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Antimicrobial sensitivity of *Escherichia coli* and *Enterococcus* species isolated from table eggs, and backyard poultry farmers' knowledge and attitudes on responsible antimicrobial use in Balfour, Dipaleseng Municipality of Mpumalanga Province, South Africa

by

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DECLARATION

I Sherpherd Mbedzi, declare that the thesis which I hereby submit for the degree MASTER OF SCIENCE, in the Faculty of Veterinary Science at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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Ethics statement

The author, whose name appears on the title page of this thesis, has obtained the required ethics approval/exemption for the research described in this work.

The author declares that they have observed the ethical standards required in terms of the University's Code of ethics for scholarly activities.

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ABSTRACT

The emergence and subsequent spread of antimicrobial resistance (AMR) in pathogenic microorganisms is an increasing and well documented global health concern (Eagar *et al*., 2012; Roth *et al.,* 2019). Widespread antimicrobial drug usage in livestock is implicated as one of the drivers of AMR (Kapena *et al*., 2020). Significant knowledge gaps exist about the efficacy of these drugs. It is crucial to carry out surveillance research in accordance with the global action plan of the World Health Organization (WHO) to combat AMR (Nulsen *et al*., 2008; Kapena *et al*., 2020). This research was conducted to determine the antibiotic sensitivity of *Escherichia coli (E. coli) and Enterococcus* isolates from chicken table eggs, as well as respondents' knowledge of antibiotics, withdrawal times and AMR in the Balfour community of Mpumalanga province. A structured questionnaire was administered in face-to-face interview format to backyard poultry farmers (n=27). Out of 27 respondents, 48.1% (13/27) indicated having an idea of what antibiotics are, while 29.6% (8/27) had an idea about withdrawal times. Only 14.8% (4/27) of respondents heard about AMR, with 51.9% (14/27) of respondents indicating they consumed eggs laid during treatment and 22.2% (6/27) would slaughter chickens for meat during treatment. Standard bacteriological methods were used to isolate *E. coli* and *Enterococci* from eggshells and egg contents. Ten *E. coli* isolates were recovered; 90% (9/10) from eggshells and 10% (1/10) from egg contents. A total of 58 *Enterococcus* isolates were recovered from eggshell swabs, and none from egg contents. Antibiotic sensitivity of the recovered bacteria was determined using the Kirby-Bauer disk diffusion method. Of the 10 *E. coli* isolates, susceptibility to ampicillin was noted to be 40%, (4/10). Susceptibility to colistin, gentamicin and sulpha-trimethoprim was 100% (10/10). Doxycycline was 40% (4/10). Susceptibility to enrofloxacin, fosfomycin and kanamycin was similar, with 80% (8/10) of isolates showing susceptibility. There were 90% (9/10) isolates susceptible to sulphonamide compound. Susceptibility to tetracycline was 60% (6/10). All (100%) of the *Enterococcus* isolates recovered, were susceptible to ampicillin. Susceptibility to doxycycline was noted in 86.2% (50/58) of isolates, with only 6.9% (4/58) susceptible to enrofloxacin. Against erythromycin, 32.8% (19/58) of isolates were susceptible. None of the *Enterococci* showed susceptibility to kanamycin, with 86.2% (50/58)

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demonstrating resistance and 13.8% (8/58) showing intermediate susceptibility. There was 100% resistance to sulphonamides. Susceptibility to sulpha-trimethoprim and vancomycin was quite high at 98.3% (57/58) and 94.8% (55/58), respectively. Susceptibility to tetracycline was 48.3%. All *Enterococcus* isolates demonstrated resistance to at least a single antibiotic, with 72.4% exhibiting MDR (resistance to 3 or more antibiotics) (Magiorakos *et al*., 2012). The outcomes of the research show that there is very little awareness about antibiotics, withdrawal periods, and AMR among the surveyed community members. These outcomes emphasize the necessity of educational initiatives and community outreach efforts to better inform the public on these issues of public health concern.

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CHAPTER 1: INTRODUCTION

The emergence and subsequent spread of antimicrobial resistance (AMR) in pathogenic microbes is a growing and well documented global health concern (Eagar *et* al., 2012; Roth *et al*., 2019). The problem is further worsened by the general lack of scientific research and manufacture of novel antimicrobial drugs (Majumder *et al*., 2020). Antimicrobial resistance results in previously treatable infections becoming difficult, or impossible, to treat with currently available drugs (Kapena *et al*., 2020; Mupfunya *et al.,* 2021; Jain and Yadav, 2017). Previously effective antimicrobials are rendered ineffective, leading to treatment failures.

Antimicrobial usage in livestock production is a widespread practice. Antimicrobial agents are used for different reasons ranging from disease prevention, treatment, and control to growth promoters to increase animal productivity (Roth *et al*., 2019). In addition, using antimicrobials in livestock production promotes animal welfare and contributes to food security (Eagar *et* al., 2012. Food animal products have been implicated as a source of AMR (Kapena *et al*., 2020).

There are significant knowledge gaps about the efficacy of many antimicrobials used in livestock after decades of their use. It is crucial to carry out surveillance research to gather data that will enable the formulation of policies to guide responsible antibiotic use to combat AMR in accordance with the global action plan of the World Health Organization (WHO) (Nulsen *et al*., 2008; Kapena *et al*., 2020). Microorganisms which exhibit resistance to at least a single antimicrobial are categorised as resistant. Microbes showing resistance to three or more different types of antimicrobial agents are described as multidrug resistant (MDR). Pan-drug resistant (PDR) microorganisms are resistant to all classes of antibiotics (Nulsen *et al*., 2008; Magiorakos *et al*., 2012).

Traditional family-based poultry rearing is widely practiced in many countries (Mack *et al*., 2005), and poultry products are the most widely consumed worldwide (Agyare *et al*., 2019). The rearing of poultry in both urban and rural areas is a common practice in many

African countries including South Africa. Poultry plays a pivotal role in providing nutrition (meat and eggs), as well as income (Ngongolo *et al*., 2021; Nkukwana, 2018).

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Backyard poultry farmers often face challenges with poultry diseases because of sub-optimal management practices. To treat their flocks, farmers purchase antibiotics over the counter. It has been reported in several countries that withdrawal periods following treatment are not adhered to, resulting in the occurrence of antibiotic residues in poultry eggs and meat (Adesiyun *et* al., 2020).

In South Africa, an estimated 8 billion eggs are consumed per year, with per capita consumption of 145 eggs (Moswane and Oladele, 2024). Like many communities across South Africa, backyard poultry rearing is a common practice in the Balfour community of Dipaleseng Municipality. There is however no available literature on the backyard poultry populations or egg consumption in the area. The aim of the study was to determine antimicrobial sensitivity of *E. coli* and *Enterococcus* isolates from chicken table eggs, and to assess backyard poultry farmers' knowledge of antibiotics, withdrawal periods and AMR.

1.1 Rationale and justification

Backyard poultry farmers in the Balfour community have access to a variety of over-the-counter antibiotics for poultry. No studies of antimicrobial resistance patterns in *E. coli* and *Enterococcus* isolates from chicken eggs in Balfour have been conducted before. Most available studies on antimicrobial sensitivity of bacteria form poultry have been focused on large commercial poultry production units (Adesiyun *et* al., 2020). Given that eggs can harbor potentially zoonotic pathogens, it is important to carry out surveillance research on the antimicrobial sensitivity profiles of bacteria from eggs (Jain and Yadav, 2017; Kapena *et al*., 2020). Furthermore, the farmers' knowledge of antibiotics and awareness of the public health implications of antibiotic use in animals remains largely unknown. It is against this background that this study was formulated.

1.2 Aims

The purpose of this study was to determine antimicrobial sensitivity of E. coli and Enterococcus isolates from chicken table eggs, and to assess backyard poultry farmers' awareness of public health implications of antibiotic use in poultry.

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1.3 Objectives

- To isolate *E. coli* and *Enterococcus* from chicken table eggs.
- To determine the antimicrobial sensitivity of the isolates using the Kirby-Bauer disk diffusion method.
- To determine poultry farmers' knowledge of antibiotics, their use and awareness of treatment period, withdrawal periods and AMR by means of a questionnaire.

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CHAPTER 2: LITERATURE STUDY

2.1 Egg consumption

Poultry eggs are the most consumed food worldwide and are an affordable source of important nutrients (Atoyebi *et al*., 2019; Kapena *et al*., 2020; Theobald *et al*., 2019). Eggs are rich in protein, lipids, vitamins, and minerals (Jain and Yadav, 2017). This rich nutritional content fosters the growth of bacteria, including harmful bacteria (Kapena *et al*., 2020). This makes them a potential source of food-borne pathogens (Atoyebi *et al*., 2019). *Escherichia coli (E. coli), Salmonella,* and *Enterococcus* are some of the pathogenic organisms that colonise eggs with ease (Kapena *et al*., 2020). People may become infected via handling of contaminated eggs, or the consumption of raw or undercooked eggs (Jain and Yadav, 2017; Kapena *et al*., 2020). Diarrheal conditions are the most common illnesses arising from the consumption of contaminated food. These afflict an estimated 550 million people globally, causing about 230 thousand deaths annually (Kapena *et al*., 2020). These largely occur following the consumption of raw or undercooked eggs (Kapena *et al*., 2020). Although the presence of potential pathogens in eggs has implications for public health, little is understood about the antibiotic sensitivity of these microbes (Atoyebi *et al*., 2019).

2.2 *Escherichia coli*

Escherichia coli is a Gram-negative, facultative anaerobic, motile rod that is oxidase negative, catalase positive, produces acid from glucose and lactose fermentation (Atoyebi *et al*., 2019; Quinn *et al*., 2011). The indole, methyl red, Voges-Proskauer and citrate utilisation tests (IMViC tests) are used in differentiating *E. coli* from other lactose fermenters (Quinn *et al*., 2011). These bacteria are part of the normal microflora of the alimentary canal of people and animals (Khan *et al*., 2015; Oguttu *et al*., 2021). *Escherichia coli* is abundant in environments inhabited by animals and is spread by the fecal-oral route (Quinn *et al*., 2011). The bacterium has a strong tendency to readily interchange genetic material, including AMR traits, horizontally with other bacteria (Agyare *et al*., 2018; Kapena *et al*., 2020; Oguttu *et al*., 2021). This makes *E. coli* a valuable sentinel or indicator microbe for the detection of AMR in bacterial populations (Oguttu *et al*., 2021).

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Some studies in South Africa have demonstrated AMR in *E. coli* isolated from poultry meat (Manie *et al*., 1998; Roth *et al.,* 2019). There is, however, scant data available on the antibiotic sensitivity profiles of *E. coli* from table eggs in resource-constrained communal farming setups (Theobald *et al.,* 2019).

Most available studies focused mainly on commercial layer and broiler units; one such study demonstrated significant levels of AMR in *E. coli* isolates from commercial farms in the Gauteng province (Adesiyun *et al*., 2020). Similar studies have revealed a significant AMR trend amongst *E. coli* isolated from table eggs in Zambia (Kapena *et al*., 2020), Nigeria (Okorie-Kanu *et al*., 2016) and Egypt (MS *et al*., 2017), with some of the isolates showing MDR patterns. Ongoing research is needed to keep abreast of the current trends of AMR in these bacteria as they are potential food-borne pathogens for humans.

2.3 *Enterococcus* species

Enterococci are Gram-positive cocci and are also found as commensals of the intestines of people and animals (Quinn *et al*., 2011). They are oxidase negative, catalase negative and are lactose fermenters. *Enterococcus faecalis* and *E. faecium* are the two most prevalent species in the genus (Agyare *et al*., 2018). *Enterococci* possess intrinsic MDR and are an important cause of nosocomial infections worldwide (Mascini and Bonten, 2005), resulting in serious, fatal infections in humans such as UTIs, bacteremia, meningitis, and endocarditis, (Agyare *et al*., 2018; Alemayehu and Hailemariam, 2020; Hollenbeck and Rice, 2012). Acquired AMR is especially important in *Enterococci, and* their ability to gain foreign genetic material (transposons, plasmids) enables them to quickly develop resistance to other antibiotics. Thus, the members of the genus are crucial to the acquisition, preservation, and spread of AMR genes to other bacteria. (Semedo-Lemsaddek *et al*., 2021).

Of greatest concern are the vancomycin resistant enterococci (VRE), which severely limit treatment options, and lead to prolonged hospitalisation and high treatment costs (Eichel *et al*., 2023; Mascini and Bonten, 2005). Vancomycin resistance develops within the hospital environment following the acquisition of *van* genes (Rodríguez-Lucas and Ladero, 2023).

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These genes result in terminal amino acid substitution in peptidoglycan precursors, resulting in altered peptidoglycan structure. This in turn leads to reduced glycopetptide affinity thus rendering the bacteria resistant to vancomycin and other glycopeptides (Ahmed and Baptiste, 2018; Rodríguez-Lucas and Ladero, 2023). Given the huge public health significance of VRE, it is very important to regularly monitor the antibiotic sensitivity profiles of these bacteria (Ahmed and Baptiste, 2018).

Little information is available on the AMR profile of *Enterococci* from backyard poultry eggs in South Africa. A recent study showed the presence of MDR in 37% of *Enterococcus* sp. isolates from poultry intestines (Molechan *et al*., 2019). Fatoba *et al*. (2022) found multidrug resistance and the presence of more than one antibiotic resistance gene in *Enterococci* isolated from soil fertilised with chicken waste. However, these studies focused on commercial poultry farms. *Enterococcus* isolates from intensively produced pigs in South Africa demonstrated significant levels of AMR, with 78% of the isolates exhibiting MDR (Badul *et al*., 2021).

These abundant commensal bacteria (*E. coli* and *Enterococcus* species) have the most diverse genetic pools in nature as well as within the host. This makes them more suitable indicators of the AMR status of microbial communities. They can give early warning of impending emergence of drug-resistant pathogens (Wang *et al*., 2012).

2.4 Mechanism of action of antibiotics

Antibiotics are chemicals that inhibit and/ or kill susceptible bacteria (Quinn *et al*., 2011). They can be naturally occurring, semi-synthetic or synthetic. They affect susceptible bacteria through various mechanisms of action (Quinn *et al*., 2011; Pulingam *et al*., 2022). These include inhibition of cell wall production, protein synthesis, nucleic acid synthesis, cellular metabolism, as well as disruption of the cytoplasmic membrane (Pulingam *et al*., 2022).

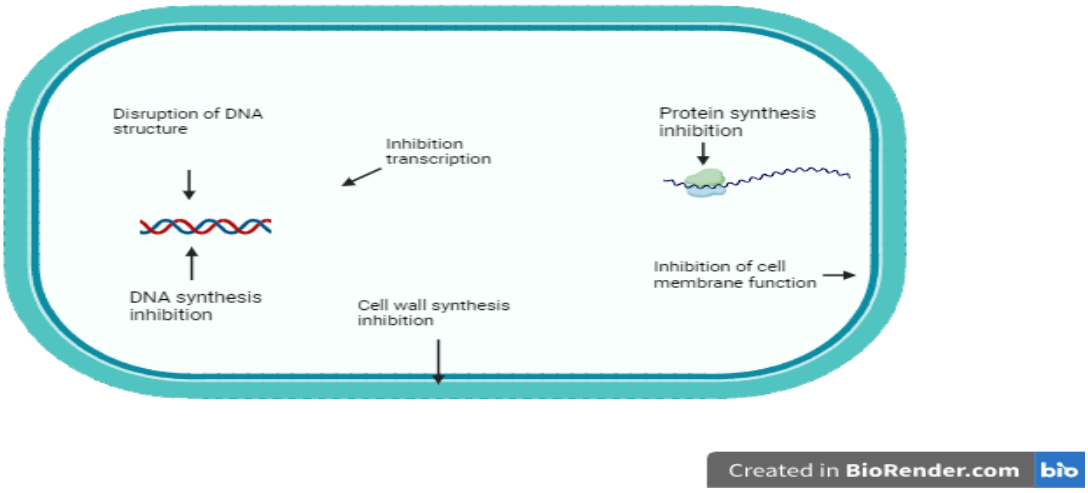

Fig 2.1. An illustration of the different modes of action and sites of action of antibacterial drugs (Created with BioRender.com).

2.5 Antimicrobial resistance

Reduced antimicrobial sensitivity or antimicrobial resistance (AMR) occurs when bacteria develop the ability to endure and survive the harmful effects of antimicrobial agents (Agyare *et al*., 2018; Pulingam *et al.,* 2022). The prevalence of AMR is on the rise, especially in developing countries. The advent of multidrug resistant (MDR), extremely drug resistant (XDR), and pan drug resistant (PDR) pathogens exacerbates the situation (Pulingam *et al.,* 2022).

Drug resistance is either innate (intrinsic) or acquired (extrinsic) (Quinn *et al.,* 2011). All microorganisms have intrinsic/ innate resistance to some types of antimicrobial agents. Most antibiotics are naturally occurring compounds produced by microbes. Bacterial species sharing the same biological niche with antibiotic-producing species have evolved defense mechanisms that enable them to withstand the effects of antibiotics (Munita and Arias, 2016). A microorganism's inherent physiological and structural characteristics give rise to its intrinsic resistance. These characteristics are encoded in the bacterial chromosomes (Reygaert, 2018).

Acquired resistance results from bacterial gene mutations, environmental transmission of resistance genes, and/or horizontal gene transfer (Agyare *et al*., 2018; Hollenbeck

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and Rice, 2012). Horizontal genetic transfer may occur in three different ways. These are: (i) transformation, which is the absorption of naked DNA; (ii) transduction, which is the process of genetic transfer between microbial cells through the action of phages; and (iii) conjugation, which happens when there is direct contact between two bacterial cells (Munita and Arias, 2016; Quinn *et al*., 2011). These genes are carried by plasmids and transposons, which serve as genetic transfer vehicles (Munita and Arias, 2016; Quinn *et al*., 2011). The loss of susceptibility to antibiotics arises through natural biological, biochemical, and genetic aspects (Pulingam *et al.,* 2022). The various mechanisms which enable bacteria to survive the action of antimicrobial agents are described as follows:

1. Decreased cell membrane permeability

   Many antimicrobial drugs exert their effect by binding to intracellular targets and thus need to penetrate the bacterial cell to reach their target sites. Bacteria counteract this by reducing the permeability of their cell membrane, thereby limiting the ability of the drugs to penetrate the cell, thus making them ineffective (Munita and Arias, 2016). Formation of physical barriers or altered porin production reduces or blocks drug molecules from penetrating into the bacterial cell (Quinn *et al*., 2011). For example, *Mycobacterium* species have a lipid-rich outer cell wall layer. This layer serves as a permeability barrier against hydrophilic drugs, giving *Mycobacterium* species natural resistance against a wide range of antibiotics (Pulingam *et al*., 2022; Reygaert, 2018). Bacteria without a cell wall (*Mycoplasma* species and related species) are naturally resistant to drugs acting on the cell wall (Reygaert, 2018). Membrane-bound porin channels in Gram-negative bacteria facilitate passage of hydrophilic molecules into the cell. Antibiotic uptake is reduced through either reduction of the number of porins present, or altered selectivity of the porin channels (Reygaret, 2018). Members of the *Enterobacteriaceae* develop resistance against carbapenems through decreased porin expression (Reygaert, 2018).

2. Antibiotic inactivation

   Several bacteria evade the effects of antibiotics by producing enzymes that alter the drug molecule and render it inactive (Agyare *et al*., 2018; Quinn *et al*., 2011). Antibiotics are rendered ineffective through degradation, or the addition of a

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chemical group, to the drug molecule (Munita and Arias, 2016). Enzymatic inactivation is achieved through hydrolysis, group transfer and reduction-oxidation reactions. Hydrolysis and group transfer are the most common, whilst redox reactions are rarely seen (Pulingam *et al*., 2022). Beta-lactamases, which provide resistance to beta-lactam antibiotics (penicillins, cephalosporins, and carbapenems), are among the drug-inactivating enzymes. They hydrolyse the beta-lactam ring within the drug molecule structure, rendering the drug ineffective (Munita and Arias, 2016; Quinn *et al*., 2011). Bacteria producing these enzymes include *E. coli, Klebsiella pneumoniae,* and *Enterobacter* species (Pulingam *et al*., 2022). Other antibiotic inactivating enzymes include acetyltransferases, phosphotransferases, thioltransferases, and aminoglycoside kinases (Munita and Arias, 2016; Quinn *et al*., 2011). Of the group transfer mechanisms, acetylation is the most common reaction, and is used against aminoglycosides, chloramphenicol, the streptogramins and fluoroquinolones (Reygaert, 2018).

3. Target modification

Bacteria have a wide range of cell components that are drug target sites. Alteration or modification of these sites makes bacteria resistant to antibiotics acting on these cell components (Munita and Arias, 2016; Reygaert, 2018). Others produce alternative functional proteins to replace those that are drug target sites, making the bacteria resistant to certain antibiotics (Pulingham *et al*., 2022). An example is the production of penicillin binding protein 2A (PBP-2A) by methicillin resistant *Staphylococcus aureus* (MRSA). Upon exposure to methicillin, MRSA down-regulates the high affinity PBPs, replacing them with extremely low affinity PBP-2A. This renders the bacteria resistant to beta-lactam antibiotics (Pulingam *et al.*, 2022; Reygaert, 2018). Similarly, *E. faecium* and *E. faecalis* also produce low-affinity PBPs (PBP5 and PBP4, respectively) making them resistant to β-lactams (Hollenbeck and Rice, 2012). The acquisition of *van* genes by *Enterococcus* species results in structural changes in the peptidoglycan precursors, leading to decreased binding capacity of vancomycin, thus making the bacteria resistant to vancomycin (Reygaert, 2018).

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4. Efflux pump activity

Efflux pumps are membrane-bound proteins that transport nutrients (Pulingam *et al*., 2022) and extrude toxic compounds (Reygaert, 2018). These pumps transport a wide range of chemicals such as dyes, bile salts, antibiotics, and detergents (Quinn *et al.,* 2011). They are an important component of bacterial resistance to different classes of antibiotics. Efflux pumps may be drug-specific or extrude a wide range of drugs; those transporting distinct types of antibiotics are known as multidrug efflux pumps (MEPs) (Pulingam *et al.,* 2022; Reygaert, 2018). The transportation of different classes of antibiotic molecules gives rise to MDR (Quinn *et al.,* 2011). Efflux pump activity effectively decreases intracellular drug concentrations, making the drug ineffective (Munita and Arias, 2016). Macrolides, beta lactams, fluoroquinolones, oxazolidinones, fourth generation cephalosporins and carbapenems are extruded from the bacterial cell through these pumps (Pulingam *et al.,* 2022).

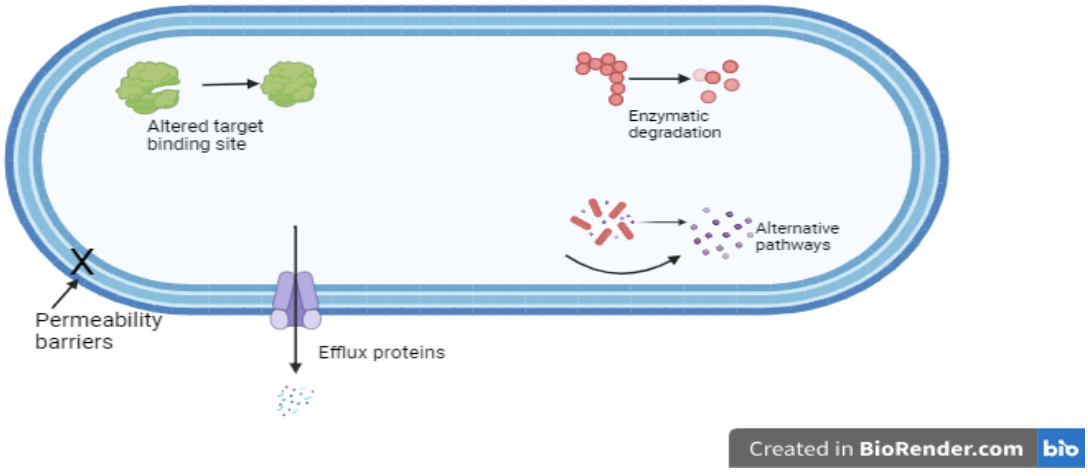

Fig 2.2 Diagram illustrating mechanisms of resistance (Created with BioRender.com)

There is variation in the type of resistance mechanisms used owing to inherent structural differences that exist between Gram-positive and Gram-negative bacteria. Gram-negative bacteria utilise all four mechanisms of resistance (Reygaert, 2018).

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Gram-positive bacteria, on the other hand, lack some types of efflux pumps. They also less commonly use drug uptake limitation as they lack the lipopolysaccharide outer membrane layer (Reygaert, 2018).

2.6 Drivers of growing antibiotic resistance

Bacteria have intrinsic natural resistance against certain antibiotics (Quinn *et al*., 2011). A variety of external factors contribute to the development of resistance in previously susceptible bacteria. These factors are largely a result of selection pressure due to increased, and often improper, use of antibiotics in the public and animal health sectors (Eagar *et* al., 2012; Reygaert, 2018).

The establishment and spread of AMR has been attributed to antimicrobial usage in livestock (Phiri *et al*., 2020). Bacteria are exposed to non-lethal drug concentrations when antimicrobials are used at subtherapeutic levels for non-therapeutic objectives like prophylaxis and growth stimulation. This leads to rapid development and spread of resistance (Kapena *et al.,* 2020; Kariuki *et al.,* 2022; Reygaert, 2018). In many countries, farmers can buy antibiotics over the counter and use them without involvement of veterinary personnel (Alonso *et al.,* 2017). In South Africa, the Fertilisers, Farm feeds, Agricultural and Stock Remedies Act (Act 36, 1947) makes provision for the sale of some antimicrobials over the counter without the need for a veterinary prescription (Theobald *et al.,* 2019). Tetracyclines are the most common and widely used as they are quite cheap and readily available. Following the administration of antibiotics to animals, some farmers do not observe the recommended withdrawal periods, leading to the occurrence of drug residues in food animal products (Manie *et al*., 1998; Phiri *et al*., 2020). These residues find their way into the human food chain, resulting in exposure of commensal bacteria and pathogens to subtherapeutic drug levels, which in turn leads to the development and spread of AMR (Manie *et al*., 1998; Reygaert, 2018). The introduction into the market of substandard and/ or counterfeit antimicrobial agents further compounds the problem (Njukeng *et al*., 2019).

In human medicine, drug choice is determined by cost; hence the more common and cheapest antibiotics are the most prescribed (Reygaert, 2018).

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Broad-spectrum antibiotics are prescribed empirically in situations where they are either not required, or inappropriate for the targeted infection (Reygaert, 2018). The use of broad-spectrum antibiotics promotes resistance in a wide range of bacterial species (Mupfunya *et al.,* 2021). In low-resource settings the situation is further worsened by lack of access to laboratory diagnostics (Aljeldah, 2022; Njukeng *et al.,* 2019). When ill, people sometimes refrain from seeking medical assistance, preferring to self-medicate at home. In such instances, the wrong medication is often used and at the improper dosage (Kariuki *et al.,* 2022). Another commonly identified problem is that of patients not completing a treatment course of prescribed antibiotics (Kariuki *et al.,* 2022).

The use of disinfectants has also been implicated as another possible contributing factor in the development and spread of AMR (Schwaiger *et al.,* 2014). Some evidence suggests that exposure of bacteria to certain disinfectant chemicals may result in reduced susceptibility to the disinfectant. This exposure may also induce cross-resistance to some antimicrobial agents. A higher level of expression of multidrug efflux pumps is believed to be the cause of decreased susceptibility to both quaternary ammonium compounds and some antimicrobials (Nhung *et al.,* 2015). In methicillin resistant *Staphylococcus aureus* (MRSA), a link was described between antibiotic resistance and reduced susceptibility to disinfectants (Schwaiger *et al.,* 2014). However, reports on links between AMR and decreased susceptibility to disinfectants are inconsistent as some researchers have reported no links between the two (Maertens *et al.,* 2019; Schwaiger *et al.,* 2014).

2.7 The socio-economic burden of antimicrobial resistance

There is widespread global distribution of antibiotic-resistant bacteria, posing a serious health concern worldwide (Perovic *et al*., 2014). Antibiotic resistance is also a serious threat to global food security and animal welfare (Selaledi *et al*., 2020).

Antibiotic resistance drastically reduces treatment options for patients with resistant bacterial infections, leading to higher morbidities and mortalities from previously curable infections (Essack *et al*., 2017; Perovic *et al*., 2014; Reygaert, 2018). This also places a huge strain on health systems (Munita and Arias, 2016; Njukeng *et al.,* 2019).

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Antimicrobial resistant infections cause about 700 thousand deaths per year worldwide (Bennani *et al*., 2020). By the year 2050, antibiotic resistant bacterial infections could kill an estimated ten million people globally (Bennani *et al.,* 2020; Njukeng *et al.,* 2019; Selaledi *et al.,* 2020). In the European Union, an estimated thirty-five thousand AMR related mortalities occur annually (Aljeldah, 2022). The CDC estimates that over two million people in the United States contract AMR infections annually, with over twenty-three thousand deaths (Selaledi *et al*., 2020; Perovic *et al*., 2018).

Due to AMR, there is an increase in prevalence of severe infections, often with prolonged disease courses that require prolonged hospitalisations and more extensive treatment (Essack *et al*., 2017; Njukeng *et al.,* 2019). There is a rapid rise in AMR in Africa, with 15% of *E. coli* isolates in private laboratories being found resistant to the third and fourth generation cephalosporins (Perovic *et al*., 2018; Njukeng *et al.,* 2019). However, the unavailability of reliable data means that the true extent of the problem remains largely unknown (Essack *et al*., 2017; Perovic *et al*., 2018).

Furthermore, there is a significant decrease in both labor supply and labor efficiency due to poor health (Bennani *et al.,* 2019; Kariuki *et al.,* 2022). These factors consequently result in the ballooning national health care costs associated with infection by drug resistant pathogens (Njukeng *et al.,* 2019; Reygaert, 2018). Global economic costs associated with AMR infections could reach a staggering US$100 trillion by the year 2050 (Kariuki *et al.,* 2022; Munita and Arias, 2016). In the United States, costs associated with such infections range from US$7 000-US$12 000 per patient (Reygaert, 2018), resulting in a cumulative cost of approximately US$55 billion (Kariuki *et al.,* 2022). In Europe, AMR accounts for approximately €2 million in associated healthcare costs (Kariuki *et al.,* 2022).

2.8 Strategies for limiting development and spread of AMR.

Antimicrobial resistance is a naturally occurring phenomenon and is not preventable. It is the spread of genes that confer AMR to previously susceptible pathogens that is of great concern (Munita and Arias, 2016). Antimicrobial resistance has far-reaching detrimental effects on public health, animal welfare and food security.

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Thus, it is important for countries to come up with and implement strategies for containing this global pandemic. Measures promoting a combination of restricted antibiotic usage and good hygiene practices may go a long way toward addressing this problem (Reygaert, 2018). This requires diligent monitoring of drug supply and usage in conjunction with risk assessments (Quinn *et al*., 2011).

The emergence and spread of AMR can be considerably reduced by limiting and/or outlawing the improper prescription and usage of antibiotics (Kariuki *et al.,* 2022). Sound therapeutic principles should always be used as guidelines for the prescription and dispensing of antimicrobials (Munita and Arias, 2016; Quinn *et al.,* 2011). As far as possible, therapy should ideally be supported by laboratory results (Pulingam *et al*., 2022; Quinn *et al*., 2011). In addition, prescribed antimicrobial drugs should be given or taken at the proper dosage for the prescribed treatment duration (Quinn *et al.,* 2011).

Non-therapeutic antimicrobial usage in livestock needs to cease (Quinn *et al*., 2011). Several countries have banned this practice (Oguttu *et al.,* 2021). Sweden banned their use for purposes of growth promotion and prophylaxis in 1986 and 1988, respectively (Agyare *et al.,* 2018). In 2010 the Netherlands banned the use of ceftiofur in hatcheries. This saw a reduction in the prevalence of resistant *E. coli* (from 20% to 2.9%) by 2014 (Bennani *et al*., 2020). In the European Union (EU) and the United States (US) the use of antibiotics as growth promoters was banned in 2006 and 2017, respectively (Roth *et al*., 2019). Globally, antibiotic usage in animals saw a 63% reduction by 2013. This was accompanied by a notable decrease in AMR in animals (Bennani *et al.,* 2020).

Farmers need to be taught and encouraged to adopt alternative husbandry practices that eliminate the need for non-therapeutic use of antibiotics. These include improving nutrition, animal welfare, on-farm hygiene, biosecurity (Mupfunya *et al*., 2021) and minimizing stress to enhance productivity. Hygienic housing of animals, regular cleaning, effective use of disinfectants and vaccinations should be implemented to reduce reliance on prophylaxis (Quinn *et al*., 2011). Farmers also need to be educated and encouraged to observe and adhere to recommended withdrawal periods following the treatment of their livestock (Kapena *et al*., 2020; Mupfunya *et al.,* 2021).

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Multidrug therapy is proposed as one of the ways of countering AMR. This method involves the alternating, or simultaneous use of two or more drugs with distinct mechanisms of action (Reygaert, 2018).

To promote responsible antimicrobial usage, antibiotic classification systems have been developed by the World Health Organization (WHO), World organization for Animal health (WOAH) and European Medicines Agency (EMA) (Gehring *et al*., 2023). For example, the EMA system puts antimicrobials into four categories A-D (European Medicines Agency, 2020). Category A antibiotics are not approved for use in veterinary medicine; Category B is highly restricted as they are considered critical in human medicine. Category C antibiotics may only be used when none of the approved first line antimicrobials are effective and must be used with caution. Category D are the approved first line antibiotics and must be used prudently (European Medicines Agency, 2020). The main goal of these antibiotic classifications is to promote effective and prudent use of antibiotics to limit the development and spread of antibiotic resistance (Gehring *et al*., 2023). Global adoption, or regional tailoring, of these antibiotic categorisation systems may add momentum in the fight against AMR.

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CHAPTER 3: MATERIALS AND METHODS

3.1 Study design and participant enrolment

A descriptive study with a cross-sectional study design was used. Eggs were purchased from randomly selected backyard poultry units in Balfour, Dipaleseng Municipality of Mpumalanga Province. There are no previous studies of antimicrobial resistance patterns in *E. coli* and *Enterococcus* isolates from chicken eggs in this area. Balfour was selected as the study site because of relative ease of access for the researcher. Random invitations were extended to community members who keep poultry in their backyards. Potential participants were selected based on word-of-mouth referrals from fellow community members who reared poultry. Only participants older than 18 years of age were selected. The aims and objectives of the study were explained to each potential participant. Those who indicated willingness to participate signed consent forms and were paid for the eggs they supplied. A closed format questionnaire was used in this study. Questions were selected to ask participants to share their personal experiences and knowledge pertaining to the use of antibiotics in poultry. The questionnaire was then administered in face-to-face interview format. Where further clarification was required, the information was relayed to the farmers in Zulu.

3.2 Egg collection

The sample size was calculated using the formula:

$$n_0 = [1.645^2 \times P_{exp} \times (1 - P_{exp})] / d^2,$$

where P_{exp} is the expected prevalence of bacteria in eggs, d' is the desired precision, and 1.645 is the z-value for 90% level of confidence, P_{exp} = 50% (unknown P_{exp}) and d = 8% (Thrusfield, 2007).

$$n_0 = [1.645^2 \times 0.5 \times (1 - 0.5)] / 0.08^2 = 106 \text{eggs}$$

Sample collections were mainly done during weekends. For this study, 4 eggs were bought from each of 25 backyard poultry units, and 3 eggs from each of the remaining

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two backyard poultry units to make a total of 106 eggs as calculated above. The collected eggs were labelled by pencil and placed in egg trays that had been previously sprayed with 70% alcohol and allowed to dry. They were then placed in a cooler box with ice, then stored in a refrigerator at 4-8^{o}C prior to transportation on ice to the DVTD Bacteriology laboratory, Faculty of Veterinary Science, University of Pretoria for processing. Eggs that were not immediately processed were stored between 4-8^{o}C in a walk-in fridge at the DVTD bacteriology laboratory.

3.3 Eggshell sampling

Eggs from each source were handled with the use of sterile gloves. The surface of each eggshell was entirely swabbed with a sterile cotton swab (LasecSA, Johannesburg, South Africa) moistened with sterile buffered peptone water (BPW) (CM 0509, Oxoid, Basingstoke, UK). Each swab was inoculated into 10ml BPW in a labeled screw-capped bottle and vortexed. The inoculated BPW bottles were then incubated for 24 hours at 37^{o}C in a walk-in incubator (Jain and Yadav, 2017).

3.4 Egg albumin and yolk sampling

Each eggshell was disinfected by spraying the entire shell surface with 70% alcohol, then left to dry at room temperature. Once dry, the eggs were opened around the air sac area using a pair of sterile scissors and forceps. The albumin and yolk of all eggs from each backyard poultry unit (n=4 for 25 units, and n=3 for the other 2 units) were pooled into a whirl pack and manually homogenized to make a composite sample of albumin and yolk. A pipette was used to transfer 1ml of the homogenate into a screw cap bottle containing 9ml of normal saline (Oxoid, BR0053G, Basingstoke, UK) and vortexed to obtain a 10^{-1} dilution. This was further serially diluted to get a final 10^{-3} dilution of egg homogenate. The screw capped bottles containing homogenate dilutions were placed in walk-in 37^{o}C incubator for 24 hours.

3.5 Isolation and Characterization of Bacteria

After a 24-hours, the inoculated bottles were each vortexed to thoroughly mix the contents. A sterile throat swab was dipped in each bottle, and excess fluid removed by firmly pressing the swab against the side of the bottle and rotating it 3-4 times. Each swab was used to create an initial inoculation pool (covering a quarter of the plate) on a correspondingly labelled Columbia blood agar plate with 5% sheep blood (SBA) (Thermofisher Scientific, Selecta media, Johannesburg, South Africa) and then

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a similarly labelled plate of MacConkey agar without crystal violet (Thermofisher Scientific, Selecta media, Johannesburg, South Africa). A 3.3µl nichrome wire loop (Prolab Diagnostics, Davies Diagnostics) was flame sterilized over a Bunsen burner (Fireboy, Integra), cooled, and used to streak out the inoculums by the quadrant streaking method to dilute the inoculum and isolate single colonies. The inoculated SBA plates were incubated in a 5% carbon dioxide incubator (Memmert, Schwabach, Germany) for 24 hours at 37°C. The MacConkey agar plates were aerobically incubated for 24 hours at 37°C in a walk-in incubator. After the 24-hour incubation period, the SBA and MacConkey plates were examined for bacterial growth. Suspected colonies of interest were identified on the basis colony morphology, hemolysis, and lactose fermentation. Suspected *E. coli* was defined as large pink colonies on MacConkey agar and large grey non-hemolytic colonies on SBA. The suspect *Enterococcus* colonies were dark pink, tiny colonies on MacConkey agar and grey tiny, round beta-hemolytic colonies on SBA. A permanent marker was used on the back of the plate to identify suspect colonies for subculturing to obtain pure colonies.

The marked suspect colonies were subcultured onto SBA and MacConkey agar without crystal violet and incubated as described above to isolate pure colonies. After incubation, the subcultured plates were examined for purity. Further subcultures were made from plates still exhibiting mixed bacterial growth. Samples from pure suspect colonies on SBA were subjected to gram staining. Gram-positive cocci and gram-negative rods were subjected to biochemical identification tests as described in the following sections

3.5.1 Identification of *E. coli*

Catalase positive and oxidase negative lactose fermenting Gram-negative rods that produced large pink colonies on MacConkey agar without crystal violet, were considered as suspect *E. coli.* Colonies were picked from the corresponding purified culture on SBA and subjected to the spot indole test (Merck, South Africa), Methyl red test (Thermofisher Scientific, Johannesburg, South Africa), Voges-Proskauer (Thermofisher Scientific, Johannesburg, South Africa) and Citrate test (Thermofisher Scientific, Johannesburg, South Africa) (IMViC tests), triple sugar iron (TSI) test (Thermofisher Scientific, Johannesburg, South Africa), lysine decarboxylase

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(Thermofisher Scientific, Johannesburg, South Africa) and urease activity test (Thermofisher Scientific, Johannesburg, South Africa). A suspension with turbidity corresponding to a 3 MacFarland Standard was used for inoculating the biochemical tests. Due to budget constraints, no further tests were done for the identification of *E. coli*.

3.5.2 Identification of *Enterococcus* species

Catalase negative and oxidase negative lactose fermenting Gram-positive cocci that produced tiny dark pink colonies on MacConkey agar without crystal violet, were considered suspect *Enterococcus* species. The corresponding suspect colonies from the SBA plates were tested with a Streptex Lancefield grouping test kit (Oxoid, Basingstoke, United Kingdom). Further testing was done on isolates that fell into Lancefield group D. A colony was picked from the corresponding pure culture on the SBA plate and transferred into a tube containing 5ml of normal saline to prepare a suspension with turbidity corresponding to a 3 MacFarland Standard. Six drops of the suspension were inoculated into 6.5% salt broth (Thermofisher Scientific, Johannesburg, South Africa) and Aesculin broth (Thermofisher Scientific, Johannesburg, South Africa) for confirmation of *Enterococcus* species. Colonies that tested positive in the salt broth and aesculin hydrolysis were identified as *Enterococcus* species. Further tests for differentiation of *Enterococcus* species were not done as this was beyond the scope of the project.

3.6 Antimicrobial susceptibility testing

The antimicrobial susceptibility assays were done using the Kirby-Bauer disc diffusion method. Bacterial suspensions with turbidity equal to a 0.5 McFarland standard of the *E. coli* and *Enterococcus* species isolates were inoculated on the surface of Mueller-Hinton (MH) agar plates (Thermofisher Scientific) to obtain a uniform 'lawn' of bacterial growth. Antibiotic impregnated discs (Oxoid, Basingstoke, United Kingdom) were placed onto the inoculated MH agar plates using a sterile pair of forceps. The antibiotic discs used were ampicillin (10µg), colistin (10µg), doxycycline (30µg), enrofloxacin (5µg), erythromycin (15µg), fosfomycin (200µg fosfomycin + 50 µg glucose-6-phosphate), gentamicin (10µg), kanamycin (30µg), sulphonamide (300µg), sulphonamide/ trimethoprim (1.25/23.7 µg), tetracycline (30µg), and vancomycin (30µg). The plates were then aerobically incubated for 24hours at 37°C. The zone of bacterial growth inhibition around each disk was then measured with the aid of a digital

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caliper (Tork Craft, Johannesburg, South Africa). Zones of inhibition were interpreted as per guidelines of the Clinical and Laboratory Standards Institute (CLSI 2018).

3.7 Permit applications

A Section 20 permit (Reference number: 12/11/1/1/6 (2862SR) for transporting samples was obtained from the Department of Agriculture, Land Reform and Rural Development (DALRRD) of the Republic of South Africa. Ethical approval for the research was obtained from the University of Pretoria Faculty of Health Sciences (Reference number: 84/2023). Ethical approval for the questionnaire survey was obtained from the University of Pretoria Faculty of Humanities (Reference number: 22908872 (HUM007/0223)). Ethical approval for the use of animals was obtained from the University of Pretoria Faculty of Veterinary Science Animal Ethics Committee and Research Ethics Committee (Reference number REC 129/22). All permits are presented in the addendum.

CHAPTER 4: RESULTS

4.1 Bacterial isolation.

A total of ten *E. coli* and fifty-eight *Enterococcus* isolates were obtained in the study (table 4.1). The *E. coli* isolates were from seven households. Nine of these were isolated from eggshell samples (9/106) while one was from a pooled sample out of a total of 27 pooled egg homogenate samples (1/27). *Enterococci* were successfully isolated from fifty-eight eggshell samples (58/106) from twenty-two (22/27) backyard poultry units (table 4.2). No *Enterococci* were isolated from pooled egg content samples.

Table 4.1. *Escherichia coli* and *Enterococcus* isolate from each backyard poultry unit.

Household	*Enterococcus* isolates	*E. coli* isolates	Household	*Enterococcus* isolates	*E. coli* isolates
1	4	3	15	0	0
2	3	2	16	0	0
3	4	0	17	0	1
4	3	0	18	2	0
5	1	0	19	4	0
6	3	0	20	3	0
7	3	0	21	1	0
8	2	0	22	2	1
9	2	0	23	4	0
10	1	0	24	0	0
11	4	0	25	1	0
12	4	1	26	2	0
13	4	1	27	0	0
14	1	1			

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Table 4.2. The number of *E. coli* and *Enterococcus* isolated from chicken eggs

	Bacterial isolates	
Sample	*E. coli*	*Enterococcus* spp.
Egg contents	1	0
Eggshell	9	58
Total	10	58

4.2 Antimicrobial sensitivity testing

4.2.1 Antibiograms for E. coli

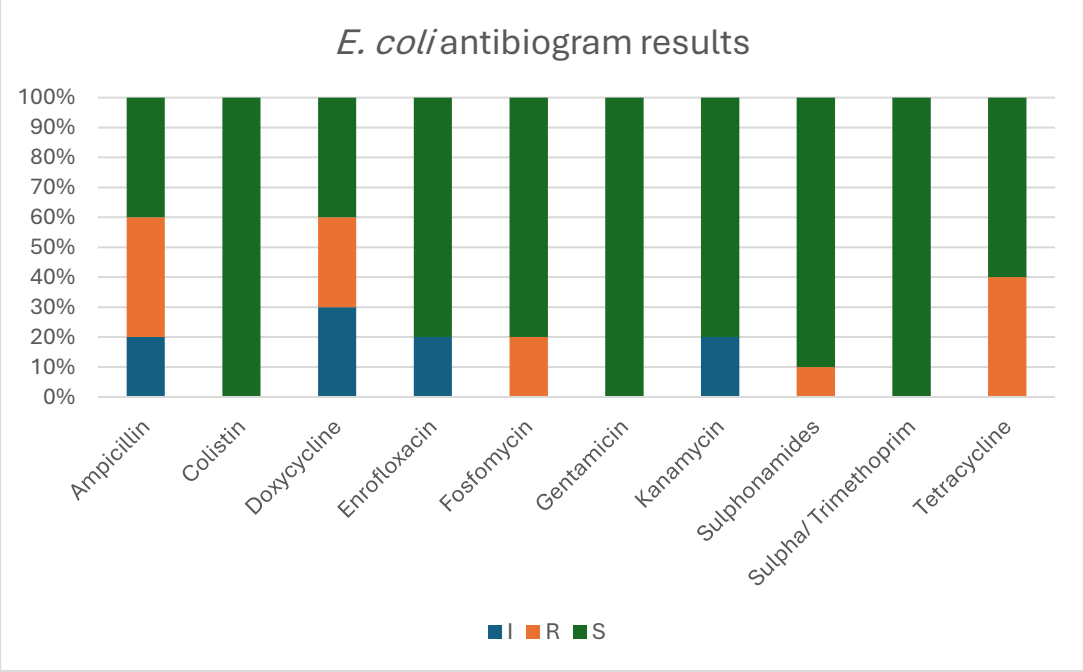

Fig 4.1 Antibiogram results of *E. coli* isolates

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Of the ten *E. coli* isolates, 80% (8/10) were resistant to at least one antibiotic. There were 40% (4/10) susceptible to ampicillin with 40% showing resistance, while 20% (2/10) showed intermediate susceptibility. There was 100% (10/10) susceptibility to colistin, gentamicin and sulpha-trimethoprim. Doxycycline susceptibility was 40% (4/10), while resistance was 30% (3/10) and the remaining 30% (3/10) were intermediate. Eighty percent of the isolates (8/10) were susceptible to enrofloxacin with 20% (2/10) showing intermediate susceptibility. When evaluated against fosfomycin, 80% (8/10) were susceptible while 20% (2/10) were resistant. Against kanamycin, 8% (8/10) isolates were susceptible and 20% (2/10) were resistant. There were 90% (9/10) isolates susceptible to, and 10% (1/10) resistant to, sulphonamides. Against tetracycline, there were 6% (6/10) susceptible and 4% (4/10) resistant isolates.

4.2.2 Antibiograms for Enterococcus isolates

The antibiogram results for Enterococcus isolates are shown in fig 4.2 below.

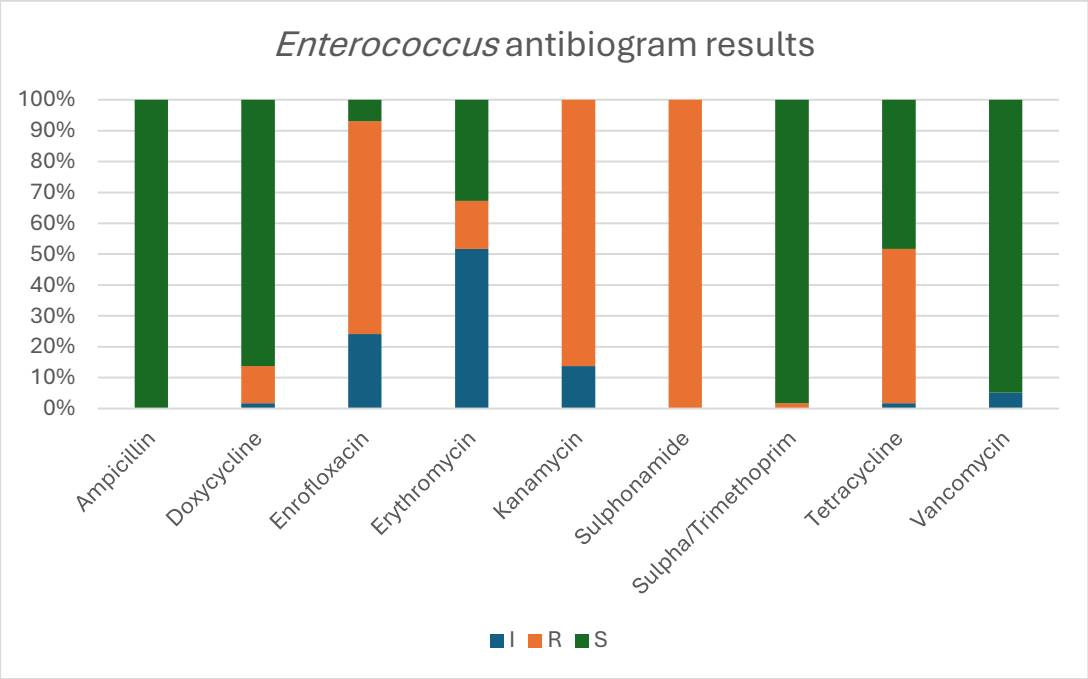

Fig 4.2: Antibiogram results of *Enterococcus* isolates

Out of the fifty-eight *Enterococcus* isolates, 100% (58/58) were susceptible to ampicillin. Susceptibility to doxycycline was 86.2% (50/58), while 12.1% (7/58) were resistant and 1.7% (1/58) showed intermediate susceptibility. Susceptibility to

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enrofloxacin was low at only 6.9% (4/58), while 69% (40/58) were resistant and 24.1% (14/58) showed intermediate susceptibility. Against erythromycin, 32.8% (19/58) of isolates were susceptible, with 51.7% (30/58) being intermediate susceptibility and 15.5% (9/58) showing resistance. None of the *Enterococci* showed susceptibility to kanamycin, with 86.2% (50/58) demonstrating resistance and 13.8% (8/58) showing intermediate susceptibility. There was 100% resistance to sulphonamides. Susceptibility to sulpha-trimethoprim was 98.3% (57/58), with 1.7% (1/58) resistance. Susceptibility to tetracycline was 48.3%, while resistance was noted in 50% (29/58) of isolates, with 1.7% (1/58) demonstrating intermediate susceptibility. Susceptibility to vancomycin was quite significant, with 94.8% (55/58) of the isolates showing susceptibility and 5.2% (3/58) showing intermediate susceptibility.

4.3 Questionnaire responses

Of the twenty-seven farmers interviewed, 81.5% (22/27) had treated their chickens before (Figure 4.3). Fifteen of the 22 farmers that had treated their chickens before used antibiotics, while seven used herbal remedies.

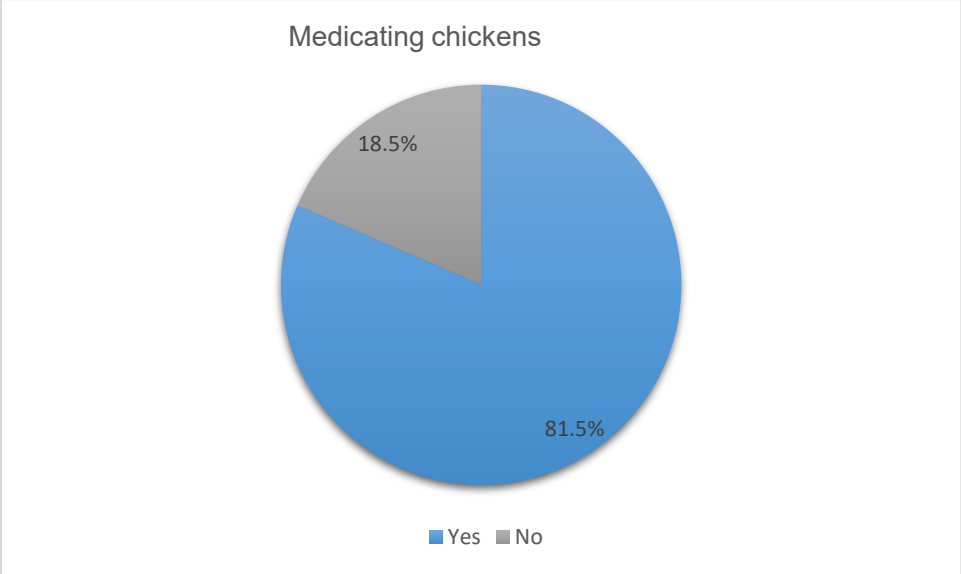

Fig 4.3: Proportion of farmers medicating their chickens

A few farmers 29.6% (8/27) had an idea about withdrawal times (Figure 4.4). Only 14.8% (4/27) of respondents had heard about AMR (Figure 4.5), while 51.9% (14/27)

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consumed eggs laid during treatment and 22.2% (6/27) would slaughter chickens for meat during treatment (Figure 4.6). Interestingly, those 77.8% (21/27) who do not slaughter sick animals for meat cited cultural and/ or religious beliefs that

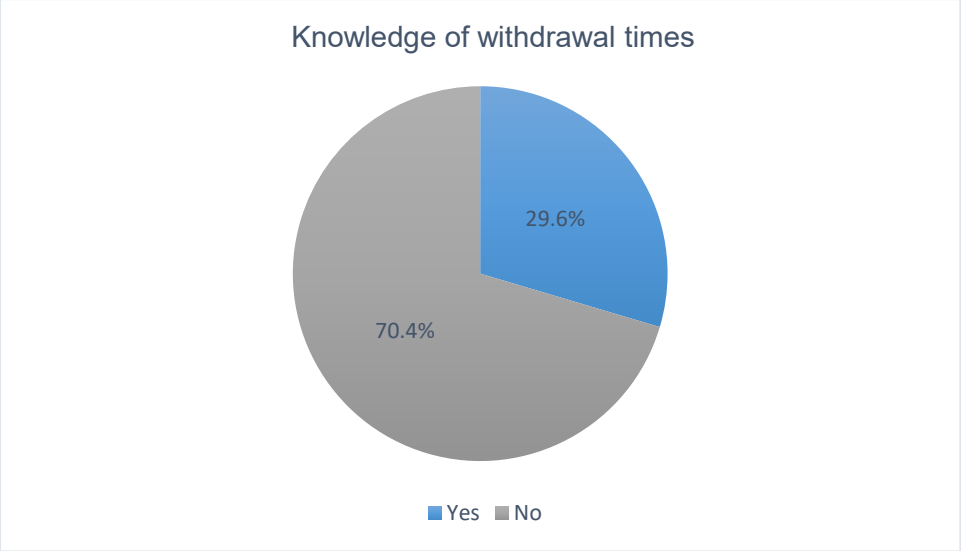

Fig 4.4: Farmers' awareness of withdrawal times

discouraged the slaughtering of sick animals as the reason for not slaughtering during treatment. Of the fifteen individuals who used antibiotics, nine disposed containers in the bin, three burnt them with the rubbish while two threw them into the bush and one into the latrine. Those who used antibiotics purchased them from the local veterinary shops, whilst one got the medicine from their neighbour. While some farmers could not remember the names of the medicines they used, some still had empty packages and others had pictures on their phones.

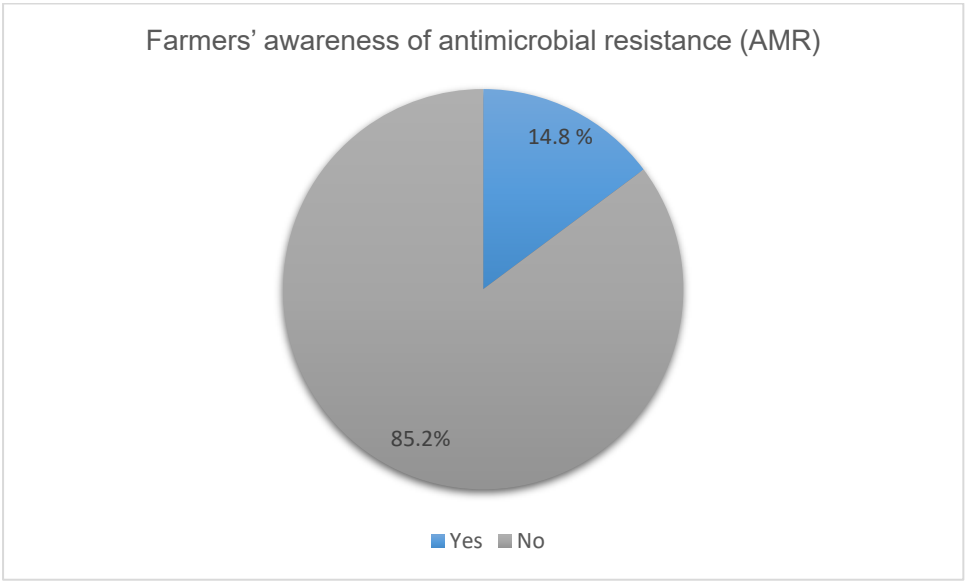

Fig 4.5: Farmers' awareness of antimicrobial resistance (AMR)

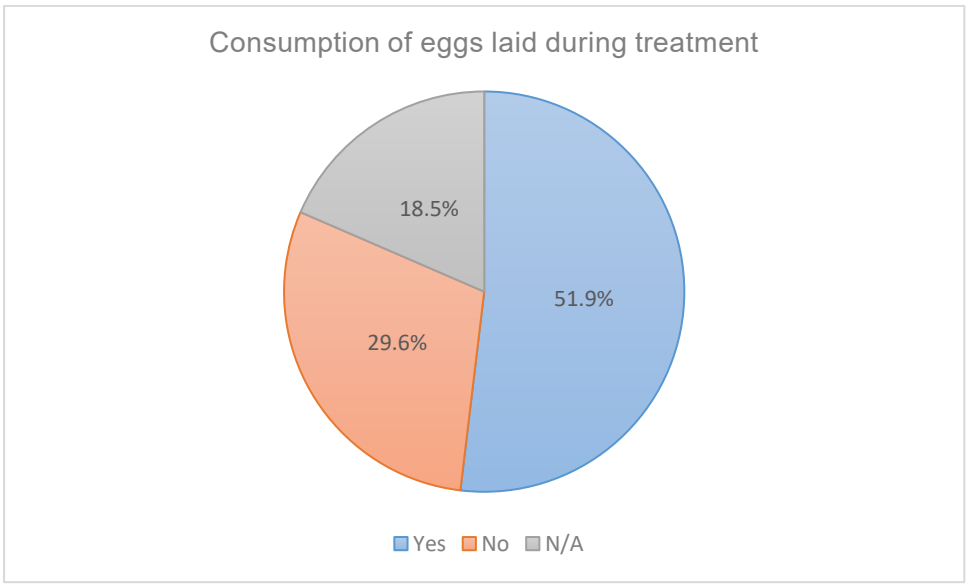

Fig 4.6: Consumption of eggs laid during the treatment

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CHAPTER 5: DISCUSSION

A descriptive study with a cross-sectional study design was conducted to investigate the antibiotic sensitivity of *E. coli* and *Enterococcus* isolates from chicken table eggs, as well as respondents' knowledge of antibiotics, withdrawal times and AMR in the Balfour community of Mpumalanga province. A structured questionnaire was administered in face-to-face interview format to backyard poultry farmers (n=27). A total of 106 eggs were purchased from the households. Eggs from brooding hens were excluded from the study. Some of the interviewed individuals (n=3) were reluctant to sign informed consent forms, and new participants had to be found to replace them.

5.1 Bacterial isolation

Eggs generally become contaminated with microflora of the oviduct during laying, or with environmental bacteria after laying (Atoyebi *et al*., 2019). In the present study, the eggshells were most contaminated compared to the egg contents. Sixty-seven (67/68) of the isolates were from eggshells while only a single (1/68) isolate was recovered from egg contents. Human hands are a possible source of contamination of eggshells. However, in this study, the use of sterile gloves during egg collection reduced the likelihood of this happening. These results are comparable with the findings of other researchers (Jain and Yadav, 2017; Kapena *et al*., 2020) who reported an almost similar pattern of contamination. The absence of microbes in egg contents may also be attributed to the natural barriers conferred by the eggshell and outer membrane (Atoyebi *et al*., 2019).

All *Enterococcus* isolates (58/58) were recovered from eggshells. Interestingly, ten (10/58) of the isolated *Enterococcus* isolates yielded unexpected results when subjected to the Lancefield grouping tests. These isolates showed Lancefield group D and group G agglutination. This may be due to the presence of *Enterococcus* strains which cross react with group D and group G antiserum, such as *Enterococcus durans* (Savini *et al*., 2014). There were limitations however with the isolation technique as the incubation period of 24hours did not cater for slow-growing enterococci, resulting in isolation of fewer isolates.

Out of the ten *E. coli* isolates obtained, nine were isolated from eggshells and one was isolated from a pooled sample of egg contents from one household. This is quite an

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interesting finding as no *E. coli,* or Enterococci were isolated from any of the eggshells from this household. The contamination is therefore unlikely to have occurred during sample processing. A possible explanation may be that the bacterium was transmitted vertically (Atoyebi *et al*., 2019).

5.2 Antimicrobial sensitivity

5.2.1 Escherichia coli

Of the ten isolates obtained, 80% were resistant to at least one antibiotic whilst 10% (1/10) was sensitive to all tested antibiotics. Only a single isolate (10%) was susceptible to nine antibiotics and showed intermediate susceptibility to one antibiotic. The susceptibility to ampicillin and doxycycline was similar at 40% (4/10) while resistance was 40% and 30%, respectively. The sensitivity of E. coli in this study has a comparable pattern to that observed by other researchers where 37.8% of isolates were susceptible and 59.5% were resistant (Kapena *et al*., 2020). All isolates (100%) were susceptible to colistin, gentamicin and trimethoprim sulphonamide. Susceptibility to non-potentiated sulphonamides was also high (90%), with intermediate resistance at 10%. The observations for sensitivity to colistin are notably different from those reported in a study in Zambia (Kapena et al., 2020) which found 94% resistance. This may be due to differences in antibiotic usage in poultry between the two countries, or a result of the comparatively smaller sample size used in this study. Susceptibility to tetracycline was 60% and resistance 40%, while Kapena et al (2020) reported 2.7% susceptibility and 83.8% resistance. The differences may also be attributable to sample size. On the other hand, resistance to tetracyclines is generally expected to be high due to their low cost, over-the-counter availability, and widespread use in the poultry industry. Susceptibility profiles for enrofloxacin and kanamycin in this study were similar (80% susceptible and 20% intermediate). Although enrofloxacin is licensed for use in poultry in South Africa, it is a prescription drug and generally expensive. It is therefore not frequently used in low resource settings. The susceptibility to fosfomycin was high (80%) and resistance low (20%). The resistance against fosfomycin was higher than the 6.7% recorded in a 2020 study in South Korea (Seok et al., 2020). Another study found a high level of fosfomycin resistance (100%) in E. coli isolates sourced from different countries across the world (Findlay et al., 2023).

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5.2.2 *Enterococcus* isolates

Resistance to at least one antibiotic was observed in all (100%) of the Enterococcus spp isolates. A greater proportion of the isolates (72.4%), (42/58) exhibited MDR (resistance to 3 or more antibiotics) (Magiorakos et al., 2012). All the Enterococcus isolates (100%) were sensitive to ampicillin. This agrees with other studies which found members of the genus susceptible to penicillins (Agyare et al., 2018). There was notable resistance against sulphonamides (100%), kanamycin (86.2%), enrofloxacin (69%). A considerable proportion of the Enterococcus isolates 98.3% (57/58) were susceptible to trimethoprim sulphonamide. The high resistance patterns observed against sulphonamides, kanamycin and enrofloxacin are expected since Enterococci are intrinsically resistant to these antibiotics (Giguere et al., 2013; Semedo-Lemsaddek et al., 2021; Miller et al., 2014). Although susceptibility to trimethoprim-sulphonamide was high, in the clinical setting this is not regarded as sensitivity since Enterococci are intrinsically resistant to both active ingredients (CLSI 2018; Giguere et al., 2013).

No vancomycin resistance was detected, with the greatest proportion of isolates (94.8%) showing susceptibility and only a marginal proportion (5.2%) showing intermediate susceptibility. This may be because vancomycin is not available for use in poultry, and/or that vancomycin resistance genes are absent in the research community area. Resistance to tetracycline was high (50%). Tetracycline resistance in Enterococci is also reported by other authors (Semedo-Lemsaddek et al., 2021). This phenomenon may be a result of cross-resistance with doxycycline, which was the most used antibiotic in the area. However, resistance to doxycycline was low (12.1%), with a higher proportion of isolates (86.2%) showing susceptibility. Resistance to erythromycin was 15.5%, with a greater proportion of isolates (51.7%) showing intermediate susceptibility and 32.8% showing susceptibility. These results reflect the acquired resistance of Enterococci to erythromycin (Kak and Chow, 2002). Resistance to antibiotics against which no intrinsic resistance exists is a possible indicator of the development of acquired resistance. Enterococci are known to get AMR genetic material from other bacteria with relative ease (Semedo-Lemsaddek et al., 2021).

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5.3 Questionnaire responses

The results of the research show that there is very little awareness about antibiotics, withdrawal periods, and AMR among the surveyed community members. The few respondents who indicated some degree of understanding of these had some background training in stockmanship. These outcomes emphasize the necessity of educational initiatives and community outreach efforts to better inform the public on the public health implications of antimicrobial usage in poultry as well as other livestock. These may encourage farmers to use antibiotics more responsibly as a result, which in turn could strengthen the ongoing fight against the AMR pandemic.

5.4 Limitations of the study

Carbapenems were excluded in the study since they are not licensed for use in animals. Their inclusion however would have added more value to the research given their importance. Carbapenems are regarded as a last resort treatment option and are effective against MDR gram-negative bacteria, including extended beta-lactamase enzyme producers, and gram-positive bacteria (Manenzhe *et* al., 2015; Meletis, 2016). Carbapenem resistance is on the rise particularly within the Enterobacteriaceae and has been identified in *E. coli* and *Klebsiella pneumoniae* (Manenzhe *et al*., 2015). Carbapenem resistant bacteria produce beta lactam enzymes capable of inactivating both carbapenems and beta-lactams. Acquired resistance occurs through gene mutations, gene acquisition or horizontal gene transfer (Meletis, 2016).

The 24-hour incubation period for the agar plates during isolation may have been too short to allow growth of slow-growing bacteria, resulting in the recovery of fewer isolates than present in the samples. During sample preparation, human error in preparing the 0.5 McFarland standards may have led to inconsistencies in the concentration of bacterial suspensions used to inoculate the Mueller-Hinton agar plates, which in turn could affect the antibiogram results. This could have been addressed using spectrophotometry in reading the turbidity to ensure uniformity.

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5.5 Conclusion

The target bacteria were successfully isolated mainly from the eggshells (100% of the *Enterococcus* isolates and 90% of the *E. coli* isolates). There was no *Enterococcus* species isolated from egg contents, while only a single *E. coli* isolate was cultured from the 27 pooled samples. The highest resistance of *Enterococcus species was* observed against sulphonamide compound (100%) while the highest sensitivity was against ampicillin (100%). Among the *E. coli isolates,* the highest resistance (40%) was recorded against ampicillin and tetracycline. The least resistance (0%) was recorded against colistin, gentamicin and sulpha-trimethoprim, to which all the isolates were completely susceptible. The use of synthetic antimicrobials has a huge role in the increased development and spread of AMR. All necessary precautions must be taken to mitigate this public health threat before it is too late.

The presence of antibiotic-resistant bacteria in eggs is a public health concern. Since the isolated bacteria are potential zoonotic pathogens, eggs pose a food safety hazard through which both pathogens and AMR transmission can occur. This highlights the importance of practicing good personal hygiene when handling and preparing food. Public awareness of the importance of thorough cooking to reduce the risk of food-borne infections is also essential.

The poultry farmers' knowledge and use of antibiotics in poultry was assessed. Although most of the participants used antibiotics to treat their chickens, their awareness of antibiotics, withdrawal periods and AMR were very low. A significant proportion of participants consumed eggs and meat from chickens receiving antibiotic treatment. This practice exposes people antimicrobial residues, which in turn heightens the risk of AMR development in human bacterial flora and subsequent AMR passage to pathogens. Farmers lack awareness of the public health implications of antimicrobial use in poultry, hence the need for awareness education as mentioned above.

In addition, there is a need to educate farmers on alternative husbandry practices that promote flock health. Improved housing with adequate ventilation will help reduce buildup of ammonia and reduce the incidence of ammonia-related respiratory conditions. Improved nutrition and use of vaccines will also help improve immunity,

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which in turn reduces disease burden and thus reduces the need for administration of antibiotics.

The onus is therefore on all the relevant stakeholders to adopt the relevant policies and action plans as recommended by international organizations such as the WHO, FAO, WOAH, and others. Only through efficient, collaborative One Health efforts can the AMR pandemic be contained.

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ADDENDUM

Section 20 permit

agriculture, land reform & rural development
Department:
Agriculture, Land Reform and Rural Development
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Land Reform and Rural Development
Private Bag X250, Pretoria 0001
Enquiries: Ms. Marna Laing · Tel: 012 319 7442 · Fax: +27 12 319 7470 E-mail: MarnaL@dalrrd.gov.za
Reference: 12/11/1/1/6 (2862SR)

Dr Sherpherd Mbedzi
5398 Zone 7 Siyathemba
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2410
Mpumalanga

E-mail: shepmbedzi@gmail.com

RE: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO. 35 OF 1984)

Dear Dr Sherpherd Mbedzi,

Your fax / memo / letter/ Email received 2023-01-26, requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers. I am pleased to inform you that permission is hereby granted to perform the following research/study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. The research project is approved as per the application form received 2023-01-26 and the correspondence thereafter. Written permission from the Director: Animal Health must be obtained prior to any deviation from the conditions approved for this research project under this Section 20 permit. Please apply in writing to MarnaL@dalrrd.gov.za;
3. All potentially infectious material utilised or collected during the study is to be destroyed at the completion of the study using the specified waste contractor (Compass Medical Waste Services). Records must be kept for five years for audit purposes. A dispensation application may be made to the Director Animal Health in the event that any of the above is to be stored or distributed;
4. Permission in terms of the Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies Act, 1947 (Act No 36 of 1947) and/or the Medicines and Related Substances Control Act, 1965 (Act No 101 of 1965) may be needed prior to the start of the study;
5. Ethics approval must be obtained prior to the start of the study;

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6. Only the samples listed, may used in this study: Chicken eggs
7. Samples may only be collected from the Dipaleseng Municipality, in the province of Mpumalanga;
8. If required, an application for an extension must be made by the responsible researcher at least one month prior to the expiry of this Section 20 permit. Please apply in writing to MamaL@dalrrd.gov.za;
9. This Section 20 approval is valid until 31 January 2026.

Title of research/study: "*Antimicrobial sensitivity of Escherichia coli and Enterococcus species isolated from table eggs in Balfour, Dipaleseng Municipality of Mpumalanga Province, South Africa.*"
Researcher (s): Dr Sherpherd Mbedzi
Institution: University of Pretoria - Faculty of Veterinary Science - Department of Veterinary Tropical Diseases
Your Ref./ Project Number: REC129-22
Our ref Number: 12/11/1/1/6 (2882SR)

Kind regards,

MMaja pp.

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 30/01/2023.

SUBJECT: RE: Permission to do research in terms of Section 20 of the ANIMAL DISEASES ACT, 1984 (ACT NO. 35 of 1984)

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Animal Ethics approval

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Faculty of Veterinary Science
Animal Ethics Committee

8 March 2023

Approval Certificate
New Application

AEC Reference No.: REC129-22
Title: Antimicrobial sensitivity of Escherichia coli and Enterococcus species isolated from table eggs in Balfour, Dipaleseng Municipality of Mpumalanga Province, South Africa
Researcher: Dr S Mbedzi
Student's Supervisor: Dr A Jonker

Dear Dr S Mbedzi,

The **New Application** as supported by documents received between 2022-12-13 and 2023-02-27 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2023-02-27.

Please note the following about your ethics approval:

1. The use of species is approved:

Species	Number
Poultry - Chickens	106
Samples	Number
Chickens - Eggs Samples from live animals	106

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2024-03-08.
3. Please remember to use your protocol number (REC129-22) on any documents or correspondence with the AEC regarding your research.
4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
5. **All incidents** must be reported by the PI by email to Ms Marleze Rheeder (AEC Coordinator) within 3 days, and must be subsequently submitted electronically on the application system within 14 days.
6. The committee also requests that you record major procedures undertaken during your study for own-archiving, using any available digital recording system that captures in adequate quality, as it may be required if the committee needs to evaluate a complaint. However, if the committee has monitored the procedure previously or if it is generally can be considered routine, such recording will not be required.

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Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

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Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

We wish you the best with your research.

Yours sincerely

Prof V Naidoo
CHAIRMAN: UP-Animal Ethics Committee

Faculty of Health Sciences Research Ethics Committee approval

Institution: The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 18 March 2022 and Expires 18 March 2027.
- IORG #: IORG0001762 OMB No. 0990-0278 Approved for use through August 31, 2023.

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Faculty of Health Sciences

Faculty of Health Sciences **Research Ethics Committee**

2 June 2023

Endorsement Notice

Dear Dr S Mbedzi

Ethics Reference No: 84/2023
Title: Antimicrobial sensitivity of Escherichia coli and Enterococcus species isolated from table eggs in Balfour, Dipaleseng Municipality of Mpumalanga Province, South Africa

The **New Application** as supported by documents received between 2023-02-21 and 2023-05-31 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2023-05-31 as resolved by its quorate meeting.

Please note the following about your ethics approval:

- Ethics Approval is valid for 1 year and needs to be renewed annually by 2024-06-02.
- Please remember to use your protocol number (84/2023) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

On behalf of the FHS REC, Dr R Sommers
MBChB, MMed (Int), MPharmMed, PhD
Deputy Chairperson *of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria*

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health).

Research Ethics Committee
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Fakulteit Gesondheidswetenskappe
Lefapha la Disaense tša Maphelo

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Faculty of Humanities approval

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Faculty of Humanities
Fakulteit Geesteswetenskappe
Lefapha la Bomotho

Humanities 100. Since 1919

3 June 2023

Dear Dr S Mbedzi

Project Title:	Antimicrobial sensitivity of Escherichia coli and Enterococcus species isolated from table eggs in Balfour, Dipaleseng Municipality of Mpumalanga Province, South Africa
Researcher:	Dr S Mbedzi
Supervisor(s):	Dr A Jonker
Department:	Veterinary Tropical Diseases
Reference number:	22908872 (HUM007/0223)
Degree:	Masters

I have pleasure in informing you that the above application was **approved** by the Research Ethics Committee on 4 May 2023. Please note that before research can commence all other approvals must have been received.

Please note that this approval is based on the assumption that the research will be carried out along the lines laid out in the proposal. Should the actual research depart significantly from the proposed research, it will be necessary to apply for a new research approval and ethical clearance.

We wish you success with the project.

Sincerely,

Prof Karen Harris
Chair: Research Ethics Committee
Faculty of Humanities
UNIVERSITY OF PRETORIA
e-mail: tracey.andrew@up.ac.za

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Faculty of Veterinary Science Research Ethics Committee approval

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Faculty of Veterinary Science
Research Ethics Committee 11 July 2025

LETTER OF APPROVAL

Ethics Reference No	**REC129-22**
Protocol Title	**Antimicrobial sensitivity of Escherichia coli and Enterococcus species isolated from table eggs in Balfour, Dipaleseng Municipality of Mpumalanga Province, South Africa**
Principal Investigator	**Dr S Mbedzi**
Supervisors	**Dr A Jonker**

Dear Dr S Mbedzi,

We are pleased to inform you that your submission conforms to the requirements of the Faculty of Veterinary Sciences Research Ethics committee.

Please note the following about your ethics approval:

1. Please use your reference number (REC129-22) on any documents or correspondence with the Research Ethics Committee regarding your research.
2. Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
3. Please note that ethical approval is granted for the duration of the research as stipulated in the original application (for Post graduate studies e.g. Honours studies: 1 year, Masters studies: two years, and PhD studies: three years) and should be extended when the approval period lapses.
4. The digital archiving of data is a requirement of the University of Pretoria. The data should be accessible in the event of an enquiry or further analysis of the data.

Ethics approval is subject to the following:

1. The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.
2. Note: All FVS animal research applications for ethical clearance will be automatically rerouted to the Animal Ethics committee (AEC) once the applications meet the requirements for FVS ethical clearance. As such, all FVS REC applications for ethical clearance related to human health research will be automatically rerouted to the Health Sciences Research Ethics Committee, and all FVS applications involving a questionnaire will be automatically rerouted to the Humanities Research Ethics Committee. Also take note that, should the study involve questionnaires aimed at UP staff or students, permission must also be obtained from the relevant Dean and the UP Survey Committee. Research may not proceed until all approvals are granted.

We wish you the best with your research.

Yours sincerely

PROF M. OOSTHUIZEN
Chairperson: Research Ethics Committee

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Farmer questionnaire

Faculty of Veterinary Science
Department Veterinary Tropical Diseases

Project title: Antimicrobial sensitivity of *Escherichia coli* and *Enterococcus* species isolated from table eggs in Balfour, Dipaleseng Municipality of Mpumalanga Province, South Africa.

Name of Researcher: Shepherd Mbedzi
Contact number: 062 839 5472.
Name of supervisor: Dr Annelize Jonker
Contact number: 012 529 8329.

A. Background information

The use of antibiotic medicines in poultry production is a common practice. These medicines are used for disease prevention and treatment, as well as for boosting egg production. In as much as their use contributes to improved productivity, their continued use carries the risk of development of antibiotic resistance by disease causing bacteria (germs). Very little information is available on the current efficacy of these antibiotics against bacteria of poultry origin. It is therefore the aim of this study to investigate the sensitivity of bacteria from chicken eggs to these antimicrobial agents. The information generated in this study will provide an important overview of the sensitivity profiles of bacteria to the tested antibiotic agents.

You are invited to volunteer for my research study for a Master of Science degree at the University of Pretoria. Please note that you may withdraw from the study at any time should you so wish, and that you will not be questioned or penalized in any way for doing so. You are required to provide a sample of eggs and answer a few questions relating to your knowledge and the use of medicines on your flock, and this will take about 45 minutes of your time.
We need a total of 106 fresh chicken eggs, with at least 4 eggs from each household for the purposes of this study. The location and date of collection will be recorded on the receptacle containers for each sample. These samples will then be transported from the collection points to the DVTD laboratory in Pretoria under a red-cross permit.

Upon reaching the laboratory, the eggs will be sampled for the bacteria on the outside shell surface as well as in the yolk and egg white. Isolated bacteria will be grown in the laboratory, after which they will be tested against several different types of antibiotic medicines. These tests will enable us to see which of these antibiotics can kill these bacteria and which ones are ineffective against the bacteria.

B. Risks to participants:
There are no risks associated with participation in the study.

C. Benefits to participants
Free advice and awareness teaching on AMR and the measures they can employ to help combat its spread, including the importance of observing manufacture's drug withdrawal periods. They will also learn improved husbandry and hygiene practices in poultry rearing.

D. Procedures regarding confidentiality

With the exception of participants wishing to have their contributions acknowledged, (kindly refer to sections 8 and 9 of the consent form), information to be used for this project is limited to species, date and place of collection. Your personal information will not be disclosed to third parties.

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E. The use of the samples, data in research, publications, sharing and archiving

All samples remaining after use in this project will be appropriately destroyed by incineration (burning at very high temperature) in terms of the Animal Diseases Act, Act 35 of 1984.
The information generated from this study, including any subsequent studies, is intended for academic purposes and publication. Owner specific data will be kept anonymous. The storage and archiving of data will be done in accordance with the processes and principles of the University of Pretoria. The data and/or documents relating to this study will be stored at the University of Pretoria Data Repository for a minimum of ten years and will be available for future use.

F. Informed consent form

_____I, the undersigned, confirm that (please tick box as appropriate):

1.	I have read and understood the information about the project, as provided in the information sheet at the back of this consent form.	☐
2.	I have been given the opportunity to ask questions about the project and my participation.	☐
3.	I voluntarily agree to participate in the project in one of the following manners. • Supply eggs from my chicken flock • Provide information about the medications that I use on my chickens for the prevention or treatment of disease	☐ ☐
4.	I understand I can stop participating at any time should I so wish and that I will not be penalized for withdrawing nor will my decision to withdraw be questioned.	☐
5.	The use of the data in research, publications, sharing and archiving has been explained in the information sheet at the back of this consent form and is clear to me.	☐
6.	The use of the data in research, publications, sharing and archiving has been explained in the information sheet at the back of this consent form and is clear to me.	☐
7.	I understand that other researchers will have access to this data only if they agree to preserve the confidentiality of the data and if they agree to the terms I have specified in this form.	☐
8.	Select only one of the following: • I agree that my name may be used in reports/ publications of this study so that my contribution to the project can be acknowledged. • I do not wish for my name to be disclosed in any part of the project.	☐ ☐
9.	Details I want to be recorded for recognition purposes, as chosen in 8 above: Name: Organization	☐
10.	I, along with the researcher/ state veterinarian, agree to sign and date this informed consent form.	☐

_____
Participant:

Name of Participant	Signature	Date

Researcher:

Name of Researcher	Signature	Date

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6. Research Questionnaire

1.	Have you ever given medicines to your chickens?	☐Yes ☐No
2.	Where do you get the medicines from?	
3.	For which of the following were you giving the medicine: a. Prevent disease b. Treat sick birds c. Increase egg production d. Increase growth rate	 ☐ ☐ ☐ ☐
4.	For how long do you give the medicine?	
5.	Do you know what antibiotic medicines are?	☐Yes ☐No
6.	Give the name of one antibiotic that you have used before	
7.	Are you aware that when giving chickens medicine, eggs from these chickens: a) cannot be used for human consumption? b) can only be eaten if they were laid after a recommended waiting period from the last day of treatment has passed?	 ☐Yes ☐No ☐Yes ☐No
8.	Do you throw away eggs laid when your chickens are taking medicine?	☐Yes ☐No
9.	Do you eat eggs laid by your chickens whilst they are taking medication?	☐Yes ☐No
10.	Have you ever slaughtered chickens for meat whilst they were taking the medication?	☐Yes ☐No
11.	Have you ever heard about antimicrobial resistance?	☐Yes ☐No
12.	How do you dispose of empty medicine packages/containers after the medicine is finished? a. Throw into the bush b. Throw into the dust bin c. Throw into a pit toilet d. Bury under the soil e. Burn with the rubbish Other (please specify)	 ☐ ☐ ☐ ☐ ☐