Assessment of commercial and wild birds as potential vectors of antibiotic resistant *Escherichia coli* and *Enterococcus* species

by

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PREFACE

The research contained in this dissertation was completed by the candidate while based in the Discipline of Microbiology, School of Life Sciences of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Pietermaritzburg, South Africa.

The contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

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Signed: Prof. Stefan Schmidt

Date: 16 July 2024

DECLARATION: PLAGIARISM

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ABSTRACT

Due to zoonotic infections resulting from antibiotic resistant (ABR) bacteria, the presence of ABR bacteria in birds is a human health concern. The aim of this study was to assess if commercial free-range ducks, wild sacred ibis, and raptors are potential vectors of ABR *E. coli* and *Enterococcus* species. Thirty phenotypically and genotypically confirmed *E. coli* and *Enterococcus* spp. strains isolated from each bird's faeces were analysed using the EUCAST disk diffusion method employing twelve (*E. coli*) and eight (*Enterococcus* spp.) appropriate antibiotics representing clinically important classes. β-Lactamase activity in isolates showing resistance to at least one of the tested β-lactam antibiotics was assessed using the nitrocefin spot test. Over 70% of *E. coli* isolates (47% from ducks; 73% from sacred ibis; 93% from raptors) were resistant to at least one of the twelve antibiotics tested, with resistance observed for penicillins, cephalosporins, aminoglycosides, and fluoroquinolones, but not for carbapenems, monobactams and tetracyclines. Additionally, two isolates each from sacred ibis' and raptor faecal samples were classified as multidrug-resistant. Over 25% of *Enterococcus* spp. isolates (0% from ducks; 33% from sacred ibis; 47% from raptors) were resistant to at least one of the eight antibiotics tested. Resistance was observed for penicillins, carbapenems, glycopeptide/lipopeptides, and oxazolidinone, but not for aminoglycosides, tetracyclines, and fluoroquinolones. While the resistance level in *E. coli* isolates from commercial duck faeces was lower than in those from wild sacred ibis and raptors, none of the *Enterococcus* spp. isolates from duck displayed resistance to any of the tested antibiotics. β-Lactamase activity was detected in nine *E. coli* isolates from sacred ibis and raptors faeces and further confirmed using UV-Vis spectrometry. Therefore, commercial, and wild birds in KwaZulu-Natal, South Africa, carry ABR bacteria and may act as a source and vector for the dispersal and transfer of these antibiotic resistant bacteria.

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TABLE OF CONTENTS

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

CHAPTER 1 : INTRODUCTION

Since its discovery in the 1920's, antibiotics have played a pivotal role in modern medicine, enabling life-saving procedures, facilitating faster recovery from bacterial infections, and contributing to overall improvements in human and animal health and longevity. However, the overuse of antibiotics over the years has come with a detrimental side effect – antibiotic resistance (ABR) in bacteria. Efforts to address ABR challenges involve the development of newer generations of antibiotics, and in severe bacterial infections, combination treatments utilizing multiple antibiotic classes simultaneously. Unfortunately, the efficacy of these drugs and treatment approaches is diminishing due to the escalating development of ABR in bacteria (Naylor *et al.*, 2018).

Adding to this challenge is the alarming fact that the pipeline of new antibiotics is drying out. The development of new antibiotics has significantly slowed, with fewer pharmaceutical companies investing in antibiotic research and development due to economic and regulatory hurdles (Cook and Wright, 2022). Consequently, the existing antibiotics are becoming less effective as bacteria rapidly evolve resistance mechanisms, further exacerbating the global health crisis.

The World Health Organization (WHO) has highlighted several critical antibiotics that are increasingly endangered due to rising resistance levels. Among these, carbapenems and colistin are particularly concerning. Carbapenems, which include imipenem, meropenem, and ertapenem, are considered last-resort antibiotics for many infections caused by multidrug-resistant (MDR) bacterial pathogens, but the emergence of carbapenem-resistant *Enterobacteriaceae* (CRE) poses a significant threat (Tilahun *et al*., 2021). CRE infections are difficult to treat and have high mortality rates because of the limited available therapeutic options. Similarly, colistin, another last-resort antibiotic used to treat infections caused by carbapenem-resistant bacteria, is now facing resistance, rendering it less effective. Colistin's use has been critically increased due to its efficacy against *Acinetobacter baumannii* and *Pseudomonas aeruginosa*, which are notoriously resistant to many other antibiotics (WHO, 2024).

The WHO's 2024 list also underscores the critical status of polymyxins (which include colistin) and carbapenems, categorizing them as "Critically Important Antimicrobials" (CIA). This category indicates that they are essential for treating serious infections in humans and that alternatives are not available or are significantly limited. The list also highlights other antibiotics, such as glycopeptides (including vancomycin), macrolides, and fluoroquinolones, which are vital for treating various severe infections. The growing resistance to these antibiotics further complicates the management of bacterial infections globally. The WHO emphasizes the urgent need for stringent measures to preserve the efficacy of these drugs, including antibiotic stewardship, surveillance of antibiotic use, and investment in new antibiotic development (WHO, 2024).

In the "WHO Bacterial Priority Pathogens List, 2024", the WHO identifies key bacterial pathogens of public health importance to guide research, development, and strategies for preventing and controlling antibiotic resistance. This list includes high-priority pathogens such as carbapenem-resistant *Acinetobacter baumannii*, carbapenem-resistant *Pseudomonas aeruginosa*, and third-generation cephalosporin-resistant *Enterobacteriaceae*, which are associated with severe, often untreatable infections (WHO, 2024). These pathogens represent a critical focus for research and development efforts aimed at creating new antibiotics and alternative treatment strategies. The WHO's list serves as a roadmap for global action to tackle the growing threat of ABR, prioritizing the most dangerous pathogens that pose significant challenges to public health. The comprehensive approach outlined by the WHO highlights the importance of global collaboration in developing effective interventions and ensuring the sustainability of current and future antibiotics (WHO, 2024).

The global impact of ABR is starkly evident, with an estimated 1.27 million people succumbing to infections caused by antibiotic resistant (also ABR) bacteria in 2019 alone (Murray *et al.*, 2022). Projections indicate a worrisome trajectory, with an anticipated ten million deaths worldwide attributed to ABR bacterial infections by 2050, underscoring the pressing need to comprehend and combat ABR in bacteria (MacLean and San Millan, 2019). Consequently, health organizations, governments,

and researchers are focused on monitoring ABR bacteria in water and food products, recognizing these as primary sources of ABR elements, which could be transferred throughout the environment. Adding to these concerns, recent research has identified wildlife as carriers of ABR bacteria, as they circulate and disseminate these bacteria in the environment, posing an increased risk of ABR bacterial infections to more vulnerable communities (De Oliveira *et al.*, 2020).

In the subsequent sections, specific aspects of antibiotic resistance will be highlighted, including its prevalence in animals and more specifically birds, particularly focusing on ABR bacteria in avian populations. The review will extend to key bacterial representatives, such as *E. coli* and *Enterococcus* species, and the antibiotics commonly employed against them. This chapter aims to provide a comprehensive understanding of the complex dynamics surrounding ABR bacteria within the bird-human interface, shedding light on critical areas that demand attention and research within this global health concern.

1.1. Antibiotic Resistance

The origin and development of antibiotic resistance (ABR) is a debated topic within the research community. Some argue that acquisition of ABR is promoted through human activities, while others point to evidence of ABR in isolated environments unexposed to modern antibiotics for centuries (Larsson and Flach, 2022). Supporting the human activity perspective, studies in the Galapagos islands revealed no naturally acquired ABR genes in bacterial isolates from terrestrial animals, suggesting potential cumulative exposure to antibiotics may result in an increase in ABR pathogens (Thaller *et al*., 2010). Conversely, a Dutch survey indicated an increased prevalence of ABR genes in post-antibiotic era soil compared to pre-antibiotic era samples (Knapp *et al*., 2010). Nevertheless, reports of ABR genes and bacteria in extreme environments like permafrost, caves, and the deep ocean suggest natural resistance in environments without previous antibiotic exposure (Bhullar *et al*., 2012; Larsson and Flach, 2022).

Whether ABR originates naturally or is promoted anthropogenically, the fact remains that ABR poses a major global health risk. Since 2015, the World Health Organisation (WHO) had established and used the Global Antimicrobial Resistance and Use Surveillance System (GLASS) to determine changes (i.e., increase, decrease, or stability) in resistance against antibiotics among common fungi and bacteria, as well as consumption of antimicrobials by humans. An annual report has been published ever since, which encapsulates summarized data from member states on detected ABR rates from countries, territories, and areas, at both national and global level (WHO, 2022). A concerning factor highlighted by the 2022 report, is that testing, and detection of ABR bacterial infections is more prevalent in higher income countries compared to lower income countries, including South Africa. Although the exact numbers are difficult to report due to the limitations within contributing countries due to accessibility and poor representation, the available data showed that higher income countries may have lower ABR infection rates due to superior healthcare systems, testing, and detection, which can reduce the rate of ABR. Another attributing factor regarding lower income countries, is the difficulty and limitations of access to antibiotics, which is a result of limited access to prescriptions due to financial constraints (WHO, 2022). This challenge creates the issue of misuse of antibiotics that are available and obtained over the counter without a prescription.

Although COVID-19 did impact the number of countries that were able to participate and submit data for the report, it is alarming that there has been an increase of more than 15% in ABR towards third generation antibiotics from 2017 to 2020 in bacteria responsible for bloodstream infections, such as pathogenic strains of *E. coli* and *Salmonella* spp. Additionally, the emergence and prevalence of carbapenem resistance, one of the last resort antibiotics, is a major global concern as this often results in treatment failure. Resistance towards carbapenems can be attributed to the increased use of carbapenems to treat *Klebsiella pneumoniae*, the third most common cause of bloodstream infections, which was indicated by an increase of more than 8% of ABR *Klebsiella pneumoniae* (WHO, 2022). Global surveillance of ABR primarily depends on standardized testing conducted at national, regional, and local levels, aiming to mitigate ABR and enhance global healthcare. Regrettably, numerous developing countries are underrepresented in this effort due to insufficient

detection and reporting of ABR bacterial infections, resulting in underestimations of global ABR cases and data (WHO, 2022). Unfortunately, developing countries are hardest hit by inadequate healthcare, exacerbated by poor sanitation and knowledge, further increasing the risk of allowing ABR to circulate in the environment, hospitals, and homes. Murray *et al.* (2022) estimated that the rate of death caused by ABR bacterial infections was highest in western Sub-Saharan Africa and lowest in Australia, with the main causes of global ABR infection fatality being (in order from highest to lowest) pathogenic strains of *E. coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*, all of which are currently on the "WHO Bacterial Priority Pathogens List, 2024" (WHO, 2024).

Outside of hospitals, ABR bacteria and antibiotic residues are circulating within the ecosystem between agriculture, animals, humans, and the environment (Figure 1.1). Antibiotics are widely used in these sectors, whether in agricultural practices like aquaculture and crop production, livestock farming, or animal and human medicine. Consequently, there is frequent contact between these domains, with animals, humans, and agricultural activities serving as sources of antibiotic exposure. This exposure fosters the development of ABR bacteria, which can then spread through various pathways, including animal waste, human sewage, and agricultural runoff, ultimately entering the environment. Once released, these antibiotic residues and ABR bacteria pose significant risks to animal and public health by contaminating soil, water sources, and even the air. The environmental repercussions are multifaceted, ranging from disruptions in microbial ecosystems to the dissemination of resistance genes, highlighting the complex interplay between human activities, antibiotic use, and ecological health (Kunhikannan *et al.*, 2021).

To effectively combat the spread of ABR across these interconnected sectors, the OECD (2023) advocates for a "One Health" approach, which recognises the interdependence of human, animal, and environmental health. This framework emphasizes coordinated efforts across sectors to address ABR holistically. By integrating surveillance systems, promoting prudent antibiotic use, and enhancing regulatory measures, the One Health approach aims to reduce the spread of ABR

bacteria and antibiotic residues. It underscores the need for collaboration between public health authorities, veterinary services, and environmental agencies to monitor and control antibiotic use and manage waste effectively. This integrated strategy not only aims to safeguard public health but also ensures the sustainability of agricultural practices and the health of the ecosystems. Embracing the One Health framework is crucial for developing comprehensive policies and interventions that address the root causes of ABR and mitigate its impact across all sectors (OECD, 2023).

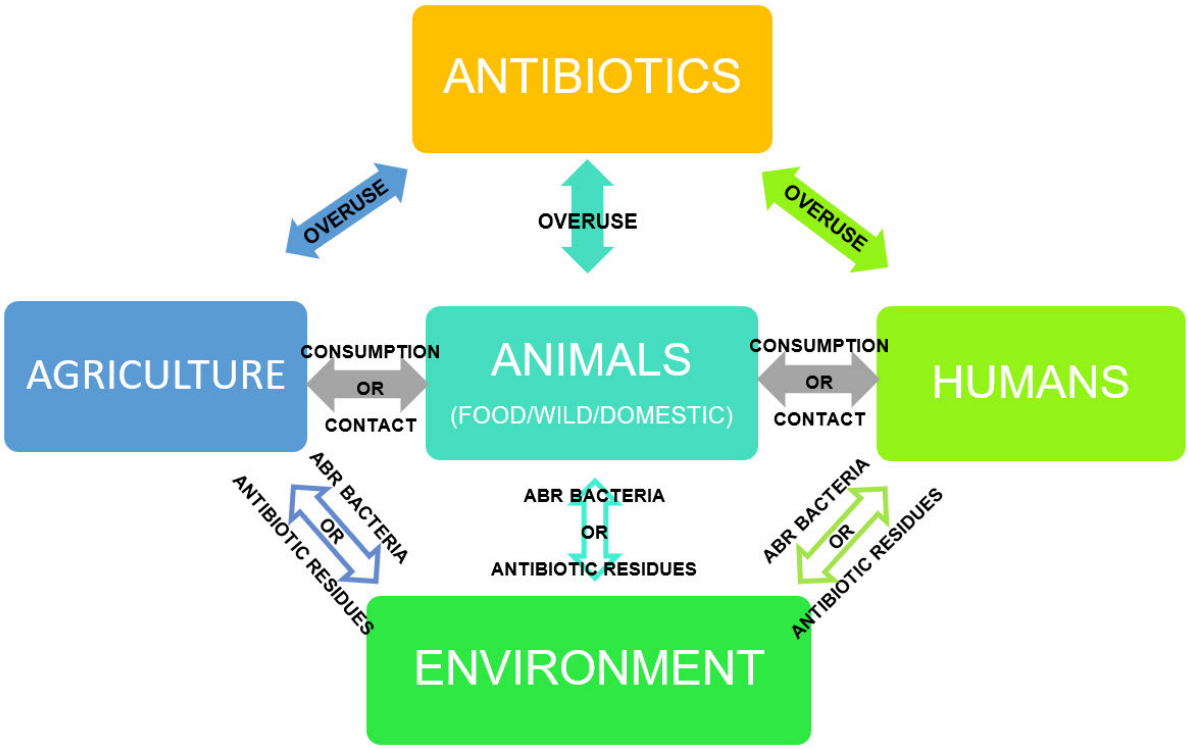

Figure 1.1. Circulation of antibiotic waste and antibiotic resistant bacteria (ABR) within the ecosystem.

Research dedicated to pinpointing antibiotic resistant (ABR) bacteria and identifying antibiotic waste in specific ecological niches, such as agricultural runoff sites, wastewater treatment plants, and natural water bodies, enhances our comprehension of how these bacteria circulate within and among ecosystems. By characterizing the sources and pathways of ABR dissemination, such as the transmission of ABR bacteria through animal waste runoff into nearby water sources or the release of

antibiotic residues from agricultural soils into the environment, this research provides crucial insights into the dynamics of ABR proliferation. Understanding these circulation patterns is essential for devising targeted interventions to mitigate the spread of antibiotic resistance and safeguard environmental and public health (Kunhikannan *et al.*, 2021). This investigative approach aligns with The One Health Approach, employing holistic monitoring and data acquisition to detect and survey ABR within ecosystems (McEwen and Collignon, 2018). The One Health Approach involves identifying ABR bacteria and genes, and their potential movement between environmental compartments, helping locate resistance reservoirs. Holistic studies exemplify this approach, revealing instances where resistance elements and ABR bacteria traverse from humans and animals through sewage systems and pit latrines to impact wildlife, irrigation, and drinking water. This perpetuates a cycle of ABR bacteria circulating through the environment, hospitals, and households (Aijuka *et al.*, 2015; Hendriksen *et al.*, 2019; Kunhikannan *et al*., 2021).

1.1.1 Animals as Vectors of Antibiotic Resistance

It is now widely acknowledged that animals function as reservoirs for antibiotic resistant (ABR) bacteria, acting as vectors for the spread and transmission of ABR bacteria and genes within the ecosystem. The increased interactions between humans and animals have consequently led to an increase in zoonotic infections and the prevalence of ABR pathogens (Ikhimiukor *et al*., 2022).

1.1.1.1 Animal food production

In the realm of commercial animal food production, antibiotics are still incorporated in many countries into animal feed under the guise of disease prevention and growth promotion. Despite the assumption that this practice fosters growth, it actually expedites the emergence of ABR bacteria, which then proliferate in the environment due to increased antibiotic waste (Tiseo *et al*., 2020). A staggering 73% of global antibiotic sales are attributed to their use in food animals (Mohsin *et al*., 2020). Projections indicate a worrisome 11.5% increase in the global sales of antibiotics

used in animal food production (including poultry, cattle, and pork) by 2030, reaching an estimated 104,079 tonnes of antibiotics (Tiseo *et al*., 2020).

In Africa, macrolides and tetracyclines stand out as the most commonly used antibiotic classes in this context (Van *et al*., 2020). Specifically, in South Africa, a report from the years 2009-2012 revealed that two-thirds of antibiotics designated for animal use were employed as growth enhancers (Eagar *et al*., 2012). Although Europe and the USA have prohibited the use of antibiotics for growth promotion, both regions continue to employ them for disease prevention, effectively sidestepping the ban. Consequently, these two entities persist as the highest users of antibiotics in food animal production (Van *et al*., 2020).

ABR bacteria and the genes responsible for their resistance, frequently originating from livestock farms, can enter the environment through runoff into water bodies, as well as via manure, soil, and horizontal gene transfer to other bacteria. The identification of identical multidrug-resistant (MDR) bacteria isolated from both livestock and humans underscores the transmission of ABR bacteria and genes from livestock to the broader ecosystem (Xu *et al.*, 2022). The widely used hygiene indicator, *E. coli*, has been detected in poultry, beef, eggs, seafood, and mutton samples from various countries worldwide. The primary resistance in these animal-derived *E. coli* exhibiting ABR is often linked to the presence of *tet*A and bla_{TEM} genes (Xu *et al.*, 2022). Moreover, environmental dispersion of ABR bacteria and antibiotic residues from livestock production occurs through the common agricultural practice of using faeces-based manure as fertilizers. This practice is cost-effective but contributes to the dissemination of ABR bacteria and antibiotic compounds into the environment (Zalewska *et al.*, 2021).

Approximately 40-90% of the consumed antibiotics are excreted in their original compound state, either as a conjugate that can be reconverted to the antibiotic precursor or as a metabolite. Notably, fluoroquinolones and tetracyclines have consistently been reported at the highest concentrations in food animal faeces over the past four decades compared to other antibiotics (Ghirardini *et al*., 2020). A study in Egypt revealed concerning findings, with 44% of meat and liver samples from fresh

chickens containing tetracycline residues up to 8148 µg/kg. Given that poultry is the second most consumed meat globally, these findings raise significant concerns (Salama *et al*., 2011). Similarly, a study conducted in China found that up to 57.1% of chicken meat samples contained detectable levels of various antibiotics, including sulfonamides and fluoroquinolones, further emphasizing the widespread issue of antibiotic residues in poultry products (Wang *et al*., 2017). Research has demonstrated that even sub-therapeutic levels of antibiotics in animal feed can promote the growth of ABR, which can be transferred to humans through the consumption of animal products or through environmental pathways (Marshall and Levy, 2011).

The Pekin duck (*Anas platyrhynchos domesticus*), originating from China, stands as one of the two most widely consumed ducks globally. While duck meat ranks as the second-largest consumed and farmed poultry in Southeast Asia, its popularity is expanding in South Africa, driven by a growing niche market, including high-end restaurants and local farmer markets. Pekin duck meat is prized for its high protein quality and serves as a rich source of essential amino acids, notably surpassing chicken meat in methionine content (Aronal *et al*., 2012).

Despite its nutritional value, duck meat, like all animal products, poses a potential source for the transmission of foodbourne pathogens from animals to humans. In a study by Darwish *et al*. (2015), five serogroups of pathogenic *E. coli* were isolated from commercially available duck meat obtained randomly from poultry stores in Egypt. Effective regulation on the use of antibiotics in food production is crucial in mitigating the prevalence of ABR bacteria and residues in animal-derived food products. High levels of antibiotic residues can favour the selection and success of ABR bacteria, making antibiotic use restrictions imperative (Tang *et al*., 2017).

1.1.1.2 Wild birds

With over ten thousand living species in the Aves class, birds exhibit varied lifestyles across different habitats, showcasing adaptability even in areas significantly influenced by human activity. This has led to a growing interest in understanding wild

birds as potential vectors of antibiotic resistant (ABR) bacteria and corresponding resistance genes (Wu *et al*., 2018). Birds play a crucial role in the One Health Approach, which recognizes the interconnectedness of human, animal, and environmental health. Their ability to move freely across various environments makes them key players in the transmission of ABR bacteria (Daoud and Rolain, 2022). By studying birds and their role in ABR transmission, researchers can gain insights into broader dynamics and develop strategies to mitigate risks across all sectors.

Birds possess unique traits that enhance their potential as carriers of ABR bacteria, distinguishing them from related species like reptiles. Their high mobility, driven by migratory patterns, enables them to traverse long distances, facilitating the spread of ABR bacteria across diverse regions and ecosystems (Wu *et al*., 2018). Additionally, birds' diets, often comprising contaminated food and water from human-influenced areas, such as waste sites, contribute to their role in ABR dissemination. Coupled with their efficient biological and ecological characteristics, such as a high metabolic rate, elevated internal body temperature, and effective digestive system, birds become particularly efficient carriers of ABR *Enterobacteriaceae* (Shobrak and Abo-Amer, 2014). Their interactions with contaminated environments, including agricultural fields and urban areas, amplify their role in disseminating ABR bacteria, as they can acquire resistant bacteria from these settings and disperse them through their faeces, thereby contaminating water sources, soil, and other animals (Laborda *et al*., 2022).

The collective body of research on ABR bacteria in birds provides a comprehensive understanding of avian populations as potential vectors of ABR, highlighting the intricate interplay between birds, their habitats, and the prevalence of ABR bacteria. Initial recognition of the vast diversity and adaptability of birds in human-influenced environments sets the stage for understanding their potential role in ABR. Studies focusing on *E. coli* present in the faeces of wild birds, such as raptors, reveal a notable frequency of Extended Spectrum β-lactamase (ESBL) producing *E. coli* isolates, underscoring the commonality of this bacterium and its significance in transmitting ABR (Costa *et al*., 2006). Subsequent investigations, such as those on yellow-legged gulls in France (Bonnedahl *et al*., 2009) and black-headed gulls

carrying ABR *Salmonella* spp. (Čížek *et al.* 2007), emphasize the high incidence of ABR bacteria in various wild bird species and the potential crossover between birds and humans. The identification of multidrug-resistant (MDR) phenotypes in Irish gulls by Carroll *et al*. (2015) further emphasizes the role of birds in disseminating ABR, necessitating continued research to mitigate the impact on both animal and human health.

An amalgamation of studies on ABR bacteria in diverse bird species underscores the global scope of avian contributions to ABR dynamics. Guenther *et al*. (2010) conducted a comprehensive analysis revealing a high prevalence of ABR *E. coli* in common European birds, including the buzzard, mute swan, barn owl, pigeon, white-throated dipper, and the garden warbler. Notably, 4.8% of isolates exhibited MDR phenotypes, showcasing resistance to fluoroquinolones, tetracyclines, β-lactams, and sulfonamides. A study in the USA by Jamborova *et al*. (2017) uncovered plasmid-mediated resistance to cephalosporins and quinolones in *E. coli* isolated from American crows, underlining the global nature of ABR dissemination in avian populations. Troxler *et al.* (2017) extended this perspective, investigating ABR in *E. coli*, *Salmonella* species, and thermophilic *Campylobacter* spp. from wild birds in the Austrian-Czech border region. Their findings revealed varying degrees of ABR, with *E. coli* and *Campylobacter* spp. displaying higher resistance than *Salmonella* spp. emphasizing the need for continued global research on ABR in wild bird populations.

More recent studies further illustrate the extent and diversity of ABR in avian species. A 2021 study by Yuan *et al*. (2021) reported the presence of carbapenem-resistant *Enterobacteriaceae* (CRE) in migratory birds in China, highlighting the potential of long-distance spread of highly resistant bacteria. Similarly, a 2022 investigation by Ribeiro-Almeida *et al*. (2022) detected colistin-resistant *E. coli* in seagulls in Portugal, which poses significant public health concerns due to colistin being a last-resort antibiotic for MDR infections. These findings underscore the evolving and expanding nature of ABR in wild bird populations across different countries.

The interface between wildlife and suburban areas is delicate and easily breached, allowing wild birds to harbour bacteria inherently resistant to clinically relevant

antibiotics, positioning them as important vectors for the dissemination of ABR bacteria at the animal-human interface (Blair *et al*., 2015). This vulnerability is exacerbated by the increased loss of natural wetlands, leading waterbirds to seek refuge in artificial habitats, including wastewater treatment plants, thereby facilitating the transmission of ABR bacteria of human origin to birds. This transmission is also notable in urban birds, which have become more accustomed to human interaction (Murray and Hamilton, 2010). Additionally, a study conducted by Chidamba and Korsten (2015) in a Gauteng suburban area of South Africa, of rainwater collected from residential roof-harvested tanks with pigeon faeces as a source of *E. coli* contamination revealed alarming findings. Approximately 53.3% of *E. coli* isolates from pigeon faeces and 67.3% from the rainwater samples exhibited antibiotic resistance to at least one of the thirteen antibiotics used in the study, underscoring the significance of ABR transmission in both wild and urban bird populations.

South Africa is home to diverse wild bird populations, with the sacred ibis (*Threskiornis aethiopicus*) being a common sight throughout the country and migrating to other sub-Saharan African countries during winter (Harrison and Cherry, 1997). All other populations outside of Africa, originate from captivity and as a result, are now successfully invasive in the USA, UAE, Taiwan, and Europe (Maillard *et al*., 2020). Their adaptable diet, including aquatic and terrestrial invertebrates, small fish, amphibians, reptiles, mammals, and birds (Yésou *et al*., 2017), coupled with their scavenging behaviour in urban and suburban areas, facilitates their success within human settlements. Frequently found near wastewater treatment plants, landfills, freshly ploughed fields, and poultry farms, the sacred ibis is exposed to antibiotic residues and waste, potentially dispersing ABR bacteria in the environment (Maillard *et al*., 2020).

Raptors, integral to South African bird diversity, are widely distributed across the country and occupy the top of the food chain. With a diet comprising of rodents, reptiles, and other smaller birds, raptors are susceptible to carrying ABR bacteria or genes. Despite their prevalence, there remains a knowledge gap regarding the potential of these birds as vectors of ABR bacteria in South Africa. However, birds of prey have been reported as potential vectors of ABR bacteria in other countries such

as Portugal (Costa *et al*., 2006), China (Wang *et al.*, 2017), and Spain (Mencía-Gutiérrez *et al*., 2021).

Research on *Enterococcus* species in birds is relatively scarce compared to the extensive studies focused on *E. coli*. While numerous investigations have explored the presence and antibiotic resistance patterns of *E. coli* in avian populations, there is a notable gap in understanding *Enterococcus* spp. in the context of birds. This research imbalance warrants increased attention to explore the prevalence, distribution, and ABR profiles of *Enterococcus* spp. in avian communities. Further studies on *Enterococcus* spp. in birds are crucial for a more holistic understanding of the role of avian populations in the dissemination of ABR and their role in the One Health continuum.

1.1.1.3 Dietary influences on ABR in birds

The diet of birds, whether in the wild or under commercial farming conditions, plays a significant role in shaping the composition of their gut microbiota and the potential development of ABR. The nutritional components, feed additives, and exposure to contaminated food sources can directly or indirectly influence the microbial communities within the avian gastrointestinal tract, which in turn affects the prevalence of ABR bacteria.

Commercial poultry diets

In commercial poultry farming, birds are often provided with formulated feeds designed to optimize growth, egg production, and overall health. These diets typically include grains, proteins, vitamins, and minerals. However, in some cases, subtherapeutic doses of antibiotics, such as tetracyclines, macrolides, or ionophores, are incorporated into feed as growth promoters, particularly in intensive farming operations (Agyare *et al*., 2018). While the use of antibiotics in animal feed has been regulated in many countries, there remains a concern that residual antibiotic levels or improper usage can contribute to the selection of ABR bacteria in the avian gut microbiome. Studies have shown that prolonged exposure to low levels of antibiotics can select for resistant strains of *Escherichia coli*, *Enterococcus* spp., and other gut

commensals that can act as reservoirs of resistance genes (Wang *et al*., 2021; Ribeiro *et al*., 2023).

In free-range systems, such as those in which commercial ducks are raised, the exposure to antibiotics may be lower, as antibiotic use is generally restricted. However, access to natural feed sources, including insects, plants, and contaminated water bodies, introduces other risks. Feed that is contaminated with antibiotic residues or pesticide runoff from nearby agricultural activities can further influence the selection of resistant bacterial populations (Agyare *et al*., 2018).

Wild bird's diets

Wild birds, including species such as sacred ibis and raptors, have a diet that is heavily influenced by their environment and food availability. Sacred ibis, for example, are known scavengers and often forage near urban environments, including landfills and wastewater treatment plants. These sites are potential hotspots for ABR bacteria due to human waste, pharmaceutical run-off, and other pollutants that contain antibiotic residues. Raptors, as apex predators, may also acquire resistant bacteria through the consumption of prey that have been exposed to environmental contaminants or human-related activities (Lagerstrom and Hadly, 2021).

Wild birds often ingest microorganisms, including resistant bacteria, directly from their food sources, which can range from small mammals to aquatic organisms and decomposing organic material. As a result, they can acquire ABR bacteria, particularly if their prey has been exposed to antibiotics through environmental contamination. Moreover, birds that feed near agricultural sites may be exposed to antibiotic-laden manure or fertilizers, further increasing their likelihood of harbouring resistant bacteria (Wu *et al*., 2018).

1.2 *Escherichia coli* and *Enterococcus* species

1.2.1 Natural Presence

E. coli, a Gram-negative bacterium, and *Enterococcus* species such as *E. faecalis*, a Gram-positive bacterium are commonly used as hygiene indicators in research studies owing to their inherent presence in the gastrointestinal tract of humans and various animals, including avian species (Silva *et al*., 2012). Functioning as reliable indicators of hygiene, these commensal bacteria form integral components of the normal gastrointestinal microbiome. They play essential roles in supporting host immunity, metabolizing nutrients, synthesizing vitamins, contributing to pH regulation, and aiding in the prevention of pathogen colonization (Silva *et al*., 2012; Dubin and Pamer, 2017).

The burden of *E. coli* and *Enterococcus* spp. in birds, both wild and domestic, is noteworthy. Studies have shown that these bacteria are frequently isolated from avian species, where they play similar roles to those observed in other animals (Radhouani *et al*., 2014). However, birds, particularly those in close contact with human environments, can act as reservoirs for pathogenic strains of *E. coli* and *Enterococcus* spp., which can be transmitted to humans and other animals. Wild birds often carry these bacteria without showing clinical signs of disease, but the potential for transmission of ABR strains is a significant concern. This is especially relevant for migratory birds, which can spread resistant strains over vast distances and across borders (Radhouani *et al*., 2014).

In comparison, domestic animals and those used in food production also harbour substantial populations of *E. coli* and *Enterococcus* spp. In livestock, these bacteria, if/when exhibiting pathogenic traits, can lead to infections that impact animal health and productivity, and they can be transmitted to humans through direct contact or consumption of contaminated animal products. The widespread use of antibiotics in agriculture has exacerbated the issue, leading to an increase in ABR strains. For instance, *E. coli* from food-producing animals often exhibit resistance to multiple

antibiotics, posing a threat to both animal and human health (Hammerum and Heuer, 2009).

While generally considered harmless commensals, for both microorganisms, *E. coli* and *Enterococcus* spp., pathogenic strains have been identified. The classification of *E. coli* into phylogenetic groups is instrumental in distinguishing between commensal and pathogenic strains of this Gram-negative bacterium. Similarly, certain strains of *Enterococcus* spp., such as *E. faecalis* and *E. faecium* can transform into nosocomial opportunistic pathogens, particularly when displaying heightened resistance to antibiotics. Notably, *E. faecalis* stands out as the predominant species responsible for human clinical enterococcal infections, making up 80-90% cases, while *E. faecium* accounts for 5-15% (Silva *et al*., 2012). The variability in virulence factors among enterococcal species is evident, with *E. faecalis* typically exhibiting a greater number of virulence traits compared to *E. faecium* (Silva *et al*., 2012).

Overall, the presence and impact of *E. coli* and *Enterococcus* spp. in birds and other animals highlight the intricate connections within ecosystems and between human and animal health. Monitoring and managing these bird-associated bacteria, especially ABR strains, is crucial for maintaining the health of diverse species and protecting public health (van den Bogaard and Stobberingh, 2000).

1.2.2 Prevalence of Antibiotic Resistance

While playing a crucial role in preserving the microbial equilibrium in the mammalian gastrointestinal tract, commensal *E. coli* and *Enterococcus* spp. possess the ability to acquire and disseminate antibiotic resistance genes, which is particularly concerning in the case of pathogenic strains. These characteristics make them valuable model organisms for understanding host-pathogen interactions and examining the prevalence of antibiotic resistance in environmental compartments.

1.2.2.1 *Escherichia coli*

Numerous studies underscore the role of both migrating and non-migrating birds as carriers of antibiotic resistant (ABR) *E. coli*. The primary transmission pathways linking resistant *E. coli* from human or veterinary sources to wild birds often involve water contact and food (Shobrak and Abo-Amer, 2014). For instance, an analysis of *Escherichia* spp. isolates obtained from cloacal swabs of wild bird species in Egypt revealed a distinct pattern: isolates from non-migrating birds exclusively showed resistance to oxacillin, while those from migrating birds exhibited resistance to an extended spectrum of antibiotics (Shobrak and Abo-Amer, 2014).

Similarly, a study in Bangladesh, focusing on *E. coli* isolates from migratory birds, uncovered a substantial prevalence of multi drug resistance (MDR), with 67.27% of isolates carrying avian pathogenic *E. coli* associated virulence genes (Islam *et al*., 2021). The wide occurrence of *E. coli* resistance is not confined to specific bird species but extends to various environments, including domestic or companion birds (Sigirci *et al*., 2020), birds of prey (wild and captive) (Shobrak and Abo-Amer, 2014), and poultry farms (Islam *et al*., 2023). These diverse examples emphasize the potential of birds, across different species and habitats, to serve as vectors for spreading ABR *E. coli.*

1.2.2.2 *Enterococcus* species

Similar to the documented antibiotic resistant (ABR) *E. coli* prevalent in birds, bird associated ABR *Enterococcus* spp. has also been reported. A study conducted in Poland investigated *E. faecalis* isolates from twenty-five wild bird species, demonstrating widespread antibiotic resistance. Specifically, all *Enterococcus* isolates (n=27) exhibited resistance to lincomycin, while 48% showed resistance to tetracycline, 44% towards erythromycin, and 22% to ciprofloxacin. Additionally, a comprehensive analysis of *E. faecalis* genetic diversity revealed high variability of resistance mechanisms, with potential implications for the transmission of ABR strains to humans, pets, and farm animals (Stępień-Pyśniak *et al*., 2018). Furthermore, another study focusing on the intensive poultry production system in KwaZulu-Natal, South Africa, evaluated ABR and virulence profiles of *Enterococcus*

spp. along the farm-to-fork production chain. The findings revealed high ABR diversity in *Enterococcus* isolates, with prevalent resistance to various antibiotics, such as tetracycline, erythromycin, ampicillin, including multi drug resistance (MDR) patterns. Resistance and virulence genes were common among isolates, emphasizing the potential transmission of ABR strains from poultry to humans through the food chain (Molechan *et al*., 2019). These studies collectively underscore the global concern of ABR in *Enterococcus* spp., emphasizing the need for continuous surveillance and management strategies in both wildlife and food animals.

1.3 Antibiotics

1.3.1 A Brief History of Discovery

The development of antimicrobial agents relies heavily on our understanding of infective target organisms and microorganisms that have the remarkable capability of naturally synthesizing antimicrobial compounds. The use of naturally occurring antimicrobial compounds dates back over 2000 years, with traditional 'healers' applying poultices made from mouldy bread and/or medicinal soil (Hutchings *et al.*, 2019). Fascinatingly, a traditional medicinal recipe dating back 1000 years ago was found to be effective against modern-day methicillin resistant *Staphylococcus aureus* (MRSA) (Harrison *et al.*, 2015).

Medicinal practices based on microbial knowledge continued through the centuries, exemplified by Paul Ehrlich's development of the first known anti-infective drugs including "salvation arsenic" (commonly known as salvarsan), used to treat syphilis (Jubilee, 1936). Ehrlich's work enabled Bayer bacteriologists to develop the sulphonamide prodrug Prontosil, based on his bacterial-selective staining dyes. Remarkably, sulphonamide prodrugs are still in use today, even after the discovery of penicillin (Koshti, 2022).

The 1920's saw the "accidental" discovery of penicillin by Sir Alexander Fleming, following the observation of a contaminated Petri dish. Sir Fleming, with the aid of colleagues from Oxford, developed penicillin as an antimicrobial drug. In 1945, Chemistry Nobel Prize winner Dorothy Hodgkin determined that penicillin had a β-lactam structure, enabling the development of derivatives to combat penicillin resistance (Lobanovska and Pilla, 2017).

With increasing discoveries of antimicrobial compounds produced by other microorganisms, Selman Waksman initiated a systematic study in the 1930's and defined an antibiotic as "a compound made by a microbe to destroy other microbes" (Waksman *et al.*, 2010; Hutchings *et al.*, 2019). Waksman's work, pivotal during the "Golden Age" of antibiotic history spanning the 1940's to 1960's, lead to the discovery of actinomycetes producing a variety of antimicrobial compounds. These discoveries led to various antibiotics and are currently used for treating tuberculosis, other microbial infections, and even cancer (Hutchings *et al.*, 2019).

Key antibiotics discovered post-1950's include tetracycline, erythromycin, and vancomycin, which have played critical roles in treating a wide range of bacterial infections. However, the peak in rapid discovery of multi-class antibiotics plateaued from the 1970's onwards, leading to increased use and misuse of existing antibiotics and the subsequent rise of antibiotic resistance (ABR) (Silver, 2011).

The decline in new antibiotic discoveries can be attributed to several factors, including economic and regulatory challenges that discourage pharmaceutical companies from investing in antibiotic research and development (Silver, 2011; Cook and Wright, 2022). As a result, scientists have focused on developing and modifying pre-existing antibiotics to combat ABR. However, slower advancements have resulted in a greater increase in ABR (Lobanovska and Pilla, 2017).

The impact of ABR extends beyond treating infections; it threatens the efficacy of modern medical practices, including surgeries, chemotherapy, and other treatments that rely on effective antibiotics. This underscores the urgent need for coordinated global efforts to combat ABR.

Current efforts to address ABR include the development of novel antibiotics, alternative therapies such as bacteriophage therapy, and global initiatives to improve antibiotic stewardship and reduce misuse. These efforts are critical in mitigating the growing threat of ABR and ensuring the continued efficacy of antimicrobial treatments.

1.3.2 Most Commonly Used Antibiotics and Relevant Resistance Mechanisms

Surveillance of antibiotic resistance (ABR) in key bacterial indicators, including faecal *E. coli* and *Enterococcus* species, is crucial for comparing resistance prevalence and identifying potential routes for resistant bacteria or resistance genes within the food chain (Vrabec *et al*., 2015). These bacteria, frequently resistant to common antibiotics, are potent opportunists in nosocomial infections. *Enterococcus* spp. exhibit inherent resistance to various antibiotics and are increasingly resistant to β-lactams, glycopeptides, aminoglycosides, and linezolid. In the context of extended-spectrum beta-lactamase (ESBL) mediated resistance, *E. coli*, traditionally associated with TEM and SHV encoded ESBL derivatives, has seen a rising prevalence of the CTX-M ESBL type globally (Vrabec *et al*., 2015).

Bacteria can possess inherent resistance to specific antibiotics and can acquire resistance through mutations and horizontal gene transfer (Blair *et al*., 2015). Acquired resistance involves mechanisms altering intracellular antibiotic concentration, modifying antibiotic targets, and inactivating antibiotics through chemical modification or hydrolysis. Altered membrane composition via modified porins and efflux pumps, can cause ABR by reducing antibiotic uptake and concentration of the antibiotic within the cell. Acquired resistance can result in altered antibiotic targets through point mutations or DNA uptake and recombination. Inactivation can occur through enzymes like β-lactamase, hydrolysing β-lactam antibiotics, or chloramphenicol acetyl transferase, which modifies the antibiotic, thus prohibiting it from binding to its target site. The emergence of ESBLs and carbapenemases, with genes like CTX-M originating from mostly clinical bacteria and

spreading through plasmids, presents significant challenges in the efficacy of currently available antibiotics (Blair *et al*., 2015).

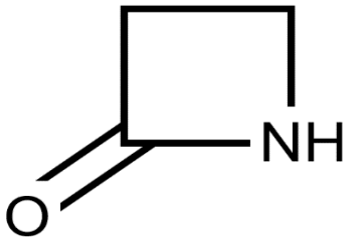

Figure 1.2. Basic structure of the β-lactam ring.

1.3.2.1 β-Lactam antibiotics

The physiochemical characteristics inherent to the key structure of β-lactam antibiotics exert a significant influence on their antibacterial spectrum of activity. In general, the hydrophilicity of these antibiotics is conducive to their action against Gram-negative bacteria, such as *E. coli*, and their lipophilicity is conducive to their action against Gram-positive bacteria, such as *Enterococcus* spp. (Fernandes *et al*., 2013). Penicillin, as the pioneering antibiotic within this group, acts by inhibiting cell wall synthesis through the targeting of penicillin-binding proteins (PBPs) involved in peptidoglycan biosynthesis. Other members of the β-lactam antibiotic family, such as cephalosporins, monobactams, and carbapenems (see Figure 1.3), also share this mechanism, collectively inhibiting the catalytic activity of bacterial transpeptidase (Fernandes *et al*., 2013). Additionally, they all feature the characteristic β-lactam ring, as illustrated in Figure 1.2.

β-lactam antibiotics are among the most widely used antibiotics globally, both in human and veterinary medicine, as well as in agriculture and livestock (Klein *et al*., 2018). Their broad spectrum of activity, effectiveness, and safety profile have made them a cornerstone in the treatment of bacterial infections. In human medicine, β-lactam antibiotics are extensively used to treat a wide variety of infections, serving as

the first-line treatment for many bacterial infections, including pneumonia, meningitis, and sepsis. Penicillins and cephalosporins are particularly common, with amoxicillin and ceftriaxone being frequently prescribed (WHO, 2022). In veterinary medicine, β-lactam antibiotics are used to treat infections in both companion animals and livestock, with applications ranging from treatment of mastitis in dairy cows to respiratory infections in pigs. In agriculture and livestock, β-lactam antibiotics are used not only for therapeutic purposes but also, in some regions, for growth promotion and disease prevention (Mohsin *et al*., 2020).

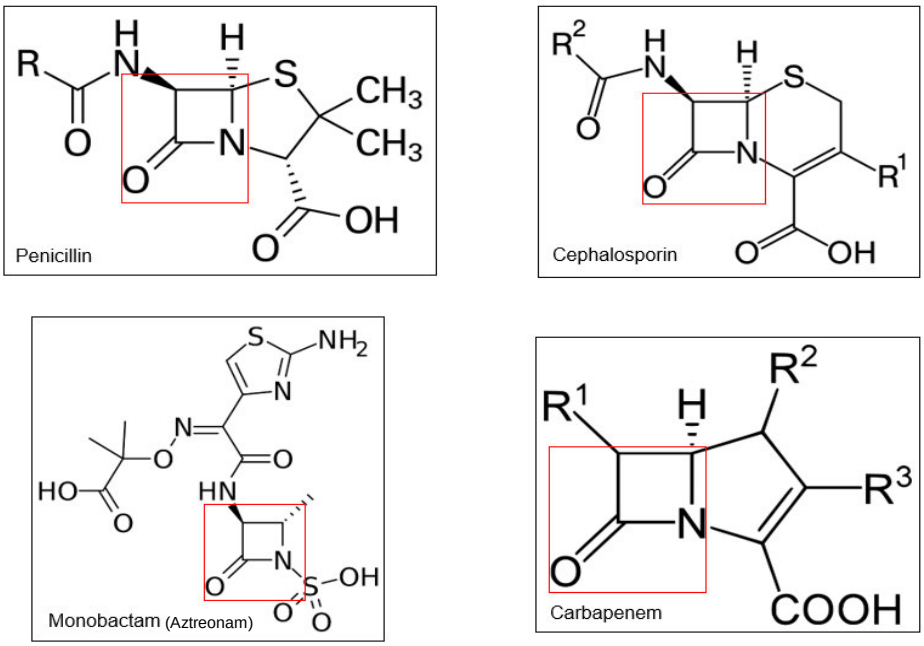

Figure 1.3. Chemical structure of representative β-lactam antibiotics.

Penicillin

The discovery of penicillin by Sir Alexander Fleming in 1928 marked the beginning of a new era in antibiotic development, though it was not until the early 1940s that its potential for large-scale production and clinical use was realized. This breakthrough was the result of the collaborative efforts of Howard Florey, Ernst Boris Chain, and their team, who successfully extracted, purified, and developed Penicillin G (Pen G) for medical use (Kardos and Demain, 2011). While Pen G, along with its oxa-homolog Penicillin V, exhibited vulnerability to hydrolysis by β-lactamases, Pen G

was also unstable in both acidic and alkaline conditions, limiting its use primarily to Gram-positive bacteria and precluding oral administration. The introduction of Pen G formulations with procaine or benzathine helped extend its efficacy, with benzathine providing prolonged serum concentrations. The identification of *Staphylococcus aureus* resistance to Pen G by Kirby in 1944 underscored the critical role of β-lactamases in rendering the antibiotic ineffective (Kirby, 1944; Lima et al., 2020).

The subsequent introduction of methicillin addressed instances of PnG-resistant *S. aureus*, followed by the development of isoxazolylpenicillins to enhance stability. The evolutionary trajectory continued with the discovery of ampicillin and amoxicillin widening the spectrum of action. Over time, the introduction of antibiotics like carbenicillin, ticarcillin, azlocillin, mezlocillin, and piperacillin expanded the efficacy against specific bacterial strains. Despite variances in stability, spectrum and administration routes, all penicillins share a common reactivity – susceptibility to nucleophilic attack due to the inherent ring tension of the β-lactam system (Kardos and Demain, 2011).

Cephalosporin

The origins of cephalosporins trace back to 1948 when Giuseppe Brotzu isolated the first cephalosporins from *Cephalosporium acremonium* cultures found in the sewage systems of Sardinia. Cephalosporin C, as the prototype antibiotic, demonstrated activity against penicillin resistant cultures, particularly *Staphylococcus aureus*. Structurally, cephalosporins are characterized by a dihydrothiazine ring fused to the β-lactam nucleus. This unique structure provides increased resistance to nucleophilic attack and hydrolysis in comparison to penicillins (Lima *et al.*, 2020).

Cephalosporins exhibit a distinctive mechanism of action against penicillin-binding proteins (PBPs), which disrupts bacterial cell wall synthesis and leads to bacterial cell death. While the initial cephalosporin C had limited application, subsequent semi synthetic cephalosporins became pivotal for drug development. First generation cephalosporins targeted primarily Gram-positive bacteria. Second generation cephalosporins, characterized by increased polarity demonstrated efficacy against Gram-negative bacteria. The third generation cephalosporins, including cefotaxime,

offered stability against non-ESBL β-lactamases and broad coverage. The fourth generation exhibited improved stability and expanded activity against both Gram-positive and Gram-negative organisms. The fifth generation, also known as anti-methicillin-resistant *S. aureus* (MRSA) cephalosporins, marked significant progress in combating resistant strains (Fernandes *et al*., 2013).

Monobactam

The exploration of monocyclic β-lactam antibiotics commenced with the unveiling of sulfazecin in a 1979 Japanese patent, followed by scientific publications in 1981 from the Takeda and Squibb laboratories (Lima *et al.*, 2020). Squibb labs introduced screening protocols utilizing soil bacteria and coined the term “monobactam” for this antibiotic class. Despite the existence of natural monobactams, the first synthetic compound to hit the pharmaceutical market was aztreonam in 1984, resulting from structural modifications aimed at enhancing antimicrobial activity. Aztreonam boasts a distinctive monobactam framework ensuring resilience against many β-lactamases. Its unique physiochemical attributes facilitate sufficient diffusion through the membranes of Gram-negative bacteria (Dean *et al*., 2018).

Aztreonam lacks oral absorption due to its non-substrate nature for the di- and tripeptide transport system. With a broad spectrum targeting various Gram-negative aerobes, aztreonam exhibits high affinity for penicillin binding protein 3 (PBP-3). Despite low oral bioavailability (1%), intramuscular injection achieves nearly 100% bioavailability, accompanied by a short half-life of 1.6 hours. Approximately 68% is excreted unaltered in urine with a minor percentage as an inactive metabolite. Notably aztreonam displays low immunogenicity, making it suitable for patients with prior allergies to penicillins or cephalosporins (Dean *et al*., 2018; Lima, *et al.*, 2020).

Carbapenem

Carbapenems emerge as a crucial class of β-lactam antibiotics celebrated for their notable resilience against most β-lactamases, showcasing the ability to function as gradual substrates or inhibitors of these enzymes. The mechanism of action involves traversing the outer membranes of Gram-negative bacteria through porin channels to

reach penicillin binding proteins (PBPs), resulting in a wide-ranging antibacterial impact on diverse Gram-negative and Gram-positive pathogens. Structurally akin to thienamycin, a β-lactamase inhibitor derived from *Streptomyces cattleya*, carbapenems feature a distinct 4:5 fused ring lactam of penicillins with a double bond between C-2 and C-3. This unconventional bioisosterism, inspired by nature, introduces a hybrid chemical reactivity compared to penicillin and cephalosporin (Fernandes *et al*., 2013).

Diverging from penicillin and cephalosporin in terms of stereochemistry at C6, carbapenem consistently presents a chiral hydroxyethyl group in S-absolute stereochemistry. This particular structural aspect contributes to its robust resistance against β-lactamase activity. Imipenem introduced as the initial carbapenem antibacterial drug, employed a non-classical isosterism strategy by replacing the primary amine with an amidine group, enhancing its chemical stability. However, it's susceptibility to renal dehydropeptidase-1 (DHP-1) necessitated co-administration with cilastatin to prevent inactivation and associated nephrotoxicity (Breilh *et al*., 2013).

Meropenem, a subsequent carbapenem, overcame DHP-1 hydrolysis, demonstrating metabolic stability attributed to a C4-(R)-methyl group. Despite limited chemical stability in concentrated solutions, meropenem exhibits efficacy against a broad spectrum of bacteria. Tebipenem, the oral carbapenem prodrug was developed with enhanced stability, mainly due to a tertiary amine group at C3, preventing self β-lactam ring cleavage. Doripenem and ertapenem, structural analogs of meropenem, showcased improved pharmacokinetics, with ertapenem featuring a meta-substituted benzoic acid group for an enhanced half-life (Breilh *et al*., 2013; Lima, *et al.*, 2020).

Biapenem, distinctive among carbapenems, provides a broad antibacterial spectrum better enzymatic stability and reduced potential for gamma-aminobutyric acid (GABA) receptor inhibition. Neurotoxicity in carbapenems is correlated with the basicity of the side chain in C2. Although bacterial resistance to carbapenems was considered relatively uncommon, it has evolved into a notable public health concern, primarily involving Gram-negative pathogens due to enzymatic inactivation by

carbapenemases, target site mutations, and efflux pumps (Breilh *et al*., 2013; Armstrong *et al*., 2021).

Mechanisms of β-*lactam resistance*

Gram-negative bacteria, like *E. coli*, resist β-lactam antibiotics through β-lactamase production, and limiting access to target PBPs. Unlike Gram-positive bacteria, the PBPs of Gram-negative bacteria are located in the periplasmic space, requiring β-lactam antibiotics to traverse the outer membrane. Changes hindering entry (porin mutation) or causing extrusion (efflux pumps) result in antibiotic resistance, potentially causing cross-resistance, where the bacteria become resistant to multiple antibiotics, often across different classes (Hobson *et al*., 2021). The primary resistance mechanisms in Gram-negative bacteria involves β-lactamase, ESBL, and carbapenemase production, and inducible AmpC type β-lactamase. The synergistic interplay of these mechanisms significantly influences the phenotypic expression of resistance (Tang *et al*., 2014).

The main factor leading to β-lactam resistance in Gram-positive bacteria, such as *Enterococcus* spp., is generally associated with the modification of penicillin-binding proteins (PBPs). Enzymatic resistance towards β-lactam antibiotics, acquired through plasmid-mediated β-lactamases via horizontal gene transfer, is rare and predominantly observed in *E. faecalis* (Tang *et al*., 2014). To combat β-lactamase mediated resistance, β-lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam have been developed. These inhibitors bind to β-lactamases, neutralizing their activity and thereby protecting the antibiotic from degradation. The combination of β-lactam antibiotics with these inhibitors enhances their efficacy against β-lactamase-producing bacteria. This approach is particularly effective in overcoming resistance in Gram-negative bacteria and has also shown potential in treating infections caused by β-lactamase-producing Gram-positive bacteria (Drawz and Bonomo, 2010).

Carbapenem resistance

Carbapenem-resistant organisms (CRO) have emerged as a global public health threat, driven primarily by the production of carbapenemases – enzymes that hydrolyze carbapenems, rendering them ineffective. These carbapenemases are classified into three main classes: Class A (e.g., *Klebsiella pneumoniae* carbapenemase, KPC), Class B metallo-β-lactamases (e.g., NDM, VIM), and Class D oxacillinases (e.g., OXA-48). Each class has its unique mechanisms, with KPC widely spread in Europe, South America, and Asia, and NDM rapidly disseminating from the Indian subcontinent to other regions. OXA-48 carbapenemases are prevalent in Mediterranean countries and Africa (Livermore *et al*., 2020). The co-existence of carbapenemases with other resistance mechanisms, such as efflux pumps and porin mutations, limits treatment options, often leaving last-resort antibiotics like colistin and tigecycline as the few viable choices.

The global distribution of carbapenemases is linked to patient movement, international travel, and the transfer of resistance genes through mobile genetic elements. Regions like Europe and the Americas have seen widespread KPC, while Asia and Africa are grappling with NDM and OXA-48. The rise of CROs increases healthcare costs, morbidity, and mortality rates, underscoring the urgent need for coordinated global efforts to improve surveillance, infection control, and antibiotic stewardship to mitigate the spread of these highly resistant pathogens (Thomas *et al*., 2022).

1.3.2.2 Fluoroquinolones

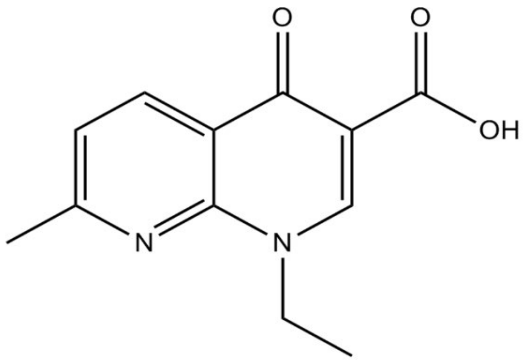

Figure 1.4. Nalidixic acid - fluoroquinolone precursor.

The founding member of this antibiotic group is nalidixic acid, characterized by a bicyclic core structure (Figure 1.4). Initially discovered in the early 1960s, nalidixic acid served as a narrow spectrum antibiotic, effective against Gram-negative bacteria (excluding *Pseudomonas* spp.) for treating uncomplicated urinary tract infections (UTIs) (Pham *et al*., 2019). Subsequent development led to fluoroquinolones, exhibiting a broader spectrum (effective against both Gram-negative and Gram-positive bacteria) and improved pharmacokinetics. Operating by inhibiting bacterial DNA synthesis, fluoroquinolones disrupt bacterial topoisomerase type II, inhibiting the catalytic activity of DNA gyrase and topoisomerase type IV (Pham *et al*., 2019). This class of antibiotics is further classified into four generations, delineated in Table 1.1 based on specificity and developmental stage.

Similar to β-lactam antibiotics, fluoroquinolones are extensively used worldwide in human and veterinary medicine, and agriculture, owing to their broad-spectrum activity and oral bioavailability. In human medicine, fluoroquinolones are used to treat a wide variety of respiratory tract, urinary tract, and gastrointestinal infections. Commonly used antibiotics of the class are ciprofloxacin, levofloxacin, and moxifloxacin (WHO, 2024). In veterinary medicine, they are used to treat severe bacterial infections in companion and livestock animals that are unresponsive to other antibiotics. Although fluoroquinolones are used in agriculture and livestock, it is

primarily for therapeutic purposes as their use in food producing animals is strictly regulated in many regions to prevent ABR (FDA, 2021).

Table 1.1. Examples of fluoroquinolone antibiotics classified by the progressive development and microbial targets (Pham *et al*., 2019).

Generation	Antibiotic	Microbial target
1	Nalidixic acid	Gram-negative pathogens, except *Pseudomonas* spp.
2a	Enoxacin Norfloxacin Ciprofloxacin	Gram-negative and some Gram-positive pathogens, *Mycoplasma pneumoniae*, and *Chlamydia pneumoniae*
2b	Ofloxacin Levofloxacin Lomefloxacin	Additionally effective against Gram-positive pathogens such as *Staphylococcus aureus*
3	Sparfloxacin Grepafloxacin Clinafloxacin Gatifloxacin	Improved coverage of Gram-positive, and atypical pathogens
4	Moxifloxacin Gemifloxacin Trovafloxacin Garenoxacin	Additional activity against anaerobes such as *Clostridium perfringens*

Mechanisms of fluoroquinolone resistance

Fluoroquinolone resistance, observed in both Gram-negative and Gram-positive bacteria, primarily stems from mechanisms that alter drug accumulation, including changes in cellular permeability and efflux enhancement, along with modifications in intracellular target proteins like DNA gyrase and topoisomerase IV (Webber and Piddock, 2001; Hooper, 2002). Mutations in DNA gyrase, particularly in its GyrA subunit, play a pivotal role in gradually increasing resistance. Additionally, reduced

fluoroquinolone accumulation involves the downregulation of biosynthesis of porin proteins and the activation of efflux pumps. High-level resistance often arises from a combination of chromosomal mutations in genes such as *gyr*A, *par*C, and those associated with efflux pumps and outer membrane permeability (Webber and Piddock, 2001). Plasmid-mediated resistance mechanisms, such as QnrA, QnrB. QnrS, and Aac(6')-Ib-cr, also contribute to resistance. These mechanisms affect the efficacy of commonly used fluoroquinolones differently; for instance, ciprofloxacin and norfloxacin are generally affected, while gatifloxacin and levofloxacin are less or unaffected (Hooper, 2002; Morgan-Linnell *et al*., 2009). Surveillance studies may underestimate these resistance mechanisms, as strains with mutations can still appear phenotypically susceptible to the antibiotic. The widespread use of fluoroquinolones in clinical and agricultural settings exacerbates bacterial resistance, given their intrinsic mutagenic properties (Drlica and Malik, 2003).

1.3.2.3 Aminoglycosides

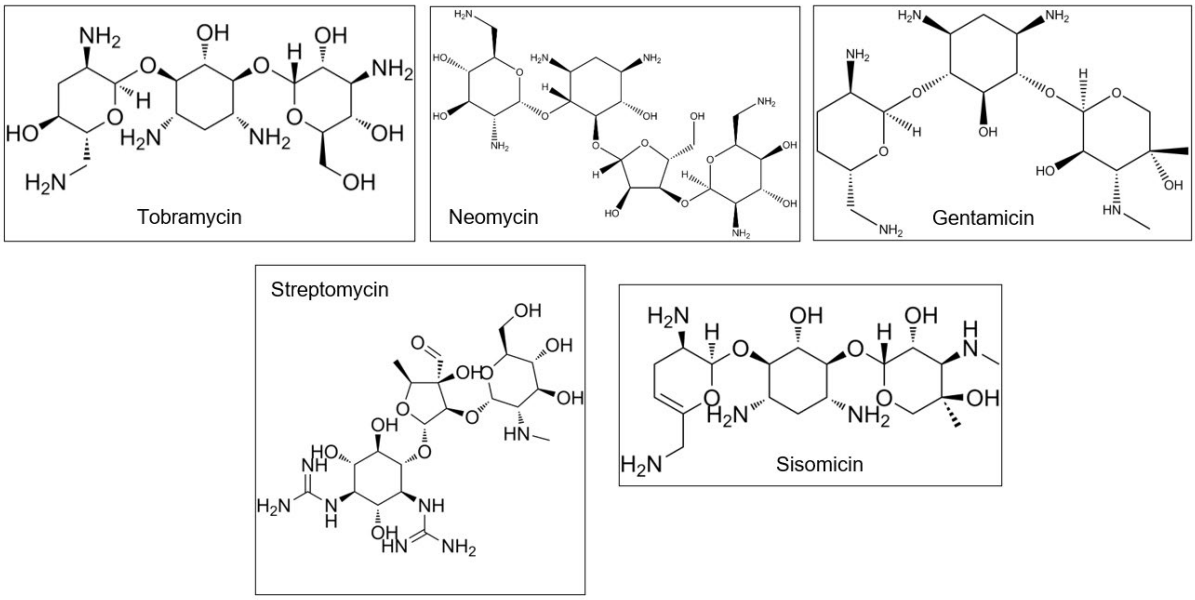

Figure 1.5. Chemical structure of representative aminoglycoside antibiotics.

Isolated in the early 1940s from *Streptomyces* and *Micromonspora*, aminoglycosides, exemplified by the first aminoglycoside antibiotic streptomycin, have a structure featuring an inositol derivative linked to at least one amino sugar with multiple amino and hydroxyl groups (Becker and Cooper, 2013). Operating by disrupting protein

translation these antibiotics interact with the RNA of the 30S subunit of the ribosome. Prominent aminoglycosides include neomycin, gentamicin, tobramycin, and sisomicin, each distinguished by its chemical structure (see Figure 1.5). Second generation aminoglycosides emerged in the 1970s in response to resistance and severe side effects. Despite the diminished use, aminoglycosides are experiencing a resurgence due to increased resistance to other antibiotics, being employed alongside them for severe infections (Becker and Cooper, 2013).

Aminoglycosides are utilized globally for their efficacy against severe infections, particularly those caused by Gram-negative bacteria. In human medicine they are often used in combination with other antibiotics to combat hospital-acquired infections, such as sepsis and pneumonia, especially when other treatments fail due to resistance (Vakulenko and Mobashery, 2003). Gentamicin and tobramycin are commonly used for their broad-spectrum activity and effectiveness against multidrug-resistant (MDR) strains of Gram-negative and Gram-positive pathogens, such as *E. coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* (Shakil *et al*., 2008). In veterinary medicine, gentamicin and neomycin are commonly used to treat respiratory, urinary, and gastrointestinal infections in both companion and livestock animals. However, concerns about ABR have led to stricter regulations and guidelines to control their use (Guardabassi *et al*., 2008).

Mechanisms of aminoglycoside resistance

Aminoglycosides are frequently employed in the treatment of infections caused by both *E. coli* and *Enterococcus* spp., along with infections caused by other Gram-negative and Gram-positive bacterial pathogens, such as *Klebsiella pneumoniae* and MDR *Mycobacterium tuberculosis* (Garneau-Tsodikova and Labby, 2016). Resistance to aminoglycosides can arise from mutations in the ribosome target, modification of the ribosome facilitated by ribosomal methyltransferase enzymes, acquired lipid modifications leading to reduced membrane permeability, or through the action of efflux pumps or aminoglycoside modifying enzymes (Garneau-Tsodikova and Labby, 2016).

The bacterial cell wall as a diffusion barrier and efflux pumps play a pivotal role in aminoglycoside resistance, exerting the impact not only on a wide range of aminoglycosides but also across other classes of antibiotics. In pathogenic bacterial species, the simultaneous presence of multiple resistance mechanisms, frequently harbored on a single plasmid magnifies their combined effects, leading to the emergence of highly resistant phenotypes against aminoglycosides. Recognizing these diverse mechanisms becomes imperative for devising effective strategies to address multifaceted bacterial resistance. Emphasizing the significance of tailored therapeutic approaches and vigilance of surveillance is crucial to curbing the proliferation of aminoglycoside resistance (Garneau-Tsodikova and Labby, 2016).

1.3.2.4 Tetracyclines

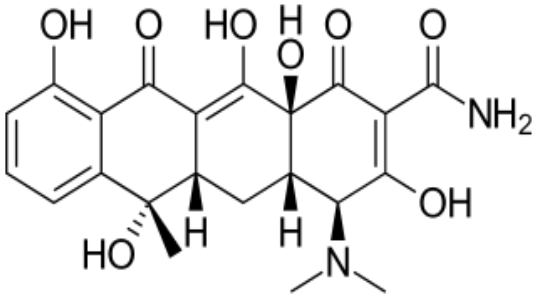

Figure 1.6. Chemical structure of tetracycline

Tetracycline antibiotics exhibit effectiveness against a broad spectrum of Gram-negative and Gram-positive bacteria, with the common structure depicted in Figure 1.6. The initial members of this group, aueromycin and terramycin, were naturally derived from Actinomycetes between the late 1940s and early 1950s. Tetracyclines exert the antibiotic effect primarily by selectively binding to bacterial ribosomes, specifically interacting with the conserved 16S ribosomal RNA (rRNA) target within the 30S ribosomal subunit. This binding disrupts translation by sterically hindering the docking of aminoacyl-transfer RNA (tRNA) during the elongation process (Grossman, 2016).

Their success paved the way for the isolation of more tetracycline antibiotics and the production of semi synthetic derivatives. Despite the proven efficacy, the emergence of bacterial resistance prompted the development of targeted derivatives in the pursuit of enhanced effectiveness, widened spectrum, reduced resistance, improved solubility, and increased bioavailability. A resurgence of interest in tetracyclines in the late 1980s led to the creation of semisynthetic derivatives, such as tigecycline (Grossman, 2016). Tigecycline, introduced into clinical use in 2005 as a last resort antibiotic for multidrug-resistant (MDR) Gram-negative and Gram-positive pathogens, continues to be crucial in treating MDR bacterial infections, with close monitoring of tigecycline resistance underway (WHO, 2024).

Tetracyclines are widely used in human medicine for treating a variety of infections, including respiratory tract infections, skin infections, sexually transmitted infections, and some zoonotic diseases. Doxycycline and minocycline are commonly prescribed due to their broad-spectrum activity and favorable pharmacokinetic properties (Chopra and Roberts, 2001). In veterinary medicine, tetracyclines, such as oxytetracycline and chlortetracycline, are particularly effective against respiratory, urinary, and soft tissue infections in animals (Eliopoulos *et al*., 2003). Additionally, tetracyclines are used to treat and prevent infections in poultry, cattle, swine, and other livestock, such as aquaculture, and sometimes unfortunately used as growth promoters to enhance growth rates and feed efficiency (Landers *et al*., 2012).

Mechanisms of tigecycline resistance

Tigecycline resistance mechanisms encompass mutations in the 16S rRNA and genes encoding ribosomal proteins along with the involvement of ribosomal protection proteins (RPPs). Target based mutations in 16S rRNA are particularly notable in bacteria with low RNA gene copy numbers. These mutations, commonly identified in both primary and secondary tetracycline binding sites, significantly impact the affinity of tetracycline for the 30S ribosomal subunit. Furthermore, mutations in ribosomal proteins S10 (rpsJ) and S3 (rpsC) have been associated with resistance to tetracycline or its derivative, tigecycline, across diverse bacterial species, influencing their interactions with both tetracyclines and 16S rRNA. RPPs disrupt the stacking interaction between tetracycline and 16S rRNA, enabling

translation to persist in the presence of tetracycline. Despite conferring resistance to certain tetracyclines, specific derivatives containing side chains at the C9 position, like tigecycline, can successfully evade the action of RPP's through enhanced interactions and static interference (Grossman, 2016).

Additionally, efflux pumps, such as *tet*A and *tet*B, contribute to resistance by significantly reducing intracellular drug concentrations, limiting its efficacy. Moreover, the presence of the *tet*X gene may result in production of tetracycline-degrading enzymes (e.g., monooxygenases) if the gene is expressed (Cheng *et al*., 2021). The expression of *tet*X leads to enzymatic modification of tigecycline, rendering it ineffective against bacterial targets (Grossman, 2016; Cheng *et al*., 2021).

1.3.2.5 Glycopeptides/Lipopeptides

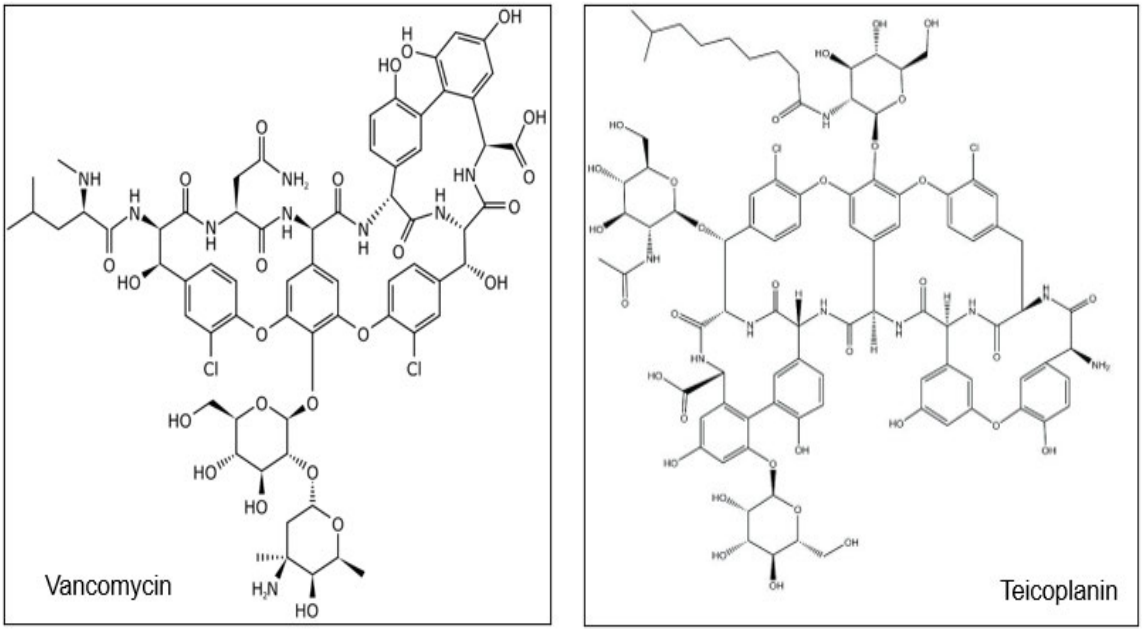

Figure 1.7. Chemical structure of the two glycopeptide antibiotics vancomycin and teicoplanin.

Glycopeptide antibiotics (GPAs) are heptapeptides that are produced nonribosomally, originating from soil-dwelling microorganisms. Characterized by an extensive cross linking of aromatic amino acid residues and adorned with various

functional groups such as sugars, acyl chains, sulfate groups and chlorine atoms (depicted in Figure 1.7), GPAs are last resort antibiotics against Gram-positive pathogens. Their primary mechanism of action involves binding to the d-Ala-d-Ala dipeptide terminus of the lipid II precursor in peptidoglycan. This binding disrupts the transglycosidase and transpeptidase enzymes crucial for bacterial cell wall synthesis, leading to cell lysis (Marschall *et al*., 2019).

Glycopeptide/lipopeptide antibiotics are primarily used to treat severe infections caused by Gram-positive pathogens that are resistant to other antibiotics. Vancomycin is essential for treating methicillin-resistant *Staphylococcus aureus* (MRSA), while teicoplanin is used to treat vancomycin-resistant *Enterococci* (VRE) infections. The use of these antibiotics is often reserved for hospital settings due to their potency and the need to limit resistance (Levine, 2006). As a result, the use of glycopeptide/lipopeptide antibiotics in veterinary medicine and agriculture is highly restricted due to the risk of developing resistance that can impact human health (Aarestrup, 2005; Guardabassi *et al*., 2008).

Mechanisms of glycopeptide/lipopeptide resistance

Gram-negative bacteria inherently resist glycopeptides/lipopeptides due to their outer membrane preventing drug permeation and target binding. In Gram-positive bacteria, particularly *Enterococcus faecalis*, the emergence of resistance to vancomycin highlighted the issue of glycopeptide antibiotic (GPA) resistance (Zeng *et al*., 2016). This concern escalated when rapid resistance developed in *Staphylococcus aureus* through horizontal gene transfer from resistant enterococci, leading to the global challenge of vancomycin-resistant *Staphylococcus aureus* (VRSA) (Marschall *et al*., 2019).

Vancomycin resistance primarily stems from the production of lower affinity binding precursors where the carboxyl-terminal d-alanine residue is substituted with either d-lactate or d-serine, resulting in diminished binding affinity. Various molecular resistance elements contribute to altering the structures of peptidoglycan precursors. For instance, Van-A type resistance involves a transposon, which encodes for the

modification of the carboxyl terminus of the peptidoglycan precursor, imparting high level resistance (Zeng *et al*., 2016).

Van-B type resistance modifies the synthesis of the depsipeptide d-Ala-d-Lac, while Van-D type resistance leads to the production of peptidoglycan precursors ending in d-Ala-d-Lac. Importantly, Van-D type resistance is conferred by the bacterial chromosome, making it non-transferable through horizontal gene transfer (Zeng *et al*., 2016).

Van-C type resistance results in the production of peptidoglycan precursors terminating in d-Ala-d-Ser. In contrast Van-E type resistance which is inducibly expressed in intrinsically resistant *E. faecalis*, shares organizational similarities with Van-C type resistance (Zeng *et al*., 2016).

Van-G type resistance involves genes from various van operons and produces d-Ala-d-Ser, conferring intermediate-level resistance. Understanding these distinct resistance mechanisms is crucial for addressing the challenges posed by GPAs and combating the emergence of antibiotic resistant bacterial strains (Zeng *et al*., 2016).

1.3.2.6 Oxazolidinones

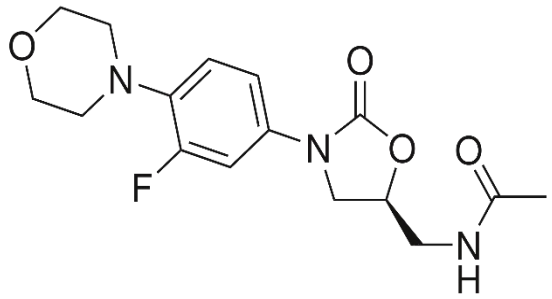

Figure 1.8. Chemical structure of the oxazolidinone linezolid.

The development of oxazolidinones, initiated in 1978, led to the synthesis of potent antibacterial compounds, including linezolid and eperezolid. The mode of action of oxazolidinones involves binding to the 50S ribosomal subunit, specifically to the P

site, with no affinity to the 30S subunit. They inhibit formation of the initiation complex, composed of the 30S subunit, fMet-tRNA, mRNA, guanosine triphosphate (GTP), and initiation factors 1-3, by binding to the 50S subunit. Additionally, oxazolidinones hinder the translocation of the peptide chain from the A site to the P site during the formation of the peptide bond (Bozdogan *et al*., 2004).

Despite the initial toxicity issues, linezolid (see Figure 1.8) emerged as effective against Gram-positive bacteria, including methicillin-resistant and susceptible *S. aureus*, coagulase-negative staphylococci, enterococci, including vancomycin-resistant *Enterococcus* spp., and *Streptococcus pneumoniae*. Linezolid's activity is not affected by methicillin resistance, and it remains effective against vancomycin-intermediate and vancomycin-resistant strains. Moreover, it demonstrates efficacy against Gram-positive anaerobic bacteria (Bozdogan *et al*., 2004).

Linezolid is used for treating nosocomial pneumonia, complicated skin and soft tissue infections and other infections caused by resistant Gram-positive pathogens (Hashemian *et al*., 2018). Due to its efficacy and oral bioavailability, linezolid is a valuable option for outpatient treatment as well (Livermore, 2003). The use of oxazolidinones in veterinary medicine and agriculture is limited due to its critical importance in treating MDR infections. There are stringent regulations and guidelines in place to prevent its use in animals to avoid the development of resistance that could impact human health (Guardabassi *et al*., 2008).

Mechanisms of oxazolidinone resistance

Gram negative bacteria exhibit intrinsic resistance to linezolid due to their efflux pump activity, preventing effective drug accumulation. On the other hand, Gram-positive bacteria, like *Enterococcus* spp., primarily facing linezolid resistance due to clinical overuse, employ various mechanisms. Mutations in the 23S ribosomal RNA gene, particularly at the 2576 position, and other point mutations, hinder binding to the antibiotic's action site, fostering bacterial resistance. Studies indicate a positive correlation between linezolid use duration and an increased number of mutations, signifying gradual resistance development. Additionally, mutations in ribosomal genes contribute to altered spatial structures, reinforcing resistance (Liu *et al*., 2020).

The *cfr* gene, initially associated with chloramphenicol resistance, emerged as another linezolid resistance mechanism. The protein expressed functions as a methyltransferase, it methylates the 23S rRNA gene, impacting the linezolid binding site. The *cfr* gene can horizontally spread through plasmids, leading to outbreaks of linezolid-resistant bacteria (Liu *et al*., 2020).

1.4 Antibiotic Resistance Screening

Bacterial resistance can be assessed through phenotypic methods, providing insights into the test organism's sensitivity to an antibiotic. Alternatively, genotypic methods can be used to identify specific genes encoding proteins that are responsible for the test organism's resistance to the antibiotic.

1.4.1 Phenotypic Detection

1.4.1.1 Disk diffusion

The disk diffusion method, established by Bauer and Kirby in 1956 and considered the gold standard for bacterial susceptibility testing to antibiotics, is a widely used and cost-effective phenotypic characterization assay. In this method, the test organism is inoculated onto Mueller-Hinton Agar, and commercially available antibiotic disks are applied at equal distances. Following overnight incubation, a visibly clear zone of inhibition around the antibiotic disk indicates growth inhibition. The diameter of the zone is measured and compared to the established break point values, allowing determination of susceptibility, intermediate status, or resistance (Reller *et al*., 2009; EUCAST, 2024). While this method is rapid and accurate it requires strict quality control and uniformity throughout the procedure (Skov *et al*., 2015). Despite its drawbacks such as semi automation and limitations with certain bacteria, recent technological advances, including instruments like Accuzone and Sirscan, contribute to result reliability by reducing variability (Khan *et al*., 2019).

1.4.1.2 Agar well diffusion

Agar well diffusion, also known as the well diffusion method, is a widely utilized technique for assessing the antimicrobial activity of various compounds. This method involves creating wells in an agar plate inoculated with a microbial strain and introducing the antimicrobial agent into these wells. The agent diffuses into the agar, inhibiting microbial growth and forming clear zones of inhibition around the wells following suitable incubation, which indicate the antimicrobial potency of the tested agent (Balouiri *et al*., 2016). This method is commonly used for screening plant extracts, chemicals, and other substances of antimicrobial properties, and for determining bacterial susceptibility to antibiotics in clinical settings. While it is simple, cost effective, and provides direct visual results, it has limitations such as dependence on the diffusion properties of antimicrobial agent in the agar and providing only a semi-quantitative assessment of antimicrobial activity (Valgas, 2007; Balouiri *et al*., 2016).

1.4.1.3 MALDI-TOF MS

Introduced in 2000, MALDI-TOF MS (Matrix-Associated Laser Desorption Ionization-Time of Flight Mass Spectrometry) stands out as a sensitive bacterial identification method, notable for its high sensitivity and accuracy in clinical applications. Its efficacy extends to discriminating strains such as methicillin-resistant or -sensitive *Staphylococcus aureus*, showcasing subtle differences in protein profiles among isogenic *Staphylococcus aureus* strains. MALDI-TOF MS proves effective in analyzing multiple targets, demonstrating efficiency in detecting vancomycin resistant *Enterococci* and various antibiotic resistant *Pseudomonas* species strains (Khan *et al*., 2019). The recently developed MBT-ASTRA (MALDI Biotyper Antibiotic Susceptibility Test Rapid Assay) offers a more straightforward and cost-effective application of MALDI-TOF MS for both antibiotic susceptibility testing and minimum inhibitory concentration (MIC) determination, as well as detection of hydrolysis of lactams. However widespread implementation is hindered by the instrument's expensive nature and maintenance requirements (Khan *et al*., 2019).

1.4.1.4 Agar and broth dilution

Dilution methods, tracing back to the 1870s, have been pivotal in microbiology, with notable contributions from pioneers like Pasteur, Lister, Kock, and Ehrlich. The two main types are broth dilution and agar dilution, employing broth or agar as media (Khan *et al*., 2019). In broth dilution, consecutive two-fold dilutions of antibiotics are made in microcentrifuge tubes, followed by incubation and turbidity examination for bacterial growth to determine breakpoints. In agar dilution, antibiotics are diluted into the agar medium, and bacterial cells are applied to the surface for growth assessment. Serial dilution, introduced in the early 20th century, saw advancements by various scientists, including Alexander Fleming's work in 1929. The standardized broth macro dilution method for Minimum Inhibitory Concentration (MIC) and antibiotic susceptibility testing was established in 1942.

In recent decades, microdilution – a miniaturized version using 96-well microtiter plates – has become widely adopted for its efficiency in handling multiple samples and its suitability for fastidious bacteria. However, both macro and micro dilution methods have certain limitations, such as requiring large volumes of reagents, significant experimental space, potential for false positives, and challenges in maintaining optimal testing conditions (Khan *et al*., 2019).

Complementing these traditional methods is the E-test method, a gradient diffusion technique that provides a quantitative MIC value. The E-test uses a plastic strip impregnated with a pre-defined antibiotic concentration gradient, placed on an agar plate inoculated with the test organism. The MIC is determined where bacterial growth inhibition intersects the strip. This method is particularly useful in clinical settings as it combines the simplicity of disk diffusion with the quantitative precision of dilution methods, offering a practical alternative for laboratories needing accurate MIC determinations (Glupczynski *et al*., 2002).

Despite certain drawbacks, including potential for cross-contamination and the need for careful experimental design, dilution methods—along with the E-test—remain integral to microbiological practice, providing essential insights into bacterial susceptibility and guiding effective clinical treatment (Khan *et al*., 2019).

1.4.1.5 Semi-automated methods

Semi-automated systems such as MicroScan, Vitek, and Phoenix have transformed antibiotic susceptibility testing (AST) by combining the precision of traditional phenotypic methods with automation, significantly enhancing laboratory efficiency. MicroScan employs broth microdilution technology to test multiple antibiotics simultaneously, providing detailed MIC values crucial for informed treatment decisions. Vitek and Phoenix utilize card-based and microtiter formats, respectively, to rapidly identify bacterial species and determine antibiotic susceptibility (Evangelista and Karlowsky, 2016). Both systems generate results in a matter of hours, making them particularly valuable in clinical settings where timely treatment is essential.

Despite their advantages, these systems come with drawbacks, including high costs associated with purchase and maintenance, which may limit accessibility for smaller laboratories or those in resource-limited settings. Additionally, while they provide quick results, confirmation through traditional methods may still be necessary for complex organisms. The interpretation of results can also be constrained by the available testing panels, which may not cover all relevant antibiotics or resistance mechanisms (Evangelista and Karlowsky, 2016). Overall, while semi-automated methods enhance the efficiency and accuracy of AST, careful consideration of their limitations is essential for effective implementation.

1.4.1.6 β-Lactamase detection

Once phenotypic resistance to any β-lactam antibiotic is detected, β-lactamase activity can be confirmed phenotypically for quick detection in laboratories. Most convenient methods involve chromogenic cephalosporins, which are hydrolysed by β-lactamase. This type of assay is more specific and reliable compared to testing based on the reduction of iodine or pH changes (Livermore and Brown, 2001). The nitrocefin assay makes use of nitrocefin, a chromogenic cephalosporin that changes from yellow to red when hydrolysed (see Figure 1.9). The nitrocefin solution is prepared by dissolving powdered nitrocefin in dimethylsulphoxide, then diluted with phosphate buffer to obtain a 0.5 mM solution. The test isolates are grown on Nutrient

Agar overnight, colonies are then suspended in 20 µL phosphate buffer, with 20 µL of the nitrocefin solution added to it. A positive test is produced within minutes, no longer than an hour, indicating β-lactamase activity (Livermore and Brown, 2001).

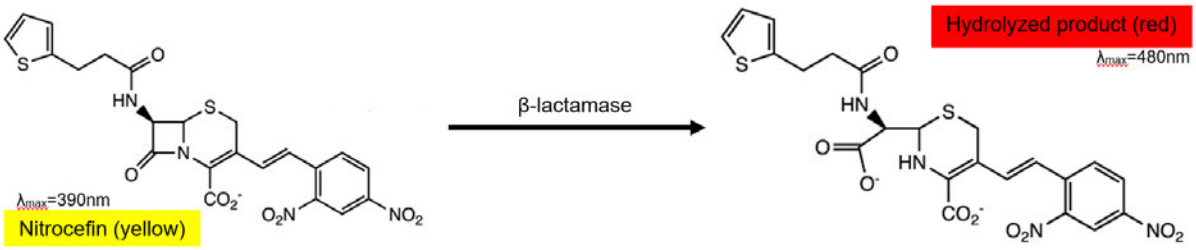

Figure 1.9. Hydrolysis of nitrocefin by β-lactamase

Phenotypic detection methods for β-lactamase resistance, including disk diffusion, dilution, and automatic detection, have been successfully used to evaluate the resistance of β-lactamase-producing bacteria to β-lactam antibiotics in clinical trials based on susceptibility testing outcomes. Despite their utility, these methods come with limitations, including prolonged detection times, complexity, labour-intensive processes, high costs, potential for false positives and comparatively lower specificity and sensitivity. Moreover, these tests may not encompass other mechanisms contributing to β-lactam resistance (Zhuang *et al*., 2023).

Hydrolysis detection is based on the ability of β-lactamase to hydrolyze the lactam ring in antibiotics, disrupting the structure, generating hydrolyzed products that no longer inhibit transpeptidase. Detection methods focus on identifying β-lactamase by recognizing antibiotic substrates or their hydrolyzed forms (Zhuang *et al*., 2023). The acid diametric approach involves detecting penicilloic acid's acidity resulting from penicillin hydrolysis. Methods include paper acid tests, pH meter techniques, biochemical tests, and biosensors. The ideometric method relies on the competitive binding of penicilloic acid to iodine starch, leading to observable color changes. Spectrophotometric methods utilize UV-Vis spectrophotometry to monitor absorbance changes at specific wavelengths due to antibiotic hydrolysis. Mass-spectrometry-based methods including MALDI-TOF MS, provide accurate and rapid detection of β-lactamase activity. Lateral flow immunoassays use monoclonal antibodies to detect

β-lactamase by binding to hydrolyzed products, with various commercial kits available. Fluorescent probes, both intensity variable and ratio metric, offer qualitative and quantitative detection based on fluorescence changes upon β-lactamase activity. Each method has specific advantages and considerations, such as sensitivity specificity and applicability in different settings, providing a diverse toolkit for basal activities detection (Zhuang *et al*., 2023).

1.4.2 Genotypic Detection

Molecular techniques, such as PCR, DNA microarrays, and loop mediated isothermal amplification (LAMP), and whole genome sequencing (WGS), can be used to rapidly detect antibiotic resistance genes (Khan *et al*., 2019). PCR, a swift molecular tool, involves cycles of denaturation, primer annealing, and DNA elongation, with subsequent confirmation through various methods (Fluit *et al*., 2001). LAMP, operating isothermally, amplifies the gene of interest at a constant temperature. DNA microarrays and chips employ cDNA probes for susceptibility screening, demonstrating success in pinpointing resistance in *Mycobacterium tuberculosis*. WGS, a more comprehensive approach, generates sequences representing the entire genome of a microorganism, providing detailed insights into the presence of known resistance genes and potential novel resistance mechanisms. WGS offers high-resolution data that can reveal the complete resistome of a pathogen, facilitating a deeper understanding of ABR patterns and transmission dynamics (Didelot *et al.*, 2012). The WGS method has been recommended by major global organizations such as the European Food Safety Authority and US Centre for Disease Control and Prevention.

Despite their speed, sensitivity, and specificity, non-WGS genotypic methods have challenges, including the need for specific assays, limited detection of relevant resistance genes, reduced sensitivity in latent infections or low-bacterial load samples, undefined genetic profiles for resistance in all bacteria, potential false positives, the requirement for expensive equipment and reagents with specific

maintenance conditions, and the dependency on skilled personnel (Khan *et al*., 2019).

1.5 Outlook

The global concerns regarding antibiotic resistance (ABR) had escalated significantly and garnered recognition as a major issue by prominent organizations, including the World Health Organization (WHO). Previously, research concentrated on ABR in clinical setting and food animals, now there is a growing interest in environmental studies, given the holistic One Health Approach. Despite this, exploration of wildlife and their role in ABR, particularly birds had been limited in the South African context.

Earlier investigations had substantiated that birds could harbor ABR bacteria and associated genes; however, studies in South Africa are limited. This study was undertaken to address this gap. The selection of ducks was strategic, targeting a bird population within food animal production and providing insights into the presence of ABR bacteria. Despite the surge in popularity and consumption of duck meat, studies on ABR bacteria in ducks are still scarce. While previous studies have explored poultry, the increasing consumer preference for duck meat makes it an important focus for ABR research. Furthermore, two distinct wild bird groups, namely the sacred ibis and various raptors, were incorporated into the study. These birds categorized by unique habitats and feeding preferences, served as subjects, offering new insights into the prevalence and transmission of ABR in South African wildlife.

By choosing *E. coli* and *Enterococcus* species, frequently utilized as hygiene indicators in surveillance studies, the objective of this study was to employ these bacteria as Gram-negative and Gram-positive representatives. The study aimed to enhance the current understanding of ABR by investigating ABR in selected bird populations, including free-range ducks, wild sacred ibis and raptors. Additionally, the study intended to verify the burden of *E. coli* and *Enterococcus* spp. in bird faecal matter and the presence of ABR strains.

1.6 Aims and Objectives

The main aim of this research was to assess commercial ducks, and wild sacred ibis and raptors, as potential vectors of antibiotic resistant *Escherichia coli* and *Enterococcus* spp.

The objectives of this study were to:

1. Enumerate and isolate *Escherichia coli* and *Enterococcus* spp. from commercial ducks, and wild sacred ibis and raptors feces.
2. Determine antibiotic susceptibility profiles of all isolates.
3. Detect and confirm β-lactamase activity.

CHAPTER 2 : MATERIALS AND METHODS

2.1 Sample Collection

In this observational study, fresh faecal samples from commercial ducks (Merriducks Farm, Howick, KwaZulu-Natal), wild sacred ibis (Darvill Wastewater Treatment Plant, Pietermaritzburg, KwaZulu-Natal), and raptors (African Birds of Prey Sanctuary, Pietermaritzburg, KwaZulu-Natal) were collected in sterile beakers. All three samples consisted of pooled faecal matter obtained from each bird group (i.e., commercial ducks, wild sacred ibis, and raptors). Additionally, as environmental controls, soil that was clearly free of faecal matter was sampled from each location within a one-meter radius of the corresponding sample site. All samples were collected (once each) between March – November 2019 (commercial ducks sampled in March; wild sacred ibis sampled in June; and wild raptors sampled in November), and after collection, sealed, placed on ice, and transported immediately to the laboratory.

All three pooled bird faecal samples and all three soil samples were initially decimally diluted by adding 10g of sample to 90mL sterile saline solution (0.85% NaCl) containing 0.1% (w/v) peptone. Thereafter, further decimal dilutions were prepared to achieve a dilution series from 10^{-2} to 10^{-10}. This was conducted for all of the three bird faecal and soil samples. All samples were transported directly to the laboratory on the same day and analysis of all samples commenced within two hours of collection.

2.2 *Escherichia coli*

2.2.1 Enumeration and Isolation

The enumeration of total coliforms, faecal coliforms, and *E. coli* was conducted once for all faecal and soil samples in accordance with the Most Probable Number (MPN) guideline method MFHPB – 19 (Health Canada, 2002). Briefly, this involved an initial quintuple inoculation of Lauryl Sulfate Tryptose (LST) broth (incubated for 24hr to

48hr at 35°C) using the prepared 10^{-1} to 10^{-10} dilution series. All gas-positive tubes were then used to inoculate Brilliant Green Lactose 2% Bile (BGLB) broth (incubated for 24hr to 48hr at 35°C), followed by *Escherichia coli* (EC) broth (incubated for 24hr to 48hr at 45°C). Generally, at each step, tubes not showing growth after 24hr were incubated for an additional 24hr to confirm result. Based on gas-positive tubes, MPN values were established in accordance with De Man (1983) and reported as log MPN/g of sample.

The last step of the MPN procedure consisted of using EC broth (incubated for 24hr to 48hr at 45°C) for the detection and enumeration of *E. coli*. Growth in EC broth indicated the presence of *E. coli*. Positive tubes were used to inoculate Eosin Methylene Blue (EMB) agar (Merck) and incubated for 24hr at 35°C. Single distinct, dark centred colonies with a green metallic sheen, indicative of *E. coli*, were selected and purified using Nutrient agar (NA) (Merck) (incubated for 24hr at 35°C). A total of thirty presumptive *E. coli* isolates from faecal matter of each bird were randomly selected. From each presumptive *E. coli* isolate culture obtained, a single distinct colony was used to inoculate 20mL Nutrient broth (Merck) and incubated for 24hr at 35°C at 120 rpm. Glycerol stock cultures (20% glycerol) were prepared for each isolate and stored at -80°C for further analysis.

2.2.2 Phenotypic Confirmation

Phenotypic confirmation of all presumptive *E. coli* isolates (30 isolates per bird, a total of 90 isolates) was conducted using the biochemical GIMViC testing as per MFHPB – 19 procedure (Health Canada, 2002). The following amendments were made:

a) Tryptone water (Oxoid) was used instead of tryptophane broth for the indole test, and
b) MRVP (Methyl Red Voges-Proskauer) broth (Oxoid) was used instead of the buffered glucose broth for the methyl red and Voges-Proskauer tests.

Salmonella Typhimurium ATCC 14028 and *Staphylococcus aureus* ATCC 6538 were used as negative controls, and *E. coli* ATCC 25922 was used as the positive control.

2.2.3 Genotypic Confirmation

Genotypic confirmation of all 30 presumptive *E. coli* isolates from faecal matter of each bird (a total of 90 isolates), was conducted using PCR amplification of the *gad*A gene (McDaniels *et al*., 1996).

2.2.3.1 DNA extraction

Isolates were grown in 20mL Nutrient broth (Merck) overnight in the dark at 35°C and 120 rpm. Following incubation, 1mL of culture was transferred to sterile Eppendorf tubes and centrifuged for 6 minutes at 13 000 x *g*. After discarding the supernatant, 100µL of sterile water was added to re-suspend the pellet. DNA was extracted using the freeze-thaw method whereby cell suspensions were subjected to five cycles of heating at 95°C for 5 minutes in a heating block, immediately followed by submerging suspensions in liquid nitrogen for 3 minutes. Suspensions were subsequently centrifuged for 6 minutes at 13 000 x *g.* The supernatant was directly used for Polymerase Chain Reaction (PCR) amplification.

2.2.3.2 PCR amplification

Amplification reactions were performed in a final volume of 25µl consisting of 1.2µl supernatant (template DNA), 12.5µl One*Taq*® 2X Master Mix (New England BioLabs), 10.3µl nuclease free water (Promega) and 0.5µl of each of the following *E. coli* specific primers (Inqaba) which target the *gad*A gene, with an expected amplicon size of 680 bp (Lee *et al.,* 2006):

Forward primer: 5'- ACCTGCGTTGCGTAAATA -3'
Reverse primer: 5'- GGGCGGGAGAAGTTGATG -3'

The PCR reaction was conducted in a Labnet MultiGene II thermal cycler with the following parameters: initial denaturation for 2min at 94°C; 30 cycles of denaturation for 30sec at 94°C, annealing for 30sec at 55°C, and extension for 1min at 72°C; final extension for 7min at 72°C; and holding temperature at 4°C until further analysis. The PCR amplification products were visualized using gel electrophoresis (1.5% agarose gel, 1×TBE buffer), and ethidium bromide staining. Sterile water and *Salmonella* Typhimurium ATCC 14028 were used as negative controls and *E. coli* ATCC 25922

was used as a positive control. Gbox Chemi XRQ (Syngene) and GeneSnap™ software was used for documentation.

2.3 *Enterococcus* Species

2.3.1 Enumeration and Isolation

The detection, phenotypic confirmation, and quantification of *enterococci* from all three bird and three soil samples were performed using a modified version of the ISO 7899-2 method (2000), involving spread plating of the diluted faecal sample from each bird instead of membrane filtration. The results were expressed as colony forming units per gram of faecal matter (CFU/g) using duplicate plates. From each decimal dilution (10^{-1} to 10^{-10}), 0.1mL was spread plated onto Slanetz and Bartley (Merck) agar plates and incubated for 44 hours at 35°C. Suspicious colonies that appeared pink/red/maroon were counted and transferred (using the colony pick-off method) to pre-heated Bile Esculin Azide (BEA) (Merck) agar plates for confirmation. After incubation (2 hours at 44°C) thirty single colonies with a dark brown to black surrounding were randomly selected and streaked onto individual Brain Heart Infusion (BHI) (Merck) agar plates for each bird faecal sample (thirty per bird; a total of 90 presumptive *Enterococcus* spp. isolates) and incubated for 24 hours at 35°C (Facklam and Collins, 1989). A single distinct colony of *Enterococcus* spp. from each plate was used to inoculate 20mL BHI broth (Merck) and incubated for 24 hours at 35°C at 120 rpm. Glycerol stock cultures (20% glycerol) were prepared for each isolate and stored at -80°C for further analysis.

2.3.2 Genotypic Confirmation

The genotypic confirmation of all 30 presumptive *Enterococcus* spp. isolates from each bird (a total of 90 isolates) was conducted using a multiplex PCR amplification for genus confirmation (expected amplicon size of 733 bp) (Deasy *et al.*, 2000), and

species-specific identification of *E. faecalis* (expected amplicon size of 360 bp) and *E. durans* (295 bp) (Jackson *et al*., 2004).

2.3.2.1 PCR sample preparation

Isolates were grown on BHI agar (Merck) overnight at 37°C. For PCR preparation, the colony pick-off method was used, whereby one single colony of each isolate was suspended in 100µL sterile water, thoroughly vortexed and directly used for PCR amplification.

2.3.2.2 PCR amplification

The use of the multiplex PCR method allowed for the confirmation of isolates at genus and species level as described by Jackson *et al.* (2004). The PCR was conducted in a final volume of 25µl consisting of 2.5µl whole cell template, 12.5µl One*Taq*® 2X Master Mix (New England BioLabs), 1µl nuclease free water (Promega), 1.5µl of each of the following *Enterococcus* genus specific primers (i.e., E_1 and E_2) (Deasy *et al.*, 2000):

Forward primer (E_1): 5'- TCAACCGGGGAGGGT -3'
Reverse primer (E_2): 5'- ATTACTAGCGATTCCGG -3'

and 1.5 µL of each of the *Enterococcus* species specific primers for *E. faecalis* (i.e., F_1 and F_2) and *E. durans* (i.e., D_1 and D_2) (Jackson *et al.*, 2004):

Forward primer (F_1): 5'- ACTTATGTGACTAACTTAACC -3'
Reverse primer (F_2): 5'- TAATGGTGAATCTTGGTTTGG -3'
Forward primer (D_1): 5'- CCTACTGATATTAAGACAGCG -3'
Reverse primer (D_2): 5'- TAATCCTAAGATAGGTGTTTG -3'

The PCR was performed in a Labnet MultiGene II thermal cycler with the following parameters: initial denaturation for 4min at 95°C; 35 cycles of denaturation for 30sec at 95°C, annealing for 1min at 52°C, and extension for 1min at 72°C; final extension for 7min at 72°C; and holding temperature at 4°C until further analysis. The PCR amplification products were visualized using gel electrophoresis (1.5% agarose gel, 1×TBE buffer) and ethidium bromide staining. Sterile water and *Staphylococcus*

aureus ATCC 6538 were used as negative controls and *Enterococcus faecalis* ATCC 29212 and *Enterococcus durans* ATCC 6056 were used as positive controls. Gbox Chemi XRQ (Syngene) and GeneSnap™ software was used for documentation.

2.4 Antibiotic Susceptibility Testing

The European Committee on Antimicrobial Susceptibility Testing (EUCAST) disk diffusion method (version 7.0) (EUCAST, 2021) was employed to determine the antibiotic resistance profiles of 90 confirmed isolates of *E. coli* and 90 confirmed isolates of *Enterococcus* spp., with 30 isolates each from commercial ducks, wild sacred ibis and raptors faecal samples. The antibiotics used were supplied as ready-to-use discs of 6 mm diameter (Oxoid).

For the screening of *E. coli* isolates, the following twelve antibiotics representing clinically relevant classes as suggested by EUCAST (2021) were tested: ampicillin (AMP – 10µg), amoxicillin-clavulanic acid (AMC – 20µg-10µg), cefotaxime (CTX – 5µg), ceftazidime (CAZ – 10µg), ertapenem (ETP – 10µg), meropenem (MEM – 10µg), aztreonam (AZT – 30µg), ciprofloxacin (CIP – 5µg), norfloxacin (NOR – 10µg), gentamicin (CN – 10µg), tobramycin (TOB – 10µg) and tigecycline (TGC – 15µg).

For the screening of *Enterococcus* spp. isolates, the following eight antibiotics as suggested by EUCAST (2021) were tested: ampicillin (AMP – 2µg), imipenem (IPM – 10µg), norfloxacin (NOR – 10µg), gentamicin (CN – 30µg), teicoplanin (TEC – 30µg), vancomycin (VAN – 5µg), tigecycline (TGC – 15µg), and linezolid (LZD – 10µg).

According to EUCAST (2021), the disk diffusion method requires an inoculation suspension of approximately 2 $\times 10^8$ cells/mL, which is equivalent to the McFarland standard of 0.5. In order to obtain this cell concentration, isolates were grown in 20mL nutrient broth (Merck) at 35°C at 120rpm overnight until a concentration of approximately 1 – 2 $\times 10^8$ cells/mL was obtained. Subsequently, 1mL of the culture was transferred to sterile Eppendorf tubes, samples were centrifuged for 8min at 10 000 × *g*. The supernatant was removed, and the pellet was re-suspended in 1mL of sterile saline solution (0.85% NaCl). Samples were washed again using the same

procedure. The cell suspensions were adjusted to an optical density at 600nm (OD_{600}) of approximately 0.1, which equates to a McFarland standard of 0.5. Thereafter, 100µL of the suspension was spread plated on Mueller-Hinton (MH) agar (Oxoid) using a sterile Drigalsky spatula. Four different antibiotic discs (6mm) were placed onto the inoculated agar surface (90mm plates, 25mL MH agar) at equal distances using sterile tweezers. All assays were performed in duplicate; plates were inverted and incubated in stacks of 4 for 20hr at 35°C (EUCAST, 2021). Following incubation, inhibition zones were measured to the nearest mm using a digital Vernier caliper (Marshall Tools). The average zone diameter of two independent experiments was used to determine resistance profiles using the EUCAST antibiotic breakpoints v14.0 (EUCAST, 2024). Isolates that are found to be resistant to three or more classes of antibiotics are classified as multidrug-resistant (MDR) (EUCAST, 2024). As prescribed by EUCAST (2021), *E. coli* ATCC 25922 and *Enterococcus faecalis* ATCC 29212 were used as quality controls for method verification.

2.5 Detection of β-Lactamase Activity

2.5.1 Nitrocefin Spot Test

All confirmed *E. coli* and *Enterococcus* spp. isolates that were resistant to at least one of the β-lactam antibiotics tested were additionally analyzed for their ability to hydrolyze nitrocefin, a chromogenic cephalosporin (Livermore and Brown, 2001). A single colony from an overnight culture (Nutrient agar, 37°C) of each isolate was transferred to individual wells of a microtiter plate that contained 20µL of a ready to use nitrocefin solution (0.5mg/mL) (Oxoid). The microtiter plate was then incubated in the dark at 30°C for 30min. A color change from yellow to red indicated a positive result (hydrolysis of nitrocefin); yellow to orange indicated partial hydrolysis; and a lack of color change indicated a negative result (no hydrolysis of nitrocefin). *E. coli* ATCC 25922 and *Salmonella* Typhimurium ATCC 14028 were used as negative controls.

2.5.2 β-Lactamase Enzyme Detection

To confirm β-lactamase activity detected with the nitrocefin spot test, UV-Vis spectrophotometry was employed (Livermore and Brown, 2001). One representative *E. coli* isolate from sacred ibis with a positive spot test and one from raptors with a negative spot test but resistance to at least one β-lactam antibiotic were selected. Isolates were grown overnight on Nutrient agar (Merk), and a single pure colony was transferred to 1mL of sodium phosphate buffer (0.05M, pH 7). Cells were harvested by centrifugation for 8min at 10 000 × *g*, washed once more with the same buffer, and centrifuged again. The supernatant was discarded, and the cells were resuspended in 900µL of sodium phosphate buffer (0.05M, pH 7) to achieve an OD_{600} of 0.1, equivalent to a cell concentration of approximately 2 $\times 10^8$ cells/mL, to which 50µL nitrocefin stock solution (0.5mg/mL) was added and incubated in the dark for 30min at 30°C. Additionally, 900µL of sodium phosphate buffer (0.05M, pH 7) with 50µL nitrocefin (0.5mg/mL) was kept on ice in the dark for 30min to serve as a control. After completion of the incubation period, the absorbance spectra for all samples were determined.

2.6 Statistical Analysis

The p-value used for evaluating the statistical significance of the obtained data was determined using the Pearson's Chi-squared (X^2) Test on Microsoft Excel (Version 2304).

CHAPTER 3 : RESULTS

3.1 Isolation and Quantification of *E. coli* and *Enterococcus* spp.

3.1.1 Quantification

Table 3.1 summarizes the results of microbial quantification in faecal samples collected from commercial free-range ducks, wild sacred ibis, and raptors, alongside corresponding control soil samples. The microbial load is expressed in logarithmic units per gram of sample (log MPN/g for total and faecal coliforms, log MPN/g for *E. coli*, and log CFU/g for *Enterococcus* species). Notably, among the avian species, raptors faecal samples exhibited the highest bacterial burden, with 8.45 log MPN/g for total coliforms, 8.11 log MPN/g for faecal coliforms, and 7.86 log MPN/g for *E. coli*.

Wild sacred ibis and commercial duck faecal samples always exhibited a slightly lower bacterial burden than raptor faecal samples and the detected values were within the same range. The established total coliforms counts were 7.23 and 7.54 log MPN/g for sacred ibis and ducks' faecal samples respectively, while values for faecal coliforms ranged from 7.15 (sacred ibis) to 6.73 (duck) log MPN/g. As expected, values for *E. coli* were lower than total and faecal coliform counts. The calculated log MPN/g value for *E. coli* was slightly lower in duck faecal samples (6.73 log MPN/g) compared to 6.90 log MPN/g from sacred ibis faecal samples.

The number of *Enterococcus* spp. was generally lower than the values calculated for *E. coli* (about 2 log), but followed a similar trend, with raptor faecal samples having the highest viable enterococci counts at 5.30 log CFU/g. The number of *Enterococcus* spp. from sacred ibis' faecal samples was 4.80 log CFU/g and duck faecal samples showed the lowest value with 4.05 log CFU/g.

All control soil samples were below the limit of detection (LOD) for the quantification of *E. coli* and *Enterococcus* spp.. However, total and faecal coliforms were detected in lower numbers, ranging from 1.89 – 2.36 log MPN/g for all three sampling sites.

Table 3.1. Quantification of total coliforms, faecal coliforms, *E. coli* and *Enterococcus* spp. from pooled faecal samples collected from commercial ducks, wild sacred ibis, raptors, and from corresponding environmental controls (soil).

	Commercial Ducks		**Wild Sacred ibis**		**Wild Raptors**	
	Faecal Sample	**Control (Soil)**	**Faecal Sample**	**Control (Soil)**	**Faecal Sample**	**Control (Soil)**
Total coliforms (log MPN/g)	7.54	2.03	7.23	2.36	8.45	2.29
Faecal coliforms (log MPN/g)	6.73	1.89	7.15	2.16	8.11	2.13
E. coli (log MPN/g)	6.73	<LOD*	6.90	<LOD*	7.86	<LOD*
Enterococcus spp. (log CFU/g)	4.05	<LOD*	4.80	<LOD*	5.30	<LOD*

*LOD = Limit of detection (i.e., 1.8 MPN/g for *E. coli* and 100 CFU/g for *Enterococcus* spp.)

3.1.2 Phenotypic and Genotypic Confirmation of Isolates

3.1.2.1 *Escherichia coli*

All ninety randomly chosen presumptive *E. coli* isolates obtained by the described MPN method (MFHB-19, Chapter 2.2.1) showed characteristic results in the GIMViC test confirming the presence of *E. coli*. To verify this assignment, all isolates were further confirmed using PCR with *E. coli* specific primers targeting the *gad*A gene. Positive results were observed for all ninety presumptive *E. coli* isolates from the birds' faecal samples, as evidenced by the presence of the expected PCR amplification product of 670bp (Figure 3.1). The reliability of the procedure was verified through the absence of any amplification product in all negative controls

employed and the presence of the expected product in the positive control, demonstrating the specificity and accuracy of the PCR assay.

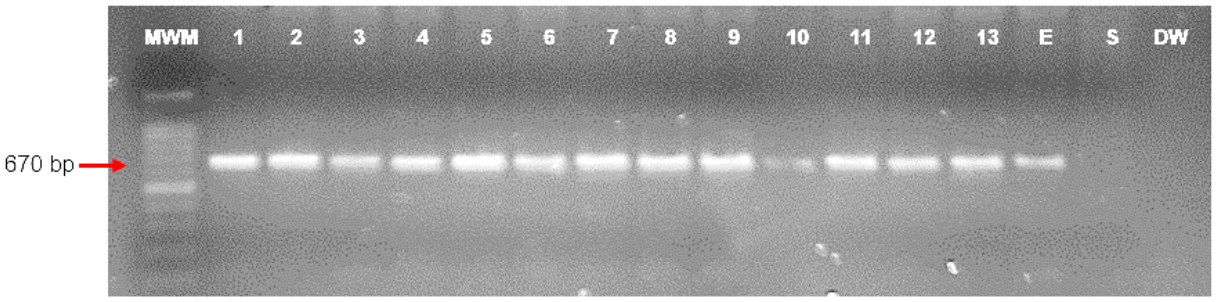

Figure 3.1. Representative 1.5% agarose gel image illustrating the results for PCR amplification of the *gad*A gene of thirteen selected *E. coli* isolates.

Key: MWM – Molecular Weight Marker (100 bp); 1 – 4 commercial ducks faecal isolates; 5 – 9 wild sacred ibis faecal isolates; 10 – 13 wild raptors faecal isolates; E – *E. coli* ATCC 25922 (positive control); S – *Salmonella* Typhimurium ATCC 14028 and DW – distilled water (negative controls).

3.1.2.2 *Enterococcus* species

The application of multiplex PCR successfully confirmed the genus *Enterococcus* for all ninety presumptive faecal enterococci isolates collected from commercial free-range ducks, wild sacred ibis, and raptors (thirty isolates from each bird) by the detection of the 733 bp amplification product specific to the *Enterococcus* genus (Figure 3.2). The reliability of the procedure was verified through the absence of any amplification product in all negative controls employed and the presence of the expected product in the positive controls, demonstrating the specificity and accuracy of the PCR assay. Simultaneously, the use of the species-specific primers for *E. faecalis* (360 bp) and *E. durans* (295 bp) allowed for further species-specific identification.

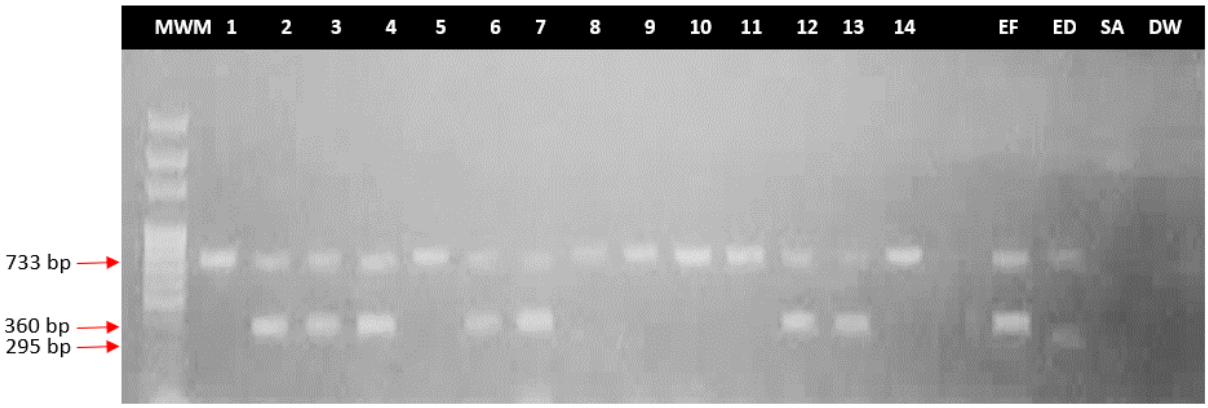

Figure 3.2. Representative 1.5% agarose gel image illustrating the results for PCR amplification of the genus *Enterococcus* (733 bp) and the species specific (*E. faecalis* 360 bp and *E. durans* 295 bp) products of fourteen selected *Enterococcus* spp. isolates.

Key: MWM – Molecular Weight Marker (100 bp); 1 – 5 wild raptors faecal isolates; 6 – 10 commercial ducks faecal isolates; 11 – 14 wild sacred ibis faecal isolates; EF – *E. faecalis* ATCC 29212 and ED – *E. durans* ATCC 6056 (positive controls); SA – *Staphylococcus aureus* ATCC 6538 and DW – distilled water (negative controls).

The comprehensive genotypic results of the multiplex PCR for all ninety *Enterococcus* spp. isolates (thirty from each bird's faecal sample) are documented in Table 3.2. Isolates from wild sacred ibis and raptors faecal samples contained more *E. faecalis* with 40% and 47% of the isolates showing positive results for *E. faecalis*, respectively, while a much lower proportion was present in commercial duck faecal samples with only 10% (n=30) of the isolates confirmed as *E. faecalis* in the PCR. None of the isolates from the different bird faecal samples were identified as *E. durans*. No further species-specific assignment could be made for the non-assigned enterococci isolates from commercial ducks (90%), wild sacred ibis (60%), and raptors (53%) faecal samples as only *E. faecalis* and *E. durans* specific primers were available for testing. Additionally, a pairwise comparison using the Pearson's chi-squared test (Table 3.3) revealed that there was no statistical significance between wild sacred ibis and raptors *E. faecalis* isolates ($p=0.6023$), however, there was statistical significance ($p<0.05$) between commercial ducks and both wild birds' *E. faecalis* isolates.

Table 3.2. Prevalence of *E. faecalis* and *E. durans* among ninety confirmed *Enterococcus* spp. isolates obtained from faecal samples from commercial ducks, wild sacred ibis, and raptors (thirty isolates each) using multiplex PCR.

	Number of isolates identified		
	Commercial Ducks	**Wild Sacred ibis**	**Wild Raptors**
E. faecalis	3 (10%)	12 (40%)	14 (47%)
E. durans	0	0	0
Other *enterococci*	27 (90%)	18 (60%)	16 (53%)
Total *Enterococcus* spp.	**30**	**30**	**30**

Table 3.3. Pairwise comparison of number of isolates positive for *E. faecalis* obtained from commercial ducks, wild sacred ibis, and raptor faecal samples using the Pearson's X^2 test.

Comparison	Pearson's X^2 p value
Commercial ducks – Wild sacred ibis	**0.0073**
Commercial ducks – Wild raptors	**0.0016**
Wild sacred ibis – Wild raptors	0.6023

*If p>0.05 there is no statistical significance, and if **p<0.05** there is **statistical significance**.

3.2. Antibiotic Susceptibility

Antibiotic resistance profiles were determined for all ninety *E. coli* and ninety *Enterococcus* spp. isolates (thirty of each from commercial ducks, wild sacred ibis, and raptors faecal samples) using the EUCAST disk diffusion assay. Results indicated a higher frequency of antibiotic resistances (ABR) present in *E. coli* isolates compared to isolates of the genus *Enterococcus* spp. (Figure 3.3). Generally, the resistance rates varied across the three different bird species sampled (Figure 3.3). Interestingly, faecal enterococci isolates from ducks displayed no resistances, while faecal enterococci isolates from sacred ibis showed a moderate resistance level of 33% (10 of 30), and 47% (14 of 30) of the faecal enterococci isolates from raptors

showed resistance to at least one of the antibiotics tested. In contrast, for *E. coli* isolates, resistance percentages were uniformly higher across all birds tested. Notably, the lowest level for resistant *E. coli* (47% of isolates obtained from duck faeces) was equivalent to the highest level of resistant enterococci isolates, found in raptor faecal samples. Faecal *E. coli* isolates from wild birds showed much higher resistance levels for faecal *E. coli*, with 73% (sacred ibis) and 93% (raptors).

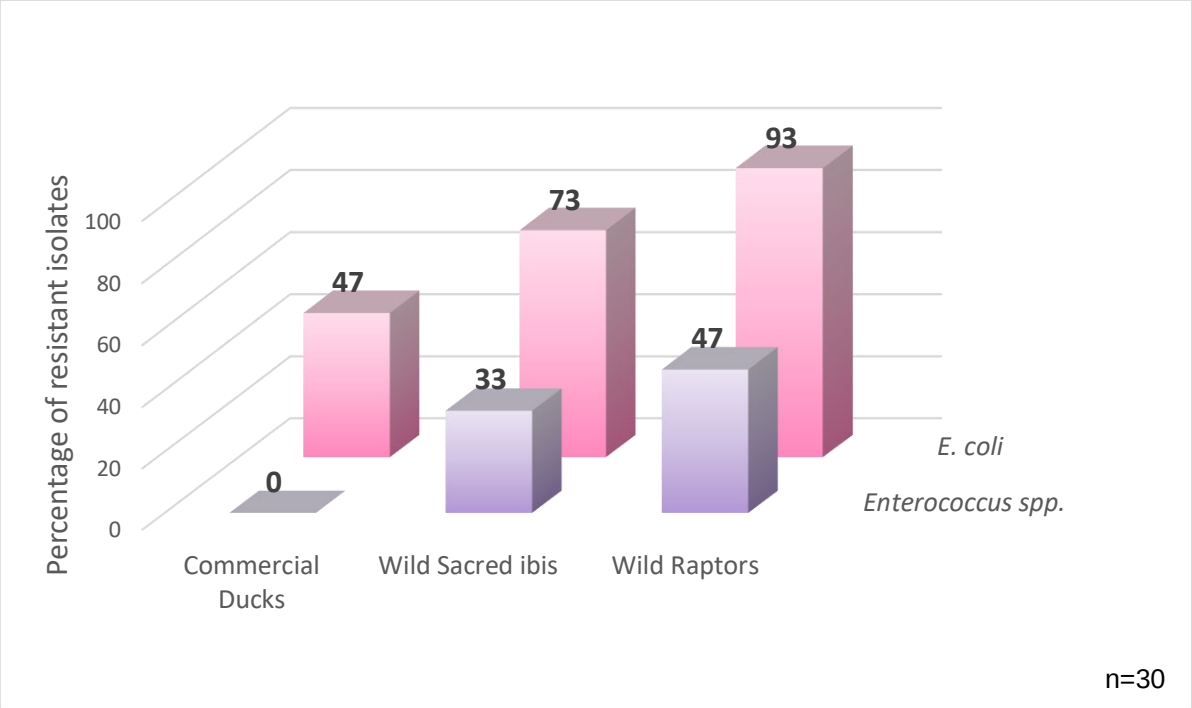

Figure 3.3. The percentage of resistant *E. coli* and *Enterococcus* spp. isolates (n=30 each) from commercial ducks, wild sacred ibis, and raptors faecal samples towards one or more antibiotics.

3.2.1. *Escherichia coli*

Antibiotic resistance (ABR) profiles of thirty confirmed *E. coli* isolates from each of the three avian species – commercial ducks, wild sacred ibis, and raptors – were examined using the disk diffusion assay, and the frequency of susceptibility, intermediate resistance, and resistance present in all *E. coli* isolates to each of the twelve clinically relevant antibiotics was determined using EUCAST 2024 breakpoints (Appendix A) (EUCAST, 2024). The intermediate resistance (susceptible, increased exposure) category is only defined by EUCAST for five out of the twelve tested antibiotics used for *E. coli* (i.e., MEM, CAZ CTX, AZT, CIP).

As seen in Figure 3.4, the incidence of *E. coli* antibiotic resistance varied between commercial and wild bird species. Among the twelve antibiotics tested, resistance in *E. coli* isolates from commercial ducks was detected against three antibiotics: amoxicillin-clavulanic acid, gentamicin, and tobramycin. In contrast, resistance in *E. coli* isolates from wild sacred ibis and raptors was detected against the same six antibiotics: ampicillin, amoxicillin-clavulanic acid, ceftazidime, gentamicin, tobramycin, and norfloxacin. No resistance to antibiotics from the tetracycline, carbapenem, and monobactam classes was detected in any of the isolates from the bird species tested. However, intermediate resistance to aztreonam (monobactam) was detected in *E. coli* isolates from all bird faeces. Additionally, intermediate resistance against meropenem (a carbapenem) was detected in two *E. coli* isolates, on from duck faeces and one from raptor faeces.

For ceftazidime, intermediate resistance was found only in isolates from duck faeces, while resistance was present in isolates from both wild birds. Furthermore, intermediate resistance to ciprofloxacin was observed only in isolates from wild birds, and cefotaxime intermediate resistance was detected only in isolates from raptors.

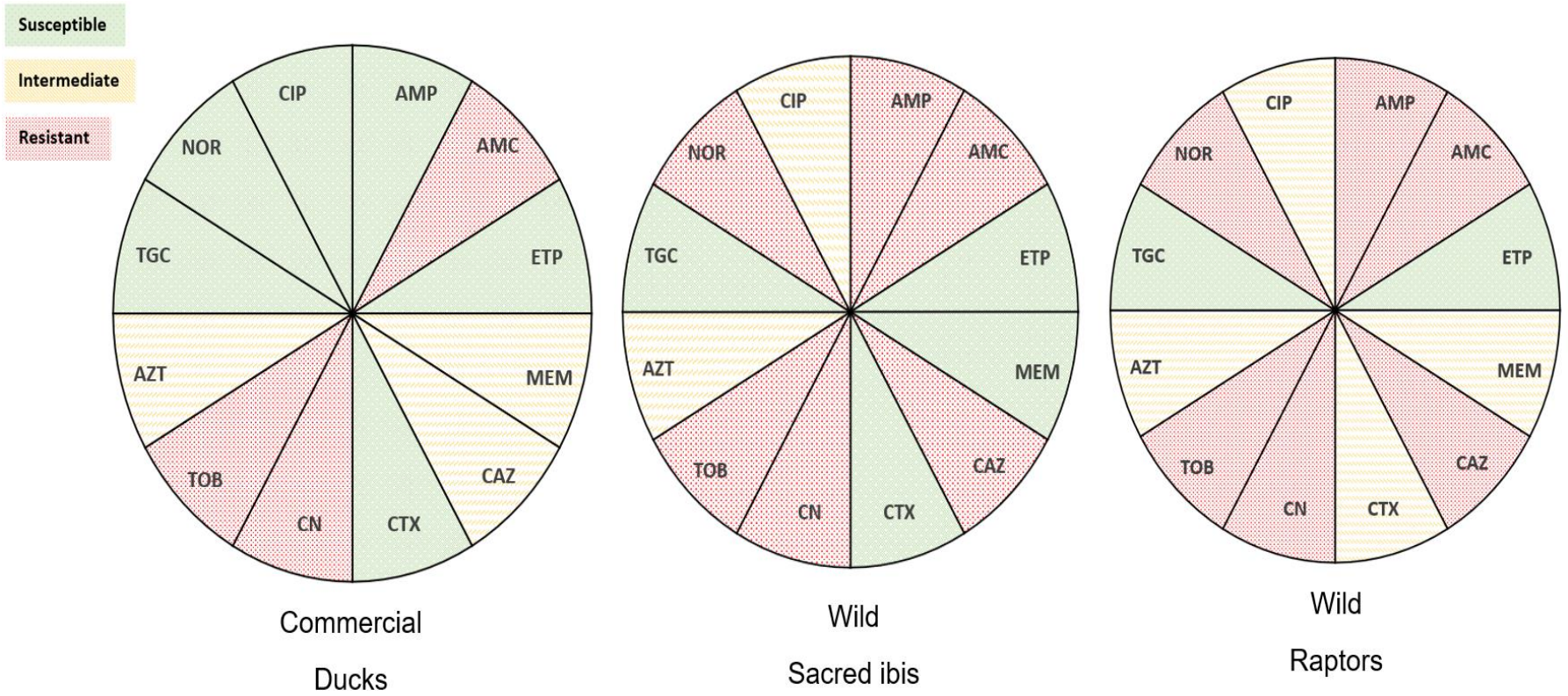

Figure 3.4. Incidence of susceptibility, intermediate resistance, and resistance of *E. coli* isolates from commercial ducks, wild sacred ibis, and raptors towards twelve antibiotics tested.

The following twelve antibiotics as suggested by EUCAST (2021) were tested: AMP (ampicillin – 10µg), AMC (amoxicillin-clavulanic acid – 20µg-10µg), ETP (ertapenem – 10µg), MEM (meropenem – 10µg), CAZ (ceftazidime – 10µg), CTX (cefotaxime – 5µg), CN (gentamicin – 10µg), TOB (tobramycin – 10µg) AZT (aztreonam – 30µg), TGC (tigecycline – 15µg), NOR (norfloxacin – 10µg), and CIP (ciprofloxacin – 5µg).

E. coli isolates obtained from commercial ducks' faeces exhibited a high level of susceptibility, with all thirty isolates (100%) showing sensitivity to ampicillin, ertapenem, cefotaxime, tigecycline, norfloxacin, and ciprofloxacin (Figure 3.5). However, intermediate resistance was observed in one isolate for meropenem (3%), ten isolates for ceftazidime (33%), and 22 isolates for aztreonam (73%). Resistances occurred in one isolate for amoxicillin-clavulanic acid (3%), six isolates for tobramycin (20%), and 10 isolates for gentamicin (33%) (Figure 3.5), covering two classes of antibiotics (penicillin and aminoglycoside).

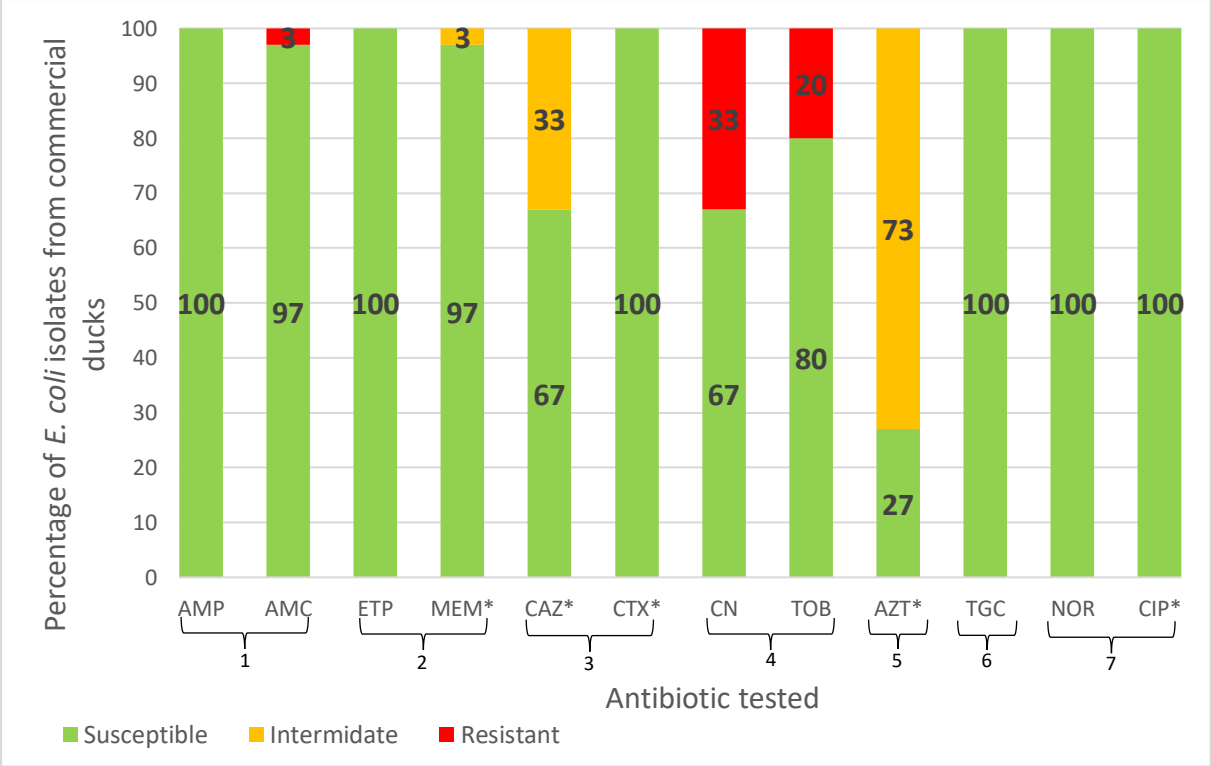

Figure 3.5. Percentage of antibiotic resistance and susceptibility to twelve selected antibiotics for confirmed *E. coli* isolates (n=30) from commercial duck faecal samples according to the EUCAST disk diffusion assay.

*intermediate resistance as defined by EUCAST breakpoints are applicable

The following twelve antibiotics as suggested by EUCAST (2021) were tested: AMP (ampicillin – 10µg), AMC (amoxicillin-clavulanic acid – 20µg-10µg), ETP (ertapenem – 10µg), MEM (meropenem – 10µg), CAZ (ceftazidime – 10µg), CTX (cefotaxime – 5µg), CN (gentamicin – 10µg), TOB (tobramycin – 10µg) AZT (aztreonam – 30µg), TGC (tigecycline – 15µg), NOR (norfloxacin – 10µg), and CIP (ciprofloxacin – 5µg).

Classes: 1. Penicillin; 2. Carbapenem; 3. Cephalosporin; 4. Aminoglycoside; 5. Monobactam; 6. Tetracycline; 7. Fluoroquinolone.

All thirty *E. coli* isolates from sacred ibis displayed susceptibility to ertapenem, meropenem, cefotaxime, and tigecycline (Figure 3.6). Intermediate resistance was observed in nine isolates (30%) towards ceftazidime, six isolates (20%) towards aztreonam, and three isolates (10%) towards ciprofloxacin. Resistance was detected in four antibiotic classes (penicillin, cephalosporin, aminoglycoside, and fluoroquinolone), with seven isolates (23%) showing resistance to ampicillin, ten isolates (33%) to amoxicillin-clavulanic acid, and fourteen isolates each (47%) to gentamicin and tobramycin. Resistance to norfloxacin and ceftazidime was lowest, with one isolate (3%) and two isolates (7%) showing resistance, respectively (Figure 3.6).

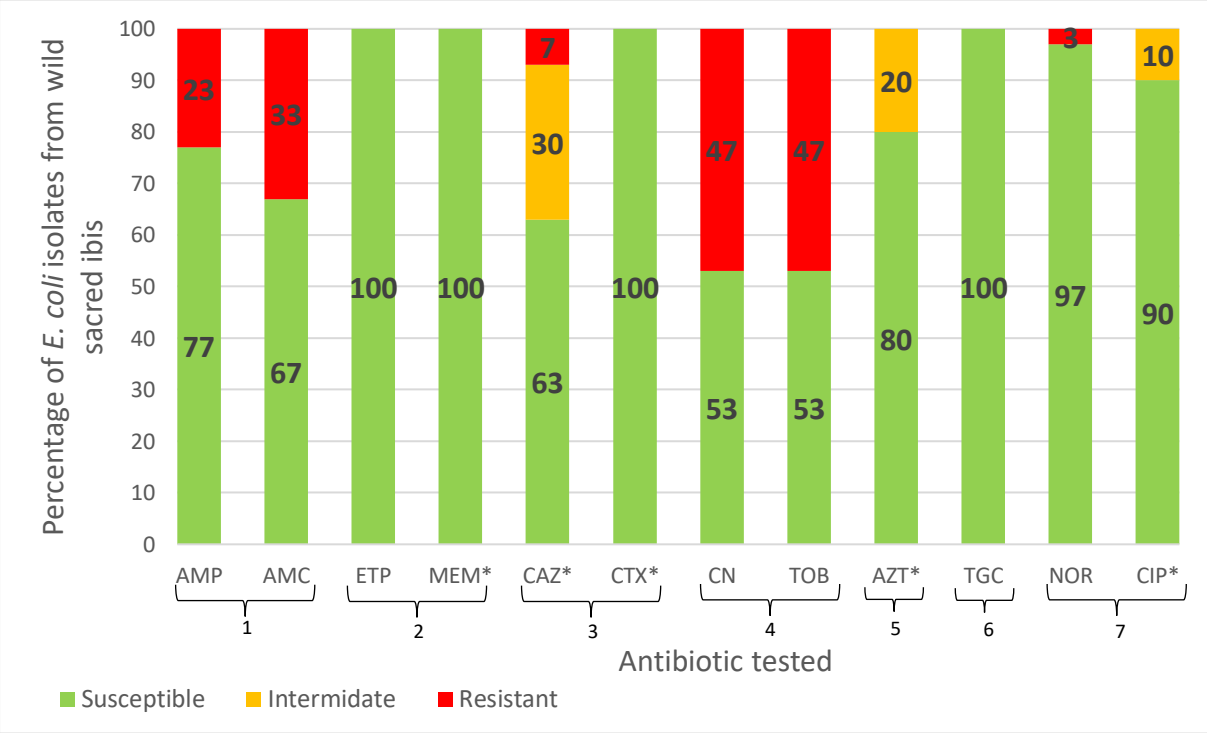

Figure 3.6. Percentage of antibiotic resistance and susceptibility to twelve selected antibiotics for confirmed *E. coli* isolates (n=30) from wild sacred ibis faecal samples according to the EUCAST disk diffusion assay.

*intermediate resistance as defined by EUCAST breakpoints are applicable

The following twelve antibiotics as suggested by EUCAST (2021) were tested: AMP (ampicillin – 10µg), AMC (amoxicillin-clavulanic acid – 20µg-10µg), ETP (ertapenem – 10µg), MEM (meropenem – 10µg), CAZ (ceftazidime – 10µg), CTX (cefotaxime – 5µg), CN (gentamicin – 10µg), TOB (tobramycin – 10µg) AZT (aztreonam – 30µg), TGC (tigecycline – 15µg), NOR (norfloxacin – 10µg), and CIP (ciprofloxacin – 5µg).

Classes: 1. Penicillin; 2. Carbapenem; 3. Cephalosporin; 4. Aminoglycoside; 5. Monobactam; 6. Tetracycline; 7. Fluoroquinolone.

Among the thirty *E. coli* isolates from raptors, full susceptibility was observed only to ertapenem and tigecycline. Intermediate resistance was detected in one isolate (3%) to meropenem, sixteen isolates (53%) to ceftazidime, two isolates (7%) to cefotaxime, eight isolates (27%) to aztreonam, and five isolates (17%) to ciprofloxacin. Notably, resistance was more frequent in raptor *E. coli* isolates than the other birds tested, with thirteen isolates (43%) resistant to ampicillin, fifteen isolates (50%) to amoxicillin-clavulanic acid, five isolates (17%) to ceftazidime, three isolates (10%) to norfloxacin, fourteen isolates (47%) to gentamicin, and fifteen isolates (50%) to tobramycin (Figure 3.7). Interestingly, resistance detected in *E. coli* isolates from raptors was against the same four classes of antibiotics as those from sacred ibis, but the number of resistant isolates was higher in raptors.

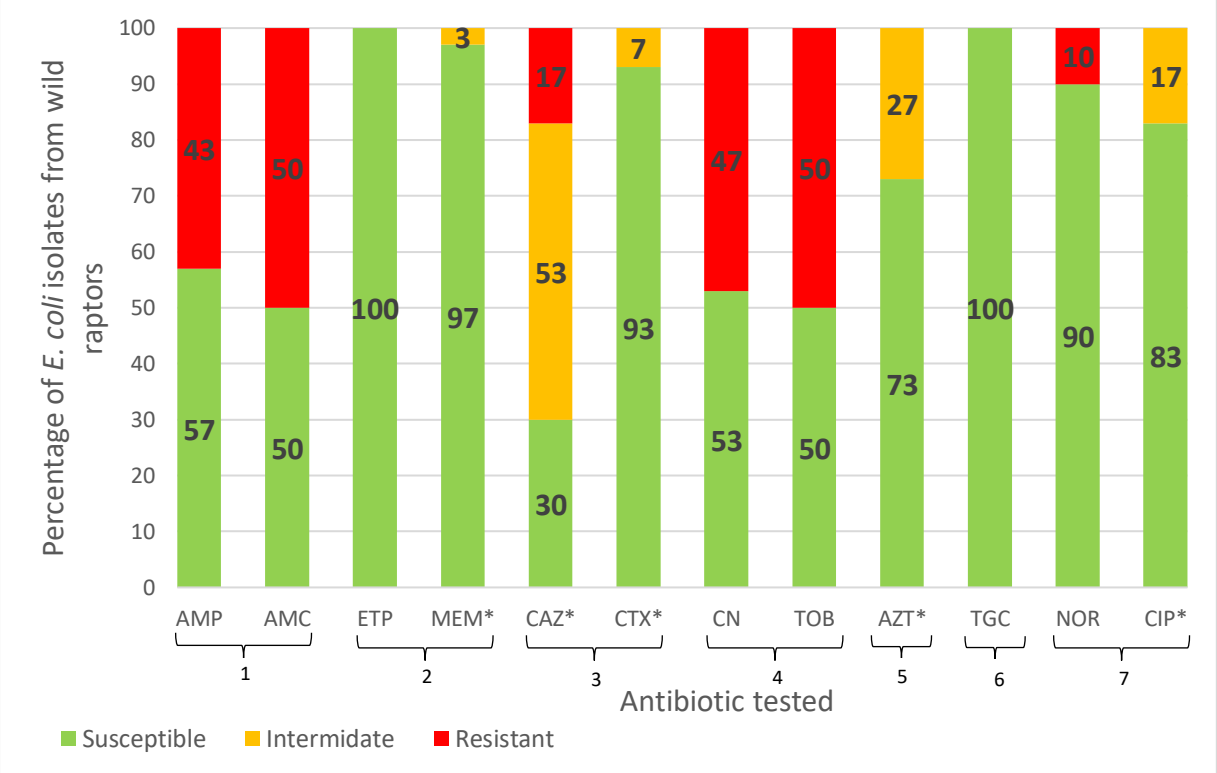

Figure 3.7. Percentage of antibiotic resistance and susceptibility to twelve selected antibiotics for confirmed *E. coli* isolates (n=30) from wild raptor faecal samples according to the EUCAST disk diffusion assay.

*intermediate resistance as defined by EUCAST breakpoints are applicable . The following twelve antibiotics as suggested by EUCAST (2021) were tested: AMP (ampicillin – 10µg), AMC (amoxicillin-clavulanic acid – 20µg-10µg), ETP (ertapenem – 10µg), MEM (meropenem – 10µg), CAZ (ceftazidime – 10µg), CTX (cefotaxime – 5µg), CN (gentamicin – 10µg), TOB (tobramycin – 10µg) AZT (aztreonam – 30µg), TGC (tigecycline – 15µg), NOR (norfloxacin – 10µg), and CIP (ciprofloxacin – 5µg). Classes: 1. Penicillin; 2. Carbapenem; 3. Cephalosporin; 4. Aminoglycoside; 5. Monobactam; 6. Tetracycline; 7. Fluoroquinolone.

The data were further categorized based on the number of antibiotics to which each *E. coli* isolate showed resistance (Table 3.4). Among the duck isolates, 53% of the isolates (16 of 30) displayed full susceptibility to the twelve antibiotics tested, however, thirteen of these were categorized as intermediate resistant (43%) as defined by EUAST (susceptible, requiring increased exposure). A total of 37% of duck isolates were resistant to only one antibiotic, and 10% of isolates were resistant to two antibiotics. None of the *E. coli* isolates from ducks showed resistance to three or more antibiotics (Table 3.4).

In contrast, fewer sacred ibis *E. coli* isolates (8 of 30; 27%) displayed susceptibility, including two isolates (7%) categorized as intermediate resistant. Notably, 30% of the isolates were resistant to one antibiotic, 17% to two antibiotics, and 27% to three or more antibiotics. Two out of thirty isolates were classified as multidrug-resistant (MDR), indicating resistance to antibiotics from three or more classes (Table 3.4).

Raptor *E. coli* isolates demonstrated the lowest susceptibility frequency with only 7% (2 of 30) falling in this category, of which all were categorized as intermediate resistant. Among the resistant *E. coli* isolates, 23% were resistant to one antibiotic and two antibiotics each, while nearly half of the isolates (47%) were resistant to three or more antibiotics. Similar to *E. coli* isolates obtained from sacred ibis, two out of thirty (7%) were also classified as MDR *E. coli* isolates (Table 3.4).

A pairwise comparison using the Pearson's X^2 test (Table 3.5) revealed that there was no statistical significance between the frequency of resistances of wild sacred ibis and raptors faecal *E. coli* isolates ($p=0.1207$). However, there was statistical significance ($p<0.05$) between commercial ducks and both wild birds' faecal *E. coli* isolates frequency of resistances (not considering the number of intermediate and MDR resistances).

Table 3.4. Frequency of *E. coli* isolates from commercial ducks, wild sacred ibis, and raptors faecal samples exhibiting susceptibility or resistance to one, two, and three or more antibiotics.

Frequency of *E. coli* resistance	Commercial Ducks		Wild Sacred ibis		Wild Raptors	
	n	%	n	%	n	%
Susceptible	16*	53	8*	27	2*	7
(of which intermediate)	(13)	(43)	(2)	(7)	(2)	(7)
Resistant to one antibiotic	11*	37	9*	30	7*	23
Resistant to two antibiotics	3*	10	5*	17	7*	23
Resistant to three or more	0*	0	8*	27	14*	47
(of which are MDR)	(0)	(0)	(2)	(7)	(2)	(7)
TOTAL	**30**	**100**	**30**	**100**	**30**	**100**

*values used for Pearson's X^2 test to determine statistical significance.

Table 3.5. Pairwise comparison of resistance frequency of *E. coli* isolates from commercial ducks, wild sacred ibis, and raptor faecal samples using Pearson's X^2 test.

Comparison	p value
Commercial ducks – Wild sacred ibis	**0.0099**
Commercial ducks – Wild raptors	**0.0001**
Wild sacred ibis – Wild raptors	0.1207

*If p>0.05 there is no statistical significance, and if **p<0.05** there is **statistical significance**.

When examining the antibiotic resistance profiles of *E. coli* isolates (Table 3.6), the most common phenotype of isolates resistant to only one antibiotic were AMC, CN, or TOB, which were common among isolates from all three birds. However, variations emerge among isolates resistant to two of the twelve antibiotics tested. The predominant phenotype combination, CN-TOB, was observed in isolates from commercial ducks (10%), wild sacred ibis (13%), and raptors (7%). Interestingly, this was the only two-combination resistance phenotype present among isolates from all three birds. Additionally, one isolate (3%) from sacred ibis exhibited AMP-AMC resistance, which was not present in other faecal *E. coli* isolates from the other two birds. Notably, raptor *E. coli* isolates displayed a wider array of two-combination resistance phenotypes, including AMP-CAZ (3%), AMC-CAZ (7%), AMC-TOB (3%), and TOB-NOR (3%).

E. coli isolates resistant to three of the twelve antibiotics tested displayed more similarity among wild sacred ibis and raptors isolates, with the following combinations present in faecal isolates from both birds AMP-AMC-CN (3% each), AMP-AMC-TOB (7% and 10% respectively), and AMP-CN-TOB (7% and 3% respectively). However, faecal *E. coli* isolates from raptors had four more three-combination resistance phenotypes (Table 3.6). Two of these isolates, which displayed resistance patterns of AMP-TOB-NOR and AMC-CN-NOR, respectively, were categorized as multidrug-resistant (MDR), as they exhibited resistance to antibiotics from three different classes, i.e., penicillin, aminoglycoside, and fluoroquinolone.

Resistance to four antibiotics was observed in faecal *E. coli* isolates from both of the wild birds tested, with the combination of AMP-AMC-CN-TOB found in one isolate (3%) from sacred ibis and two isolates (7%) from raptors. However, a different combination of four antibiotics – AMP-AMC-CAZ-CN – was found in one isolate (3%) from sacred ibis, which was categorized as MDR due to resistance to three antibiotic classes: penicillin, cephalosporin, and aminoglycoside.

One isolate (3%) from sacred ibis even displayed resistance to six antibiotics – AMP-AMC-CAZ-CN-TOB-NOR – from four different classes: penicillin, cephalosporin, aminoglycoside, and fluoroquinolone, which was therefore also categorized as MDR.

Table 3.6. The antibiotic resistance phenotype of *E. coli* isolates from commercial ducks, wild sacred ibis, and raptors faecal samples, towards one or more of the twelve selected antibiotics.

***E. coli* resistance phenotype**	**Commercial Ducks (n=30)**		**Wild Sacred ibis (n=30)**		**Wild Raptors (n=30)**	
	No. of resistant isolate/s	%	No. of resistant isolate/s	%	No. of resistant isolate/s	%
AMC	1	3	1	3	2	7
CN	7	23	4	13	4	13
TOB	3	10	4	13	1	3
CN-TOB	3	10	4	13	2	7
AMP-AMC	-	-	1	3	-	-
AMP-CAZ	-	-	-	-	1	3
AMC-CAZ	-	-	-	-	2	7
AMC-TOB	-	-	-	-	1	3
TOB-NOR	-	-	-	-	1	3
AMP-AMC-CN	-	-	1	3	1	3
AMP-AMC-TOB	-	-	2	7	3	10
AMC-CN-TOB	-	-	2	7	1	3
AMP-CN-TOB	-	-	-	-	3	10
AMP-AMC-CAZ	-	-	-	-	2	7
***AMP-TOB-NOR**	-	-	-	-	1*	3
***AMC-CN-NOR**	-	-	-	-	1*	3
AMP-AMC-CN-TOB	-	-	1	3	2	7
***AMP-AMC-CAZ-CN**	-	-	1*	3	-	-
***AMP-AMC-CAZ-CN-TOB-NOR**	-	-	1*	3	-	-

*MDR (multidrug-resistant) isolate

AMP (ampicillin – 10µg), AMC (amoxicillin-clavulanic acid – 20µg-10µg), CAZ (ceftazidime – 10µg), CN (gentamicin – 10µg), TOB (tobramycin – 10µg), TGC (tigecycline – 15µg), and NOR (norfloxacin – 10µg).

3.2.2. *Enterococcus* species

Antibiotic resistance (ABR) profiles of thirty confirmed *Enterococcus* spp. isolates from each of the three avian species – commercial ducks, wild sacred ibis, and raptors – was analyzed using the disk diffusion assay, and the frequency of susceptibility, intermediate resistance, and resistance in all *Enterococcus* spp. isolates to the eight antibiotics were determined using EUCAST 2024 breakpoints (Appendix B) (EUCAST, 2024). Notably, the intermediate resistance category (susceptible, increased exposure) is only specified for ampicillin and imipenem.

As seen in Figure 3.8, the incidence of *Enterococcus* spp. antibiotic resistances varied between commercial and wild bird species, with no resistances detected among isolates from commercial duck faeces. However, intermediate resistance was detected towards two antibiotics (i.e., ampicillin and imipenem). In contrast to this, faecal *Enterococcus* spp. isolates from both wild birds, sacred ibis and raptors, showed resistance to the same five antibiotics (i.e., ampicillin, imipenem, teicoplanin, vancomycin, and linezolid).

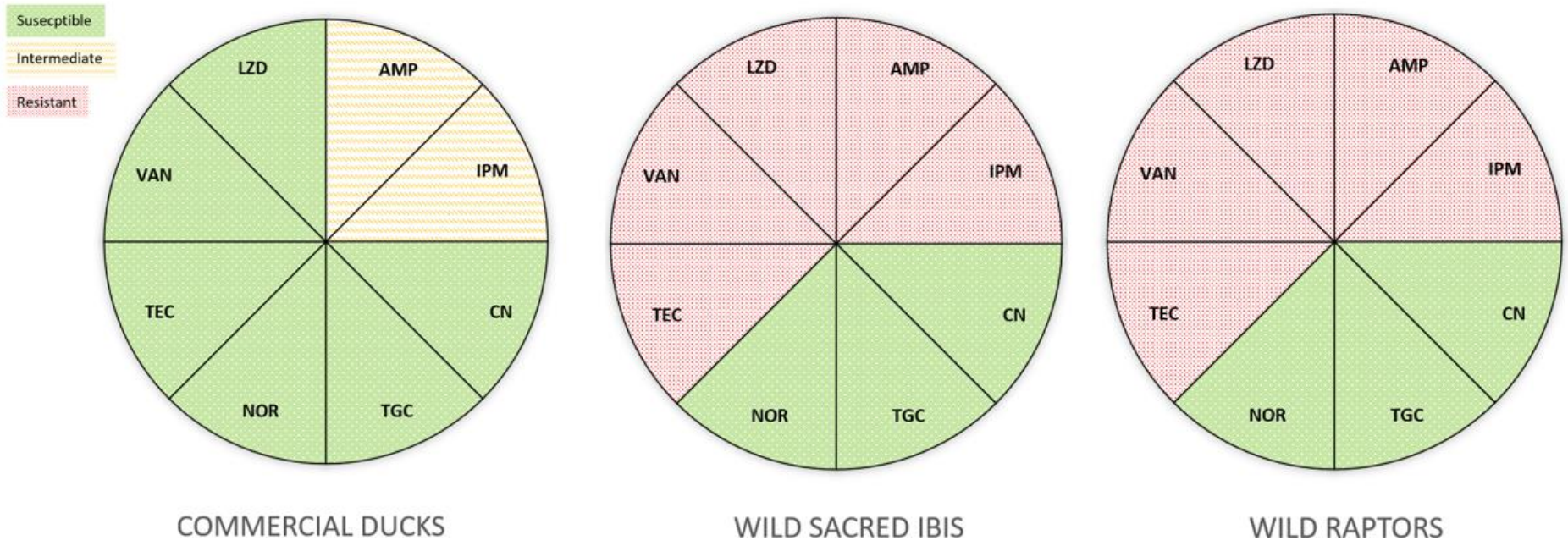

Figure 3.8. Incidence of susceptibility, intermediate resistance, and resistance of *Enterococcus* spp. isolates from commercial ducks, wild sacred ibis, and raptors towards eight antibiotics tested.

The following twelve antibiotics as suggested by EUCAST (2021) were tested: AMP (ampicillin – 2µg), IPM (imipenem – 10µg), CN (gentamicin – 30µg), TGC (tigecycline – 15µg), NOR (norfloxacin – 10µg), TEC (teicoplanin – 30µg), VAN (vancomycin – 5µg), and LZD (linezolid – 10µg).

Enterococcus spp. isolates obtained from commercial ducks exhibited high level of susceptibility, with all thirty isolates (100%) showing sensitivity to gentamicin, tigecycline, norfloxacin, teicoplanin, vancomycin, and linezolid. However, intermediate resistance as defined by EUCAST (2024) was observed for three isolates to ampicillin (10%) and for all isolates to imipenem (100%) (Figure 3.9).

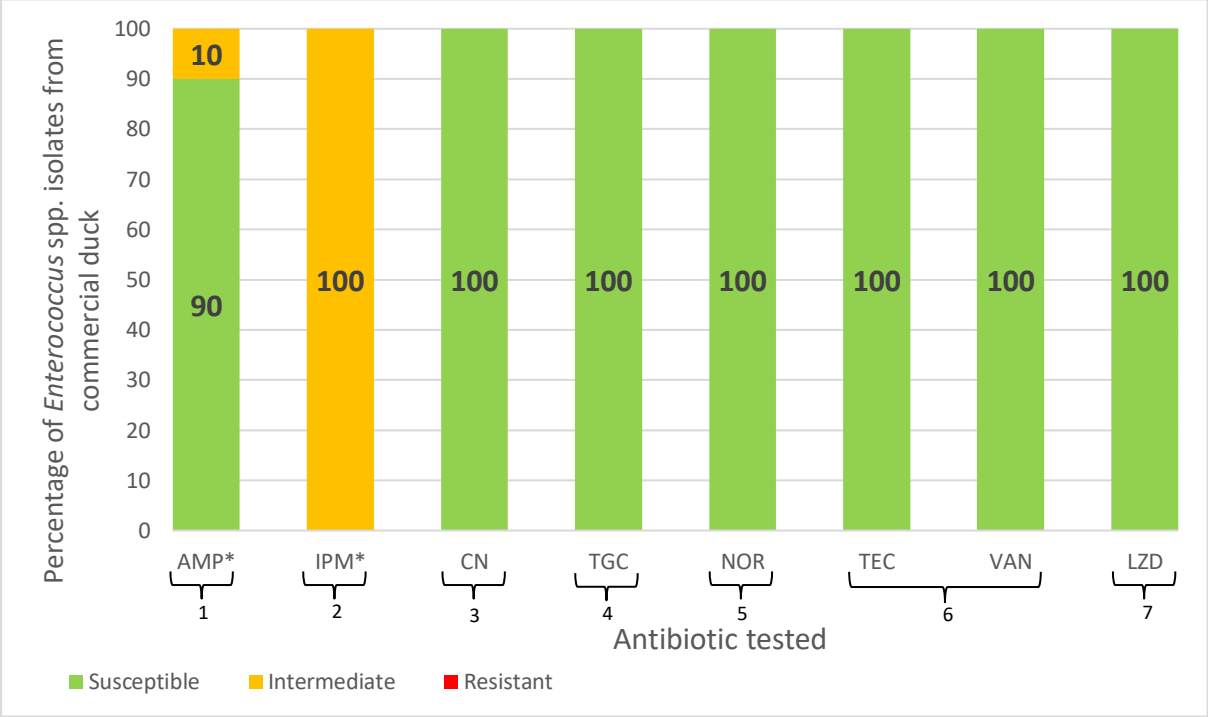

Figure 3.9. Percentage of antibiotic resistance and susceptibility to eight selected antibiotics for thirty confirmed Enterococcus spp. isolates (n=30) from commercial duck faecal samples according to the EUCAST disk diffusion assay.

*intermediate resistance according to EUCAST breakpoints are applicable

The following twelve antibiotics as suggested by EUCAST (2021) were tested: AMP (ampicillin – 2µg), IPM (imipenem – 10µg), CN (gentamicin – 30µg), TGC (tigecycline – 15µg), NOR (norfloxacin – 10µg), TEC (teicoplanin – 30µg), VAN (vancomycin – 5µg), and LZD (linezolid – 10µg).

Classes: 1. Penicillin; 2. Carbapenem; 3. Aminoglycoside; 4. Tetracycline; 5. Fluoroquinolone; 6. Glycopeptide/lipopeptide; 7. Oxazolidinone.

As shown in Figure 3.10, all thirty sacred ibis *Enterococcus* spp. isolates displayed susceptibility towards gentamicin, tigecycline, and norfloxacin. Only one isolate (3%) was resistant to imipenem, while all other isolates (97%) were categorized as intermediate. Additionally, intermediate resistance and resistance was detected in three isolates (10%) each towards ampicillin. Resistances were also detected in one isolate (3%) to teicoplanin, in five isolates (17%) to vancomycin, and in two isolates (7%) to linezolid.

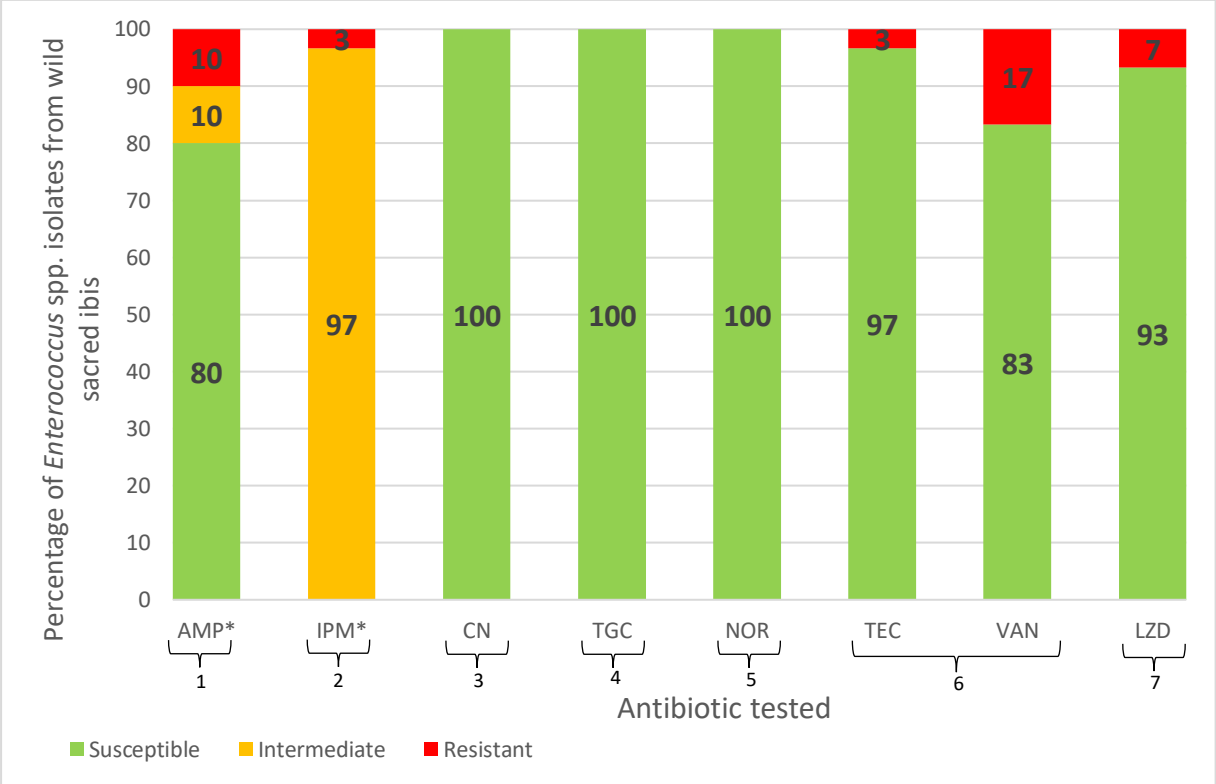

Figure 3.10. Percentage of antibiotic resistance and susceptibility to eight selected antibiotics for thirty confirmed *Enterococcus* spp. isolates (n=30) from wild sacred ibis faecal samples according to the EUCAST disk diffusion assay.

*intermediate resistance according to EUCAST breakpoints are applicable

The following twelve antibiotics as suggested by EUCAST (2021) were tested: AMP (ampicillin – 2µg), IPM (imipenem – 10µg), CN (gentamicin – 30µg), TGC (tigecycline – 15µg), NOR (norfloxacin – 10µg), TEC (teicoplanin – 30µg), VAN (vancomycin – 5µg), and LZD (linezolid – 10µg).

Classes: 1. Penicillin; 2. Carbapenem; 3. Aminoglycoside; 4. Tetracycline; 5. Fluoroquinolone; 6. Glycopeptide/lipopeptide; 7. Oxazolidinone.

The frequency of resistances in *Enterococcus* spp. isolates from raptors showed similar trends as detected for sacred ibis isolates (Figure 3.11). All thirty isolates (100%) were also susceptible to gentamicin, tigecycline, and norfloxacin. Two isolates (7%) were resistant to imipenem, while all other isolates (93%) were categorized as intermediately resistant to imipenem. Additionally, intermediate resistance and resistance was detected in four isolates (13%) each towards ampicillin. Furthermore, faecal *Enterococcus* spp. isolates from raptors displayed the highest resistance prevalence, with two isolates (7%) resistant to teicoplanin, eight isolates (27%) resistant to vancomycin, and one isolate (3%) resistant to linezolid.

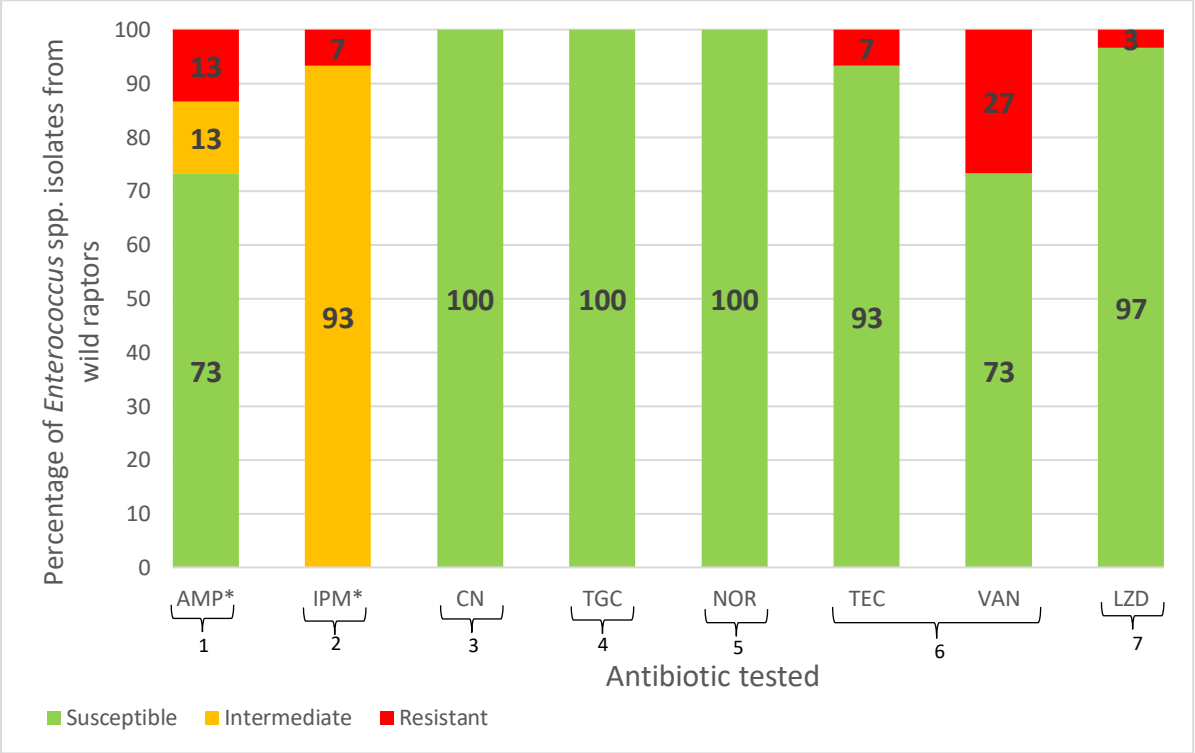

Figure 3.11. Percentage of antibiotic resistance and susceptibility to eight selected antibiotics for thirty confirmed *Enterococcus* spp. isolates (n=30) from wild raptor faecal samples according to the EUCAST disk diffusion assay.

*intermediate resistance according to EUCAST breakpoints are applicable

The following twelve antibiotics as suggested by EUCAST (2021) were tested: AMP (ampicillin – 2µg), IPM (imipenem – 10µg), CN (gentamicin – 30µg), TGC (tigecycline – 15µg), NOR (norfloxacin – 10µg), TEC (teicoplanin – 30µg), VAN (vancomycin – 5µg), and LZD (linezolid – 10µg).

Classes: 1. Penicillin; 2. Carbapenem; 3. Aminoglycoside; 4. Tetracycline; 5. Fluoroquinolone; 6. Glycopeptide/lipopeptide; 7. Oxazolidinone.

Further categorization of the faecal *Enterococcus* spp. isolates was based on the number of antibiotics to which each *Enterococcus* spp. isolate exhibited resistance, as summarized in Table 3.7. Among the duck faecal isolates (n=30) all displayed no resistance to any of the eight antibiotics tested. In contrast to duck isolates, 67% (20 out of 30) of *Enterococcus* spp. isolates obtained from sacred ibis exhibited susceptibility, while 27% of isolates were resistant to one antibiotic, and only two isolates (7%) to two antibiotics. However, none displayed resistance to three or more antibiotics. As seen for *E. coli* isolates, faecal *Enterococcus* spp. isolates from raptors (n=30) demonstrated the lowest frequency of susceptibility (no-resistance), with just over half of the isolates (53%) falling into this category. Additionally, 37% (11 of 30) of isolates from raptors were resistant to one antibiotic, and 10% (3 of 10) of isolates to two antibiotics. Similar to isolates from sacred ibis, none of the raptor isolates exhibited resistance to three or more antibiotics. Therefore, MDR (classified as resistant to three or more classes of antibiotics) was not detected for any of the ninety *Enterococcus* spp. isolates.

A pairwise comparison using the Pearson's X^2 test (Table 3.8) revealed that, similar to *E. coli* isolates, there was no statistical significance between the frequency of resistance of wild sacred ibis and raptors faecal *Enterococcus* spp. isolates ($p>0.5717$). However, there was statistical significance ($p<0.05$) between commercial ducks and both wild birds' faecal *Enterococcus* isolates frequency of resistance (not considering the number of intermediate and MDR resistances).

Table 3.7. Frequency of antibiotic resistance profiles of *Enterococcus* spp. isolates from commercial ducks, wild sacred ibis, and raptors faecal samples exhibiting susceptibility or resistance to one, two, and three or more antibiotics.

Frequency of *Enterococcus* spp. resistance	Commercial Ducks		Wild Sacred ibis		Wild Raptors	
	n	%	n	%	n	%
Susceptible	30*	100	20*	67	16*	53
(of which intermediate)	(30)	(100)	(20)	(67)	(16)	(53)
Resistant to one antibiotic	0*	0	8*	27	11*	37
Resistant to two antibiotics	0*	0	2*	7	3*	10
Resistant to three or more	0	0	0	0	0	0
(of which are MDR)	(0)	(0)	(0)	(0)	(0)	(0)
TOTAL	**30**	**100**	**30**	**100**	**30**	**100**

*values used for Pearson's X^2 test to determine statistical significance.

Table 3.8. Pairwise comparison of resistance frequency of *Enterococcus* spp. isolates from commercial ducks, wild sacred ibis, and raptor faecal samples using Pearson's X^2 test.

Comparison	p value
Commercial ducks – Wild sacred ibis	**0.0025**
Commercial ducks – Wild raptors	**0.0001**
Wild sacred ibis – Wild raptors	0.5717

*If p>0.05 there is no statistical significance, and if **p<0.05** there is **statistical significance**.

The established antibiotic resistance phenotypes of faecal *Enterococcus* spp. isolates from commercial ducks (n=30), wild sacred ibis (n=30), and raptors (n=30) have been summarized in Table 3.9. None of the duck isolates displayed resistance to any of the eight antibiotics tested. Single antibiotic resistances to ampicillin, vancomycin, and linezolid were found in both wild bird species isolates, while single resistance to imipenem was found only in raptors isolates. The two-antibiotic resistance combination phenotypes were the same for faecal isolates from both wild sacred ibis and raptors faeces, which were teicoplanin – vancomycin (3% and 7% respectively), and one isolate (3%) each with ampicillin – imipenem phenotype.

Table 3.9. The antibiotic resistance phenotype of *Enterococcus* spp. isolates from commercial ducks, wild sacred ibis, and raptor faecal samples, towards one or more of the eight selected antibiotics tested.

***Enterococcus* spp. resistance phenotype**	**Commercial Ducks (n=30)**		**Wild Sacred ibis (n=30)**		**Wild Raptors (n=30)**	
	No. of resistant isolate/s	%	No. of resistant isolate/s	%	No. of resistant isolate/s	%
AMP	0	0	2	7	3	10
VAN	0	0	4	13	6	20
LZD	0	0	2	7	1	3
IPM	0	0	0	0	1	3
TEC-VAN	0	0	1	3	2	7
AMP-IPM	0	0	1	3	1	3

AMP (ampicillin – 2µg), IPM (imipenem – 10µg), TEC (teicoplanin – 30µg), VAN (vancomycin – 5µg), and LZD (linezolid – 10µg).

3.3 β-lactamase Activity Using the Nitrocefin Spot Test

The nitrocefin hydrolysis assay was conducted on thirty-one *E. coli* isolates (one isolate from duck, ten from sacred ibis, and twenty from raptors) and eight *Enterococcus* spp. isolates (three from sacred ibis, and five from raptors) that displayed phenotypic resistance to at least one of the β-lactam antibiotics according to the determined antibiotic resistance profiles. Verification of the assay was conducted using *E. coli* ATCC 25922 and *Salmonella* Typhimurium ATCC 14028 as negative controls, which resulted in no hydrolysis of the nitrocefin.

The results from the nitrocefin hydrolysis assay for *E. coli* isolates are presented in Table 3.10. For *E. coli* isolates, the one tested isolate with only AMC-resistance from commercial ducks' faeces did not exhibit β-lactamase activity. Of the ten *E. coli* isolates from wild sacred ibis faecal samples tested, only one isolate (10%) showed no activity, while 30% (three of ten) showed full hydrolysis, and 60% (six of ten) showed partial hydrolysis. Similar results were obtained for faecal isolates from wild raptors, with 30% (six of twenty) and 60% (twelve of twenty) of the twenty tested isolates showing full and partial hydrolysis, respectively. However, two of the twenty isolates (10%) showed no nitrocefin hydrolysis activity, indicating an absence of β-lactamase.

Table 3.10. Detection of β-lactamase activity in *E. coli* isolates (exhibiting resistance to at least one β-lactam antibiotic) from ducks, sacred ibis, and raptors faecal samples, based on nitrocefin hydrolysis.

***E. coli* isolate**	**Antibiotic resistance phenotype**	**Nitrocefin result**
Duck		
$D_{EC}13$	AMC	No hydrolysis
Sacred ibis		
$S_{EC}3$	AMP-AMC-CN	Full hydrolysis
$S_{EC}7$	AMC	No hydrolysis
$S_{EC}9$	AMP-AMC-TOB	Partial hydrolysis
$S_{EC}13$	AMC-CN-TOB	Partial hydrolysis
$S_{EC}17$	AMP-AMC	Partial hydrolysis
$S_{EC}18$	AMC-CN-TOB	Partial hydrolysis

$S_{EC}20$	AMP-AMC TOB	Partial hydrolysis
$S_{EC}25$	AMP-AMC-CN-TOB	Full hydrolysis
$S_{EC}27$	AMP-AMC-CAZ-CN	Partial hydrolysis
$S_{EC}28$	AMP-AMC-CAZ-CN-TOB-NOR	Full hydrolysis
Raptor		
$R_{EC}3$	AMP-AMC-CN	No hydrolysis
$R_{EC}4$	AMP-CN-TOB	Full hydrolysis
$R_{EC}5$	AMC	Partial hydrolysis
$R_{EC}6$	AMP-AMC-CN-TOB	Full hydrolysis
$R_{EC}8$	AMP-CAZ	Partial hydrolysis
$R_{EC}9$	AMP-AMC-CAZ	Full hydrolysis
$R_{EC}10$	AMC-CAZ	Partial hydrolysis
$R_{EC}11$	AMP-AMC-CN-TOB	Partial hydrolysis
$R_{EC}13$	AMP-AMC-TOB	Partial hydrolysis
$R_{EC}14$	AMP-TOB-NOR	Partial hydrolysis
$R_{EC}15$	AMC	No hydrolysis
$R_{EC}16$	AMP-CN-TOB	Partial hydrolysis
$R_{EC}17$	AMP-CN-TOB	Partial hydrolysis
$R_{EC}18$	AMC-CN-NOR	Partial hydrolysis
$R_{EC}20$	AMC-CN-TOB	Full hydrolysis
$R_{EC}21$	AMC-CAZ	Full hydrolysis
$R_{EC}22$	AMP-AMC-TOB	Partial hydrolysis
$R_{EC}24$	AMC-TOB	Partial hydrolysis
$R_{EC}25$	AMP-AMC-TOB	Partial hydrolysis
$R_{EC}28$	AMP-AMC-CAZ	Full hydrolysis

AMP (ampicillin – 10µg), AMC (amoxicillin-clavulanic acid – 20µg-10µg), CAZ (ceftazidime – 10µg), CTX (cefotaxime – 5µg), CN (gentamicin – 10µg), TOB (tobramycin – 10µg), AZT (aztreonam – 30µg), TGC (tigecycline – 15µg), NOR (norfloxacin – 10µg), and CIP (ciprofloxacin – 5µg).

As none of the faecal *Enterococcus* spp. isolates from commercial ducks displayed resistance against any of the tested β-lactam antibiotics, none were tested. Results for the faecal *Enterococcus* spp. isolates from sacred ibis (three) and raptors (five) are shown in Table 3.11. In contrast to *E. coli* isolates obtained from wild sacred ibis and raptors, none of the faecal *Enterococcus* spp. isolates showed full hydrolysis activity. However, partial hydrolysis was observed for two of the three sacred ibis

Enterococcus spp. isolates (67%) tested and three of the five tested raptor isolates (60%) tested. A total of three enterococci isolates (one from sacred ibis, and two from raptors) showed no color chance, indicating the absence of nitrocefin hydrolysis activity.

Table 3.11. Detection of β-lactamase activity in *Enterococcus* spp. isolates (exhibiting resistance to at least one β-lactam antibiotic) from ducks, sacred ibis, and raptors faecal samples, based on nitrocefin hydrolysis.

***Enterococcus* spp. isolate**	**Antibiotic resistance phenotype**	**Nitrocefin result**
Sacred ibis		
$S_{ET}15$	AMP-IPM	Partial hydrolysis
$S_{ET}16$	AMP	No hydrolysis
$S_{ET}25$	AMP	Partial hydrolysis
Raptor		
$R_{ET}7$	AMP	No hydrolysis
$R_{ET}15$	AMP	Partial hydrolysis
$R_{ET}24$	AMP-IPM	Partial hydrolysis
$R_{ET}25$	IPM	No hydrolysis
$R_{ET}30$	AMP	Partial hydrolysis

AMP (ampicillin – 2µg), and IPM (imipenem – 10µg)

3.3.1 β-lactamase Activity Confirmation

To validate the hydrolysis of nitrocefin by β-lactamase activity, a representative faecal *E. coli* isolate from sacred ibis ($S_{EC}25$) with a positive result (full hydrolysis) in the nitrocefin spot test was analysed using UV-Vis spectrophotometry. As a negative control, an *E. coli* isolate from raptors faecal sample ($R_{EC}3$), resistant to more than one β-lactam antibiotic but with a negative nitrocefin spot test result (no hydrolysis), was selected. As depicted in Figure 13, the presence of nitrocefin in the control sample (sodium phosphate buffer and 50µL nitrocefin (0.5mg/mL)) resulted in an absorbance maximum at 390 nm. A similar absorbance maximum was present after

incubation of the sample with the faecal *E. coli* isolate ($R_{EC}3$) from raptors, confirming no hydrolysis of nitrocefin as seen in the nitrocefin spot test. However, the change in the spectrum to an absorbance maximum at 480 nm in the sacred ibis *E. coli* isolate ($S_{EC}25$) was indicative of the hydrolytic cleavage of nitrocefin by β-lactamase. The results obtained for these samples provide confirmation of the nitrocefin spot test.

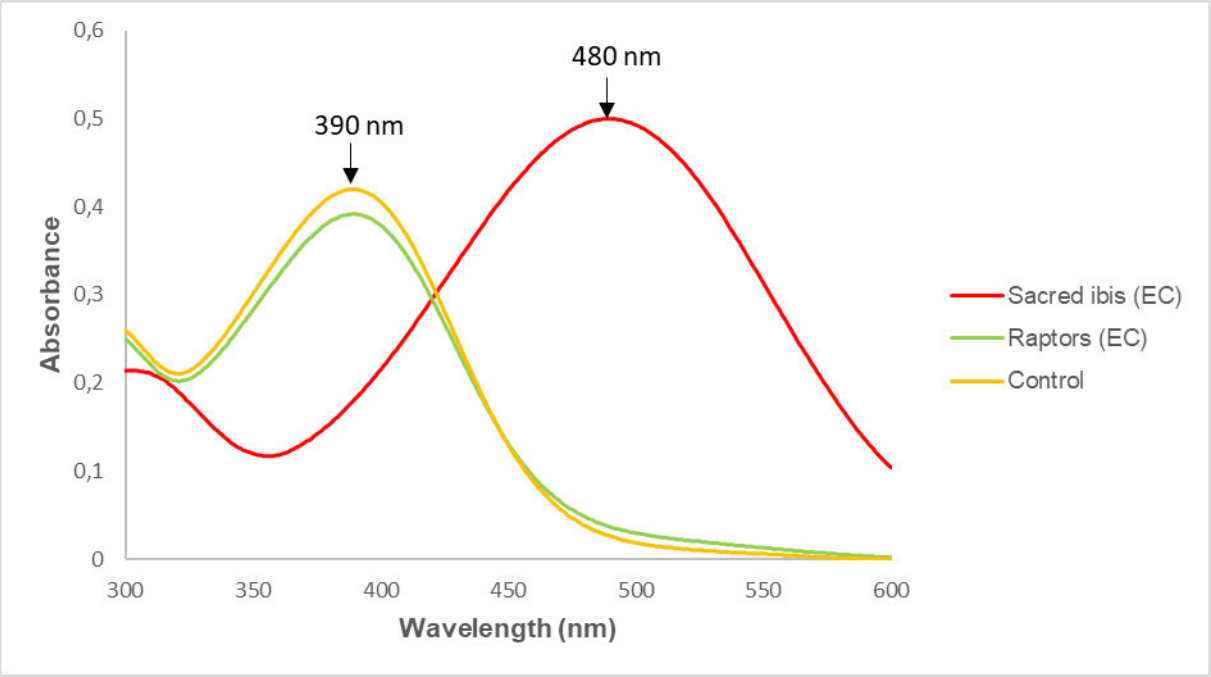

Figure 3.12. UV-Vis spectra analysis of the β-lactamase activity assay of two selected *E. coli* isolates (SEC25 and REC3)

$S_{EC}25$: *E. coli* isolate from sacred ibis with full hydrolysis in the nitrocefin spot test (resistant to the β=lactams ampicillin and amoxicillin-clavulanic acid).

$R_{EC}3$: *E. coli* isolate from raptors with no detectable hydrolysis in the nitrocefin spot test (resistant to the β=lactams ampicillin and amoxycillin-clavulanic acid)

Control: sodium phosphate buffer and 50µL nitrocefin (0.5mg/mL).

Test conditions: the absorbance was determined following incubation of cell suspensions at 30°C for 30min in the dark in the presence of nitrocefin.

CHAPTER 4 : DISCUSSION

4.1 Microbial Burden

This study investigated the microbial burden in faecal samples from commercial free-range ducks, wild sacred ibis, and raptors, revealing significant differences between these three avian groups. These differences provide insights into the potential health risks and environmental impacts associated with microbial contamination from these birds. The findings are supported by and contrasted with existing literature, highlighting the complex interplay between bird species, their habitats, and microbial exposure.

Total and Faecal Coliforms

The results indicate that wild raptors had the highest faecal microbial load of 8.45 log MPN/g for total coliforms and 8.11 log MPN/g for faecal coliforms. In contrast, commercial ducks and wild sacred ibis exhibited lower but comparable levels of microbial burden, with total coliforms at 7.54 log MPN/g and 7.23 log MPN/g respectively, and faecal coliforms ranging from 6.73 log MPN/g in ducks to 7.15 log MPN/g in sacred ibis.

These findings align with studies reporting high bacterial counts in bird faeces with varied diets and exposure to contaminated environments. For example, Giacopello *et al*. (2016) observed that raptors had a higher prevalence of Enterobacteriaceae (59% of total isolates) than waterbirds (22%) and passerines (19%), consistent with the variations of total and faecal coliform levels found in this study. Similarly, Moriarty *et al*. (2011) reported that wild ducks had total coliform counts ranging from 5.8 to 8.0 log CFU/g and faecal coliform counts between 4.4 to 8.0 log CFU/g, which matches the values observed in both the wild birds and commercial ducks in this study. Trawińska *et al*. (2016) also found that laying hens in a farm setting had total coliform levels ranging from 5.8 to 6.4 log CFU/g, highlighting that birds in managed environments like commercial farms might have lower coliform levels compared to wild birds, again matching the results of the current study.

Escherichia coli

The *E. coli* load was highest in faecal samples from raptors with 7.86 log MPN/g, followed by sacred ibis with 6.90 log MPN/g and ducks with 6.73 log MPN/g. The *E. coli* loads observed in commercial ducks are similar to the range, albeit slightly higher, reported by Trawińska *et al*. (2016), who found *E. coli* levels from 4.5 to 6.4 log CFU/g in faecal samples from a laying-hen farm.

For wild birds, Telesford-Checkley *et al*. (2017) reported *E. coli* levels in egrets, which share similar lifestyles to sacred ibis, ranging from 4 to 9 log CFU/g, which corresponds to the *E. coli* load established for faecal samples from sacred ibis. Malekian *et al.* (2021) documented *E. coli* levels in wild birds such as rooks, starlings, sparrows, and gulls ranging from 4 to 7 log CFU/g. The elevated level in raptors suggests that this bird population are likely to encounter environments with greater bacterial burden, possibly due to their predatory nature and scavenging habits, which expose them to a wider range of this hygiene indicator species.

***Enterococcus* spp.**

Enterococcus spp. counts were also highest in raptors (5.30 log CFU/g), compared to sacred ibis (4.80 log CFU/g) and ducks (4.05 log CFU/g). This pattern suggests that raptors carry a higher burden of enterococci and shed larger quantities into the environment via faeces. This in turn, like for *E. coli*, can contribute to the contamination of water bodies (Telesford-Checkley *et al*., 2017) and even fresh produce (Smith *et al*., 2020). Studies support these findings, with Vela *et al*. (2015) reporting *Enterococcus* spp. as a predominant bacterium in avian samples, especially in species that have known interactions with various environments, such as crop and livestock farms. Moriarty *et al*. (2011) found that wild ducks had enterococci counts ranging from 4.4 to 8.0 log CFU/g, which is consistent with the levels found in the three bird species examined in this study.

PCR results showed a higher prevalence of *E. faecalis*, an opportunistic pathogen, in wild sacred ibis (40%) and raptors (47%), compared to only 10% in commercial ducks. Indeed, the statistical analysis revealed significant differences between commercial ducks and both wild birds ($p<0.05$), but not between the wild sacred ibis

and raptors (p=0.6023). This suggests a potential shared ecological or dietary exposure to *E. faecalis* in wild birds, which may not be as prominent in commercial settings. Middleton and Ambrose (2005) found that faecal samples from wild migratory geese in Canada contained between 2 to 7 log CFU/g enterococci, which indicates a considerable presence and variance of these bacteria in wild birds.

Control soil samples

Control soil samples showed markedly lower microbial counts, with total and faecal coliforms ranging from 1.89 to 2.36 log MPN/g, and being below the detection limit for *E. coli* and *Enterococcus* spp. This disparity underscores the substantial impact of avian faecal matter on environmental microbial pollution. According to Meerburg *et al*. (2011), faeces from birds such as wild gulls and geese, can contain *E. coli* levels reaching 10 to 11 log CFU/g of excrement, illustrating the significant contamination risk posed by high microbial loads in avian faeces. Consequently, these findings highlight the potential for water sources used in the irrigation of ready-to-eat produce to be contaminated by avian-origin microorganisms, emphasizing the importance of mitigating this risk through appropriate agricultural practices and monitoring measures.

Species-specific findings

Raptors exhibited the highest microbial burden across all measured parameters (i.e., total, and faecal coliforms, *E. coli*, and *Enterococcus* spp.). Their diet, which includes carrion and potentially contaminated and deteriorating prey, likely contributes to this increased faecal bacterial burden observed. Benskin *et al*. (2009) noted that raptors and scavenging birds often have higher pathogen loads due to their feeding habits and exposure to human-associated waste, which is consistent with the high microbial counts observed in this study. Giacopello *et al*. (2016) reported that raptors showed higher prevalence of Enterobacteriaceae, with *E. coli* carriage rates of 53.1% (n=32).

Wild sacred ibis displayed a slightly lower microbial burden than raptors but still carried elevated levels of total and faecal coliforms, *E. coli* and *Enterococcus* spp. The sacred ibis' feeding behaviour, which includes scavenging in urban environments and foraging in polluted wetlands, and wastewater treatment plants,

likely contributes to their microbial load. This finding is consistent with Maraci *et al*. (2022), who showed that birds in urban settings have higher counts of Enterobacteriaceae, with counts in urban birds being up to 4 log higher than in rural birds, likely due to greater exposure to human-associated waste, and therefore different diets.

Commercial ducks had the lowest microbial burden among the studied birds, although still substantial. Their lower exposure to varied and potentially contaminated diets compared to wild birds likely contributes to this. Studies on commercial birds, such as by Younessi *et al*. (2022), report coliform counts in industrial poultry ranging from 6.67 to 8.75 log CFU/g, which is slightly higher than the values observed in the free-range ducks in this study. However, the diet and environment typical of free-range organic farming may contribute positively to lower microbial faecal loads overall.

A One-Health outlook

The higher faecal microbial loads of wild raptors and sacred ibis highlight the importance of monitoring these birds as vectors for microbial contamination and pathogen transmission, which can impact both the environment, wildlife, and human health. Meerburg *et al*. (2011) emphasized the potential for bird faeces to contaminate water sources, presenting a risk for pathogen spread via overhead irrigation. Effective management strategies, including habitat management and waste control, are crucial in mitigating these risks. Benskin *et al*. (2009) further noted that birds feeding at human-provided sources can carry bacteria like *Salmonella* and pathogenic strains of *E. coli*, which can pose risks to public health.

This study highlights substantial differences in the microbial burden among different bird species, with wild birds showing higher levels of hygiene indicator bacteria such as *E. coli*, compared to commercial ducks. These findings underscore the importance of monitoring hygiene indicators across various One Health compartments – humans, animals, and the environment – to detect potential transmission scenarios linked to bird populations. Implementing targeted surveillance and management strategies is

crucial for mitigating microbial contamination in diverse environments that might affect human health.

4.2 Antibiotic Resistance

To understand the patterns of bacterial antibiotic resistance (ABR) in different avian populations, this study assessed ninety *E. coli* and ninety *Enterococcus* spp. isolates from the faeces of commercial ducks, wild sacred ibis, and raptors. Using the EUCAST disk diffusion assay (EUCAST, 2021), notable differences in resistance profiles between the confirmed *E. coli* and *Enterococcus* spp. isolates from different birds were observed. *E. coli* demonstrated a higher frequency of ABR across all bird species compared to *Enterococcus* spp. *Enterococcus* spp. isolates from commercial duck faeces showed no ABR, whereas isolates from wild sacred ibis and raptors exhibited moderate to high antibiotic resistance levels (33% and 47%, respectively) categorized according to the European Food Safety Authority (EFSA, 2021). For *E. coli*, resistance levels (i.e., ABR to at least one of the tested antibiotics) in isolates from ducks were 47%, while *E. coli* isolates from sacred ibis and raptors showed much higher levels at 73% and 93%, respectively. The findings highlight substantial disparities in ABR between *E. coli* and *Enterococcus* spp., with a pressing issue of high ABR in *E. coli* from wild birds and a lower prevalence in commercial free-range ducks compared to wild bird populations.

4.2.1 *Escherichia coli*

The resistance patterns observed in this study reveal significant differences between *E. coli* isolates from commercial ducks and wild birds ($p<0.05$). Specifically, *E. coli* from ducks exhibited resistance to three out of the twelve tested antibiotics (amoxicillin-clavulanic acid, gentamicin, and tobramycin) and showed intermediate resistance to three others (meropenem, ceftazidime, and aztreonam). In contrast, *E. coli* isolates from wild birds, including sacred ibis and raptors, demonstrated resistance to a broader range of antibiotics (i.e., ampicillin, amoxicillin-clavulanic-

acid, ceftazidime, gentamicin, tobramycin, and norfloxacin) and exhibited intermediate resistance to ciprofloxacin and aztreonam. Additionally, among the wild birds, isolates from raptors showed intermediate resistance to meropenem, a carbapenem, and cefotaxime, a third-generation cephalosporin. These findings highlight very notably distinct antibiotic resistance profiles, indicating that wild birds, particularly, raptors, may harbour *E. coli* strains with a higher propensity for ABR compared to those from commercial ducks.

The results of the pairwise comparison's using Pearson's X^2 test further support these observations, revealing statistically significant differences in resistance frequencies between *E. coli* isolates from commercial ducks and those from both wild sacred ibis ($p=0.0099$) and raptors ($p=0.0001$). However, no statistically significant difference was observed between resistance frequencies in wild sacred ibis and raptors ($p>0.05$), indicating a higher level of similarity in resistance patterns of *E. coli* within the two wild bird groups.

These findings highlight the critical need to monitor and manage antibiotic resistance in both commercial and avian populations to mitigate potential public health risks, as *E. coli* exhibiting resistance to clinically important antibiotics is listed on the World Health Organization's (WHO) list of priority ABR pathogens (WHO, 2024).

4.2.1.1 β-Lactam resistance

The investigation into *E. coli* resistance to four classes of β-lactam antibiotics provided valuable insights into the prevalence and patterns of resistance within each antibiotic category. The classes tested included penicillins (ampicillin and amoxicillin-clavulanic acid), carbapenems (ertapenem and meropenem), cephalosporins (ceftazidime and cefotaxime), and monobactam (aztreonam). The data from this study indicated that β-lactam antibiotic resistance is present across all bird categories, however with varying degrees. *E. coli* isolates from wild raptors exhibited the highest β-lactam resistance frequencies, followed by wild sacred ibis, and then commercial ducks.

Commercial birds

It was found that the *E. coli* isolates from the commercial ducks exhibited low antibiotic resistance to amoxicillin-clavulanic acid (3%) and intermediate resistance to meropenem (3%), and ceftazidime (33%), the intermediate resistance to aztreonam was notably higher at 73%. This finding suggests that while resistance to β-lactam antibiotics like aztreonam is not widespread among *E. coli* from commercial ducks, there is a notable presence of intermediate resistance levels. No resistance (100% susceptibility) was observed towards ampicillin, ertapenem, and cefotaxime.

Comparatively, Martínez-Álvarez *et al*. (2022) have reported higher resistance frequencies for ampicillin (>40%) in poultry settings, with no resistance to carbapenems or cephalosporins. Additionally, research by Jung *et al*. (2023) in integrated broiler chicken operations in Korea highlighted β-lactam antibiotics as the most frequently prescribed, resulting in substantial proportions of β-lactam resistant *E. coli* in faecal samples (95%) and farm dust samples (61%). Moreover, these authors found that β-lactam resistant *E. coli* isolates were more likely to exhibit multidrug-resistance (MDR) than non-β-lactam resistant isolates.

These differences underscore the impact of β-lactam antibiotic usage practices in different farming systems. More intensive antibiotic use in commercial large scale poultry farms, as seen in integrated operations, can contribute to higher levels of resistance compared to free-range systems observed in the current study site with the free-range ducks. Understanding these patterns is crucial for developing strategies to mitigate ABR in agricultural operations and to control its potential spread into public health and the environment, which is a key aspect of the One Health concept (Daoud and Rolain, 2022).

Wild birds

E. coli isolates from wild sacred ibis exhibited notable resistance to several β-lactam antibiotics, including 23% resistance to ampicillin, 33% to amoxicillin-clavulanic acid, and 7% to ceftazidime, a third-generation cephalosporin. High levels of intermediate resistance were observed for ceftazidime (30%) and aztreonam (20%). No resistance

(100% susceptibility) was observed towards the carbapenems. Similarly, isolates from wild raptors demonstrated the highest resistance frequencies among the studied wild bird groups, with 43% resistance to ampicillin, 50% to amoxicillin-clavulanic acid, and 17% to ceftazidime. Intermediate resistance was noted for meropenem (3%), ceftazidime (53%), cefotaxime (7%), and aztreonam (27%). This is concerning due to the potential of emerging resistance among such isolates, as the intermediate category means that such strains can easily develop resistance mechanisms towards the particular antibiotic upon exposure (WHO, 2024). No resistance (100% susceptibility) was observed ertapenem. These findings indicate distinct β-lactam resistance profiles and a higher prevalence of β-lactam resistance compared to commercial ducks, possibly influenced by environmental factors and diet.

Available studies support varying degrees of resistance in *E. coli* from wild birds. Nowaczek *et al*. (2021) found 28% ampicillin resistance in *E. coli* isolates from wild birds in Poland, which aligns with the current study's findings from sacred ibis *E. coli* isolates. Ahmed and Gulhan (2024) reported 28% ampicillin resistance in *E. coli* from wild gulls and pigeons, which share similar lifestyles to sacred ibis. Raptors may face increased exposure to resistant bacteria due to their predatory diet and environment, as supported by Prandi *et al*. (2023), who found a higher prevalence of ABR *E. coli* in raptors (74%) compared to synanthropic species (26%). Furthermore, ABR *E. coli* from raptors demonstrated elevated resistance to cephalosporins, such as cephalexin (85%), and penicillins, such as amoxicillin-clavulanic acid (40%) (Prandi *et al*., 2023).

Additionally, Ong *et al*. (2020) discovered that *E. coli* isolates from wild birds in Singapore exhibited remarkably higher resistance frequencies to antibiotics compared to *E. coli* isolates from rodents. The most common resistances were against ampicillin, followed by amoxicillin-clavulanic acid, with no resistance to meropenem, consistent with the findings of this study for *E. coli* isolates from the wild bird groups. These results underscore the role of wild birds, such as raptors and sacred ibis, in the possible long-distance dissemination of β-lactam resistant *E. coli* compared to rodents traditionally recognized as local carriers of pathogens (Ong *et al*.,2020).

4.2.1.2 Aminoglycoside resistance

The investigation into *E. coli* resistance to two aminoglycoside antibiotics, gentamicin, and tobramycin, revealed notable differences in resistance patterns between *E. coli* isolates from commercial ducks and wild birds (sacred ibis and raptors).

Commercial birds

In commercial ducks, the resistance of *E. coli* to gentamicin was found to be 33%, making it the highest resistance observed for *E. coli* isolates from this group. Tobramycin resistance was established as 20%. These results indicate a moderate level of resistance to aminoglycosides in commercial ducks.

Interestingly, Assoumy *et al*. (2021) reported a higher gentamicin resistance (45%) in *E. coli* from poultry farms in Côte d'Ivoire, highlighting a stark contrast to the 33% observed in this study. However, these authors found an even higher resistance (71%) to streptomycin, an older aminoglycoside not tested in this study, suggesting that resistance levels can vary significantly depending on the specific aminoglycoside tested. Indeed, streptomycin is frequently used in agriculture (Assoumy *et al*., 2021).

Cox *et al*. (2022) found co-resistance with gentamicin and β-lactam antibiotics in chicken-source *E. coli* isolates, which might explain the observed higher intermediate resistance to aztreonam in the commercial duck isolates in this study. Shrestha *et al*. (2022) reported *E. coli* aminoglycoside resistance in commercial Canadian turkey flocks at 45% in antibiotic-treated flocks and 32% in untreated flocks, indicating that the resistance observed in commercial ducks in the current study, falls within a comparable range given the free-range system.

In contrast, Dreyer *et al*. (2022) found no *E. coli* resistance to gentamicin or tobramycin in wild ducks in Germany, and Kocúreková *et al.* (2021) reported less than 3% resistance to these antibiotics in commercial broilers in Slovakia. These differences underscore the potential influence of ABR *E. coli* occurrence within duck populations, local farming practices, and veterinary antibiotic use on resistance patterns.

Wild birds

E. coli isolates from wild sacred ibis showed a higher frequency of resistance at 47% to both gentamicin and tobramycin. For wild raptors, the gentamicin resistance frequency was also 47%, while tobramycin resistance was even higher at 50%, the highest resistance frequency observed for this particular antibiotic. These results align with findings by Suárez-Pérez *et al.* (2021), who reported 50% resistance to gentamicin and 33% tobramycin resistance for *E. coli* isolated from vultures in Spain.

In contrast, Musa *et al.* (2023) observed a lower gentamicin resistance frequency (10%) in *E. coli* from wild birds (including raptors) in Italy, which is substantially lower than the 47% found in the isolates from the wild birds of this study. Similarly, Rybak *et al*. (2022) reported 27% resistance to aminoglycosides (including gentamicin) in *E. coli* from free-living birds in human habitats in Poland, which is lower than the resistance levels of *E. coli* isolates observed in sacred ibis in the current study, which share a similar lifestyle.

However, a study in South Africa, which assessed ABR *E. coli* isolated from roof-harvested rainwater contaminated by pigeon faeces, found that the second and third most common phenotypic resistance detected was towards gentamicin and amikacin, although at lower levels of 24% (Chidamba and Korsten, 2015). Despite the aminoglycoside resistance being 24%, it was the second most prevalent frequency observed in that study. Conducted in suburban areas of Gauteng, South Africa, the same country as this study, there might be a link to shared environmental factors contributing to the observed resistance frequencies, although the gentamicin resistance in this study was higher.

The findings of this study, when compared to existing studies, reveal varying frequencies of aminoglycoside resistance in *E. coli* across different bird populations. The higher resistance levels observed in *E. coli* isolates from the wild birds of this study are concerning and suggest that environmental exposure and lifestyle play an important role in ABR development, while the moderate resistance levels for aminoglycosides in *E. coli* isolates from the commercial ducks align with findings from regions with varied farming practices and environments.

4.2.1.3 Tetracycline resistance

The investigation into *E. coli* resistance to the tetracycline antibiotic, tigecycline, revealed complete susceptibility (0% resistance) across *E. coli* isolates from all bird categories studied, including commercial ducks, wild sacred ibis, and raptors. This finding is particularly noteworthy given tigecycline's status as a last-resort antibiotic, used primarily for the treatment of clinical multidrug-resistant (MDR) infections (WHO, 2024). The absence of resistance to tigecycline observed in this study is encouraging. It suggests that the current use of tigecycline, typically in clinical settings, has not led to resistance development in *E. coli* from these bird populations. This finding has important public health implications, indicating that tigecycline resistance is currently not disseminated by the three bird groups analyzed in the current study.

Commercial birds

Mohsin *et al*. (2021) reported the presence of the plasmid-mediated tigecycline resistance gene *tet*(X4) in *E. coli* from commercial poultry in Pakistan. However, only phenotypic resistance to tetracycline, not tigecycline, was detected, although it was reported as tigecycline-resistant. This discrepancy underscores the importance of assessing phenotypic resistance rather than solely relying on genotypic traits, as tetracycline resistance-encoding genes, such as the monooxygenase encoded by *tet*(X4), do not necessarily confer tigecycline resistance. The absence of tigecycline resistance in the current study aligns with the need for careful interpretation of resistance reports and highlights the robustness of tigecycline's efficacy, even among *E. coli* isolates from commercial settings.

Wild birds

Chen *et al*. (2019) investigated tigecycline resistance mechanisms in *E. coli* strains from migratory birds in China. They found that three tetracycline-resistant *E. coli* strains from egrets were also resistant to tigecycline and, interestingly, florfenicol, and sulfamethoxazole-trimethoprim, antibiotics representing different antibiotic classes. This finding contrasts with the current study's results, which showed no tigecycline resistance in *E. coli* isolates from wild birds. The difference underscores the necessity for continuous surveillance of resistance patterns in birds, as

environmental factors and geographic locations can significantly influence resistance profiles.

4.2.1.4 Fluoroquinolone resistance

Bird associated *E. coli* resistance to two fluoroquinolone antibiotics, norfloxacin and ciprofloxacin, was additionally investigated. The findings highlighted varying degrees of resistance, with *E. coli* isolates from commercial ducks showing complete susceptibility to these crucial antibiotics, while sacred ibis, and raptors exhibited some degree of resistance and intermediate resistance. Specifically, *E. coli* isolates from sacred ibis showed 3% resistance to norfloxacin and 10% intermediate resistance to ciprofloxacin, while raptor derived *E. coli* exhibited 10% resistance to norfloxacin and 17% intermediate resistance to ciprofloxacin. The presence of intermediate resistance in wild birds, particularly in raptors, is concerning, as it suggests potentially increased resistance development over time.

<u>Commercial birds</u>

Aworh *et al*. (2023), who investigated fluoroquinolone-resistant *E. coli* in poultry environments in Nigeria, found a substantially higher resistance frequency of 29% for ciprofloxacin, which contrasts with the current study, as no resistance to the tested fluoroquinolones was observed in *E. coli* isolates from commercial ducks. This disparity could be attributed to differences in in antibiotic usage practices and environmental exposure.

Perrin-Guyomard *et al*. (2020) demonstrated that decreased fluoroquinolone usage in poultry farming in France, led to a reduction in ciprofloxacin-resistant *E. coli* from 21.3% to 15.9% in turkeys between 2011 and 2018. The absence of fluoroquinolone resistance in commercial ducks in this study aligns with these findings, highlighting the positive impact of reduced antibiotic use in farming practices. The free-range environment of the ducks in this study, without known exposure to these antibiotics, likely contributed to the observed 100% susceptibility.

Wild birds

Varying levels of fluoroquinolone resistance in *E. coli* isolates from Australian wild birds were reported by Mukerji *et al*. (2020), with 47% in silver gulls' isolates, notably higher than the resistance observed in *E. coli* isolates from wild sacred ibis (3%) and raptors (10%) in this study. Additionally, these authors found 8.8% resistance to fluoroquinolones, such as ciprofloxacin, among *E. coli* isolates from feral pigeons, and 7% in penguin isolates, which is similar to the frequency observed for isolates from raptors (Mukerji *et al*., 2020). This suggests differences in exposure to fluoroquinolones due to inhabiting different ecological niches among different bird species.

Islam *et al*. (2021) reported high levels of ciprofloxacin resistance (50.9%) in *E. coli* from wintering migratory birds in Bangladesh. This is in stark contrast with the 0% ciprofloxacin resistance found in wild birds in the present study, indicating the importance of regional differences in antibiotic exposure and resistance development.

Haenni *et al*. (2020) observed 13.6% and 5.7% fluoroquinolone resistance in ESBL-producing *E. coli* from wild birds admitted to a rescue hospital, using nalidixic acid and enrofloxacin. While different fluoroquinolones were used, the resistance levels are notable due to the intermediate resistance seen in wild raptors in this study, which can lead to the rapid development of resistance if exposure to fluoroquinolones is increased.

Ong *et al*. (2020) found varying degrees of fluoroquinolone resistance among wild birds, with 11.5%, 7.7%, and 19.2% resistance to ciprofloxacin, norfloxacin, and nalidixic acid, respectively. This matches the fluoroquinolone resistance results obtained for *E. coli* isolates from sacred ibis and highlights the importance of continuous surveillance. Especially considering that birds, such as the sacred ibis, often frequent wastewater treatment plants and are potentially exposed to untreated hospital effluent, which may contain fluoroquinolone residues given the clinical use of fluoroquinolone antibiotics (Telesford-Checkley *et al*., 2017).

4.2.1.5 Multidrug resistance (MDR)

Upon analyzing the observed antibiotic resistance (ABR) phenotypes among *E. coli* isolates from commercial ducks, wild sacred ibis, and raptors, it became evident that the commercial duck isolates exhibited no multidrug-resistance (MDR) patterns, which is a positive finding. These *E. coli* isolates primarily showed resistance to gentamicin and tobramycin, with no MDR phenotypes detected, highlighting the possible benefits of free-range farming practices.

In contrast, *E. coli* isolates from wild sacred ibis exhibited a broader spectrum of antibiotic resistance, while those from wild raptors displayed the highest diversity in resistance phenotypes. Notably, MDR isolates were identified in both wild bird populations (7% MDR profiles for *E. coli* isolates from each bird group), with varied resistance phenotypes observed. The two MDR isolates from sacred ibis displayed an AMP-AMC-CAZ-CN resistance profile and an AMP-AMC-CAZ-CN-TOB-NOR resistance profile, whereas the two MDR isolates from raptors displayed AMP-TOB-NOR and AMC-CN-NOR resistance profiles. This broader spectrum of resistance observed in sacred ibis MDR isolates is likely attributable to environmental conditions and antibiotic residue exposure due to close proximity to human-influenced areas, and contact with the local wastewater treatment plant.

These findings align with reported MDR *E. coli* in wild birds, although at lower frequencies. For instance, Yuan *et al*. (2021), investigated migratory wild birds in China and found that the proportion of MDR phenotypes among all *E. coli* isolates was 15.3%. Similarly, Mohamed *et al*. (2022) reported MDR *E. coli* isolates in the range of 33% to 100% from wild birds in six different villages in Malaysia, indicating a large presence of MDR *E. coli* phenotypes in various wild bird populations within close proximity to human-influence.

Dietary habits of raptors may also influence the variability of ABR phenotypes. Suárez-Pérez *et al*. (2021) reported that 45% of *E. coli* isolates from raptors were classified as MDR, which might explain the high diversity of resistance phenotypes observed in the raptor isolates.

In summary, while the commercial duck *E. coli* isolates generally showed low frequencies of ABR and no MDR profiles, the wild bird *E. coli* isolates demonstrated a higher frequency of ABR phenotypes and even MDR patterns. These patterns are comparable to findings in other regions and matching bird groups, highlighting the critical role of environmental factors and human activities in the development and spread of ABR within and among wild bird populations.

4.2.2 *Enterococcus* spp.

Enterococcus spp. isolates from the faeces of commercial ducks displayed no resistance to any of the eight antibiotics tested (ampicillin, imipenem, gentamicin, tigecycline, norfloxacin, teicoplanin, vancomycin, and linezolid). However, intermediate resistance was observed against ampicillin (10%) and imipenem (100%) among the enterococci isolates from commercial ducks. The lack of resistance among these isolates suggests that the controlled environment and regulated use of antibiotics in commercial free-range duck farming may limit the development and spread of ABR *Enterococcus* strains.

In contrast, *Enterococcus* spp. isolates from both wild bird species (sacred ibis and raptors) exhibited resistance (i.e., at least one isolate) to the same five antibiotics (ampicillin, imipenem, teicoplanin, vancomycin, and linezolid). This notable resistance frequency indicates a higher level of ABR among enterococci in the faeces of wild birds compared to the commercial ducks, reflecting the birds' exposure to diverse environmental sources of antibiotic contamination, including natural habitats and human-influenced areas such as wastewater treatment plants.

The statistical analysis using Pearson's X^2 test further supports these findings. A significant difference in resistance frequencies was observed between *Enterococcus* spp. isolates from commercial ducks and wild sacred ibis ($p=0.0025$), and between isolates from commercial ducks and wild raptors ($p=0.0001$). However, no significant difference was found between the isolates from wild birds (i.e., sacred ibis and raptors; $p=0.5717$). These results demonstrate the significant disparity in ABR

frequency between enterococci from commercial and wild bird populations, while the similarity between sacred ibis and raptors suggests comparable environmental pressures and lifestyles contributing to ABR.

4.2.2.1 β-Lactam resistance

In this study, the β-lactam antibiotics ampicillin (penicillin class) and imipenem (carbapenem class) were used to assess β-lactam resistance in *Enterococcus* spp. The high incidence of enterococci isolates being classified as intermediate resistant to imipenem is particularly concerning and warrants further investigation.

Commercial birds

In commercial ducks, the absence of ampicillin resistance (0%) with only a small proportion of isolates showing intermediate resistance (10%) indicates a relatively low level of β-lactam resistance. Similarly, while no faecal *Enterococcus* spp. isolates were resistant to imipenem, all isolates from ducks exhibited intermediate resistance (100%).

Comparing these findings with the existing literature, Noh *et al*. (2020) also reported lower levels of β-lactam resistance in *E. faecalis* from broiler farms in Korea, with 8.3% resistance to penicillin and 3.5% to ampicillin. Farming practices at those sample sites were akin to standard commercial farming practices, which may have influenced the prevalence of β-lactam resistance, albeit still at low levels. Alzahrani *et al*. (2022) found higher levels of ampicillin resistance (30%) in backyard chicken flocks in Saudi Arabia, potentially due to closer human interaction and possibly environmental contamination. Zowalaty *et al*. (2023) observed 10% ampicillin resistance and 5% imipenem resistance, with 95% intermediate imipenem resistance among enterococci isolates from small-scale livestock farms (including ducks), in South Africa, which aligns with the current findings.

Wild birds

Enterococcus spp. isolates from wild sacred ibis exhibited 10% resistance and 10% intermediate resistance to ampicillin, with a smaller proportion of resistance to

imipenem (3%) and a high level of intermediate resistance (97%) observed. Isolates from wild raptors showed the highest levels of β-lactam resistance among the three bird groups studied, with 13% resistance and 13% intermediate resistance to ampicillin, and 7% resistance and 93% intermediate resistance to imipenem observed.

These results match with the findings from Cagnoli *et al*. (2022), who found 12% and 11% ampicillin resistance in enterococci isolates from wild raptors and synanthropic birds, respectively. However, Kwit *et al*. (2023) reported no ampicillin resistance in enterococci from free-living birds in Poland. In contrast, Islam *et al*. (2021) found significantly higher resistance levels in migratory birds in Bangladesh, with 100% of *Enterococcus* spp. isolates resistant to ampicillin and 50% resistant to meropenem, like imipenem a carbapenem antibiotic.

4.2.2.2 Aminoglycoside resistance

In this study, the aminoglycoside antibiotic gentamicin was tested against *Enterococcus* spp. isolates. Remarkably, no resistance was detected among any of the ninety isolates, indicating 100% susceptibility across isolates from commercial ducks, wild sacred ibis, and raptors. This absence of resistance towards a clinically important antibiotic is a promising finding for these bird populations.

In commercial ducks, the lack of gentamicin resistance (100% susceptibility) contrasts sharply with findings from Ahmed *et al*. (2020), who reported high resistance levels (72% - 100%) in *Enterococcus* spp. from large-scale commercial poultry farms in Egypt. This discrepancy likely stems from differing farming practices, environmental conditions, and antibiotic usage protocols.

Similarly, in the wild birds, sacred ibis and raptors, no gentamicin resistance (thus 100% susceptibility) was found among *Enterococcus* spp. isolates. This aligns with Smith *et al*. (2022), who observed no resistance in *Enterococcus* spp. from wild Australian shorebirds and terns. It suggests that these Australian wild bird

populations had limited exposure to aminoglycoside antibiotics and possibly minimal human influence, which likely contributed to lower resistance levels.

The association between increased aminoglycoside resistance and higher levels of human interaction is supported by findings of Cabral *et al*. (2022), who reported 11% high-level gentamicin (120µg) resistance (HLGR) in *Enterococcus* spp. isolated from companion birds (captive parrots). Interestingly, HLGR has been reported to be more frequently found in *E. faecalis* than other *Enterococcus* spp. (Cabral *et al*, 2022). This finding underscores the impact of different environments and management practices, including veterinary interventions, on aminoglycoside resistance patterns in bacteria from different bird populations.

4.2.2.3 Tetracycline resistance

In this study, tigecycline, a last-resort tetracycline derivative used in human medicine, was tested against *Enterococcus* spp. isolates. Remarkably, no resistance was detected in any of the ninety isolates, indicating 100% susceptibility across isolates from commercial ducks, wild sacred ibis, and raptors. This is again a promising finding, matching the tigecycline resistance results obtained for *E. coli* isolates from the three birds of the current study, particularly since tigecycline is used to treat infections caused by bacteria resistant to other tetracyclines (WHO, 2024).

<u>Commercial birds</u>

The absence of tigecycline resistance (100% susceptibility) in enterococci from commercial ducks aligns with Rajendiran *et al*. (2022), who found no resistance among *Enterococcus* spp. isolates from poultry farm environments in Malaysia. This result is encouraging, as it eliminates the risk of poultry acting as potential vectors of tigecycline-resistant *Enterococcus* spp.

However, this finding contrasts with Kim *et al*. (2021), who reported tigecycline resistance among *Enterococcus* spp. isolates (5.5% resistance in *E. faecium* and 10.6% resistance in *E. faecalis*) from healthy chickens on poultry farms in South Korea. The difference may be attributed to variations in antibiotic usage practices

and regional differences in poultry farming patterns, underscoring the necessity for continued surveillance efforts.

Wild birds

In wild sacred ibis and raptors, the absence of tigecycline resistance (100% susceptibility) in *Enterococcus* spp. is indicative of tigecycline's effectiveness against ABR bacteria from bird faeces analysed in the present study. However, it differs significantly from the findings of Cagnoli *et al*. (2022), who reported a high level of tigecycline resistance (41%) in wild birds in central Italy. This discrepancy may also be due to environmental factors or human influences (e.g., interaction, contact, waste), considering the popularity of central Italy among tourists year-round.

The limited number of studies on tigecycline resistance in *Enterococcus* spp. among wild bird populations supports the notion that it remains an effective antibiotic, without the concern of wildlife as potential vectors as yet. Most studies focus on tetracycline resistance, with tigecycline resistance primarily discussed and reported in clinical contexts where tetracycline resistance has already developed. However, given the reports of tetracycline resistance in various environments, such as faecal enterococci tetracycline resistance (33%) from wild birds on remote islands (Santos *et al*., 2013), continued surveillance is essential to monitor emerging resistance patterns.

4.2.2.4 Fluoroquinolone resistance

The fluoroquinolone representative antibiotic used in this study was norfloxacin. Notably, no resistance was detected among any of the ninety *Enterococcus* spp. isolates, indicating 100% susceptibility across isolates from commercial ducks, wild sacred ibis, and raptors. This is an encouraging result, due to the clinical use of fluoroquinolone antibiotics in the treatment of serious infections and infections caused by MDR bacteria (WHO, 2024).

Commercial birds

The absence of norfloxacin resistance (100% susceptibility) in commercial ducks is a positive outcome, especially when compared to findings in other studies. For

instance, Osman *et al*. (2019) found 94%-100% norfloxacin and ciprofloxacin resistance among *Enterococcus* spp. isolates from chickens and ducks raised in small-scale backyard farming schemes in Egypt, suggesting a substantial influence of human contact and associated antibiotic exposure in such settings.

Similarly, Lee *et al*. (2023) reported high levels of ciprofloxacin resistance (73%-100%) among *Enterococcus* spp. isolates from five different integrated broiler operations in South Korea. Ciprofloxacin, like norfloxacin, is a fluoroquinolone, and the high resistance levels observed highlight the impact of inappropriate fluoroquinolone use in commercial farming practices. The lack of norfloxacin resistance in the current study reflects positively on the free-range farming practices, suggesting that minimal or no fluoroquinolone usage in these settings helps to prevent resistance development.

Wild birds

In wild sacred ibis and raptors, the absence of norfloxacin resistance (100% susceptibility) is consistent with the findings of Russo *et al*. (2022), who also reported the absence of fluoroquinolone resistance (0%; using ciprofloxacin) in *Enterococcus* spp. isolates from wild birds in Southern Italy. This indicates minimal exposure to fluoroquinolone antibiotics in wild bird populations, reducing the threat of these birds acting as vectors of fluoroquinolone-resistant *Enterococcus* spp. even across different countries.

The limited reports available on norfloxacin resistance in *Enterococcus* spp. in the literature, combined with the findings of this study, suggest that fluoroquinolone antibiotics, specifically norfloxacin, remain an effective antibiotic class for usage against Gram-positive bacterial infections, such as those caused by *Enterococcus* spp.

4.2.2.5 Glycopeptide/Lipopeptide resistance

The two representative antibiotics used in this study were teicoplanin and vancomycin. The results revealed varying levels of *Enterococcus* spp. resistance,

with isolates from the wild bird populations showing a higher resistance frequency compared to isolates from commercial ducks from free-range farming, where no resistance was detected.

Commercial birds

The absence of resistance to teicoplanin and vancomycin in *Enterococcus* spp. isolates from commercial ducks (0% resistance) is a notable finding, particularly when compared to other studies. For instance, Ahmed *et al*. (2020) found high levels of resistance (45% teicoplanin and 43% vancomycin) in *Enterococcus* spp. from poultry in small-scale backyard farms in Egypt. This stark difference may be attributed to the farming environment, as the current study's free-range duck farming practices likely minimize antibiotic exposure and subsequent selection pressure.

In contrast, Mwikuma *et al*. (2023) reported elevated vancomycin resistance levels of 33%-44% in *Enterococcus* spp. isolates from poultry in Zambia. This further underscores the impact of different farming practices on ABR. Makarov *et al*. (2022), however, found no glycopeptide resistance (using vancomycin as a representative) in *Enterococcus* spp. isolates from chicken and duck farms in Russia, aligning with the current study's findings and suggesting that certain farming practices can effectively mitigate resistance development.

Wild birds

In contrast, wild birds showed higher levels of resistance against the tested glycopeptide/lipopeptide antibiotics. In wild sacred ibis *Enterococcus* spp. isolates, 3% resistance to teicoplanin and 17% resistance to vancomycin was detected. In wild raptor isolates, the resistance levels were even higher, with 7% resistance to teicoplanin and 27% resistance to vancomycin recorded. These findings again highlight that the environmental exposure, bird lifestyle, potential human influence and interaction can affect wild bird populations in view of ABR.

Cagnoli *et al*. (2022) reported similar findings, albeit at a higher frequency, in *Enterococcus* spp. from wild birds of different species in central Italy, with 17% and 37% resistance to teicoplanin and vancomycin, respectively. This suggests a

widespread issue of glycopeptide/lipopeptide resistance in wild bird populations across different regions. Conversely, Sigirci *et al*. (2021) found no vancomycin resistance in enterococci from wild birds, including raptors, in Turkey, indicating possible regional variations, and environmental exposure, affecting resistance patterns.

Smith *et al*. (2022) found lower resistance levels (10% teicoplanin and 1% vancomycin) in *Enterococcus* spp. isolates from Australian shorebirds and terns, suggesting that these populations have less exposure to this class of antibiotics. Anders *et al*. (2020) reported 6% vancomycin resistance in starlings that frequented livestock feeding stations, illustrating how proximity to human activities and livestock can influence ABR levels in *Enterococcus* spp. from wild birds.

4.2.2.6 Oxazolidinone resistance

In this study, linezolid was tested against *Enterococcus* spp. isolates. The results revealed varying levels of resistance, with no resistance detected in isolates from commercial ducks, and low levels of resistance observed in isolates from wild bird populations.

Commercial birds

The absence of linezolid resistance (100% susceptibility) in *Enterococcus* spp. isolates from commercial ducks in this study aligns with findings from Alzahrani *et al*. (2022), who also reported no linezolid resistance among *Enterococcus* spp. isolates from small backyard chicken flocks in Saudi Arabia. Conversely, Yoon *et al*. (2020) found a range of 0%-11% linezolid resistance in *Enterococcus* spp. isolates from chickens on eight different broiler breeder farms in Korea, indicating variability in resistance levels possibly influenced by regional differences and farming practices. Mudenda *et al*. (2022) reported a higher linezolid resistance level of 30% among *Enterococcus* spp. isolates from commercial poultry farms in Zambia, further highlighting the potential impact of intensive farming practices, and possibly unrestricted associated antibiotic usage on ABR.

Wild birds

In wild sacred ibis, 7% of *Enterococcus* spp. isolates were resistant to linezolid, while in wild raptors, 3% resistance was observed. These levels are relatively low but notable, indicating some exposure to oxazolidinone antibiotics or related compounds, especially at wastewater treatment plants often frequented by sacred ibis. However, dietary influences could also affect the development of ABR in bacteria, especially in birds with close human associations or influence.

Yahia *et al*. (2018) and Kwit *et al*. (2023) found no linezolid resistance among *Enterococcus* spp. isolates from wild birds in Tunisia and free-living birds in Poland, respectively. These findings suggest that in certain regions, wild bird populations have minimal exposure to oxazolidinone antibiotics.

In contrast, Cagnoli *et al*. (2022) reported a high resistance level of 42% among *Enterococcus* spp. isolates from wild birds in central Italy. This dramatic difference underscores the variability in resistance patterns across different regions and highlights the potential, albeit not well established, influence of environmental factors and human activities, on ABR enterococci in wild bird populations.

The variability in resistance levels documented in the available literature, alongside the comparatively low levels of resistance observed in *Enterococcus* spp. isolated from wild birds in the current study, underscores the need for ongoing surveillance and monitoring of oxazolidinone resistance in both commercial and wild bird populations. This is particularly crucial considering the significance of linezolid in treating MDR bacterial infections in human medicine (WHO, 2024).

4.3 β-Lactamase Activity

β-Lactamase activity in *E. coli* and *Enterococcus* spp. isolates from the studied birds was evaluated using the nitrocefin hydrolysis spot test as reported (Livermore and Brown, 2001). The nitrocefin spot test was applied to isolates displaying phenotypic

resistance to at least one β-lactam antibiotic. UV-Vis spectrometry was additionally employed to verify this enzymatic reaction for selected isolates.

In commercial ducks, one *E. coli* isolate was tested, which showed no hydrolysis activity. Among wild sacred ibis *E. coli* isolates, one showed no hydrolysis, six showed partial hydrolysis, and three exhibited full hydrolysis. Wild raptor isolates exhibiting β-lactam-resistance displayed β-lactamase activity more frequently, with two showing no hydrolysis, twelve showing partial hydrolysis, and six showing full hydrolysis.

No *Enterococcus* spp. isolates from commercial ducks were tested due to the absence of resistance to β-lactam antibiotics. In wild sacred ibis, one *Enterococcus* spp. isolate, with resistance to the β-lactam antibiotic, ampicillin, showed no visible hydrolysis, while two isolates, with the AMP-IPM and AMP resistance phenotypes, showed partial hydrolysis. Among wild raptors, two isolates, with the AMP and IPM resistance phenotype, showed no visible hydrolysis, and three isolates, with the AMP and/or AMP-IPM resistance phenotype, exhibited partial hydrolysis.

Commercial birds

Although no β-lactamase activity was observed among *E. coli* and *Enterococcus* spp. isolates from commercial ducks in this study, varying β-lactam resistance levels have been reported in the literature. Sharmila *et al*. (2024) reported that 45% of *E. coli* isolates from a small-scale poultry farm in India were phenotypically classified as ESBL-producers, though not all possessed ESBL-genes. However, β-lactamase activity was not verified. Similarly, Mahadewaswami *et al*. (2022) found 48% of *E. coli* isolates from broiler chickens to be phenotypically ESBL-producers, however less than half of the β-lactam-resistant isolates (48%) possessed β-lactamase genes. Conversely, Noenchat *et al*. (2022) reported that none of the *Enterococcus* spp. isolates that were phenotypically resistant to β-lactam antibiotics possessed β-lactamase genes. This highlights the complexity of β-lactam resistance, which can arise from factors other than β-lactamase production, such as porin mutations induced by selective pressure (Tang *et al*., 2014).

Wild birds

Previous studies on β-lactamase activity in wild birds vary, but the available reports align with the current findings from this study, showing that detection levels are low and β-lactamase activity is not present in all isolates that are phenotypically resistant to β-lactam antibiotics. Parker *et al*. (2016) found ESBL-producing *E. coli* in two crows of urban-origin, suggesting possible human influence on resistance patterns. Zurfluh *et al*. (2019) identified 2% of ABR *E. coli* isolates from wild birds in Switzerland as ESBL-producers. Russo *et al*. (2022) found no ESBL-positive *Enterococcus* spp. isolates from phenotypically β-lactam-resistant isolates from wild birds, similar to the current findings on *Enterococcus* spp. isolates. However, partial hydrolysis of nitrocefin was detected, suggesting a low level of β-lactamase activity, highlighting the sensitivity of the nitrocefin spot test.

Of the phenotypically β-lactam-resistant isolates tested, four *E. coli* isolates (one from ducks, one from sacred ibis, and two from raptors), and three *Enterococcus* spp. isolates (one from sacred ibis, and two from raptors), showed no visible hydrolysis of nitrocefin. This was confirmed by UV-Vis spectrometry using a representative isolate. The absence of nitrocefin hydrolysis indicates that these isolates might be employing alternative β-lactam-resistance mechanisms, such as porin mutations, efflux pumps, or modification of the penicillin-binding proteins (PBPs), which inhibit the entry or expel the β-lactam antibiotic from the bacterial cell, thereby conferring phenotypic β-lactam resistance (Tang *et al*., 2014; Prajapati *et al*., 2021). Therefore, utilizing chromogenic compounds, like nitrocefin, is an effective tool to distinguish between enzymatic β-lactamase based resistance and other resistance mechanisms, providing valuable insight into the underlying resistance strategies that may be overlooked if genotypic analysis is only performed.

Overall, these findings underscore the importance of assessing phenotypically β-lactam-resistant bacterial isolates from birds for β-lactamase activity as a means to elucidate the underlying resistance mechanism. A recent study by D'humières *et al*. (2024) demonstrated the utility of nitrocefin as a chromogenic substrate to detect β-lactamase activity in the faeces of patients treated with β-lactam antibiotics. Patients who underwent a three-day course of cefotaxime or ceftriaxone, exhibited

measurable nitrocefin hydrolysis activity in their faeces, with a notable increase in β-lactamase from day zero to ten days post-antibiotic exposure (D'humières *et al*, 2024). This highlights the potential of chromogenic substrates like nitrocefin for ABR research and underscores the risk of developing β-lactam-resistance in gut microbiota via exposure to antibiotics, including that of birds, when exposed to these antibiotics through environmental sources such as wastewater effluent and discarded medications.

4.4 Limitations

While this study provided valuable insights into ABR in *E. coli* and *Enterococcus* spp. among commercial free-range ducks and wild birds, several limitations should be acknowledged. First, the sample size may limit the generalizability of the findings, as a larger sample would provide a more comprehensive view of ABR patterns across different populations and environments. Additionally, the study focused primarily on specific bacterial isolates, which may not fully represent the broader microbial diversity present in the sampled environments. Future research could address this limitation by incorporating a larger and more diverse sample size, as well as exploring additional bacterial species to gain a more complete understanding of ABR dynamics. Furthermore, the environmental factors influencing ABR in wild birds were not exhaustively analysed; thus, future studies should consider a more in-depth examination of environmental contaminants and their potential contributions to resistance patterns. By recognizing these limitations and implementing suggested solutions, researchers can enhance the robustness of future studies in this important area of public health.

4.5 Conclusion

<u>Microbial burden</u>

This study has provided valuable insights into the microbial burden in faecal samples from commercial free-range ducks, wild sacred ibis, and raptors from the Midlands area of KwaZulu-Natal, South Africa. The variability of the data between wild and commercial birds, underscore the complex interplay between bird species, their

habitat, and microbial exposure, with important implications for environmental health and public safety.

The higher microbial load observed in wild raptors can be attributed to their predatory nature and scavenging habits, exposing them to a wider range of bacteria. Sacred ibis, while slightly less burdened than raptors, still showed elevated faecal microbial levels, likely due to their scavenging in urban environments, polluted wetlands, and wastewater treatment plants. In contrast, commercial ducks, despite having substantial microbial loads, showed lower levels compared to their wild bird counterparts, reflecting their managed free-range farming conditions and controlled diets.

The substantial microbial burden in wild birds' faeces, particularly raptors and sacred ibis, highlights their potential role as vectors of microbial contamination of soil or waterbodies, posing risks to both environmental and human health. Effective control measures, including habitat management and stringent waste control, are essential to mitigate these risks. From a One Health perspective, continuous monitoring and targeted interventions are necessary to prevent pathogen and ABR transmission, emphasizing the interconnectedness of human, animal, and environmental health. Understanding microbial dynamics in different bird species can inform better biosecurity measures for both wildlife and commercial poultry farming, ensuring comprehensive management of microbial contamination.

Antibiotic resistance

The resistance patterns observed in this study reveal substantial differences between *E. coli* and *Enterococcus* spp. isolates from faecal samples of commercial ducks and wild birds (sacred ibis and raptors). The findings are underpinned by statically significant differences in resistance frequencies identified through Pearson's X^2 test.

For *E. coli*, isolates from commercial ducks exhibited resistance to a limited range of antibiotics, with some intermediate resistance detected. In contrast, isolates from wild birds demonstrated a broader and more diverse resistance profile. This highlights the possible impact of environmental factors and human activities on the spread of ABR

bacteria. The investigation into β-lactams, aminoglycoside, and fluoroquinolone resistance, as well as multidrug-resistance patterns, underscores the need for continuous monitoring and targeted interventions to mitigate the public health risks associated with ABR in *E. coli*. Notably, the absence of tigecycline resistance across all bacterial isolates from bird categories suggests that this last-resort antibiotic remains effective, and is not yet affected or under threat via bird associated ABR.

For *Enterococcus* spp., the present study revealed distinct ABR profiles between isolates from commercial ducks and wild birds (sacred ibis and raptors). Isolates from commercial ducks did not exhibit complete resistance to the antibiotics tested, with only intermediate resistance to ampicillin and imipenem. This suggests that controlled farming practices and regulated antibiotic use in free-range, organic farming effectively limits the development and spread of resistant enterococci strains in commercial settings. In contrast, *Enterococcus* spp. isolates from wild birds showed resistance to several antibiotics, indicating higher ABR levels due to exposure to diverse environmental sources of antibiotic contamination, such as polluted natural habitats and human-impacted areas like wastewater treatment plants.

The study's statistical analysis using Pearson's X^2 test highlighted significant differences in resistance frequencies between commercial ducks and wild birds. No significant difference was found between the two wild bird species, suggesting similar environmental pressures influencing ABR in microorganisms carried by sacred ibis and raptors. The widespread intermediate bacterial resistance to imipenem across all bird groups warrants further investigation. The complete susceptibility of all isolates to gentamicin, tigecycline, and norfloxacin is promising, indicating these antibiotics remain effective against *Enterococcus* spp.

Comparative analysis using existing literature revealed that resistance levels in both *E. coli* and *Enterococcus* spp. can vary significantly depending on farming practices, environmental exposure, and geographic locations. These findings emphasize the need for robust biosecurity measures, responsible antibiotic usage, and comprehensive management strategies to control the spread of ABR in both

agricultural and wild bird populations, ensuring the protection of public health and environmental integrity. The higher resistance levels observed in wild birds underscore the importance of ongoing surveillance and targeted interventions to address the environmental and human influences of ABR development.

β-Lactamase activity

The evaluation of β-lactamase activity in *E. coli* and *Enterococcus* spp. isolates from commercial ducks and wild birds (sacred ibis and raptors) using the nitrocefin hydrolysis spot test provided valuable insights into the underlying mechanisms of phenotypic β-lactam resistance. Notably, no β-lactamase activity was observed in *E. coli* and *Enterococcus* spp. isolates from commercial ducks, which aligns with the available literature suggesting β-lactam resistance can arise from mechanisms other than β-lactamase production, such as porin-mutations and efflux pumps (Prajapati *et al*., 2021).

In contrast, wild bird isolates displayed varying levels of β-lactamase activity. Some *E. coli* isolates from sacred ibis and raptors showed partial or full hydrolysis of nitrocefin, indicating β-lactamase activity, while other isolates that did not exhibit any hydrolysis, suggest the presence of alternative resistance mechanisms.

These findings are consistent with previous research on wild birds, demonstrating low detection levels of β-lactamase activity and the presence of alternative resistance mechanisms. This highlights the complexity of β-lactam-resistance and underscores the importance of using phenotypic analysis, such as the nitrocefin spot test, to distinguish between enzymatic activity and other underlying resistance strategies. The observed differences in β-lactamase activity between commercial and wild birds underscore the influence of environmental factors, such as exposure to wastewater effluent, on resistance patterns.

Overall, the application of the nitrocefin spot test, complemented by UV-Vis spectrometry, proved to be an effective method for detecting β-lactamase activity and provided a deeper understanding of the resistance mechanisms in phenotypically β-

lactam-resistant isolates. These findings emphasize the need for comprehensive phenotypic and genotypic analysis to fully understand and identify the resistance mechanisms in bacteria carried by avian populations, which is crucial for informing strategies to mitigate the spread of ABR bacteria by both commercial and wild birds.

Future studies

For future studies, there are several key avenues to explore based on the findings of this research on microbial burden and ABR bacteria carried by avian populations. Longitudinal and seasonal studies would be valuable to track climate and environmental associated variations and temporal changes in microbial burden and ABR dynamics among both commercial and wild bird populations. These studies, which was not conducted in the current study, could provide insights into how environmental factors, such as habitat changes or seasonal fluctuations, influence microbial communities and resistance profiles over time.

Additionally, employing metagenomic approaches would enhance the understanding of the microbiota of birds, by examining the entire microbial community present in bird faecal samples. This method would enable researchers to identify non-culturable organisms and their potential roles in ABR dissemination within avian habitats. Genomic analysis could further elucidate the genetic mechanisms underpinning ABR in *E. coli* and *Enterococcus* spp. including the detection of specific resistance genes and mobile genetic elements contributing to resistance phenotypes, as this was not part of the present study.

The process that promotes ABR in bacteria carried by birds are not yet well understood. It is unclear whether the primary drivers are the uptake of ABR residues from the environment, horizontal gene transfer of ABR genes, or the acquisition of ABR bacteria through their diet and lifestyle. Future research should investigate these pathways to clarify the mechanisms behind the development and spread of resistant bacteria among avian populations. This understanding could be achieved through studies focusing on the interaction between birds and their environments,

including the analysis of environmental samples for antibiotic residues and resistance genes.

Environmental monitoring efforts should expand to assess the presence and persistence of antibiotic residues and resistant bacteria across different avian habitats, ranging from wetlands to agricultural areas and urban environments. Such studies would provide crucial data on environmental reservoirs and transmission pathways of ABR bacteria in avian populations. Comparative studies across diverse geographic regions would also be beneficial to explore regional variations in microbial burden and resistance profiles, considering factors like climate, land use practices, and regulatory measures.

Furthermore, investigating the impact of specific management practices, such as antibiotic usage protocols and habitat conservation efforts, could reveal their effectiveness in mitigating ABR bacteria in both wild and commercial bird populations. Embracing One Health approaches that integrate human, animal, and environmental health perspectives would be essential for a more comprehensive understanding and management of ABR bacteria carried by avian species.

Developing surveillance systems and early warning indicators to detect emerging ABR trends in avian populations, both commercial and wild (including migratory) birds would enable proactive interventions and policy decisions. By addressing these research priorities, future studies can contribute notably to developing evidence-based strategies aimed at mitigating ABR risks in avian populations, thereby safeguarding environmental health and promoting sustainable practices in agriculture and wildlife management.

Recommendations

Based on the findings of this study, several recommendations can be made to address the challenges posed by microbial burden and ABR in avian populations. First, it is crucial to implement robust monitoring programs for both wild and commercial birds to track microbial loads and resistance patterns over time. Such

programs should incorporate seasonal and longitudinal studies to understand the dynamics of ABR in relation to environmental changes and anthropogenic influences.

Second, the establishment of guidelines for responsible antibiotic use in poultry farming should be prioritized to minimize the selection pressure for resistance. This includes promoting alternative management practices that reduce the reliance on antibiotics for growth promotion.

Additionally, enhancing public awareness and education about the risks associated with ABR in wildlife and its potential impact on human health is vital for fostering community engagement in conservation efforts. Collaborative efforts between veterinarians, environmental scientists, and public health officials are essential to develop integrated One Health strategies that address the interconnectedness of human, animal, and environmental health. By prioritizing these recommendations, stakeholders can work towards mitigating the risks of ABR and protecting both public health and ecological integrity.

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APPENDIX A:

The following tables provide the antibiotic resistance profiles of all *E. coli* isolates. All zone diameters are the average of duplicate measurements (i.e., two independent measurements) and have been rounded to the nearest millimetre (mm).

Please make note of the following keys:

SUSCEPTIBLE	INTERMEDIATE	RESISTANT

Also note that the isolate numbers that have been shaded in light grey indicate an isolate is resistant to at least one antibiotic and dark grey to indicate an isolate as multidrug-resistant.

A 1. Zone diameters for *E. coli* isolates from commercial duck faecal samples

	Penicillin		Carbapenem		Cephalosporin		Aminoglycoside		Monobact	Tetracyclir	Fluroquinolone	
D (EC)	AMP	AMC	ETP	MEM	CAZ	CTX	CN	TOB	AZT	TGC	NOR	CIP
1	15	19	25	21	21	23	16	16	25	19	29	32
2	17	21	24	26	24	22	18	16	24	20	29	31
3	16	20	25	25	22	23	18	17	24	21	29	33
4	17	20	28	23	21	23	17	16	23	19	29	32
5	15	19	23	26	22	23	18	17	24	18	24	25
6	15	19	28	24	23	24	17	16	23	19	26	26
7	16	20	27	25	22	24	18	15	25	19	29	32
8	16	20	26	27	23	24	17	16	24	20	31	33
9	16	21	28	26	23	25	16	16	26	20	30	32
10	17	20	25	24	21	22	16	16	24	19	28	31
11	16	19	26	26	22	22	16	16	26	19	25	28
12	15	19	28	26	23	24	16	15	25	20	26	27
13	15	18	28	27	23	24	17	16	26	19	27	30
14	16	19	27	27	21	25	18	17	25	21	25	29
15	16	19	28	26	23	25	17	16	26	19	30	30
16	16	21	28	28	23	24	18	16	26	19	30	32
17	18	22	28	26	22	24	16	16	25	20	31	34
18	18	21	26	27	21	24	16	15	24	20	30	31
19	17	19	27	24	21	24	16	16	24	19	31	31
20	18	21	27	25	22	23	17	16	25	19	30	33
21	18	21	27	27	23	23	17	18	25	19	30	31
22	19	20	28	27	21	24	17	15	25	19	30	32
23	17	21	28	25	23	23	16	15	26	19	29	30
24	16	21	26	25	21	23	16	16	23	20	31	32
25	16	21	28	26	22	24	17	16	26	20	28	31
26	18	22	26	27	21	23	17	18	26	19	30	30
27	17	21	27	27	22	24	17	17	24	20	30	31
28	16	22	27	27	21	24	18	15	25	19	24	30
29	18	23	28	28	23	23	18	17	25	20	31	30
30	18	22	27	27	23	24	17	16	24	19	32	31

A 2. Zone diameters for *E. coli* isolates from wild sacred ibis faecal samples

	Penicillin		Carbapenem		Cephalosporin		Aminoglycoside		Monobact	Tetracyclir	Fluroquinolone	
SI (EC)	AMP	AMC	ETP	MEM	CAZ	CTX	CN	TOB	AZT	TGC	NOR	CIP
1	18	19	31	29	25	24	14	20	29	20	27	32
2	16	21	29	27	22	25	16	16	28	21	25	28
3	13	16	29	27	21	22	16	16	23	23	27	28
4	18	21	29	29	24	29	17	18	29	21	29	30
5	19	21	29	29	26	27	17	17	29	20	28	28
6	15	20	29	27	23	25	17	16	28	21	27	30
7	14	18	29	29	23	25	19	16	30	21	31	32
8	14	20	31	28	24	24	18	16	28	20	25	28
9	13	18	30	27	24	25	18	15	28	24	27	30
10	16	20	30	26	23	25	16	15	25	22	27	29
11	14	19	33	27	24	22	18	17	27	22	30	29
12	14	19	30	28	24	26	16	18	27	23	28	28
13	16	18	26	26	22	25	15	15	28	21	31	32
14	15	19	30	27	23	26	18	14	28	21	29	30
15	15	20	29	28	22	24	16	15	27	21	27	29
16	16	19	28	27	23	25	17	14	27	21	31	30
17	12	17	28	28	23	25	17	16	27	21	26	28
18	14	18	27	27	23	25	15	15	27	21	28	28
19	14	19	28	26	20	22	17	16	25	22	28	27
20	12	15	27	28	21	22	17	15	25	19	24	24
21	15	19	28	28	21	24	16	16	27	21	27	29
22	16	20	27	27	22	23	19	15	28	21	25	25
23	19	20	29	27	22	24	18	17	29	20	26	27
24	18	20	28	27	21	27	18	16	27	21	26	27
25	12	16	27	25	21	22	15	15	27	18	25	24
26	14	20	30	27	21	25	16	15	27	22	26	30
27	13	17	29	26	18	23	16	16	24	22	24	26
28	13	17	28	25	18	23	15	15	22	21	23	22
29	17	19	28	24	21	25	16	15	28	21	25	27
30	14	20	27	28	20	25	17	15	26	23	25	29

A 3. Zone diameters for *E. coli* isolates from wild raptors faecal samples

	Penicillin		Carbapenem		Cephalosporin		Aminoglycoside		Monobact	Tetracyclir	Fluoroquinolone	
R (EC)	AMP	AMC	ETP	MEM	CAZ	CTX	CN	TOB	AZT	TGC	NOR	CIP
1	15	20	28	23	23	23	16	14	27	22	30	26
2	14	19	26	23	19	22	18	16	27	22	26	25
3	12	15	25	21	20	22	15	16	22	19	25	23
4	13	21	27	24	19	24	15	15	26	23	27	26
5	15	15	23	22	19	26	17	16	28	20	24	28
6	13	17	25	23	19	24	16	14	22	19	25	27
7	15	19	26	25	21	28	14	17	30	21	25	25
8	13	19	24	26	18	23	17	18	21	22	27	26
9	12	16	27	24	17	19	19	16	28	20	28	28
10	16	18	25	26	18	25	17	16	26	20	25	24
11	10	15	26	23	19	27	15	15	30	23	24	25
12	14	21	30	25	20	24	18	16	31	19	26	29
13	10	16	28	27	22	26	19	14	26	22	28	22
14	13	20	26	26	20	24	17	16	28	21	23	27
15	14	17	26	28	22	23	20	18	23	23	25	25
16	13	19	29	22	23	23	14	15	27	20	29	26
17	11	19	25	24	21	26	15	14	26	19	28	28
18	14	18	27	26	20	25	16	16	29	20	23	25
19	15	20	30	24	22	22	21	14	27	20	25	26
20	14	15	28	27	21	21	16	14	23	22	26	24
21	14	17	25	23	18	24	17	18	24	23	28	27
22	12	16	25	28	19	18	19	14	28	20	30	29
23	14	19	29	26	23	22	15	16	30	19	29	25
24	14	18	27	27	20	22	21	13	32	22	29	28
25	13	15	25	23	21	25	20	14	26	21	27	25
26	14	20	27	22	22	23	14	17	25	20	25	26
27	14	19	29	25	23	24	18	15	32	23	23	28
28	11	18	26	26	18	20	17	16	25	19	25	23
29	15	20	29	28	19	26	14	16	30	21	28	29
30	16	21	30	22	22	22	16	14	29	20	26	26

APPENDIX B:

The following tables provide the antibiotic resistance profiles of all *Enterococcus* spp. isolates. All zone diameters are the average of duplicate measurements (i.e., two independent measurements) and have been rounded to the nearest millimetre (mm).

Please make note of the following keys:

SUSCEPTIBLE	INTERMEDIATE	RESISTANT

Penicillin
Carbapenem
Glycopeptide/lipopeptide
Aminoglycoside
Oxazolidinone
Tetracycline
Fluoroquinolone

Also note that the isolate numbers that have been shaded in light grey indicate an isolate is resistant to at least one antibiotic.

B 1. Zone diameters for Enterococcus spp. isolates from commercial duck faecal samples

D (ET)	AMP	IPM	CN	TGC	NOR	TEC	VA	LZD
1	16	30	14	24	16	17	15	22
2	18	25	16	25	12	19	13	21
3	12	29	17	25	14	19	13	21
4	13	30	15	27	12	22	14	24
5	19	30	12	26	14	18	12	23
6	12	24	14	24	17	20	14	20
7	17	29	15	20	14	21	12	20
8	20	25	14	22	13	17	14	22
9	20	25	12	24	15	19	13	25
10	12	24	15	25	13	22	15	23
11	16	23	15	27	16	21	12	26
12	16	26	13	26	14	21	14	24
13	9	24	14	24	17	23	13	22
14	14	30	15	25	18	20	14	22
15	14	28	14	25	12	19	12	24
16	15	27	14	27	14	22	14	23
17	14	26	16	21	14	18	15	20
18	15	22	12	26	13	17	13	23
19	10	25	12	24	17	20	14	22
20	9	26	15	23	13	23	14	25
21	16	24	14	20	12	19	13	24
22	17	24	17	24	14	17	14	26
23	20	27	16	25	15	20	15	25
24	15	30	14	22	16	18	14	22
25	19	23	13	26	12	21	16	22
26	8	24	14	27	14	19	14	21
27	16	24	13	23	13	22	15	25
28	17	28	14	22	14	20	15	23
29	14	25	12	20	18	18	13	23
30	14	24	11	24	13	21	12	21

B 2. Zone diameters for Enterococcus spp. isolates from wild sacred ibis faecal samples

SI (ET)	AMP	IPM	CN	TGC	NOR	TEC	VA	LZD
1	11	28	10	20	15	18	11	23
2	9	25	10	23	17	18	12	21
3	11	23	12	24	12	19	14	20
4	13	25	12	23	16	21	14	20
5	14	24	14	20	16	20	13	22
6	14	25	13	21	12	19	14	24
7	11	24	15	21	13	22	13	21
8	14	27	15	25	16	17	14	23
9	15	25	14	23	15	17	14	24
10	16	21	10	24	13	19	12	24
11	8	23	9	22	14	15	11	20
12	12	21	10	23	13	16	12	21
13	14	23	13	20	15	16	14	21
14	14	24	12	24	12	20	13	18
15	7	20	15	25	13	18	12	23
16	7	26	14	22	14	22	12	21
17	10	22	10	22	13	16	13	24
18	17	22	9	24	13	19	12	20
19	16	21	10	20	14	20	10	22
20	13	22	14	25	14	17	13	21
21	15	28	9	20	14	22	14	19
22	16	22	10	20	13	21	12	24
23	13	24	13	21	13	18	13	24
24	13	24	13	23	12	17	12	20
25	7	25	15	21	16	20	15	23
26	11	26	14	24	16	18	14	21
27	16	22	11	22	15	22	11	25
28	12	21	11	22	13	17	11	20
29	8	26	13	25	15	17	14	22
30	15	22	13	23	13	16	15	21

B 3. Zone diameters for Enterococcus spp. isolates from wild raptors faecal samples

R (ET)	AMP	IPM	CN	TGC	NOR	TEC	VA	LZD
1	14	24	12	21	19	19	15	22
2	8	22	16	20	15	18	11	22
3	11	24	13	20	18	19	11	20
4	10	26	12	22	14	20	13	23
5	13	25	14	24	17	21	12	19
6	15	28	15	23	15	20	14	24
7	7	21	11	20	18	19	15	23
8	16	29	12	22	16	15	11	20
9	12	23	16	24	20	18	14	20
10	15	26	15	21	15	18	15	24
11	17	22	12	23	19	17	11	20
12	9	25	13	25	16	19	13	20
13	14	27	17	22	15	16	14	21
14	19	29	12	20	17	21	14	23
15	7	21	15	23	14	20	15	24
16	13	25	16	21	18	20	13	20
17	16	24	10	25	15	19	11	22
18	15	28	13	20	12	15	10	21
19	11	26	11	24	16	18	14	21
20	9	23	15	21	13	16	10	23
21	10	23	15	20	15	17	13	24
22	14	26	16	23	20	17	12	22
23	17	24	13	25	16	21	15	22
24	7	20	10	21	19	19	14	20
25	11	19	11	20	12	18	12	21
26	19	22	15	20	14	20	16	23
27	17	25	16	23	17	16	13	24
28	15	29	14	25	13	16	12	22
29	9	22	16	22	15	18	11	20
30	7	21	12	20	18	20	14	24