethazine showed increased sorption on soil material with decreasing pH values (Park and Huwe, 2016).

The important parameter for evaluating the level to which an environment is exposed to antibiotics is the sorption coefficient (Koc) (Cycoń et al., 2019). Sorption coefficients of antibiotics have been reported to range from 0.6 l/kg to 6000 l/kg (Pikkemaat et al., 2016). Antibiotics with Koc < 15 l/kg are highly mobile and easily degraded in soil (with low persistence and half-life < 5days) (Cycoń et al., 2019). On the other hand, antibiotics characterised by values of Koc > 4000 l/kg are non-mobile and very persistent in soils because they degrade to a very low degree, and it takes more than 60 days for half (50%) of an initial dose of such antibiotics to be degraded (Cycoń et al., 2019). It is generally believed that tetracyclines have a high affinity for soil components, exhibiting higher adsorption percentages close to 100% and desorption percentages less than 10% in most cases, while fluoroquinolones and macrolides exhibit significant sorption potential (Figueroa-Diva et al., 2010; Pan and Chu, 2016; Conde-Cid et al., 2020). On the contrary, sulphonamides express weak adsorption and high desorption and are, regarded as the most mobile antibiotics in the soil (Figueroa-Diva et al., 2010; Pan and Chu, 2016). The low affinity of some antibiotics to various organic and non-organic soil components may be associated with their relatively low persistence in soils (Cycoń et al., 2019). Pan and Chu (2016), in a study on adsorption of five different antibiotics in sterilised and non-sterilised agricultural soil in Hong Kong, showed that their adsorption affinities on soil assumed a descending order of tetracycline > norfloxacin > erythromycin > chloramphenicol > sulfamethazine. In the study, tetracycline had the highest adsorption coefficient (1093 l/kg) while sulfamethazine had the lowest (1.365 l/kg). The adsorption coefficients of the five antibiotics suggest that sulfamethazine was the most mobile antibiotic in the soil, while tetracycline was the least mobile among the five (Pan and Chu, 2016). The

sulfamethazine converts from its cationic to a neutral form in the natural soil environment, resulting in lower adsorption than other classes of antibiotics (Figueroa-Diva et al., 2010). The low affinity or interaction of sulphonamides with soils is mainly because its molecules only possess aniline and amide groups (Conde-Cid et al., 2020). In contrast to the sorption behaviour of sulphonamides, tetracyclines and norfloxacin (fluoroquinolones) molecules have multiple functional groups, whose combined actions result in a high affinity for various organic and non-organic components of the soil. This is facilitated through multiple adsorption mechanisms, such as cation exchange, surface complexation, cationic bridging and hydrogen bond, which are not important in sulphonamides (Pan and Chu, 2016; Conde-Cid et al., 2020).

2.5.2.1 Degradation (transformation)

The degradation of antibiotics in the soil can occur via biotic (biodegradation) and abiotic processes the most important of which are hydrolysis, biodegradation, and photodegradation (Conde-Cid et al., 2020). The extent to which each of these processes degrades antibiotics depends largely on the antibiotics' molecular structure and physicochemical properties. These include the stability, solubility and hydrophobicity, environmental factors (temperature, intensity of solar radiation, rainfall), and physical, chemical, and microbiological properties of the soil (soil type, organic carbon content, pH, presence of specific degrading bacteria) (Pan and Chu, 2016; Pikkemaat et al., 2016; Conde-Cid et al., 2020). Since the degradation of antibiotics depends on numerous biotic and abiotic factors, the degradation rates indicated in literature for different antibiotics vary widely, with half-lives (DT50) ranging between <1 and 3, 466 days (Cycoń et al., 2019). A study conducted in Italy found that monocyclic β-lactams antibiotics were quickly degradable in soils at field capacity, with a half-life of 0.03 to 2.5 days (Braschi et al., 2013). On the contrary, Walters et al. (2010), in an outdoor mesocosm study, observed long-term persistence of tetracycline, azithromycin, and ofloxacin in soils, with half-lives of 578, 408-3466, and 866-1733 days, respectively. It is worth noting that the half-life values of antibiotics in the same class or group may vary significantly. However, the differences observed in the persistence of similar antibiotic compounds may be due to variations in soil properties and compositions, the concentrations of antibiotics, and the conditions used in these studies. Moreover, literature has shown that tetracyclines,

fluoroquinolones, and macrolides are distinguished by high half-live values (Cycoń et al., 2019).

2.4.3.1.1 Hydrolysis

Hydrolysis is a chemical reaction in which a compound reacts with water to produce other compounds. It involves splitting a bond and the addition of the hydroxide anion and the hydrogen cation from the water. Hydrolysis is generally considered one of the most important abiotic degradation pathways in the environment for some compounds like esters and amides (Mitchell et al., 2015; Conde-Cid et al., 2020). The common reaction sites for hydrolysis in antibiotics include labile carbonyl moieties like lactams, lactones, and esters (Waterman et al., 2002; Mitchell et al., 2014). Hydrolysis products of amides and esters may bioaccumulate at a lesser degree compared to the parent compound because of polarity and solubility (Mitchell et al., 2014). *B*-lactams are highly susceptible to hydrolysis, while sulphonamides and macrolides are less susceptible (Braschi et al., 2013). Tetracyclines are also susceptible to hydrolysis but to a lesser degree than β-lactams, because tetracycline compounds are stable at acidic pH values but unstable under alkaline conditions and form salts in both conditions (Pikkemaat et al., 2016).

The most important environmental factors that affect hydrolysis rates are temperature and pH (Braschi et al., 2013; Mitchell et al., 2014). High temperatures and alkaline conditions have been reported to accelerate the hydrolysis of antibiotics. Mitchell et al. (2014) found that hydrolysis rates under ambient alkaline conditions (pH 9 and 25^{o}C) were significantly greater than acid and neutral pH hydrolysis rates for cefalotin, cefoxitin and, ampicillin with half-lives 1.4, 6.6, and 6.7 days, respectively. It was also reported that hydrolysis rates increased from 2.5- to 3.9-fold for a 10^{o}C increase in temperature, suggesting that β-lactam antibiotic hydrolysis is relatively sensitive to temperature changes. Mitchell and colleagues (2014) indicated that the generated hydrolysed lactam and amide moieties might pose a minimal threat to the environment because hydrolysis of the functional groups reduces the antimicrobial activity.

2.4.3.1.2 Photodegradation

Photodegradation is another important abiotic degradation process that breaks down antibiotics in the environment due to solar radiation (Du and Liu, 2012). It occurs mainly on the soil surface and is mediated by humic substances (Thiele-Bruhn and Peters, 2007; Du and Liu,

2012). The majority of the widely used antibiotics in veterinary medicine, such as macrolides, tetracyclines, sulphonamides, *β*-lactams, and fluoroquinolones, are susceptible to photodegradation (Batchu et al., 2014; Timm et al., 2019), albeit tetracyclines are more susceptible to photodegradation than sulphonamides. The photodegradation rate of each class of antibiotics is highly dependent on pH, with the process being favoured in alkaline conditions (Conde-Cid et al., 2020). Even though photodegradation plays a major role in reducing antibiotics on soil and water surfaces, the persistence of photodegradable antibiotics in soils is affected or depends on agricultural practices, such as application depth and time of manure application and ploughing (Conde-Cid et al., 2020). Antibiotics are also protected from photodegradation when adsorbed to soil particles or penetrate soil pores or certain depths. Timm and colleagues (2019), in a photodegradation study conducted in Germany, observed that all the investigated *β*-lactam antibiotics (amoxicillin, ampicillin, penicillin V, and piperacillin) were photodegraded by simulated sunlight ($1kW/m^2$) with half-lives between 3.2 and 7.0 hours. Structure elucidation of the transformed antibiotics products revealed that the primary transformation of the antibiotics was due to the hydrolysis of the *β*-lactam ring, followed by the elimination of carboxylic and dimethyl thiazolidine carboxylic acid (Timm et al., 2019). A study from Miami, Florida, on photodegradation in both pure and natural waters (Fresh and saltwater) under irradiation of different light sources, showed that the tested antibiotics degraded fastest under ultraviolet light of 254 nm, followed by 350 nm and simulated solar radiation (Batchu et al., 2014). Under simulated solar radiation, the authors reported that ciprofloxacin and sulfamethoxazole degraded relatively quickly with half-lives of 0.5 and 1.5 hours, respectively, while roxithromycin and erythromycin were persistent with a half-life of 2 to 10 days (Batchu et al., 2014). Study on photodegradation of nine antibiotics, spread on a sterilised layer of unfertilised agricultural soils and incubated for 28 days in Germany, showed a reduced concentration of the antibiotics recovered from the samples exposed to natural sunlight compared to the light exclusion experiments in which degradation was not observed (Thiele-Bruhn and Peters, 2007). The authors reported that photodegradation was strongest for the tetracyclines, while degradation of the sulphonamides, *p*-aminobenzoic acid, and benzimidazole fenbendazole was much lesser (Thiele-Bruhn and Peters, 2007)

2.4.3.1.3 Biodegradation

Soil microorganisms play vital roles in the maintenance of soil health and quality. They play a major role in organic matter turnover, nutrient release, and stabilisation of soil structure and fertility (Cycoń et al., 2019). In addition to abiotic processes, biodegradation contributes to

removing antibiotics from the soil environment (Pan and Chu, 2016). Soil biodegradation is mainly driven by the activities of indigenous soil microorganisms, including bacteria, fungi, or algae (Pikkemaat et al., 2016). Biodegradation of antibiotics in soils depends on many factors such as oxygen, temperature, soil properties, microbial population, accessibility of nutrients, degree of adaptation, and physicochemical properties of the antibiotics, such as solubility, chemical structure, adsorption capacity, and capacity of fixation to soil pores (Selvam and Wong, 2017). Studies have shown that antibiotics can be susceptible to enzymatic transformation and have reported the isolation of bacteria capable of degrading antibiotics from antibiotics-contaminated soils (Leng et al., 2016; Zhang et al., 2017; Wen et al., 2018). Erythromycin-degrading *Ochrobactrum* spp. isolated from antibiotic-contaminated soil from a pharmaceutical factory in China was reported to grow in a medium containing erythromycin A as the only carbon source at an optimal growth temperature and pH of 32^{o}C and 6.5 respectively (Zhang et al., 2017). Similarly, bacteria belonging to the genera *Stenotrophomonas* (Leng et al., 2016), *Burkholderia* (Zhang and Dick, 2014), *Microbacterium* (Topp et al., 2013), *Labrys* (Mulla et al., 2018), and *Escherichia* (Wen et al., 2018), were able to degrade tetracycline, penicillin G, sulfamethazine, sulfamethoxazole and doxycycline in liquid cultures respectively.

Studies conducted on sterile and non-sterile soils confirmed the key role of microorganisms in antibiotic degradation in soil, in that the half-life values of antibiotics in sterilised soils were higher than the values obtained in soils with autochthonous microorganisms (Accinelli et al., 2007; Pan and Chu, 2016). A study conducted on agricultural soil obtained from an organic farm in Hong Kong, showed that all the tested antibiotics (tetracycline, erythromycin, chloramphenicol, norfloxacin, sulfamethazine) were susceptible to microbial degradation under aerobic conditions, with half-lives values in non-sterilised soil (2.9 to 43.3 days) lower compared to the sterilised soil (40.8 to 86.6 days). In the study, a higher degradation rate was observed under aerobic conditions, but the antibiotics were relatively persistent in anaerobic conditions due to a lower degradation rate. It was also reported that the antibiotics exhibited different biodegradation behaviour under the same experimental conditions and soil incubation due to their physicochemical properties (Pan and Chu, 2016). Similarly, Accinelli et al. (2007) stated that the addition of liquid swine slurry (LSS) to soil led to a significant increase in the degradation rates of sulfamethazine compared to the unamended soil. The authors attributed the increase in the rate of sulphonamides degradation and its lower persistence in the LSS-amended soil to the addition of a large microbial population in the LSS and the stimulation of microbial activity due to the availability of LSS-derived nutrients (Accinelli et al., 2007). On

the contrary, the microbial degradation assessment of 18 antibiotics (including tetracyclines, sulphonamides, macrolides, aminoglycosides, lincosamides, quinolones, and β-lactams) conducted in a closed bottle test in Germany showed that none of the antibiotics were readily biodegradable within the 28 days test period (Alexy et al., 2004). This result suggests that anaerobic conditions may not support microbial degradation. The differences in the results on the biodegradability of the antibiotics may be due to the differences in the methods used to assess the degradation, experimental conditions, and the inoculum used in the case of laboratory experiments.

The increasing number of ARB and ARGs in the environment is becoming a global threat to public health because of its potential to spread to other environmental compartments and/or subsequently reach the human and animal population via the food chain. The excessive use and misuse of antibiotics and subsequent antibiotic resistance in food animals are recognized as a major factor contributing to the overall burden of AMR in agricultural soil fertilized with animal manure. Contamination of the environment with antibiotics, ARB and ARGs, can accelerate the development and spread of antibiotic resistance in the environment (Wellcome Trust, 2018). A continuous release of antibiotics into the environment can lead to a high concentration of residual antibiotics that can exert selective pressure for ARB and ARGs in diverse ecosystems and alter the microbiota of plants, animals, and the environment they live in. Besides, ARGs are recognised as environmental pollutants whose presence in different ecosystems can aid their entrance into the food chain (Iwu et al., 2020).

Even though AMR is considered a "One Health" issue that recognizes that humans, animals, and the environment are interconnected, there is a scarcity of information on the resistance burden posed by the release of antibiotics, ARB, and ARGs in the environments. Even though AMR exists in the three-domain of the One Health and the WHO has recommended a One Health approach as the Global Action Plan (GAP) for handling AMR, most of the surveillance and research on AMR focuses on the human (Mbelle et al., 2019) and animal ("farm to fork") (Molechan et al., 2019; Theobald et al., 2019; McIver et al., 2020) components of One Health, with fewer studies on the environment (Heuer et al., 2011b; Tang et al., 2015). This study addresses the environmental dimensions of ABR by investigating the impact of manure application on the soil resistome, mobilome and virulome in *E. coli* and *Enterococcus* spp. as indicator bacteria to inform interventions to mitigate ABR in the environment.

2.6 Manure management

The presence of antibiotic residues, ARB, and ARGs in animal manure poses a significant challenge to the application of animal manure to agricultural soil; thus, there is an urgent need for the control and elimination of antimicrobial resistance in animal manure (Li et al., 2020; Liu et al., 2021). Composting (aerobic) and anaerobic digestion are eco-friendly techniques suitable for reducing ARB, ARGs, MGEs, and concentrations of antibiotic residues in manure which could be transferred to soil (Collignon and McEwen, 2019). Composting is a biological aerobic digestion process that involves the decomposition and mineralization of degradable organic substances by environmental microorganisms (Fan et al., 2020; Liu et al., 2021). This process is an effective method of disposing animal manure because it creates a high-temperature environment that reduces or eliminates pathogenic bacteria, ARGs, and antibiotic residues with environmental, public health, and economic advantages (Lima et al., 2020; Xie et al., 2019). Composting also reduces the volume of animal waste and results in the formation of stable organic products (compost), rich in nutrients and beneficial organisms like fungal mycelium and worms (Lima et al., 2020; Xie et al., 2019).

Several studies have effectively reduced some ARB, ARGs, MGEs, and antibiotic residues content during composting, albeit the conclusions are not consistent. In ten weeks, composting of poultry manure resulted in a significant reduction of *tetA, tetB tetK, tetM, tetQ, tetS, tetW, ermB qnrS, and bla*$_{\text{TEM}}$ genes, but an increased abundance of *aadA, sul1, sul2* and *tetY* genes was observed in the study (Esperon et al. 2020). Similarly, a twenty-six days study on chicken manure composting with bamboo charcoal showed a significant decrease in the abundance of *tetW, tetX, tetG, drfA1, drfA7, ermB, ermF, ermQ, ermX sul2*, and *intI1* genes while *sul1* increased (Li et al., 2020). Furthermore, a 45% reduction in the absolute abundance of *intl1* and *intl2* MGEs and different *tet, erm, sul* genes were observed in 40 days of pig manure composting with cotton stalks (Duan et al., 2019). Composting manure under thermophilic conditions (44 ^{o}C to 65 ^{o}C) has been shown to completely remove *mcr-1* gene in livestock manure after twenty-two days (Gao et al., 2019). Zheng et al. 2016 also showed that high temperature effectively reduced the abundance of *bla*$_{\text{TEM}}$, *blaSHV*, and *qnrS* genes with a removal rate of 98% during thermophilic composting of pig manure. Additionally, the abundance of *sul3, ermB*, and *bla*$_{\text{TEM}}$ genes during chicken manure composting significantly decreased compared to simple storage for six weeks (Le Devendec et al., 2016).

Studies have also reported efficient removal of antibiotic residues during composting. Liu et al. 2021, in a 40 day aerobic composting of pig manure, showed an efficient reduction of

tetracycline (89.2%), oxytetracycline (87.8%), doxycycline (98.6%), and enrofloxacin (89%) at high temperature (50 – 70 ^{o}C). Similarly, Esperon et al. 2020 observed a 90% decrease in the concentration of ciprofloxacin and doxycycline in antibiotic-spiked poultry manure after composting for three weeks.

Anaerobic digestion is also widely used to manage animal manure, and it involves the decomposition of organic matter in the absence of oxygen by anaerobic microorganisms to produce methane-rich biogas, a renewable energy source (Xie et al., 2019). Anaerobic digestion can reduce organic matter pollution, antibiotic residues, ARGs, and pathogenic bacteria. Studies have shown that temperature is a significant factor in anaerobic digestion, as changes in temperature can cause variations in microbial community succession and ARGs content (Xie et al., 2019). Zou et al. 2020, in 60 days anaerobic digestion of swine manure at thermophilic and mesophilic conditions, showed the abundance of all the five evaluated ARGs (*sul1, sul2, tetA) tetO, and tetX*) and *intI1* decreased at the thermophilic temperature. However, only half of the ARGs (*intI1, sul1,* and *tetO*) reduced at the mesophilic temperature while *sul2, tetA,* and *tetX* increased. The authors also observed that ARB numbers were decreased by 4-log CFUs per gram of dry manure during the thermophilic temperature (55°C) but approximately 1-log CFU at the mesophilic (35°C) temperature (Zou et al., 2020). Thermophilic (55 °C) anaerobic digestion of dairy manure efficiently reduced the quantity of *tetM, tetQ, gyrA,* and *sul1 genes while tetC, tetM, tetQ, tetX,* and *sul1 genes* increased under moderate (20 °C), mesophilic (35 °C) temperatures (Sun et al., 2016). The thermophilic digestion also reduced the number of potential pathogens and mesophilic bacteria (Bacteroidetes and Proteobacteria) but not the moderate and mesophilic digestion (Sun et al., 2016).

In general, composting and anaerobic digestion plays a vital role in the reduction and elimination of ARB, ARGs, and antibiotic residues in animal manure and reduces the risk of antimicrobial resistance dissemination to the environment. These provide a feasible way to efficiently reduce antimicrobial resistance in animal manure and its impact on environmental and public health.

3.0 Aim and objectives

3.1 Aim

This study aimed to ascertain the impact of chicken litter application on the resistome, virulome, mobilome, clonality, and phylogenies of *Escherichia coli* and *Enterococcus* spp. in agricultural soil in order to inform public health interventions.

3.2 Specific objectives

1) To enumerate the *E. coli* and Enterococci in the soil before and after chicken litter amendment and in the heap of chicken litter using the Colilert® -18 / Quantiti-Tray® 2000 system and the Enterolert® -18® Quantiti-Tray®/2000 systems, respectively.
2) To isolate and confirm *E. coli* and *Enterococcus* spp. using selective media and real-time polymerase chain reaction (RT-PCR)
3) To determine the antibiotic susceptibility profile of the confirmed *E. coli* and *Enterococci* spp. isolates using the Kirby-Bauer disk diffusion method according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI) and/or the European Committee on Antimicrobial Susceptibility Testing (EUCAST).
4) To identify the antibiotic resistance genes and the virulence genes harboured by the isolates using whole-genome sequencing (WGS) and bioinformatics tools such as ResFinder, Comprehensive Antibiotic Resistance Database (CARD), and VirulenceFinder.
5) To determine the genetic environment and the MGEs associated with the ARGs and virulence genes using MobileElementFinder, Rapid Annotation using subsystem Technology (RAST), and NCBI Prokaryotic Genome Annotation Pipeline (PGAP).
6) To ascertain the clonal relatedness and phylogeny of the studied isolates using MLST and bioinformatics tools such as CSI Phylogeny pipeline, Phandango and Figtree and to compare the isolates from this study with other isolates deposited in a public repository.

4.0 Synopsis of methodology

4.1 Ethical approval

This study was part of a larger project for which ethical approval had been received from the Biomedical Research Ethics Committee (Reference: BCA444/16) of the University of KwaZulu-Natal. The study is also on record at the South African National Department of Agriculture, Forestry, and Fisheries (Reference: 12/11/1/5 (879)).

4.2 General methodology

Soil samples were collected at five sequential visits (D1, D2, D3, D5, D9) before chicken litter application and on days 0, 1, 3, 7, 14, 21, and bi-monthly thereafter for three months after the chicken litter application between October 2018 and February 2019 from a sugarcane field located in uMshwathi Local Municipality under uMgungundlovu District of KwaZulu-Natal, South Africa. The chicken litter applied on the field originated from an intensive poultry production system in uMgungudlovu District, South Africa, and it was a mixture of raw chicken faeces and wood shavings. Isolation and enumeration of *E. coli* and E*nterococci* were carried out using the Colilert® -18 / Quantiti-Tray® 2000 system and the Enterolert® -18® Quantiti-Tray®/2000 system, respectively. Ten randomly picked positive wells from the the Colilert® -18 / Quantiti-Tray® 2000 system and the Enterolert® -18® Quantiti-Tray®/2000 system were streaked on Eosin Methylene Blue (Oxiod, Hampshire, England) and Bile Aesculin agar plates (Lab M, Lancashire, UK) respectively. The presumptive *E. coli* and E*nterococci* isolates were confirmed by identifying the *uidA* and *tuf* genes, respectively, using real-time polymerase chain reaction (RT-PCR).The antibiotic susceptibility profiles of the confirmed *E. coli* and *Enterococci* spp. were determined using the Kirby-Bauer disk diffusion method. Whole-genome sequencing (WGS) and bioinformatics tools (ResFinder, VirulenceFinder, PlasmidFinder, NCBI PGAP) were used to determine the resistome, virulome, mobilome, clonal lineages, and phylogenies of the representative isolates circulating between the agricultural soil and the chicken litter.

5.0 Thesis outline

This thesis is written in the form of manuscripts prepared for submission to suitable journals. It comprises of six chapters as shown below:

- Chapter 1- Introduction and Literature review.
  Antibiotic resistance in agricultural soil and animal manure as well as bacterial mechanism of resistance to antibiotics were reviewed in this chapter.
- Chapter 2- Manuscript 1
  Rethinking Manure Application: Increase in Multidrug-Resistant *Enterococcus* spp. in Agricultural Soil Following Chicken Litter Application. This paper has been published in Antibiotics. The prevalence, speciation, and antibiotic resistance profiles of *Enterococcus* spp. are described in this manuscript.

- Chapter 3- Manuscript 2

  Transmission of Antibiotic-Resistant *E. coli* from Chicken Litter to Agricultural Soil (Proposed Journal: Frontiers in Environmental Science): Prevalence and antibiotic susceptibility profiles and multiple antimicrobial resistance index of *E. coli* are described in this manuscript.

- Chapter 4- Manuscript 3

  Genomic Analysis of Antibiotic-Resistant *Enterococcus* spp. reveals Novel Enterococci Strains and the Spread of Plasmid-Borne *Tet*(*M*), *Tet*(*L)* and *Erm(B)* Genes from Chicken Litter to Agricultural Soil in South Africa (Proposed Journal: Frontiers in Environmental Science). The manuscript highlights the presence and co-occurrence of ARGs and MGEs and the potential transmission of litter-borne ARGs to soil bacteria.

- Chapter 5-Conclusion, Limitations, and Recommendations

  This chapter presents the findings of this study in light of the overall objectives. The significance and limitations of the study are highlighted, as well as recommendations for further work.

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CHAPTER TWO

Rethinking Manure Application: Increase in Multidrug-Resistant *Enterococcus* spp. in Agricultural Soil Following Chicken Litter Application.

AUTHOR CONTRIBUTIONS

- Dorcas O. Fatoba: co-conceptualized the study, undertook sample collection and processing, performed the laboratory experiments, analysed the data, and wrote the paper
- Akebe L. K. Abia: supervised and co-conceptualized the study, and undertook a critical revision of the manuscripts
- Daniel G. Amoako: co-analysed the data and undertook a critical revision of the manuscripts
- Sabiha Y. Essack: supervised and co-conceptualized the study, facilitated ethical approval and sample collection, vetted the data analysis and undertook a critical revision of the manuscript.

Objective(s) covered: This paper has been published in Microorganisms and addresses objectives 1, 2, and 3

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Rethinking Manure Application: Increase in Multidrug-Resistant *Enterococcus* spp. in Agricultural Soil Following Chicken Litter Application

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Abstract: The current study investigated the impact of chicken litter application on the abundance of multidrug-resistant *Enterococcus* spp. in agricultural soil. Soil samples were collected from five different strategic places on a sugarcane farm before and after manure application for four months. Chicken litter samples were also collected. Enterococci were enumerated using the Enterolert®/Quanti-Tray 2000® system and confirm and differentiated into species using real-time PCR. The antibiotic susceptibility profile of the isolates was determined using the disk diffusion method following the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. The overall mean bacterial count was significantly higher ($p < 0.05$) in manure-amended soil (3.87×10^7 MPN/g) than unamended soil (2.89×10^7 MPN/g). Eight hundred and thirty-five enterococci (680 from soil and 155 from litter) were isolated, with *E. casseliflavus* being the most prevalent species (469; 56.2%) and *E. gallinarum* being the least (16; 1.2%). Approximately 56% of all the isolates were resistant to at least one antibiotic tested, with the highest resistance observed against tetracycline (33%) and the lowest against chloramphenicol (0.1%); 17% of *E. faecium* were resistant to quinupristin-dalfopristin. Additionally, 27.9% (130/466) of the isolates were multidrug-resistant, with litter-amended soil harbouring more multidrug-resistant (MDR) isolates (67.7%; 88/130) than unamended soil (10.0%; 13/130). All isolates were susceptible to tigecycline, linezolid and gentamicin. About 7% of the isolates had a multiple antimicrobial resistance index > 0.2, indicative of high antibiotic exposure. Although organic fertilizers are regarded as eco-friendly compared to chemical fertilizers for improving soil fertility, the application of untreated animal manure could promote the accumulation of antibiotics and their residues and antibiotic-resistant bacteria in the soil, creating an environmental reservoir of antimicrobial resistance, with potential human and environmental health risks.

Keywords: animal manure; antibiotic resistance; *Enterococcus* spp.; chicken litter; environmental reservoirs; multidrug resistance; public health; agricultural soil

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1. Introduction

Poor soil fertility is a significant challenge for small and large-scale farming systems in sub-Saharan Africa, and chemical and organic fertilizers are frequently added to soil to improve its quality, texture, and crop yield [1]. However, chemical fertilizers affect beneficial microorganisms in the soil, cause an imbalance in soil pH, contaminate groundwater through leaching without fully benefiting plants, and cause plant disease [2]. On the other hand, organic manure adds nutrient-rich organic matter, which improves soil fertility, texture, water-holding capacity, and imparts resistance to wind and water erosion [3,4]. Thus, applying animal manure to soil has become common in agricultural farms in many countries, including South Africa [1,5], as it improves soil properties and increases productivity [6].

Although the use of antibiotics in livestock farming has proven to be beneficial for economic reasons, their use as growth promoters for prophylaxis, metaphylaxis and treatment establishes a reservoir of antibiotic-resistant bacteria (ARB), including multidrug-resistant (MDR) ones and antibiotic resistance genes (ARGs) in the gastrointestinal tract of livestock, and subsequently their waste [7]. The addition of such animal waste as manure to the soil, without treatment, may contribute to the transmission of antibiotic resistance to soil bacteria and pose serious environmental risks [8]. This agricultural practice has resulted in the contamination of soil, surface water, groundwater, and the food chain with antibiotic residues and ARB, posing a severe public health concern associated with farm produce such as raw vegetables [5,9].

Enterococci are Gram-positive natural commensals inhabiting humans and animals' digestive systems with a wide range of species such as *E. faecalis*, *E. faecium*, *E. casseliflavus*, *E. gallinarum*, *E. durans*, *E. munditi*, *E. hirae*, and *E. avium* [10]. The abundance of *Enterococcus* spp. in animal and human faeces and their prolonged survival in the environment have made them a popular indicator of faecal contamination in the environment [11,12]. Although considered a commensal in humans, certain *Enterococcus* species have been identified as high-ranking (second to staphylococci) agents causing nosocomial infections in humans [13]. These bacteria, especially multidrug resistance ones, in animal manure applied to agricultural fields represent a significant environmental and public health concern that needs considerable attention through continuous monitoring.

Therefore, this study investigated the prevalence of antibiotic-resistant *Enterococcus* spp. in soil amended with chicken litter on a sugarcane farm in KwaZulu-Natal, South Africa. Such information would guide decision making regarding the use of manure and emphasise the importance of antibiotic stewardship in agricultural practices, thus protecting human and environmental health.

2. Materials and Methods

2.1. Ethical Clearance

This study was part of a larger project for which ethical approval had been received from the Animal Research Ethics Committee (Reference: AREC073/016PD) and the Biomedical Research Ethics Committee (Reference: BCA444/16) of the University of KwaZulu-Natal. The study was also approved by the South African National Department of Agriculture, Forestry, and Fisheries (Reference: 12/11/1/5 (879).

2.2. Study Location

This study was carried out for four months, between October 2018 and February 2019, on a sugarcane farm in the uMgungundlovu District, KwaZulu-Natal, South Africa (Figure 1). The district has a population of 1,069,657, with a population density of 110.7 persons/km^2. The area is home to the bulk of food animal production firms (pigs and poultry) and agriculture, mainly sugarcane, pear, and vegetable farms. Some of the water bodies around the farm in this locality include Mqeku, Mkabela, Mbhava, and Sterkspruit, which are small rivers that drain into the main Umgeni River (the primary source of drinking water for the people living in Pietermaritzburg). The sugarcane is planted between September and November and becomes fully mature in about 12 to 14 months. Soil amendment with chicken litter (a mix of chicken manure and wood shavings) is a common agricultural practice in the locality because of its availability, cost-effectiveness, and efficiency in improving soil quality. Chicken litter is usually randomly spread over the soil surface about ten days before planting. Urea is also applied to the soil seven days after manure application.

Figure 1. Map of the study site, including the sampling points (A, B, C, D, E and manure heap). Source: Google Earth.

2.3. Sample Collection

Soil samples were collected from five points of the sugarcane farm (Figure 1) on days 1, 2, 3, 5, and 9 before manure application. Following manure application, samples were collected on the day of application (day 0), then days 1, 3, 7, 14, 21, and bi-monthly after that for three months after the chicken litter application. Samples were collected until the farm became inaccessible due to the height of the plants.

Using a sterile hand shovel, ≈50 g of soil was aseptically collected within the top 5 cm of the soil and transferred into sterile ziplock bags. The same quantity of chicken litter was also collected from a heap of unapplied manure around the farm (Figure 1). All the soil samples were collected in duplicates and the chicken manure in triplicates. Samples were transported on ice packs to the laboratory for analyses within 6 h from collection. A total of 275 samples (82 chicken litter and 193 soil) were collected throughout the study.

2.4. Sample Processing and Enumeration of Enterococcus spp.

The Enterolert®-18® Quanti-Tray®/2000 system (IDEXX Laboratories (Pty) Limited, Totowa, NJ, United States) was used to enumerate *Enterococcus* spp. Soil and chicken litter samples were processed for analysis, as previously described by Abia et al. [14]. Briefly, 5 g of sample was transferred from a well-shaken zip-lock bag into a sterile bottle containing 5 mL of sterile distilled water, giving a 1:1 (v/v) dilution. The mixture was shaken vigorously to dislodge the bacteria from the soil into the water. The bottle was

allowed to settle for 20 min, and 1 mL of the supernatant was extracted, topped up to 100 mL with sterile distilled water, and processed following the IDEXX protocol for water sample analysis (IDEXX Laboratories (Pty) Ltd., Johannesburg, South Africa). Ten positive wells in the quanti-tray (those that fluoresced under the UV light) were randomly picked, and their content was streaked onto Bile Aesculin agar plates (Lab M, Lancashire, UK) and incubated at 44 ± 0.5 °C for 24 h to obtained pure colonies. One pure isolate was collected per plate and stored in Trypticase soy broth (Oxiod, Hampshire, England) with 20% glycerol at −80 °C for further analysis.

2.5. DNA Extraction, Molecular Confirmation and Differentiation Enterococcus Species

Stored isolates were resuscitated by culturing them on nutrient agar (Lab M, Lancashire, UK) at 41 °C for 24 h. Colonies were then transferred to sterile Eppendorf tubes containing 200 µL of sterile distilled water and the DNA was extracted using the boiling method as previously described [15]. The supernatant was then used as the DNA template for the PCR assays.

Real-time polymerase chain reaction (RT-PCR) was used to confirm the isolates to genus level and distinguish between the species on a QuantStudio® 5 Applied Biosystems (Applied Biosystems, ThermoFisher, Waltman, MA, USA) real-time PCR machine. The confirmation to genus level was carried out by targeting the *tuf* gene [16], using cycling conditions previously described by Molechan et al. [17]. All the confirmed *Enterococcus* isolates were further screened to speciate them as *E. casseliflavus*, *E. faecalis*, *E. faecium*, and *E. gallinarum*, using species-specific primers (Table S1) and PCR conditions previously described [17]. *Enterococcus* isolates that did not fall within the four species categories were tagged as *Enterococcus* spp. All primers were supplied by Inqaba Biotech Industries Ltd., Pretoria, South Africa. All reactions contained a positive control (Table S1) and a No Template Control (reaction mixture but no DNA). Melt curves were analysed using the QuantStudio™ Design and Analysis Software v.1.3.1 (Applied Biosystems, ThermoFisher Waltman, MA, USA).

2.6. Antibiotic Susceptibility Testing

The confirmed *Enterococcus* isolates were subjected to antibiotic susceptibility testing using the disk diffusion method on Mueller–Hinton agar (Lab M, Lancashire, UK) according to the Clinical and Laboratory Standards Institute [18] and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [19] for breakpoints absent in the CLSI guidelines. The isolates were tested against 16 antibiotics in 12 antibiotic classes. These included ampicillin (AMP, 10 µg), teicoplanin (TEC, 30 µg), vancomycin (VAN, 30 µg), streptomycin (STR, 300 µg), linezolid (LZD, 30 µg), imipenem (IPM, 10 µg), erythromycin (ERY, 15 µg), ciprofloxacin (CIP, 5 µg), levofloxacin (LEV, 5 µg), nitrofurantoin (NIT, 300 µg), gentamicin (GEN, 120 µg), chloramphenicol (CHL, 30 µg), sulfamethoxazole-trimethoprim (SXT, 25 µg), tetracycline (TET, 30 µg) and tigecycline (TGC, 15 µg) (Oxoid, Hampshire, England). *E. faecium* was additionally tested against quinupristin-dalfopristin (QD, 15 µg). *E. faecalis* ATCC 29,212 was used as a positive control. The inhibition zones' diameters were measured in millimetres and interpreted as susceptible (S), intermediate (I), or resistant (R) [18]. EUCAST was used for three antibiotics (TGC, 15 µg, SXT, 25 µg, and IMP, 10 µg). Isolates resistant to one or more antibiotics in three or more different antibiotics classes were classified as MDR.

The multiple antibiotic resistance index (MARI) of each isolate was calculated as a/b (a: number of antibiotics to which the isolates were resistant, b: number of antibiotics against which the isolates were tested) [20]. The MARI of each sample group was calculated using the formula a/(bc), where "a" represents the aggregate antibiotic resistance score of all *Enterococcus* isolates from each sample group, "b" is the number of antibiotics tested against the isolates, and "c" represents the total number of isolates per sample group [20].

2.7. Data Analysis

All statistical analyses were performed using Microsoft Excel 2016 and the Statistical Package for the Social Science (SPSS v26, IBM Corporation, Armonk, NY, USA). Before analysis, enterococci counts were log-transformed, and the geometric means were used to describe the microbial concentration in soil and chicken litter. To calculate the log counts and the geometric means, all values > 2419.6 were approximated to the nearest whole number (2420), and values < 1 were considered as 1. One-way analysis of variance (ANOVA) and the Games–Howell post hoc test were conducted to compare the mean *Enterococcus* counts and the number of antibiotic-resistant species between the chicken litter, litter-amended soil and soil samples collected before litter application. Results were considered statistically significant if the *p*-value was <0.05.

3. Results

3.1. Quantification of Enterococcus spp.

All the soil and chicken litter samples from the various sample groups and points tested positive for *Enterococcus*. The highest mean count (5.68×10^7 MPN/g) per sample round was observed in the chicken litter (Table S2). The overall mean bacterial count was higher in litter-amended soil (3.87×10^7 MPN/g) than unamended soil (2.89×10^7 MPN/g) (Figure 2).

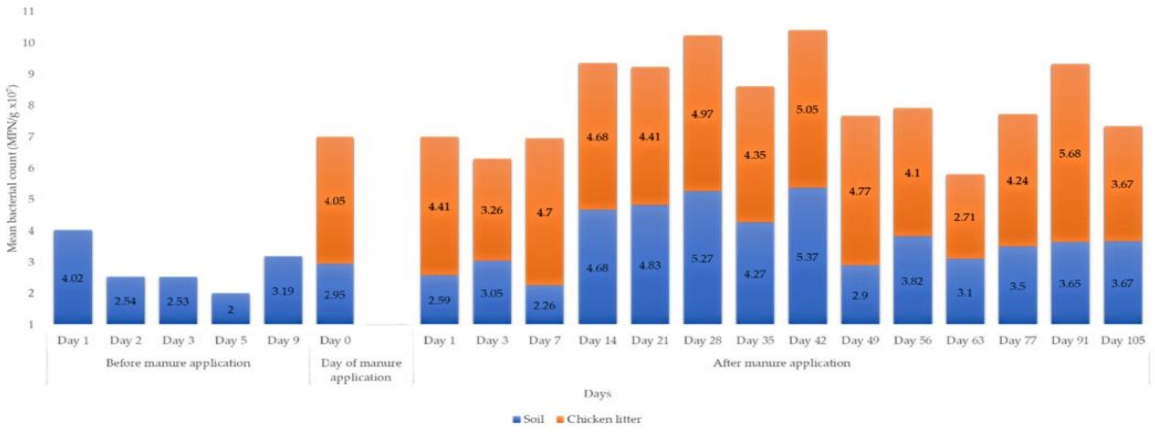

Figure 2. Mean *Enterococcus* spp. count per sampling source and sampling day. No litter sample collected before the litter application. The farm was burnt on day 2 in preparation for harvest. Rainfall events were recorded on day 3 (before litter application) and day 14, 42 and 77 after the litter amendment. Urea was applied to the farm on day 7.

There was an overall statistically significant difference ($p = 0.000$; $p < 0.05$) in *Enterococcus* count between the three sample groups (Table 1). Games–Howell post hoc test indicated that the overall *Enterococcus* mean count in the chicken litter was significantly higher than the litter-amended soil ($p = 0.01$, $p < 0.05$), and unamended soil ($p < 0.001$, $p < 0.05$). A statistically significantly higher mean *Enterococcus* count was observed in the litter-amended soil than unamended soil ($p = 0.01$, $p < 0.05$).

3.2. Prevalence of Enterococcus spp. Isolates in Soil and Chicken Litter

A total of 835 enterococci (680 from soil and 155 from chicken litter) isolates were confirmed. *E. casseliflavus* was the most prevalent species (56.17%), and *E. gallinarum* as the least prevalent (1.9%) (Figure 3). *E. faecium* and *E. gallinarium* were not detected in unamended soil samples; 12.2% of the isolates could not be classified into any of the four *Enterococcus* species.

Table 1. Statistical comparison of mean *Enterococcus* counts between sample sources.

Group	N	Overall Mean *Enterococcus* Count (MPN/g × 10^7)	Overall *p*-Value	Pair Wise Comparison (Games–Howell)		95% Confidence Interval for Mean (log MPN/g)		*p*-Value
					Mean Difference (±SE)	Lower Bound	Upper Bound	
SBL	48	2.89 (±0.92) *	0.000 ***	SBL vs. LAS	−0.98 (±1.18) *	−1.40	−0.57	0.000 *
LAS	145	3.87 (±1.43) *		SBL vs. CL	−1.52 (±0.19) *	−1.98	−1.06	0.000 *
CL	44	4.41 (±0.92) *		LAS vs. CL	−0.54 (±1.18) *	−0.97	−0.10	0.011 *

* $p < 0.05$; *** $p < 0.001$; SBL: soil before litter application; LAS: litter-amended soil; CL: chicken litter.

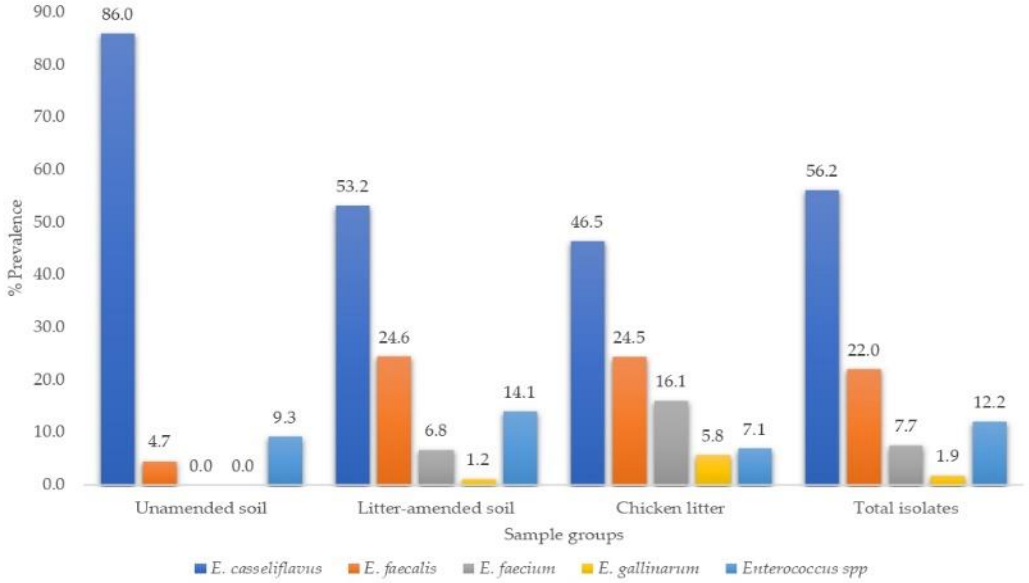

Figure 3. Distribution of *Enterococcus* spp. across sample types.

3.3. Antibiotic Susceptibility Profiles of Enterococcus spp.

Overall, 466 (55.8%) of 835 *Enterococcus* isolates in this study were resistant to at least one antibiotic, of which 321 (68.9%) were from litter-amended soil, 93 (19.9%) from chicken litter, and 52 (11.2%) from unamended. Overall, the highest resistance observed was against tetracycline (58.2%) and the lowest against chloramphenicol (0.2%) (Figure 4). None of the isolates were resistant to tigecycline, linezolid, and gentamicin. The variation in the number of resistant isolates with sampling days is shown in Figure S1 (Supplementary Materials). The susceptibility profile of quinupristin-dalfopristin was only reported for *E. faecium*, and 19% were resistant isolates. Only *E. casseliflavus* (2%) and *E. gallinarum* (4%) species showed resistance to vancomycin while *E. faecalis* (7%) were of intermediate susceptibility (Table S3; Supplementary Materials).

Although there was an overall increase in the number of resistant isolates following the chicken litter amendment, this was not statistically significant (Table 2) in the number of resistant isolated between the three sample groups.

3.4. Prevalence of Multidrug Resistance and Calculation of MARI

Multidrug resistance was observed in 27.8% (130/466) of the resistant enterococci isolates. Among these MDR, the litter-amended soil isolates had the highest percentage, 67.7% (88/130), followed by the chicken litter 22.3% (29/130) and unamended soil 10% (13/130) (Table S3). The division of the MDR into species revealed that *E. faecium* had the highest rate (41%, 26/64) of MDR compared to other species, with the least MDR observed in *E. faecalis* (13%, 23/184).

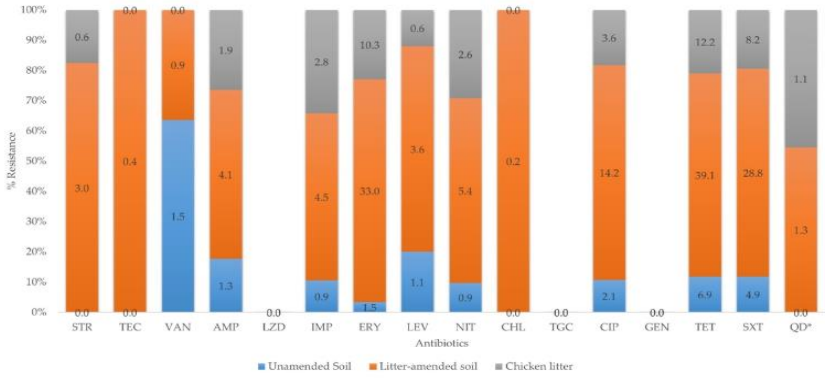

Figure 4. Prevalence of antibiotic-resistant *Enterococcus* in the soil and chicken litter. AMP = ampicillin, TGC = tigecycline, TET = tetracycline, CHL = chloramphenicol, GEN = gentamicin, TEC = teicoplanin, VAN = vancomycin, STR = streptomycin, LZD = linezolid, IPM = imipenem ERY = erythromycin, CIP = ciprofloxacin, LEV = levofloxacin, NIT = nitrofurantoin, SXT = sulfamethoxazole/trimethoprim, QD = quinupristin-dalfopristin. QD* is reported only for *E. faecium* isolates.

Overall, 63 MDR patterns were observed across the enterococci isolates, the most prevalent phenotype being ERY-TET-SXT (Table S3). At the species level, *E. casseliflavus*, *E. faecium*, *E. faecalis*, other *Enterococcus* spp. and *E. gallinarum* showed 40, 23, 12, and 12 MDR patterns, respectively.

The isolates' MAR indices ranged from 0.13 (resistance to two antibiotics) to 0.44 (resistance to seven antibiotics) (Figure 5). In total, 12.1% (56/466) of the resistant isolates had a MARI > 0.2. Of these, 58.9% (33/56) was from the litter-amended, 26.8% (15/56) of the isolates from the chicken litter, and 14.3% (8/56) from the soil before the litter amendment. The average MAR indices value according to the sample groups revealed that the chicken litter had the highest value of 0.09 compared to the litter-amended soil (0.08) and the soil samples before litter-amendment (0.06) (Table S4).

Table 2. Statistical comparison of the prevalence of resistant-*Enterococcus* isolates between sample sources.

Group	N	Overall Mean Difference (±Standard Deviation) of Resistant *Enterococcus*	Overall ANOVA *p*-Value	Pairwise Comparison (Games–Howell)		95% Confidence Interval for Mean		*p*-Value
						Lower Bound	Upper Bound	
					Mean Difference (±Standard Error)			
SBL	107	0.49 (±0.50)	0.184	SBL vs. LAS	−0.08 (±0.53)	−0.20	0.05	0.324
LAS	573	0.56 (±0.50)		SBL vs. CL	−0.11 (±0.63)	−0.26	0.03	0.165
CL	155	0.60 (±0.49)		LAS vs. SBL	0.08 (±0.53)	−0.05	0.20	0.324

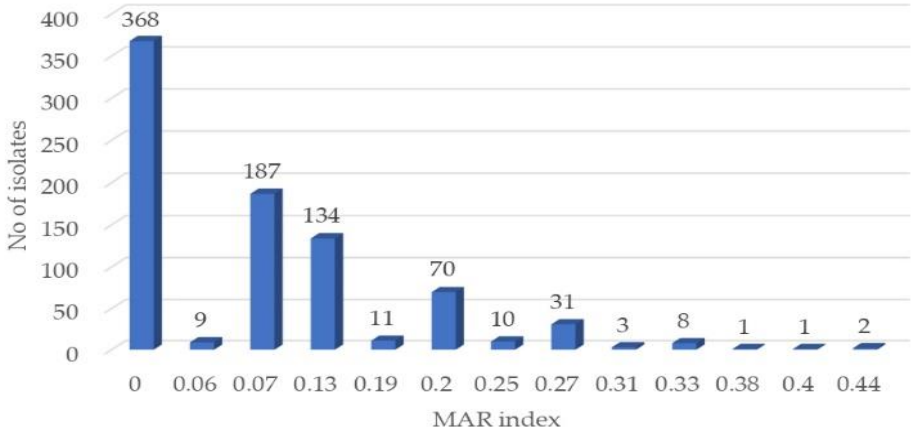

Figure 5. Multiple antibiotic resistance (MAR) indices of all the *Enterococcus* isolates.

4. Discussion

Although animal manure is regarded as an organic approach to soil fertility improvement, its application to soil may introduce numerous ARB to the environment. Thus, we investigated the impact of chicken litter application on the prevalence of antibiotic-resistant *Enterococcus* spp. in agricultural soils. There was a marked increase in *Enterococcus* counts in the soil following manure application, with some species only identified in manure-amended soils and chicken litter but not in the soil before manure application. The *Enterococcus* spp. were resistant to tetracycline, erythromycin, trimethoprim-sulfamethoxazole, and fluoroquinolone, commonly used in poultry production in South Africa. Approximately 28% of the resistant enterococci were MDR, with a substantial percentage of them having a MARI > 0.2.

4.1. Enumeration of Enterococcus before and after Manure Application

Enterococcus has been widely used as a faecal bacterial indicator in the environment. In the current study, the mean *Enterococcus* count in the litter-amended soil was statistically significantly higher than in the soil before amendment (Table 1), indicating that manure application impacted the soil bacterial abundance. This could have happened in two ways. Firstly, chicken manure is exceptionally nutrient-rich, and its application on the farm resulted in the enrichment of indigenous bacteria, including *Enterococcus*. The use of animal manure for soil fertilization has been shown to enhance resident soil bacteria's proliferation in agricultural soil [21]. Secondly, *Enterococcus* is a normal flora of human and animal intestines [11,12]. Therefore, manure application resulted in the direct introduction of *Enterococcus* into the soil. The second argument is supported by the fact that the chicken litter recorded a statistically significantly higher bacterial count than the soil before manure application and manure-amended soil. Consistent with our findings, Marti and colleagues [21] reported that soil fertilisation with swine and dairy manure increased the count of viable bacteria in the soil.

Although there were fluctuations in the *Enterococcus* counts during the different sampling rounds after chicken litter application, an overall decline to baseline values was observed by the last sampling date (day 105). Some studies have indicated that bacteria of animal manure origin only survive in the soil environment for a short period (days to few months) [22–24], while others have indicated that enterococci could persist in manured soil environments for up to a year [25]. Cools et al. [24] demonstrated that *Enterococcus* spp. derived from pig manure could survive in the soil for 54 days in a study conducted in Belgium. Contrarily, [25] reported in a USA study that enterococci concentration in the swine manured soil decreased to concentrations equivalent to the no-manure soil after one year of manure amendment. Therefore, although the sampling in the current study ended on day 105, the manure's effect could be felt far beyond the sampling period. This could explain the presence of *Enterococcus* in the soil before manure application in this study. The long-term persistence of enterococci in the litter-amended soil is worrying, as the potentially pathogenic strains could enter the food chain or get washed during rainfall events to nearby surface water bodies. This also implies that yearly manure application could maintain high enterococci concentrations in the soil environment, with potential environmental and human health implications.

4.2. Prevalence of Different Enterococcus Species

According to species, the molecular characterization of the enterococci revealed differences between the soil before and after the litter amendment. Although *E. casseliflavus* was present in all sample sources, its high prevalence and dominance in the soil before manure was expected as this species is plant-associated [26]. Similar findings had earlier been reported in the USA [27]. Contrarily, there was a low *E. faecalis* prevalence in the soil before manure (Figure 3). No other species were identified in this soil. However, after manure application, the prevalence increased in the soil, indicating the manure's impact. Most importantly, *E. faecium* and *E. gallinarum* were only identified in the litter-amended soil.

These species were absent in soil before manure application but present in the chicken-litter samples, further highlighting the chicken litter's significant impact on the soil *Enterococcus* abundance. These observations corroborate a field experiment in China, which observed a significant increase in bacterial diversity in soil following chicken manure amendment [28]. The appearance of *E. faecium* and *E. gallinarum* as well as the increased detection of *E. faecalis*, which are characteristic of warm-blooded animals in the soil after the litter amendment, implies that these two important human infectious agents may be potential indicators of transmission dynamics between the soil environment and humans, directly through exposure to contaminated soil or indirectly through consumption of poorly washed crops from manure fertilized farms.

4.3. Antibiotic Resistance of Enterococcus Species

Antibiotic-resistant *Enterococcus* spp. was detected in all the sample groups with the highest resistance percentage in the litter-amended soil (Figure 3). The isolates from the soil before amendment were mostly resistant to tetracycline and trimethoprim-sulfamethoxazole, while the litter-amended soil and chicken litter isolates expressed high-level resistance to tetracycline, erythromycin, and trimethoprim-sulfamethoxazole. The extensive administration of sub-therapeutic doses of antibiotics overtime in food animal production creates a selective advantage for the emergence of ARB in their intestines, which invariably ends up in manure and the environment [29–31]. This was reflected in the high percentage resistance to tetracycline, erythromycin, and trimethoprim-sulfamethoxazole among the litter-amended soil and the chicken litter enterococci isolates in the current study. These antibiotics or their homologues are used in the South African poultry industry [32], despite the prohibited use of several critically important antibiotics for humans in other parts of the world such as Europe [33]. The increased frequency of erythromycin-resistant enterococci in the soil after the litter amendment and the high intermediate susceptibility of the isolates to erythromycin and ciprofloxacin observed could have resulted from the addition of tylosin and enrofloxacin to the chicken's feed and water, and their use for treating infections in the animals [17,32]. A previous study on antibiotic-resistant enterococci in chicken litter in Canada reported a higher prevalence of resistance to tetracycline and ciprofloxacin [34], attributed to the large quantities of antibiotics used for growth promotion in broiler chicken farms [34]. Similarly, a previous study of antibiotic-resistant *Enterococcus* spp. from farm-to-fork conducted in uMgungudlovu District, South Africa, also indicated a higher level of resistance to tetracycline and erythromycin and high intermediate resistance to ciprofloxacin [17].

The literature has shown that animals excrete as much as 90% of the antibiotics administered orally or added to the feeds through faeces or urine [35]. Although the use of chicken litter as organic fertilizer is a common agricultural practice in South Africa [1], no previous study has examined the impact of chicken litter application on soil antimicrobial-resistant microorganisms in South Africa. In the current study however, the litter amendment increased the number of antibiotic-resistant enterococci in the soil, which could be attributed to the enrichment of resident resistant enterococci in the soil and the addition of resistant species directly to the soil. This is supported by the increase in the number of resistant enterococci in the soil on the day of chicken litter application (Figure S1; Supplementary Materials). Previous studies have reported animal manure as a reservoir of ARB and a source of environmental (soil) contamination with ARB [9,26]. For example, a study on agricultural soil fertilized with swine manure in Iowa State, USA, showed that the concentration of antibiotic-resistant enterococci in the soil with manure was greater than the control soil that was not treated with manure [25]. Similarly, a practical survey of ARB in chicken manure-amended soil and manure-free soil carried out in China reported a significantly higher prevalence of cultivable ARB in the manure-amended soil than the count in the manure-free soil samples [8].

Although the prevalence of resistant *Enterococcus* spp. was generally higher in the litter amended soil, this was not statistically significant (Table 2), as some external factors

played a significant role. For example, a major reduction in the number of resistant *Enterococci* was observed on day 7 (after the chicken litter amendment), which happened to be the day urea was applied to the field. Urea application has been shown to decrease the soil microbial population and diversity [36,37]. The number of antibiotic-resistant enterococci in the litter-amended soil decreased to levels comparable to the soil resistance before amendment at 28 days after the chicken litter amendment, suggesting the depletion of manure nutrient that enhanced the growth of resident bacteria and the death of the litter-borne enterococci [38].

It should, however, be noted that there was also a high tetracycline and trimethoprim-sulfamethoxazole percentage resistance observed in the soil before the chicken litter amendment. These antibiotics or their residues should also be considered regarding the selection pressure they may exert on soil bacterial populations. While some of the antibiotics such as erythromycin and tylosin completely biodegrade in soil within 30 days at 20 °C to 30 °C, only a small amount of antibiotics such as ciprofloxacin, tetracycline, and some sulphonamides degrade even after 30 to 80 days [35,39,40].

The MARI is used in differentiating between bacteria from low- and high-health risk sources. A MARI value greater than 0.2 indicates that such bacterial isolates originate from a source with high antibiotics use contamination [20]. The impact of manure on the presence of antibiotic-resistant *Enterococcus* spp. was further demonstrated here as isolates from the litter-amended soil had a higher percentage of isolates with a MARI > 0.20. Additionally, although the sample groups' MAR indices were <0.20, the comparable MAR index of the chicken litter (0.09) and the litter-amended soil (0.08) suggests the transmission of ARB from sources of frequent antibiotic use, such as on intensively produced chicken as was the litter source here. Furtula and colleagues [34] reported high average MAR indices for the enterococci isolated from the chicken litter samples and attributed their observation to different antibiotic usage levels in the poultry systems from which the litter was sourced.

5. Conclusions

Chicken litter application increased the abundance and diversity of *Enterococcus* species in agricultural soil. There was also an increase in antibiotic-resistant enterococci species, including MDR ones, in the litter-amended soil, suggesting the possibility of the transfer of ARB in the chicken litter to the soil. This was further supported by the overall higher MARI of litter-amended soil than unamended soil. A higher percentage resistance was observed against tetracycline, erythromycin, and sulfamethoxazole-trimethoprim. A substantial number of the isolates from chicken litter shared similar resistance patterns to litter-amended soil isolates, suggesting a possible transfer of ARB (or ARGs) of chicken litter to the agricultural soil. The persistence of antibiotic-resistant enterococci species in the manured soil and the heap of chicken litter throughout this study highlights the risk of antibiotic resistance exposure when humans and animals consume contaminated farm produce. This study delineated chicken litter as a "hotspot" of antibiotic-resistant enterococci species that can contaminate the soil fertilized with it and pose a public health threat from its incorporation into plants, run-off to water sources and direct contact in occupationally exposed workers. It is, therefore, necessary to rethink the use of animal manure for soil fertilization. Since composting has been reported to reduce the number of ARB in animal manure, this should be carried out on chicken litter before its application to the soil to minimize soil contamination with ARB and reduce the possible dissemination of antibiotic resistance from chicken to farm produce. Policies on the prudent use of antibiotics in animal production is also required.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/microorganisms9050885/s1, Figure S1: Variation in antibiotic-resistant *Enterococcus* to at least one antibiotic throughout sample collection, Table S1: List of genus and species-specific primers and control strains used in this study, Table S2: Enumeration of *Enterococcus* in soil and chicken litter over the sampling period, Table S3: Multidrug-resistant pattern of the *Enterococcus* spp. isolates, Table S4:

Percentage of *Enterococcus* isolates that were resistant to at least one antibiotic at each sample point and the multiple antibiotic resistance index.

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Supplementary Tables

Table S1: List of genus and species-specific primers and control strains used in this study

Control Strain	Primer	Primer sequence 5'-3'	Product size (bp)	Reference
E. faecalis ATCC 51299	ENT1	TACTGACAAACCATTCATGATG	112	(Ke et al., 1999)
	ENT2	AACTTCGTCACCAACGCGAAC		
E. faecalis ATCC 51299	FA1	ACTTATGTGACTAACTTAACC	360	(Jackson et al., 2004)
	FA2	TAATGGTGAATCTTGGTTTGG		
E. faecium ATCC 35667	FM1	GAAAAAACAATAGAAGAATTAT	215	
	FM2	TGCTTTTTTGAATTCTTCTTTA		
E. gallinarum Field strain (NHLS)	GA1	TTACTTGCTGATTTTGATTCG	173	
	GA2	TGAATTCTTCTTTGAAATCAG		
E. casseliflavus ATCC 700327	CA1	TCCTGAATTAGGTGAAAAAAC	288	
	CA2	GCTAGTTTACCGTCTTTAACG		

*****Field strains were provided by the National Health Laboratory Services (NHLS), South Africa.

Table S2: Multidrug-resistant pattern of the *Enterococcus* spp. isolate

Antibiogram	**Frequency**	**E. casseliflavus (n = 469)**	***E. faecalis* (n=184)**	***E. faecium* (n = 64)**	***Enterococcus* spp. (n = 102)**	**E. gallinarum (n = 16)**	**Sample source**		
							Unamended soil (n = 107)	**Litter-amended Soil (n = 573)**	**Chicken Litter (n = 155)**
DAY 3 SAMPLING									
CIP-TET-SXT	**4**	2	1	1	0	0	**1**	**1**	**2**
LEV-CIP-TET-SXT	**1**	1	0	0	0	0	**1**	**0**	**0**
DAY 5 SAMPLING									
ERY-TET-SXT	**31**	17	8	3	3	0	**1**	**25**	**5**
VAN-IPM-ERY-LEV-SXT	**1**	1	0	0	0	0	**1**	**0**	**0**
DAY 7 SAMPLING									
NIT-CIP-TET-SXT	**3**	2	0	1	0	0	**2**	**0**	**1**
VAN-LEV-TET-SXT	**1**	1	0	0	0	0	**1**	**0**	**0**
AMP-NIT-TET	**1**	1	0	0	0	0	**1**	**0**	**0**
LEV-TET-SXT	**2**	1	0	1	0	0	**1**	**1**	**0**
VAN-ERY-CIP-TET	**1**	1	0	0	0	0	**1**	**0**	**0**
VAN-CIP-TET	**1**	1	0	0	0	0	**1**	**0**	**0**
VAN-AMP-TET-SXT	**1**	1	0	0	0	0	**1**	**0**	**0**
VAN-ERY-CIP-TET-SXT	**1**	1	0	0	0	0	**1**	**0**	**0**
DAY 0 OF MANURE APPLICATION									
ERY-CIP-TET	**9**	5	1	1	2	0	**0**	**8**	**1**
NIT-TET-SXT	**2**	1	1	0	0	0	**0**	**1**	**1**
ERY-NIT-TET	**3**	2	1	0	0	0	**0**	**3**	**0**
TEC-VAN-ERY-NIT	**1**	1	0	0	0	0	**0**	**1**	**0**
ERY-LEV-TET	**1**	1	0	0	0	0	**0**	**1**	**0**

TEC-VAN-NIT-SXT	**1**	1	0	0	0	0	**0**	**1**	**0**
ERY-CIP-TET-SXT	**4**	2	0	1	1	0	**0**	**3**	**1**
STR-ERY-CIP-TET	**1**	0	1	0	0	0	**0**	**1**	**0**
STR-LEV-CIP-TET	**1**	0	1	0	0	0	**0**	**1**	**0**
STR-ERY-TET-SXT	**7**	0	5	1	1	0	**0**	**6**	**1**
STR-AMP-ERY-TET-SXT	**1**	1	0	0	0	0	**0**	**1**	**0**
STR-AMP-ERY-TET	**1**	0	1	0	0	0	**0**	**1**	**0**
ERY-NIT-TET-SXT	**4**	2	1	1	0	0	**0**	**3**	**1**
STR-ERY-QD-CIP-TET-SXT	**1**	0	0	1	0	0	**0**	**0**	**1**
AMP-IPM-ERY-LEV-QD	**1**	0	0	1	0	0	**0**	**0**	**1**
DAY 1 SAMPLING AFTER MANURE APPLICATION									
ERY-NIT-SXT	2	1	0	0	1	0	**0**	**2**	0
LEV-NIT-CIP-TET-SXT	1	1	0	0	0	0	**0**	**1**	0
AMP-TET-SXT	2	2	0	0	0	0	**0**	**1**	**1**
AMP-ERY-SXT	1	0	0	0	1	0	**0**	**1**	0
STR-AMP-IPM-ERY-TET-SXT	1	0	0	1	0	0	**0**	**1**	0
AMP-ERY-NIT-TET	1	1	0	0	0	0	**0**	**1**	0
AMP-ERY-TET	1	1	0	0	0	0	**0**	**1**	0
IPM-ERY-TET	3	1	0	1	1	0	**0**	**2**	**1**
IPM-ERY-CIP-TET	3	1	0	1	1	0	**0**	**2**	**1**
STR-IPM-ERY-CIP-TET-SXT	1	1	0	0	0	0	**0**	0	**1**
AMP-IPM-ERY-LEV-TET	1	1	0	0	0	0	**0**	0	**1**
DAY 3 SAMPLING AFTER MANURE APPLICATION									
VAN-ERY-TET	1	1	0	0	0	0	**0**	**1**	**0**
IPM-ERY-NIT	1	1	0	0	0	0	**0**	**1**	**0**
IPM-ERY-LEV-NIT-TET	1	0	0	1	0	0	**0**	**1**	**0**
AMP-LEV-CIP-SXT	1	0	1	0	0	0	**0**	**1**	**0**
AMP-ERY-TET-SXT	2	1	1	0	0	0	**0**	**2**	**0**

AMP-ERY-NIT-TET-SXT	1	0	0	0	1	0	**0**	**0**	**1**
ERY-QD-TET-SXT	1	0	0	1	0	0	**0**	**0**	**1**
ERY-QD-TET	2	0	0	2	0	0	**0**	**1**	**1**
DAY 14 SAMPLING AFTER MANURE APPLICATION									
ERY-CIP-TET-QD	1	0	0	1	0	0	**0**	**1**	**0**
IPM-CIP-TET	1	0	0	0	1	0	**0**	**0**	**1**
IPM-QD-CIP-TET	1	0	0	1	0	0	**0**	**0**	**1**
AMP-IPM-ERY-NIT	1	1				0	**0**	**0**	**1**
DAY 21 SAMPLING AFTER MANURE APPLICATION									
IPM-ERY-LEV-CIP-SXT	1	0	0	0	1	0	**0**	**1**	**0**
IPM-LEV-CIP-SXT	1	0	0	1	0	0	**0**	**1**	**0**
IPM-TET-SXT	1	0	0	0	1	0	**0**	**1**	**0**
NIT-QD-SXT	1	0	0	1	0	0	**0**	**1**	**0**
ERY-LEV-CIP-TET-SXT	1	1	0	0	0	0	**0**	**1**	**0**
ERY-NIT-CIP-TET-SXT	1	0	0	1	0	0	**0**	**0**	**1**
IPM-ERY-NIT-SXT	1	0	0	1	0	0	**0**	**0**	**1**
IPM-ERY-LEV-QD-CIP-TET-SXT	1	0	0	1	0	0	**0**	**1**	**0**
AMP-LEV-TET	1	1	0	0	0	0		**0**	**1**
DAY 28 SAMPLING AFTER MANURE APPLICATION									
STR-ERY-TET	1	1	0	0	0	0	**0**	**1**	**0**
DAY 77 SAMPLING AFTER MANURE APPLICATION									
STR-ERY-SXT	1	1	0	0	0	0	**0**	**1**	**0**
STR-AMP-ERY	1	1	0	0	0	0	**0**	**1**	**0**
STR-CIP-SXT	1	1	0	0	0	0	**0**	**1**	**0**
Total 16% (130/835)	**130**	**66**	**23**	**26**	**15**	**0**	**12% (13/107)**	**15% (88/573)**	**19% (29/155)**

CHAPTER THREE

Transmission of Antibiotic-Resistant *E. coli* from Chicken Litter to Agricultural Soil

AUTHOR CONTRIBUTIONS

- Dorcas O. Fatoba: co-conceptualized the study, undertook sample collection and processing, performed the laboratory experiments, analysed the data, and wrote the paper
- Akebe L. K. Abia: co-conceptualised the study, supervised the project, and undertook a critical revision of the manuscripts
- Daniel G. Amoako: co-analysed the data and undertook a critical revision of the manuscripts
- Sabiha Y. Essack: supervised and co-conceptualized the study, facilitated ethical approval and sample collection, vetted the data analysis and undertook a critical revision of the manuscript.

Objective(s) covered: This manuscript addresses objectives 1, 2, and 3

This manuscript will be submitted to Frontiers in Environmental Science

Transmission of Antibiotic-Resistant *E. coli* from Chicken Litter to Agricultural Soil

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Keywords: antibiotics; antibiotic resistance; *E. coli*; multidrug-resistance; chicken litter; litter-amended soil.

Abstract

A growing concern regarding the use of animal manure as fertilizer is the contamination of soil, plants, and the environment with a variety of antibiotic-resistant and pathogenic bacteria. This study quantified and characterized the antibiotic resistance profiles of *Escherichia coli* in soil before and after chicken litter application to determine the impact of manure on the soil resistome. Litter and soil samples were collected from a sugarcane field before and after litter application. *E. coli* was isolated and quantified using the Colilert®-18/ Quanti-tray® 2000 and 10 randomly selected isolates from the positive wells of each Quanti-tray were putatively identified on eosin methylene blue agar. Real-time PCR was used to confirm the isolates by targeting the *uidA* gene. Antibiotic susceptibility test against 18 antibiotics was conducted using the disk diffusion method, and the multiple antibiotic resistance index (MARI) was calculated. Soil amendment with chicken litter significantly increased the number of antibiotic-resistant *E. coli* in the soil. Among the 126 *E. coli* isolates purified from all the samples, 76% showed resistance to at least one antibiotic, of which 54.2% were multidrug-resistant (MDR). The highest percentage resistance was to tetracycline (78.1%), with the least percentage resistance (3.1%) to imipenem, tigecycline, and gentamicin. The isolates also showed resistance to chloramphenicol (63.5%), ampicillin (58.3%), trimethoprim-sulfamethoxazole (39.6%), cefotaxime (30.2%), ceftriaxone (26.0%), cephalexin (20.8%), cefepime (11.5%), amoxicillin-clavulanic acid (11.5%), cefoxitin (10.4%), Nalidixic acid (9.4%), amikacin (6.3%), and ciprofloxacin (4.2%). Of the 54.2% (52/96) of MDR isolates, the highest number isolated from the litter-amended soil (61.5%) and the least in isolates from soil samples collected before litter application (1.9%). The relatively higher mean MAR index of the litter-amended soil (0.14), compared to the soil before the amendment (0.04), suggests soil pollution with antibiotic-resistant *E. coli* from sources of high antibiotic use. *E. coli* could only be detected in the soil up to 42 days following manure application, making it a suitable short-term indicator. Notwithstanding its relatively short detectability/survival, the application of chicken litter appeared to transfer antibiotic-resistant *E. coli* to the soil, enhancing the soil resistome and highlighting the consequences of such agricultural practices on public health.

1.0 Introduction

The increasing prevalence of antibiotic-resistant bacteria (ARB) in the environment is a growing global threat to public health in the 21st century (Udikovic-Kolic et al., 2014, Wellcome Trust, 2018). The misuse and overuse of antibiotics in food-animal production contributes to the emergence and subsequent spread of antibiotic resistance from animals to the environment (Laxminarayan et al., 2013, WHO 2020). A significant route by which ARB enter the environment and the food chain is through manure from antibiotic-treated animals applied to agricultural soil (Heuer et al., 2011; Marti et al., 2013). However, the impact of this agricultural practice on the soil resistome is not well known, particularly in African countries.

Animal manure is often applied to agricultural soil as a substitute for inorganic fertilisers to meet the growing demand for crops and improve soil fertility, particularly in organic farming (Jechalke et al., 2013; Marti et al., 2013; Atidégla et al., 2016). Also, the application of chicken litter to agricultural soil as organic fertiliser is the cheapest means of disposing of the large volumes of poultry waste generated from the rapidly growing poultry industry worldwide (Kyakuwaire et al., 2019). Furthermore, chicken litter, a mixture of chicken faeces, waste feed, wood shavings, and other small invertebrates, is a means of soil amendment that improves and maintains the chemical, physical, and biological soil properties (Brye et al., 2004).

Despite their increased use, a growing concern about the application of untreated animal manure to agricultural soil is the possibility of contamination with pathogenic ARB and antibiotic resistance genes (ARGs), as animal manure is considered a significant reservoir of both enteric and pathogenic ARB and ARGs (Robins-Browne, 2005; Johnson et al., 2016). The addition of ARB of animal origin to the soil can also lead to horizontal transfer of ARGs between the manure-borne bacteria and the indigenous soil bacteria through mobile genetic elements (MGEs) (Heuer et al., 2011). Studies investigating the impact of animal manure on soil resistome have shown that applying animal manure to soil increased the abundance of ARB and the diversity of ARGs in soils. Although most studies have indicated that such increases are temporal because bacteria from manure are less adapted to soil environments (Sengeløv et al., 2003b; Heuer and Smalla, 2007; Binh et al., 2008; Marti et al., 2014; Muurinen et al., 2017), other studies have found certain ARB to survive in manure-amended soil for extended periods (Islam et al., 2004; Merchant et al., 2012; Çekiç et al., 2017). These ARB and ARGs can subsequently enter the food chain through contaminated farm produce or spread to surface water bodies as run-off from the soil (Marti et al., 2013), posing severe human health risks.

Several infection outbreaks have been linked to *E. coli* in food contaminated by animal manure (Atidégla et al., 2016; Yang et al., 2017; Shonhiwa et al., 2019).

There is a paucity of information on the environmental dimensions of AMR in Africa, as most of the AMR surveillance and research focuses on the prevalence of ARB in humans and food animals (farm-to-fork) (Mbelle et al., 2019; McIver et al., 2020; Abdalla et al., 2021). However, a substantial number of ARB can be transferred to the soil via animal manure application, and attempts to identify them may not be financially and technologically feasible. Therefore, identifying an organism suitable to be an indicator of such pollution is necessary. *E. coli* has been used as an indicator of faecal pollution for centuries. Recently, the World Health Organization (WHO) has recommended using *E. coli* to trace AMR because its molecular mechanisms of resistance are well characterised (WHO, 2020). However, several studies have repprted on the relatively shorter duration of survival of *E. coli* in the environment compared with other organisms (Sengeløv et al., 2003a; Bolton et al., 2011; Abia et al., 2015b). In addition, a previous study showed that *Enterococcus* could be found in litter amended soils up to 105 days following manure application (Fatoba et al., 2021). Therefore, this study investigated the potential transmission of antibiotic-resistant *E. coli* from chicken litter to agricultural soil and sought to determine how long *E. coli* could be detected in litter amended soil following chicken litter application. This study evaluated the prevalence, antibiotic resistance profiles, and the MAR indices of *E. coli* isolated from chicken litter and the soil of a sugarcane field before and after chicken litter application.

2.0 Materials and methods

2.1 Ethical clearance

This study was approved by the Biomedical Research Ethics Committee (Reference: BCA444/16) of the University of KwaZulu-Natal.

2.2 Study site and sample collection

This study was carried out on a sugarcane field located in uMshwathi Local Municipality in uMgungundlovu District, KwaZulu-Natal, South Africa, fertilized with chicken litter. The

study site and its surroundings have previously been described (Fatoba et al., 2021). The sample collection was carried out for one hundred and sixteen days (October 2018 to February 2019). Soil samples were collected for five days before manure application (i.e. days 1, 2, 3, 5, and 9). Samples were also collected on the day of manure application over 111 days. Samples were collected until no microbial counts were recorded in three consecutive sampling rounds. The chicken litter was a mixture of raw chicken faeces and wood shavings from a large-scale chicken farm that supplements feed and water with antibiotics in the uMgungudlovu District. The poultry farmer uses zinc bacitracin, olaquindox, and avilamycin, for growth promotion. Doxycycline, macrolide-lincosamides (tylosin, kitasamycin), enrofloxacin, sulfadiazine-trimethoprim, and zinc bacitracin are used for therapeutic purposes (personal communication with the farmer). A detailed sampling regime has previously been described (Fatoba et al., 2021).

2.3 Quantification and purification of *E. coli*

E. coli was detected and quantified using the Colilert®-18 / Quanti-Tray® 2000 system (IDEXX Laboratories (Pty) Ltd., Johannesburg, South Africa) according to the manufacturer's guidelines. All the samples collected were processed as previously described by Abia et al. (2015a). Briefly, approximately 5g of homogenised soil or litter samples were resuspended in sterile distilled water, and the supernatant analysed using the IDEXX defined substrate multiple tube technique as recommended by the manufacturer. The most probable number (MPN) of *E. coli* in 100 mL of sample (MPN/100mL) was calculated as recommended by IDEXX.

Following incubation, pure *E. coli* isolates were obtained by subculturing the content of fluorescent quanti-trays several on Eosin Methylene Blue agar plates (Oxiod, Hampshire, England) and incubating at 37 °C for 24 h as previously described (Abia et al., 2015b). The presumptive pure, distinct colonies obtained from the selective media plates were stored in trypticase soy broth (TSB) with 20% glycerol at -80^{o}C for further analysis.

2.4 Molecular confirmation of the *E. coli* isolates

DNA extraction was carried out on overnight grown *E. coli* cultures using the boiling method previously described (Dashti et al., 2009). All the *E. coli* isolates were then confirmed by real-time polymerase chain reaction using specific primers sets that targeted the *uidA* (encoding

beta-glucuronidase) gene as described by López-Saucedo et al. (2003) using the forward and reverse primers 5'-AAAACGGCAAGAAAAAGCAG-3' and 5'-ACGCGTGGTTAACAGTCTTGCG-3', respectively. The positive control used was *E. coli* ATCC 25922 (American Type Culture Collection, Manassas, VA, USA), and the no-template control was the reaction mixture without template DNA. The PCR protocols were as previously described (Abia 2015b).

2.5 Antibiotic susceptibility testing

The antibiotic susceptibility test of the *E. coli* isolates was carried out according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2020) using the disk diffusion method on a panel of 18 antibiotics (Figures 2 & 3). Zones of inhibition were interpreted according to CLSI breakpoints except for tigecycline (15 µg) and cephalexin (30 µg), where the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints were used. Multidrug resistance is defined as resistance to at least one drug in three or more different classes of antibiotics. *E. coli* ATCC 25922 was used as the control strain. The multiple antibiotic resistance (MAR) index of each isolate was calculated as a/b, where a is the number of antibiotics to which a tested isolate expressed resistance, and b is the number of antibiotics to which the isolate has been evaluated for susceptibility (Krumperman, 1983).

2.6 Statistical Analysis

The data on *E. coli* counts were log-transformed and analysed using Microsoft Excel 2016 and Statistical Package for the Social Science SPSS version 26 (IBM Corporation, Armonk, New York, USA). One-way analysis of variance (ANOVA) and Games-Howell Post-hoc test was used to check for any significant differences in the mean counts of *E. coli* and the number of antibiotic-resistant *E. coli* in the soil before and after litter amendment and the chicken litter. All statistical tests were considered significant at $p<0.05$. For ease of data presentation, the most probable number per gram (MPN/g) of samples with values <1 was considered as 1 for log-transformation and average calculations.

3.0 Results

3.1 Mean concentrations of *E. coli*

A total of 193 samples (45 chicken litter and 148 soil) were collected. Among the three sample groups, chicken litter had the highest (4.09E + 07) *E. coli* counts per sample round (**Table 1**). The overall mean count of *E. coli* in the chicken litter (2.11 x 10^7 ±1.29 MPN/g) was significantly higher than the litter-amended soil (p = 0.020), and the soil samples collected before the litter amendment (p = 0.023) (**Table S1**). There was no statistically significant difference (p = 0.999) in the overall mean count of *E. coli* in the litter-amended soil (1.51 (±0.99) x 10^7 MPN/g) and the soil samples collected before the litter amendment (1.52 (±0.72) x 10^7 MPN/g) (**Table S1**). The *E. coli* counts in the soil and the stored chicken litter fluctuated throughout the sampling period. No *E. coli* was detectable in the soil 49 days after the litter amendment.

3.2 Prevalence and Antibiotic susceptibility profiles of the *E. coli* isolates

A total of 126 *E. coli* isolates were recovered from all the positive samples, with 88 from the litter-amended soil, 10 from soil samples before the litter amendment, and 28 from the chicken litter. Seventy-six percent (96/126) of the *E. coli* isolates displayed resistance to at least one of the antibiotics tested. The highest number was recorded in the litter-amended soil (71.9%, 69/96), followed by the chicken litter (27.1%, 26/96) and in soil samples collected before the litter amendment (1%, 1/96). The highest percentage resistance was to tetracycline (78.1%), while the least (3.1%) was to imipenem, tigecycline, and gentamicin (3.1%) (**Fig 1**). In addition, all the isolates were susceptible to meropenem and ceftazidime. Notably, there was an increased detection of *E. coli* isolates resistant to tetracycline, ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole in the soil after the chicken litter application (**Fig 2**).

The overall prevalence of antibiotic-resistant *E. coli* in the litter-amended soil was statistically significantly higher than in the soil samples before amendment (p = 0.001) and chicken litter (p = 0.001) (**Table S2**).

3.3 Multidrug resistance

Multidrug resistance was evident, and the predominant resistance patterns were ampicillin-chloramphenicol-tetracycline and chloramphenicol-tetracycline-trimethoprim-sulfamethoxazole (**Table 2**). In total, 54.2% (52/96) of the isolates were multidrug-resistant, grouped into 21 different resistance patterns. The highest prevalence of MDR was detected in the litter-amended soil (61.5%) and the least in isolates from soil samples collected before litter application (1.9%) (**Table 2**). Interestingly, two isolates, one from each of the chicken litter and litter-amended soil, displayed resistance to ten antibiotics that belong to 6 and 4 classes of antibiotics, respectively.

3.4 Multiple antibiotic resistance (MAR) index of the *E. coli* isolates

The MAR index of all the isolates ranged between 0.11 and 0.56, representing resistance to two and ten antibiotics, respectively (**Table 3**). Overall, 38.5% (37/96) of the resistant isolates had a MARI > 0.2 with the highest rate (51.4%) in the litter-amended soil and the least in the soil before litter amendment (2.7%).

4.0 Discussion

An anthropogenic activity like the application of manure from food animals exposed to antibiotics to soil can increase the burden of AMR in the soil environment, thereby posing a public health threat, particularly when potential pathogenic ARB like *E. coli* enter the food chain. This study investigated the potential transmission of antibiotic-resistant *E. coli* from chicken litter to agricultural soil in KwaZulu-Natal, South Africa. The chicken litter amendment increased the bacterial count and the number of antibiotic-resistant isolates in the soil. Antibiotic-resistant *E. coli* was detected in all the sample points and the three sample groups with the highest prevalence in the litter-amended soil. The isolates displayed high percentage resistance to tetracycline, ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole commonly used in poultry farms. Also, 54.2% of all the isolates were MDR. The relatively high percentage (51.4%) of isolates with MAR index > 0.2 in the litter-amended soil compared to the soil before the litter amendment (2.7%) indicated that the litter amendment results in soil contamination with *E. coli* from sources with high use of antibiotics.

4.1 Mean concentrations of *E. coli*

E. coli is an established indicator of faecal contamination because of its ubiquitous presence in the intestines of animals and its prominence in faecal-contaminated environments (Aarestrup et al., 2008). This explains the highest *E. coli* count recorded in the chicken litter, making it a potential source of *E. coli* contamination to the receiving environment. There was an increase in *E. coli* count in the soil at the time (day 0) the chicken litter was applied and days (D3, D14, and D21) after the soil amendment (**Table 1**). The litter amendment could have contributed to the observed increase, as previous studies have indicated that the addition of animal manure to soil increases the number of viable bacteria in soil by the enrichment of indigenous soil bacteria or addition of manure-borne bacteria (Zhu et al., 2013).

In this study, a short-term (42 days) persistence of *E. coli* in the soil was observed following the amendment suggesting that *E. coli* can serve as a suitable indicator of short-term faecal pollution in agricultural soil. This finding is consistent with previous reports (Sengeløv et al., 2003a; Binh et al., 2008; Bolton et al., 2011), that indicated that some bacteria from animal manure are less adapted to the soil environment and only survive for a short time (9 days to 11 weeks). Also, several indicator organisms like enterococci, *E. coli*, faecal coliforms, and *Clostridium perfringens* are the commonly tested faecal pollution indicators. However, the limitations and strength of each of these indicators suggest that none of these indicator organisms should be used in isolation for predicting the impact of faecal pollution in any environment (Tyagi and Chopra, 2006). *E. coli* hardly survive under environmental stress such as limited moisture, low organic matter, high and low temperatures (Berry and Miller 2005; Williams et al., 2005). Contrary to *E. coli*, high densities of enterococci in soils has been attributed, in part, to the more excellent survival abilities of Gram-positive bacteria (e.g., enterococci and staphylococci) than of Gram-negative bacteria (e.g., *E. coli*, and *Pseudomonas* spp.) in the face of environmental stresses, particularly desiccation and cellular injury (Bale et al., 1993, Byappanahalli et al., 2012). As such, the long-term persistence of antibiotic-resistant enterococci in soils may increase the chance of AMR transmission in the agricultural soil environment.

Fatoba et al. 2021 showed that resistant enterococci were still detectable in the litter-amended soil even at 105 days after the litter application. However, *E. coli* was no longer detectable after day 42 in the current study, suggesting that the long-term impact and accurate monitoring of

the soil environment for bacterial contamination from manure-based fertilizers requires a more persistent indicator organism alongside *E. coli*. Supporting this finding, a previous study of faecal pollution in riverbed sediments in South Africa recommended using *Clostridium perfringens* and *E. coli* as indicators of soil faecal pollution (Abia et al., 2015b). Although *E. coli* was not recorded in the amended soils after 42 days, some isolates were still recovered from the unapplied chicken litter heap. These isolates also displayed multidrug resistance. This could be because of the rich nutrient content in the chicken litter, while the disappearance in the amended soil could have also been influenced by other farm practices like urea application.

4.2 Antibiotic susceptibility profiles of the *E. coli* isolates

The use of antibiotics in food animal production has been beneficial for economic and animal health reasons. Thus, different antibiotic classes are used in food-animal production, depending on the purpose (prophylaxis, metaphylaxis, treatment, or growth promotion), the kind of animal, and the country's policies. However, their overuse and misuse have led to increased detection of ARB in manure, which in most cases is released into the environment for soil quality improvement (Looft et al., 2012; Johnson et al., 2016). Additionally, antibiotic administration patterns and quantities used in food animal production vary considerably from country to country, region to region, and farm to farm, resulting in substantial differences in the rate of resistance recorded in many studies (Van Boeckel et al., 2015). Studies conducted within South Africa and other countries have reported varying levels of *E. coli* resistance to the antibiotics included in the current study.

Overall, the *E. coli* isolates examined in this study expressed the highest percentage of resistance to tetracycline (78.1%), followed by chloramphenicol (63.5%), ampicillin (58.3%), and trimethoprim-sulfamethoxazole (39.6%), correlating with the frequent use of doxycycline and trimethoprim-sulphadiazine in poultry farms in KwaZulu-Natal Province, South Africa. Furthermore, two surveys carried out in South Africa on antimicrobial use in food animals showed that the highly consumed antibiotics in food animal production in South Africa include the macrolides, tetracyclines, sulphonamides, and the penicillins (Henton et al., 2011; Eagar et al., 2012). Therefore, the increased detection of *E. coli* resistant to these four antibiotics (tetracycline, ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole) in the soil after the litter amendment suggests emergence on the farm followed by transmission of antibiotic-resistant *E. coli* and/or ARGs from the chicken litter to the soil.

The high prevalence of chloramphenicol resistance was not expected. This antibiotic is not permitted for use in food-animal production in South Africa (Eagar et al., 2012), neither is it among the antibiotics used on the poultry farms in KwaZulu-Natal (personal communication). Therefore, the high chloramphenicol resistance in the absence of chloramphenicol selection pressure may be due to co-selection and/or co-transmission of chloramphenicol resistance due to genetic linkage to genes conferring resistance to antibiotics that are commonly used in poultry farms. For example, the co-selcetion of chloramphenicol resistance with resistance to sulfamethoxazole, tetracycline, and kanamycin due to frequent use of sulphonamides, aminoglycosides, and tetracyclines in food animals has been reported in the United States (Bischoff et al., 2005) who demonstrated the conjugative transfer of the chloramphenicol resistance gene *CmlA* with both sulphonamide (*sul*) and aminoglycoside (*aadA*) resistance genes on class 1 integrons from swine-borne *E. coli* donors to the recipient *E. coli* strains (Bischoff et al., 2005). Since aminoglycosides are not used in food-animal production in South Africa, the common use of sulphonamides may be responsible for the spread of chloramphenicol resistance among the isolates. Although the resistance of *E. coli* to the third-generation cephalosporins (cefotaxime and ceftriaxone) was relatively low compared to tetracycline, chloramphenicol, ampicillin, and trimethoprim-sulfamethoxazole, the high frequency observed in chicken litter isolates needs urgent monitoring as cephalosporins are important front-line antibiotics widely used to treat infections caused by Gram-negative bacteria in humans. Thus, to curb the spread of AMR in food animal production and the environment, there is a need to implement policies that will ensure strict and proper use of available antibiotics.

In this study, the chicken litter application significantly increased the detection of antibiotic-resistant *E. coli* in the soil. This could be ascribed to the combined proliferation of indigenous soil *E. coli* and exogenous manure-borne ones, both of which were enhanced by the added nutrients from the chicken litter. These findings are consistent with previous studies that have indicated that land application of animal manure can result in the introduction of new ARB and ARGs of animal manure origin to the soil or increase the quantity of already existing soil ARB and ARGs (Sengeløv et al., 2003a; Udikovic-Kolic et al., 2014). Udikovic-Kolic et al. (2014), in a field experiment conducted in the USA, showed that cow manure amendment increased *β*-lactam-resistant bacteria in the manured soil. The increase was attributed to the enrichment of the ARB initially present in the soil. Another field experiment conducted on four farms in Denmark indicated that the temporary increase in tetracycline-resistant Gram-negative bacteria

in the soil after the spread of pig manure slurry was due to resistant intestinal bacteria of manure origin (Sengeløv et al., 2003a).

E. coli isolates were only detectable until 42 days following manure application. This short-term detection suggests that *E. coli* is only suitable as a good indicator of recent or short-term AMR pollution in agricultural soil environments. Although *E. coli* was not found in the soil after 42 days following manure amendments, it is not certain if its resistance genes were still present in the litter-amended soil. Thus, studies to determine if *E. coli*-associated resistance genes would survive long in the environment should be conducted, as these could be transferred horizontally to closely related bacteria (Poole et al., 2017).

4.5 Multidrug resistance and Multiple antibiotic resistance (MAR) index of the *E. coli* isolates

In this study, 41% of the *E. coli* isolates were MDR, with the highest rate (61.5%) in the litter-amended soil. The highest number and most diverse resistance patterns in soil were recorded on the day of litter application (day 0), suggesting a major influx of MDR *E. coli* from the litter into the soil environment. Furthermore, similar resistance patterns in the litter-amended soil and the chicken litter indicates possible transmission and mobility of ARGs between the litter-borne *E. coli* and the *E. coli* present in soil throughout sample collection. Therefore, the presence of MDR *E. coli* up to 77 days in the stored chicken litter heap on the sugarcane field is of great concern, as it can be a source of continuous MDR *E. coli* contamination to the soil environment, plants, the drainage channel on the field and surrounding water bodies through run-off.

The MAR indexing method is a simple and cost-effective indicator of ABR trends (Osundiya et al., 2013; Sandhu et al., 2016). This study showed that 60%, 22%, and 10% of the isolates from the chicken litter, litter-amended soil, and soil before litter amendment had a MARI > 0.2, indicating that they originated from environments of high antibiotic exposure (Krumperman, 1983). The average MAR index of 0.25 observed in the chicken litter isolates in this study further attests to the high usage of antibiotics in the poultry farm where the chicken litter was obtained. Furthermore, the relatively high percentage (51.4%) of isolates with MAR index > 0.2 in the litter-amended soil compared to the soil before the litter amendment (2.7%) shows that the application of chicken litter resulted in soil contamination with *E. coli* from sources with high use of antibiotics. The MAR indices intimate that the litter-amended soil and

the chicken litter should be considered significant reservoirs of MDR *E. coli* and should undergo pre-treatment (e.g., composting) before it is used as fertiliser.

5.0 Conclusion

The present study show that chicken litter is a major reservoir of antibiotic-resistant *E. coli* that can be transferred to soil. The increase in the number of antibiotic-resistant *E. coli* immediately following litter application suggests a significant influx of resistant *E. coli* from the chicken litter to litter-amended soil. The increased detection of resistance to antibiotics commonly used for treatment and growth promotion in poultry farms in KwaZulu-Natal in the soil after the amendment further attests to the transmission of litter-borne *E. coli* to the soil. The higher number of isolates with MAR index > 0.2 in the litter-amended soil compared to the soil before the litter amendment indicates soil contamination with *E. coli* from sources with high use of antibiotics such as the chicken litter. Finally, relying on *E. coli* alone to predict the effect of chicken litter application on AMR in the environment would only provide short-term evidence; other organisms like enterococci should be included in monitoring schemes to understand the long-term effects. The presence of *E. coli* with resistance to antibiotics of clinical importance in the agricultural environment could pose a severe risk to public health. Thus, biosecurity measures that will ensure prudent use of antibiotics in food animal production and pre-treatment of animal manure (composting or anaerobic digestion) which reduces AMR in manure should be put in place in South Africa to curb the spread of ABR.

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Table 1: Mean *E. coli* counts ((MPN/g) throughout the sample collection

Sample collection day	Mean *E. coli* count (MPN/g) per sample point						The geometric mean of *E. coli* count (MPN/g)/ Sample group		
	A	B	C	D	E	H	Soil before litter amendment	Litter-amended soil	Chicken litter
D1	1.30E + 07	1.85E + 07	2.58E + 07	2.61E + 07	-	-	1.99E + 07	-	-
D2[b]	1.88E + 07	2.31E + 07	2.61E + 07	2.01E + 07	1.00E + 00	-	1.84E + 07	-	-
D3[r]	1.00E + 00	1.00E + 00	1.00E + 00	1.00E + 00	1.00E + 00	-	1.00E + 00	-	-
D5	1.00E + 00	1.00E + 00	1.00E + 00	1.00E + 00	1.46E + 07	-	1.07E + 07	-	-
D9	2.92E + 07	1.94E + 06	1.00E + 00	1.00E + 00	1.00E + 00	-	1.38E + 07	-	-
D0	2.61E + 06	1.40E + 07	1.15E + 07	3.45E + 06	1.45E + 07	1.63E + 07	-	1.72E + 07	1.56E + 07
D1	1.00E + 00	1.77E + 07	1.89E + 07	1.00E + 00	2.03E + 06	2.15E + 07	-	1.40E + 07	2.13E + 07
D3	1.00E + 00	2.29E + 06	2.27E + 07	1.82E + 07	2.27E + 07	1.84E + 07	-	1.68E + 07	1.52E + 07
D7u	1.00E + 00	1.00E + 00	1.00E + 00	1.00E + 00	1.65E + 07	1.80E + 07	-	1.09E + 06	1.76E + 07
D14[r]	2.76E + 07	2.10E + 07	1.15E + 07	1.15E + 07	1.00E + 00	3.93E + 07	-	1.45E + 07	3.76E + 06
D21	3.60E + 07	1.40E + 07	1.00E + 00	2.80E + 07	2.99E + 08	3.41E + 07	-	1.89E + 07	4.09E + 07
D28	1.00E + 00	1.00E + 00	1.00E + 00	1.00E + 00	1.46E + 07	1.20E + 07	-	1.07E + 07	1.92E + 07
D35	1.40E + 07	1.00E + 00	1.00E + 00	1.00E + 00	1.00E + 00	1.60E + 07	-	1.06E + 07	1.41E + 06
D42r	1.00E + 00	1.00E + 00	1.00E + 00	1.00E + 00	2.84E + 07	2.83E + 07	-	1.17E + 07	2.57E + 07

D49[d]	1.00E + 00	1.00E + 00	1.00E + 00	1.00E + 00	1.00E + 00	3.28E + 07	-	1.00E + 00	3.23E + 07
D56	-	-	-	-	-	1.00E + 00	-	-	1.00E + 00
D63	-	-	-	-	-	1.00E + 00	-	-	1.00E + 00
D77[r]	-	-	-	-	-	2.29E + 07	-	-	1.67E + 07
D91	-	-	-	-	-	1.81E + 07	-	-	1.51E + 07
D105	-	-	-	-	-	1.26E + 07	-	-	1.21E + 07
Overall Mean count	1.63E + 07	1.47E + 07	1.38E + 07	1.52E + 07	1.58E + 07	2.11E + 07	1.52E + 07	1.51E + 07	2.11E + 07

- Sample not collected. The farm was burnt (b) on day 2 (D2), chicken litter, and urea (u) salt was applied on day 0 (D0) and day 7 (D7) respectively.

E. coli reduced to detection limit in soil on day 49 (D49).

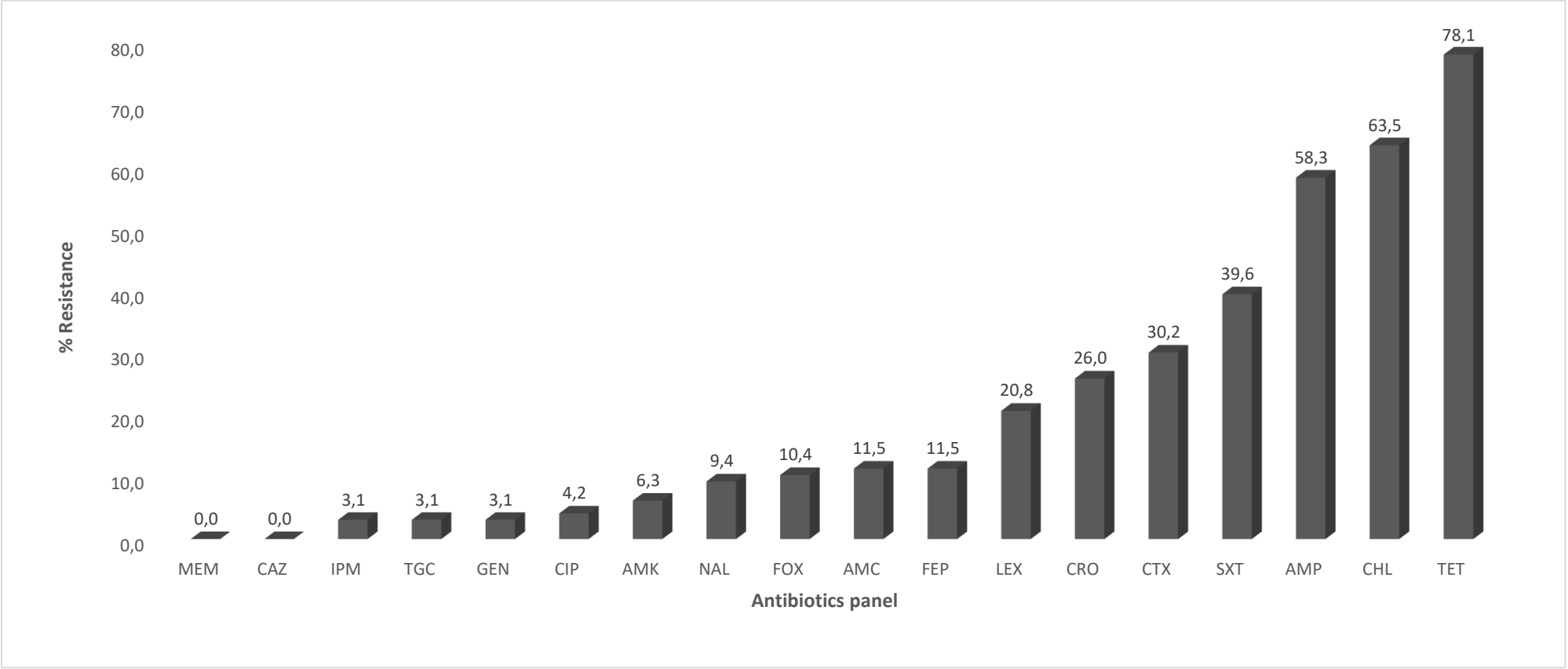

Fig 1: Percentage resistance of the *E. coli* isolates.

LEX-Cefalexin, FEP- Cefepime, CTX-Cefotaxime, CAZ-Ceftazidime, AMP-Ampicillin, FOX-Cefoxitin, TGC-Tigecycline, CIP-Ciprofloxacin, TET-Tetracycline, SXT- Trimethoprim-sulfamethoxazole, AMC-Amoxicillin-clavulanic acid, CHL- Chloramphenicol, GEN- Gentamicin, AMK-Amikacin, MEM-Meropenem, CRO-Ceftriaxone, IPM-Imipenem, NAL-Nalidixic acid.

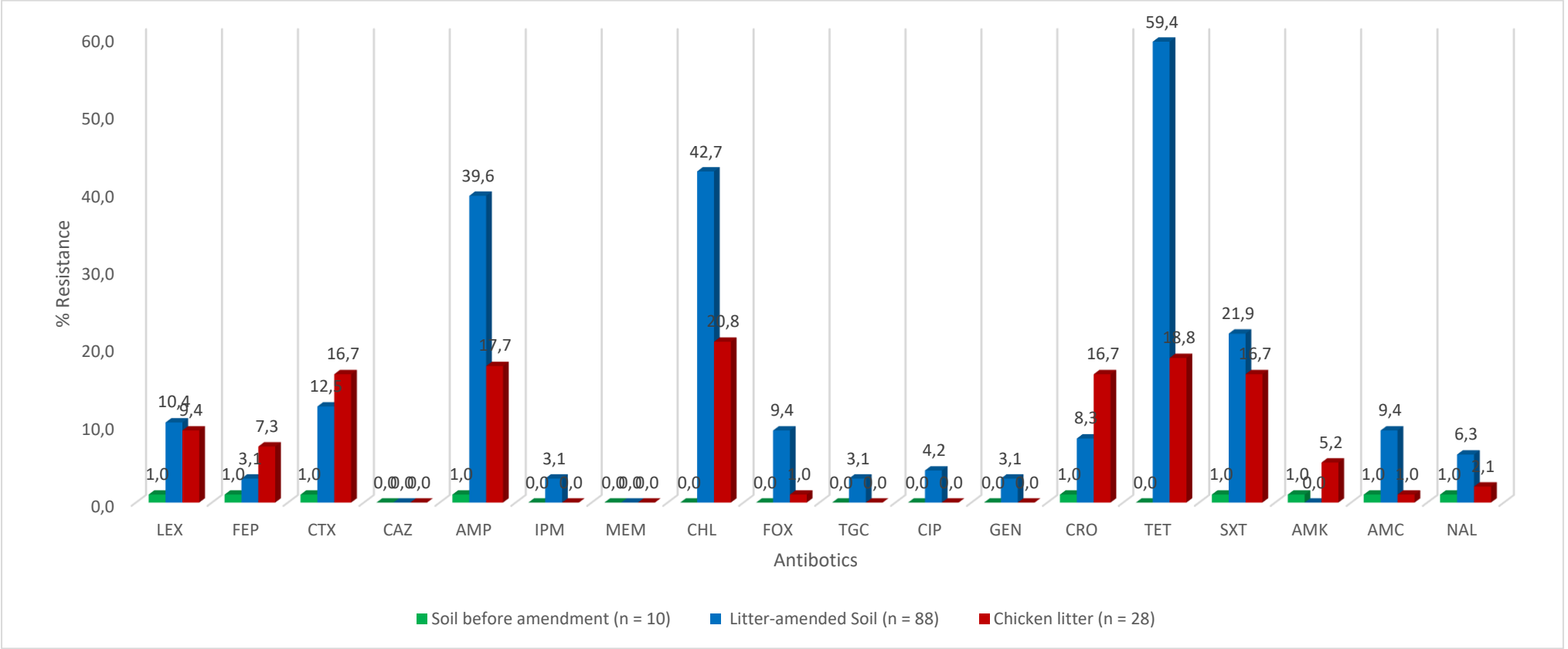

Fig. 2: Percentage resistance of *E. coli* stratified by sample source

LEX-Cefalexin, FEP- Cefepime, CTX-Cefotaxime, CAZ-Ceftazidime, AMP-Ampicillin, FOX-Cefoxitin, TGC-Tigecycline, CIP-Ciprofloxacin, TET-Tetracycline, SXT- Trimethoprim-sulfamethoxazole, AMC-Amoxicillin-clavulanic acid, CHL- Chloramphenicol, GEN- Gentamicin, AMK-Amikacin, MEM-Meropenem, CRO-Ceftriaxone, IPM-Imipenem, NAL-Nalidixic acid.

Table 2: Multidrug-resistance patterns of the *E. coli* isolates

Antibiogram	Soil before litter (n = 10)	Litter- amended Soil (n = 88)	Chicken litter (n = 28)	Total
FARM BURNING DAY (D2)				
AMP-AMC-LEX-CTX-CRO-FEP-AMK-SXT	1	0	0	1
DAY 0 OF LITTER APPLICATION				
AMP-CHL-TET	0	8	0	8
AMP-CTX-CRO-NAL-CHL-TET	0	1	0	1
AMP-CTX-CRO-CHL-TET	0	2	5	7
AMP-CHL-TET-SXT	0	3	0	3
AMP-CTX-CHL-TET	0	1	0	1
AMP-CHL-CIP-GEN-TET-SXT	0	1	0	1
AMP-NAL-CIP-GEN- CHL-TET-SXT	0	1	0	1
AMP-TET-SXT	0	7	0	7
DAY 3 AFTER LITTER APPLICATION				
AMP-AMC-LEX-FOX-CTX-CRO-FEP-TET-SXT	0	1	0	1
AMP-AMC-LEX-FOX-CTX-CRO-TET-SXT	0	1	0	1
DAY 7 AFTER LITTER APPLICATION				
AMP-AMC-LEX-FOX-CTX-CRO-FEP-TET-TGC-SXT	0	1	0	1
DAY 14 AFTER LITTER APPLICATION				

AMP-LEX-NAL-CIP-GEN-CHL-TET	0	1	0	1
AMP-LEX-CTX-CRO-FEP-NAL-CIP-TET-SXT	0	1	0	1
DAY 21 AFTER LITTER APPLICATION				
AMP-AMC-TET-SXT	0	1	0	1
CHL-TET-SXT	0	1	7	8
AMP-AMC-FOX-TET-SXT	0	1	0	1
DAY 49 AFTER LITTER APPLICATION				
AMP-LEX-CTX-CRO-FEP-NAL-AMK-CHL-TET-SXT	0	0	1	1
AMP-LEX-CTX-CRO-FEP-CHL-TET-SXT	0	0	4	4
AMP-LEX-CTX-CRO-FEP-NAL-AMK-SXT	0	0	1	1
DAY 77 AFTER LITTER APPLICATION				
AMK-CHL-TET-SXT	0	0	1	1
Total MDR Isolates (n = 52, 54.2%)	1 (1.9%)	32 (61.5%)	19 (36.5%)	52

LEX-Cefalexin, FEP- Cefepime, CTX-Cefotaxime, CAZ-Ceftazidime, AMP-Ampicillin, FOX-Cefoxitin, TGC-Tigecycline, CIP-Ciprofloxacin, TET-Tetracycline, SXT- Trimethoprim-sulfamethoxazole, AMC-Amoxicillin-clavulanic acid, CHL- Chloramphenicol, GEN- Gentamicin, AMK-Amikacin, MEM-Meropenem, CRO-Ceftriaxone, IPM-Imipenem, NAL-Nalidixic acid

Table 3: Multiple antibiotic resistance (MAR) index of the isolates

MAR index	No. of isolates	Percentage
0.11	26	27.1%
0.17	25	26.0%
0.22	10	10.4%
0.28	9	9.4%
0.33	4	4.2%
0.39	2	2.1%
0.44	8	8.3%
0.50	2	2.1%
0.56	2	2.1%

Supplementary Materials

Table S1: Summary of statistical analysis of differences in *E. coli* count in the soil and chicken litter

Group	N	*E. coli* count (log MPN/g)		Overall ANOVA p-value	Pair wise comparison (Games-Howell)		95% confidence interval for mean (log MPN/g)		p-value	Statistical Test used
		Mean	±SD			Mean difference (±SE)	Lower bound	Upper bound		
1	48	1.52	0.72	0.003*	1 vs 2	0.01 (± 0.14)	-0.33	0.35	0.999	One way ANOVA; Games Howell
2	100	1.51	0.99		1 vs 3	-0.59 (± 0.22)*	-1.11	-0.07	0.023*	
3	45	2.11*	1.29		2 vs 1	-0.01 (± 0.14)	-0.35	0.33	0.999	
					2 vs 3	-0.60 (± 0.22)*	-1.12	-0.08	0.020*	
					3 vs 1	0.59 (± 0.22)*	0.07	1.11	0.023*	
					3 vs 2	0.60 (± 0.22)*	0.08	1.12	0.020*	

*P<0.05; 1- Soil before litter application; 2- Litter-amended soil; 3- Chicken litter

Table S2: Summary of statistical analysis of differences in the number of antibiotic-resistant *E. coli* in the soil and chicken litter

Group	N	Antibiotic resistant *E. coli*		Overall ANOVA p-value	Pair wise comparison (Hochberg's GT2)		95% confidence interval for mean (log MPN/g)		p-value	Statistical Test used
		Mean	±SD			Mean difference (±SE)	Lower bound	Upper bound		
1	10	0.10	0.32	<0.001**	1 vs 2	-0.68* (0.11)	-0.97	-0.39	<0.001*	One way ANOVA; Games-Howell Post Hoc test
2	88	0.78	0.41		1 vs 3	-0.83* (0.11)	-1.12	-0.54	<0.001*	
3	28	0.93*	0.26		2 vs 1	0.68* (0.11)	0.39	0.97	<0.001*	
Total	126	0.76	0.43		2 vs 3	-0.14 (0.07)	-0.30	0.01	0.082	
					3 vs 1	0.83* (0.11)	0.54	1.12	<0.001*	
					3 vs 2	0.14 (0.07)	-0.01	0.30	0.082	

*P<0.05; 1- Soil before litter application; 2- Litter-amended soil; 3- Chicken litter

Table S3: Percentage resistance and multiple antibiotic resistance index per sample point

Sample point	No. of isolates	AMP	AMC	LEX	FOX	CTX	CAZ	CRO	FEP	IPM	MEM	NAL	CIP	GEN	AMK	TET	TGC	CHL	SXT	MAR index
A	17	6.25	1.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.08	2.08	2.08	0.00	14.58	0.00	11.46	4.17	0.13
B	12	3.13	0.00	2.08	0.00	1.04	0.00	1.04	1.04	0.00	0.00	2.08	2.08	1.04	0.00	3.13	0.00	2.08	2.08	0.09
C	13	8.33	5.21	5.21	4.17	4.17	0.00	2.08	1.04	2.08	0.00	1.04	0.00	0.00	1.04	5.21	2.08	4.17	3.13	0.20
D	12	5.21	0.00	0.00	0.00	4.17	0.00	3.13	0.00	0.00	0.00	1.04	0.00	0.00	0.00	11.46	0.00	9.38	1.04	0.16
E	44	17.71	4.17	4.17	5.21	4.17	0.00	3.13	8.33	1.04	0.00	1.04	0.00	0.00	0.00	26.04	1.04	15.63	12.50	0.12
Heap 1	28	17.71	1.04	9.38	1.04	16.67	0.00	17.71	1.04	0.00	0.00	2.08	0.00	0.00	5.21	17.71	0.00	20.83	16.67	0.25

LEX-Cefalexin, FEP- Cefepime, CTX-Cefotaxime, CAZ-Ceftazidime, AMP-Ampicillin, FOX-Cefoxitin, TGC-Tigecycline, CIP-Ciprofloxacin, TET-Tetracycline, SXT- Trimethoprim-sulfamethoxazole, AMC-Amoxicillin-clavulanic acid, CHL- Chloramphenicol, GEN- Gentamicin, AMK- Amikacin, MEM-Meropenem, CRO-Ceftriaxone, IPM-Imipenem, NAL-Nalidixic acid

CHAPTER FOUR-MANUSCRIPT 3

Genomic Analysis of Antibiotic-Resistant *Enterococcus* spp. reveals Novel Enterococci Strains and the Spread of Plasmid-Borne *Tet*(*M*), *Tet*(*L*) and *Erm(B)* Genes from Chicken Litter to Agricultural Soil in South Africa

AUTHOR CONTRIBUTIONS

- Dorcas O. Fatoba: co-conceptualised the study, performed the experiments, analysed the data, and wrote the paper
- Daniel G. Amoako: co-supervised the project, co-analysed the data, and undertook a critical revision of the manuscripts
- Akebe L. K. Abia: co-conceptualised the study, supervised the project, undertook a critical revision of the manuscripts
- Sabiha Y. Essack: supervised and co-conceptualized the study, facilitated ethical approval and sample collection, vetted the data analysis and undertook a critical revision of the manuscript.

Objective(s) covered: This manuscript addresses objectives 5, 6, and 7

Genomic Analysis of Antibiotic-Resistant *Enterococcus* spp. reveals Novel Enterococci Strains and the Spread of Plasmid-Borne *Tet*(*M*), *Tet*(*L*) and *Erm(B)* Genes from Chicken Litter to Agricultural Soil in South Africa

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Abstract

Manure from food animals exposed to antibiotics is often used as soil fertiliser, potentially releasing antibiotic-resistant bacteria (ARB) with diverse antibiotic-resistance genes (ARGs) into the soil. To determine the impact of chicken litter application on soil resistome, *Enterococcus* spp. isolated from chicken litter and soil samples collected before and after the soil amendment with chicken litter were characterised, using whole-genome sequencing and bioinformatics tools. Nineteen *Enterococcus* spp. isolates from unamended soil, litter-amended soil and chicken litter were sequenced on illumina Miseq platform to ascertain the isolates' resistome, mobilome, virulome, clonality, and phylogenomic relationships. The multilocus sequence typing (MLST) analysis revealed eight novel sequence types (STs) (ST1700, ST1752, ST1753, ST1754, ST1755, ST1756, ST1004, and ST1006). The isolates harboured multiple resistance genes including those conferring resistance to *inter alia* macrolides-lincosamide-streptogramin (*erm(B)*, lnu(B), *lnu(G)*, *lsaA*, *lsaE*, *eat(A)*, *msr(C)*), tetracycline (*tet(M)*, *tet(L)*, *tet(S)*), aminoglycosides (*aac(6')-Ii*, *aac(6')-Ii*h, *ant(6)-Ia*, *aph(3')-III*, ant(9)-Ia), fluoroquinolones (*efmA*, and *emeA*), vancomycin (*VanC {VanC-2*, *VanXY*, *VanXY*C-3, *VanXY*C-4, *VanRC*}), and chloramphenicol (*cat*). The litter-amended soil harboured new ARB (particularly *E. faecium*) and ARGs (*ant(6)-Ia*, *aac(6')-Ii*, *aph(3')-III*), *lnu(G)*, msr(C), and eat(A), *efmA)* that were not detected in the soil before the amendment. The identified ARGs were associated with diverse mobile genetic elements (MGEs) such as insertion sequences (IS6, ISL3, IS256, IS30), transposons (Tn*3* and Tn*916*) and plasmids (repUS43, repUS1, rep9b, and rep 22). Twenty-three different virulence genes encoding adherence/biofilm formation (*ebpA*, *ebpB*, *ebpC*), antiphagocytosis (*elrA*) and bacterial sex pheromones (*Ccf10*, *cOB1*, *cad*, and *camE*), were detected in the genomes of the isolates. Phylogenomic analysis revealed a close relationship between a few isolates from litter-amended soil and the chicken litter isolates. The differences in the ARG and ARB profiles in the soil before and after the litter amendment and their association with diverse MGEs indicate the mobilisation and transmission of ARGs and ARB from the litter to the soil.

Keywords: Antibiotic resistance genes, soil, *Enterococcus* species, chicken litter, whole-genome sequencing.

1.0 Introduction

The use of animal manure as organic fertiliser to increase soil fertility and crop yield is a typical farming practice in Africa. However, these fertilisers could be sources of pollutants as antibiotics are used in livestock production for growth promotion, prophylaxis, metaphylaxis, and therapeutic purposes (Yang et al., 2017). Animal manure is an important reservoir of antibiotic residues, antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) that could be transferred to manure-fertilised soil (Heuer et al., 2011; Chen and Jiang, 2014; He et al., 2014). Mobile genetic elements (MGEs) such as plasmids, transposons, integrons, integrative and conjugative elements in manure have been implicated in the horizontal transfer of ARGs between manure-borne bacteria and indigenous soil bacteria (Binh et al., 2008; Gillings et al., 2015). The ARGs in manure-fertilised soil can subsequently be transmitted to humans through the food chain, highlighting the importance of tracking ARB and ARGs in the soil environment from a public health perspective.

Enterococcal species are opportunistic pathogens in animals and humans. Their ability to acquire ARGs presents a challenge for the treatment and control of enterococcal infections. *Enterococcus faecium* is known for its unique multidrug-resistant ability, while *Enterococcus faecalis*, in addition to its antibiotic resistance traits, harbours unique virulence factors, including an advanced pheromone system that facilitates the transfer of ARGs and virulence genes (Sharifi, 2012; Lins et al., 2013). Antibiotic-resistant *Enterococcus* spp. have been reported in food animals and their production systems, farm produce, the environments (soil), and clinical settings (Hammerum, 2012; Ben Said et al., 2015; Rehman et al., 2018; Molechan et al., 2019; Ekwanzala et al., 2020; Zaheer et al., 2020). Despite the potential public health impact of antimicrobial resistance in the soil environment, the dissemination and fluidity of ARB and ARGs from manure-based fertilisers into soil resistome are not well characterised. This study used whole-genome sequencing and bioinformatics analysis to investigate the impact of chicken litter application on agricultural soil resistome. To the best of our knowledge, this is the first study ascertaining the potential impact of this farming practice in Africa.

2.0 Materials and methods

2.1 Ethical clearance

This study was part of a larger project for which ethical approval was received from the Biomedical Research Ethics Committee (Reference: BCA444/16) of the University of

KwaZulu-Natal. The study was also placed on record with the South African National Department of Agriculture, Forestry and Fisheries (Reference: 12/11/1/5 (879)).

2.2 Sampling site, sample collection, bacterial identification and susceptibility testing

The soil samples were collected from a sugarcane field in the uMgungudlovu District, South Africa. The soil has been cultivated for several years, with a known history of yearly chicken litter amendment. The soil samples were collected from the four corners and centre (approximately) of the assigned plot of the sugarcane farm on 20 field visits (Oct 2018 - Feb 2019). A detail description of the sampling site, sample collection and processing have been previously described (Fatoba et al. 2021). Briefly, *Enterococcus* spp. were isolated using the Enterolert® -18®/Quanti-Tray® 2000 system (IDEXX Laboratories (Pty) Ltd., Johannesburg, South Africa) and purified on chromocult enterococci-agar (Merck Microbiology, Darmstadt, Germany) and bile esculine azide agar (Lab M, Lancashire, UK). The molecular confirmation of the isolates was carried out by real-time polymerase chain reaction of the *tuf* gene as previously described (Molechan et al., 2019). All the PCR assays were carried out in QuantStudio 5 Applied Biosystems (Applied Biosystems, ThermoFisher Waltman Massachusetts, USA) real-time PCR. The confirmed *Enterococcus* isolates were subjected to antibiotic susceptibility tests using the disk diffusion method on Mueller-Hinton agar against 15 antibiotics as previously described (Fatoba et al., 2021). *E. faecalis* ATCC 29212 was used as a positive control. Isolates resistant to one or more antibiotics from three or more classes of antibiotics were classified as multi-drug resistant (MDR) *Enterococcus*.

2.3 Whole-genome sequencing (WGS) and Bioinformatics analysis

2.3.1. Bacterial selection, DNA extraction and genome sequencing

A total of 22 antibiotic-resistant *Enterococcus* isolates were randomly selected per sample collection day (round) and site of collection for WGS. Genomic DNA (gDNA) was extracted from a single colony from an overnight culture on nutrient agar (Oxoid, Basingstoke, Hampshire, England) using the GenElute Bacterial Genomic DNA kit (Sigma Aldrich, St. Louis, USA) according to the manufacturer's instructions. The extracted gDNA was quantified on a Nanodrop 8000 (Thermo Scientific, Waltham, MA) at 260/280 nm wavelength. Multiplexed paired-end libraries (2 x 300 bp) were prepared using the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, United States). The whole genome was sequenced on an Illumina Miseq platform (Illumina, San Diego CA, USA) with 100X coverage at the National Institute of Communicable of Disease Core Sequencing Facility, South Africa.

The quality of the raw reads was checked and trimmed using Sickle v1.33 (https://github.com/najoshi/sickle). SPAdes version 3.11 (H et al., 2012) was used for the *de novo* assembly of the raw reads. All the contiguous sequences were submitted to GenBank for gene annotation using NCBI Prokaryotic Genome Annotation Pipeline (PGAP). After WGS, three isolates failed NCBI sequence quality check and could not be assigned accession numbers. The remaining nineteen isolates were selected for further analysis, of which five were from each of the litter and unamended soil and nine were from litter-amended soil. These included *E. faecium* (n = 7), *E. casseliflavus* (n = 7), *E. faecalis* (n = 4), and *E. durans* (n = 1).

2.3.2 Molecular typing and confirmation of *Enterococcus* Isolates

Multilocus sequence typing (MLST) of the assembled genome sequences was carried out in-silico using the WGS data online platform tool PubMLST database (https://pubmlst.org/databases/), which predicted the allelic profiles of the seven *E. faecalis* (*gdh, gyd, pstS, gki, aroE, xpt, yqiL*) and *E. faecium* (*adk, atpA, ddl, gdh, purk, gyd, pstS*) housekeeping genes. The isolates that did not match any of the known sequence types were submitted to the PubMLST database to be assigned new sequence types (STs).

2.3.3 Resistome analysis

To confirm the phenotypic resistance profiles of the *Enterococcus* isolates, the ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/) using default threshold ID (90%) and minimum length (60%) values, and the Comprehensive Antibiotic Resistance Database (CARD; https://card.mcmaster.ca/analyze/rgi) (Jia et al.,2017) using the default criteria (perfect and strict hits only), were used for ARG annotation and identification. Both platforms were used to accommodate the shortfalls of each platform. Genetic mutations encoding fluoroquinolone resistance in the genomes were manually curated using NCBI's *BLA*STn (https://*bla*st.ncbi.nlm.nih.gov/*Bla*st.cgi?PAGE_TYPE=*Bla*stSearch).

2.3.4 Virulome and Pathogenicity

VirulenceFinder 2.0 (https://cge.cbs.dtu.dk/services/VirulenceFinder/) and PathogenFinder v1.1 (https://cge.cbs.dtu.dk/services/PathogenFinder/) were used to identify the virulence factors and predict the pathogenicity of the isolates respectively.

2.3.5 Identification of mobile genetic elements

Plasmid replicons were identified using PlasmidFinder 2.1 (https://cge.cbs.dtu.dk/services/PlasmidFinder/). The insertion sequences (ISs) were predicted using the ISFinder database (http://isfinder.biotoul.fr/) and MobileElementFinder database v1.0.3 (https://cge.cbs.dtu.dk/services/MobileElementFinder/). The ICEfinder database (https://db-mml.sjtu.edu.cn/ICEfinder/ICEfinder.html) was used to determine the presence of transposons or integrative conjugative elements (ICE). Intact prophages were identified using the PHASTER database (http://phaster.ca). MobileElementFinder v1.0.3 (https://cge.cbs.dtu.dk/services/MobileElementFinder/) was also used to illustrate the insertion sequence (ISs) and transposons flanking the resistance genes and the plasmid replicon associated with the ARGs. The genetic environment of the ARGs was determined using the NCBI annotations.

2.3.6 Phylogenomic analyses

The phylogeny of the *Enterococcus* isolates was characterised using CSI Phylogeny-1.4 (https://cge.cbs.dtu.dk/services/CSIPhylogeny/), which identifies single-nucleotide polymorphisms (SNPs), filters and validates the SNP positions, before deducing a phylogeny based on the concatenated alignment of the high-quality SNPs. The SNPs selections were carried out using default parameters: minimum distance between SNPs (prune) at 10bp, minimum depth at SNP positions of 10x, minimum mapping quality of 25, a minimum Z-score of 1.96, and minimum SNP quality of 30. Phandango was used to visualise and edit the generated phylogenomic tree associated with the isolates' source, MLST typing, resistance determinants, and plasmid metadata (Hadfield et al., 2018).

To ascertain the relationships if any of isolates from this study relate with other enterococci, globally (*E. casseliflavus* and *E. durans*) and in Africa (including South Africa), enterococci isolates curated at the PATRIC website (https://www.patricbrc.org/) were downloaded together with the study isolates for phylogenomic analysis. Figtree (http://tree.bio.ed.ac.uk/software/figtree/) was used to edit and visualise the phylogenomic tree. The genome of *Enterococcus faecium* ATCC 8459 (accession number: PRJNA206348) and *Enterococcus faecalis* ATCC 29212 (accession number: ALOD00000000) were used as the out-group to root the tree for easy configuration of phylogenetic distance between the strains on the branches.

2.3.7 Accession Numbers

The sequences were deposited at GenBank under the Bioproject PRJNA609650. The accession numbers are provided in Table S1

3.0. Results

3.1. Genomic characteristics

The genomic characteristics of the isolates in terms of N_{50}, L_{50}, coding sequences, and coverage are shown in Supplementary **Table S1**. The draft genome size of the isolates ranged from 2.6 Mb to 3.8 Mb, with a GC content of 37.2 – 42.5 (**Table S1**).

3.2. Phenotypic profiles and genetic determinants of antibiotic resistance

The resistance patterns displayed by the selected isolates from the different time points and sampling sites are shown Table 1. A total of 21 different ARGs conferring resistance to aminoglycosides (*aac(6')-Ii*, *aac(6')-Ii*h, *ant(6)-Ia*, *aph(3')-III*, ant(9)-Ia), macrolide-lincosamide-streptogramin AB (MLS_{AB}) [*erm(B)*, lnu(B), *lnu(G)*, *lsaA*, *lsaE*, *eat(A)*, *msr(C)*], trimethoprim-sulfamethoxazole (*dfrE*, and *dfrG*), tetracycline (*tet(M)*, *tet(L)*, and *tet(S)*), fluoroquinolones (*efmA*, and *emeA*), vancomycin (*VanC {VanC-2*, *VanXY*, *VanXY*C-3, *VanXY*C-4, *VanRC*}), and chloramphenicol (*cat*), were detected (**Table 1)**. Genes encoding high-level gentamicin resistance (HLGR) and resistance to nitrofurantoin, teicoplanin, linezolid , and tigecycline were not detected. The most frequently detected ARGs included *tet(M)* (n = 9, 47%), *erm(B)* (n = 8, 42%), *tet(L)* (n = 7, 37%), *eat*(*A*) (n = 7, 37%), *aac(6')-Ii* (n = 7, 37%), *VanC* (n = 7, 37%) and *msr*(*C*) (n = 6, 32%). **(Table 1)**. *Tet(L)*, *tet(M)*, and *erm(B)* genes were mobilised by diverse plasmids, transposons and insertion sequences. The litter-amended soil harboured new ARGs (*ant(6)-Ia*, *aac(6')-Ii*, *aph(3')-III*), *lnu(G)*, msr(C), and eat(A), *efmA)* and ARB (particularly *E. faecium*) that were not detected in the soil before the litter amendment **(Table 1)**. Analysis by species, showed that *E. faecium* harboured the highest number of ARGs (15) compared to *E. faecalis* (5), *E. casseliflavus* (3), and *E. durans* (6) **(Table 1)**. The *tet(M)* gene was detected in all the *Enterococcus* spp. except *E. durans* **(Table 1)**.

The phenotypes were not corroborated by ARGs in some cases as some isolates expressed resistance to antibiotics in the absence of the associated resistance determinants (**Table 1 &**

S2). For example, tetracycline resistance genes *tet(M), tet(L),* or *tet(S)* were found in 10 of 11 (91%) isolates that were phenotypically resistant to tetracycline, while isolate 6L3-36, which harboured *tet(M),* and *tet(L),* were susceptible to tetracycline. The MLS_B resistance determinant *erm(B)* and *msr(C)*) were identified in 9/11(82%) of the phenotypically resistant isolates. The trimethoprim-sulfamethoxazole resistance gene *dfrE* and *dfrG* were found in 28% (4/14) of the trimethoprim-resistant isolates. The mechanism of trimethoprim-sulfamethoxazole resistance in six *E. casseliflavus,* three *E. faecium* , and *E. durans* were not detected in the ResFinder and CARD databases.

Nine and seven putatively novel mutations in the DNA-*gyrA*se [*gyrA*] and topoisomerase IV [*parC*] regions respectively, were identified in 5 out of 8 (63%) fluoroquinolone-resistant isolates (Table 2). The nine mutations in the DNA-*gyrA*se included I354T*, N708D*, A626S* I259L*, I306V*, D759N*, A811V*, G819A*, and S820T* while the seven mutations in the *parC* consisted of A391V*, V307I*, G59S*, K228R*, I699V*, E707D*, and L773I* (**Table S3**). Furthermore, multi-drug efflux systems such as *emeA* and *efmA* were found in 5/8 (one *E. faecalis* and four *E. faecium*) fluoroquinolone-resistant isolates respectively (**Table 1 & S2**). The efflux genes were also detected in 6 out of 10 isolates that were susceptible to at least one of the tested fluoroquinolones antibiotics.

Chromosomal point mutations (14 known and 10 putative novel) conferring resistance to *β*-lactams (ampicillin and imipenem) were detected in 5 of the 10 (50%) isolates that were phenotypically resistant (**Table S2 & S3**). Only the *E. faecium* isolates had the mutations. Isolate 7L3-69 and 10L1-121 from chicken litter had similar mutations but were susceptible to all the tested *β*-lactam antibiotics. Additionally, two isolates from chicken litter [7L3-69 (ST 1700) and 10L1-121 (ST1756)] had a mutation (E192G) in the *liaS* gene, which encodes resistance to daptomycin **(Table S3)**.

3.4. Mobilome and the genetic synteny of the ARGs

The PlasmidFinder platform revealed 11 different plasmid replicon types in diverse permutations and combinations **(Table 1)**, the most prevalent of which were repUS1 (n = 9), repUS43 (n = 8), and repUS15 (n = 7) while the least detected were rep9a (n = 1), rep9b (n = 1), rep11a (n = 1), rep21 (n = 1), rep29 (n = 1) **(Table 1)**. Nine (47%) isolates from chicken litter (n = 4), litter-amended (n = 4) and unamennded soil (n = 1) had more than one plasmid replicon, while plasmids were absent in two litter-amended soil isolates (20D1-63 and 20B1-9). The MobileElementFinder showed that the tetracycline resistance gene *tet(L)* and *tet(M)*

were located on the repUS43, rep9b, and rep 22 plasmids and the *erm(B)* gene was mostly carried on the repUS1 plasmid (**Table 1**). The only disinfectant resistance gene *qacG* (quaternary ammonium compound efflux SMR transporter) detected among the isolates (8D1-48) was located on rep21 plasmid (**Table 1 and 2**). Plasmid repUS1 and repUS43 were detected across the *Enterococcus* spp. *E. faecium* harboured the highest (9) plasmid replicon types dominated by repUS15 (n = 6), rep1 (n = 5), and repUS43 (n = 5). The *E. casseliflavus* and *E. faecalis* isolates showed lower plasmid diversity carrying mainly the repUS1 and rep9 plasmids, respectively.

Across the sample groups, isolates from the chicken litter and litter-amended soil showed greater diversity in plasmid profiles, i.e. 8/11 (72.7%) and 7/11 (63.6%) plasmid replicon types respectively compared with isolates from the unamended soil (3/11, 27.3%) and had six plasmid replicon types in common. The predominant plasmid types in the litter-amended soil were repUS1, repUS15, and repUS43, while repUS1 predominated the unamended soil. The most prevalent replicon types in the chicken litter were repUS15 and repUS43.

Three different groups of transposons were found in 53% (10/19) of the isolates; 26% (5/19) contained the *Tn916* family of integrative conjugative elements, including two putatively novel (*Tn644* & *Tn645*) and two known (*Tn659* & *Tn6009*) *Tn916*/Tn*1545*-like transposons (**Table 1**). Some of the isolates had more than one transposon. The litter-amennded soil isolate 13D1-56 which harboured *tet(M)* gene and repUS43 plasmid, also harboured the Tn*6009* element (**Table 1**). The tetracycline resistance gene *tet(M)* was associated with *Tn659* and *Tn6009* transposons in isolate 13D1-56 and 7A2-40 from the litter-amended soil, and 6L3-36, and 7L3-69 from chicken litter (**Table 1**). *Tet(M)* and *erm(B)* genes (adjacent position) were both associated with the *Tn644* transposon (**Fig 1**) in isolate 4A2-6 (unamended soil), 7A2-40, 6L3-36 and 7L3-69 (**Table 1**). None of the ARGs was associated with the *Tn645* transposon. Across the species, three *E. faecium* (48%) and two *E. casseliflavus* (14%) had the *Tn916*-like element. The *Tn917* transposon of the *Tn3* family was detected in three (16%) isolates (**Table 1**). The *Tn917* transposon was associated with the *erm(B)* gene in isolate 10B2-36 and 8L1-82 from the litter-amended soil and chicken litter respectively (**Table 1 & 2**). Four different putative composite transposons Tn*1855*, Tn*2426*, Tn*46199* and Tn*5509* flanked on both side by ISEfa11 (ISL3), ISEfm1 (IS982), ISS1N (IS6) and IS1062 (IS30) insertion sequences respectively were identified in isolate 3C1-8 from the unamended soil, and isolate 5D2-10, 7A2-40, and 10B2-36 recovered from the litter-amended soil (**Table 1**).

A diversity of IS types belonging to different IS families were found across the isolates. The most frequently detected IS families included IS982, ISL3, IS6, IS5, IS3, IS256, and IS30 (**Table 1**). IS30 was mostly associated with the MLS_B resistance gene *erm(B)* in 7 isolates (**Table 1 & 2**) while the *IS6* (ISEnfa1) was associated with the *Tet*(*M*), *Tet*(*L*) and *erm*(*B*) genes in isolates 7L3-69, 8D1-48, and 6L3-36 respectively (**Table 1 & 2**). The *VanC2* ARG was found in association with ISL3 (ISEfa11) in isolate 5D2-51 from the unamended soil, while *VanC4XY* was associated with IS256 (ISLgar5) and IS982 (ISEfm1) in isolate 16B1-19 from the litter-amended (**Table 1**). The virulence genes were not associated with any of the ISs.
A total of fourteen intact prophages (**Table S4)** were detected in the genomes of 13/19 (68%) isolates. The most predominant prophages were Entero_phiFL4A (n = 3), Bacill_vB_BhaS_171 (n = 3), and Lactoc_50101 (n = 3). Only the lincosamide resistance gene *lnu*(*G*) was associated with the phage integrase family **(Tabl**e **2**).

The genetic synteny of ARGs showed that the majority of the ARGs were co-carried on plasmids or associated with insertion sequences and/or transposons located on large conjugative plasmids (**Tables 2**) with 92.36% to 100% sequence identity similarity with target sequences in the GenBank database. The most frequently detected and closest nucleotide sequence was the *E. hirae* strain HDC14-2 plasmid pHDC14-2.133K (CP042290.1), *E. faecium* isolate P39 transposon Tn6247 (KP345886.1), and *E. faecalis 28157_4#18* genome assembly, plasmid: 2 *(LR962490.1)*.

3.5. Pathogenicity and Virulome

PathogenFinder predicted 15 out of 19 isolates as human pathogens with a mean probability ranging from 0.694 to 0.896 (**Table 1**). All but one isolate (8/9) from the litter-amended soil (16B1-19) and two isolates (2/5) from the unamended soil were predicted as human pathogens. All but four of the *E. casseliflavus* isolates had a probability of being pathogenic to humans (**Fig S1**). Virulence genes were only detected in the *E. faecalis* and *E. faecium* where 23 different putative virulence genes associated with biofilm formation, adhesion, sex pheromone, gelatinase, host immune evasion, oxidative stress response, and hyaluronidases were detected across the two species (**Fig S1**). The *E. faecalis* isolates harboured a higher number (21/23, 91%) of virulence genes than *E. faecium* (2/23, 9%). The litter-amended soil had more (6/9, 66.7%) potential pathogenic isolates compare to the unamended soil (1/5, 20%) **(Fig S1)**. The most frequently detected virulence genes were *acm* and *efaAfm* in six isolates each. The virulence genes were species-specific in that isolates of the same species harboured the same

repertoire of virulence genes (**Fig S1**). Isolate 13D2-63 from the litter-amended soil had the highest number (19/23, 83%) of virulence genes. None of the virulence genes was associated with any MGE except the *agg* gene, which encodes aggregation protein found in association with rep9a plasmid replicon type in isolate 13D2-63 (**Fig S1**).

3.6. Multilocus sequence typing (MLST)

MLST analysis detected ten different sequence types (STs), which included two known STs (ST10 and ST271) and eight novel STs (**ST1700, ST1752, ST1753, ST1754, ST1755, ST1756, ST1004, and ST1006**) assigned to nine isolates **(Table 1)**. Two isolates belonged to ST1006 with singletons of the other STs. The seven sequenced *E. faecium* belonged to seven different ST types (six novel STs and one known ST (ST10 (clonal complex 17)). The four *E. faecalis* isolates belonged to three different STs (ST271, ST1004, and ST1006), but no ST could be assigned to the *E. casseliflavus* (n = 7), and *E. durans* (n = 1) isolates that accounted for 42% (n = 8) of the isolates **(Table 1)**. The litter-amended soil isolates showed greater diversity in STs types (5) than the unamended soil (1). The chicken litter isolates comprised of 4 different MLST types of *E. faecium* and *E. faecalis.*

3.7. Phylogenomics and Metadata Insights

Phylogenomic analysis coupled with the metadata showed general clustering of the isolates according to their species and sequence types. For instance, two litter-amended soil isolates (20B1-9 and 20D1-63) that both belonged to the same ST (ST1006) clustered together **(Fig 2)**. Moreover, the isolates clustered according to their sample group. For example, isolate 3C1-8, 5D2-10, and 5D2-51 from the unamended soil formed a unique clade. Chicken litter isolate 6L3-36 [ST1752] & 11L2-121 [ST1754] also clustered together **(Fig 2)**. However, isolates from different sample groups clustered together in few instances. Isolate 7A2-40 (ST10) from the litter-amended soil clustered with two isolates from chicken litter [7L3-69 (ST 1700) and 10L1-121 (ST1756)]. Overall, the isolates clustered according to their species **(Fig 2)**.

Comparison of the studied *E. faecium* isolates with genomes of isolates from Africa, showed that the *E. faecium* from this study, clustered with clinical and animal isolates (**Fig. 3a**). For example, the *E. faecium* isolate 10B2-36 (ST1755) from the litter-amended soil clustered together with three clinical isolates (1352.4198 (ST97), 1352.4197 (ST822), and 1352.4196

(ST636)) from rectal samples of patients from hospitals at uMgungundlovu District, and one isolate from an aviary (1352.8216 (ST29)) in Angola (**Fig. 3a**).

Phylogenomic analysis of *E. faecalis* with isolates from Africa revealed that *E. faecalis* from this study were more closely related to clinical and animal isolates (**Fig. S2**). *E. faecalis* 13D2-63 (ST1004) from the litter-amended soil clustered with urine isolate 1351.637 (ST6) from hospital patient in Pretoria and two clinical isolates (1351.1373 (ST6) and 1351.1371 (ST563)) from rectal samples of patients from a hospital in uMgungundlovu District (**Fig. S2**). Isolate 3A2-2 (ST271) from the unamended soil also clustered with two *E. faecalis* (1351.1812 (ST476) and 1351.2011 (ST476)) isolated from animal (chicken faeces and meat) sources in Tunisia (**Fig. S2**).

Comparison of *E. casseliflavus* with global isolates showed that the isolates from this study were more related to *E. casseliflavus* of human and animal sources **(Fig. 4)**. *E. casseliflavus* 13D1-56 from the litter-amended soil clustered with human faecal isolate 37734.84 from the United Kingdom, and isolate 37734.93 (human gut) and 37734.37 (human faecal sample) from China **(Fig. 4)**. Unamended soil isolate 4A2-6 clustered with isolate 37734.122 of animal (cow urine) origin from Brazil **(Fig. 4).**

The only *E. durans* isolate (8L1-82) in this study closely clustered with isolates of animal origin (cow and chicken) from South Africa (53345.56) and USA (53345.33) **(Fig. S3)**.

4.0 Discussion

The impact of animal manure application on the soil resistome and the potential dissemination routes of ARGs from manure to agricultural soil is not well characterised. Whole-genome sequencing and bioinformatics analysis were used to examine the changes in soil resistome and the associated mobilome before and after chicken litter amendment using *Enterococcus* spp. as an indicator. Overall, the genomic analysis of the enterococci isolates showed that the litter amendment resulted in the transmission of antibiotic-resistant *E. faecium* and plasmid-borne *tet*(*M*), *tet*(*L*), and *erm*(B) genes from the chicken litter to the litter-amended soil. The identified ARGs were located on plasmids, and/or associated with transposons or ISs across the three sample groups indicating the potential mobilization of ARGs between the litter and the soil isolates. The diversity of plasmids, transposons, and ISs in the genomes provide evidence of the plasticity of the bacterial genomes and the potential for horizontal dissemination of the

ARGs. The diversity of clonal lineages, resistome, mobilome, and virulome of the isolates highlights the agricultural soil fertilized by animal manure as a milieu for the exchange of ARB and ARGs that can affect occupationally exposed workers, contaminate farm produce, or the surrounding water bodies through run-offs. To our knowledge, this is the first genomic report on the impact of chicken litter application on the soil resistome in South Africa.

4.1 Phenotypic profiles and genetic determinant of antibiotic resistance in the isolates

There was a short-term (35 days) increase in the frequency and diversity of ARGs that conferred resistance to multiple classes of antibiotics in the soil following the litter amendment. Before the litter amendment, the ARGs detected in the soil were mainly genes that confer intrinsic resistance to antibiotics in individual *Enterococcus* spp. like the *vanC* gene, which encodes resistance to vancomycin in *E. casseliflavus* (dominant species in the sample group). On the other hand, the litter-amended soil harboured new ARB (particularly *E. faecium*) and new ARGs that confer resistance to aminoglycosides (*ant(6)-Ia*, *aac(6')-Ii*, and *aph(3')-III*), and MLS_B (*lnu(G)*, msr(C), and eat(A)), as well as antimicrobial efflux pump gene *efmA* (macrolides and fluoroquinolones resistance) (**Table 1**). The litter-amended soil isolates also harboured genes (*VanC* operon genes, *dfrE*, and *emeA*) that were not found in the unamended soil isolates **Table 1**). This shows that the litter-amended soil also retained ARGs that were present in the soil prior to the litter amendment. The increased detection of ARGs indicated direct addition of enterococci carrying ARGs from the chicken litter, potential horizontal transfer of transposon and plasmid-borne resistant genes, or the proliferation of indigenous resistant enterococci due to soil enrichment (Marti et al., 2013; Udikovic-Kolic et al., 2014; Lopatto et al., 2019; Zhang et al., 2019). Studies have shown that land application of untreated animal manure can introduce new ARB, ARGs, and MGEs of animal manure origin to the soil, thereby increasing the diversity of soil ARB and ARGs (Su et al., 2014; Chen et al., 2016; Cheng et al., 2019). Chen et al. (2019) in a 120-day soil microcosm study in United States, showed that manure amendment significantly increased the ARG relative abundance and diversity in the soil (2.21 x controls) compared to the unamended soils (controls). Chen and colleagues (2016) observed a significant increase in ARGs and bacterial diversity in manure-amended soil following chicken manure application. The soil amendment increased the abundance of tetracycline resistance genes and caused over 100-fold enrichment of *ermB*, and *floR* genes in the soil (Chen et al., 2016).

Even though studies have indicated an increased level of ARGs and ARB diversity in the soil following manure application. It has also been reported that the increase is only for a while because ARB of manure origin are less adapted to the soil environment and only survive for a short time (Sengeløv et al., 2003; Heuer et al., 2011; Miller et al., 2020). As expected, the detection of many ARGs in the litter-amended soil decreased over time (day 35 to day 105) to types and levels observed in the unamended soil at the study's inception and mostly consisted of ARGs conferring intrinsic resistance. All the ARGs identified in the soil after the litter amendment were no longer detected in the soil isolates from day 56 till the end of sample collection on day 105. The decrease may be due to nutrient depletion, soil matrix dilution, breakdown of resistant DNA, death of some bacteria harbouring the ARGs in the soil, environmental stress or dissemination to other compartments of soil environment (Marti et al., 2014; Muurinen et al., 2017; Miller et al., 2020). The decrease in ARGs observed in this current study could be further explained by the urea salt added to the soil on day 7 after litter amendment in the current study. Urea has been shown to reduce the abundance and diversity of soil microorganisms in that it favours the growth of nitrifying bacteria over others (Staley et al., 2018). Consistent with our finding, a field study conducted in Southern Finland reported a decrease in the number and abundance of manure-associated ARGs and MGEs in soil amended with dairy and swine manure at two and six weeks after manure application (Muurinen et al., 2017). These findings suggests that increases in ARG and ARB abundance in the soil following manure application are temporary and only occur annually during manure application (Muurinen et al., 2017).

In correlating the resistance phenotypes and genotypes, an absence of known erythromycin, trimethoprim-sulfamethoxazole ampicillin, imipenem, and fluoroquinolone ARGs was observed despite the phenotypic resistance observed in the *E. casseliflavus* isolates and postulated that this could be due to novel or uncharacterised mechanisms of resistance (Beukers et al., 2017; Yamanaka et al., 2019) as reported in a Japanese study where *E. casseliflavus* isolated from fresh faeces of laboratory mice purchased from four different commercial mouse breeding companies showed resistance to both erythromycin and ciprofloxacin in the absence of macrolide and fluoroquinolone resistance genes, including mutations in *gyrA* and *parC*. Beukers et al. (2017) also reported intermediate resistance to erythromycin in one *E. casseliflavus* isolate with no resistance genes for macrolides. The discordance between the trimethoprim-sulfamethoxazole resistance phenotype and the genotype in other *Enterococcus* spp. could be due to impaired penetration of the antibiotic, hetero-resistance, multidrug-

resistant efflux pumps which is common in environmental bacteria or limitations of the current traditional phenotypic (antimicrobial susceptibility testing) methods (Doyle et al., 2020; Yee et al., 2021). The molecular mechanism of resistance to nitrofurantoin was not found in the nitrofurantoin-resistant isolates. The complete susceptibility of isolates to ampicillin and imipenem antibiotics despite the presence of chromosomal mutations that encode resistance to *β*-lactams may be a result of silent mutations.

4.2 Mobilome and the genetic synteny of the ARGs

Plasmid-mediated transfer of ARGs has been reported as the dominant ARGs dissemination pathway in animals, humans and the environment (Liu et al., 2020). In this study, some of the identified ARGs were associated with transposons or ISs on plasmids (**Table 2**), indicating the important role plasmids play in the spread of ARGs in the soil environment (Beukers et al., 2017; Rehman et al., 2018; Fogler et al., 2019; Smith et al., 2019). The genetic context of *tet(L)* and *tet(M)* genes was consistent in tetracycline-susceptible isolate 6L3-36 (chicken litter) and all the five tetracycline resistant isolates (3A2-2, 8D1-48, 10B2-36, 7L3-69, 10L1-121) from chicken litter, the unamended and litter-amended soil (**Table 2**). *Tet(L)* and *tet(M)* genes were consistently associated with plasmid recombination protein, *tet* resistance determinant, conjugal transfer protein, and IS6 family or Tn*3* family transposase (few instances) on plasmids (protein rep:plasmid recombination protein:*tet*(*L*):*tet* resistance efflux:*tet*(*M*):*tet* resistance determinant: conjugal transfer protein (**Table 2)**). This genetic context found in five *E. faecium* recovered from the litter-amended soil (8D1-48, 10B2-36) and chicken litter (6L3-36, 7L3-69, 10L1-121) had high nucleotide identity similarity with transposon Tn*6247* (*E. faecium* P39 transposon Tn*6247* (KP345886.1)) and plasmid (*E. hirae* strain HDC14-2 plasmid pHDC14-2.133K (CP042290.1*)*) sequences deposited in GenBank (**Table 2**) indicating that the two litter-amended soil isolates might have originated from the chicken litter, or the genes might have been horizontally acquired from the chicken litter isolates through plasmids or transposon (**Table 2**). The absence of phenotypic resistance to tetracycline in isolate 6L3-36 harbouring the *tet(M)* and *tet*(*L*) genes in the same genetic context as the other resistant isolates could be due to lack of expression of these genes in the isolate. A similar genetic context of *tet(L)* and *tet*(*M*) observed in *E. casseliflavus* 13D1-56 and *E. faecalis* 3A2-2 (**Table 2**) indicates the mobilisation of the two tetracycline resistance genes in different *Enterococcus* species through plasmids (**Table 1**).

In addition to the mobilisation of the *tet(M)* gene on plasmids, it was also associated with *Tn916*-like transposons in some isolates (**Table 1**). Co-carriage of *tet(M)* and *erm*(*B*) genes by a putatively novel integrative conjugative element Tn*644* related to the *Tn916* family transposons (**Table 1 & Fig 1**) detected in three *E. faecium* isolate 7A2-40, 6L3-36, and 7L3-69 from the litter-amended soil and chicken litter could have also aided the mobility of the ARGs. The *Tn916* family are broad host range conjugative transposons carrying clinically relevant resistance genes (e.g. *tet*(*M*)) that could be transferred between different bacterial genera (Flannagan et al., 1994; Hegstad et al., 2010). The transposon encodes all the information needed for their excision, conjugation, and integration into a new bacterium host (Hegstad et al., 2010; Rehman et al., 2018). Consistent with our finding, Rehman and colleagues (2018) reported the co-carriage of *erm(B)* and *tet(M)* on *Tn916*-like element in *E. faecium* and *E. faecalis* isolated from antibiotic-treated broilers litters in Canada. The carriage of *tet(M)* gene by repUS43 plasmid and the occurrence of conjugative Tn*6009* transposon observed in *E. casseliflavus* 13D1-56 was recently reported in one vancomycin-resistant *E. faecalis* isolate from the same district (uMgungundlovu), albeit in a hospitalised patient (Founou et al., 2021). These reports suggest the spread of tetracycline resistance gene *tet(M)* mediated by repUS43 plasmid in multiple *Enterococcus* species in the district. To the best of our knowledge, this is the first report of a tetracycline-resistant *E. casseliflavus* harbouring both Tn*6009* and the repUS43 plasmid in South Africa.

The presence of transposons and insertion sequence elements in bacterial genomes is crucial for intracellular transposition transfer (Flannagan et al., 1994). In this study, the *erm(B)* gene was consistently associated with TetR/AcrR family transcriptional regulator gene, *IS30* family, IS6-like family insertion sequences, recombinase family protein, and Tn*917* of *Tn3* family transposase (Table 3), which could aid their transposition to different locations of the bacterial genome.

Notably, the co-carriage of seven different ARGs including the MLSB resistance gene *erm(B)* and genes encoding resistance to aminoglycosides (*ant(6)-Ia*, *ant*(*9*)), lincosamides-streptograminA (*Lsa*(*E*)), lincosamides (*lnu*(*B*)) streptothricin (*satA*), and chloramphenicol (*catA*), with IS1182, IS6, ISL3, and IS30 insertion families on a single plasmid in isolate 7L3-69 from chicken litter is of great concern (**Table 2**). This genetic context could facilitate the horizontal transfer of multiple ARGs from the chicken litter isolates to resident soil bacteria via plasmid. Furthermore, the chloramphenicol resistance gene *catA* inserted into a region flanked by two *IS6-like* IS1216 family ISs (**Table 2**) can also transpose out from the plasmid

by IS1216-mediated transposition to the chromosome. IS1216 has been reported to play a major role in the spread of ARGs in Gram-positive bacteria (Partridge et al., 2018). MGEs and accessory genes contribute to bacterial genome plasticity and the differences in microbial genomes (Dobrindt et al., 2004; Li and Wang, 2021). They also play an important role in microbial evolution and environmental adaption of bacteria (Dobrindt et al., 2004; Li and Wang, 2021). They are widely distributed in pathogenic and non-pathogen bacteria, including *Enterococcus* (Dobrindt et al., 2004; Laverde Gomez et al., 2011; Boyd et al., 2016; Li and Wang, 2021).

4.3 Pathogenicity and Virulome

Several virulence genes known to contribute to the pathogenicity of enterococci were detected in the genomes of the studied *E. faecalis* and *E. faecium* isolates (**Fig. S1**). The increased detection of potential pathogenic isolates in soil following the chicken litter application suggests that the chicken litter introduces pathogenic ARB into the soil. None of the virulence genes (except the aggregation substance *agg* gene) were mobilised on any MGE, and this explains the specificity of the virulence factors to individual species. The virulence genes detected in this study have been found in various *Enterococcus* spp. isolated from poultry litter in South Africa and Canada, with *E. faecalis* having a higher prevalence of virulence genes than other species (*E. faecium , E. casseliflavus,* and *E. durans*) (Rehman et al., 2018; Molechan et al., 2019).

4.4 Multilocus sequence typing (MLST)

The MLST analysis revealed the multiclonal nature of the *Enterococcus* spp. isolated from the litter amended soil and the chicken litter (**Table 1**). The presence of the STs that were absent in the unamended soil in the litter-amended, further attest to the influx of manure-borne ARB into the soil. Notably, the globally distributed ST10 of clonal complex 17 (CC17) recovered from the litter-amended soil (7A2-40). The *E. faecium* CC17 is considered a high-risk enterococcal clonal complex (HiRECC) associated with nosocomial infections and outbreaks, mainly recovered from hospitalised patients (Leavis et al., 2006). This finding indicates that this human clonal lineage may be circulating between humans, chicken, and the soil. Detected in the genome of the ST10 isolate were the *acm* and *efaAfm* genes that enhance pathogenicity in the species. This host-specific CC17 is widespread and has been isolated from non-hospital sources like chicken, pig excrement, and environmental piggery samples (De Leener et al., 2005; Freitas et al., 2009; Getachew et al., 2013). The study reported novel STs assigned to the

six *E. faecium* , and three *E. faecalis* isolates. Even though the novel isolates belonged to multiple clones, they harboured the same ARG types. The absence of ST assignment for some isolates suggests that there are STs yet to be designated within the MLST database. This study recommends establishing an MLST scheme for *E. casseliflavus* and *E. durans* for the identification of clonal lineages.

4.5 Phylogenomics

The phylogenomic analysis further confirmed the diversity among the isolates within and across the species and sample sources. The majority of the isolates from the soil before the amendment differed from litter-amended soil isolates and clustered as separate clades, indicating a change in soil bacterial diversity as a result of the chicken litter application (**Fig 2**). The close phylogenomic relation of the *E. faecium* isolates from litter-amended soil (7A2-40 and 8D1-48) and the chicken litter isolates (10L1-121 and 7L3-69) further suggests that 7A2-40 and 8D1-48 originated from chicken litter (**Fig 2**). The phylogenomic comparisons with isolates from South Africa and other countries showed that the isolates from the current study mostly clustered with isolates from hospitalised patients and food animal sources (**Fig. 3, 4, S2, S3**). Eight enterococci isolates (*E. faecium* (1), *E. faecalis* (1), and *E. casseliflavus* (6)) clustered together with human isolates and four isolates (*E. durans* (1), *E. faecium* (1), *E. faecalis* (1), and *E. casseliflavus* (1)) were closely related with animal isolates. The close relationship between the isolates in this study and isolates from clinical/human, and food animal sources from South Africa and other countries suggests an intra- and inter-country and international spread of similar *Enterococcus* spp. in two of the three-domain of One Health.

Overall, the detection of multiple ARGs harboured by *Enterococcus* spp. in the soil after the chicken litter application is of great concern as the amendment of agricultural soil with ARB- and ARG-contaminated chicken litter could increase the risk of farm produce contamination. This could pose a risk to humans who may consume contaminated plant food, or drink from untreated surface waters contaminated with run-off from these farms, thereby causing widespread community-acquired enterococcal infections. These ARB could also pose a threat to occupationally exposed workers through direct contact with contaminated soil.

5.0 Conclusion

This study revealed the mobilisation of ARGs, MGEs, and multiple *E. faecium* clones from the chicken litter to the soil. The *Enterococcus* isolates harboured an extensive ARG repertoire mobilised on diverse MGEs. The ARGs were borne on plasmids, flanked by transposons and/or

ISs in isolates from the litter-amended soil and the chicken litter, indicating a possible exchange of genetic materials among the isolates. This study provides evidence on the mobility and fluidity of ARB, ARGs and MGEs from chicken litter to the soil and identifies both chicken litter and litter-amended soil as reservoirs of potentially pathogenic MDR *Enterococcus* spp. that could threaten public health. It is therefore important to employ policies that promote prudent use of antibiotics in animal production and pre-treatment of animal wastes such as composting to reduce the ARGs and ARB load. It is important to note that since soil is a community of heterogeneous bacteria, more robust surveillance on the impact of animal manure application on soil resistome, including a larger number of isolates and more farms covering larger geographical locations, may be helpful in future monitoring of AMR pollution in the environment.

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Table 1: Antibiotic resistance genes, mobile genetic elements, phenotypes, sources, and pathogenicity score of Enterococci

Isolate ID	Source	Species	MLST	Sample collection day	Resistance Phenotype	ARGs Detected	Plasmid replicon type	Transposon/ICE/IME	Insertion sequences	Pathogenicity Score (no. of pathogenic family)
Unamended Soil Isolates										
3C1-8	Soil	*E. casseliflavus*	-	Day 3	LEV-CIP-TET-SXT	*VanC-2*, *VanXY*, *VanRC*, *tet(S)*	repUS1	Cn2426*	ISL3, ISKra4, IS982	0.533(4)_NHP
3A2-2	Soil	*E. faecalis*	ST271	Day 3	CIP-TET-SXT	*lsaA*, *tet(M)*, *tet(L)*, *dfrE*, *emeA*	rep9b, repUS43		ISL3, IS1595, IS5	0.887 (60)_HP
4A2-6	Soil	*E. casseliflavus*	-	Day 5	VA-IMP-ERY-LEV-SXT	*VanC-2*, *VanXY*, *tet(M)*,*erm* (B)	repUS43	Tn644* (IME)	ISL3, IS91, IS1380, IS21	0.852(5)_HP
5D2-10	Soil	*E. casseliflavus*	-	Day 9	VA-LEV-TET-SXT	*VanC-2*, *VanXY*, *tet(S)*	repUS1	Cn1855*; Cn2426*	ISL3, ISKra4, IS982, ISNCY	0.478(4)_NHP
5D2-51	Soil	*E. casseliflavus*	-	Day 9	VA-AMP-TET-SXT	*VanC-2*, *VanXY*, *tet(S)*	repUS1		ISL3, ISKra4, ISNCY	0.478(4)_NHP
Litter-amended Soil Isolates										
7A2-40	Soil	*E. faecium*	ST10 (CC17)	Day 1	STR-AMP-IMP-ERY-TET-SXT-QD	*aac(6')-Ii*, *ant(6)-Ia*, *aph(3')-III*, *erm(B)*, *lnu(G)*, *eat(A)*, *tet(M)*, *tet(L)*, *dfrG*, *efmA*	rep2, rep22, repUS15	Tn644*, Tn659, & Cn5509*	IS1380, IS982, IS3, IS30	0.711(9)_HP
8D1-48	Soil	*E. faecium*	**ST1753***	Day 3	IMP-ERY-LEV-NF-TET	*aac(6')-Ii*, *erm(B)*, *msr (C)*, *tet(L)*, *tet(M)*,*eat(A)*, *efmA*	rep1, rep21, rep22, repUS1, repUS15, repUS43	-	IS3, IS6, IS256, IS1595, IS30	0.745(8)_HP
10B2-36	Soil	*E. faecium*	**ST1755***	Day 14	IMP-ERY-CIP-TET	*aac(6')-Ii*, *erm(B)*, *msr(C)*, *tet(L)*, *tet(M)*, *eat(A)*,*efmA*	rep1, rep2, repUS15, repUS43	Tn917 & Cn46199*	IS982, IS6, IS3, IS1182, IS30	0.705(7)_HP
13D2-63	Soil	*E. faecalis*	**ST1004***	Day 35	ERY-NF	*dfrE*, *lsaA*, *emeA*	rep9a (pAD1)	-	IS91, IS5, IS1595	0.898(56)_HP
13D1-56	Soil	*E. casseliflavus*	-	Day 35	TET	*VanXYC-3*, *vanRc*, *tet(M)*	repUS43, repUS1	Tn6009	ISL3, IS200/IS605, IS1182, IS3	0.749(8)_HP
16B1-19	Soil	*E. casseliflavus*	-	Day 56	AMP-SXT	*VanXYC-4*, *VanRc*	repUS1	-	IS256, IS982, IS91, IS3	0.49(3)_NHP
18C-21	Soil	*E. casseliflavus*	-	Day 77	STR-ERY-SXT	*VanXYC-4*, *VanRc*	repUS1	-	ISKra4, ISNCY, IS21, IS1595	0.71(4)_HP

20D1-63	Soil	*E. faecalis*	**ST1006***	Day 105	SXT	*dfrE, lsaA, emeA*	-	-	IS3, IS1595, IS5, IS982	0.896(65)_HP
20B1-9	Soil	*E. faecalis*	**ST1006***	Day 105	IMP-SXT	*dfrE, lsaA, emeA*	-	-	IS3, IS1595, IS5, IS982	0.896(65)_HP
Chicken litter isolates										
6L3-36	Chicken litter	*E. faecium*	**ST1752***	Day 0	AMP-IMP-ERY-LEV-QD	*aac(6')-Ii, erm(B), msr (C), tet(L), tet(M),eat(A), efmA*	repUS1, repUS43,	Tn644* & Tn659	IS982, IS5, IS6, IS256, IS30	0.884(6)_HP
7L3-69	Chicken litter	*E. faecium*	**ST1700***	Day 1	STR-ERY-TET-SXT	*aac(6')-Ii, ant(6)-Ia, ant(9)-Ia, erm(B), lnu(B), lsaE, msr(C), cat, tet(L), tet(M), eat(A)*	rep22, repUS15, repUS43	Tn644* Tn645 & Tn659	IS982, IS5, IS6, IS256, IS30	0.75(8)_HP
8L1-82	Chicken litter	*E. durans*	-	Day 3	AMP-ERY-NF-TET-SXT	*aac(6')-Iih, ant(6)-Ia, ant(9)-Ia, erm(B), lnu(B), lsaE*	repUS1	Tn917	IS6, IS256, ISL3, IS5, IS30	0.865(4)_HP
10L1-121	Chicken litter	*E. faecium*	**ST1756***	Day 14	ERY-CIP-TET-SXT	*aac(6')-Ii, erm(B), msr(C), tet(L), tet(M), eat(A), efmA*	rep2, repUS15, repUS43	-	IS6, IS256, IS3, ISL3, IS982, IS30	0.694(6)_HP
11L2-121	Chicken litter	*E. faecium*	**ST1754* (CC94)**	Day 21	IMP-ERY-NF-SXT	*aac(6')-Ii, msr(C), eat(A)*	rep1, rep11a, rep29, repUS15	-	IS256, ISL3, IS5, IS6	0.73(6)_HP

*Novel sequence types in bold font

AMP-Ampicillin, TET-Tetracycline, SXT- Trimethoprim-sulfamethoxazole, CHL- Chloramphenicol, GEN- Gentamicin, VAN-Vancomycin, STR-Streptomycin, IMP- Imipenem, ERY- Erythromycin, CIP- Ciprofloxacin, LEV- Levofloxacin, NIT- Nitrofurantoin, QD- Quinupristin-dalfopristin (QD was reported for only *E. faecium* isolates).

Table 2: The resistance genes and associated MGEs detected in the Enterococci

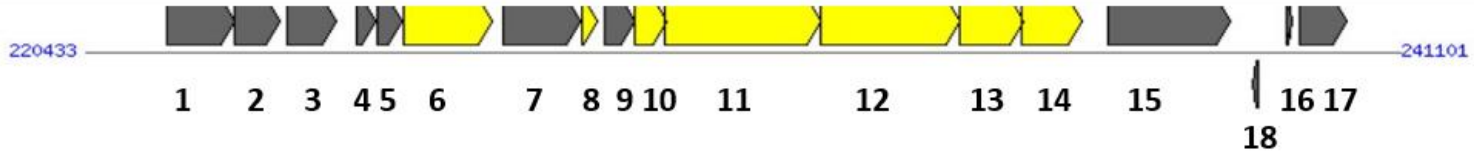
Isolate ID	Species	Contig	Synteny of the resistance genes	Plasmid/chromosomal sequence with the closet nucleotide homology (accession number)
3C1-8	*E. casseliflavus*	1	*tet(S)*:conjugal transfer protein::::::site-specific integrase	ND
3A2-2 (ST271)	*E. faecalis*	7	Tn3 family transposase:recombinase::::::::::::plasmid recombination protein:*tet(L)*:tet resistance efflux:*Tet(M)*:tet resistance determinant:conjugal transfer protein	*E. faecalis* strain EGM183 plasmid pEGM183 (CP050492.1)
4A2-6	*E. casseliflavus*	3	elongation factor Tu (EF-Tu):translation elongation factor G (EF-G):30S ribosomal protein S7:30S ribosomal protein S12:ribose-5-phosphate isomerase RpiA:recombinase family protein	*E. casseliflavus* strain EGM182 chromosome (CP050490.1)
5D2-10	*E. casseliflavus*	1	*tet(S)*:conjugal transfer protein::::::site-specific integrase	ND
5D2-51	*E. casseliflavus*	1	*tet(S)*:conjugal transfer protein::::::site-specific integrase	ND
7A2-40 (ST10) Clonal complex: CC17	*E. faecium*	30	*dfrG*:insertion element protein	*E. faecium* strain LAC7.2 plasmid pI (CP045013.1)
		42	*lnu(G)*:transposase:phage integrase family	*E. faecium* strain LS170308 chromosome (CP025077.1)
		45	recombinase family protein::::*erm(B)*:::::::*TetR/AcrR* family:::IS30 family::*Erm(B)*:ErmL	*E. faecium* isolate E0595 plasmid: 2 (LR135180.1)

		51	recombinase family:::methyltransferase:*ant(6)-Ia*	*E. faecium* isolate E1774 plasmid: 3 (LR135183.1)
		86	*Erm(B)*:methyltransferase::*aph(3')-III*	*E. faecium* isolate E8407, plasmid: 2 (LR536659.1)
		107	"*Tet(L)*/*Tet*(*K*)/*Tet*(*45*) family tetracycline efflux MFS transporter"	*E. faecium* strain E211 plasmid pE211-2 (MK465704.1)
8D1-48 (ST1753)	*E. faecium*	27	IS6 family::protein rep:plasmid recombination protein:*Tet(L)*:tet resistance efflux:*Tet(M)*:tet resistance determinant:conjugal transfer protein	*E. faecium* isolate P39 transposon Tn6247 (KP345886.1)
		36	IS256-like element IS256 family:::RepR protein::IS6 family transposase:::recombinase family protein::::::*erm(B)*::::*TetR/AcrR* family:::IS30 family::IS6 family transposase	*E. faecium* pVEF2 plasmid (AM410096.1)
		80	*qacG*: rep protein	*S. saprophyticus* strain UTI-035 plasmid pUTI-035-3 (CP054437.1)
10B2-36 (ST1755)	*E. faecium*	31	protein rep:plasmid recombination protein:*Tet(L)*:tet resistance efflux:*tet(M)*:tet resistance determinant:conjugal transfer protein	*E. hirae* strain HDC14-2 plasmid pHDC14-2.133K (CP042290.1)
		38	IS30 family:::*erm(B)*:recombinase family:Tn3 family transposase	*E. faecalis* strain 28157_4#18 genome assembly, plasmid: 2 (LR962490.1)

13D1-56	*E. casseliflavus*	1	conjugal transfer protein:tet resistance determinant:*tet(M)*:conjugal transfer protein:::::recombinase/integrase	ND
		23	transposase:*VanC:VanXY-C:VanT:VanRc*	*E. casseliflavus* strain EC291 chromosome (CP046123.1)
		33	IS6-like (ISEnfa1 family):::*TetR/AcrR* family:::IS30 family:::rRNA methyltransferase:*erm(B)*	*E. faecalis* plasmid pTW9 DNA (AB563188.1)
		24	protein rep:plasmid recombination protein::*tet(L)*:tet resistance efflux:*tet(M)*:tet resistance determinant:conjugal transfer protein	*E. hirae* strain HDC14-2 plasmid pHDC14-2.133K (CP042290.1)
7L3-69 (ST 1700)	*E. faecium*	25	adenine phosphoribosyltransferase:*ant(9)-Ia*:::recombinase:*Lsa(E)*:*lnu(B)*:ISL3 family:site-specific recombinase:::::*ant(6)-Ia*:streptothricin acetyltransferase (*satA*)::IS1182 family:::*Erm(B)*:::::::*TetR/AcrR* family:::IS30 family::IS6-like IS1216 family:Rep initiation protein:*catA*:IS6-like IS1216 family::::IS30 family	*E. faecalis* plasmid pKUB3007-1 KUB3007 DNA (AP018544.1)
		29	IS6 family::protein rep:plasmid recombination protein:*Tet(L)*:tet resistance efflux:*Tet(M)*:tet resistance determinant:conjugal transfer protein	*E. faecium* isolate P39 transposon Tn6247 (KP345886.1)
8L1-82	*E. durans*	4	ISL3 family::::transposase::::transporter permease:::*aac(6')-Ii*h:rRNA methyltransferase	ND

		16	*TetR/AcrR* family:::IS30 family:::rRNA methyltransferase:*erm(B)*:recombinase family protein:Tn3 family transposase	*E. saigonensis* VE80 plasmid pVE80-1 DNA (AP022823.1)
		17	methyltransferase:*Ant(6)-Ia*:adenine phosphoribosyltransferase:*ant(9)-Ia*:::recombinase family:*Lsa(E)*:*Lnu(B)*	*E. faecium* isolate E4457 genome assembly, plasmid: 3 (LR135260.1)
10L1-121 (ST1756)	*E. faecium*	27	protein rep:plasmid recombination protein:*tet(L)*:tet resistance efflux:*tet(M)*:tet resistance determinant:conjugal transfer protein	*E. hirae* strain HDC14-2 plasmid pHDC14-2.133K (CP042290.1)
		39	IS30 family:::*erm(B)*:recombinase family	*E. faecalis* strain 28157_4#18 genome assembly, plasmid: 2 (LR962490.1)

ND: Not determined



#	Locus tag (Gene)	Coordinates [+/-], size (bp)	Protein GI	Product	Component
1	*SPPN_01400*	221719..222780 [+], 1062	342162945	methionine import ATP-binding protein MetN	
2	*SPPN_01405*	222782..223474 [+], 693	342162946	ABC transporter permease	
3	*SPPN_01410*	223605..224372 [+], 768	342162947	transcriptional activator Rgg/GadR/MutR family protein	
4	*SPPN_01415*	224688..225002 [+], 315	342162948	hypothetical protein	
5	*SPPN_01420*	225018..225404 [+], 387	342162949	hypothetical protein	
6	*SPPN_01425*	225433..226818 [+], 1386	342162950	cell division FtsK/SpoIIIE protein	Orf21_Tn
7	*SPPN_01430*	226996..228201 [+], 1206	342162951	Cro/CI family transcriptional regulator	
8	*SPPN_01435*	228244..228465 [+], 222	342162952	Tn916 ORF19 protein	Orf19_Tn
9	*SPPN_01440*	228582..229079 [+], 498	342162953	Tn916 ORF18 antirestriction (ArdA) protein	
10	*SPPN_01445*	229054..229560 [+], 507	342162954	Tn916 ORF17 signal peptide containing protein	Orf17_Tn
11	*SPPN_01450*	229544..231991 [+], 2448	342162955	hypothetical protein	Orf16_Tn
12	*SPPN_01455*	231994..234171 [+], 2178	342162956	Tn916 ORF15 signal peptide containing protein	Orf15_Tn
13	*SPPN_01460*	234168..235169 [+], 1002	342162957	Tn916 ORF14 extracellular hydrolase/peptidase	Orf14_Tn
14	*SPPN_01465*	235166..236101 [+], 936	342162958	Tn916 ORF13 protein	Orf13_Tn
15	*SPPN_01470*	236509..238428 [+], 1920	342162959	tetracycline resistance protein TetM	
16	*SPPN_01475*	238774..238857 [-], 84	342162960	hypothetical protein	
17	*SPPN_01480*	239327..239410 [+], 84	342162961	leader peptide	
18	*SPPN_01485*	239535..240272 [+], 738	342162962	erythromycin ribosome methylase	

Fig 1: Location of *tet*(*M*) gene on a putative Tn*916*-like (Tn*644*) transposon linked with *erm*(*B*) gene

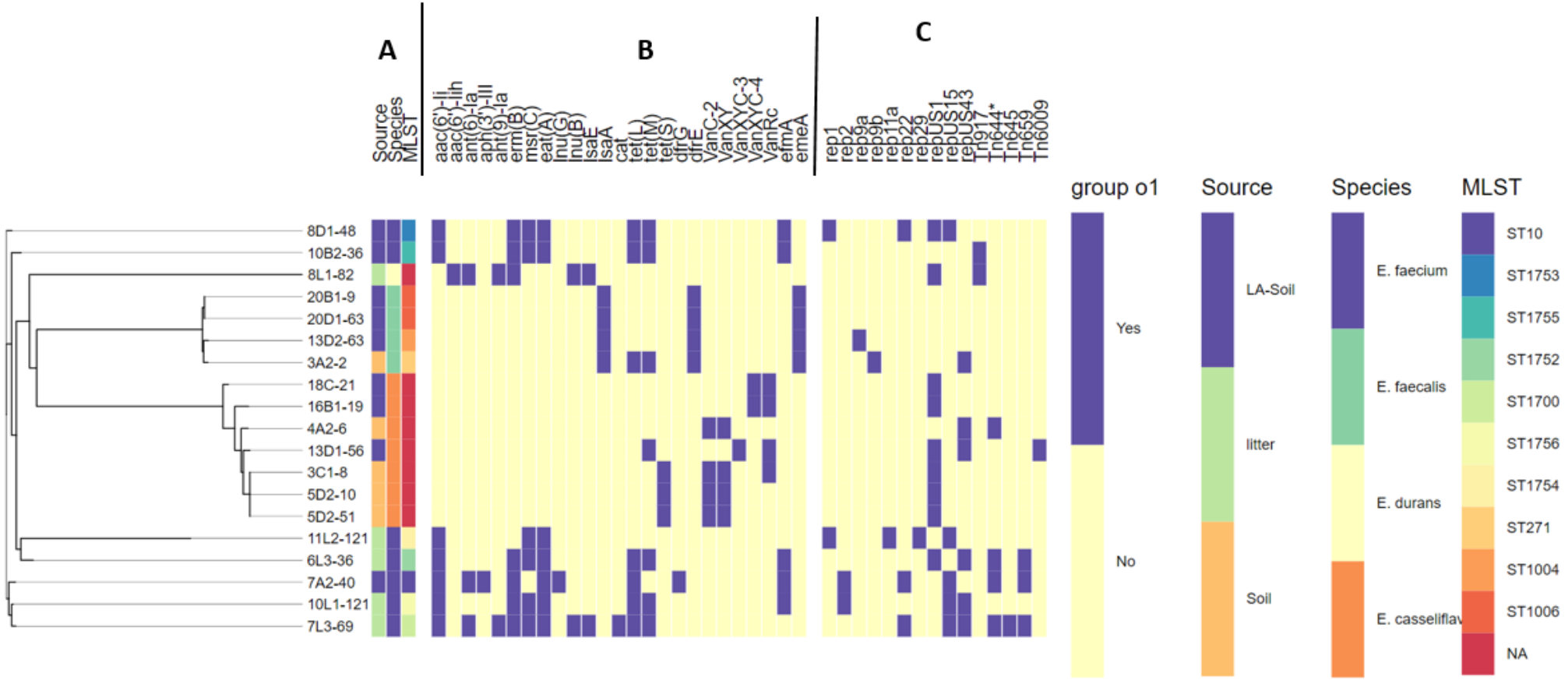


Figure 2: Phylogenomic tree of Enterococci with the metadata (source, species, WGS insilicotyping, antibiotic resistance genes and the associated plasmid type and transposon). **A**- Isolate information and molecular typing, **B**- Resistance genes, **C**- Plasmid replicons, **LA-soil**-litter-amended soil.

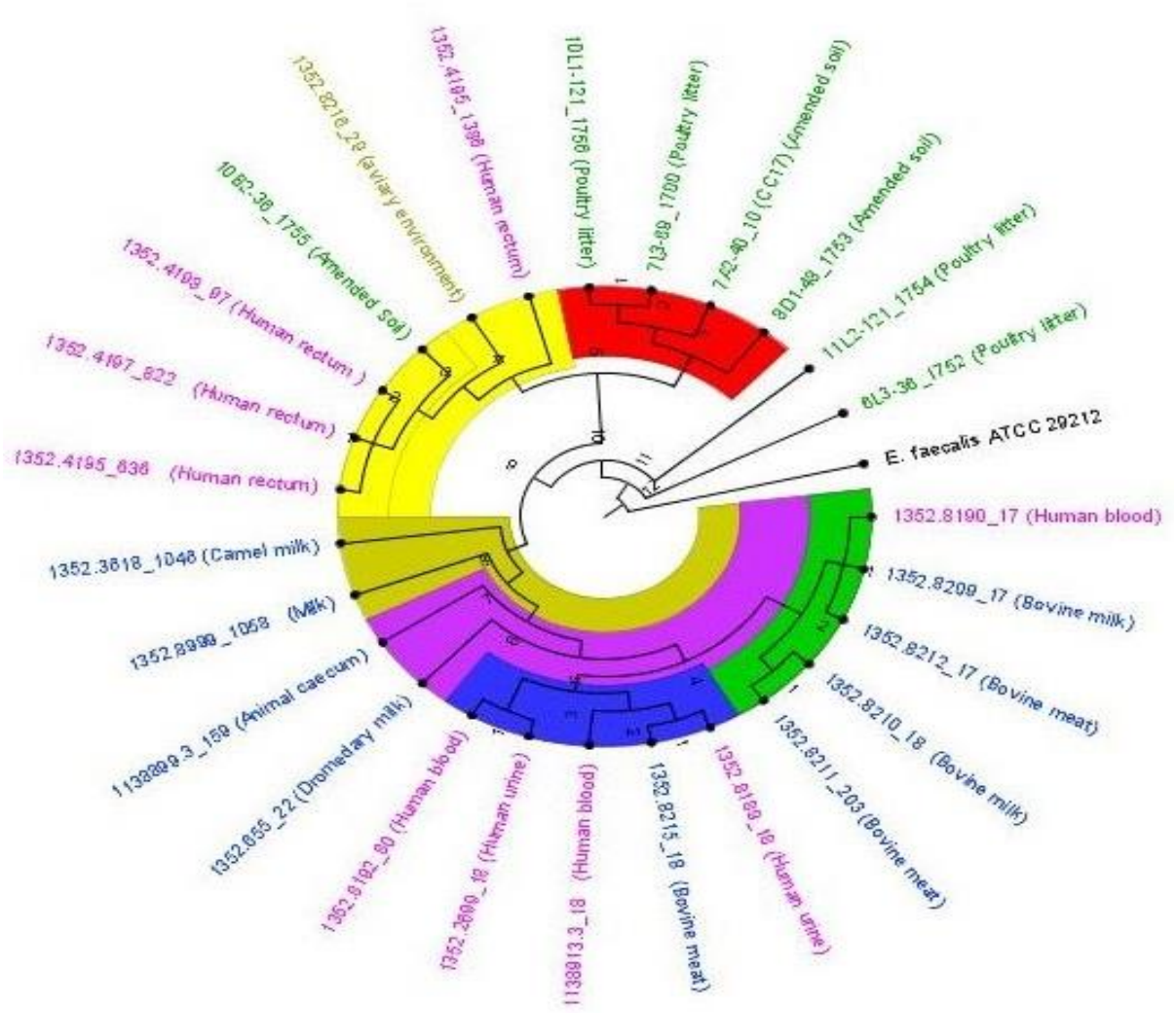


Figure 3: Phylogeny of *E. faecium* based on analysis of single-nucleotide polymorphisms (SNPs) of the core genes. The origin of isolates are indicated in the figures and grouped by colour into humans (pink), animals (blue) and the environment (gold) groups. The isolates from this study were coloured green.

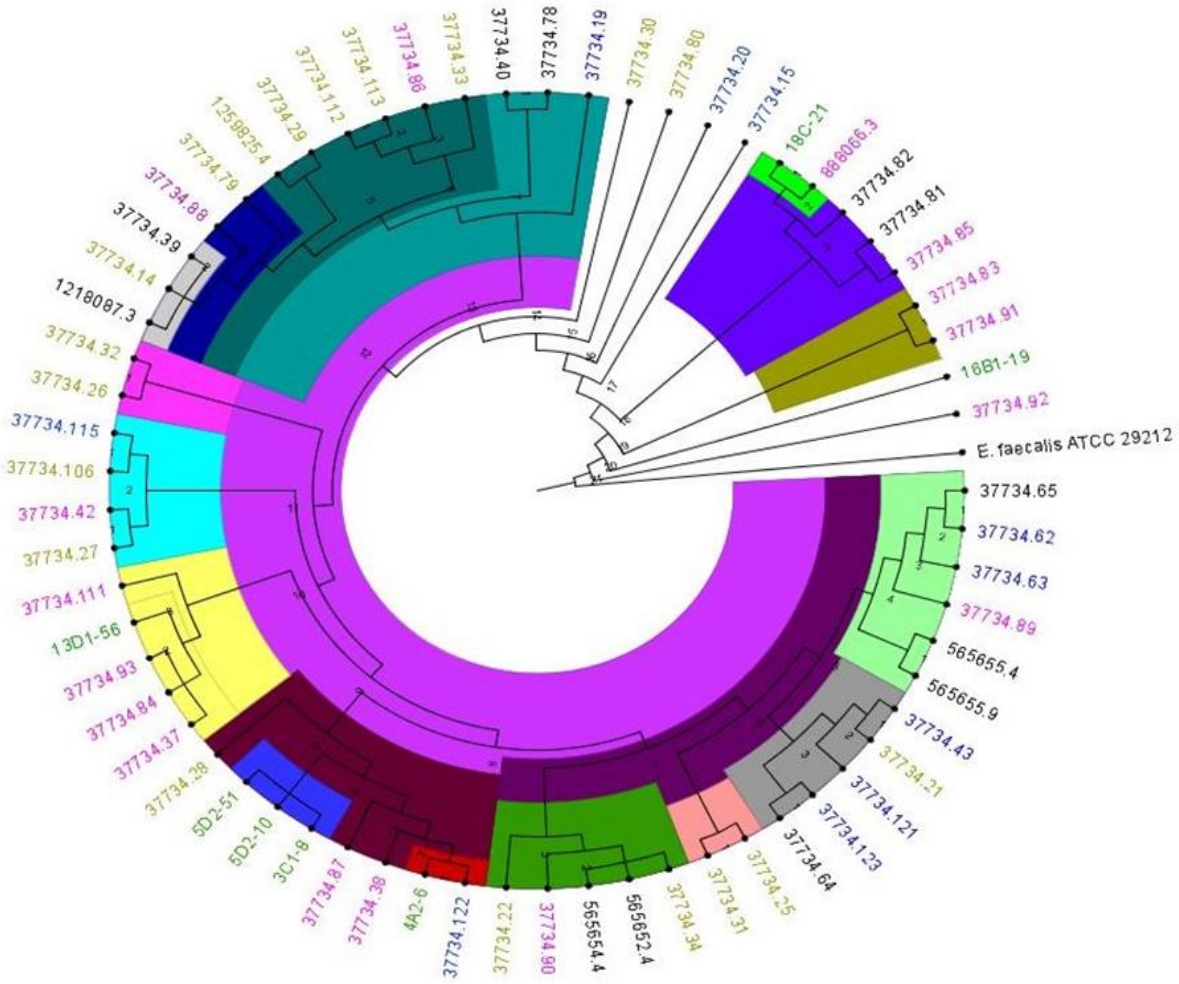


Figure 4: Phylogeny of *E. casseliflavus* based on analysis of single-nucleotide polymorphisms (SNPs) of the core genes. The source of isolates are indicated by colour in the figures {humans (pink), animals (blue), unavailable source (*bla*ck) and the environment (gold)}. The isolates from this study are coloured green.

Supplementary materials

Table S1: Genomic characteristics of the Enterococci isolates from chicken litter, the unamended and litter-amended soil

Isolate ID	Accession no	Sample source	Species	Size (Mb)	GC%	No. of Contigs	No. of RNAs	No of coding Sequences	*N50* (bp)	*L50*	Coverage (X)
3C1-8	JAAMSL000000000	Soil	*E. casseliflavus*	3.6	42.4	108	48	3634	279,907	5	38
3A2-2	JAAMSK000000000	Soil	*E. faecalis*	2.7	37.6	13	53	2518	355,199	2	93
4A2-6	JAAMSJ000000000	Soil	*E. casseliflavus*	3.8	42.2	19	57	3738	582,543	3	64
5D2-10	JAAMSG000000000	Soil	*E. casseliflavus*	3.5	42.4	45	48	3531	279,907	5	86
5D2-51	JAAMSE000000000	Soil	*E. casseliflavus*	3.5	42.4	45	48	3531	279,907	5	77
7A2-40	JAAMSC000000000	Soil	*E. faecium*	2.6	38.0	134	60	2613	58,739	14	82
8D1-48	JAAMSA000000000	Soil	*E. faecium*	2.6	37.9	101	61	2637	88,123	10	72
10B2-36	JAAMRX000000000	Soil	*E. faecium*	2.7	38.0	63	61	2717	84,487	10	90
13D1-56	JAAMRU000000000	Soil	*E. casseliflavus*	3.4	42.5	46	51	3480	145,906	6	72
13D2-63	JAAMRT000000000	Soil	*E. faecalis*	2.8	37.5	18	48	2717	579,081	2	156
16B1-19	JAAOLQ000000000	Soil	*E. casseliflavus*	3.5	42.4	14	53	3437	562,111	3	70
18C-21	JAAOLS000000000	Soil	*E. casseliflavus*	3.7	42.3	21	53	3549	295,202	4	93
20D1-63	JAAOLU000000000	Soil	*E. faecalis*	2.9	37.2	13	50	2771	1,463,532	1	128
20B1-9	JAAOLV000000000	Soil	*E. faecalis*	2.9	37.2	12	50	2771	1,511,308	1	86
6L3-36	JAAMSD000000000	Chicken litter	*E. faecium*	2.7	38.0	46	62	2653	132,199	7	90
7L3-69	JAAMSB000000000	Chicken litter	*E. faecium*	2.7	37.9	78	63	2717	93,555	10	107
8L1-82	JAAMRZ000000000	Chicken litter	*E. durans*	3.0	37.8	26	60	2900	282,203	4	103
10L1-121	JAAMRW000000000	Chicken litter	*E. faecium*	2.6	38.1	71	62	2592	99,677	9	120
11L2-121	JAAMRV000000000	Chicken litter	*E. faecium*	2.8	38.2	57	66	2592	110,725	9	104

Table S2: Antibiotic susceptibility profile of Enterococci from chicken litter, the unamended and litter-amended soil.

Isolate ID	Species	Category	STR	GEN	TEC	VAN	AMP	IPM	ERY	LEV	CIP	NIT	CHL	LZD	TET	TGC	SXT	QD
Isolates from unamended soil																		
3C1-8	*E. casseliflavus*	MDR	S	S	S	I	S	S	I	R	R	I	I	S	R	S	R	-
3A2-2	*E. faecalis*	MDR	S	S	S	S	S	S	I	I	R	S	I	S	R	S	R	-
4A2-6	*E. casseliflavus*	MDR	S	S	I	R	S	R	R	R	I	I	S	S	I	S	R	-
5D2-10	*E. casseliflavus*	MDR	S	S	S	R	S	S	S	R	S	S	S	S	R	S	R	-
5D2-51	*E. casseliflavus*	MDR	S	S	S	R	R	I	I	S	I	S	S	S	R	S	R	-
Isolates from litter-amended soil																		
7A2-40	*E. faecium*	MDR	R	S	S	S	R	R	R	I	I	S	S	S	R	S	R	R
8D1-48	*E. faecium*	MDR	S	S	S	S	S	R	R	R	I	R	S	S	R	S	S	I
10B2-36	*E. faecium*	MDR	S	S	S	S	S	R	R	S	R	I	I	S	R	S	S	I
13D2-63	*E. faecalis*	-	S	S	I	I	S	I	R	S	I	R	S	S	I	S	S	-
13D1-56	*E. casseliflavus*	-	S	S	S	S	S	S	S	S	I	S	S	S	R	S	S	-
16B1-19	*E. casseliflavus*	-	S	S	S	I	R	S	I	S	I	S	I	S	S	S	R	-
18C-21	*E. casseliflavus*	MDR	R	S	S	I	S	S	R	S	I	S	S	S	I	S	R	-
20D1-63	*E. faecalis*	-	S	S	S	I	S	S	I	I	I	S	S	S	S	S	R	-
20B1-9	*E. faecalis*	-	S	S	S	I	S	R	I	I	I	I	S	S	S	S	R	-
Chicken litter isolates																		
6L3-36	*E. faecium*	MDR	S	S	I	S	R	R	R	R	S	S	S	S	S	S	S	R
7L3-69	*E. faecium*	MDR	R	S	S	S	S	S	R	I	I	S	I	S	R	S	R	I
10L1-121	*E. faecium*	MDR	S	S	S	S	S	I	R	I	R	S	S	S	R	S	R	I
11L2-121	*E. faecium*	MDR	S	S	S	S	S	R	R	S	I	R	S	S	S	S	R	I
8L1-82	*E. durans*	MDR	S	S	S	S	R	S	R	S	S	R	S	S	R	S	R	-

AMP-Ampicillin, TGC-Tigecycline, TET-Tetracycline, SXT- Sulfamethoxazole-Trimethoprim, CHL- Chloramphenicol, GEN- Gentamicin, TEC-Teicoplanin, VAN-Vancomycin, STR-Streptomycin, LZD- Linezolid , IPM- Imipenem, ERY- Erythromycin, CIP- Ciprofloxacin, LEV-Levofloxacin, NIT- Nitrofurantoin, QD- Quinupristin-dalfopristin (QD* was reported for only *E. faecium* isolates).

Table S3: Point mutations in the *gyrA*, *parC* and *pbp5* region of the Enterococci isolates.

Isolate ID	***gyrA***	***parC***	***pbp5***	***liaS***
3A2-2	-	G59S*, V307I*	-	-
7A2-40	N708D*	A391V*	V24A, S27G, R34Q, G66E, E100Q, K144Q, T172A, L177I, A216S, T324A, N496K, A499I, E525D, T25A*, S39T*, A401S*, D644N*	-
8D1-48	N708D*, I354T*	-	V24A, S27G, R34Q, G66E, E100Q, K144Q, T172A, L177I, A216S, T324A, N496K, A499I, E525D,T25A*, S39T*, S358G*, A401S*, D644N*	-
10B2-36	N708D*	-	V24A, S27G, R34Q, G66E, E100Q, K144Q, T172A, L177I, A216S, T324A, N496K, A499T, E525D, T25A*, S39N*, D644N*	-
13D2-63	A626S*	K228R*	-	-
6L3-36	N708D*	-	V24A, S27G, R34Q, G66E, E100Q, K144Q, T172A, L177I, A216S, T324A, N496K, A499T, E525D, T25A*, S39N*, D644N*	-
7L3-69	-	A391V*	V24A, S27G, R34Q, G66E, E100Q, K144Q, T172A, L177I, A216S, T324A, N496K, A499I, E525D, A19V*, T25A*, S39T*, A401S*, D644N*	E192G*
10L1-121	-	A391V*	V24A, S27G, R34Q, G66E, E100Q, K144Q, T172A, L177I, A216S, T324A, N496K, A499I, E525D, T25A*, S39T*, A401S*, D644N*	E192G*
11L2-121	I259L*,I306V*, N708D*,D759N*, A811V*, G819A*, S820T*	I699V*, E707D*, L773I*	V24A, S27G, K144Q, T324A, T25A*, S39T*, A73T*, S133Y*, K318R*, D644N*	-

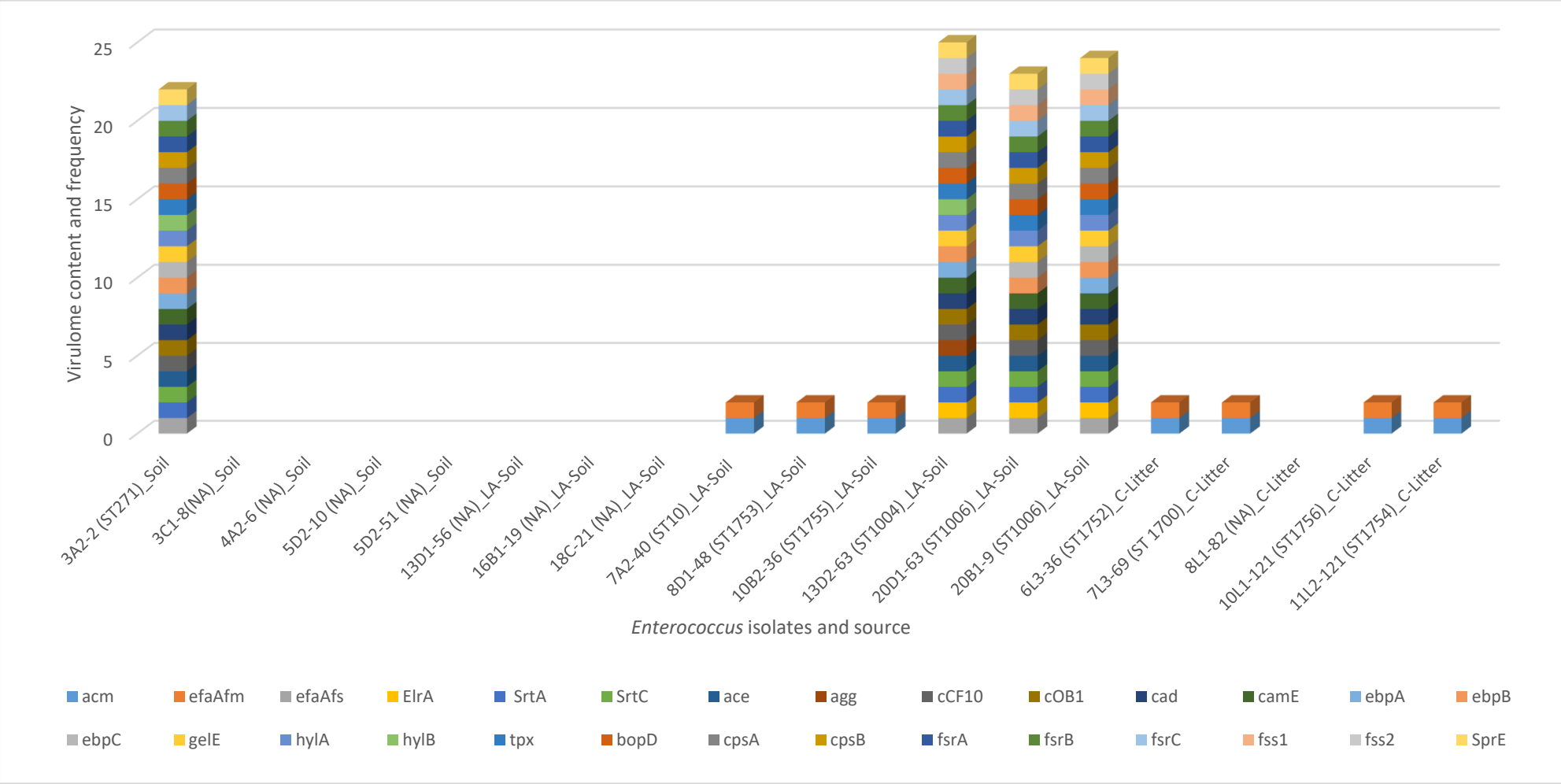


Figure S1: Distribution of virulence genes among the enterococci isolates

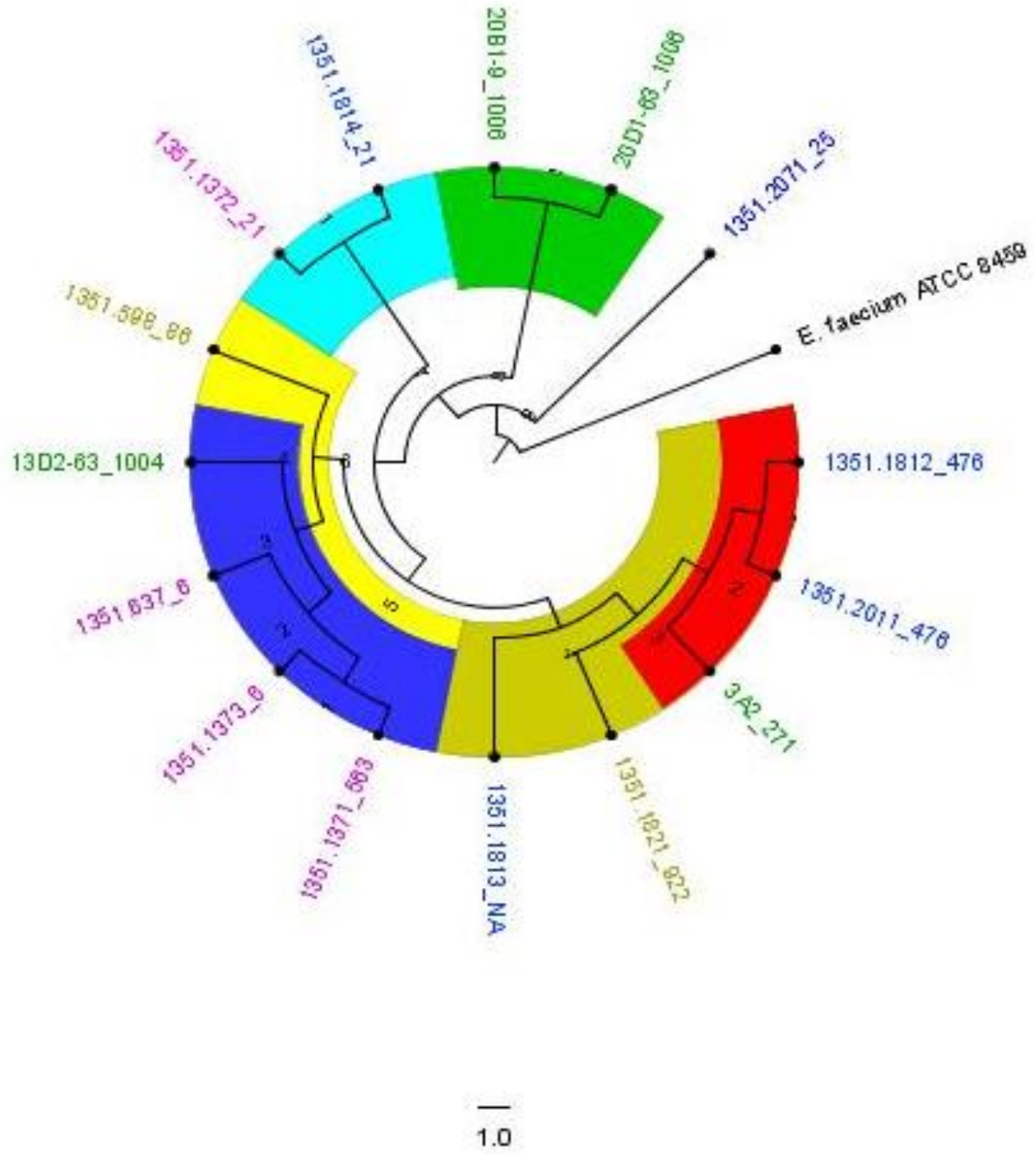


Figure S2: Phylogeny of *E. faecalis* based on analysis of single-nucleotide polymorphisms (SNPs) of the core genes. Isolates source are indicated and grouped by colour into humans (pink), animals (blue) and the environment (gold) groups. The isolates from this study were coloured green.

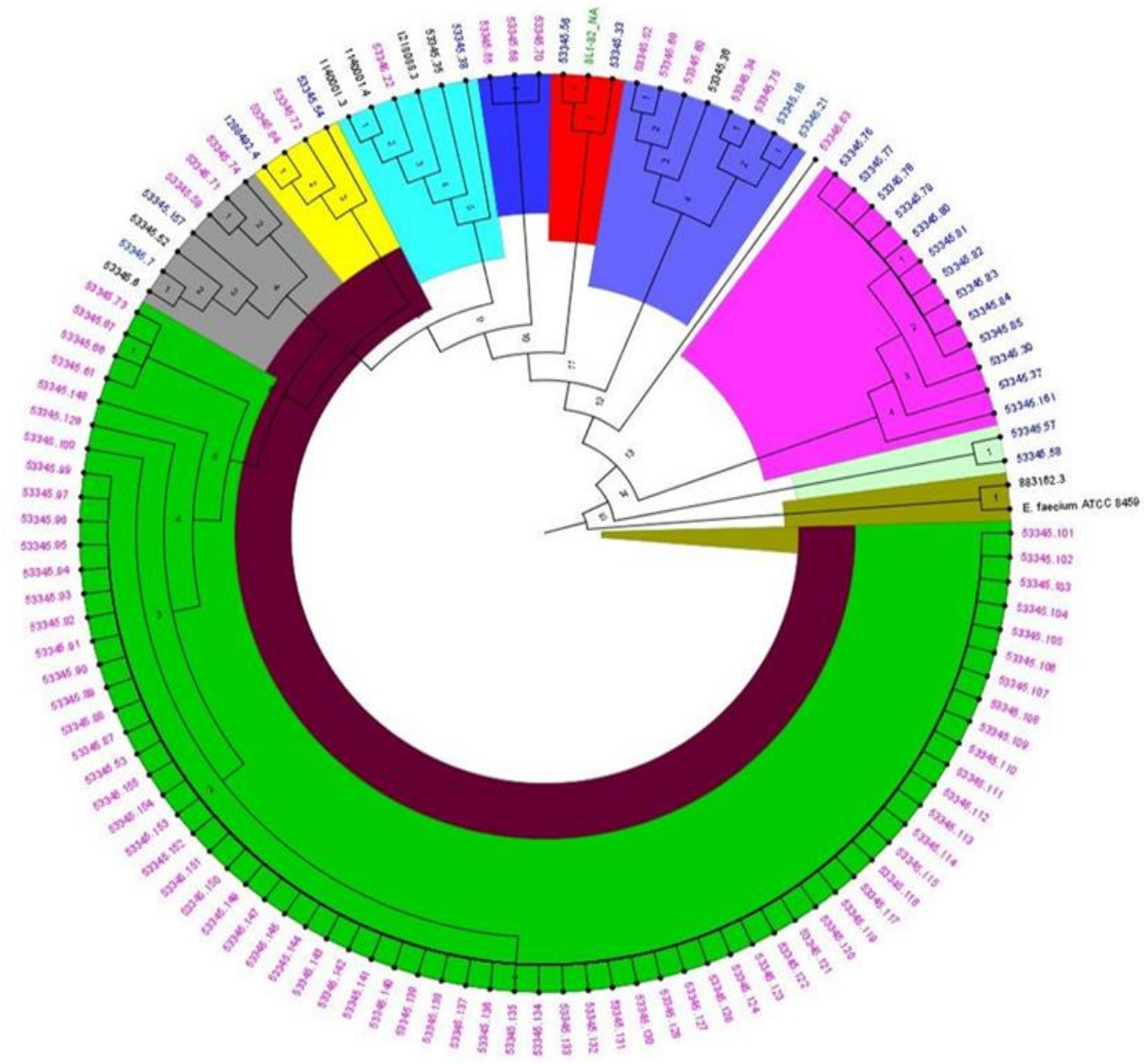


Figure S3: Phylogeny of *E. durans* based on analysis of single-nucleotide polymorphisms (SNPs) of the core genes. Isolates source are indicated by colour in the figures {humans (pink), animals (blue), unavailable source (black) and the environment (gold)}. The isolates from this study are coloured green.

Table S4: Distribution of intact prophages among Enterococci from soil and chicken litter.

Isolate ID	Intact Prophages
Isolates from background soil	
3C1-8	1. Entero_phiFL4A
	2. Bacill_vB_BhaS_171
	3. Lactoc_50101
4A2-6	1. Lactob_PLE3
5D2-10	1. Entero_phiFL4A
	2. Bacill_vB_BhaS_171
	3. Lactoc_50101
5D2-51	1. Entero_phiFL4A
	2. Bacill_vB_BhaS_171
	3. Lactoc_50101
Isolates from litter-amended soil	
7A2-40	1. Entero_SANTOR1
8D1-48	1. Entero_Ec_ZZ2
10B2-36	1. Entero_SANTOR1
13D2-63	1. Entero_phiFL3A
	2. Staphy_80
13D1-56	1. Strept_phiARI0746
	2. Strept_phiARI0131
Chicken litter isolates	
6L3-36	-
7L3-69	1. Strept_phiARI0131
	2. Strept_9871
8L1-82	1. Entero_Ec_ZZ2
10L1-121	1. Lister_2389
	2. Staphy_SPbeta_like
11L2-121	1. Strept_9872

CHAPTER FIVE

5.0 CONCLUSIONS, LIMITATIONS, RECOMMENDATIONSAND SIGNIFICANCE OF STUDY

This study describes the molecular epidemiology of antibiotic-resistant *E. coli* and *Enterococcus* spp. from agricultural soil fertilized with chicken litter.

5.1 Conclusions

The following were the main findings from this study according to the study objectives:

- To enumerate the *Escherichia coli* and Enterococci in the soil before and after chicken litter amendment and in the heap of chicken litter using the Colilert® -18 / Quantiti-Tray® 2000 system and the Enterolert® -18® Quantiti-Tray®/2000 systems, respectively.

***Enterococcus* spp.**

- The highest concentrations of *Enterococcus* spp. was observed in the chicken litter, indicating that chicken litter could be an important source of *Enterococci* contamination to the receiving soil.
- Enterococci count in the litter-amended soil (3.87 (±1.43) × 10^7 MPN/g) was significantly higher than the unamended soil (2.89 (±0.92) × 10^7 MPN/g), reflecting the impact of chicken litter application on the soil.
- There were fluctuations in enterococci count in the litter-amended soil. The count in the litter-amended soil decreased to levels comparable with the count in the soil before the amendment after 50 days of chicken litter amendment but persisted till the end of the sample collection period.

E. coli

- The overall mean count of *E. coli* in the chicken litter (2.11 (±1.29) x 10^7 MPN/g) was significantly higher than the litter-amended soil ($p = 0.020$), and the soil samples collected before the litter amendment ($p = 0.023$)
- *E. coli* count in the litter-amended soil (1.51 (±0.99) x 10^7 MPN/g) was not significantly different from the unamended soil (1.52 (±0.72) x 10^7 MPN/g)
- There were fluctuations in *E. coli* counts in the soil throughout the sampling period until it was no longer detectable in the soil at 49 days after the litter amendment.

- The increase and short-term detection of *E. coli* in the soil following the chicken litter application suggest that *E. coli* could serve as a suitable indicator of short-term pollution in the agricultural soil environment.

❖ To isolate and confirm *Escherichia coli* and *Enterococcus* spp. using selective media and real-time polymerase chain reaction (RT-PCR).

- All the samples (soil and chicken litter) collected from the various sample groups and points tested positive for *Enterococcus*.
- All the samples were not positive for *E. coli*
- The overall prevalence of enterococci was 835 (soil before litter amendment (107), litter-amended soil (573), and 155 from chicken litter). Analysed by species: *E. casseliflavus* was the most prevalent species at 469 (56.2%), followed by *E. faecalis* 184 (22%), *E. faecium* 64 (8%), *E. gallinarum* 16 (1.9%), and other *Enterococcus spp* 102 (12%).
- A total of one hundred and twenty-six *E. coli* was isolated from all the samples (98 isolates from the soil and 28 from the chicken litter).

❖ To determine the antibiotic susceptibility profile of the confirmed *E. coli* and *Enterococcus* spp. isolates using the Kirby-Bauer disk diffusion method according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI) and/or the European Committee on Antimicrobial Susceptibility Testing (EUCAST)

***Enterococcus* spp.**

- The enterococci isolates were mostly resistant to tetracycline (33%), erythromycin (25%), and trimethoprim-sulfamethoxazole (23%) antibiotics which are commonly used in poultry farms in the uMgungundlovu District of KwaZulu-Natal, South Africa.
- Lower levels of resistance were evident to ciprofloxacin (11%), nitrofurantoin (5%), imipenem (5%), ampicillin (4%), levofloxacin (3%) streptomycin (2%), vancomycin (1%), teicoplanin (0.2%) and chloramphenicol (0.1%) were also observed in the isolates.
- All the isolates were susceptible to tigecycline, linezolid, and gentamicin.
- Multidrug resistance was recorded in 27.8% (130/466) of the resistant enterococci isolates, of which the litter-amended soil isolates had the highest percentage, 67.7% (88/130), followed by the chicken litter 22.3% (29/130) and unamended soil 10% (13/130). MAR indices ranging from 0.13 (resistance to

seven antibiotics) to 0.44 (resistance to seven antibiotics). Sixty-three different resistance patterns were recorded in the MDR enterococci isolates, indicating high resistance to several antibiotics.

E. coli

- The *E. coli* isolates displayed resistance to tetracycline (60%), chloramphenicol (48%), ampicillin (44%), trimethoprim- sulfamethoxazole (30%), cefotaxime (23%), ceftriaxone (20%), cefalexin (16%), cefepime (9%), amoxicillin-clavulanic acid (9%), cefoxitin (8%), nalidixic acid (7%), amikacin (5%), ciprofloxacin (3%) with the least percentage resistance (2%) in imipenem, tigecycline, and gentamicin.
- The *E. coli* isolates expressed 100% susceptibility to ceftazidime and meropenem.
- Multidrug-resistance was evident in 41% of *E. coli*, with the highest prevalence observed in the litter-amended soil (61.5%) and the least in soil samples collected before litter application (1.9%), suggesting an influx of MDR into the soil via the litter amendment. Twenty-one different resistance patterns and MAR indices range of 0.11 to 0.56 (resistance to ten antibiotics) were observed in the isolates
- Overall, resistance to antibiotics commonly used as growth promoters and for treatment in poultry farms in uMgungundlovu District of KwaZulu-Natal, South Africa, was evident

❖ To identify the antibiotic resistance genes (ARGs) and the virulence genes harboured by the isolates using whole-genome sequencing (WGS) and bioinformatics tools (ResFinder, CARD, and VirulenceFinder).

- Genes conferring resistance to aminoglycosides (*aac(6')-Ii*, *aac(6')-Ii*h, *ant(6)-Ia*, *aph(3')-III*, ant(9)-Ia), macrolide-lincosamide-streptogramin AB (MLS_{AB}) [*erm(B)*, lnu(B), *lnu(G)*, *lsaA*, *lsaE*, *eat(A)*, *msr(C)*], trimethoprim-sulfamethoxazole (*dfrE*, and *dfrG*), tetracycline (*tet(M)*, *tet(L)*, and *tet(S)*), fluoroquinolones (*efmA*, and *emeA*), vancomycin (*VanC {VanC-2*, *VanXY*, *VanXY*C-3, *VanXY*C-4, *VanRC*}), and chloramphenicol (*cat*) were detected in the isolates. There were discrepancies between the phenotypes and the genotypes in some instances.

- Nine putatively novel mutations in the *gyrA* and seven in *parC* genes were found in the isolates. Fourteen known mutations conferring resistance to *β*-lactams and ten putative novel mutations were also identified in the isolates.
- The litter-amended soil harboured new ARB (particularly *E. faecium*) and ARGs (*ant(6)-Ia*, *aac(6')-Ii*, *aph(3')-III*), *lnu(G)*, msr(C), and eat(A), *efmA)* that were not detected in the soil before the litter amendment, indicating the impact of manure application on soil resistome.
- Twenty-three different putative virulence genes associated with adherence/biofilm formation (*ebpA*, *ebpB*, *ebpC*), sex pheromone (*Ccf10*, *cOB1*, *cad*, and *camE*), gelatinase (*gelE*), anti-phagocytosis (*elrA*), oxidative stress response (*tpx*) and hyaluronidases (*hylA*, *hylB*) were found in *E. faecalis* and *E. faecium*

❖ To determine the genetic environment and the MGEs associated with the ARGs and virulence genes using MobileElementFinder, RAST, and NCBI PGAP.

- Several MGEs, including insertion sequences (IS982, ISL3, IS6, IS256, IS30), plasmids (repUS15, rep1, repUS43), *Tn3* family, and *Tn916*-like transposons were associated with the identified ARGs.
- *Tet(L)* and *tet(M)* genes were mostly associated with repUS43, rep9b, and rep 22 plasmids while the *erm(B)* gene was found in association with repUS1 plasmid that could aid their horizontal transfer in soil bacteria.
- *Tet(M)* and *erm(B)* genes (adjacent position) were also associated with a novel integrative conjugative element Tn*644* transposon in some isolates.

❖ To ascertain the clonal relatedness and phylogeny of the studied isolates using MLST and bioinformatics tools such as CSI Phylogeny pipeline, Phandango and Figtree and to compare the isolates from this study with other isolates deposited in a public repository.

- The MLST analysis revealed the multiclonal nature of the enterococci isolates MLST analysis detected ten different sequence types (STs), which included two known STs (ST10 and ST271) and eight novel STs (**ST1700, ST1752, ST1753, ST1754, ST1755, ST1756, ST1004, and ST1006**) assigned to nine isolates.

- The close phylogenomic relationship of the *E. faecium* isolates from litter-amended soil and the chicken litter isolates highlights the potential transfer of these ARB (from chicken litter to the litter-amended soil.
- Most isolates from this study clustered with human/clinical and animal isolates previously reported in South Africa, Angola, and Tunisia.

5.2 Limitations and Recommendations

Limitations

- The sampling strategy and the small number of *E. coli* isolates obtained per sample group/source may have affected the study's statistical power, notwithstanding the critical baseline information on the prevalence and resistance pattern of *Enterococcus* and *E. coli* in agricultural soil and chicken litter is provided by the study.
- This study would have been more enhanced if samples from occupationally exposed workers on the poultry and sugarcane farms were included, as this could have provided a holistic impact of this study in one health triad perspective.
- The high cost of WGS prompted the selection of a small number of enterococci isolates and prevented the inclusion of the WGS of *E. coli* isolates in this present study. As such, inclusive comparison of enterococci isolates from the three sample groups was limited due to the small number.

Recommendations

- Since soil is a community of heterogeneous bacteria, it is recommend that more robust surveillance on the impact of animal manure application on soil resistome, including more bacterial species, a larger number of isolates and more farms covering larger geographical locations be carried out to monitor AMR pollution in the environment.
- Future studies should consider the use of metagenomics approach as this is highly promising. It unravels multiple ARGs and the genetic elements in which it is embedded, gives insight into the composition of the microbial community, and the interactions between different microbial groups in the soil and chicken litter.
- There should be increased education and awareness about antibiotic resistance, its spread to the environment, and its potential risks to public health. Farmers should be encouraged to pre-treat animal manure before its application to farms.

5.3 Significance of the study

This study describes the impact of the chicken litter application on agricultural soil resistome in uMshwathi Local Municipality under uMgungundlovu District of KwaZulu-Natal, South Africa. The amendment of the agricultural soil with chicken litter increased the number of antibiotic-resistant *E. coli* and *Enterococcus* spp., attributable to the proliferation of indigenous or influx of litter-borne ARB. This study highlights chicken litter and litter-amended soil as reservoirs of potential pathogenic MDR *E. coli* and *Enterococcus* spp. that could contaminate fresh farm produce, surrounding water bodies and occupationally exposed workers. WGS and bioinformatics analyses of the enterococci revealed diverse ARGs mobilized on several MGEs that could facilitate the spread of AMR in the environment. The results highlight the potential public health risks associated with the high prevalence of such potential pathogenic MDR bacteria in the agricultural soil environment and provides preliminary evidence for policy-makers to consider surveillance of antibiotic-resistant bacteria as part of strategies needed to monitor AMR pollution in the agricultural soil environment. These findings also call for urgent implementation of policies that will ensure prudent use of antibiotics in poultry farms, provision of standard microbial load limit in animal waste intended for use as fertilizer and the efficient treatment of animal waste before their application to the soil.

APPENDICES:

APPENDIX I:

Ethical Approval

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

17 March 2017

Prof SY Essack
Department of Pharmaceutical Sciences
School of Health Sciences
essacks@ukzn.ac.za

Dear Prof Essack

Title: One Health approach to the containment of antibiotic resistance.
Degree: Non-degree
BREC Ref No: BCA444/16

CLASS APPROVAL

The Biomedical Research Ethics Committee (BREC) has considered the abovementioned application at a meeting held on 13 September 2016.

The study was provisionally approved by BREC pending appropriate responses to queries raised. Your responses dated 28 February 2017 to queries raised on 19 September 2016 have been noted and approved by the Biomedical Research Committee at a meeting held on 14 March 2017.

This approval is valid for one year from **17 March 2017.** To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

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SOUTH AFRICAN NATIONAL DEPARTMENT OF AGRICULTURE, FORESTRY AND FISHERIES

REGISTRATION

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Henry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HenryG@daff.gov.za
Reference: 12/11/1/5 (879)

Professor Sabiha Yusuf Essack
Antimicrobial Research Unit
College of Health Sciences
University of KwaZulu-Natal
Tel: 031 260 7785
E-mail: ESSACKS@ukzn.ac.za

Dear Prof Essack,

RETROSPECTIVE APPLICATION UNDER SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984) FOR: "ANTIBIOTIC RESISTANCE AND ONE HEALTH", SPECIFICALLY PERTAINING TO THE STUDY CONDUCTED IN INTENSIVELY PRODUCED POULTRY

Your application received on 30 August 2018 for a Section 20 permit for the above mentioned study refers. Unfortunately, Section 20 approval cannot be given retrospectively for a study that has already proceeded or concluded, but we hereby take note of the information divulged in the application.

We take note of the letter of apology dated 28 August 2018, as well as the letter dated 13 September 2018 where you undertake to comply with Section 20 of the Animal Diseases Act, 1984 (Act No. 35 of 1984) and relevant processes in future.

Kind regards

[redacted]

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2018 -09- 25

APPENDIX II

List of Conferences and Trainings

1. Norad Conference on Antibiotic Stewardship and Conservation in Africa held, University of KwaZulu-Natal, Durban, South Africa, 20th – 23rd October 2019
2. Wellcome Genome Campus Advanced Courses on Genomics and Clinical Microbiology (Virtual), 25th – 29th January 2021.
3. SEQAFRICA virtual training in Whole Genome Sequencing (WGS) for antimicrobial research: Module 1- Introduction to WGS in antimicrobial research and surveillance, 15th to 26th February, 2021. Module 2- WGS workflow from bacteria isolation to fully analyzed genomes, 22nd to 29th, March 2021.
4. 31st European Congress of Clinical Microbiology & Infectious Diseases (ECCMID), virtual, 9th to 12th July, 2021.

1: CERTIFICATE OF ATTENDANCE FOR THE NORAD CONFERENCE

2: CERTIFICATE OF ATTENDANCE FOR TRAINING ON GENOMICS AND CLINICAL MICROBIOLOGY

WELLCOME GENOME CAMPUS
CONNECTING SCIENCE
ADVANCED COURSES+ SCIENTIFIC CONFERENCES

Certificate of Attendance

To whom it may concern

Wellcome Genome Campus Advanced Course
Genomics and Clinical Microbiology (Virtual)
25 – 29 January 2021

This is to confirm that: **Dorcas Fatoba**

Attended The Wellcome Genome Campus Advanced Course, 'Genomics and Clinical Microbiology' (Virtual) Course from 25 – 29 January 2021.

The Wellcome Genome Campus Advanced Courses are intensive courses and I would estimate that this represents in excess of 37 hours contact time.

Please find attached a summary of the topics covered.

Yours faithfully

[redacted]

Dr Darren Hughes
Scientific Programme Manager

Wellcome Genome Campus Advanced Courses and Scientific Conferences, Hinxton Cambridge CB10 1SA
T +44 (0)1223 496910 www.wellcomegenomecampus.org/coursesandconferences

Connecting Science is part of Genome Research Limited. Genome Research Limited, a charity registered in England with number 1021457 and a company registered in England with number 2742969, whose registered office is 215 Euston Road, London, NW1 2BE.

3A: CERTIFICATE OF ATTENDANCE FOR SEQAFRICA VIRTUAL TRAINING ON WHOLE GENOME SEQUENCING (MODULE 1)

The Fleming Fund
Regional Grants

CERTIFICATE OF COMPLETION

This is to certify that

Dorcas Fatoba

has completed a SEQAFRICA Virtual Course in **Introduction to Whole Genome Sequencing in AMR Surveillance.**

The **Consortium of SEQAFRICA**

awards this certificate

8 March 2021

[redacted]

Rene Hendriksen
SEQAFRICA Technical Lead
Technical University of
Denmark

NATIONAL INSTITUTE FOR COMMUNICABLE DISEASES
Division of the National Health Laboratory Service

UNIVERSITY OF IBADAN

KCRI Kilimanjaro Clinical Research Institute

NOGUCHI MEMORIAL INSTITUTE FOR MEDICAL RESEARCH
UNIVERSITY OF GHANA, LEGON

SEQAFRICA

DTU

UKaid
from the British people

3A: CERTIFICATE OF ATTENDANCE FOR SEQAFRICA VIRTUAL TRAINING ON WHOLE GENOME SEQUENCING (MODULE 2)

4: CERTIFICATE OF ATTENDANCE FOR THE 31ST ECCMID

ESCMID

Online
9 – 12 July 2021

31st ECCMID EUROPEAN CONGRESS OF CLINICAL MICROBIOLOGY AND INFECTIOUS DISEASES

CERTIFICATE OF ATTENDANCE

This is to certify that

Dorcas Oladayo Fatoba
(dorcas4c@gmail.com)

participated in the

31st European Congress of Clinical Microbiology & Infectious Diseases

that took place online from

9 – 12 July 2021

Yours sincerely,

[redacted]

Jacob Moran-Gilad
ECCMID Programme Director