Antibiotic resistant bacteria prevalent in livestock and wildlife species in South Africa

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Pectora roborant cultus recti

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DECLARATION

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SUMMARY

Much research has focussed on the fate of antibiotics in clinical settings whereas research of antibiotics in natural environments has been comparatively limited. It has been hypothesised that wildlife could play a significant role in the development of antibiotic resistant bacteria in nature as a variety of wildlife species carry antibiotic resistant bacteria and cover a large territory throughout their lifespan The aim of this study was to determine whether wild ungulates, namely, African buffalo (*Syncerus caffer*), black wildebeest (*Connochaetes gnou*), blue wildebeest (*Connochaetes taurinus*), bontebok (*Damaliscus pygargus*), eland (*Taurotragus oryx*), fallow deer (*Dama dama*), impala (*Aepyceros melampus*) and springbok (*Antidorcas marsupialis*), host antibiotic resistant bacteria, specifically, *Escherichia coli*, *Enterococcus faecalis* and *Staphylococcus aureus*, from various South African farms. The Kirby-Bauer disk diffusion method was used according to the Clinical and Laboratory Standards Institute 2018 guidelines. Overall, antibiotic resistance among the wild ungulate species was low towards the selected antibiotics. On average, the antibiotic resistance levels were 8% *E. coli* (N= 353), 4% *E. faecalis* (N= 194) and 22% *S. aureus* (N= 106). The highest antibiotic resistance was towards antibiotics which are of natural origin, namely the β-lactams and streptomycin. These antibiotics are found in the soil microbiome, produced by Actinobacteria. In addition, certain resistant genes were detected using the polymerase chain reaction in isolates which showed phenotypic resistance. The resistant genes *sul*1 (40%), *sul*2 (80%), *sul*3 (0%), *bla*CMY (98%), *tet*A (63%), *tet*B (75%), *tet*C (0%) and *aad*A (98%) were detected in resistant *E. coli* isolates (N= 44); *tet*K (7%), *tet*L (100%), *tet*M (100%), *bla*Z (100%), *van*A (95%) and *van*B (10%) in resistant *S. aureus* (N= 5) and *E. faecalis* (N= 22) isolates. The results of this study indicate that wildlife can be considered a natural reservoir of antibiotic resistant genes. The wildlife were also found to be more multi-drug resistant than the livestock. Thus it is speculated that these resistant genes are picked up from the soil and the surrounding environment and are spread by the animals as well as by other natural vectors like the wind and flies. Various factors and agricultural practices were found to influence the antibiotic resistance of the bacteria harboured by the wildlife species, namely, co-grazing with livestock, the practice of wildlife supplementary feeding and farm history of antibiotic use. Bacteria isolated from game meat was frequently more antibiotic resistant than bacteria from the faeces, indicating human cross-contamination during slaughter. The level of antibiotic resistance determined in this study from the bacteria of the wildlife from pristine areas, could serve as a baseline for monitoring the influence of human activities on the development of antibiotic resistance in various environments, which this study contributed towards.

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OPSOMMING

Heelwat navorsing fokus op die lot van antibiotika in kliniese omgewings terwyl navorsing van antibiotika in natuurlike omgewings relatief beperk is. Daar word veronderstel dat wild ‘n betekenisvolle rol kan speel in die ontwikkeling van antibiotika weerstandbiedende bakterieë in die natuur aangesien verskeie wildspesies draers van antibiotika weerstandbiedende bakterieë is en tydens hul lewensduur ‘n groot area dek. Die doel van hierdie studie was om te bepaal of wilde hoefdiere, naamlik Afrika-buffel (*Syncerus caffer*), swart wildebees (*Connochaetes gnou*), blou wildebees (*Connochaetes taurinus*), bontebok (*Damaliscus pygargus*), eland (*Taurotragus oryx*), hert (*Dama dama*), rooibok (*Aepyceros melampus*) en springbok (*Antidorcas marsupialis*), antibiotika weerstandbiedende bakterieë huisves, spesifiek, *Escherichia coli*, *Enterococcus faecalis* en *Staphylococcus aureus*, van verskeie Suid-Afrikaanse plase. Die Kirby-Bauer skyf diffusie metode is gebruik volgens die “Clinical and Laboratory Standards Institute” 2018 riglyne. Oor die algemeen was antibiotika weerstandbiedendheid onder die wild hoefdierspesies laag in terme van die geselekteerde antibiotika. Die gemiddelde antibiotika weerstandvlakke was 8% *E. coli* (N= 353), 4% *E. faecalis* (N= 194) en 22% *S. aureus* (N= 106). Die hoogste antibiotika weerstandbiedendheid was teenoor antibiotika van natuurlike oorsprong, naamlik die β-laktame en streptomisien. Hierdie antibiotika word gevind in die grondmikrobioom, en word geproduseer deur Aktinobakterieë. Daarbenewens is sekere weerstandbiedende gene opgespoor met behulp van die polimerase kettingreaksie in isolate wat fenotipiese weerstand getoon het. Die weerstandbiedende gene *sul*1 (40%), *sul*2 (80%), *sul*3 (0%), *bla*CMY (98%), *tet*A (63%), *tet*B (75%), *tet*C (0%) en *aad*A (98%) is opgespoor in weerstandbiedende *E. coli* isolate (N= 44); *tet*K (7%), *tet*L (100%), *tet*M (100%), *bla*Z (100%), *van*A (95%) en *van*B (10%) in weerstandbiedende *S. aureus* (N= 5) en *E. faecalis* (N= 22) isolate. Die resultate van hierdie studie dui aan dat wild beskou kan word as ‘n natuurlike reservoir vir antibiotika weerstandbiedende gene. Daar is ook bevind dat wild meer weerstandig is teen veelsoortige antibiotika as wat vee is. Daar word dus gespekuleer dat hierdie weerstandbiedende gene opgetel word uit die grond en die omliggende omgewing en dan versprei word deur die diere sowel as ander natuurlike vektore soos die wind en vlieë. Daar is gevind dat verskeie faktore en landboupraktyke die antibiotika weerstandbiedendheid van die bakterieë wat deur die wildspesies gehuisves word beïnvloed, naamlik, mede-weiding met vee, die gebruik van aanvullende voeding vir wild en geskiedenis van antibiotika gebruik op die plaas. Bakterieë wat uit wildvleis geisoleer is, was dikwels meer weerstandbiedend teen antibiotika as bakterieë wat afkomstig is van ontlasting. Dit dui op menslike kruiskontaminasie tydens die slagproses. Die vlak van antibiotika weerstandbiedendheid wat in hierdie studie bepaal is vanaf die bakterieë van die wild van ongerepte gebiede, kan as basis dien vir die monitering van die invloed van menslike aktiwiteite op die ontwikkeling van antibiotika weerstandbiedendheid in verskeie omgewings, waartoe hierdie studie bygedra het.

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NOTES AND PUBLICATIONS

The language and style used in this thesis is in accordance with the requirements of the *International Journal of Food Science and Technology*. This thesis represents a compilation of manuscripts where each chapter is an individual entity and some repetition between the chapters has, therefore, been unavoidable, especially in terms of the materials and methods sections.

Results from this study have been published or presented in the following scientific peer-reviewed journal or symposiums:

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"Messieurs, c'est les microbes qui auront le dernier mot."

(Gentlemen, it is the microbes who will have the last word)

Louis Pasteur

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ABBREVIATIONS

ABR	Antibiotic resistance
AGP	Antimicrobial growth promoter
AIDS	Acquired immune deficiency syndrome
AMP	Ampicillin
AMR	Antimicrobial resistance
API	Analytical Profile Index
ANOVA	Analysis of Variance
AST	Antibiotic susceptibility testing
BPA	Baird Parker agar
C	Chloramphenicol
CA-MRSA	Community acquired- methicillin resistant *Staphylococcus aureus*
CAZ	Ceftazidime
CDC	Centers of Disease Control and Prevention
CLSI	Clinical and Laboratory Standards Institute
DAFF	Department of Agriculture, Forestry and Fisheries
DLA	Deoxycholate Lactose Agar
DoH	Department of Health
E	Erythromycin
EMBA	Eosin Methylene Blue Agar
ETEC	Enterotoxigenic *Escherichia coli*
ESBL	Extended spectrum beta-lactamase
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FDA	Food and Drug Administration
FOX	Cefoxitin
GARP	Global Antibiotic Resistance Partnership
GAP	Good Agricultural Practices
GBD	Global Burden of Disease
GMP	Good Manufacturing Practices
HACCP	Hazard Analysis Critical Control Points
HA-MRSA	Hospital-acquired methicillin resistant *Staphylococcus aureus*
HGT	Horizontal gene transfer
HIV	Human Immunodeficiency Virus

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I	Intermediate resistance
ICC	Intraclass correlation coefficient
LSM	Least Squares Mean
LA-MRSA	Livestock-acquired methicillin resistant *Staphylococcus aureus*
MACA	MacConkey Agar
MALDI-ToF	Matrix Assisted Laser Desorption/Ionization- Time of Flight
MBC	Minimum Bactericidal Concentration
MDR	Multidrug-resistant
MHA	Mueller Hinton Agar
MIC	Minimal Inhibitory Concentration
MRSA	Methicillin resistant *Staphylococcus aureus*
MSA	Mannitol Salt Agar
MSSA	Methicillin susceptible *Staphylococcus aureus*
NA	Nalixidic acid
NA	Nutrient Agar
NAMC	National Agricultural Marketing Council
NGS	Next-Generation Sequencing
ORSA	Oxacillin-resistant *Staphylococcus aureus*
OX	Oxacillin
P	Penicillin
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
PK-PD	Pharmacokinetic/ pharmacodynamic
PSS	Physiological Saline Solution
R	Resistant
RT-PCR	Real-time Polymerase chain reaction
S	Susceptible
ST	Streptomycin
SAASP	South African Antibiotic Stewardship Programme
SANS	South African National Standards
SANVAD	South African National Veterinary Surveillance and Monitoring Programme for Resistance to Antimicrobial Drugs
SCC	Staphylococcal cassette chromosome
SF	Sulphonamide
STEC	Shigatoxigenic *Escherichia coli*

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TE	Tetracycline
TB	Tuberculosis
TSB	Tryptone Soy Broth
UK	United Kingdom
US	United States
VA	Vancomycin
VRBA	Violet Red Bile Agar
VRBDA	Violet Red Bile Dextrose Agar
VRBGA	Violet Red Bile Glucose Agar
VRE	Vancomycin-resistant *Enterococci*
VRSA	Vancomycin-resistant *Staphylococcus aureus*
WGS	Whole- genome sequencing
WHO	World Health Organisation
XDR	Extensively drug resistant
YLL	Years of life lost

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CHAPTER 1

Introduction

1.1. Background

Antibiotic agents are considered one of modern medicine's greatest discoveries, saving millions of lives annually (Ventola, 2015). Unfortunately, we are currently in danger of entering a 'post-antibiotic era', where the efficacy of antibiotics may be significantly compromised by the emergence of antibiotic resistant (ABR) bacteria (Morley *et al.*, 2005; van Hoek *et al*., 2011). This would pose a major global threat to human health. Increased treatment failures, increased infection severity and increased occurrence of infections that would not otherwise have occurred in both animals and humans, will become evident (Walsh & Fanning, 2008; Capita *et al*., 2016).

This phenomenon emerges largely as a result of a selective pressure created in a bacterial community, which creates a 'survival of the fittest' evolutionary change (Szmolka & Nagy, 2013). The antimicrobial agent exerted onto the bacterial population creates a stressed environment where all the susceptible bacteria are killed and the few resilient bacteria are able to survive and resist the antimicrobial action (Levy, 1998; Capita *et al.*, 2016). The resistant bacteria that survived the treatment then go on to replicate and, over time, produce a larger resistant population (Wiuff *et al*., 2005; Capita *et al.*, 2016).

The misuse, overuse and inappropriate use of antimicrobials in human medicine and animal medicine and husbandry creates an enormous selection pressure (Carlet *et al*., 2012; Ventola, 2015). This increases and accelerates the likelihood that bacteria will adapt and multiply to produce a more resistant population. The epidemic of antibiotic resistance was initially bound to hospital environments but has since emerged into community-acquired and livestock-acquired resistant bacteria, for example methicillin-resistant *Staphylococcus aureus* (Appelbaum, 2006: Purrello *et al*., 2014; Ventola, 2015).

It is not just resistant pathogens which are a concern but also the resistant non-pathogenic commensal bacteria. In addition, resistant commensals create a large resistance gene pool where resistant traits can be readily transferred, even to pathogenic bacteria (Blake *et al.,* 2003; Landers *et al*., 2012).

The effect of antimicrobial resistance on human and animal health is not well known nor properly understood at this point (Morley *et al.,* 2005; Mc Nulty *et al.,* 2016). This may well be due to the complexity of the problem, with a long list of factors that play a role in the development and transfer of ABR bacteria in the environment (Wallmann, 2006). There is therefore a need for research in this area to assist in documenting and reporting the antimicrobial resistance situation in various environments across the globe (Mc Nulty *et al.,* 2016).

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1.2. Focus on antibiotic resistance

Opportunities exist to make important contributions towards mitigating the risk of a "post-antibiotic era" through building up knowledge of the many ways in which resistance to antibiotics may emerge, including in the agricultural sector. The farming industry has come under scrutiny over recent years for being considered a major contributor to the emergence of antibiotic resistance worldwide, followed closely by the human medicine industry (Morley *et al.,* 2005; Gilchrist *et al*., 2007). This arises in part because antimicrobials are used to a greater extent in the agricultural industry than in human medicine, as the former relies heavily on antimicrobials to improve animal health and productivity, especially in intensively reared species (Moyane *et al*., 2013; Woolhouse *et al*., 2015). It is time to review agricultural practices in this regard.

Specifically, antibiotics are used in the farming industry to prevent, control and treat diseases in food-producing animals and, in some instances, to improve feed efficiency for growth promotion (Gaskins *et al*., 2002; Phillips *et al.,* 2004). Prophylaxis and metaphylaxis expose bacterial populations to small doses of antibiotics over extended time periods, which are often administered to an entire herd (Rolain, 2013). This kind of application of antibiotics is of more concern over therapeutic use, which generally involves higher dose administration over a short period, to the specific diseased animal/s (Phillips *et al.,* 2004). Although the use of antibiotics as growth promoters has now been banned in the European Union since 2006, they are still used by many other countries worldwide, including South Africa (Szmolka & Nagy, 2013). The application of antibiotics as growth promoters and at sub-therapeutic doses in food-producing animals results in the creation of a constant selection pressure in bacterial communities, which, in turn, favours the emergence of ABR bacteria (Catry *et al.,* 2003; Gilchrist *et al.,* 2007).

The potential for transmission of zoonotic pathogenic and commensal bacteria to humans, either by direct contact with animals or through indirect contact via the food chain, presents a major challenge to the future of human health (Mc Nulty *et al.,* 2016). Antibiotic resistant bacteria can be transferred to humans through consumption of contaminated meat that is improperly cooked and/or inappropriately handled during food preparation (Essack & Bester, 2010; Lerma *et al.,* 2014).

1.3. Epidemiology of antibiotic resistance

Antibiotic resistant cases in the farming industry are commonly documented in intensive animal production, such as broiler chickens and feedlot cattle and pigs, where the use of antibiotics is evident and regularly employed (Phillips *et al.,* 2004; Gilchrist *et al.,* 2007). However, research on extensively produced food animals, such as free-range livestock and game species, is narrowly documented (Szmolka & Nagy, 2013), and is the area of study in this present work. This scarcity of research in these environments may be due to low, or negligible, expectations of intrinsic and acquired resistance patterns in these natural environments. But microbial ecosystems in nature are never isolated and thus extensive gene exchange occurs in nature, usually via mobile genetic elements (Woolhouse *et al.,* 2015).

There are numerous vectors of antibiotic resistance transfer throughout the environment, from agriculture to the food industry, which result in a constant release of low level antibiotic concentration into the water and soil through wastewater treatment plant effluents, sewage, agricultural waste and bio-solid application to fields, among others (Dias *et al.*, 2015). Other vectors of antibiotic resistance transfer include wind, crops and flies (Heuer *et al.,* 2011; Mercat *et al.*, 2016). Various mechanisms of transfer of antibiotic resistance among different industries (Figure 1.1) indicate that these sectors are, indeed, interlinked.

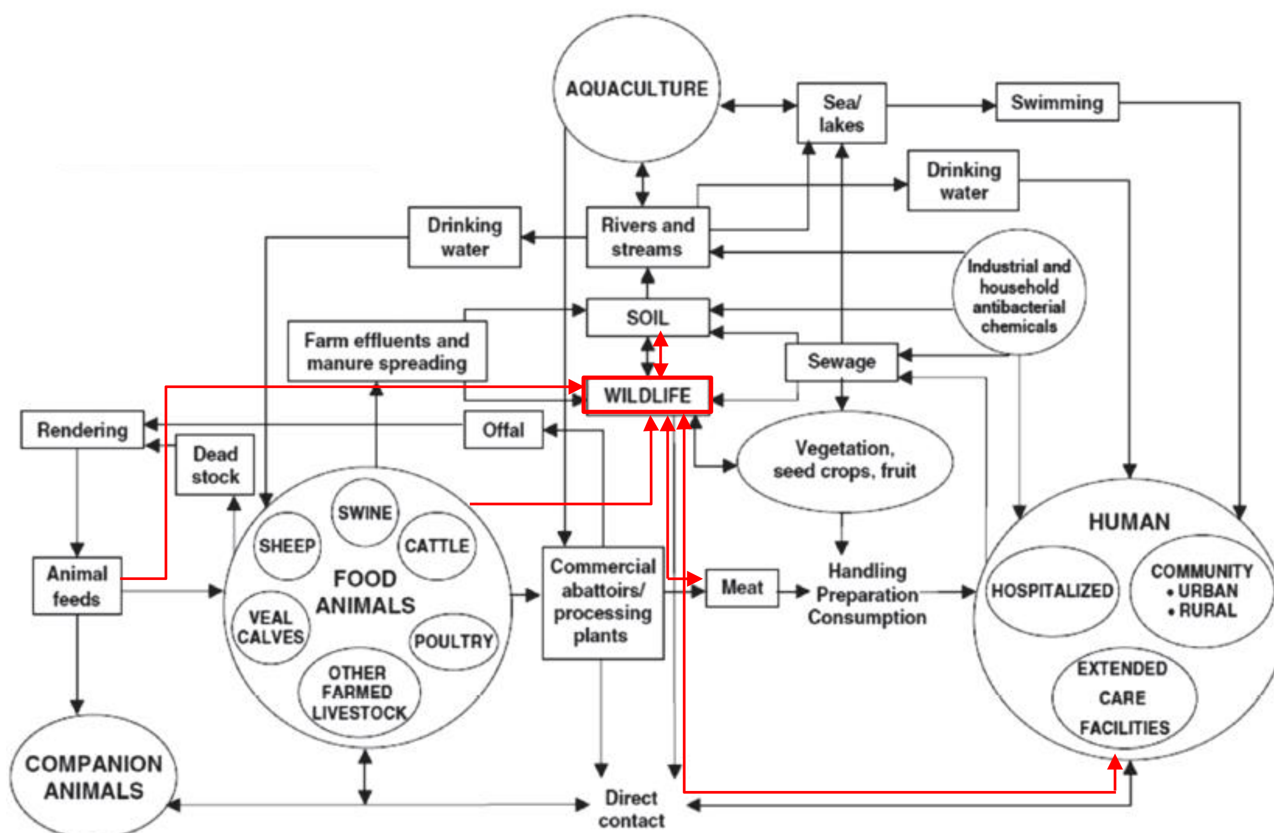

Figure 1.1 Epidemiology of antimicrobial resistance (adapted from Landers *et al.,* 2012). With a focus on the wildlife sector in this study (highlighted in red).

This environmental pollution, together with cohabitation of livestock and wild animals and increased human contact with animals, allows antibiotic compounds to reach many natural habitats over vast areas. This, in turn, alters the population dynamics of microorganisms, facilitating the emergence of antibiotic resistance in microbiological communities of wild animals through increased selection pressure (Martínez, 2008; Dias *et al.*, 2015).

Despite the perception of a low risk of antibiotic resistance developing in natural environments, studies, as listed by Vittecoq *et al.* (2016), have shown that antibiotic resistance among wild animals is a growing public health issue, due to increased wildlife contact between humans, livestock and domestic animals, as well as increased co-habitation with other animals, among others. In addition, there is a rising trend of consumption of game meat (Sousa *et al.*, 2014; Dias *et al.*, 2015). There is therefore a potential likelihood

of ABR traits being found in food-producing animals, leading to the distribution of antibiotic resistance genes in other, more widespread, populations, such as humans through the food chain and into the broader environment (Aminov & Mackie, 2007).

It has been hypothesised that wildlife could play a significant role in the development of ABR bacteria in nature. This is due to the fact that a variety of wildlife species carry ABR bacteria intrinsically and cover a large territory throughout their lifespan (Vittecoq *et al.*, 2016). Recent studies have demonstrated that wild animals and their surrounding environments can become important reservoirs of ABR bacteria (Karesh *et al*., 2012; Guerrero-Ramos *et al.,* 2016; Vittecoq *et al*., 2016).

Resistance genes transferred to the commensals of wildlife species could evolve into more harmful variants and be passed on to pathogenic bacteria. From there, they could be transferred back into the human and domestic animal environments, generating a host of major health issues in the future (Mercat *et al.*, 2016).

1.4. Study Aim

A better understanding of antibiotic resistance in natural environments may help to predict and counteract the emergence and development of resistance (Martínez, 2008).

To the author's knowledge, limited research has been conducted on the antibiotic resistance profiles of extensive livestock and wild ungulates, especially in developing countries such as South Africa. Despite conflicting results shown in previous studies, the availability of data on the antibiotic resistance levels and transfer to wild species is limited (Katakweba *et al.*, 2015). It is often assumed that antibiotic resistance development is unlikely to occur in wild animals as they are known to be free-ranging animals which graze on open pastures. However, with the increase in more intensive production systems for various wildlife species in South Africa, the feeding of artificial feed, often containing antibiotics, has become a more common practice (without many producers understanding the impact that this might have on the microbiota of the wild species) (Bekker *et al.,* 2011). Filling this knowledge gap will enable greater understanding of this phenomenon among the farming community in South Africa. This new knowledge will enable the agricultural sector to take some preventative measures.

The overall aim of this study was to determine whether livestock and wild ungulates host antibiotic resistance bacteria, specifically *Escherichia coli, Enterococcus faecalis* and *Staphylococcus aureus,* on various South African farms. These microorganisms being analysed, are emerging resistant commensal and pathogenic bacteria and are commonly found on and in humans and animals (Wallmann, 2006). *Escherichia coli* and *E. faecalis* are commonly used by other researchers as indicator organisms of antibiotic resistance in an environment. This study strives to make a contribution to a greater global understanding of how resistance to antibiotics may increase in future through highlighting key agricultural processes and distribution patterns of the spread of ABR bacteria from natural, more remote areas.

To achieve this, a comparative study between co-grazing and non-co-grazing wildlife and livestock and supplementary fed and non-supplementary fed wild ungulates was conducted. The results could determine

if co-grazing, and both wildlife supplementary feeding and human contact increases bacterial resistance and facilitates antibiotic resistance transfer. In addition, the study would show if any intrinsic resistance was present in the free-range/ non-supplementary fed animals.

The prevalence of ABR bacteria on game meat was also conducted to assess the potential risk to the food chain and thus to human health. Finally, common resistant genes were detected to confirm phenotypic resistance and to gain a better understanding of the mechanism of resistance used by the resistant bacteria.

The conclusions of this study could enable better understanding of the origins and spread of ABR bacteria from agriculture and the environment in South Africa (and in countries with similar conditions). It could also lead to the development of new monitoring systems to track and analyse the distribution of ABR bacteria through the interlocking systems of farms, food production and food consumption and thus to lower the risk of mass increases of this phenomenon in future, especially during a time when game meat is in a greater demand and thus could be a major risk factor.

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CHAPTER 2

Literature Review

Antibiotic resistance in livestock and wildlife farming in South Africa[1]

2.1. Background

Microbial resistance to antibiotics is growing rapidly. This has brought about increased public concerns and scientific interest over the last decade (Wellington *et al*., 2013; Fair & Tor, 2014; Tanwar *et al*., 2014). Antimicrobial resistance (AMR) of bacterial origin, known as antibiotic resistance (ABR), of zoonotic food-borne pathogens, is now considered by the World Health Organisation (WHO) to be one of this century's leading global health challenges (Marshall & Levy, 2011; Mc Nulty *et al.,* 2016).

Antimicrobial resistance is the ability of a microorganism to resist the action of an antimicrobial that has been designed to kill it through targeting different parts of the microorganism's cell which are essential for growth and survival (CDC, 2018). The antibiotic resistant microorganism is thus able to survive and grow, despite the presence of an antimicrobial. If antimicrobials lose their ability to effectively kill microorganisms which are causing an infection, the infection cannot be treated, creating a major health threat (CDC, 2018).

Increasing levels of resistance, and the simultaneous decline of new antimicrobial development pose a major threat to global health, leading to a higher rate of treatment failure, increased infection, severity of infection, and a rising occurrence of infections that would have otherwise not occurred in animals and humans. Taken together, these could precipitate a 'post-antibiotic era', resulting in ineffective antibiotic use, whereby diseases caused by resistant bacteria would become untreatable with the traditional range of known antibiotics (Capita *et al*., 2016). This would create a burden on the public health system. More people would require hospitalisation while the likelihood of rising mortality rates would increase (Angulo *et al*., 2004).

It should be noted at the same time, that AMR also impacts on the general maintenance of animal health, especially in commercial animal husbandry where heightened animal density accelerates the spread of bacterial diseases.

The phenomenon of ABR has received substantial attention over recent years. This is due to the rapid emergence of antibiotic resistant pathogenic bacteria, including both Gram-positive and Gram-negative bacteria (Magiorakos *et al*., 2011; Mc Nulty *et al.,* 2016). Of special concern are the so-called "superbugs".

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[1] This chapter has been partially published in:
van den Honert M.S., Gouws, P.A. and Hoffman, L.C. (2018). Importance and implications of antibiotic resistance development in livestock and wildlife farming in South Africa. *South African Journal of Animal Science*, **48**, 401 – 412.

These are multidrug-resistant (MDR) pathogens and they pose a threat to the treatment of life-threatening infections, since little or no effective antibiotics currently exist (Aminov & Mackie, 2007; Sandora & Goldmann, 2012).

Although there is not a universally defined definition of 'multidrug resistance' (MDR), many researchers have classified it as resistance to three or more antimicrobial classes (Magiorakos *et al*., 2011). This seems a reasonable benchmark. An example of a MDR pathogen is methicillin-resistant *Staphylococcus aureus* (MRSA) which has developed since the introduction of methicillin into the clinical environment. This led to the first cases of MRSA infections in patients in European hospitals in the early 1960s and this has since spread worldwide, well beyond the carefully controlled hospital environment (Chambers, 1997; Sandora & Goldmann, 2012). Likewise, MDR *Enterococci* are becoming a clinical challenge. They have developed resistance to a vast number of antimicrobials currently used in clinical settings due to their ability to harbour a diverse array of genetic strategies (Miller *et al*., 2014).

Although there has been an effort to decrease the emergence of AMR development in the farming industry in the European Union (EU) through the banning of nontherapeutic use of antimicrobials from 2006, in hope that other countries will follow and end the practice, there is still much debate on the matter, largely associated with the cost/benefit ratio through the use of antibiotics for nontherapeutic purposes (Marshall & Levy, 2011).

What's more, the control of food-borne illnesses has been significantly challenged by the development of antimicrobial resistance, as resistant food-borne pathogens have the potential to be transferred to humans as food contaminants (Nyenje & Ndip, 2013). This latter possibility reinforces the urgency of addressing this growing problem in animal food production.

Although there is still no consensus on the degree to which antibiotic usage in animals contributes to the development and spread of AMR in humans, experimental evidence exists that suggests a relationship between antimicrobial use and AMR development in animals and the spread to humans, especially via the food chain (van Vurren, 2001). This is therefore one important area in which to focus academic attention.

2.2. Development of antibiotic resistance

The discovery and production of antibiotics in the early 1900s contributed to a significant increase in the human lifespan (van Hoek *et al.*, 2011). Sir Alexander Fleming discovered the first antibiotic, Penicillin, in 1928.

Initially, it was believed that ABR development would be minor, based on the assumption that the emergence of antibiotic resistant mutants would be negligible (van Hoek *et al*., 2011). Bacteria's ability to interchange genes through a process of horizontal gene transfer (HGT) was largely unforeseen. Likewise, a microorganism's ability to adapt to environmental conditions was greatly overlooked and underestimated.

In nature, it became clear, there exists a harmonious balance between antibiotic levels produced by antibiotic-producing bacteria and antibiotic resistant bacteria in an ecological population (Rolain, 2013). Antibiotics produced by organisms in the natural environment serve the purpose of cell-to-cell signalling

networks and furthering homeostasis of bacterial communities, over and above, that is, the use of antibiotics to increase the chances of survival in a comprising or competitive environment (Rolain, 2013).

The rise in antibiotic resistant bacterial species is a result of a complex mix of factors. Some factors are unavoidable and inherent in nature, such as the ability of bacteria to adapt rapidly to changing environmental conditions because of their short generation time and the intrinsic resistance of certain bacteria (Woodford & Ellington, 2007; Laximinarayan *et al*., 2013). But some variables are human made, such as the extensive use of antibiotics as growth promoters in the farming industry (Lowy, 2003; Ventola, 2015).

Furthermore, selection pressure, mainly caused by the use of antibiotics, determines the rate and extent of ABR emergence in an environment as it excludes susceptible bacteria and promotes the development of resistant bacteria (Catry *et al.,* 2003).

In general, the prevalence and persistence of AMR is a result of a complex interaction between the microorganisms, antimicrobials, the host and the surrounding environment (Catry *et al.,* 2003). It has been found that after removal of an antimicrobial agent and thus the selection pressure in an environment, the resistance persists for different periods of time depending on the type of antimicrobial agent and type of microorganism involved. For example, tetracycline resistance is more persistent that gentamycin resistance (Catry *et al.,* 2003).

Even longer periods of persistence can occur if the resistant bacteria obtain a selective growth advantage over the susceptible bacteria and thus continue to thrive in the environment. This selective growth advantage is often linked with genes that encode for survival, virulence and multiplication characteristics, for example, biocide resistance (Catry *et al.,* 2003). This relationship between virulence and antibiotic resistant genes has been demonstrated in some studies in which strains of Enterotoxigenic *Escherichia coli (*ETEC) have higher resistance levels than non-ETEC strains of *E. coli* (Catry *et al.,* 2003).

The most concerning and most common development of ABR involve bacteria, due to their inherent ability to rapidly and easily multiply. They are also the leading cause of food-borne illnesses (Nyenje & Ndip, 2013). When a stress factor like an antimicrobial compound is exerted on a bacterial population, all the susceptible bacteria are killed, whereas the few intrinsically resistant bacteria are able to survive (Capita *et al.*, 2016).

Antibiotics kill bacteria via various mechanisms. Some are cell wall active agents whereas some interfere with protein synthesis or DNA synthesis (Miller *et al.*, 2014). For example, ampicillin antibiotics target the cell wall by preventing the synthesis of peptidoglycan and quinolones target enzymes that play a part in DNA replication (Miller *et al.*, 2014). By a selective pressure created by the antibiotic, the 'fitter' bacteria that survived the treatment multiply. In doing so, they produce resistant offspring which essentially replaces the previously susceptible bacterial population, creating a larger antibiotic resistant population than before (Capita *et al.*, 2016). The resistant organisms develop resistance that is specific to a particular antibiotic (Khachatourians, 1998). For example, tolerance to ampicillin is associated with the presence of a gene that encodes for low binding affinity for the antibiotic, thus requiring a higher concentration in order to saturate the

active site (Miller *et al.*, 2014). Furthermore, a resistance mechanism to quinolone involves the presence of efflux pumps which externalises the antibiotic (Miller *et al.*, 2014).

The development of AMR is inherent and unavoidable and can be described as a Darwinian process – that is, selection of the fittest (Capita *et al.*, 2016). Antimicrobial resistance can be viewed as failure of a given antimicrobial treatment where a microorganism has a temporary or permanent ability to remain viable under conditions that would otherwise have destroyed or inhibited other sensitive members (Anon., 2006). More specifically, ABR involves resistant bacteria to an antibiotic compound and it is denoted in the laboratory through a Minimal Inhibitory Concentration (MIC) value or zone if inhibition that exceeds a pre-determined threshold value (Anon., 2006). Chapman (1998) defined AMR to a certain antimicrobial compound if it exhibits significantly reduced susceptibility when compared to a group of sensitive strains (Anon., 2006).

Bacteria exhibit a number of ABR mechanisms, including (i) efflux pumps which actively pump out the antibiotic from the bacterial cell and thus decrease the intracellular antibiotic concentration, (ii) enzyme modification of the antibiotic which renders it ineffective, (iii) degradation of the antimicrobial compound, (iv) the use of alternative metabolic pathways to those inhibited by the antibiotic, (v) overproduction of the target enzyme, (vi) modification of antibiotic targets, and lastly, (vii) alteration of cell wall permeability, which stops the antimicrobial agent entering the cell and reaching the target sites (Bhullar *et al.*, 2012).

A microorganism may even be resistant to antimicrobials to which it has never been exposed. This is known as intrinsic, or innate, resistance (Anon., 2006). Intrinsic resistance is naturally occurring and is related to physiological attributes, such as the complexity of the cell wall, as in Gram-negative bacteria (Anon., 2006). Gram-negative bacteria usually have a higher level of intrinsic ABR than Gram-positive bacteria, owing to their impermeable double membrane in the cell wall, making them innately resistant to penicillin, since the latter cannot enter their cell wall (Anon., 2006). Other intrinsic resistant traits include poor cell envelope permeability, production of enzymes that inactivate antibiotics and the presence of efflux systems that can decrease the intracellular antibiotic concentration (Bhullar *et al.*, 2012). Another characteristic of intrinsic resistance is the ability of some microorganisms to form a biofilm which protects the microbial cells through secretion of exopolysaccharides (Anon., 2006).

Temporary intrinsic resistance, known as 'adaption', can occur through activation of silent resistant genes in stressful situations where selective pressure increases due to, low or high temperatures, competing bacteria or low nutrient environments. Once environmental conditions become favourable for growth, then the bacterial cells once again become susceptible to the antimicrobial agents (Anon., 2006). 'Back mutations' occur when conditions become favourable again because resistant attributes generally have fitness costs associated with them (Anon., 2006).

Intrinsic resistance is shown in various studies where ABR was detected in environments in which antibiotics have never been used before (Jeters *et al.,* 2009). For example, a microbiome in an isolated cave in New Mexico exhibited ABR to several commercially available antibiotics, indicating that ABR is prehistoric. It appears to occur naturally in order to increase the chance of survival of microorganisms in a compromising environment (Bhullar *et al.*, 2012).

One of the first antibiotic-resistant bacterium studied in wildlife was found in wild pigeons around 1975, when strains of *E.coli* were found to be resistant to various antibiotics (Bonnedahl & Järhult, 2016). However, this naturally existing ABR gene pool is not a major threat to human and animal health as it contributes to a minute portion of ABR in populated environments (Davies & Davies, 2010).

Of greater concern over intrinsic resistance is the ability of bacteria to *acquire* resistance, which is widely considered the most important form of ABR development. Acquired resistance occurs either through genetic mutations or horizontal transfer of genetic material via plasmids or transposons from other resistant microorganisms (Capita *et al.*, 2016).

2.3. Antibiotic resistance transfer

Bacteria can develop resistance to antibiotics via mutation or the acquisition of resistant genes from other bacteria (von Wintersdorff *et al.*, 2016). The development of ABR is associated with the use, particularly misuse and overuse, of antibiotics in agriculture and human medicine, which creates selection pressure that favours the development of resistant bacterial strains. Antibiotic resistance can even develop towards antibiotics that have not been used, if the resistance determinants are genetically related (Phillips *et al*., 2004).

Acquired ABR develops as a result of the transfer of resistant determinants between bacteria. Transfer of antibiotic resistant genetic material through HGT is of greater concern than random genetic mutations, because it can occur more often with a more specific outcome. In addition, HGT can occur between bacterial strains, species and even between genera that share the same ecological niche, and, more worryingly, from non-pathogenic to pathogenic bacterial strains (Capita *et al*., 2016).

There are various ways in which HGT can take place, namely, conjugation, transduction and transformation (Capita *et al.*, 2016). HGT is an indirect antimicrobial resistant transfer mechanism whereby mobile genetic elements that carry resistant genes can be transferred from non-pathogenic to pathogenic bacteria (Capita *et al.*, 2016). This genetic transfer can occur almost anywhere throughout the food chain, such as in food producing animals or in the environment, for example, in soil (Capita *et al.*, 2016).

Certain conditions and factors favour the probability of the resistant genes being transferred in an ecological niche. The larger the pool of resistance genes in a certain bacterial population, the greater the likelihood of transfer among the bacterial population (Capita *et al.*, 2016). Normal gut flora in humans and animals represent an example of a large pool of resistance genes owing to their high bacterial density (Capita *et al.*, 2016). For this reason, *E. coli* is most commonly used as an ABR indicator organism owing to its abundance in mammalian gut flora and its ability to exchange genetic material (Adefisoye & Okah, 2016).

Furthermore, certain resistance genes are more likely to be transferred than others. This includes those that lead to target modifications, pump efflux synthesis and genes that encode for enzymes that modify the antibiotic structure (Capita *et al.*, 2016). In addition, certain microorganisms are more likely to develop ABR than others. Of particular concern here are the bacterial pathogens (Ashbolt *et al.*, 2013).

2.3.1. Conjugation

The conjugational pathway, illustrated in Figure 2.1, constitutes a direct cell-to-cell exchange of genetic material through enzyme activities involving a donor and a recipient cell (Essack & Bester, 2010). The transfer of genetic material between the two cells can occur within species or between species (Essack & Bester, 2010). The exchange of genetic material occurs via a specific hair-like temporary attachment called a "pilus" on the surface of the donor cell which creates a bridge between the two cells (Capita *et al.*, 2016). Then enzymes initiate the transportation of a single DNA strand to the recipient which synthesises a double DNA strand to create a copy of the antibiotic resistant gene (Essack & Bester, 2010). Not only can chromosomal DNA be transferred to other hosts but so can plasmids, transposons, integrons and insertion sequences which all bear one or more genes from the donor cell (Levy, 1998). The exchange of conjugative plasmids is the most efficient and common form of genetic transfer. This is because of independent replication in the host cell. This can therefore be considered as the most likely way in which ABR is acquired (Davies & Davies, 2010; Essack & Bester, 2010). Figure 2.1 illustrates the mechanism of conjugation.

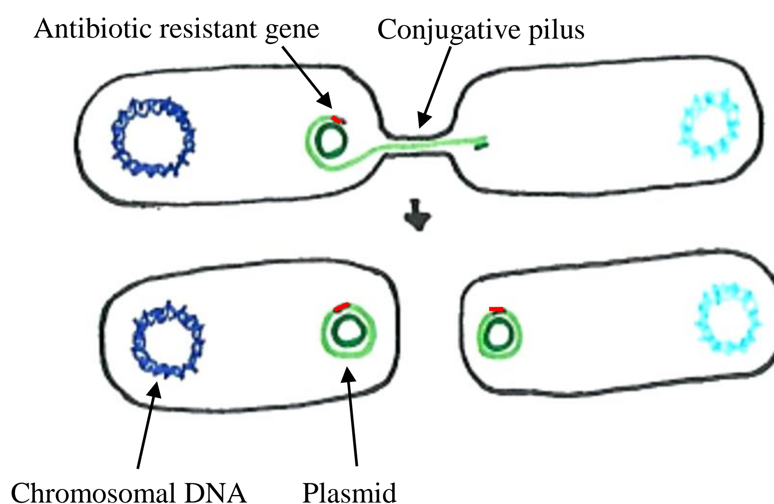

Figure 2.1 The exchange of conjugative plasmids (adapted from von Wintersdorff *et al.*, 2016).

2.3.2. Transduction

Transduction involves injection of genetic material into a host cell by making use of a "vector" (Capita *et al.*, 2016). Genetic material can be passed onto a suitable bacterial host by a bacteriophage that has previously replicated in another bacterium and contains random DNA fragments, such as insertion elements, plasmids and chromosome fragments. Antibiotic gene transfer of *tet*M (tetracycline) and *ant*2-1 (gentamicin) via transduction has previously been reported between *Enterococci* strains and species (Fard *et al.,* 2011).

Transduction appears to play a key part in bacterial evolution due to the transfer of virulence and antibiotic resistant genes to new bacterial hosts by bacteriophages that are highly abundant in nature (Muniesa *et al.,* 2013). Transduction has been considered a rare event in the past but due to the abundance of phages and

hosts in many environments and due to the additional fact that phages have excellent survival capabilities, genetic transfer via transduction has been postulated to contribute significantly to ABR in microbial communities (Muniesa *et al.,* 2013). However, gene transfer via transduction is still at this stage considered a minor mechanism compared to other genetic transfer routes such as conjugation and transformation. Figure 2.2 below demonstrates the transfer of DNA between bacteria via phages (Balcazar, 2014).

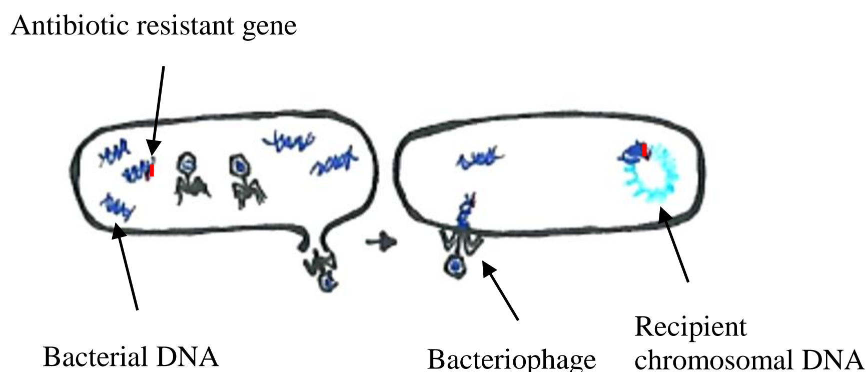

Figure 2.2 The transfer of DNA between bacteria via phages, known as transduction (adapted from Balcazar, 2014).

2.3.3. Transformation

The third type of transmission mechanism of bacterial genetic material in addition to conjugation and transduction is transformation, shown in Figure 2.3. This occurs via the uptake of free DNA from a bacterium cell close to a donor cell (Levy, 1998). The donor cell in such a scenario- is usually dead and releases its plasmids or short fragments of DNA into the environment, allowing its genetic material to be directly acquired by a healthy bacterium cell through its cell wall (Capita *et al.*, 2016). This type of genetic transfer has limitations and can only effectively occur in certain bacteria and in those that have the genetic capacity to absorb the loose DNA (Essack & Bester, 2010). Additionally, the DNA must be compatible with the recipient (Essack & Bester, 2010). This type of ABR transmission has been demonstrated in penicillin-resistant *Streptococcus pneumonia*, where penicillin-binding protein (PBP) genes encode for penicillin-insensitive enzymes by recombination from *Streptococcus mitis* (Blair *et al.,* 2015). Another example is the attainment of the staphylococcal cassette chromosome *mec* (SCC*mec*) element by which MRSA develops (Blair *et al.,* 2015). The *mec*A gene is carried on the SCC*mec*, which encodes for the PBP2a protein that enables cell wall synthesis to continue despite the inhibition exerted by the antibiotic penicillin (Blair *et al.,* 2015).

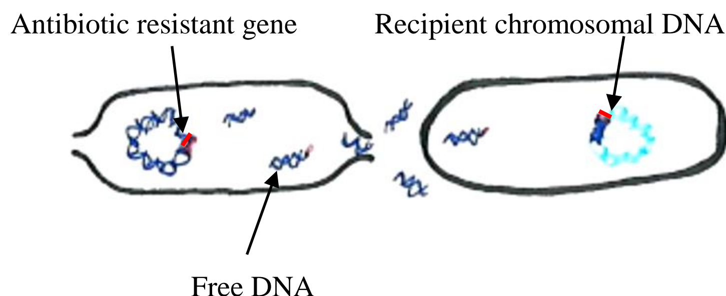

Figure 2.3 Uptake of free DNA from a nearby bacterium cell, known as transformation (adapted from Fronzes *et al*., 2009).

2.4. Existing antibiotic resistant bacteria

The potential danger of the development of ABR was first noted by Sir Alexander Fleming in the early 1900s. He predicted that if penicillin were to be misused following its introduction, then *S. aureus* mutants could develop that would build resistance to the antimicrobial compound (Capita *et al.*, 2016). Quite correctly, only a few years later, a significant amount of *S. aureus* strains were resistant to the treatment of penicillin due to the development of resistant strains capable of inactivating the drug, proving Fleming to be prescient (Davies & Davies, 2010). This lead to the development of β-lactam antibiotics, such as cephalosporins, which are penicillin derivatives that functions by inhibiting bacterial cell wall biosynthesis (Li *et al.,* 2009).

More recent instances involving the development of antibiotic resistant microorganisms include MRSA and vancomycin-resistant *Enterococci* (VRE), among others (Capita *et al.*, 2016). MRSA is considered as *S. aureus* strains that have developed a resistance to β-lactam antibiotics. Methicillin-resistant *S. aureus* began as a hospital-acquired infection, known as hospital-acquired MRSA (HA-MRSA) but has evolved to include community-acquired (CA-MRSA) and LA-MRSA (Anderson *et al.,* 2003; Smith *et al.*, 2013; Smith & Wardyn, 2015). Methicillin-resistant *S. aureus* isolates can be considered MDR as it predicts non-susceptibility to all antimicrobial categories of β- lactams (Magiorakos *et al.,* 2011).

Since the mid-2000s, livestock has become a primary reservoir of MRSA strains. A new variant, CC398, first noted in Finland in 2007, has recently been found in animals, especially intensively reared production animals like pigs, cattle and poultry, and can be transmitted to humans (Salmenlinna *et al*., 2010). Transmission is mainly via occupational exposure, resulting in mild to serious infections and even death (Anderson *et al.,* 2003; Salmenlinna *et al*., 2010; Smith & Wardyn, 2015; Grøntvedt *et al.,* 2016).

Furthermore, high level vancomycin-resistant *Enterococcus fecalis* and *Enterococcus faecium* were first documented in the 1980s (Cetinkaya *et al.*, 2000). *Enterococci* are common causes of hospital-acquired infections, notably due to their ability to acquire resistance to most antibiotics and are also intrinsically resistant to numerous commonly used antibiotics (Miller *et al.*, 2014). They can also become the dominant flora under antibiotic pressure which encourages MDR development. This can also be attributed to their ability to attain many ABR mechanisms and to survive in various different environments, such as nosocomial environments and the gastrointestinal tract (Miller *et al.*, 2014). The possible transfer of vancomycin-resistant genes to other

Gram-positive bacteria raises a further concern about the development of vancomycin-resistant *S. aureus* (VRSA) (Cetinkaya *et al.*, 2000). VRSA carry a transposon Tn1546 which encodes for high-level vancomycin-resistance in *Enterococci*, which may be considered as acquired from vancomycin-resistant *E. fecalis* (Gardete & Tomasz, 2014).

Other significant MDR microorganisms include *S. enteric*a ser. Typhimurium strains which became resistant to chloramphenicol, ampicillin and trimethoprim from the late 1980s. They were consequently responsible for numerous outbreaks of typhoid fever (Rowe *et al.*, 1997; Magiorakos *et al.,* 2011). Likewise, MDR *Acinetobacter baumannii* is a concern in the hospital environment due to its ability to survive there and cause various infections, including pneumonia, meningitis and wound infections. It also displays a persistence on surfaces for extensive time periods (Manchanda *et al.,* 2010).

Selection of resistant mutants and the uptake of antibiotic resistant genes from the environment has led to the recent development of extended spectrum beta-lactamase (ESBL)-producing *Enterobacteriaceae*, frequently found in *Escherichia coli* and *Klebsiella pneumonia* (Hawkey, 2008). They are antibiotic resistant bacteria that produce β-lactamase enzymes that can hydrolyse β-lactam antibiotics such as penicillins and cephalosporin antibiotics thus rendering them resistant (Rupp & Fey, 2003). Extended spectrum beta-lactamases are often located on plasmids which are easily transferred via HGT to other bacteria (Reinthaler *et al.,* 2010). The prevalence of ESBL- producing *Enterobacteriaceae* has been increasing since the 1980s and has been found in healthy food animals and even in wild animals (Poeta *et al.,* 2009). It is thought that the emergence of resistance caused by ESBL Enterobacteriacae is related to the spread from animal reservoirs via the environment and food (Anon., 2011b). Extended spectrum beta-lactamases now compromise third generation cephalosporins for treatment of Gram-negative infections. They have caused many infections, for example urinary tract infections. They have become a serious worldwide problem in nosocomial environments (Hawkey, 2008).

Over 400 β-lactamases have been described where the most dominant ESBL producing *E.coli* causing infections are of enzyme types CTX-M and TEM (Melzer & Peterson, 2007). TEM β-lactamase catalyses the hydrolysis of ampicillin and related antimicrobials and is associated with a transferable plasmid, causing transfer among *Enterobacteriaceae* and *Pseudomonas aeruginosa* (Hawkey, 2008). This drove the succeeding development of third generation cephalosporins where cefotaxime was first introduced. However, subsequent mutation of the SHV-1 β-lactamase encoding gene, namely the $bla_{\text{CTX-1}}$ gene, resulted in resistance to cefotaxime by allowing hydrolysis of the molecule to occur (Hawkey, 2008).

The CTX-M ESBLs produce enzymes that hydrolyse cefotaxime and are dominant in human *Enterobacteriaceae*. These genes are plasmid-associated and thus are highly transmissible which allows resistance to easily spread in the environment, often resulting in MDR (Anon., 2011b). Carapenems and fluroquinolones have been suggested for treatment of ESBL-producing Gram-negative pathogens but are becoming increasingly resistant to fluroquinolones which are the drugs of choice to treat urinary tract infections (Lee *et al.,* 2012). For this reason, ESBL-producing bacteria are frequently multi-resistant due to the presence and ease of uptake of multiple resistance mechanisms (Anon., 2011b).

Multidrug resistance poses a major worldwide health threat as the effectiveness of the primary antimicrobials used to treat serious infections is declining.

2.5. Important microorganisms in antimicrobial resistance development

Although viruses are responsible for many food-borne illnesses, bacterial infections cause most of them, accounting for up to 75% of infectious diseases in humans (Nyenje & Ndip, 2013; McNulty *et al*., 2016). The main causative bacterial agents include *Staphylococcus, Salmonella, Shigella, Clostridium, Campylobacter, Listeria, Vibrio, Bacillus* and enteropathogenic *E. coli* species (Nyenje & Ndip, 2013; FDA, 2017). It is therefore important to analyse the prevalence of ABR in food-borne pathogens with regard to human health (Wallmann, 2006).

Additionally, antibiotic susceptibility testing (AST) of indicator organisms *E. coli* and *Enterococcus* species is commonly used to determine the prevalence of acquired ABR. These commensal bacteria are seen as an indicator of ABR in bacterial populations, as they are known to form a reservoir of mobile resistance genes that can spread to other bacteria (Mc Nulty *et al.,* 2016). In addition, the South African National Veterinary Surveillance and Monitoring Programme for Resistance to Antimicrobial Drugs (SANVAD) has noted that *E.coli* and *Enterococcus* species have shown increased ABR, which is consistent with findings from its European counterparts (Eager *et al.,* 2012).

There are several bacterial pathogens that are well known for acquiring antibiotic resistant genes, which can also be used to evaluate the microbial risk of antibiotic resistant bacterial hazards in a human health risk assessment (Ashbolt *et al.*, 2013). The food-borne and water-borne faecal pathogens include *Salmonella enterica* and *Escherichia coli* and the environmental pathogens include *Legionella pneumophila, Staphylococcus aureus,* and *Campylobacter jejuni* (Ashbolt *et al.*, 2013).

Magwedere *et al*. (2012) and McNulty *et al.* (2016) recommended that the current major zoonotic food-borne pathogens *Salmonella* and *Campylobacter* should be monitored in food animals, as well as emerging zoonotic pathogens such as MRSA, ESBL-*Enterobacteriaceae* and *L. monocytogenes*. Additionally, monitoring of commensal bacteria is recommended as they give good indications of ABR levels in the environment (McNulty *et al*., 2016).

The WHO has listed ‘priority antibiotic resistant pathogens’ where there is urgent need to develop new antibiotics to fight against these emerging resistant pathogens, which pose a major threat to human health. The critical priority pathogens include carbapenem-resistant *Acinetobacter baumannii*, carbapenem-resistant *Pseudomonas aeruginosa* and lastly, carbapenem-resistant and ESBL-producing *Enterobacteriaceae*. The high priority pathogens include vancomycin-resistant *Enterococcus faecium*, vancomycin-resistant and methicillin-resistant *Staphylococcus aureus*, clarithromycin-resistant *Helicobacter pylori*, fluoroquinolone-resistant *Campylobacter* species and *Salmonellae* and lastly, fluoroquinolone-resistant and cephalosporin-resistant *Neisseria gonorrhoeae* (WHO, 2017).

2.5.1. *Staphylococcus* species

Staphylococcus species are Gram-positive, facultative anaerobic cocci that belong to the *Staphylococcaceae* family (Blunt, 2000). They are commonly found as part of the natural microbiota of the skin and mucous membranes of humans and animals and also colonise the intestinal tract (Sousa *et al.*, 2014). *Staphylococcus* species can be classified into two major classes based on their ability to produce coagulase. *Staphylococcus aureus,* the most notorious Gram-positive superbug, is a commensal and pathogenic coagulase-positive *Staphylococci* strain. It produces an enterotoxin, responsible for food poisoning and a wide variety of illnesses, ranging from minor skin infections to life-threating diseases such as pneumonia with a high impact on human and livestock health (Davies & Davies, 2010; Nyenje & Ndip, 2013; Luzzago *et al.*, 2014).

Staphylococcus species have shown increasing resistance since antimicrobials have been used, especially in hospital-acquired infections. They are already resistant to multiple antibiotics, except vancomycin which is known as the drug of last resort (Levy, 1998; Blunt, 2000; Doyle *et al.*, 2013). *S. aureus* have shown resistance towards many new antibiotics via acquiring resistance determinants (Smith *et al.*, 2013). Following the introduction of methicillin, it seemed that infections caused by penicillinases produced by bacteria were controlled, but within just three years, MRSA strains subsequently developed (Davies & Davies, 2010). The major concerns of diseases caused by *Staphylococcus* species are those caused by methicillin-resistant *S. aureus* (MRSA) (Chambers & DeLeo, 2009). Methicillin-resistant *S. aureus* has been found to be associated with domestic animals, food-producing animals and various food items such as meat and milk (Porrero *et al.*, 2014). Contamination of meat with MRSA can result from cross contamination of the carcass during the slaughtering and processing from the animal itself or from the people involved in the meat handling (Nyenje & Ndip, 2013). Many reports revealing an animal-to-human transmission of MRSA have raised concerns about the threat to human health, especially due to animal populations being potential reservoirs of MRSA (Smith *et al.*, 2013). This poses a threat to human health because MRSA is responsible for many nosocomial infections worldwide and could be transferred to humans via the food chain (Nyenje & Ndip, 2013).

2.5.2. *Enterococcus faecalis*

Enterococci are catalase-negative Gram-positive bacteria (Anderson *et al.*, 2016). They are able to survive harsh environments and are found in a variety of environments such as water, soil, food, plants and are predominant in the gastrointestinal tract of humans and animals (Giraffa, 2002; Hammerum, 2012). With their increase in ABR and their implication in life-threatening human diseases, much interest has been paid in identifying the reservoirs of the antibiotic resistant strains. Enterococci have a unique ability to exchange genetic material and are intrinsically resistant to various antibiotics such as cephalosporins, lincosamides, and low levels of aminoglycosides and beta-lactams (Giraffa, 2002; Hammerum, 2012). Increased acquired ABR towards ampicillin, tetracyclines, aminoglycosides and glycopeptides over the last two decades complicates clinical treatment (Giraffa, 2002; Hammerum, 2012). Vancomycin-resistant Enterococci are the most serious

concern with regards to acquired resistance where the vanA and vanB genes are most commonly associated (Giraffa, 2002).

Enterococcus faecalis and *E. faecium* are the most important Enterococcus pathogens, as they are associated with life-threatening infections in humans, such as bacteraemia and endocarditis (Hammerum, 2012).

2.5.3. *Escherichia coli*

Escherichia coli is a Gram-negative rod and a member of the family *Enterobacteriaceae*. They are commensal bacteria of the gastrointestinal tract of animals and humans. Consequently, they are commonly used as an indicator organism for faecal contamination (Mirzaagha *et al.*, 2009). The majority of *E .coli* exist as harmless commensals but some strains are pathogenic and can lead to serious illnesses (Cheney *et al.*, 2015). Their pathogenic traits are mainly attributed to virulent characteristics such as flagella, lipopolysaccharides, capsules and enterotoxins (Borriello *et al.,* 2012).

There are six types of intestinal pathogenic *E. coli* strains, classified according to specific virulence factors and phenotypic traits they exhibit. They include (i) shigatoxigenic *E. coli* (STEC), which causes haemolytic-ureamic syndrome and haemorrhagic colitis, (ii) enterotoxigenic *E. coli* (ETEC) which causes diarrhoea via ingestion of the enterotoxin, (iii) enteropathogenic *E. coli*, (iv) enteroaggregative *E. coli*, (v) enteroinvasive *E. coli*, which causes dysentery-like diseases and lastly, (vi) diffusely adherent *E. coli* (Nyenje & Ndip, 2013).

Shigatoxigenic *E. coli*, also referred to as enterohemorrhagic *E. coli* or verocytotoxin *E. coli*, produces a shiga toxin or shiga-like toxin (a verotoxin). It has been the most commonly associated pathogenic strain to food-borne outbreaks with serotype O157: H7 emerging as a major pathogen, causing gastrointestinal illnesses which can progress to haemolytic uremic syndrome (Heijnen & Medema, 2006). Outbreaks are mainly caused by the consumption of raw, or undercooked, contaminated beef, or contaminated water (Heijnen & Medema, 2006). Numerous reports have suggested that the application of some antibiotics, especially fluoroquinolones, induces and enhances production of the shiga toxin, resulting in serious illnesses or even death in STEC infected patients (Zhang *et al*., 2000).

Escherichia coli is commonly used as an indicator of ABR within microbial populations because it readily exchanges genetic material with other bacterial species and has the ability to harbour several resistant determinants. The prevalence of extended spectrum beta-lactamase (ESBL)-producing *Enterobacteriaceae* has been increasing since the 1980s and has been found in healthy food animals and even in wild animals (Poeta *et al*., 2009). The ESBLs now compromise third-generation cephalosporins for treating gram-negative infections (Hawkey, 2008).

In addition, *E. coli* can be carried asymptomatically by most livestock species and are commonly found in diverse hosts and environments (Catry *et al.,* 2003; Alexander *et al*., 2008; Adefisoye & Okoh, 2016). What's more, *E. coli* can effectively acquire conjugative plasmids that carry resistance genes and thus easily spread resistance to other enteric bacteria (Cheney *et al*., 2015). Monitoring of resistance patterns in indicator

bacteria are a good sign of the resistance situation in the bacterial population. Monitoring also pre-empts the overestimation of resistance levels (Catry *et al.,* 2003).

2.5.4. *Salmonella*

Salmonella ABR is a major global health concern owing to the increase in resistance to conventional antibiotics and the rise in MDR in recent years (Su *et al*., 2004; Alali *et al*., 2010). Transmission of *Salmonella* to humans most commonly takes place via contaminated meat, where poultry remains the major transmission vehicle (Alali *et al*., 2010).

Salmonella enterica subsp. *enterica* is the most common serovar and is the leading cause of foodborne salmonellosis (DiMarzio *et al*., 2013). Isolates resistant to first-line treatment antibiotics have been recorded since the 1980s and today, third-generation cephalosporins and azithromycin are used to treat resistant infections (Klemm *et al*., 2018).

2.5.5. *Clostridium* species

Clostridium difficile is considered the most important *Clostridium* specie with regard to emerging antibiotic resistant bacteria, according to the Centre of Disease Control and Prevention (CDC, 2013). The future of human health is threatened by the emergence of these antibiotic resistant strains, which is coupled with the rise of hypervirulent strains that cause severe infections, and their ability to spread rapidly (CDC, 2013; Spigaglia, 2016).

2.5.6. *Campylobacter* species

Campylobacter species have shown increased resistance to fluoroquinolones and macrolides, which is recognised as an emerging public health problem. Isolates resistant to macrolides have been found to be of animal origin, where *C. coli* has been most commonly found in pigs and *C. jejuni* in chickens (Nyenje & Ndip, 2013).

2.5.7. *Listeria* species

Listeria species are distributed widely in the environment. Only *Listeria monocytogenes* (*L. monocytogenes*) is pathogenic to humans and has emerged as a major food-borne pathogen (Teuber, 1999). Listeriosis is an often fatal disease for which antimicrobial treatment is essential, the main transmission route to humans being food borne (Nyenje & Ndip, 2013).

2.5.8. *Vibrio* species

Vibrio species are found in marine environments. Seafood-associated infections are usually caused by *V. cholera*, *V. parahaemolyticus* and *V. vulnificus* (Raissy *et al*., 2012). Recent studies have shown that these species have become multidrug resistant (MDR), owing to the misuse of antibiotics in aquaculture production,

and are thus major health threats to the public who can become infected with seafood-borne diseases causes by antibiotic resistant *Vibrio* species (Elmahdi *et al*., 2016).

2.5.9. *Bacillus* species

Bacillus species are used widely as probiotics and animal feed additives because of their ability to stimulate the immune system (Adimpong *et al.*, 2012). This has raised concern about the possibility of these feed additives acting as reservoirs of antibiotic resistant genes because *Bacillus* species have been shown to be resistant to several antibiotics (Adimpong *et al.*, 2012).

2.5.10. *Legionella pneumophila*

Legionella pneumophila is a gram-negative pathogen commonly found in water environments. It can cause pneumonia and upper respiratory tract infections (Aras & Saym, 2015). *Legionella pneumophila* colonises water systems, where exposure to veterinary and medical run-offs can occur easily, thus creating concern on the development antibiotic resistant *L. pneumophila*. Macrolides, fluoroquinolones, and rifampicin are the antimicrobials most commonly used in the treatment of legionellosis.

2.5.11. *Acinetobacter baumannii*

Acinetobacter baumannii is a gram-negative nosocomial pathogen which has shown increased ABR worldwide in hospitals, especially in intensive care units (Perez *et al*., 2007). *Acinetobacter baumannii* have become resistant to a wide range antibiotics, owing to its ability to easily exchange genetic material (Perez *et al*., 2007). Currently only carbapenems are still effective in treating *A. baumannii* infections but resistance to this class of antibiotic is also rising (Chen *et al*., 2017).

2.5.12. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a gram-negative pathogen which can colonise a wide range of environments, including hospitals, animals and aquatic environments (Yayan *et al.,* 2015). It is the pathogen which is most commonly responsible for community or hospital- acquired pneumonia. *Pseudomonas aeruginosa* has unique intrinsic antibiotic resistant mechanisms and metabolic versatility over other gram-negative pathogens, which has resulted in the emergence of multi-drug resistant strains (Cabot *et al.,* 2016).

2.5.13. *Shigella*

Shigella is responsible for the foodbourne illness “shigellosis”, which is infection of the colon causing bloody diarrhoea (Warren *et al.,* 2007). This pathogen has been labelled by the CDC as the third most common bacterial pathogen which caused disease (Warren *et al.,* 2007). Shigella finds its way most commonly into the food chain via poor hygiene practices of an infected food handler or via contaminated water (Warren *et al.,* 2007). Shigella have become resistant to the antibiotic drugs which are used to treat Shigellosis and are thus a

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huge threat to human health (Puzari *et al*., 2017). Currently, ceftriaxone and azithromycin are used to treat Shigellosis but isolates resistant to these antibiotics have already been reported (Puzari *et al*., 2017).

2.5.14. Enterococci species

Enterococci are Gram-positive commensals that live in the gastrointestinal tract of humans and animals As well as in soil and water environments. Enterococci are used as ABR and faecal contamination indicators, along with *E. coli* (Ünal *et al*., 2017). *Enterococcus feaecium* and *Enterococcus faecalis* are the most commonly isolated *Enterococus* species from the gastrointestinal tract. Enterococci were once not considered a major concern to human health (Huycke *et al.,* 1998). But *E. faecium* and *E. faecalis* and staphylococci are actually leading causes of nosocomial infections (Miller *et al*., 2014). *Enterococcus faecium* is of more concern over *E. faecalis* with regards to ABR, as *E. faecium* has shown much higher rates of vancomycin resistance, 80% versus 5% (Miller *et al*., 2014). This is due to its unique characteristics, such as hypermutability, which enables it to genetically adapt to multiple selective pressures (Miller *et al*., 2014).

2.5.15. *Helicobacter pylori*

Helicobacter pylori is a gram-negative pathogen that produces diseases of the upper gastrointestinal tract in humans (Thung *et al*., 2015). Multi-antibiotic therapies, involving various combinations of only six antibiotics, are employed to fight *H. pylori* infections. For example, triple therapy involves the administration of a protein pump inhibitor, clarithromycin and amoxicillin (Alba *et al*., 2017). However, resistance to these currently utilised antibiotics is becoming an emerging problem in the treatment of *H. pylori* infections (Mégraud, 2004).

2.5.16. *Neisseria gonorrhoeae*

The treatment of gonorrhoea, which is the second most prevalent sexually transmitted bacterial infection, is becoming a major public health concern due to the fact that it may become untreatable (Unemo & Shafer, 2011). *Neisseria gonorrhoeae* has an adaptive ability to avoid host defence systems and to acquire resistant genetic determinants and has shown to be resistant to previously and currently used antibiotics over the last 70 to 80 years (Unemo & Shafer, 2014).

2.6. Epidemiology of antibiotic resistance

Antibiotic resistance in the environment occurs in many sectors, from aquaculture, food processing and healthcare to wildlife, companion animals and food animals (Landers *et al*., 2012; Wegner, 2012). Figure 2.4 displays a comprehensive schematic of pathways, transfer vectors and practices which are all involved in the dissemination of ABR. The areas of focus in this study include the role that wildlife plays in the epidemiology of ABR (highlighted in green).

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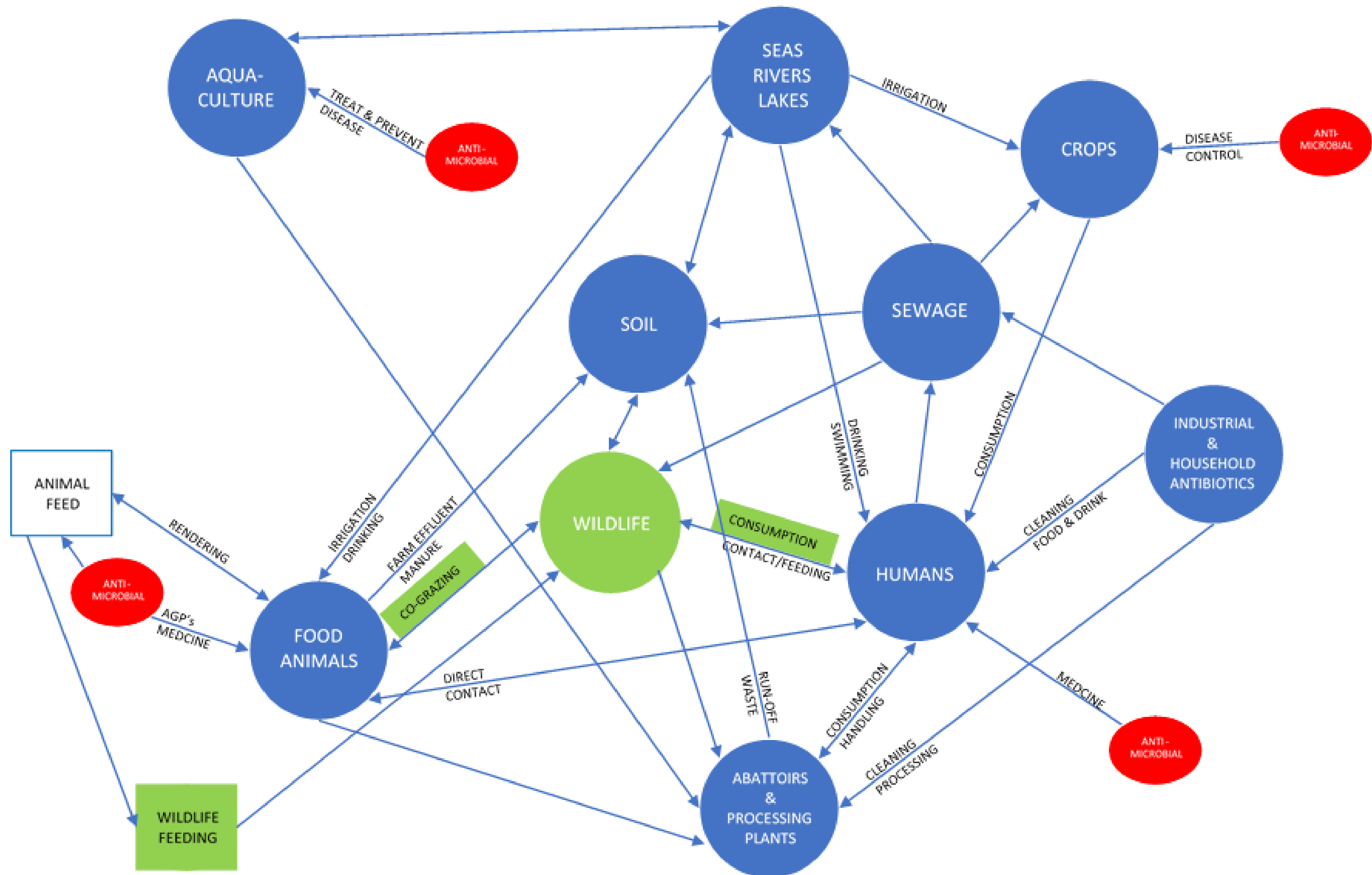

Figure 2.4 Epidemiology of antibiotic resistance (modified from Anon., 2006).

There are various stages and interactions in the food production chain where antibiotic resistant bacteria can enter. Linking resistant bacteria from animals to humans is not simple as there are many possible routes of transmission besides food animals (Phillips *et al*., 2004). Antibiotic resistant zoonotic food- borne pathogens in food-producing animals can spread to humans via consumption of contaminated food or water, as well as through direct contact with animals (Mc Nulty *et al.,* 2016). Antibiotic resistant bacteria can enter the food chain in different ways. First, the physical environment, such as soil, air and water, resistant organisms can spread from animals. An example is the consumption of water that is contaminated because of resistant bacteria from animal waste that is used as fertilizer (Landers *et al*., 2012). On the farm, there could be more direct transfer of resistant bacteria between animals in a herd and those in close proximity, as well as between farmers and their animals (Landers *et al*., 2012).

During food production, there are processing steps such as farming activities, slaughtering practices, meat processing and transportation of food animals which could possibly introduce resistant bacteria. Likewise, consumption of contaminated meat and other food products by consumers can result in a more direct transmission route of antibiotic resistant bacteria (Landers *et al.,* 2012).

2.6.1. Antibiotic resistance in food animals

Antibiotics are defined as compounds that have an inhibitory or lethal effect against sensitive bacteria which are used in human medicine and animals. Antibiotics can be of natural, semi-synthetic or synthetic origin (Rolain, 2013). Several purposes of antibiotics in the animal food industry include treatment of bacterial infections, prevention of infections, known as metaphylaxis, and growth promotion (Rolain, 2013; Mc Nulty *et al.,* 2016).

The human population is rapidly growing and is forecast by some demographic experts, to reach eight billion by 2030. This will be accompanied by a parallel increase in the global demand for food. Food demand on this scale requires industrial scale production of food to meet the demand.

However, with industrialisation of food production comes increased risks of contamination of food with pathogens (Koluman & Dikici, 2013). Various approaches have been used to combat these food-borne pathogens such as Good Manufacturing Practices (GMP), Hazard Analysis Critical Control Points (HACCP) and Good Agricultural Practices (GAP) (Koluman & Dikici, 2013).

To keep up with the enormous scale of global food production and limited resources, food producers have applied new technologies and alternative production methods to industrialise food production (Koluman & Dikici, 2013). An example involves the use of alternative feed additives to enhance the growth of animals, which are known as growth promoters (Koluman & Dikici, 2013). The mechanisms of growth promoters are still largely unknown, but it has been hypothesised that they work by altering microintestinal flora, which results in more efficient digestion and metabolism, as well as disease and pathogen suppression (Phillips *et al*., 2004).

More specifically, growth promotion is achieved through four main mechanisms: (i) enhancement of protein metabolism which improves digestion, (ii) stabilisation of the gut microflora by supressing pathogens,

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(iii) increased vitamin production by gastrointestinal microorganisms and (iv) increasing feed efficiency by increasing intestinal absorption of nutrients (Essack & Bester, 2010).

Antibiotics used for growth promotion such as bambermycins and bacitracin, have little or no application in human medicine, whereas some antibiotics commonly used therapeutically and for prophylaxis in livestock are closely related to those used in human medicine. These include β-lactams, sulphonamides, tetracyclines, macrolides, glycopeptides, quinolones, streptogramins and lincosamides (Phillips *et al*., 2004). Even though the same antibiotics are not necessarily used in humans and livestock, they may share the same class function with the same mode of action. Thus the resistance mechanisms developed are often the same.

Consequently, in 2006, Europe banned several antibiotics as growth promoters as a precautionary step (Phillips *et al*., 2004; Moyane *et al.,* 2013). Although this has commonly resulted in an increase in antibiotics for metaphylactic and prophylactic use, some countries, such as Denmark, have reported a large reduction in antimicrobial usage without a loss in productivity (Moyane *et al.,* 2013; Woolhouse *et al.,* 2015). To demonstrate, the banning of avoparcin, chemically similar to vancomycin, as a growth promoter in animal feed in Europe, has resulted in a decrease in vancomycin-resistant *Enterococci* in food products and healthy humans (Woolhouse *et al.,* 2015).

The agricultural industry relies greatly on the use of antimicrobials to improve animal health and productivity, especially in intensively reared species (Moyane *et al.,* 2013; Woolhouse *et al.,* 2015). Therefore, the volume of antimicrobial use in the industry is comparable with that of medicine (Anon., 2015). Worryingly, many antimicrobials that are regarded as important to human health are used in animal food production, such as tetracyclines, penicillins, and sulphonamides (Landers *et al.*, 2012).

But the use of antibiotics in the agricultural industry has increasingly come under scrutiny due to their overall impacts implication on human health (Catry *et al.,* 2003; Moyane *et al.,* 2013). Food animals have been considered an important source of resistant bacteria that can affect humans (Phillips *et al*., 2004). Furthermore, the use of antibiotics in agriculture which are also used in human medicine poses a threat to human medicine. Drugs considered important to human health are restricted for use in veterinary medicine in most countries (Catry *et al.,* 2003; Woolhouse *et al.,* 2015). Examples of antibiotics used in human medicine but not veterinary medicine include, fourth-generation cephalosporins, extended-spectrum penicillins, carbapenems, sulfones, cephamycins, monobactams etc.

Table 2.1 lists some of the antibiotic classes which are used both in human and veterinary medicine. Those underlined are those selected for testing in this study.

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Table 2.1 The most common antibiotic classes and their applications

Antibiotic class	**Antibiotic agents***	**Human medicine**	**Veterinary medicine**
Aminoglycosides	Gentamicin, neomycin, <u>streptomycin</u>	Yes	Yes
Penicillins	Amoxicillin, <u>ampicillin</u>, <u>penicillin G</u>, <u>methicillin</u>	Yes	Yes
Glycopeptides	<u>Vancomycin</u>	Yes	Yes
Macrolides	<u>Erythromycin</u>, tylosin	Yes	Yes
Quinolones	Enrofloxaxin, <u>nalidixic acid</u>	Yes	Yes
Streptogramins	Virginiamycin	Yes	Yes
Sulfonamides	Sulfamethazine, <u>sulphafurazole</u>	Yes	Yes
Tetracyclines	Chlortetracycline, <u>tetracycline</u>, oxytetracycline	Yes	Yes
Polypeptides	Bacitracin	Yes	Yes
Lincosamides	Lincomycin	Yes	Yes
Babermycin	Flavomycin	No	Yes
Ionophores	Monensin, salinomycin	No	Yes
Monobactams	Aztreonam	Yes	No
Cephalosporins	Cefadroxil, cefuroxime, <u>ceftazidime</u>	Yes	No
Carbapenems	Ertapenem	Yes	No
Lipopeptides	Daptomcyin	Yes	No
Oxazolidinones	Linezolid	Yes	No
Polypeptides	Bacitracin	Yes	Yes
Chloramphenicol	<u>Chloramphenicol</u>	Yes	No

*Antibiotics underlined are those selected for testing in this study

As pointed out earlier, the misuse, overuse and inappropriate use of antimicrobials in human medicine and animal husbandry creates an enormous selection pressure, which causes bacteria to adapt in response to the selective pressure, resulting in a more resistant population. This is therefore a key factor that drives the development of ABR (Lerma *et al.*, 2014; Mc Nulty *et al.,* 2016). Consequently, the farming industry has been established as an ABR reservoir 'hotspot' (Essack & Bester, 2010).

Due to the fact that many food-borne illnesses in humans are commonly associated with animal species and the surrounding environment, food-producing animals are of particular relevance to the emergence and transfer of AMR to humans via the food chain (Karesh *et al*., 2012; Mc Nulty *et al.,* 2016). For example, a multi-state outbreak of *E. coli* O157:H7 in 2006 in the United States (US) was related to consumption of spinach and the same isolates were found in wild pig and cow faeces and in a stream on one of the spinach farms (Karesh *et al*., 2012).

The potential threat to human health from the misuse of antibiotics in food animals is significant. Its impact has not been fully comprehended because of inadequate research and documentation (Landers *et al*., 2012). However, numerous studies associate ABR in food animals with antibiotic resistant infections in humans (Marshall & Levy, 2011; Landers *et al*., 2012; Chang *et al*., 2015). The most commonly identified food-borne pathogens that have been associated with bacterial resistance genes in humans and farm animals include MRSA, *E. coli*, *Salmonella and Enterococcus* species (Marshall & Levy, 2011). Zoonotic diseases, for example anthrax and rabies, account for up to 60% of infections in humans (Karesh *et al*., 2012). Furthermore, resistant microorganisms have been detected in environments surrounding livestock farms. For example, Graham *et al*. (2009) found that *Enterococci* and *Staphylococci* isolates from flies near poultry farms carried similar antibiotic susceptibility patterns to the isolates from poultry litter on the farms.

Moreover, bacteria in food that is ABR are likely to be more persistent in food commodities owing to their overall resistance to other environmental stressors, such as preservatives and disinfectants (McMahon *et al*., 2007). This is due to induction of the mar operon as a result of exposure to environmental stress. The mar operon regulates the expression of many genes, including those that encode for various antibiotic resistant mechanisms (McMahon *et al*., 2007; Anon., 2016). Therefore, the resistance of bacteria to antibiotics can have an influence on their persistence of bacteria owing to their abilities to withstand the effects of various environmental stressors.

There are numerous studies associating ABR in food animals with antibiotic-resistant infections in humans (Landers *et al.,* 2012). Furthermore, resistant microorganisms have also been detected in environments surrounding livestock farms where isolated resistant bacteria have been detected. Some of these instances are listed in Table 2.2.

Table 2.2 Studies of resistant microorganisms detected in environments surrounding livestock farms (adapted from Landers *et al.,* 2012)

Study author, year, country	Study findings
van den Bogaard *et al.,* 1997, Netherlands	VRE isolated from 50% of the turkey samples, 39% of farmers and 20% of turkey slaughterers. VRE more prevalent among turkeys fed avoparcin
van den Bogaard *et al.,* 2002, Netherlands	Resistant traits from *Enterococci* in chickens were transferred to *Enterococci* in humans. Resistant *Enterococci* more prevalent among broiler chickens and their farmers than among laying hens and their farmers.
Cui *et al*., 2005, United States	Resistant *Campylobacter* and *Salmonella* more prevalent among conventional chicken than organic chicken isolates.
Gundogan *et al*., 2005, Turkey	Resistant *Staphylococcus aureus* isolates were recovered from retail calf, lamb and chicken products
Ramchandani *et al*., 2005, United States	Resistant *E. coli* isolates from humans of a multistate outbreak of urinary tract infections suggested to originate from food animals and acquired through the food chain
Rule *et al.,* 2008, United States	Air and surface samples collected behind vehicles transporting poultry contained resistant bacteria
Graham *et al.,* 2009, United States	*Enterococci* and staphylococci from flies carried resistant traits similar to those from poultry litter
Smith *et al.,* 2009, United States	MRSA isolated from swine workers were closely related to MRSA isolates from swine and were different to strains commonly found in humans

As a result of the findings summarised in Table 2.2, it is clear that the food-borne route is where the most resistance can be transferred from animals to humans. Most infections with bacterial pathogens, we know, occur via the food chain (Wegner, 2012). A number of recent antibiotic resistant pathogens have emerged in the food chain, namely, extended β-lactamase producing *Salmonella* and *E. coli*, quinolone-resistant *Salmonella* and *E. coli* and also livestock-acquired methicillin-resistant *S. aureus* (LA-MRSA) (Wegner, 2012). These cases are associated with antimicrobial use in food animals. The use of antibiotics used in food-producing animals is becoming highly important for human health and consequently needs to be much more strictly controlled (Wegner, 2012).

Linking resistant bacteria from animals to humans is not simple as there are many possible routes of transmission other than food animals. Nonetheless, the food chain has been hypothesised as the main route of transmission to humans.

2.6.2. Antibiotic use in the agricultural industry in South Africa

Two acts regulate the use of antibiotics for animals in South Africa. The first is the Fertilisers, Farm Feeds, Agricultural Remedies and Stock Remedies Act (Act 36 of 1947), administered by the Department of Agriculture (DAFF). The second is the Medicines and Related Substances Control Act (Act 101 of 1965), administered by the national Department of Health (DoH) (Henton *et al.,* 2011).

Antibiotics that are available to the public and are sold over the counter are registered under Act 36. However, records of use are not kept. Antibiotics available only via prescription by a veterinarian are registered under Act 101, which also controls human medicines (Henton *et al.,* 2011). Antibiotics registered under Act 36 are purchased and administered mainly by farmers. This is contrary to the WHO guidelines, which suggest that all antibiotics should be administered and used only by licenced professionals (Henton *et al.,* 2011).

Data on the sales and use of antibiotics in livestock production are scarce in South Africa. Therefore, it is difficult to identify trends and patterns of antibiotic consumption. Eagar *et al.* (2012) showed that there are several antibiotic classes that are commonly used in the agricultural farming industry as in-feed growth promoters (Figure 2.5), which constitutes about 68% of the total antimicrobial forms sold for food animals, as opposed to water medication, parenterals, topicals and intramammaries. These antibiotics can be viewed in Figure 2.6 below, as the top six in-feed antibiotic classes sold in South Africa between 2002 and 2004 (Eager *et al.,* 2012).

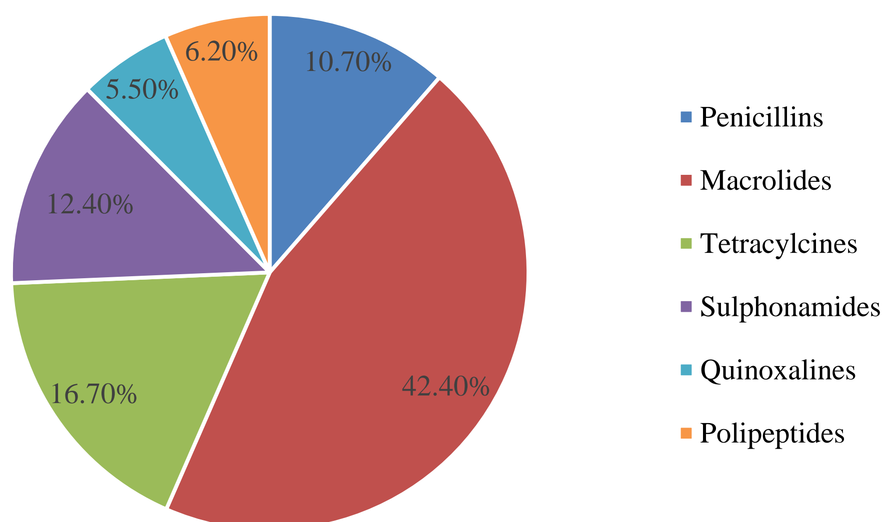

Figure 2.5 Percentages of volume (kg) for sales of classes of antimicrobials for the period 2002-2004 in South Africa (adapted from Eager *et al*., 2012).

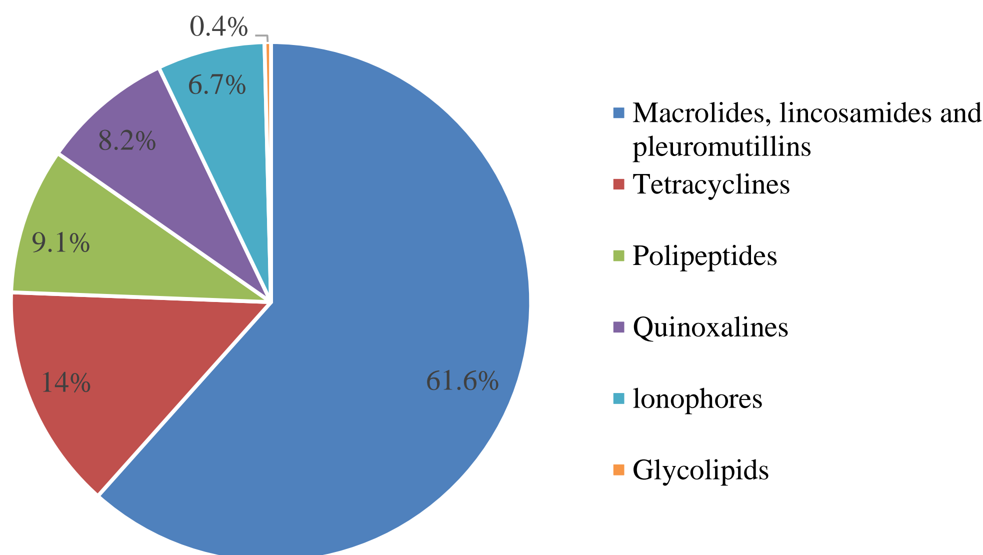

Figure 2.6 Percentages of volume (kg) for sales of in-feed antimicrobials for the period 2002-2004 in South Africa (adapted from Eager *et al*., 2012).

All the classes are authorised for use in food animals. Eagar *et al*. (2012) reported that tylosin was the most widely sold antibiotic as a growth promotor in South Africa. This is one of the four growth promoters banned in Europe, followed by sulphonamides, penicillins and tetracyclines (Moyane *et al.*, 2013). The highest ABR in the farming industry was reported by SANVAD for tetracyclines and sulphonamides (Eager *et al.,* 2012). Chloramphenicol and the nitrofurans are the only antimicrobials that are not available to use in food animals in South Africa (Henton *et al.,* 2011).

Antimicrobials administered through water are of less concern than in-feed antimicrobials as the antimicrobial concentration effectively eliminates the bacterial disease over a short period of time, usually three to seven days (Eager *et al.,* 2012). In contrast, in-feed medications are administered continuously over a large portion of the animal's life span at low doses (Eager *et al.,* 2012).

The greatest volume of antibiotics used for food animals occur in intensively reared poultry and pigs, followed by feedlot cattle and dairy cows. Extensively farmed sheep, goats and cattle use the least amount of antibiotics of other food animals, as they have lower herd densities, feed mainly on grass, and are considered healthier (Henton *et al.,* 2011).

2.6.3. Antibiotic resistance in wildlife

The application of antibiotics in numerous different situations, from agriculture to the food industry, results in a constant release of low level antibiotic concentration into the water and soil through wastewater treatment plant effluents, sewage, agricultural waste and bio-solid application to fields, among others (Dias *et al.*, 2015). This environmental pollution allows antibiotic compounds to reach other natural habitats. This, in turn, alters the population dynamics of microorganisms, encouraging the development of ABR in microbiological

communities of wild animals through heightened selection pressure of resistance (Martínez, 2008; Dias *et al.*, 2015).

Much research has focussed on the fate of antibiotics in clinical settings, whereas research of antibiotics in natural environments has been comparatively limited (Martínez, 2008; Vittecoq *et al*., 2016). Microbial ecosystems in nature are not isolated and thus extensive gene exchange occurs in nature. The use of antibiotics in animals can lead to the distribution of antibiotic resistant genes into the broader environment (Aminov & Mackie, 2007).

Most antibiotics used today are naturally produced by microorganisms or are synthetic derivatives of them (Holmes *et al*., 2016). Studies which have analysed soil microbiome have found a large diversity of antibiotic resistant genes (Holmes *et al*., 2016). Numerous studies have shown that bacterial ABR exists in nature all over due to the intrinsic resistant nature of various bacteria (Jeters *et al.,* 2009). The primary role of antibiotics in the natural environment, produced by antibiotic-producing bacteria, is to inhibit the growth of competitors when a bacterial community is exposed to a stressful environment (Martínez, 2008). The concentration of antibiotics in the soil is sub-lethal but this still has a significant effect on bacterial physiology by acting as signalling molecules and increasing the rate of microbial adaptive evolution through gene expression modification (Holmes *et al*., 2016). The inherent ability of microorganisms to become resistant to antibiotics, though, can serve a variety of natural purposes in nature, such as signal trafficking, enhanced virulence and processes including detoxification of metabolic intermediates (Martínez, 2008).

Antimicrobial resistance in humans is inter-linked with AMR in other populations and in the wider environment. Resistance, specifically mobile genetic elements, can pass between these different populations in nature (Woolhouse *et al.,* 2015). Water, soil and manure can be considered major potential pathways of ABR transfer from animals to humans, along with other vectors such as wind, crops and flies (Heuer *et al*., 2011). There are other situations which can also select for ABR in nature. For example, polluted environments create stressful conditions for microbiological communities and this encourages HGT so that antibiotic resistant genes can be transferred (Martínez, 2008). It has also been speculated that heavy metal pollution also selects for ABR (Martínez, 2008). Thus, ABR in the natural environment varies depending on geographical location, species and ecosystems (Katakweba *et al*., 2015).

A concern with regards to the spread of ABR in nature is the fact that 30-90% of antibiotics are often excreted unchanged and thus can easily enter the environment to be spread among exposed bacterial populations (Marshall & Levy, 2011). This leaching into the environment via animal manure exposes an enormous number of environmental organisms to small quantities of antibiotics which can promote selection of bacteria with resistant mutations among a diverse array of bacterial types (Marshall & Levy, 2011).

Manure, for example, is a large reservoir of antibiotic compounds and resistant bacteria. It has been shown to promote HGT in soil, as it carries large amounts of broad-host-range plasmids which are major vectors of HGT (Heuer *et al*., 2011; Woolhouse *et al*., 2015). Most of the global biomass of microorganisms resides in the soil (Woolhouse *et al*., 2015).

Bacteria in the soil produce antibiotic compounds and thus the soil can be considered a major reservoir of resistant bacteria (Woolhouse *et al*., 2015). Studies have indicated HGT between soil bacteria and pathogens (Woolhouse *et al*., 2015). Therefore, manure and soil can be considered major potential pathways of ABR transfer from animals to humans, along with other vectors, of course, such as water, wind, crops and flies (Heuer *et al.*, 2011). Antibiotic resistance transfer can also take place between neighbouring animals in nature by sharing of pastures and water sources (Mercat *et al.*, 2016).

In addition, microorganisms that are, or become, resistant to an exposed antibiotic can become resistant to other antibiotics, even unrelated antibiotics (Anon., 2006). This most commonly occurs when the microorganism is exposed to sub-inhibitory concentrations of an antimicrobial which can trigger intrinsic resistance mechanisms. This is when the microorganism develops resistance to the exposed antibiotic as well as to others (Anon., 2006). This co-selection of developing resistance to unrelated antimicrobials can also occur if different resistance genes, conferring resistance to different antibiotics, are transferred together to the bacterium (Marshall & Levy, 2011). If resistant genes are transferred to a bacterium which exhibits resistance via a specific mechanism, then cross-resistance can occur. When this happens, bacteria can become resistant to multiple antibiotics which have the same molecular targets (Anon., 2006). For example, the use of avoparcin as a growth promoter in livestock production often results in VRE found in the commensal flora and meat of the exposed food animals (Marshall & Levy, 2011). Avoparcin and vancomycin are both glycopeptides and have the same mode of action against Gram-positive bacteria which is inhibition of proper cell wall synthesis (van den Bogaard *et al.,* 2000).

Although ABR in wild animals is expected to be low owing to low-level exposure to antibiotics, studies have shown that 75% of zoonoses related to emerging human infectious diseases are associated with wildlife animals (Magwedere *et al*., 2012). The variety of human activities in natural habitats has increased over recent years through the construction of game reserves, conversion of land for crops to wildlife habitats, and the expansion of communities, causing an increased risk of disease transmission to and from wildlife (Magwedere *et al*., 2012; Katakweba *et al*., 2015). It is evident from these studies that ABR among wild animals is becoming a public health concern owing to increased wildlife contact with humans, livestock and domestic animals, as well as increased co-habitation with other animals. This increased contact and co-habitation intensifies the likelihood of antibiotic resistant traits in microorganisms transferring among ecosystems (Sousa *et al.*, 2014). Indirect contact between wildlife species and humans can occur among those that live near rearing estates or if contaminated food or meat is consumed by humans (Magwedere *et al*., 2012). Direct contact occurs most frequently with hunters, trappers and veterinarians (Vittecoq *et al*., 2016).

Moreover, various studies have found that antibiotic resistant bacteria in wildlife species are often resistant to antibiotics of natural origin, and thus can be considered natural reservoirs and transfer vectors of antibiotic resistant bacteria of environmental origin (Cole *et al.,* 2005; Jeters *et al*., 2009; Guenthler *et al.,* 2010; Wellington *et al*., 2013). The origin of many antibiotic resistant genes seems to reside in naturally occurring antibiotic synthesising organisms. Alternatively, the antibiotic resistant genes are native to organisms where the resistant gene has a physiological function, but is 'silent' in the sense of not showing a

detectable form of resistance, since its function is to protect the host's own metabolism (Gilmore *et al*., 2008). For example, the chromosomal penicillin binding proteins that confer resistance to penicillins were originally involved in bacterial cell wall synthesis. Resistance is due to a mutation or overproduction of penicillin binding proteins (Chadha, 2012).

It has been hypothesised that wildlife plays a significant role in the development of antibiotic resistant bacteria in nature because a variety of wildlife species carry antibiotic resistant bacteria and cover large territories throughout their lifespan (Magwedere *et al*., 2012; Vittecoq *et al*., 2016). Various wild animals could be considered natural reservoirs and potential spreaders of antibiotic resistant microorganisms throughout their environment, including birds of prey, wild ungulates, arctic birds, and wild rodents (Kozak *et al*., 2009; Sousa *et al.,* 2014).

Emergence of ABR in wildlife species would affect the South African game meat industry critically. It would also then threaten human health, while impacting the broader environment in terms of biodiversity and the further spread of ABR (Mercat *et al*., 2016).

It can be concluded that there are many different pathways that antibiotic resistant bacteria can enter and spread in the natural environment. However, this study has identified some of the major pathways to take into account when seeking to limit the further spread of ABR.

2.7. Factors that influence antibiotic resistance transfer and development in animal production

2.7.1. Intensive farming

Intensive production involves the confinement of a large number of animals on small areas of land and which are given pre-mixed feed. Often, the animal feed contains growth promoting substances to increase production and weight gain. The use of antibiotics in the food production chain is usually seen as important for continuing a consistent supply of healthy and substantial animals, leading to greater profitability and efficient production (Capita *et al*., 2016). The application of mass medication, known as metaphylaxis, and the use of broad-spectrum antibiotics in animal husbandry is common, because it is often impractical to treat animals individually (Capita *et al*., 2016).

The benefits of growth promotants was first discovered in the 1940s when chickens were observed to grow faster when they were fed by-products of tetracycline fermentation (Phillips *et al*., 2004). The mechanisms of growth promotants are still largely unknown but it has been hypothesised that what happens is that an alteration of the microintestinal flora results in more efficient digestion and metabolism as well as disease and pathogen suppression (Phillips *et al*., 2004).

One of the first consequences from the misuse of antimicrobials in the agricultural industry was documented in 1963 when an increased level of resistance in *S. enteric*a ser. Typhimurium was observed in several feedlots in the United Kingdom (UK) and several resistant isolates were identified (Khachatourians, 1998).

Numerous studies have shown that the use of antibiotics in intensive food-production animals, especially for nontherapeutic use, is linked to resistance seen in people on farms and even in the general population via the food chain (Marshall & Levy, 2011). Although the topic is still controversial, recent studies have demonstrated that intensive food animal farming results in higher ABR as well as increased virulence genes, compared to free-range or organic farming (Luangtongkum *et al.,* 2006; Millman *et al.,* 2014; Koga *et al.*, 2015). There is also concern with respect to the spread of antibiotic resistant bacteria from the farms into the nearby environment via ground water contamination.

The exploitation of antibiotics at low concentrations over extended time periods favours the emergence of ABR. This is due to accelerated HGT and mutation, where its spread is facilitated by the high density of animals involved in primary production (Aminov & Mackie, 2007).

2.7.2. Wildlife feeding

Wildlife supplementary feeding is the practice of laying out animal food into the animal's environment to act as a regulated source of food (Felton *et al*., 2017).

Supplementary feeding of game wildlife has become a common practice over the last few decades, most commonly in Europe and North America. Supplementary feeding is seen as a conservation and management tool to help conserve threatened species and to also boost growth, winter survival and reproduction rates. Secondary benefits include improved nutritional value, increased quality of trophies, easier attraction for hunting purposes, prevention of agricultural and forestry depletion and damage and to divert animals away from unwanted areas (Selva *et al.,* 2014; Felton *et al*., 2017). In Africa, particularly South Africa, supplementary wildlife feeding has become a more common practice in recent years due to occurrences of drought. (Selva *et al.,* 2014).

Although supplementary wildlife feeding has obvious benefits in terms of growth and survival, there are also secondary changes that occur, such as animal behavioural and ecological characteristics (Felton *et al*., 2017). These indirect effects of wildlife feeding are still not well understood due to limited studies.

Some notable negative consequences of wildlife feeding include changes in trophic cascades which, in turn, can alter the ecosystem, population dynamics and vegetation (Selva *et al.,* 2014; Felton *et al*., 2017). For example, if a wildlife specie is able to reproduce more frequently and survives longer than that which would occur without supplementary feeding, then its natural prey could also increase. Additionally, frequent crowding of animals at feed points, and increased concentration of scavengers at the feed points, can increase disease transmission, and thus also ABR transfer.

Limited research has been conducted on the effect of wildlife feeding on the transfer and level of ABR in bacterial isolates harboured by the wildlife.

2.7.3. Antibiotic resistance at the livestock/ wildlife interface

Livestock production utilizes the largest land resources in the agricultural industry, accounting for approximately 70% of agricultural land in South Africa (Meissner *et al.,* 2013). Additionally, livestock farming is one of the fastest growing agricultural industries in developing countries (Thornton, 2010). But livestock production results in water pollution and depletion, and land degradation, and has a negative impact on biodiversity if farming practices are not managed correctly (Thornton, 2010; Meissner *et al*., 2013).

Many farms in South Africa contain mixed species of livestock and wildlife for diversification and for economic reasons (Furstenburg, 2010). Otieno & Muchapondwa (2016) predicted that the performance of integrated farms would be higher than pure livestock farming. This approach helps to maintain biodiversity and conserve wildlife species and is less vulnerable to climate change (Ntiamoa-Baidu, 1997; Taylor *et al*., 2015; Otieno & Muchapondwa, 2016). Game farming helps to conserve water, because wild animals can utilize semi-arid and arid environments more effectively than livestock (Ntiamoa-Baidu, 1997). The land is better conserved because game disperse over a larger land area (Ntiamoa-Baidu, 1997). Importantly for the farmer, integrated farming can have economic advantages (Taylor *et al*., 2015). Farmers have better financial security as they have two income sources and do not have to rely on one income source because pure game farming is seasonal and pure livestock farming is affected by drought (which can occur frequently in South Africa) (Taylor *et al*., 2015).

Various studies indicate that wild animals that reside near livestock farms are more likely to carry antibiotic resistant microorganisms than those that are not exposed to food animals, because domestic animals are more frequently exposed to antibiotics (Kozak *et al.*, 2009). More specifically, the presence of nearby farms, human density and proximity, ABR levels carried by the livestock, and interaction with farm waste, all influence the ABR seen in the nearby wild ungulates (Navarro-Gonzalez *et al*., 2013). These factors also play a role in the transmission and emergence of diseases. It has been recently reported that about 70% of emerging zoonotic diseases originate from wildlife (Hassel *et al.,* 2017). Furthermore, the increase of human-ecosystem interactions, along with other factors, such as international trade and travel, antimicrobial drug usage and intensive animal production can explain why up to 80% of emerging diseases are zoonotic (Hassel *et al.,* 2017).

To the author's knowledge, the ABR transfer between livestock and co-grazing ungulates is scarcely researched and documented. This is a situation in nature which has the potential to spread ABR into the broader environment while also implicating the game meat industry and thus human health. It represents a clear threat to human health.

Common transfer pathways of mobile genetic materials and organisms between the interface of wild ungulates and domestic animals include sharing of water points and pastures (Mercat *et al.*, 2016). Furthermore, farms where wildlife species live in close proximity to livestock have increased risk of reciprocal disease transmission (Magwedere *et al*., 2012).

With regard to the effect of ABR transfer on co-grazing and co-habitation of livestock and wildlife species, a limited number of studies have been conducted, particularly in South Africa. Navarro-Gonzalez *et*

al. (2013) studied the ABR in *E. coli* isolates at a free-ranging livestock/ wild ungulate interface in a national game reserve situated in Spain. Low ABR levels were detected in both the wild ungulates and in the free-ranging livestock, indicating that the free-ranging livestock were not an important source of ABR with regards to the wild ungulate ABR in this study (Navarro-Gonzalez *et al.*, 2013). But perhaps higher resistance levels in the wild ungulates would be seen if intensively reared livestock were studied instead of free-range. It was found that the wild ungulates showed a resistance to cephalosporin and fluoroquinones, which are important antibiotics in human medicine, indicating that the antibiotic resistant bacteria carried by the wild ungulates is a concern to public health and that natural environments are not antibiotic resistant- free (Navarro-Gonzalez *et al.*, 2013).

Mercat *et al.* (2016) investigated the ABR in *E. coli* isolates at a cattle and wild ungulate interface in and around a national park in Zimbabwe. Higher ABR was shown in the ungulates at the interface than the ungulates that had no contact with cattle (Mercat *et al.*, 2016). This suggests that some ABR transfer occurred across the interface of these animals. Also, the cattle showed higher ABR than the ungulates, possibly due to administration of antibiotics to the cattle to treat infections (Mercat *et al.*, 2016). It was hypothesised that the resistant strains were spread across the interface and emerged in the wild because the ABR found in the ungulates was also found in the cattle (Mercat *et al.*, 2016).

Katakweba *et al.* (2015) researched the ABR in *E. coli* and *Enterococci* at a wild ungulate and cattle interface in Tanzania. It was found that co-grazing between wild ungulates and cattle did not result in significant transfer of resistant bacteria or genes (Katakweba *et al.*, 2015). Furthermore, there were no significantly higher levels of ABR in the wildlife at the interface as compared to the isolated ungulates (Katakweba *et al.*, 2015). It was noted that these findings should be validated with a larger study and that the drought may have caused some of the isolated wild ungulates to venture out of the park boundaries to find water where they may have come into contact with livestock and/or human environments and thus picked up antibiotic resistant genes from another environment (Katakweba *et al.*, 2015).

It can therefore be noted that ABR transfer and development in natural environments is a complex occurrence with many influencing factors. However, this phenomenon should not be over-looked and more information is needed to obtain a better understanding of the risks so that preventative measures can be employed to mitigate or minimise distribution of ABR in the environment.

2.8. The South African game meat industry

Game meat refers to the flesh of wild ungulates from Africa that are suitable for human consumption and utilised as food. Examples of typical South African game meat are blesbok, wildebeest, kudu and springbok (Siegfried & Brown, 1992). Hoffman and Bigalke (1999) view game meat as an organic product, as it is free of growth hormones and antibiotics and originates from wild, free-running animals. Also, game meat has a lower fat content and contains more minerals and protein than other mass-produced meat such as beef. For all these reasons, game meat may be considered a healthier meat source alternative. It should therefore rightly be considered as a luxury product.

There is a growing demand for meat products, which has been indicated by a four- to fivefold increase in meat production in the last 50 years, owing to a rapidly increasing human population (Meissner *et al*., 2013; Cawthorn & Hoffman, 2014; OECD, 2014). Parallel to this, there is a growing trend of consumption of wild game meat and meat from farmed game, with the game farming industry growing at 2.5% per annum (Hoffman, 2007; Hoffman & Cawthorn, 2012).

Game farming is unique in South Africa due to species diversity and abundance. Additionally, there is a high demand for organic and natural products, indicated by the rise in South Africa game meat exports, which are valued at between R60 million and R200 million (Magwedere *et al*., 2012; Cloete, 2015; Sanchez, 2015). The National Agricultural Marketing Council (NAMC) 2006 estimates that game farming is increasing by 5% per annum in South Africa (Meissner *et al*., 2013). As a result, game ranching could result in larger profits for farmers than livestock farming (Bekker *et al*., 2011). The use of wild animals for meat products began in the 1800s, when eland and buffalo were domesticated in South Africa (Ntiamoa-Baidu, 1997). Other sources of income from game ranching include sport and trophy hunting, live animal sales and tourism (Taylor *et al*., 2015). The South African game meat industry is unique because of species diversity and abundance. It is also unique because utilization of game animals is largely a private industry, with twice the number of privately owned game ranches as public parks (Cloete, 2015). In South Africa, very few large production plants of game meat exist compared to international production.

Game ranching in Africa is practised predominantly in southern Africa, namely in South Africa, Zimbabwe and Namibia (Ntiamoa-Baidu, 1997). Commercial wildlife ranches constitute 16.8% of the total agricultural land in South Africa, of which approximately 30% consists of mixed ranches (Dry, 2011; Otieno & Muchapondwa, 2016). Bekker *et al*. (2011) found that 85.7% of game on farms in South Africa were free roaming, and 14.3% were semi-extensive. In periods of drought, 90% of farmers provide supplemented feed to game.

The control of game meat throughout the supply chain is regulated by several national, provincial and local standards and regulations (Bekker *et al*., 2011). The two national departments involved in the control of game meat are the DoH, which is responsible for the game meat after it leaves the abattoir, and the DAFF, which is responsible for the slaughter of animals and the import and export of game meat. Bekker *et al.* (2011) found that most environmental health practitioners that are trained in the meat inspection of domesticated animals are not properly trained in game meat inspection. Additionally, there is lack of disease surveillance and management in the South African game meat industry. Consequently, the recent outbreaks of swine flu and Rift Valley fever in animals from South Africa have led to concern by consumers over meat safety (Bekker *et al*., 2011). Currently, it is thought that local legislation for game meat sales with regard to quality and production standards is inadequate. This holds true for ABR monitoring, as Magwedere *et al.* (2015) found that ABR tests were not taken routinely at all of the facilities they visited in Namibia during their research.

At this stage, there is not much control on the production of game meat. There is thus great opportunity for growth in the South African game meat industry which is likely to be accompanied by an increase in exports as more people around the world realise the health benefits of game meat. Meat for export is subject to strict

quality and safety controls where each country has its own specific requirements. There are also international standards to take into account. On the other hand, game meat used for the local market in South Africa has no regulations or safety guidelines for the carcasses (van der Merwe *et al.*, 2011). Regulations for the export of game meat states that no growth stimulants or promoters may be on the premises or used on the premises, either for game or domestic species (Anon., 2016).

The South African game meat industry can be seen as mainly a free-market system. Currently, it is thought that, local legislation for game meat sales with regards to quality and production standards is inadequate (Van der Merwe *et al.*, 2011). This holds true for AMR monitoring, as it was found by Magwedere *et al.* (2015) that AMR tests were not taken routinely at all of the facilities they visited in South Africa during their research.

The growth of this industry highlights added the importance of developing regulations and surveillance programmes to monitor ABR levels and to research the ABR transfer among game species. This is critical due to the possible transfer or uptake of antibiotic resistant microorganisms from nearby animals and the environment as well as the possible intrinsic resistance harboured by wild species (Klein, 2005).

2.9. Consequences of antibiotic resistance and human health

Antibiotic resistant genes occur in nature, humans, food animals, fish and plants. It is most commonly transmitted to pathogens in humans by either direct contact or indirect contact through the consumption of contaminated foods and environmental spread of faecal waste via, for example, contaminated irrigation water (Rolain, 2013; Mc Nulty *et al.,* 2016).

One of the first fears about ABR in humans was addressed in Denmark in 1994 due to the development of vancomycin-resistant *Enterococcus faecium* in humans. This occurrence was associated with the use of avoparcin, a chemically related antimicrobial agent which was used as a growth promoting agent in food animals such as pigs and poultry (Mc Nulty *et al.,* 2016).

A concern to human health is the transfer of antibiotic resistant bacteria in food to humans and subsequent colonization of the human intestine (Bester & Essack, 2010; Founou *et al*., 2016). For example, the food-borne pathogens, *Salmonella* and *Campylobacter*, cause an estimated 410,000 antibiotic-resistant infections in the US alone each year (CDC, 2013).This highlights the importance of correct food handling and preparation by consumers to avoid transmission. The presence of antibiotic resistant bacteria could affect the future of human health adversely as certain infections become more difficult to treat or infections occur if pathogenic antibiotic resistant bacteria are ingested (Bester & Essack, 2010; Founou *et al*., 2016).

During food production, processing steps such as farming activities, slaughtering practices, meat processing and transportation of food animals could introduce resistant bacteria into the food chain (McEwen & Fedorka-Cray, 2002; Landers *et al*., 2012; Founou *et al*., 2016). Spraying meat carcasses with organic acid solutions to decontaminate them may result in the survival of acid-resistant pathogens. This could consequently cross-contaminate food and 'colonize' the food-manufacturing environment, undermining cleaning and

sanitisation effectiveness (Berry & Cutter, 2000; Anon., 2006). Moreover, contamination during food preparation and consumption of contaminated meat and other food products results in a more direct transmission route of antibiotic resistant bacteria (Landers *et al*., 2012). Residual antibiotics in raw meat could result in the development of antibiotic resistant bacteria in the human gut (Kjeldgaard *et al*., 2012).

The greatest human health consequences of antibiotic resistant microorganisms are that disease and infection treatments fail more frequently, infections are more severe and their duration increases. Infections that would not have otherwise occurred, take place much more often as a result of antibiotic resistant organisms (Angulo *et al.*, 2004; Mc Nulty *et al.,* 2016). This, in turn, creates a burden on the public health system since more people require hospitalisation and the likelihood of rising mortality rates increases (Angulo *et al.*, 2004).

Humans who are the most vulnerable to the consequences caused by ABR infections, which include those with weakened immune systems, such as human immunodeficiency virus (HIV)-positive or cancer patients (Capita *et al.*, 2016). This is a concern in South Africa, as the Global Burden of Disease 2015 study lists HIV/AIDS as the number one cause of mortality in South Africa, followed by tuberculosis (TB) (Murray *et al*., 2016).

Antibiotic resistance can also create complications to medical procedures such as organ or prosthesis implants (Capita *et al.*, 2016). In South Africa, the GBD 2015 listed lower respiratory infection and diarrhoea in the top ten leading causes of 'years of life lost' (YLL), both of which are often attributable to bacterial infections caused commonly by *S. pneumonia* and *E. coli* respectively (Murray *et al*., 2016).

Thus, in summary, the emergence of antibiotic resistant bacterial pathogens in South Africa is of significant concern to public health and clearly has the potential to undermine the treatment of some common infections.

In the EU and European Economic Area, more than 25 000 people a year die from infections caused by antibiotic resistant bacteria (Mc Nulty *et al.,* 2016). There are many documented cases where there has been a direct spread of ABR from animals, namely livestock, to nearby humans, such as farm workers or residents living nearby. This type of situation was first described by Levy *et al.* (1976), who reported that tetracycline-resistant *E. coli* strains present in the chickens that were fed tetracycline in their feed were also present in the workers on the chicken farm. There have been numerous infection outbreaks as a result of antimicrobial resistant microorganisms used in food animals that caused food-borne infections in humans (Angulo *et al.*, 2004). For example, the use of fluoroquinolones in dairy cows in the UK was linked to an infection outbreak in humans caused by nalidixic acid-resistant *S. enteric*a ser. Typhimurium DT104 (Angulo *et al.*, 2004).

The same safety and hygiene procedures that are used to prevent and control the spread of bacteria via food in the food industry will help to control the spread of antibiotic-resistant pathogenic bacteria in the food chain (Capita *et al.*, 2016).

Poor regulation, control and over-use of antibiotics in medicine and agriculture has added to the emergence of antibiotic resistant bacteria reservoirs. It is therefore important to enforce strict regulation procedures which will also assist in preventing the spread of antibiotic resistant bacteria (Khachatourians,

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1998). The development of standards which are legally enforceable becomes important to provide a policy and legal framework for reducing the risk of the phenomenon spreading in South Africa.

The main threat of ABR to human health is the transfer of resistant bacteria between animals and humans. Case studies have reported evidence of resistant *Campylobacter*, *Salmonella*, MRSA and *E. coli* causing diseases in humans that originated from animals (Phillips *et al*., 2004; Bengtsson & Greko, 2014). It is alarming that the same classes of antibiotics are used for veterinary and human medicine, namely β-lactams, sulphonamides, tetracyclines, macrolides, lincosamides, streptogramins, and quinolones (Phillips *et al*., 2004). Efforts should focus on minimizing the transmission of food-borne pathogens via the food chain, despite their antibiotic susceptibility profile by adopting good hygiene practices at all stages of food production, including food marketing and food preparation by the consumer.

2.9.1. Consequences of antibiotic resistance transfer to the broader environment

The fate of antibiotic resistant bacteria in remote ecosystems is largely unknown but the emergence of resistance genes in these remote environments has the potential to decrease antibiotic efficacy in human medicine and agriculture. It has already been noted that wild animals and their surrounding environments have been shown to be important reservoirs of antibiotic resistant bacteria. Furthermore, biodiversity in the natural, more remote environments, could be threatened by ABR pollution (Mercat *et al.*, 2016). It is not just a human health issue.

The main sources of ABR spreading into the broader environment include leakage and spread of animal manure via the soil from manure lagoons and the transfer of resistant mobile genetic elements from farm animals to wild animals via co-habitation and/ or co-grazing (Heuer *et al.*, 2011). As much as 90% of antibiotic compounds ingested by animals are excreted with manure (Heuer *et al.*, 2011). Physical environmental factors can also play a role in the transmission of resistant elements throughout the environment, like wind or water which assists in the transport of small particles like soil containing antibiotic resistant bacteria to other locations (Heuer *et al.*, 2011). It has been suggested that numerous wildlife species are implicated in the development of antibiotic resistant bacteria at the interface between humans and natural environments (Vittecoq *et al.*, 2016).

Bhullar *et al.* (2012) noted that ABR occurs in all types of environments, even in those that are isolated from anthropogenic antibiotic use. This ubiquity increases the risks being discussed in this chapter. Research has shown that environmental organisms that are non-pathogenic can be a reservoir of resistance genes that could potentially be transferred to pathogens (Bhullar *et al.*, 2012). These reservoirs of antibiotic resistant genes in bacterial communities have been shown to be stable even when there is no selective pressure created by the presence of antibiotics (Looft *et al.*, 2012). It is therefore considered important to not misuse the application of antibiotics so that selection of existing resistant elements, and their subsequent movement through microbial communities, can be avoided, or at the very least, hindered (Bhullar *et al.*, 2012).

A variety of human activities within natural habitats have increased over recent years through the construction of game reserves and the expansion of communities, causing an increased risk of disease transmission to, and from, wildlife (Katakweba *et al.*, 2015).

2.9.2. Consequences of banning growth-promoting antibiotics

There have been recommendations to ban the use of nontherapeutic antibiotics in food animals. However, this has been difficult to implement in many countries, although the EU has adopted this policy (Davies & Davies, 2010). Alternatively, some suggest that strict controls be put in place to control the use of antibiotics in animal husbandry (Davies & Davies, 2010). Others suggest that reduced antimicrobial consumption would be more effective than a complete ban of antimicrobial usage, as this can potentially cause negative repercussions on animal health, productivity and thus also on food prices (Woolhouse *et al.,* 2015).

A decrease in the total consumption of antibiotics in animal husbandry could lead, unfortunately, to a subsequent increase in usage of antibiotics for therapeutic uses. That's because animal infections could increase, leading to a decline in production, weight gain and an increase in mortality rates (Capita *et al.*, 2016). Furthermore, the prevalence of *Salmonella* and *Campylobacter* has been documented to have increased in food animals due to the fact that a farm of animals not given growth promoters tend to have large variations in size, leading to more frequent rupture of the gastrointestinal tract during slaughter and consequent contamination with both pathogens (Capita *et al.*, 2016). These adverse effects were observed following the ban of growth promoters in Denmark in 1994 (Capita *et al.*, 2016).

Typically, the use of antibiotics for veterinary use increases following a ban of growth-promoting antibiotics, and these antibiotics are also commonly used in human medicine, such as tetracyclines (Capita *et al.*, 2016). This subject is still being debated in the EU, as other countries have only reported a small increased consumption of therapeutic antibiotics, which tends to decline after two or three years following removal of growth promoting antibiotics (Capita *et al.*, 2016).

Some countries have even reported a decline in antibiotic resistant bacteria after the ban. This happened for example, in Denmark where VRE in Danish broilers decreased from 80% to 3% (Capita *et al.*, 2016).

Doyle *et al.* (2013) suggests that at least five years are needed after a ban of growth promoting antibiotics in order to reduce resistance levels in animals by significant amounts. In order to improve animal welfare following a ban of growth promoters, it is important to improve farm hygiene and to implement good farming practices and production conditions in order to prevent less infections from occurring and spreading thereof (Capita *et al.*, 2016). Some examples of farm hygiene and good farming practices are, (i) having a planned herd health programme such as vaccinations and parasite control, (ii) frequent inspection of animal health status, (iii) regular cleaning of animal housing areas, (iv) keeping records of animal medications and feeds, (v) keeping an appropriately-sized herd and (vi) ensuring clean, uncontaminated water supplies (Anon., 2003).

If these steps are actively followed, it has been forecasted that comparatively good production can be obtained without the use of growth promoters in animal feed (Capita *et al.*, 2016).

2.10. Current state of antibiotic resistance in South Africa

Environmental and clinical studies indicate that ABR rates in South Africa are high (Moyane *et al.*, 2013). Infections of greatest concern with regards to South Africa's health status are HIV and TB, which dominate the healthcare sector (Eager *et al.*, 2012). A high burden of infection is accompanied by an equally high burden of antimicrobial use and, consequently, ABR, followed by the emergence of MDR bacteria (Mendelson & Matsoso, 2015). Outbreaks of MDR bacteria do exist, causing high morbidity and mortality rates. For example, the WHO has estimated that 8.5% of tuberculosis cases are from MDR tuberculosis (Mendelson & Matsoso, 2015). Also, more than half of hospital-acquired *S. aureus* that were isolated from sick patients in public hospitals in 2010 were MRSA (Mendelson & Matsoso, 2015). In addition, up to 75% of *K. pneumoniae* isolated from hospitalised patients in 2010 and 2012 were ESBL-producing bacteria (Mendelson & Matsoso, 2015).

In developing countries, antibiotic use is poorly controlled, and antibiotics that are used in agriculture such as penicillins are often used for human therapy (Levy, 1998; Grace, 2015; Founou *et al.*, 2016). In animal health, currently there is little published data on resistance rates in food animals. However, SANVAD highlighted high rates of resistance to tetracycline and sulphonamide, two commonly used growth promoters, in *E. coli, Salmonella* and *Enterococcus* species between 2002 and 2004 (Mendelson & Matsoso, 2015).

In response to the rise in outbreaks of MDR-bacterial infections in South Africa, various organisations were established in an attempt to curb the ABR situation. For example, the South African Antibiotic Stewardship Programme (SAASP) was formed which consists of various experts in different healthcare sectors who aim to promote appropriate antibiotic prescribing and education on the matter (Mendelson & Matsoso, 2015). SAASP has recently produced a set of treatment guidelines for antibiotic prescribing for adults in South Africa, which is freely available to the public.

South Africa has also been part of the Global Antibiotic Resistance Partnership (GARP) since 2010. Its aim is to address and recommend solutions towards the AMR situation in South Africa, together with the other three participating countries, namely India, Vietnam and Kenya (Moyane *et al.*, 2013). South Africa has also been enrolled in the Global Antimicrobial Resistance Surveillance System (GLASS) since 2016. It aims to support global surviellence and research on antimicrobial resistance and advises on global action plans.

A recent partnership between the Foundation of Innovative New Diagnostics (FIND) and the South African Medical Research Council (SAMRC) has also been established in 2018 in an effort to promote the development of diagnostic testing and surveillance.

Additionally, the medicines committee of the South African veterinary association with the faculty of veterinary science has developed technical guidelines for the responsible use of antimicrobials in veterinary medicine in South Africa.

In addition, the DoH has developed an implementation plan for the AMR strategy framework in the country for the period 2014-2019. Its three main objectives are (i) to better control infections, predominantly through vaccinations, and the prevention of infection through for example, improved water sanitation, (ii) to enhance antimicrobial surveillance and documenting and (iii) to implement antimicrobial stewardship (Mendelson & Matsoso, 2015). South Africa has recently had its National Action Plan approved using the One Health approach. The WHO regional office for Africa has made efforts to improve AMR surveillance and lab capacity building.

With regard to antimicrobial surveillance in the animal health industry, Eager *et al.* (2012) compared different antimicrobial surveillance systems for veterinary practice from other countries, and concluded that a combination of the Australian and United Kingdom surveillance systems would be the best approach to apply to the animal health industry in South Africa, because the national AMR surveillance programme of antibiotic usage and ABR on food- producing animals has not yet been established (Eager *et al.*, 2012; Moyane *et al.*, 2013). This type of surveillance system would include the volumes of veterinary antimicrobials consumed together with a veterinary AMR surveillance and monitoring programme (Eager *et al.*, 2012).

The lack of an efficient ABR surveillance system in South Africa makes it difficult to obtain data on the quantities of antimicrobials that are sold by veterinary pharmaceutical companies. This indicates that a good surveillance programme needs to be established that will aid in the administration and tracking of antimicrobials throughout the country. Eager *et al.* (2012) noted that resistant *S. aureus* mastitis is a current problem in the South African farming industry, followed by increased resistant *E.coli* and *Enterococcus* species to tetracyclines, fluoroquinolones, sulphonamides, amoxicillin and trimethoprim-sulpha combinations (Henton *et al.,* 2011). Furthermore, high resistance in *S. aureus, Camphylobacter jejuni* and some *Listeria* and *Salmonella* species was reported for tetracycline from a poultry abattoir (Henton *et al.,* 2011). High resistance was also reported in *S. aureus* isolates for penicillin and amoxicillin in cattle which has mastitis (Henton *et al.,* 2011).

Additionally, it was found that antibiotic usage in the agriculture industry has been increasing over the last decade (WHO, 2015). Antibiotic growth promoters are still being used in South Africa which have been banned in the EU since 2006, such as tylosin, due to their structural relatedness to antimicrobials used in human medicine (Moyane *et al.*, 2013). Inappropriate use of antibiotics drives the selection of ABR and in South Africa, in-feed antimicrobials constitute 68.5% of the total antimicrobials sold in South Africa Eager *et al.* (2012). Consequently, South Africa has recently been considered by van Boeckel *et al*. (2014) as a major contributor to the increase in antibiotic use worldwide (Mendelson & Matsoso, 2015). Significant research gaps on the ABR situation in South Africa urgently need to be filled (Mendelson & Matsoso, 2015).

2.11. Curbing the spread of antibiotic resistance in agriculture

Numerous techniques can be implemented to help reverse or slow down the development of ABR in various environments, which has largely been caused by the misuse of antibiotics (Khachatourians, 1998; Mc Nulty *et al.,* 2016). Therefore, strategies aimed at reducing exposure to antibiotics and strict control of existing

antibiotics should have the greatest effect curbing the emergence of resistance (Wallmann, 2006; Sandora & Goldmann, 2012).

As already discussed, the development of ABR is caused by a complex set of factors and thus can only be solved through an interdisciplinary effort (Wallmann, 2006). The antibiotics used in agriculture for prophylactic and therapeutic purposes are often also used for human therapy, such as penicillins and tetracyclines (Levy, 1998; Phillips *et al.,* 2004). The exploitation of antibiotics selects for resistant bacteria, as we well know, resulting in ineffective drugs and a rise in infections and illnesses.

Increasing hygiene and good herd management in livestock farming could reduce the need for antibiotics to treat diseases. The occurrence of diseases, and its spread within farms, would be reduced. This could be further improved by reducing animal density on farms (Anon., 2006).

Furthermore, survival of susceptible bacteria is slightly favoured in the absence of antibiotics, as the upkeep of resistant traits requires energy which could otherwise be used for reproduction (Levy, 1998). This would eventually result in a diminishment of resistant bacteria over time.

Vaccines, too, can reduce the need for antibiotics by preventing diseases from occurring. Immune enhancers can also be administered to further prevent illnesses from occurring (Anon., 2006). Furthermore, inclusion of probiotic bacteria in livestock feed can potentially reduce the occurrence of pathogenic bacteria in the gut by competitive exclusion, thereby reducing the chances of development and spread of antibiotic resistant pathogens (McEwen & Fedorka-Cray, 2002). Genetic modification of farm animals to produce animals that are resistant to certain infections would no longer require antibiotics for the treatment of infections. This strategy has proven successful, for example, in the development of transgenic chickens that do not transmit avian influenza (Woolhouse *et al.,* 2015).

Mass administration of antibiotics in animal husbandry, known as metaphylaxis and administration during high-risk periods of infectious disease should be avoided wherever possible, as this creates unnecessary selection of antibiotic resistant bacteria in the environment (McEwen & Fedorka-Cray, 2002).

Additionally, antibiotic use needs to be used correctly and responsibly. This involves maximising the therapeutic effect of antibiotics, while *minimising* the potential for resistant bacteria to develop (McEwen & Fedorka-Cray, 2002). Restriction on the critically important antibiotics would help to conserve the effectiveness of these drugs (Doyle *et al.,* 2013).

Due to the fact that 70- 90% of an antibiotic which is administered to animals remain unchanged in their faeces or urine, treatment of raw animal manure before application can significantly reduce the spread of antimicrobial resistant bacteria and genes throughout the environment (Masse *et al.,* 2014). Treatment options include composting and anaerobic digestion.

Monitoring systems can aid in the control of antibiotic usage by recording resistant patterns and emerging resistant pathogens so that an effective combat and control strategy can be developed (Doyle *et al.,* 2013). Additionally, an effective wild game traceability system in southern Africa should be developed at all harvesting levels. Improvement of current surveillance and control strategies should be employed. This would assist in monitoring the ABR and disease situation to assist in better control of emerging diseases in food

animals. Animals at the wildlife-livestock interface, free-roaming wildlife that are harvested for trophies or meat, and those captured from translocation would provide the most suitable samples for a representation of the human health risks associated with wildlife and livestock production (Magwedere *et al*., 2012).

The development of new antibiotics would help to prevent the use of existing antibiotics. But the development of new antibiotics has become saturated and most new antibiotics are structurally similar to existing antibiotics. Unfortunately, most resistant bacteria would already have resistant mechanisms against them (Levy, 1998).

An alternative approach to developing new antibiotics is the development of medicines specifically targeting the resistant mechanisms in resistant bacteria, thus resulting in the bacteria becoming susceptible to existing antibiotics once again (Levy, 1998).

Consequently, to correct for the ABR problem that has grown over the recent years, correct management and strict control of antibiotic use, together with a reduction in the widespread use of antibiotics and restoration of the bacterial environment needs to be achieved (Mc Nulty *et al.,* 2016). However, the solution to this worldwide epidemic is not simple and will involve a system of complex measures to achieve an effective outcome (Mc Nulty *et al.,* 2016). Monitoring ABR is a key part in risk management and involves collecting, analysing and reporting of results (Wallmann, 2006). Thus, research on current antibiotic resistant traits, patterns and development in all sectors will prove vital in the search for an effective national and global solution.

2.12. Concluding remarks

The misuse, overuse and inappropriate use of antimicrobials in human medicine and animal husbandry creates an enormous selection pressure that increases and accelerates the likelihood that bacteria will adapt and multiply to produce a more resistant population. This is therefore a key causal factor that drives the development of ABR (Lerma *et al.*, 2014). The exploitation of antibiotics at low concentrations over extended time periods favours the emergence of ABR. This is due to accelerated HGT and gene mutation. Its spread, as previously noted, is facilitated by the high density of animals involved in primary production, given that there is a larger number of animals that can potentially host the resistant bacteria (Aminov & Mackie, 2007).

In addition, it has been hypothesised that wildlife could play an important role in the development of antibiotic resistant bacteria in nature as a variety of wildlife species have been considered reservoirs of antibiotic resistant bacteria (Vittecoq *et al.*, 2016). To the author's knowledge, the ABR transfer between livestock and nearby, co-grazing ungulates is scarcely researched and documented, especially in South Africa. This is a situation in nature which has the potential to spread ABR into, and throughout, the broader environment and constitutes a real growing threat to human and animal health. At the same time, this study is highly relevant to the expanding game meat industry. Exporting of local game meat, in particular, requires strict regulations to ensure quality and safety. Yet standards are equally important in production of game meat for the domestic market.

Although there is growing evidence to support the existence of antibiotic resistant bacteria in wildlife species, studies remain limited, especially in developing countries such as South Africa (Vittecoq *et al.*, 2016). This study will therefore focus on antibiotics used in livestock and in nearby, co-grazing ungulates in the farming industry in South Africa, in order to fill this research gap. In addition, it will be instructive to shed light on the specific contribution of antibiotic usage in livestock as a selection pressure for the development of ABR and as a potential ABR reservoir hotspot in the environment.

2.13. References

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CHAPTER 3

Literature Review

Methods for determining the presence of antibiotic resistant bacteria

3.1. Detection of antibiotic resistant bacteria

There are two types of testing methods that can be used to assess a bacterium's antibiotic resistance profile namely, phenotypic and genotypic testing (see Table 3.1). Phenotypic testing determines the level of susceptibility a bacterium has towards an antibiotic. Genotypic testing, on the other hand, determines the presence of mutations that are known to confer ABR (Hanna & D'Aquila, 2001). There are advantages and disadvantages to using phenotypic and genotypic methods to assess the ABR profile of bacteria.

Due to limitations of both methods, it has been recommended to use a combination of phenotypic and genotypic methods to obtain a reliable and accurate result. Usually this would involve the use of phenotypic methods to screen for ABR, followed by confirmation of resistance and determination of resistance mechanisms using genotypic methods (Georgios *et al*., 2014). Figure 3.1 depicts this schematically.

There are various molecular techniques which are currently being used to detect resistant genes, namely, probe technology, DNA sequencing, microarrays and polymerase chain reaction (PCR) (Fluit, 2001; Healy *et al*., 2005). PCR is one of the most commonly used methods in laboratories to detect resistant genes as it is a reliable and quick detection method for genes whose sequence is known. Its popularity as a method is also due to its ease of use and its cost effectiveness (Lister, 2002; Gorski & Csordas, 2010).

Whole genome sequencing (WGS) has gained recent interest in antimicrobial resistance surveillance (Oniciuc *et al*., 2018). It has numerous benefits over PCR, whose ability is limited to detection of only known sequences. WGS is capable of detecting new resistant genes and covers the entire genome so that hundreds of resistant genes are detected and not just a select few as with PCR (Tyson *et al*., 2015).

Table 3.1 Advantages and disadvantages of phenotypic vs genotypic methods for detection of antibiotic resistant bacteria (Hanna & D'Aquila, 2001; Georgios *et al*., 2014; Anjum, 2015)

	Advantages	**Disadvantages**
Phenotypic tests	Can detect degree of susceptibility	Labour- intensive
	Can detect resistance to an antibiotic without prior knowledge of resistant mechanisms	More time- consuming (a pure culture first needs to be obtained and then grown in the presence of an antibiotic)
		Classification of resistance is dependent on what drug concentration is considered significant according to standards
		Difficult to test low-level resistance
		in vitro selective pressures
Genotypic tests	Rapid (testing can be conducted directly on the specimen)	Cannot detect susceptibility, only resistance
	The resistance mechanism can be determined	Often more expensive
	Minority resistant populations can be detected	Only known resistance genes can be detected (except when using NGS)
		False-negative results due to detection of incorrect gene/s as phenotypic resistance can be a result of numerous mutations
		False-positive results due to contamination in the laboratory

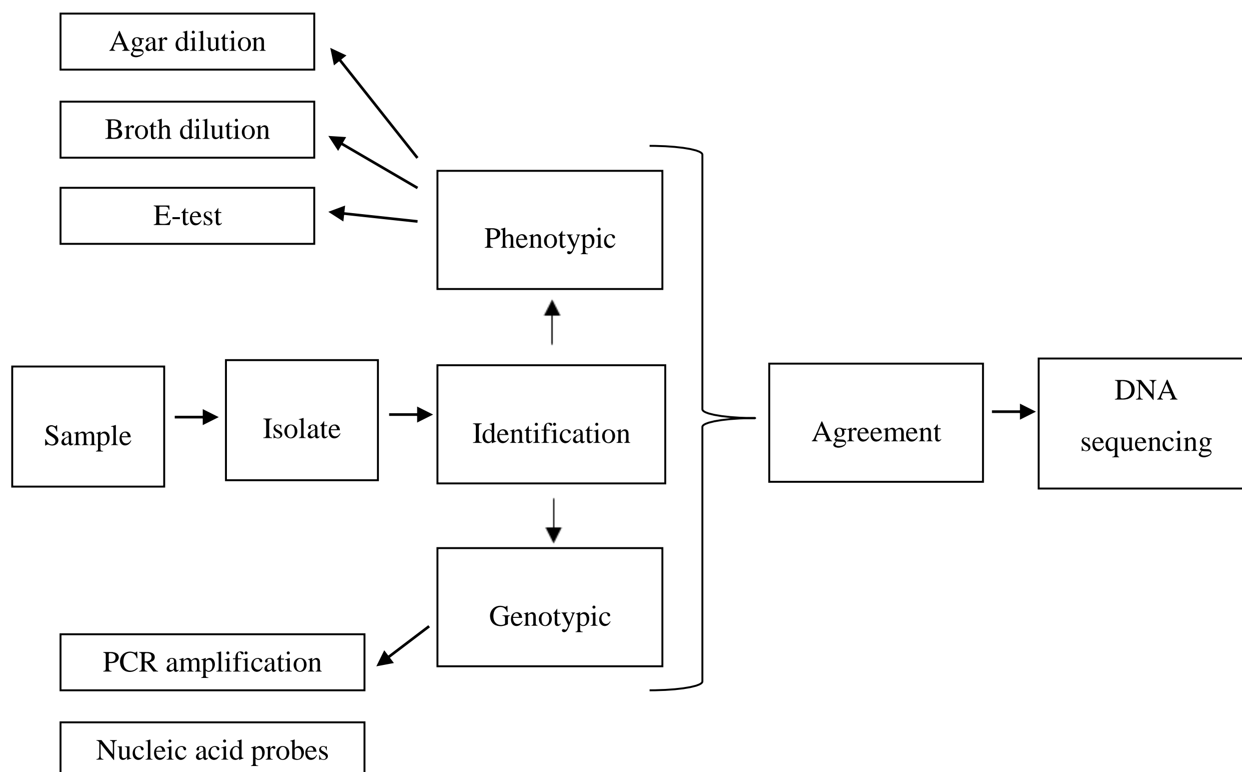

Figure 3.1 Schematic of antibiotic resistance testing methods (adapted from Merlino, 2012).

3.2. Isolation of target bacteria

Before phenotypic antimicrobial susceptibility testing (AST) can be performed, bacteria from the sample needs to isolated. There are various techniques and selective media that are available to effectively isolate bacteria from environmental or food samples. In addition, including a resuscitation step using enrichment media followed by incubation overnight before using selective media, allows for better recovery of bacterial cells, especially if subjected to stressful storage conditions such as freezing (Bloch *et al.,* 1996).

In this study, an environmental bacterial pathogen, Gram-positive *Staphylococcus aureus* and a food-borne faecal pathogen, Gram-negative *Escherichia coli,* as well as a commensal and pathogenic Gram-positive bacteria, *Enterococcus faecalis,* were chosen for AST analysis. These pathogens are commonly found in humans, animals and food and are consequently responsible for many food-borne illnesses. They have shown increased ABR over the past decade (Wallmann, 2006). They are also on the priority antibiotic resistant pathogen WHO list.

3.2.1. *Escherichia coli*

There are various selective media's which can be used to enumerate *E. coli*. These include, but are not limited to, Violet Red Bile Agar (VRBA), Violet Red Bile Glucose Agar (VRBGA), Deoxycholate Lactose Agar (DLA), MacConkey Agar (MACA) and Eosin Methylene Blue Agar (EMBA).

Violet Red Bile Agar is a selective medium used to detect and enumerate lactose-fermenting coliform microorganisms (Anon., 2005a). The medium contains bile salts and crystal violet which inhibit the growth of gram-positive microorganisms. Characteristic colonies of lactose- positive *Enterobacteriaceae*, including *E. coli*, are red, indicated by the pH indicator neutral red and are surrounded by a reddish zone of precipitated bile (Anon., 2005a). Lactose-negative microorganisms produce colourless colonies (Anon., 2005a).

A modification of VRBA was introduced by Mossel in the 1960s. It is known as VRBDA, which contains added glucose that is degraded to acid, indicated by a colour change to red/ purple, with a red/ purple zone of precipitated bile surrounding the colonies (Anon., 2005b). All *Enterobacteriaceae* degrade glucose to acid and thus this selective medium gives a presumptive coliform count. Some other microorganisms, such as *Aeromonas* also show these reactions. Thus, confirmation of *Enterobacteriaceae* should be achieved by further tests (Anon., 2005b).

Deoxycholate Lactose Agar is a selective medium for the isolation of coliform bacteria (Anon., 2005c). It is recommended for use with specimens likely to contain mixed microbial flora since it allows a preliminary grouping of enteric, and other gram-negative bacteria, based on its ability to degrade lactose. Lactose-positive microorganisms have red colonies due to the presence of neutral red pH indicator with a zone of bile acid precipitate surrounding the colony (Anon., 2005c).

MacConkey Agar is a selective medium designed to isolate and differentiate *Enterobacteriaceae* based on their ability to ferment lactose (Anon., 2005d). Bile salts and crystal violet inhibit the growth of Gram-positive organisms. Organisms that are lactose- positive will appear pink due to production of acid. It is widely used due to its low cost and high selectivity. However, many species of *Enterobacteriaceae* can grow on MACA and the colony characteristics are not sufficient to distinguish species. Therefore, further identification is required to isolate *E. coli.*

Further identification of *E. coli* can be achieved with EMBA as *E. coli* colonies show characteristic growth, differentiated based on the ability to ferment lactose and sucrose. Eosin Methylene Blue Agar was proposed by Holt-Harris and Teague as a selective and differential medium used to isolate faecal coliforms (Anon., 2005e). Eosin Y and methylene blue are pH indicator dyes which combine to form a dark purple precipitate at low pH and also inhibit the growth of most Gram-positive organisms. Characteristic colonies of *E. coli* have a greenish, metallic sheen in reflected light.

Additional confirmation of the identity of characteristic *E. coli* colonies can be tested by performing a Gram stain, Analytical Profile Index (API) 20E, oxidase test and PCR.

3.2.2. *Staphylococcus aureus*

Effective media for the enumeration of *S. aureus* include but is not limited to, Mannitol Salt Agar (MSA) and Baird parker Agar (BPA).

Mannitol Salt Agar is a selective agar for detecting pathogenic staphylococci. Only salt-tolerant microorganisms can grow on this medium. *S. aureus* degrades mannitol to acid, resulting in colonies surrounded by a bright yellow zone. Mannitol-negative microorganisms, such as *Staphylococcus epidermis*

remain red and usually exhibit poorer growth (Anon., 2005f). Further confirmation tests such as the catalase test and coagulase test are recommended.

Confirmation of the presence of *S. aureus* can be achieved using BPA. The high selectivity of the medium is due to lithium chloride and potassium tellurite which inhibits the growth of organisms other than staphylococci. Glycine and sodium pyruvate stimulate the growth of staphylococci. The differentiation between coagulase-positive and negative staphylococci can be attributed to the reduction of potassium tellurite by coagulase-positive staphylococci, resulting in black colonies. Staphylococci that contain lecithinase break down the egg yolk, resulting in clear zones around the colonies. Characteristic *S. aureus* colonies are black, shiny and convex with a clear halo (Anon., 2005g).

Further confirmation of the identity of characteristic *S. aureus* colonies can be tested by performing a Gram stain, API-Staph and PCR.

3.2.3. *Enterococcus faecalis*

Enterococci grow well under standard laboratory media such as blood agar, tryptone soy agar and chocolate agar. For selective isolation, Bile Esculin Azide agar, 6.5% sodium chloride agar or MSA can be used (Anon., 1996a).

The azide added to Bile Esculin agar inhibits the growth of gram-negative bacteria. The selectivity of this agar is due to the unique ability of Enterococcus species to hydrolyse esculin into esculetin and dextrose which reacts with ferric citrate to give a positive reaction of black precipitate seen surrounding the colonies (Anon., 2018).

Enterococcus species can be differentiated from non-enterococcal group D Streptococci using 6.5% sodium chloride agar (Anon., 1996b). Those that are salt tolerant would be capable of fermenting dextrose which produces an acid, turning the bromcresol purple indicator yellow (Anon., 1996b). Salt tolerant species which will give a positive colour change reaction include *E. faecalis*, *E. zymogenes*, *E. liquifaciens* and *E. durans* (Anon., 1996b).

Enterococci are mannitol positive- they ferment mannitol and produce lactic acid which results in yellow colonies on MSA (Anon., 2005f). *Staphylococcus aureus* also produces yellow colonies and can be differentiated from Enterococcus by the catalase test.

3.3. Phenotypic antimicrobial susceptibility testing

3.3.1. Testing methods

Reliable and accurate testing of antimicrobial susceptibility is important for detecting resistance or for confirming susceptibility in bacterial isolates. Clinically, AST is used to determine drug potency against specific pathogenic bacteria. This can determine whether a new drug will be effective and may be used to establish the best course of action for treating infected patients (Mc Nulty *et al.,* 2016). The most commonly used manual susceptibility testing methods are either based on diffusion of the antimicrobial agent in agar

(Stokes method and Kirby-Bauer method), dilution in broth or agar or a combination (E-test method). Automated instrumental techniques are also based on variations of diffusion and/ or dilution. They can produce results in shorter time periods and are more sensitive than manual readings because of optical detection systems capable of detecting subtle changes in bacterial growth (Jorgensen & Ferraro, 2009). Examples of such automated systems include Vitek AST cards, Autobac and Abbott MS-2 system (Kelly *et al*., 1982).

The two most commonly used international antimicrobial susceptibility phenotypic testing methods and standards are the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Clinical and Laboratory Standards Institute (CLSI) (Silley *et al*., 2012). These standards categorise the susceptibility results based on breakpoints, which determine whether a bacterium is considered resistant or susceptible. These breakpoints are set by international agencies and are regularly revised. The EUCAST breakpoints are based on epidemiological MIC cut-offs and pharmacokinetic-pharmacodynamic (PK-PD) properties. The CLSI breakpoints are based on MIC distributions, PK-PD properties and mechanisms of resistance (Kassim *et al*., 2016). Additional differences in the two standards include different breakpoint values, incubation times and conditions and media.

In this study, the CLSI guidelines were used as they have breakpoints for antimicrobials which are used in human and veterinary medicine, whereas the EUCAST guidelines are limited to antimicrobials important to human medicine (Silley *et al*., 2012).

3.3.1.1. Disk Diffusion

The Kirby-Bauer disk diffusion method, which is widely used in France and Sweden, is one of the most prevalent AST methods in laboratories. It is simple, well standardised and recommended by the CLSI and EUCAST (Mc Nulty *et al.,* 2016; Schlegelova *et al*., 2001). The method is performed by applying a standardised bacterial suspension to a Mueller-Hinton agar plate and then applying paper antibiotic disks which contain a fixed concentration of antibiotic (Jorgensen & Ferraro, 2009).

Furthermore, disk diffusion methods interpret the diameter of the zone of inhibition which is the area around the disc where no growth occurred. The results classify the zone of inhibition into categories of susceptibility according to criteria published by the CLSI and the EUCAST (Jorgensen & Ferraro, 2009). The CLSI is approved by the Food and Drug Administration (FDA) and is recommended by the WHO. The results are reproducible and accurate. They are qualitative but could be converted into MIC breakpoint values using standard curves (Jorgensen & Ferraro, 2009; Mc Nulty *et al.,* 2016). This method is the least expensive and offers simplicity and flexibility, allowing the categorical results to be easily interpreted by members of the public (Jorgensen & Ferraro, 2009). The disk diffusion method also gives insight into the heterogeneity of the cell population of the bacterial isolate (Schlegelova *et al.,* 2001). For these reasons, the disk diffusion method is seen as the most practical method that yields clinically relevant results. It is also most commonly employed by laboratories today.

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3.3.1.2. Dilution

Dilution methods are used to determine the minimal concentration of antimicrobial to inhibit or kill the microorganism. They are commonly used in Dutch, Danish and Norwegian AMR monitoring systems (Jorgensen & Ferraro, 2009; Mc Nulty *et al.,* 2016). This is important to know in diagnosing treatment of serious clinical infections. Dilution methods yield quantitative results via interpretation of the MIC, which is determined as the lowest antibiotic concentration inhibiting the growth of the organism, as judged by the lack of turbidity in the test tube. An advantage of the broth dilution is that the Minimum Bactericidal Concentration (MBC) can easily be obtained by sub-culturing the tubes that showed no growth onto a suitable medium (Jorgensen & Ferraro, 2009). The highest dilution showing at least 99% inhibition is then the MBC.

The broth dilution method is used to test a small number of bacterial isolates in a simple manner. A standard bacterial suspension is inoculated into prepared stock dilutions of antibiotic at varying concentrations (Jorgensen & Ferraro, 2009). This method is tedious and time-consuming and also forfeits high precision due to manual preparation of antibiotic dilutions. Some authors have found that higher MIC values are obtained in broth than in solid media due to stronger expression of resistance (Schlegelova *et al.,* 2001). Although the dilution method has been shown to be more accurate than disk diffusion, studies have revealed an excellent correlation between the values obtained from disk diffusion and dilution methods (Mc Nulty *et al.,* 2016).

The agar dilution method involves diluting the antibiotic into the agar and then inoculating the plates with a standard bacterial suspension. Multiple organisms can be applied to each plate by inoculating the plate in spots. The MIC is determined as the plate with the lowest antibiotic concentration showing more than 99% inhibition (Schlegelova *et al.,* 2001).

3.3.1.3. E-test

The epsilometer (E-test) is a testing method that uses both dilution of the antibiotic and diffusion of the antibiotic into the medium, creating an exponential gradient (Jorgensen & Ferraro, 2009). The method uses a thin test strip impregnated with a predefined antimicrobial gradient which is applied onto an inoculated agar plate. The intersection of the ellipse shaped inhibitory zone edge with the test strip indicates the MIC value. This allows testing over a wide concentration range. This method is generally used in particular instances such as organisms requiring a special incubation atmosphere as this is an expensive method. It can sometimes result in systematic biases with certain antibiotic-organism combinations (Jorgensen & Ferraro, 2009).

3.3.1.4. Mechanism-specific tests

There are tests available that can phenotypically detect the presence of an ABR mechanism. This method is limited to only a few resistance mechanisms, including the chromogenic cephalosporinase test for beta-lactamase detection and the chloramphenicol acetyltransferase kit for detecting the chloramphenicol modifying enzyme (Anon., 2011a).

3.3.2. Antibiotic agents used for phenotypic testing

The antibiotics selected for testing in this study were based on a selection of the top antibiotic classes sold in South Africa used in food-producing animals. Specific antibiotics within each class where selected based on those commonly used in livestock globally, those showing emerging resistance against or cross resistance development and those which are active against *E. coli* and *S. aureus*, as well as those which are used both in human and veterinary medicine. All of the selected antibiotics are considered critically or highly important for human medicine by WHO (WHO, 2011).

The antibiotic classes in which the selection of antibiotics fall under include: beta-lactams, glycopeptides, macrolides, sulphonamides. tetracyclines, quinolones and aminoglycosides. More specifically, *E. coli* ABR was analysed using nalixidic acid, ampicillin, tetracycline, streptomycin, ceftazidime and sulphafurazole. *S. aureus* ABR was tested using tetracycline, pencillin, oxacillin, cefoxitin, erythromycin and vancomycin.

There are many other classes of antibiotics which are used in veterinary medicine. Some of the other commonly used antibiotic classes include lincosamides, polymyxin, polipeptides, ionophores (Moyane *et al.,* 2013; De Briyne *et al*., 2014).

3.3.2.1. Beta-lactams

Beta-lactams are a class of broad spectrum antibiotics and are commonly used in beef cattle, dairy cows, sheep, poultry, swine and sheep. They include penicillins and cephalosporins such as ceftazidime and cefoxitin (Kahn & Line, 2010). Their mode of action against bacteria is inhibiting cell wall synthesis by hindering the formation of peptidoglycan crosslinks in the cell wall, resulting in cell death (Miller *et al*., 2014). The continued development of extended-spectrum β-lactamases produced by emerging resistant bacteria has resulted in decreased effectiveness of β-lactam antibiotics (Giguère *et al*., 2013). Cefoxitin is classified under the second generation cephalosporins and is used for treating infections resistant to first generation cephalosporins (Giguère *et al*., 2013). They have a broad spectrum of activity with greater activity against Gram-negative bacteria than the first generation cephalosporins (Kahn & Line, 2010). Common susceptible bacteria include *Staphylococcus*, *Enterococci* and *Streptococcus* (Kahn & Line, 2010). Ceftazidime is a third generation cephalosporin antibiotic that also has broad spectrum activity with even greater activity against Gram-negative bacteria than the second generation (Giguère *et al*., 2013). Ceftazidime, as well as other new generation beta-lactam antibiotics, are commonly used to screen for possible ESBL-producing bacteria (Rawat & Nair, 2010).

Penicillins, such as penicillin G, ampicillin and oxacillin have the same mode of action as the beta-lactams. Penicillin G, also known as benzylpenicillin, is the original penicillin found in 1928 (Giguère *et al*., 2013). It is a naturally occurring narrow spectrum antibiotic which is active against Gram-positive bacteria and only a few Gram-negative bacteria (Kahn & Line, 2010). Susceptible bacteria include *S. aureus* and *L. monocytogenes* (Kahn & Line, 2010). Resistance to Penicillin G is widespread due to extensive use since its

discovery (Kahn & Line, 2010). Ampicillin was the first broad-spectrum penicillin antibiotic and was developed in 1961 (Giguère *et al*., 2013).

In contrast to penicillin, the amino group present in the compound assists in penetration of the outer membrane of Gram-negative bacteria, thus allowing it to be active against a wider range of Gram-negative bacteria such as *Neisseria meningitis* and *Haemphilus influenza.* Ampicillin is also active against Gram-positive bacteria such as *Streptococcus pneumoniae*, *Streptococcus pyogenes,* and *Staphylococcus aureus* (Kahn & Line, 2010). However, it is not effective against penicillin-resistant or methicillin-resistant strains (Kahn & Line, 2010). Oxacillin is similar to, and has replaced methicillin (Giguère *et al*., 2013). It is a semi-synthetic beta-lactamase antibiotic that is used to treat penicillin-resistant *S. aureus* infections. MRSA or oxacillin-resistant *Staphylococcus aureus* (ORSA) is emerging which is treated with vancomycin-the drug of last resort (Kahn & Line, 2010). Often, MRSA/ORSA are also resistant to many other commonly used antibiotics, such as erythromycin (Giguère *et al*., 2013).

3.3.2.2. Glycopeptides

Glycopeptides, for example vancomycin, also inhibit cell wall synthesis but are only active against Gram-positive bacteria (Giguère *et al*., 2013). This is achieved by binding to the amino acids within the cell wall, thus preventing the addition of new units onto the peptidoglycan chains (Miller *et al*., 2014). More specifically, vancomycin functions by binding to the precursors of the peptidoglycan layer in the cell walls, preventing cell wall synthesis (Miller *et al*., 2014). Vancomycin is the drug of choice for treatment of MRSA infections and other serious Gram-positive infections that are resistant to other antibiotics (Kahn & Line, 2010). Possibly due to it limited use, resistance has not been shown to readily develop. However, the use of avoparcin in food animals which is a similar glycopeptide, has been shown to increase the development of resistance to vancomycin (Kahn & Line, 2010).

3.3.2.3. Macrolides

Macrolides, such as erythromycin are commonly used as growth promoters in beef cattle, swine and poultry. They function by preventing protein synthesis as their mode of action by inhibiting ribosmomal translation by binding to the 50S ribosome subunit and preventing peptidyltransferase from adding peptide to the next amino acid, thus inhibiting protein synthesis (Konah & Rubin, 2010). Macrolides are active against most Gram-positive bacteria such as *Staphylococcus* and *Streptococcus* and are commonly used as alternatives for penicillin and for infections of penicillin-resistant bacteria (Kahn & Line, 2010). Erythromycin is commonly used to treat mastitis in cattle (Kahn & Line, 2010). Resistant strains of *Streptococcus* have been reported as well as the development of cross-resistance to other macrolides (Giguère *et al*., 2013).

3.3.2.4. Sulphonamides

The sulphonamides, like sulphafurazole, are the oldest and most extensively used antimicrobials in veterinary medicine and are commonly used in beef cattle, dairy cows, swine, poultry and aquaculture and inhibit folate

synthesis by acting as a competitive inhibitor of the enzyme dihyropteroate synthase (Madigan *et al.,* 2012). Folic acid is needed for a variety of important cellular functions such as synthesis of the nucleic acids which are essential building blocks of DNA and RNA (Miller *et al*., 2014). Sulphafurazole is very water-soluble and is thus excreted unchanged in urine (Giguère *et al*., 2013). It has a wide range of action against Gram-positive and Gram-negative bacteria, including *Staphylococcus* and *E. coli*. Resistance is widespread among numerous animal species and cross-resistance is common where resistance is often linked to ampicillin and tetracycline resistance in Gram-negative intestinal bacteria (Giguère *et al*., 2013).

3.3.2.5. Tetracyclines

The tetracyclines are broad spectrum antibiotics used typically as growth promoters in beef cattle, dairy cows, honey bees, swine, poultry, swine and aquaculture and inhibit protein synthesis as their mode of action by reversibly binding to the 30S ribosomal subunit on the mRNA-ribosome complex, preventing ribosomal translation (Speer *et al.*, 1992). Resistance tends to develop slowly but is widespread among numerous bacteria such as *Staphylococcus*, *Streptococcus* and *Enterbacteriaceae* due to the extensive use of tetracyclines at low doses (Kahn & Line, 2010). Resistance is usually acquired via HGT (Kahn & Line, 2010).

3.3.2.6. Quinolones

Quinolones, such as nalixidic acid, are widely used in veterinary medicine. They function by interfering with DNA synthesis, through inhibition of the enzymes DNA gyrase and topoisomerase IV, which, in turn, are responsible for transcription and the replication of the genome before cell division (Miller *et al.*, 2014). Resistance to quinolones can develop rapidly and numerous pathogens, including *E. coli*, commonly exhibit resistance (Kahn & Line, 2010).

Nalixidic acid was the first drug developed of the quinolone class and has been available for many years (Kahn & Line, 2010). It has a narrow spectrum of activity, primarily against Gram-negative bacteria and was commonly used to treat urinary tract infections caused by bacteria such as *E. coli, Shigella, Enterobacter*, and *Klebsiella*. However, it is not commonly utilised nowadays due to its limited therapeutic use, the rapid development of resistance since its approval and because less toxic and more effective agents are currently available (Kahn & Line, 2010). These include the fluroquinones, which have a broader spectrum activity against bacteria such as *E. coli, Staphylococcus aureus* and *Streptococcus pneumoniae*. Fluoroquinones, such a chloroamphenicol, function by inhibiting protein synthesis. They do this by directly interfering with substrate binding (Kahn & Line, 2010). It should be noted, however, that the use of fluroquinones has been banned by the FDA for use on food-producing animals. Thus its use in veterinary medicine is highly restricted (Kahn & Line, 2010). It has been shown that some *E. coli* strains develop spontaneous resistance to fluoroquinones (Giguère *et al*., 2013).

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3.3.2.7 Aminoglycosides

Streptomycin is an aminoglycoside antibiotic and functions by irreversibly binding to the bacterial 30S ribosomal subunit, which inhibits the translocation of the peptidyl-tRNA so that the bacterium is unable to synthesise proteins (Miller *et al.*, 2014). Streptomycin was the first drug of the aminoglycoside class to be discovered and is commonly used in veterinary medicine against Gram negative bacteria in large animals such as cattle (Kahn & Line, 2010).

3.4 Genotypic detection of antibiotic resistance

Antibiotic resistant traits can develop in bacteria by chromosomal DNA mutations or by the acquisition of DNA material coding for resistance traits from other bacteria (van Hoek *et al.*, 2011; Holmes *et al.*, 2016; Munita & Arias, 2016). The acquisition of new DNA material from other bacteria most commonly happens through horizontal gene transfer, where the new DNA material can be transferred directly between chromosomes or via mobile genetic elements, such as plasmids, transposons and integrons (Munita & Arias, 2016).

It should be noted that the presence of a resistance gene does not always result in phenotypic resistance and hence treatment failure, because phenotypic resistance is also dependent on the mode and level of expression of the resistant genes (Fluit *et al*., 2001).

3.4.1. Testing methods

3.4.1.1. Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) is the most commonly used molecular technique for detecting resistant genes. This method amplifies a specific DNA sequence, which is known to encode for a resistant gene, in a DNA sample (if present).

Real-time PCR (RT-PCR) has more recently been used to not only detect resistant genes but to also quantify them. This method is based on a comparison of the number of DNA copies obtained when bacteria are cultivated in breakpoint-equivalent concentration of antibiotics after, and before, incubation and the control, that is, those grown without the presence of antibiotic (Rolain, *et al.,* 2004). If a resistant bacterium is resistant, the number of DNA copies would increase in a similar fashion to the control. Conversely, if the bacteria is susceptible to the antibiotic, then the number of DNA copies would remain similar to those before incubation in the presence of the antibiotic (Rolain, *et al.,* 2004). Other techniques utilising RT-PCR include quantification of gene copy number and gene expression analysis. PCR methods allows fairly rapid determination of antimicrobial susceptibly but at a cost, likewise for phenotypic automated systems (Rolain, *et al.,* 2004).

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3.4.1.2. DNA hybridisation

DNA hybridisation involves using a probe with a known DNA sequence that can pair up with open DNA from a sample. If hybridisation occurs (the resistant gene of interest is present in the sample) then a detectable signal will occur (Fluit *et al*., 2001). DNA arrays and DNA chips are based on the principle of DNA hybridisation. They are devices which can detect a large number of DNA sequences at once (Fluit *et al*., 2001).

3.4.1.3. Next Generation Sequencing

DNA sequencing can detect the presence of antibiotic resistant genes within the whole-genome sequence of an isolate by comparing the sequence against known gene sequences using an AMR reference gene database (Anjum, 2015). Therefore, specific primers are not needed, and this enables a much more comprehensive detection of ABR genes. This technique also enables to discovery of novel antibiotic resistant genes if the target sequence is incomplete or has low similarity to existing antibiotic resistant genes in a database (Gupta *et al.,* 2014).

3.4.2. Genes encoding for resistance

Antimicrobial agents function by interfering at bacteria's target sites that are vital for growth and survival, resulting in inhibitory or lethal effects on the bacterial cells (Lambert, 2005). Development of resistance to an antimicrobial agent cannot simply take place by avoiding their action because its target sites involve vital cellular functions (Lambert, 2005). Hence, resistance development occurs via mutational changes in the target sites but while still maintaining cellular functioning (Lambert, 2005). Specific resistance genes encode for these mutational changes, which can be transferred via HGT if they are located on mobile genetic elements.

Detection of resistance genes in bacterial isolates is useful in confirming phenotypic ABR achieved via AST, as well as to better understand the mechanism of resistance the bacterial isolate harbours as resistant genes encode for specific resistant mechanisms.

Multiplex PCR is a technique that is commonly used to detect antibiotic resistant genes (Strommenger *et al.,* 2003). This technique amplifies multiple different DNA sequences simultaneously using multiple primers in a single reaction, allowing a much larger set of genes to be detected that would otherwise take several separate single test runs to obtain with conventional PCR (Elnifro *et al.,* 2000; Ng *et al.,* 2001). Thus multiplex PCR saves time and cost of analysis if multiple target genes are to be identified and thus is an efficient method for the detection of various antibiotic resistant genes present in bacterial isolates (Strommenger *et al.,* 2003). For multiplex PCR to be successful, the annealing temperatures for each primer set must all work in the same reaction conditions. In addition, the amplicon sizes of each of the target genes should be different enough to form distinct bands when visualised by gel electrophoresis (Ng *et al.,* 2001).

3.4.2.1. Escherichia coli

Escherichia coli have predominantly built up resistance to antimicrobial agents that have been used for the longest time in human and veterinary medicine (Wilkerson & Samadpour, 2004). These include: streptomycin, which was introduced in 1943, sulfonamides introduced in 1936, ampicillin introduced in 1961 and tetracyclines which were introduced in 1948 and constitutes the most prevalent resistance found in *E. coli* (Boerlin *et al.,* 2005; Tadesse *et al.,* 2012).

In streptomycin-resistant *E. coli,* the *str*A-*str*B gene pair and the *aad*A gene cassette have been found to be the most common streptomycin resistant genes (Boerlin *et al.,* 2005; Sunde & Norström, 2005). The *str*A-*str*B gene pair mediates resistance by inactivation of the antibiotic by two phosphtransferase enzymes and has been located in bacteria colonising plants, animals, farmed fish and humans (Sunde & Norström, 2005). The *aad*A gene cassette encodes resistance to streptomycin by inactivation by aminoglycoside adenyltransferases and have been isolated from humans, wild animals, poultry and farmed fish (Sunde & Norström, 2005).

Tetracycline resistance is one of the most common types of resistance observed in *E. coli* animal isolates. This is due to its widespread use in both therapeutic and growth-promoting activities in animal production systems since its approval in 1948 (Tadesse *et al.,* 2012). There are six genes that have been identified in tetracycline-resistant *E. coli* strains with the major resistance genes for tetracycline being *tet*A, *tet*B and *tet*C (Wilkerson & Samadpour, 2004). These three genes encode for tetracycline-specific efflux pumps, which is one of the main resistance mechanisms seen in *E. coli.* The *tet*B gene has been found to be the most dominant (Tuckman *et al*., 2007; Wilkerson & Samadpour, 2004). This is due to the fact that the *tet*B gene resides on highly mobile genetic elements that can readily transfer between different bacterial genera and thus has a wide host range (Tuckman *et al*., 2007). The efflux pumps function by transporting tetracycline via proton exchange to reduce its intracellular concentration in the bacterial cell (Tuckman *et al*., 2007).

Sulphonamides were introduced in the 1930s and are among the most commonly used drugs in animal production systems, either alone or in combination with diaminopyrimidines. Consequently, resistance to sulphonamides is common and increasing. It is often associated with the *sul*1 and *sul*2 resistance genes which, in turn, are commonly associated with mobile genetic elements (Tadesse *et al.,* 2012). They encode for dihydropteroate synthases which are not inhibited by sulphonamide antibiotics and thus folate synthesis can continue as normal (Frank *et al*., 2007). The *sul*1 gene is found exclusively on large conjugative plasmids and the *sul*2 gene is usually located on plasmids (Frank *et al*., 2007).

Beta-lactam resistant *E. coli* is primarily achieved by the use of β-lactamase enzymes, which hydrolyses the β-lactam ring of the antibiotic, thus inactivating it (Lachmayr *et al.,* 2009). Researchers have found the *bla*TEM1 gene to be the most common determinant observed in ampicillin-resistant *E. coli* of animal origin and is located on the TnA transposon (Mirzaagha *et al.,* 2011). The TEM β-lactamases represent one of the most clinically significant families of β-lactamases and has become the most commonly encountered β-lactamase and is ubiquitous among *Enterobacteriaceae* (Lachmayr *et al.,* 2009).

Over recent years, gram-negative bacteria have developed resistance to the higher generation beta-lactam antibiotics by mutations of beta-lactamases, called extended-spectrum beta-lactamases. These are a group of enzymes which are able to break down penicllin and cephalosporin antibiotics, rendering them ineffective. The most common ESBLs are 1) CTX-M, which hydrolyses cefotaxime 2) SHV-1 confers resistance to broad-spectrum penicillines such as ampicillin 3) TEM-1 can also hydrolyse penicillines and first generation cephalosporins 4) OXA-1 can hydrolyse oxacillin (Shaikh *et al*., 2015).

3.4.2.2. Staphylococcus aureus

Well-known resistant genes that are associated with phenotypic resistance in *S. aureus* include *mec*A, *van*A, *van*B (Jorgensen & Ferraro, 2009). The emergence of *S. aureus* resistance was first demonstrated in the 1940s as infections caused by penicillin-resistant *S. aureus* in hospitals increased (Chambers & DeLeo, 2009).

Resistance to penicillin is most commonly attained by production of the penicillinase enzyme, encoded by the *bla*Z gene, which inactivates the antibiotic through hydrolysis of the β-lactam ring rendering the drug inactive (Lowy, 2003; Jensen & Lyon, 2009). The majority of penicillin-resistant strains also exhibit resistance to other antimicrobial agents, such as heavy metal ions, and are found to carry β-lactamase plasmids, although some strains the genes are located on the chromosome (Jensen & Lyon, 2009). Penicillin resistance can also occur due to overproduction or formation of PBPs which leads to reduced affinity towards penicillin, thus rendering the antibiotic less effective (van Hoek *et al.,* 2011).

The second event of *S. aureus* resistance emergence was in 1961, when the first strain of *S. aureus* that was resistant to methicillin was reported (Chambers & DeLeo, 2009). Resistance mechanism of MRSA is due to the alteration of transpeptidase through acquisition of the *mec*A gene which encodes the expression of a PBPs, PBP2a (Lambert, 2005). Unlike the other four PBPs in *S. aureus*, PBP2a has very low-affinity binding to β-lactam antibiotics and thus allows MRSA to continue cell wall synthesis despite inhibitory concentrations of β-lactam antibiotics (Berger-Bächi, 1999; Katayama *et al.,* 2000). The *mec*A gene is carried on a mobile genetic element called the SCC*mec*, which is believed to have been acquired from a coagulase-negative *Staphylococcus* species, possibly from the pathogen *Staphylococcus sciuri* via HGT (Lambert, 2005). The *fem*A chromosomal gene is necessary for the expression of the *mec*A gene and is believed to be unique to *S. aureus* (Turutoglu *et al*., 2009). Additionally, the *fem*A and 16 S rRNA genes have been reported as valuable tools for the identification of MRSA and can also be used for detection of *S. aureus* at the genus level to genotypically confirm colony identity (Turutoglu *et al*., 2009). Additionally, the *fem*B gene has been used in conjunction with the *mec*A gene to confirm MRSA (Paterson *et al.,* 2012). The *fem*B gene codes for an enzyme involved in the cross-linking of peptidoglycan in *Staphylococcus aureus*, and is thus used to confirm the identity of *S. aureus* (Jonas *et al*., 2002).

It has been reported by several researchers that some *S. aureus* strains which had phenotypic resistance to oxacillin did not carry the *mec*A gene and suggest that these resistant strains have alternative resistance mechanisms to oxacillin other than the production of PBP2a. Other mechanisms that may lead to the expression

of methicillin resistance include alteration of PBP subtypes or production of novel methicillinase (Turutoglu *et al*., 2009). Alternatively, the absence of the *mec*A gene could be due to the presence of the newly discovered *mec*C gene, a divergent homologue of *mec*A which also encodes for a PBP, has been found in MRSA isolates believed to have originated from dairy cattle and transferred to humans (Paterson *et al.,* 2012).

Vancomycin is the drug of choice for the treatment of MRSA infections but its increased use over the recent years has led to the emergence of two types of glycopeptide-resistance in *S. aureus* (Périchon & Courvalin, 2009). High level resistance was first reported in *Enterococci* in 1988, 30 years after its introduction into clinical practice (Périchon & Courvalin, 2009). Vancomycin resistant *S. aureus* (VRSA) have increased cell wall thickness, reduced peptidoglycan cross-linking and an increased proportion of peptidoglycan stem peptides than vancomycin- susceptible strains (Lambert, 2005). These changes in cell wall metabolism confer a resistance to vancomycin by reducing the number of vancomycin molecules that can reach the cytoplasmic membrane and thus reduce the concentration of vancomcyin in the cell (Lambert, 2005). VRSA acquire the *van*A operon carried by the Tn1546 transposon located on the chromosome or plasmid (Périchon & Courvalin, 2009).It is the most well-known gene and results in the substitution of polypeptides in peptidoglycan synthesis which greatly reduces the affinity of peptidoglycan precursors for glycopeptide antibiotics (Périchon & Courvalin, 2009).

Macrolide resistance can occur due to various mechanisms, the most common being target modification by the enzyme methylase (Schmitz *et al.,* 2000). Other resistant mechanisms include conformational change in the ribosome and macrolide efflux pumps (Schmitz *et al.,* 2000). In *S. aureus*, erythromycin resistance is usually either due to ribosomal modification by 23S rRNA methylases mediated primarily by *erm*A, *erm*B or *erm*C or by active efflux by an ATP-dependent pump mediated by *msr*A (Nicola *et al.,* 1998). It has been found that the *erm*A and *erm*C genes are the most common genes responsible for erythromycin resistance in *S. aureus* isolates where *erm*A gene more common in MRSA isolates than in methicillin susceptible *S. aureus* (MSSA) isolates and *erm*C more common in MSSA (Schmitz *et al.,* 2000). The *erm*C gene has only recently become prevalent in the *S. aureus* population where the *emr*A gene was solely responsible for erythromycin resistance until the 1970s (Schmitz *et al.,* 2000).

Tetracycline resistant isolates are widespread among Gram-positive and Gram-negative organisms and are often found in MDR bacteria (Ng *et al.,* 2001). Tetracycline resistance occurs via three mechanisms namely, (i) efflux of the antibiotic, (ii) altering the ribosome to prevent tetracycline from binding and (iii) production of enzymes that inactivate it (Ng *et al*., 2001). The two common resistance mechanisms identified in *S.aureus* are active efflux of the antibiotic mainly via *tet*K and *tet*L genes and ribosomal protection via *tet*M (Trzcinski *et al.,* 2000). Tetracycline efflux is mostly mediated by *tet*K. The *tet*M gene has been identified on the chromosome or plasmids and the *tet*K gene has only be identified on plasmids (Trzcinski *et al.*, 2000). This explains why some resistant strains carry both and some carry only one of the resistance genes (Ng *et al.,* 2001). It has been found that the *tet*M gene confers resistance to all antibiotics belonging to the tetracycline group whereas the *tet*K gene has only been described as resistant to tetracycline (Trzcinski *et al.,* 2000).

3.4.2.3. Enterococcus faecalis

Enterococci are known to be good indicators of the ecology of ABR genes due to their proficient ability to transfer genetic material horizontally and their ubiquitous nature in the gastrointestinal tract of animals and humans (Macovei & Zurek, 2006). The mobile genetic element, transposon *Tn1546* which confers vancomycin resistance in *E. faecalis*, has been transferred to *Staphylococcus aureus* (Macovei & Zurek, 2006).

Enterococci which are resistant to tetracycline and vancomycin have been recovered from clinical settings. Tetracycline resistance is most commonly due to the presence of the *tet*M gene (Santiago-Rodriguez *et al*., 2013; Jia *et al*., 2014). Vancomycin resistance has been acquired via eight different genes, *van*A, *van*B, *van*D, *van*E, *van*L, *van*M and *van*N but the *van*A and *van*B genes are the most common (Kristich *et al*., 2014; Ünal *et al*., 2017). The *van*A gene confers high level resistance to vancomcyin and teicoplanin and is located on the Tn1546 or similar transposon. The *van*B gene confers moderate to high level vancomycin resistance but no resistance towards teicoplanin and occurs on plasmids or chromosome (Kristich *et al*., 2014).

Enterococci are intrinsically resistant to clinical concentrations of aminoglycosides but high level resistance is acquired (Niu *et al*., 2016). Acquired resistance is most commonly acquired via genes that encode for enzymes which modify the aminoglycoside, thus rendering it ineffective (Chow, 2000). The most common genes include *aac*(6')/*aph*(2') which confers resistance to virtually all aminoglycosides except streptomycin, *aph*(3'), *ant*(6), *ant*(2") and *ant*(4', 4") (Chow, 2000; Jia *et al.,* 2014).

Enterococci are intrinsically non-susceptible to various beta-lactam antibiotics but the extent of resistance is variable, depending on the Enterococcal species and the class of beta-lactam (Kristich *et al*., 2014). Penicillin offers the highest activity against Enterococcus with *E. faecium* exhibiting higher resistance than *E. faecalis*. The cephalosporins have the least activity against Enterococci, followed by carbapenems (Kristich *et al*., 2014).

Macrolides are not used to treat Enterococcal infections but interestingly their resistance is still widespread (Kristich *et al*., 2014). The most common mechanism of acquired erythromycin resistance is production of an enzyme which reduces the binding affinity of the antibiotic to the ribosome. This mechanism is encoded by the *erm*B gene (Jia *et al*., 2014). Lower level macrolide resistance is conferred by the *mef*A gene which encodes an efflux pump (Kristich *et al*., 2014).

3.5 Conclusion

It is recommended that phenotypic methods be used in conjunction with molecular techniques to determine antimicrobial susceptibility of bacterial isolates as only a limited number of resistance genes are firmly associated with phenotypic resistance and there are too many resistance mechanisms associated with each ABR to be detected by current molecular methods (Jorgensen & Ferraro, 2009).

Ultimately, the results from an antimicrobial susceptibility test lead to a conclusion as to whether or not the microorganism under testing is susceptible, or resistant, to an antibiotic, at a certain concentration. The term 'susceptible' indicates that the microorganism should be inhibited by the antibiotic at a concentration that

normally would be sufficient (Jorgensen & Ferraro, 2009). The term 'resistant' on the other hand, means that the microorganism has an ability to survive the antibiotic at concentrations that should usually inhibit its growth (Jorgensen & Ferraro, 2009). Some microorganisms can be classified as having intermediate resistance, which indicates that they could survive antibiotic concentrations slightly higher than susceptible microorganisms but are not completely resistant (Jorgensen & Ferraro, 2009).

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CHAPTER 4

Escherichia coli and *Staphylococcus aureus* antibiotic resistance patterns of co-grazing and non-co-grazing livestock and wildlife on South African farms

4.1. Summary

The correlation between livestock and wildlife species who co-graze and those that do not co-graze together in terms of their antibiotic resistance (ABR) patterns was determined. The Kirby-Bauer disk diffusion method was used according to the CLSI 2018 guidelines. *Escherichia coli* (N= 150) and *Staphylococcus aureus* (N= 80) were isolated from the faeces and skin of various wildlife and livestock species to assess the antimicrobial susceptibility patterns. There is significant evidence to suggest that the practice of wildlife and livestock co-grazing has the potential to serve as an antibiotic resistance vector between animal species. Overall, the wildlife species were classified as significantly more extensively-drug resistant (resistant to more than one antibiotic class) than the livestock species, which suggests that wildlife can be considered a reservoir of antibiotic resistant traits. Twelve percent of all the *E. coli* isolates from this study were classified as resistant, 23% intermediately resistant and 65% susceptible to the range of antibiotics tested. Twenty one percent of all the *S. aureus* isolates from this study were classified as resistant, 18% intermediately resistant and 62% susceptible to the range of antibiotics tested. More specifically though, the livestock were significantly more resistant to ampicillin, tetracycline and vancomycin. The wildlife species were significantly more resistant to cefoxotin and streptomycin. The *E. coli* isolates from the non-co-grazing livestock and wildlife had significant differences in their antibiotic susceptibility patterns. The *E. coli* and *S. aureus* isolates from the wildlife and livestock which co-grazed together showed no significant differences in antibiotic resistance patterns. This suggests that there is bi-directional exchange of antibiotic resistant traits between livestock and wildlife who co-graze together which can act as a vector in the geographical spread of antibiotic resistant bacteria.

4.2. Introduction

It cannot be assumed that microbial communities in natural, more remote environments are isolated from external activities and commercial settings. Correspondingly, wildlife species, although not perceived to host antibiotic resistant bacteria, can be exposed to antibiotic resistant vectors and sources, such as co-grazing with livestock or by living in close proximity to human activities and contaminated sources and thereby develop antibiotic resistance (Aminov & Mackie, 2007; Mariano *et al*., 2009; Wellington *et al*., 2013).

Thus it is hypothesised that the bacteria from the wildlife and livestock species who co-graze together would have more similar antibiotic resistance (ABR) patterns to each other as a result of a bi-directional transfer than those which do not co-graze together. Furthermore, it is also hypothesised that the bacteria from the wildlife who co-graze with livestock would have a higher ABR profile than those that do not co-graze, as

found by Mercat *et al.* (2016). It is reasoned that a higher ABR level would be seen in the livestock species due to medication interventions by humans than in the wildlife, which would be transferred to the co-grazing wildlife, resulting in an increased ABR level.

Bekker (2011) found that 65.7% of game farmers in South Africa only farm with game species on their property while the remaining (34.3%) practice co-grazing of livestock and game. The wildlife-livestock interface is becoming a more common occurrence in animal farming which is in part, due to the increased demand for food and land, as a result of an increasing human population, as well as a the shift to more holistic farming, thereby allowing original ingenious wildlife species to return to the farmland (Craft, 2015; LC Hoffman 2018, personal communication, 3 October). This potentially increases the likelihood of disease transmission between livestock and wildlife, where wildlife is said to play an increasing role in the occurrence of livestock diseases (Miller *et al.,* 2013). It was found that 41% of farmers in South Africa who farm with livestock and wildlife do not have any control measures to prevent animal interaction in order to prevent disease transmission (Bekker, 2011). This is a public health issue as about 60% of diseases in humans are zoonotic- of which 72% are of wildlife origin (FAO, 2010).

During co-grazing, direct contact can occur through interspecies contact or indirectly, through shared space and mobile transfer vectors (Rhyan & Spraker, 2010). Differences in contact rates can influence the transmission rate of diseases and bacteria between livestock and wildlife species. For example, in periods of drought, there is more frequent contact at existing water points (Morgan *et al*., 2006). The same principles hold true for the exchange of antibiotic resistant bacteria between co-grazing wildlife and livestock.

The bi-directional exchange of antibiotic resistant traits between livestock and wildlife can act as a vector in the geographical spread of antibiotic resistant bacteria which can negatively affect either population viability (Morgan *et al*., 2006).

Furthermore, antibiotic resistant cases in the farming industry are commonly documented in intensive animal production systems, such as broiler chickens and feedlot cattle and pigs, where the use of antibiotics is evident and regularly employed (Phillips *et al.,* 2004; Berglund, 2015). However, research on extensively produced food animals, such as free-range livestock and game species, has been barely documented up until now and is one key area of study in this present work (Szmolka & Nagy, 2013).

4.3. Materials & Methods

4.3.1. Study area

Samples from wildlife and livestock species were collected from different farms in Southern Africa between 2016 and 2018. A summary of the sample species, farm location and type of farm is shown in Table 4.1 whilst Figure 4.1 displays the location of the farms across South Africa where sample collections occurred. Samples were collected from a farm in Bredasdorp which hosts livestock and wildlife species, namely, Merino sheep (*Ovis aries*), Angus cattle (*Bos taurus*), eland (*Taurotragus oryx*) and black wildebeest (*Connochaetes gnou*). The wildlife graze on pastures and are fed supplementary feed in summer, due to low rainfall. The wildlife are

separated from the livestock by a fenced off region but the cattle are occasionally allowed to co-graze with the wildlife. The livestock are fed a premixed feed on a daily basis.

More samples were collected from a farm in Witsand between March and June 2016. Springbok (*Antidorcas marsupialis*) and fallow deer (*Dama dama*) are free to co-graze with the livestock species, namely Angus cattle (*Bos taurus*) and Merino sheep (*Ovis aries*), particularly the deer move between both the cattle and sheep whilst the springbok co-graze more often with the sheep. Both the livestock and wildlife graze and drink on the farm's natural resources, although in times of drought, the livestock are supplied with supplementary feed.

For comparison of free-range versus organic livestock and wildlife (see section 3.4.2.5.), samples of springbok (*Antidorcas marsupialis*) and Merino sheep (*Ovis aries*) were collected from a sheep farm in Sutherland. The sheep are kept in a fenced-off region but the springbok occasionally co-mingle with the sheep due to their nature of jumping fences. The sheep and springbok only graze and drink on the farm's natural resources and are not given any medication or supplementary feed. The free-range animals in this comparison, were those from the Witsand and Bredasdorp farm.

Table 4.1 Details of the wildlife and livestock samples used in this study

Wildlife species	Farm location	Farm type	Faecal samples	Skin swabs
Eland	Bredasdorp	No co-grazing, free-range	5	5
Black Wildebeest	Bredasdorp	No co-grazing, free-range	5	5
Sheep	Bredasdorp	No co-grazing, free-range	5	5
Cattle	Bredasdorp	No co-grazing, free-range	5	5
Springbok	Witsand	Co-grazing, free-range	5	5
Deer	Witsand	Co-grazing, free-range	5	5
Sheep	Witsand	Co-grazing, free-range	5	5
Cattle	Witsand	Co-grazing, free-range	5	5
Springbok	Sutherland	Organic	5	-
Sheep	Sutherland	Organic	5	-

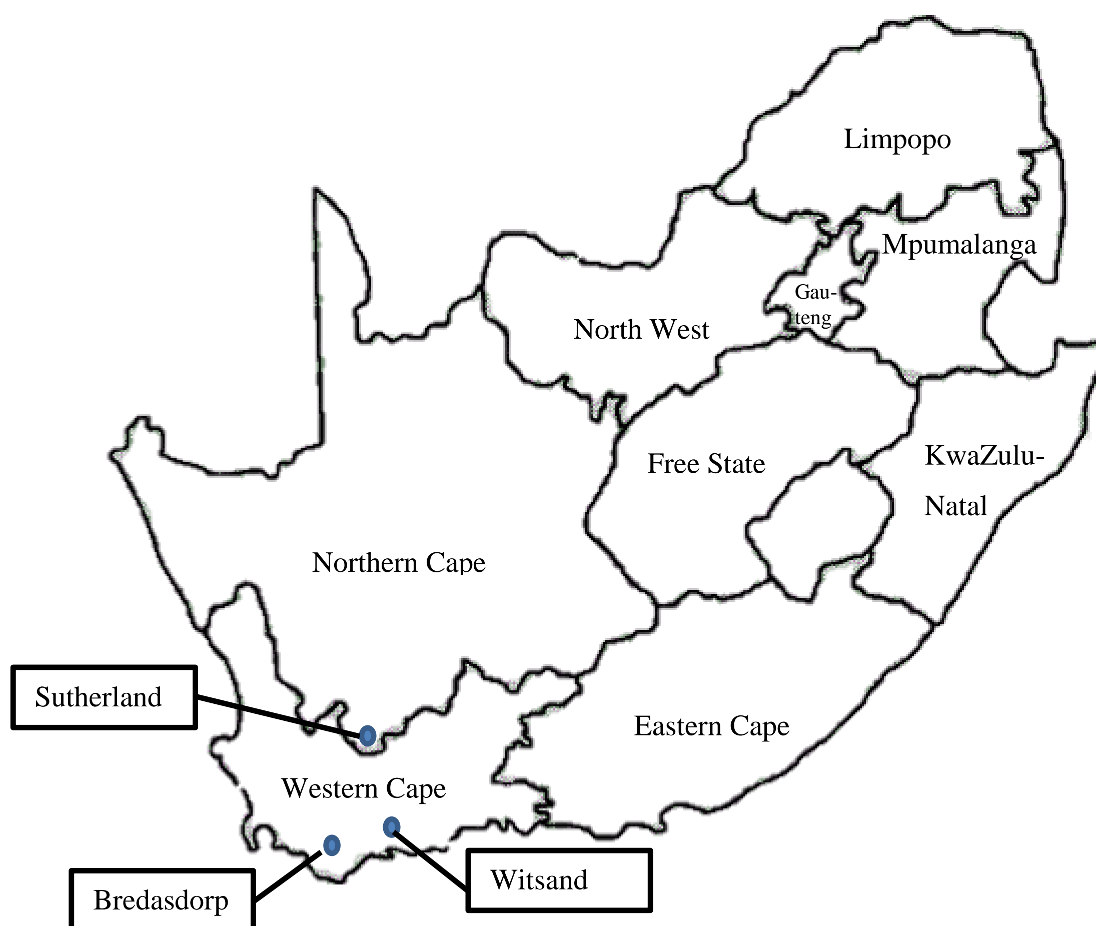

Figure 4.1 A map of South Africa which shows where sample collection took place.

4.3.2. Sample collection

Approximately 20 g of faecal matter was collected from livestock from the ground shortly after deposition in sterile sample containers that were labelled with unique identifying codes for each animal. To avoid sampling from the same animal more than once, faecal samples were selected a distance apart (≥10 m) or immediately after deposition from the specific animal. Additionally, all samples taken from the same farm were collected on the same day during the same time period to avoid sampling the same animal more than once.

Wildlife faecal samples were collected from the middle of the small intestine from recently slaughtered animals. Approximately 20 g of fresh faecal matter was located in the small intestines after evisceration and collected in a sterile sample container using a clean, disinfected knife and a new set of gloves for each animal.

Skin samples were also collected for isolation of *S. aureus*. For the livestock species, a sterile wooden cotton ear bud applicator with a cap was swabbed against the animal's skin over its back over a 5 x 5 cm area using a template, while wearing new gloves for each animal.

Wildlife skin samples were taken after slaughter before removal of their skin, using a sterile wooden cotton ear bud applicator with a cap. The applicator was swabbed against the animal's skin on its lower

hindquarter region over a 5 x 5 cm area using a template, while wearing new gloves for each animal. The lower hindquarter region was selected as an appropriate sampling area as this is the area that is the least likely to be contaminated by workers and exposed to the environment during transport of the animals from the field to the slaughterhouse, as the area is covered by the leg.

After collection, all samples were stored in a cooler box with ice at ~4°C and transported to the university's laboratory freezer and stored there at -20°C.

4.3.3. Enumeration of bacteria

Faecal and skin swab samples were defrosted at room temperature for 2h before analysis commenced. A 10^{-1} dilution of the faecal sample was made by adding 10 g faecal matter to 90 mL Buffered Peptone Water (Biolab, South Africa). The 10^{-1} faecal dilutions were homogenised using a Stomacher (Interscience) for 2 min and incubated at 35°C for 12-14 h for enumeration of *E. coli*. A 10^{-1} skin sample dilution was made by breaking off the cotton bud and placing it into 9 mL Tryptone Soya Broth (TSB) (Oxoid, South Africa) with 2.5 % sodium chloride (Biolab, South Africa) for enumeration of *S. aureus*. The 10^{-1} skin swab dilutions were vortexed for 2 min and incubated at 35°C for 12-14 h. This overnight incubation resuscitation step assists in recovery of the bacterial cells to allow for more effective enumeration using selective agar media.

After incubation, 10^{-4} and 10^{-5} serial dilutions were prepared using Physiological Saline Solution (PSS) according to the South African National Standards method 6887-1 (SANS, 1999) in 9 mL units. The pour plate technique was used by pipetting 1 mL from the dilutions onto petri dishes (Willey *et al*., 2011a). After this step, selective agar was poured over and swirled in a "figure of 8" motion. Baird-Parker Agar (BPA) (Oxoid, South Africa) supplemented with Egg Yolk Tellurite Emulsion (Oxoid, South Africa) was used for enumeration of *S. aureus*. Violet Red Bile Dextrose Agar (VRBDA) (Bioloab, South Africa) was used to select for *E. coli*. Once the petri dishes were set, they were inverted and incubated overnight at 35°C.

Following incubation, the streak plate technique was used to streak three random colonies per animal onto three selective agar petri dishes. Therefore, the antibiotic susceptibility test (AST) was performed in triplicate per animal sample. BPA was again used for *S. aureus* and Eosin Methylene Blue Agar (EMBA) (Oxoid, South Africa) was used for *E. coli.* This step isolates the specific bacteria so that individual colonies can be selected. At the same time, one can presumptively identify the bacteria by the appearance of the colonies on the selective agar. The petri dishes were inverted and incubated overnight at 35°C.

One colony per plate was then streaked onto Nutrient Agar (NA) (Biolab, South Africa) plates to yield three pure cultures per animal. The plates were then inverted and incubated overnight at 35°C. These plates were stored at 4°C for further use for up to five days.

4.3.4. Bacterial species confirmation

Gram's stain (Lasec, South Africa) was performed on all colony isolates which showed characteristic growth, using the method described in the manufacturer's instruction manual. A drop of distilled water was placed on

a microscope slide using a cooled sterile loop. The inoculation loop was sterilised through a flame and cooled and a small amount of bacterial colony from the NA plates was picked up. The bacteria were stirred into the water droplet to create a bacterial emulsion. The smear was left to air dry and then heat-fixed two to three times through a flame with the smear side facing up. The smear was flooded with crystal violet for 1 min and then rinsed with distilled water. The smear was then flooded with iodine for 1 min and then rinsed with distilled water. The smear was decolourised with alcohol until it ran clear and then was rinsed with distilled water. Lastly, the smear was flooded with safranin for 45 s and rinsed with distilled water. The smear was then gently blotted dry and observed under a microscope (Nikon YS100) on x1000 with immersion oil (Willey *et al.,* 2011b).

Eosin Methylene Blue Agar (Oxoid, South Africa) is a highly selective medium that produces characteristic colony growth specific to *E. coli*. Other lactose-fermenting gram negative rods that can also show the characteristic green metallic sheen are some species of *Citrobacter* and *Enterobacter*. Therefore, the citrate utilisation test was performed on presumptive *E. coli* isolates using Simmons Citrate agar (Oxoid). *E. coli* is citrate-negative, whereas *Citrobacter* and *Enterobacter* are both citrate-positive (Oxoid).

To confirm the presence of *S. aureus* isolated from the skin samples, the Staphylase test (Oxoid) and catalase test was performed according to the manufacture's instruction to ensure only colonies of *S. aureus* were selected from the BPA plates for antibiotic susceptibility testing. The Staphylase test identifies *S. aureus* by its unique ability to produce free and bound coagulase. *S. aureus* are catalase positive.

After colony identification was confirmed, stock cultures were made and stored in the freezer at -20°C until further use. A loop full of bacterial colony was picked with a sterile loop from the NA plates and transferred into a sterile test tube containing 3 mL TSB. The bacterial suspension was vortexed and incubated overnight at 35°C. After incubation, each test tube was vortexed and 0.75 mL bacterial suspension was pipetted into a 2 mL microtube containing 0.75 mL sterile 50% glycerol (Fluka Analytical, Germany) (Gorman & Adley, 2004). This long-term preservation technique has been commonly used for the successful preservation of numerous bacterial species (Gorman & Adley, 2004).

4.3.5. Antibiotic susceptibility testing

The Kirby-Bauer disk diffusion method was used according to the Clinical and Laboratory Standards Institute (CLSI) 2018 guidelines using Mueller-Hinton agar (Biolab, South Africa). The direct colony suspension method was used to prepare the inoculum suspension, using colonies from fresh NA plates. Five colonies per animal for each organism was selected randomly using an inoculating loop and suspended in 0.75 mL PSS in 2 mL Eppendorf tubes.

The antibiotic disks were applied within 15 min of inoculating the MHA plate. A 6-disk dispenser (Oxoid, South Africa) was used to place the disks onto the surface of the MHA plates. The disks were checked to ensure that they were firmly placed on the agar surface. Tables 4.2 and 4.3 show the antibiotic disks applied

for *E. coli* and *S. aureus,* respectively. The plates were inverted and incubated at 35°C for 16-18 h and 24 h for cefoxitin, oxacillin and vancomcyin discs, respectively (CLSI, 2018).

Table 4.2 Selected antibiotic disks for *Escherichia coli*

Antimicrobial agent	Disk content	Antibiotic class
Ampicillin (AMP)	10 µg	Penicillin
Chloramphenicol (C)	30 µg	Macrolide
Nalidixic acid (NA)	30 µg	Quinolone
Streptomycin (ST)	10 µg	Aminoglycoside
Sulphafurazole (SF)	300 µg	Sulfonamide
Tetracycline (TE)	30 µg	Tetracycline

Table 4.3 Selection of antibiotic disks applied to *Staphylococcus aureus*

Antimicrobial agent	Disk content	Antibiotic class
Cefoxitin* (FOX)	30 µg	Penicillin
Erythromycin (E)	15 µg	Macrolide
Oxacillin (OX)	1 µg	Penicillin
Penicillin (P)	10 U	Penicillin
Tetracycline (TE)	30 µg	Tetracycline
Vancomycin (VA)	30 µg	Glycolipid

*Cefoxitin disc is used to confirm oxacillin resistance

After incubation of the MHA plates, the diameter of the zones was measured to the nearest millimetre using a digital calliper (0-150 mm). The zones, except for oxacillin and vancomycin, were measured using reflected light. This was achieved by measuring the zones from the back of the plate with light shining from above and looking directly above the plate. The oxacillin and vancomycin zones were measured using transmitted light by holding the plate up towards the light to measure the zone diameter (CLSI, 2018).

Zone diameters were classified as either resistant (R), intermediate (I) or susceptible (S), according to the CLSI zone diameter interpretive standards for each microorganism (Tables 4.4 and 4.5) (CLSI, 2016). If a bacteria is classified as resistant, then it is able to grow in the presence of the antibiotic, rendering it ineffective. It is likely that these bacteria have developed specific resistance mechanisms. If a bacteria is classified as intermediate it means that a higher concentration is needed to inhibit growth and thus the response rate is lower than for susceptible isolates. Lastly, if a bacteria is classified as susceptible it means that it can't grow in the presence of the antibiotic, and thus the antibiotic is still effective (CLSI, 2018).

Table 4.4 Zone diameter interpretive standards for Enterobacteriaceae (CLSI, 2018)

Antimicrobial agent	**Disk content**	**Zone diameter, nearest whole mm**			**Comments**
		R	**I**	**S**	
Ampicillin (AMP)	10 µg	≤ 13	14- 16	≥ 17	Class representative for ampicillin and amoxicillin.
Chloramphenicol (C)	30 µg	≤ 12	13- 17	≥ 18	-
Nalidixic acid (NA)	30 µg	≤ 13	14- 18	≥ 19	-
Streptomycin (ST)	10 µg	≤ 11	12- 14	≥ 15	-
Sulphafurazole (SF)	300 µg	≤ 12	13- 16	≥ 17	Represents any of the currently available sulfonamides.
Tetracycline (TE)	30 µg	≤ 11	12- 14	≥ 15	Organisms that are susceptible to tetracycline are also considered susceptible to doxycycline and minocycline.

4.3.6. Statistical analysis

Statistical analysis was performed using Statistica 13.2 software (StatSoft, USA). The data was analysed using one-way analysis of variance (ANOVA). Levene's test was applied to determine homogeneity of variance. The main effects were the practice of co-grazing and no co-grazing; livestock and wildlife; and organic and free-range. If the group means were significantly different within the groups, post hoc tests were performed to determine where the differences occurred within each group. Significant results were identified by least significant means (LSM) by using a 95% confidence interval i.e. a 5% significance level ($p \leq 0.05$) as a guideline.

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Table 4.5 Zone diameter interpretive standards for *Staphylococcus* species (CLSI, 2016 & 2018)

Antimicrobial agent	Disk content	Zone diameter, nearest whole mm			Comments
		R	I	S	
Cefoxitin (FOX)	30 µg	≤ 24	-	≥ 25	The cefoxitin disk test is the preferred method of testing for prediction of mecA-mediated resistant to oxacillin for S. aureus.
Erythromycin (E)	15 µg	≤ 13	14- 22	≥ 23	-
Oxacillin (OX)	1 µg	≤ 10	11- 12	≥ 13	Based on the cefoxitin result, report oxacillin as susceptible or resistant.
Penicillin (P)	10 U	≤ 28	-	≥ 29	Penicillin-resistant, oxacillin-susceptible strains of S. aureus produce β–lactamase.
Tetracycline (TE)	30 µg	≤ 14	15- 18	≥ 19	Organisms that are susceptible to tetracycline are also considered susceptible to doxycycline and minocycline.
Vancomycin (VA)	30 µg	-	-	≥ 15	MIC tests should be performed to determine vancomycin susceptibility

4.4. Results & Discussion

4.4.1. Cefoxitin and oxacillin correlation

The cefoxitin disk diffusion AST is used to detect *mec*A-mediated oxacillin resistance of *S. aureus*, which represents methicillin resistance (Swenson *et al*., 2005). The results of the cefoxitin and oxacillin ASTs had good correlation, indicated by the high Intraclass Correlation Coefficient (ICC) agreement value (0.843) and r- value (0.8427). The CLSI recommends using the cefoxitin disk to confirm oxacillin resistance. It can be confirmed from these results that the cefoxitin disk is a reliable alternative for testing oxacillin resistance in the disk diffusion method and it is easier to read, producing better outcomes.

4.4.2. Antibiotic susceptibility

It should be noted that the ABR profiles of the *E. coli* isolates towards nalidixic acid and chloramphenicol were not included in the statistical analysis due to negligible resistance levels as shown in Table 4.6, leading to no variance in the data.

The highest resistance of the *E. coli* isolates was towards streptomycin from the wildlife and streptomycin and ampicillin from the livestock.

Table 4.6 A summary of the disc diffusion antibiotic susceptibility test results for *E. coli* isolates

			Ampicillin	Chloram-phenicol	Nalidixic acid	Strepto-mycin	Sulpha-furazole	Tetra-cycline
Wildlife	**(n=60)**	**Resistant**	0%	0%	0%	38%	3%	0%
		Intermediate	24%	5%	11%	45%	18%	25%
		Susceptible	75%	93%	89%	16%	78%	71%
Livestock	**(n=60)**	**Resistant**	7%	0%	0%	7%	6%	13%
		Intermediate	40%	0%	7%	71%	7%	23%
		Susceptible	52%	100%	93%	22%	87%	66%

Nalidixic acid was the first quinolone antibiotic used in animals but is no longer clinically used due to its toxicity, resistance emergence and development of more effective agents (WHO, 1998). Nalidixic acid is used as a predictive indicator for resistance against all fluroquinolones as bacteria are found to be cross-resistant to the other fluroquinolone antibiotics (Ito *et al*., 2008). Therefore, it can be concluded that the *E. coli* isolates in this study are susceptible to fluroquinolones. Resistance to quinolones develops via a step-wise chromosomal mutation and not from acquired genes carried on plasmids (Hooper, 1998). Thus it can be hypothesised that there is no significant quinolone selective pressure present in the farming environments of Bredasdorp, Witsand and Sutherland to promote resistance development. Silva *et al*. (2010), Costa *et al*. (2008) and Lillehaug *et al*. (2005) reported similar low ABR levels to nalidixic acid, ranging from 0-14% resistance in wild animals. Rolland *et al*. (1985) also reported that nalidixic acid resistance was uncommon in the wildlife isolates.

Chloramphenicol is derived from *Streptomyces venequelae* (Schwarz *et al*., 2004). It is prohibited for use in food-producing animals due to its severe side-effects in humans (Schwarz *et al*., 2004). Likewise with nalidixic acid, resistance to chloramphenicol develops slowly in a step-wise manner where a sufficient selective pressure is needed for resistance to develop (Sompolinsky & Samra, 1968; Schwarz *et al.,* 2004). This suggests that chloramphenicol is not being used on the Bredasdorp, Witsand and Sutherland farms and their surrounds as no selective pressure is evident. The very low resistance in this study is consistent with other studies which detected 0-7% resistance to chloramphenicol in various wild animals and reported that resistance to chloramphenicol is rare (Rolland *et al*., 1985; Lillehaug *et al*., 2005; Costa *et al*., 2008; Silva *et al*., 2010).

A summary of the disc diffusion AST results for *S. aureus* is shown in Table 4.7. Overall, the resistance was the highest towards penicillin and the lowest to vancomycin.

In this study, methicillin (oxacillin disk) resistance was reported as cefoxitin resistance, as the cefoxitin disk is used to confirm methicillin resistance. Therefore, methicillin resistance was only reported when the disk diffusion results revealed resistance against both oxacillin and cefoxitin.

Table 4.7 A summary of the disc diffusion antibiotic susceptibility test results for *S. aureus* isolates

			Tetracycline	Erythromycin	Vancomycin	Penicillin	Cefoxitin
Wildlife	**(n=50#)**	**Resistant**	8%	10%	8%	50%	19%
		Intermediate	25%	4%	-*	-*	-*
		Susceptible	67%	86%	92%	50%	71%
Livestock	**(n=30#)**	**Resistant**	1%	0%	16%	43%	40%
		Intermediate	50%	28%	-*	-*	-*
		Susceptible	49%	72%	84%	57%	60%

*No intermediate classification zone diameter guidelines available

#*S. aureus* was not recovered from the all the skin swab samples, leading to a lower number of isolates

4.4.2.1. Overall resistance

Antibiotic resistant bacteria were found in both the livestock and wildlife species, irrespective of whether they co-grazed or not, suggesting that antibiotic resistant bacteria are present in natural environments, possibly originating from the natural reservoirs in the soil, or even transferred from other nearby reservoirs, such as commercial farm effluent and then transmitted to the natural environment by numerous vectors like birds and the wind (Kozak *et al*., 2009; Sousa *et al*., 2014).

Figure 4.2 shows the overall ABR levels of the *E. coli* (N= 120) and *S. aureus (*N= 80) isolates from the Bredasdorp and Witsand farms. On both farms the livestock are free-ranging in extensive systems and were only fed standard feed where the inclusion of antibiotics was not evident. Therefore, it was expected that the overall antibiotic resistance levels would be fairly low. This is due to the fact that there were no major sources of antibiotic resistance selective pressures occurring predominantly from one side, such as typically found in intensively reared livestock which are fed growth promoters (Navarro-Gonzalez *et al.,* 2013).

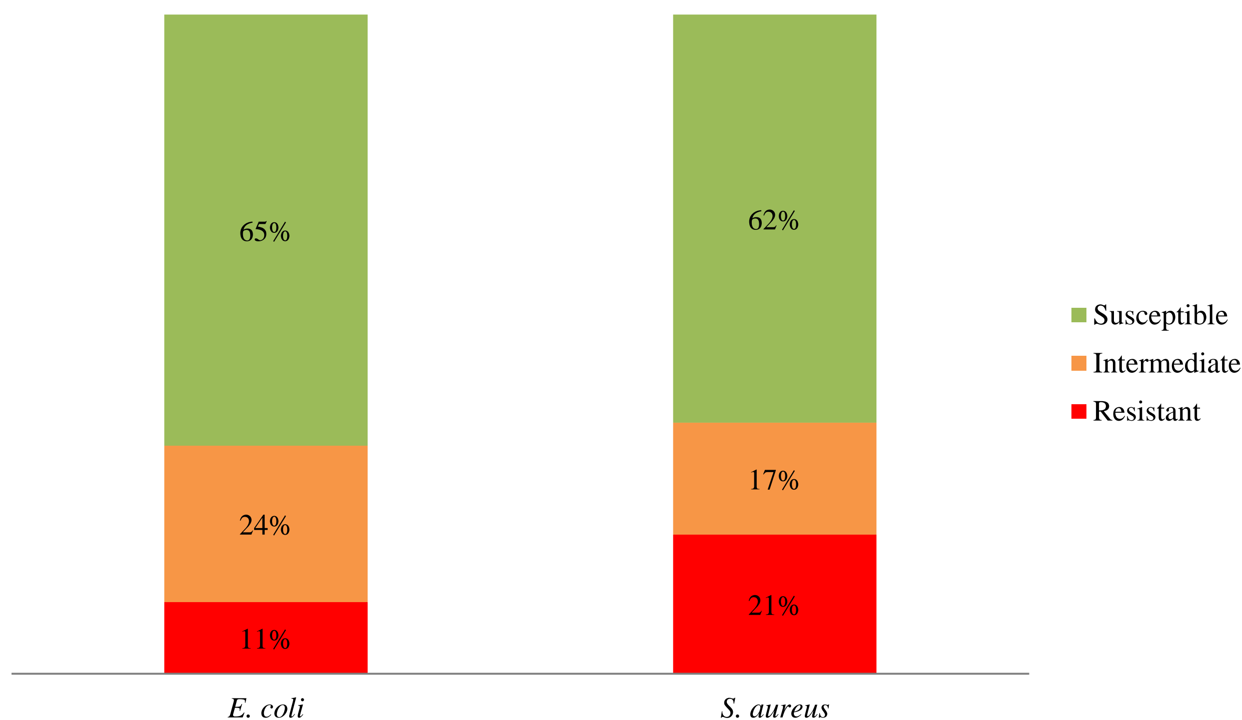

Figure 4.2 Overall antibiotic resistance levels of *E. coli* (N= 120) and *S. aureus* (N= 80) from the wildlife (eland, black wildebeest, springbok and deer) and livestock (cattle and sheep) on the Bredasdorp and Witsand farms.

When comparing the overall ABR levels between the two farms (Figure 4.3), the livestock isolates from the Bredasdorp farm were significantly more resistant than the livestock isolates from the Witsand farm, for *E. coli* and *S. aureus*. This could be due to the fact that on the Bredasdorp farm, the livestock were a mixture of stud and commercial livestock and thus the livestock management system would be more intensive than that on the Witsand farm. This would likely result in a higher level of antibiotic usage as preventative medication on the Bredasdorp farm, and thus, encouraging the development of antibiotic resistant bacteria. However, no significant differences in the ABR levels were observed between the wildlife isolates. The theory hypothesised from Mercat *et al.* (2016), who stated that the bacteria from the wildlife who co-graze with livestock would have a higher ABR profile than those that do not co-graze with livestock, is not true in this study. However, this could be due to the overall low resistant level seen in the livestock isolates from the Witsand farm. Thus there is no significant source of selective pressure coming from the livestock to be transferred to the co-grazing wildlife. Additionally, the higher (but not significantly) ABR of the wildlife isolates on the Bredasdorp farm could be due to the practice of supplementary feeding (see Chapter 5), leading to the development of ABR and thus resulting in a similar level of ABR to the wildlife on the co-grazing farm (Witsand).

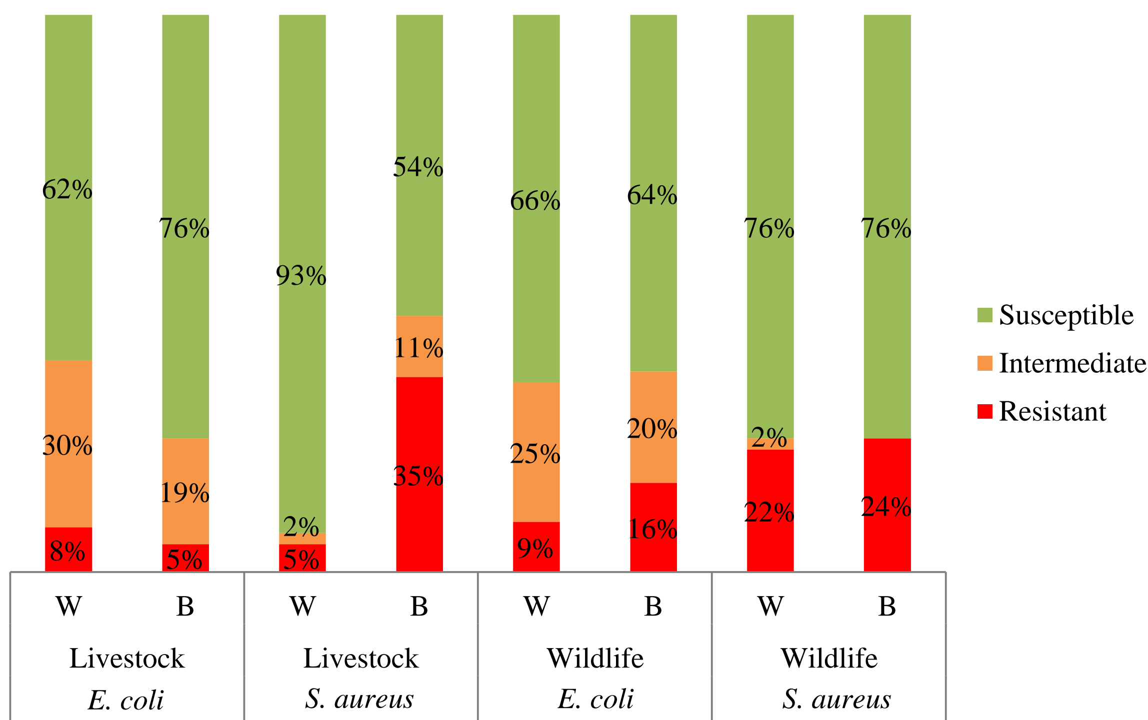

Figure 4.3 The averaged categorical antibiotic resistance levels of the livestock (cattle and sheep) isolates between Witsand (W) and Bredasdorp (B) farms (*E. coli* $p \leq 0.05$; *S. aureus* $p \leq 0.05$) and the wildlife (springbok and deer; eland and black wildebeest) isolates between Witsand (W) and Bredasdorp (B) (*E. coli* $p > 0.05$; *S. aureus* $p > 0.05$).

4.4.2.2. Affect of co-grazing

Wildlife, livestock, diseases and the environment form a complex multi-host system, where there are a large number of shared pathogens and diseases (Gortazar *et al*., 2016). Within this tightly interconnected system, the intestinal bacteria of wildlife species are densely concentrated and can easily be disseminated into different environments through the soil, water, food and other animals. This is the reason they often serve as a transfer vehicle for pathogens (Costa *et al*., 2008). Just as easily, antibiotic resistant elements and bacteria can be acquired by naturally occurring wildlife species via horizontal gene transfer (HGT), thus forming a reservoir of antibiotic resistant determinants (Radhouani *et al*., 2014).

Due to the extensive movement of antibiotic resistant genes and antibiotic compounds in nature via the wind, water, birds and animals, it is unlikely that any environment can be considered completely remote (Allen & Donato, 2010). The natural environment contains a large array of bacteria and thus also a huge pool of antibiotic resistant genes, and those which are on mobile genetic elements are available for transfer to bacteria into other environments (Cantas *et al.,* 2013).

The overall ABR levels of *E. coli* and *S. aureus* isolated from the various animals on the Bredasdorp farm are displayed in Figure 4.4. The eland and wildebeest were grouped together as 'wildlife' due to no differences ($p > 0.05$) in their antibiotic susceptibility profiles. The sheep and cattle were kept separately due

to differences (p≤ 0.05) in their antibiotic susceptibility profiles. There were differences (p≤ 0.05) between the non-co-grazing livestock and wildlife *E. coli* isolates, but no significant differences were observed for the *S. aureus* isolates.

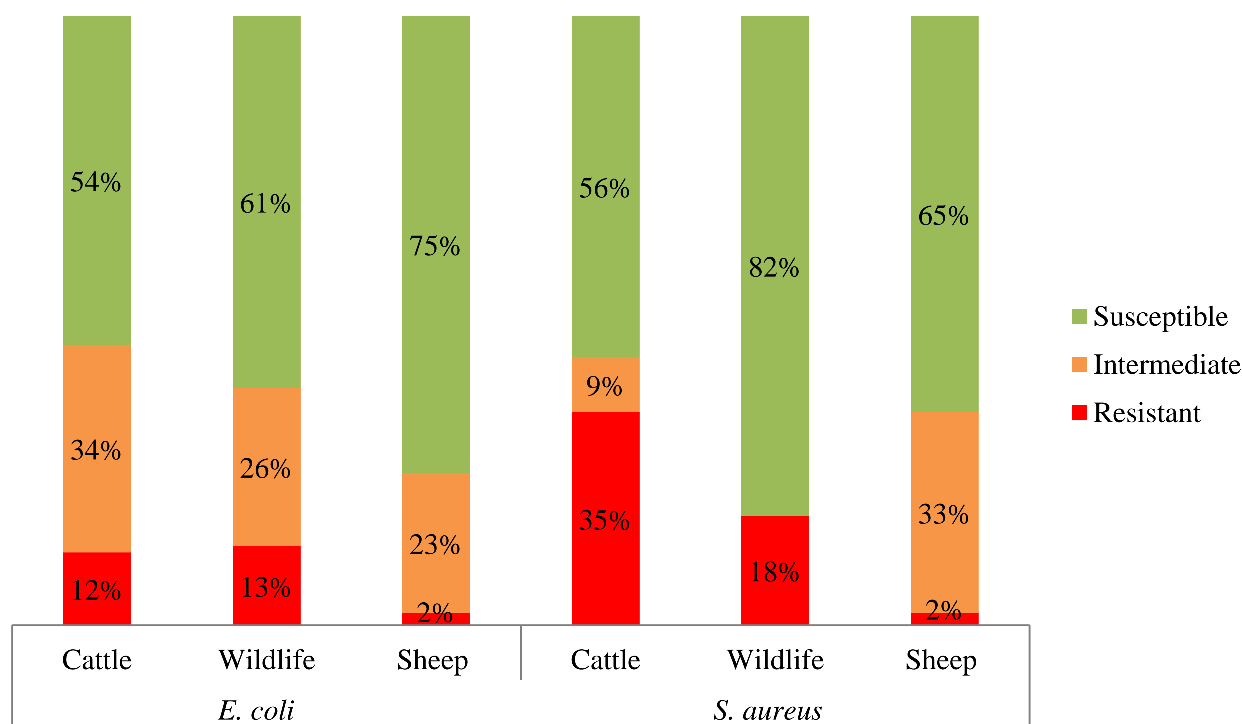

Figure 4.4 The averaged categorical antibiotic resistance levels of the bacterial isolates of non-co-grazing wildlife (eland and black wildebeest) and cattle (*E. coli* p≤ 0.05; *S. aureus* p> 0.05) and non-co-grazing wildlife and sheep (*E. coli* p≤ 0.05; *S. aureus* p> 0.05).

These observations highlight the effect of co-grazing on the transfer of diseases and antibiotic resistant bacteria between animals which are in more frequent contact with each other where genetic exchange could be occurring from both sides, whether via direct or indirect contact. Moreover, there are many pathogens that occur at the interface of humans and animals which cause shared diseases known as zoonoses, such as ESBL-producing *E. coli* and *Bacillus anthracis* (Gortazar *et al*., 2016).

The insignificant differences in the ABR levels of the *S. aureus* isolates between the non-co-grazing livestock and wildlife could be due to indirect transfer of ABR traits between species who live near to each other but do not come into contact with each other through vectors such as birds, rodents and farmers.

Although on average there was no significant ABR differences between the *S. aureus* isolates from the livestock and wildlife species, the livestock isolates showed to be more resistant (p≤ 0.05) towards erythromycin and penicillin than the wildlife isolates. Penicillin is the most widely used antibiotic in both human and veterinary medicine, explaining the higher penicillin resistance in the livestock specie isolates. Erythromycin, sulphonamides and tetracyclines, among others, are also commonly used in livestock farming

and thus the possible use of these antibiotics could explain the higher resistance observed (Economou & Gousia, 2015).

The *E. coli* isolates had significant differences in resistance between the non-co-grazing livestock and wildlife against the individual antibiotics, except for tetracycline (Figure 4.5). Although the wildlife isolates showed an overall higher resistance (but not significantly higher) than the livestock, the cattle isolates were the only group which had completely resistant isolates (4%), likely due to the extensive use of tetracycline in propylaxis and infection treatment in livestock, resulting in significant tetracycline resistance in food animals (Fan *et al*., 2006).

The wildlife isolates were significantly more resistant towards sulphafurazole and streptomycin than the livestock isolates. Other studies have also found *E. coli* isolates originating from natural environments to be highly resistant to sulfonamides due to the presence of a resistant enzyme, even though sulphonamides are synthetic antibiotics (Wise *et al*., 1975).

Many indirect factors, such as the presence of heavy metals, can be a causative factor for the onset of sulfonamide resistant bacteria in nature (Na *et al*., 2018). Sulfonamide resistant bacteria could also find their way into wildlife territory via indirect ways, such as run off or the application of manure from intensive farming that can be dissipated into the ground, as resistance to sulfonamides is fairly common in farm animals (Kozak *et al*., 2009).

A possible direct cause of sulfonamide resistance development in the wildlife is due to the fact that sulphonamide is a common antibiotic used in wildlife supplementary feed. These feeds are given on a "free-choice" basis which leads to incorrect dosages of the medicated feeds, promoting the development of drug resistance (Love *et al*., 2011). The wildlife on this farm were fed a commercial high energy and protein supplement during periods of drought but the exact composition is unknown. Unfortunately, this information was not disclosed to the researchers by the farmers/ managers; it is postulated that they did not know what is in the pre-mix added to the feed as these are typically commercial products and the manufacturers' thereof are reluctant to disclose the exact composition of these due to propriety rights.

Streptomycin on the other hand, is a naturally-produced antibiotic and is abundantly found in the soil, produced by *Actinobacteria* (Cantas *et al.,* 2013). Streptomycin resistance is prevalent worldwide, as streptomycin is one of the most commonly used antibiotics in agriculture, in use since 1936. Reports have indicated that resistance to streptomycin in clinical isolates has already been evident since the late 1940s (Chadha, 2012).

Thus it can be postulated that the wildlife species, due to their grazing nature, can easily pick up streptomycin resistant bacteria and the streptomycin resistant genes that are naturally found in the soil. However, due to the low antibiotic concentrations found in nature, it is believed that that does not provide a sufficient selective pressure to create resistant bacteria. Rather, it is thought that the complexity of the natural environment plays a role in the proliferation of antibiotic resistant bacteria in nature (Na *et al*., 2018).

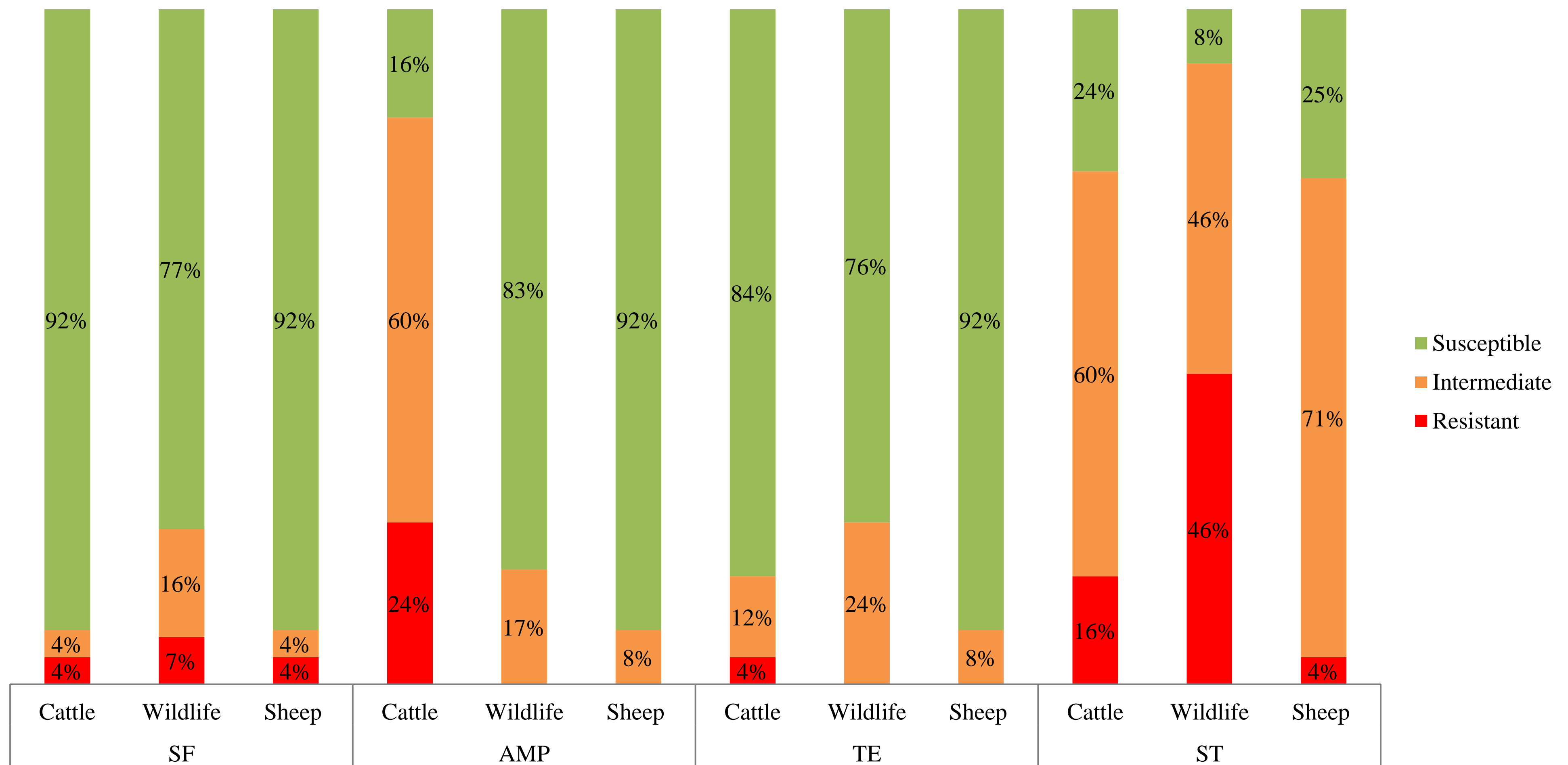

Figure 4.5 The averaged categorical antibiotic resistance levels of the *E. coli* isolates on the Bredasdorp farm of the non-co-grazing wildlife (eland and black wildebeest) and cattle against SF (sulphafurazole; $p \leq 0.05$), AMP (ampicillin; $p \leq 0.05$), TE (tetracycline; $p > 0.05$) and ST (streptomycin; $p \leq 0.05$) and non-co-grazing wildlife and sheep against (sulphafurazole; $p \leq 0.05$), AMP (ampicillin; $p > 0.05$), TE (tetracycline; $p > 0.05$) and ST (streptomycin; $p \leq 0.05$).

The cattle *E. coli* isolates (24% resistant) were more ($p \leq 0.05$) resistant towards ampicillin than the wildlife isolates (0% resistant). Ampicillin is one of the most commonly used antibiotics in livestock farming. Thus it can be postulated that the significantly higher resistance seen in the cattle is a direct result of antibiotic use in livestock farming. Ampicillin is used in cattle for therapeutic purposes to treat respiratory tract infections (Anon., 2007).

The overall ABR levels of *E. coli* and *S. aureus* isolated from the various animals on the Witsand farm, who all co-graze on the same farm, is displayed in Figure 4.6. The springbok and deer were grouped together as 'wildlife' due to no differences ($p > 0.05$) in their antibiotic susceptibility profiles. Similarly, the sheep and cattle were grouped together as 'livestock' due to no differences ($p > 0.05$) in their antibiotic susceptibility profiles. There were no overall significant differences in the ABR profiles between the livestock and wildlife *E. coli* and *S. aureus* isolates. This indicates that HGT occurs between the different species, most probably through the sharing of pastures and water points (Bengis *et al.*, 2002; Mercat *et al.*, 2016).

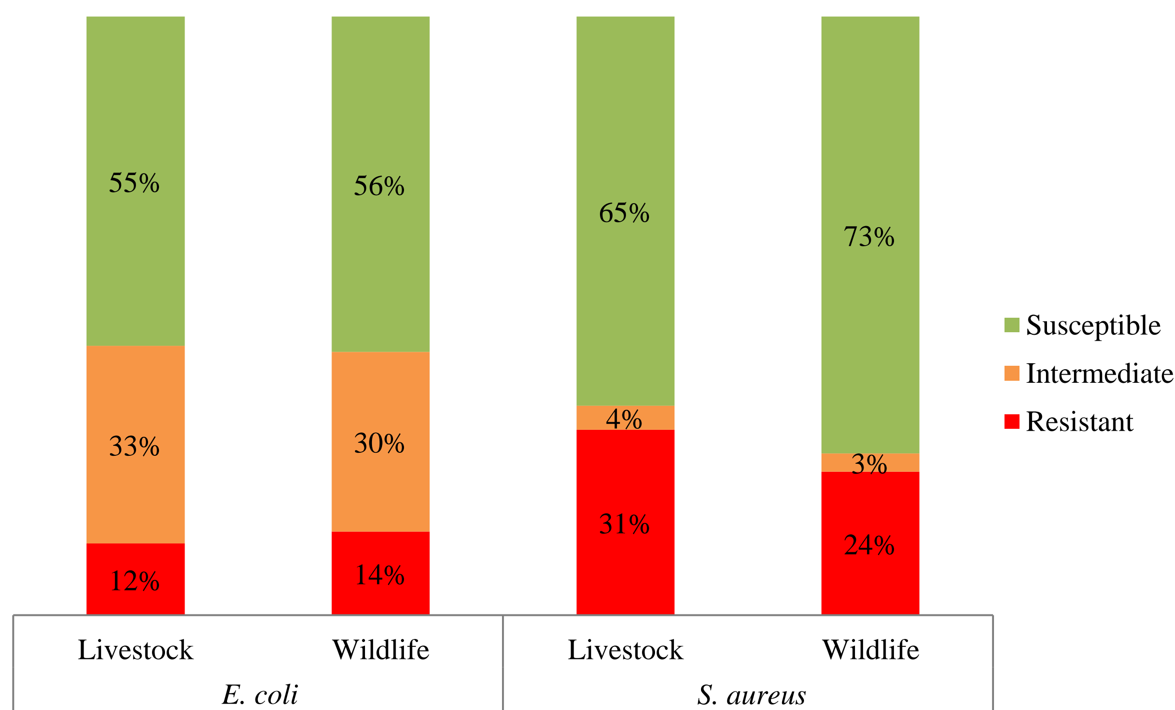

Figure 4.6 The averaged categorical antibiotic resistance levels of the *E. coli* ($p > 0.05$) and *S. aureus* ($p > 0.05$) isolates from the co-grazing livestock (cattle and sheep) and wildlife (springbok and deer) species on the Witsand farm.

Transmission of ABR as well as diseases are bi-directional at the livestock/ wildlife interface (Bengis *et al.,* 2002; Pesapane *et al*., 2013). There is evidence that transmission of microorganisms, whether it be antibiotic resistant or disease-causing microorganisms, does occur between livestock and wildlife, demonstrated by numerous outbreaks of wildlife-associated diseases that have spread to domestic livestock (Bengis *et al.,* 2002; Miller *et al*., 2003). Examples are foot-and-mouth disease and African swine fever. These diseases have been

transferred due to shared resources and land, or via indirect contact through mobile vectors such as ticks or biting flies (Bengis *et al.,* 2002; Miller *et al*., 2003).

Navarro-Gonzalez *et al.* (2013) and Kataweba *et al.* (2015) did not find any significant differences in the ABR of bacteria between co-grazing wildlife and wildlife species that were isolated (non-co-grazing). Whereas Mercat *et al.* (2016) did find that wildlife at the interface of livestock had higher resistance levels than those with no contact with livestock, suggesting a transfer of antibiotic resistance through co-grazing. These disputed points highlights the complexity of ABR and transfer in nature, a highly complex ecosystem.

4.4.2.3. Wildlife vs. livestock

There were significant differences in ABR between the livestock and wildlife *E. coli* and *S. aureus* isolates towards the individual antibiotics as shown in Figures 4.7 and 4.8. The wildlife and livestock groups originate from the Bredasdorp and Witsand farm.

The *E. coli* isolates from the livestock species were significantly ($p \leq 0.05$) more resistant than the wildlife species against ampicillin (livestock, 6%; wildlife, 0%) and tetracycline (livestock - 1% vs. wildlife - 0%) - both antibiotics commonly used in livestock farming. SANVAD reported that the highest ABR in the farming industry in South Africa is towards tetracycline (Eager *et al.,* 2012). The wildlife were more ($p \leq 0.05$) resistant towards sulfonamide (wildlife - 21%; vs. livestock - 17%) and streptomycin (wildlife - 33% vs. livestock - 14%).

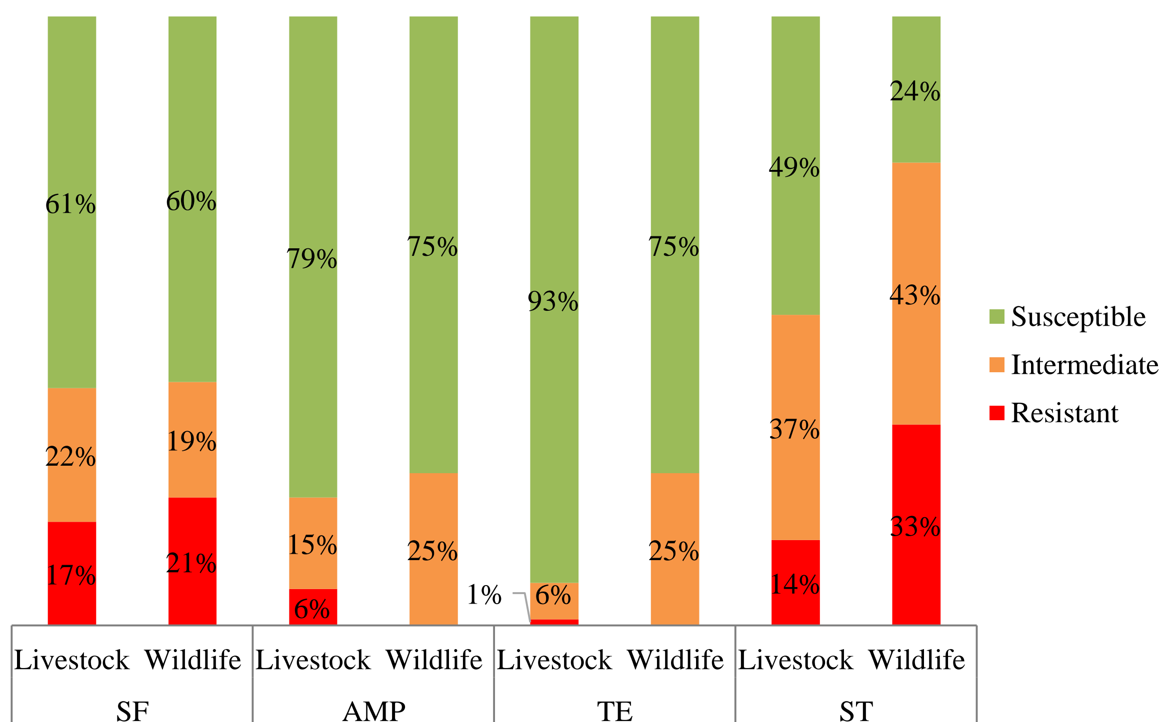

Figure 4.7 The averaged categorical antibiotic resistance levels of *E. coli* between livestock (cattle and sheep) and wildlife (springbok and deer) against SF (sulphonamide; $p \leq 0.05$), AMP (ampicillin; $p \leq 0.05$), TE (tetracycline; $p \leq 0.05$) and ST (streptomycin; $p \leq 0.05$).

Moreover, Figure 4.8 shows that there were no significant differences in resistance towards erythromycin, vancomycin and penicillin. Both groups showed the highest resistance towards penicillin (wildlife - 50% vs. livestock - 43%). This was expected, as resistance to penicillin is now widespread in humans and animals since the 1960s, in both community and hospital staphylococcal isolates (Lowy, 2003; Appelbaum, 2007; Chambers & DeLeo, 2009).

Unexpectedly, the *S. aureus* isolates from the wildlife were significantly more resistant to tetracycline than the livestock isolates. The tetracycline resistance found in the wildlife (26%) is higher than that which was found in wildlife (American Bison) (13.4%) by Anderson *et al.* (2008). The high tetracycline resistance found in the wildlife isolates mainly originates from the Bredasdorp farm and could be as a result of supplementary feeding of the wildlife on this farm, as tetracycline is commonly added to these feeds (see Chapter 5).

The *S. aureus* isolates originating from the livestock group were more resistant ($p \leq 0.05$) to cefoxitin (methicillin) than the *S. aureus* isolates from wildlife group. Methicillin-resistant *S. aureus* (MRSA) was first detected only in hospital settings where it remained confined there until the 1900s. MRSA has now spread to community and animal reservoirs, especially livestock. Livestock-associated MRSA cases have increased over

recent years and has thus been labelled as an important zoonotic pathogen (Aires-de-Sousa, 2016; Sharma *et al*., 2016).

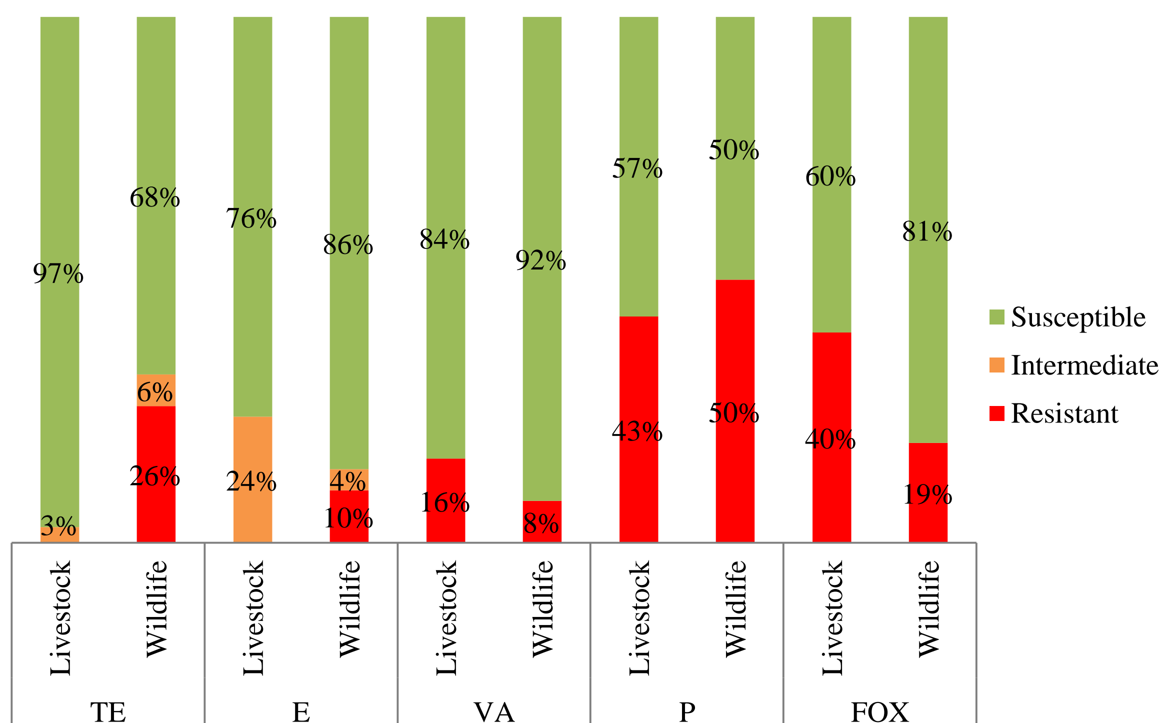

Figure 4.8 The averaged categorical antibiotic resistance levels of *S. aureus* between livestock (cattle and sheep) and wildlife (springbok and deer) against TE (tetracycline; $p \leq 0.05$), E (erythromycin; $p > 0.05$), VA (vancomycin; $p > 0.05$), P (penicillin; $p > 0.05$) and FOX (cefoxitin; $p \leq 0.05$).

Moreover, the observed resistance in the wildlife species indicates that wildlife can be considered a reservoir of antibiotic resistant genes, which is consistent with other studies on wildlife species and other species in remote areas (Silva *et al.,* 2010; Dias *et al.,* 2015). King and Schmidt (2017) concluded that various wildlife species in South Africa can be considered a potential reservoir and vector of antibiotic resistant *E. coli* strains. Studies continue to label wildlife species as a reservoir and vector of antibiotic resistant determinants, just as they have been associated with zoonotic diseases (Vittecoq *et al.,* 2016).

Other studies have also found resistant genes in wildlife species and the natural environment, along with more frequent documentation of zoonotic disease infections, and suggest that they could serve as a reservoir and transfer vector of ABR of environmental origin (Cole *et al.,* 2005; Mariano *et al*., 2009; King & Schmidt, 2017). For example, the zoonotic diseases such as swine flu, human immunodeficiency virus (HIV) and influenza have emerged as human pathogens (Pesapane *et al*., 2013). It was also found that the same CTX-M type ESBL-producing *E. coli* strains were found in blackheaded gulls and in human isolates in Sweden (Wellington *et al.,* 2013).

4.4.2.4. Extensive- and multi- drug resistance

Extensively-drug resistant (XDR) microorganisms are those that are resistant to two different classes of antibiotics. If a microorganism is resistant to three or more different classes of antibiotics then it is classified as multi-drug resistant (MDR) (Magiorakos *et al*., 2011; King & Schmidt, 2017). These MDR microorganisms therefore harbour a diverse array of genetic strategies that allow them to become resistant to a vast number of antimicrobials (Marshall & Levy, 2011). It is therefore reasonable to identify them as "superbugs", as there are few to no antibiotics that are effective against them (Aminov & Mackie, 2007).

Interestingly, the *E. coli* and *S. aureus* isolates from the wildlife were more ($p \leq 0.05$) XDR than the livestock isolates, as shown in Figure 4.9. A similar pattern was also found by King and Schmidt (2017), who concluded that *E. coli* from wildlife (Zebra) were 47% MDR compared to 7% MDR in livestock (farmed pig). As previously mentioned, this suggests that wildlife can be considered a reservoir of antibiotic resistant genes (Silva *et al.,* 2010; Dias *et al.,* 2015).

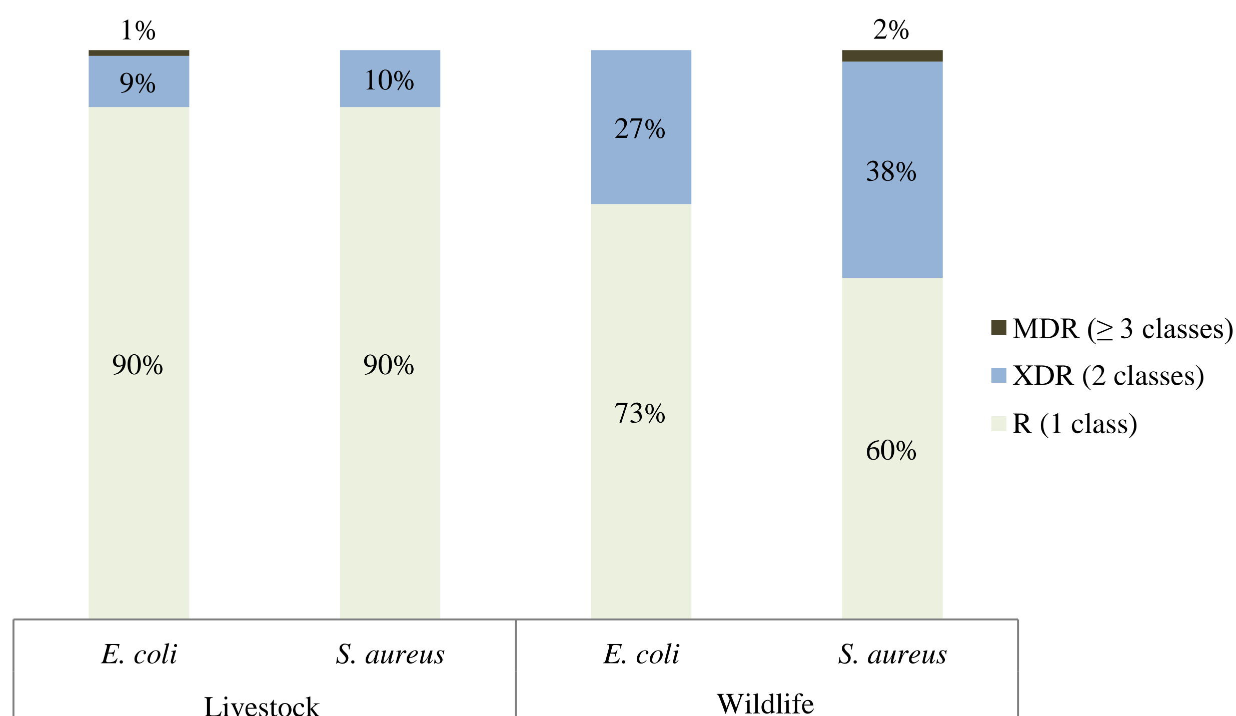

Figure 4.9 Average antibiotic resistance classification of the livestock (cattle and sheep) vs. wildlife (eland, black wildebeest, springbok and deer) species on the Bredasdorp and Witsand farm for *E. coli* (R: $p > 0.05$; XDR: $p \leq 0.05$; MDR: $p > 0.05$) and *S. aureus* (R: $p > 0.05$; XDR: $p \leq 0.05$; MDR: $p > 0.05$). R= resistant; XDR= extensively-drug resistant; MDR= multidrug resistant.

The environmental microbiota possess a diverse array of resistance genes, which has existed for many years before the 'antibiotic era' even began (Aminov, 2009; Allen & Donato, 2010; Berglund, 2015). For example, fungi and bacteria in the soil, such as *Actinobacteria*, naturally produce antibiotic compounds which serve

several purposes in nature other than self-protection from antibiotic action, such as signalling molecules, regulation of metabolic pathways and detoxification (Allen & Donato, 2010; Berglund, 2015). The *Actinobacteria* are abundant in the soil and are responsible for producing many antibiotics of clinical significance, such as tetracycline, erythromycin, streptomycin and vancomycin (Cantas *et al.,* 2013). Additionally, the resistant elements naturally present in the soil are often found to be related to resistance seen in clinical settings (Chait, 2012).

Although wildlife are not exposed to therapeutic antibiotic concentrations, they can be subjected to low concentrations of antibiotics that are secreted into the environment as a result of clinical and agricultural use of antibiotics via many natural vectors (Mariano *et al.,* 2009; Dias *et al.,* 2015).

Moreover, the practice of wildlife supplementary feeding can also expose the wildlife bacteria to sub-inhibitory concentrations of antibiotics, if included in the feed. These feeds are given on a "free-choice" basis which leads to incorrect dosing levels of the medicated feeds, promoting the development of drug resistance (Love *et al*., 2011). Additionally, the wildlife could be exposed to an ABR selective pressure from eating the livestock feed, due to co-grazing on the farm and the ability of the wildlife to jump fences. These feeds can be another potential source of antibiotics, due to the widespread use of antibiotics in livestock production where antibiotics are added to the feed as growth promoting and disease prevention agents (Landers *et al.,* 2012).

Sub-inhibitory concentrations can also naturally exist in the environment due to the production of antibiotic compounds by soil bacteria (D' Costa *et al*., 2006; Martinez, 2012). The environmental microbiota possess a diverse and large array of resistance genes. These resistance genes are thought to persist in nature due to their functions other than ABR, such as intercellular signalling, detoxification and virulence (Aminov, 2009). It has been found that antibiotic resistant genes in the soil are similar to those found in clinical settings in human pathogens, suggesting that one of the main originators of ABR is, in fact, the environmental microbiota (Martinez, 2012; Bemier & Surette, 2013). Antibiotic resistant genes have been found in wild animals and isolated environments that have never had contact with antibiotics, suggesting that antibiotic resistant genes have existed before the use of antibiotics and are widespread and continue to evolve in nature (Baltz, 2008; Mariano *et al*., 2009; Martinez, 2012).

Another reasoning for the higher XDR bacteria found in the wildlife could be due to the fact that livestock typically have a shorter generation interval than wildlife, who are generally left to die from old age as opposed to livestock which are slaughtered at weaning or bred till the age of, at most, six years old for sheep and nine years old for cattle. The longer lifespan of wildlife could allow more time for antibiotic resistant bacteria to evolve in the intestines and thus develop into XDR bacteria.

In this study, the *S. aureus* isolates showed a higher overall XDR than the *E. coli* isolates. This could be attributed to the presence of MRSA, which were documented at a notable level in this study (average 25%). MRSA are known to be multidrug resistant and would most likely show resistance to other antibiotic classes (Sandora & Goldmann, 2012). Of the MRSA isolates, 88% were resistant to atleast one other different antibiotic class.

4.4.2.5. Free-range vs. organic wildlife and livestock

The free-range sheep and springbok used in this study are those from the Witsand farm (co-grazing farm). The sheep and springbok samples taken from the organic farm are located on a farm in Sutherland that has been classified as organic for 30 years and is isolated from regular human activities. Only *E. coli* was isolated from the organic sheep and springbok and thus a comparison for *S. aureus* was not attainable.

The free-range sheep *E. coli* isolates were more resistant ($p \leq 0.05$) to ampicillin and tetracycline-antibiotics commonly used in livestock farming, compared to the sheep from the organic farm (Figure 4.10).

On the other hand, the *E. coli* from the organic sheep were more resistant ($p \leq 0.05$) to streptomycin then the springbok isolates from the free-range farm. Additionally, the *E. coli* from the springbok from the organic farm were more resistant ($p \leq 0.05$) to sulphafurazole than the springbok isolates from the free-range farm (Figure 4.11). This indicates possible intrinsic resistance to these two antibiotics in nature.

Although significant differences were observed between the free-range and organic animals, the average level of resistance was below the intermediate level for both groups, except for streptomycin and sulphafurazole resistance, where the average resistance level was above the intermediate level.

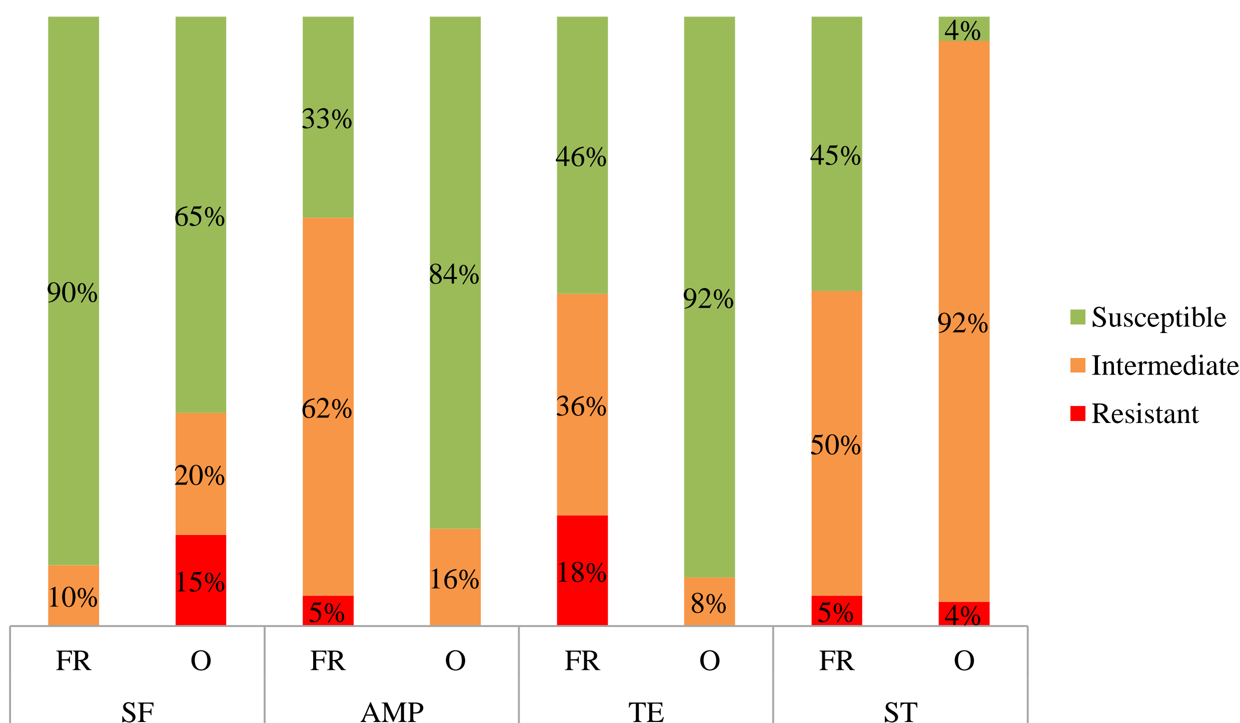

Figure 4.10 *E. coli* antibiotic resistance differences of sheep from a free-range vs. organic farm towards AMP SF (sulphonamide; $p > 0.05$), (ampicillin; $p \leq 0.05$), TE (tetracycline; $p \leq 0.05$), ST (streptomycin; $p \leq 0.05$). O= organic, FR= free-range.

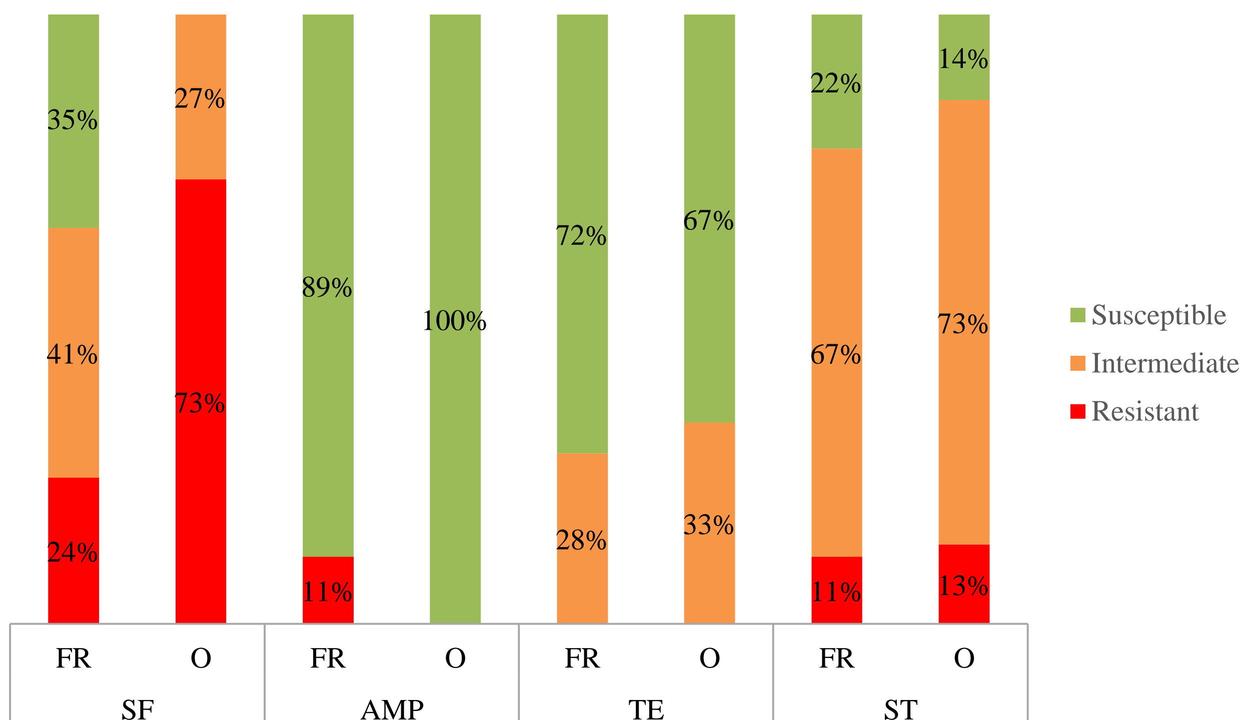

Figure 4.11 *E. coli* antibiotic resistance differences of springbok from a free-range vs. organic farm towards SF (sulphonamide; $p \leq 0.05$), AMP (ampicillin; $p > 0.05$), TE (tetracycline; $p > 0.05$), ST (streptomycin; $p > 0.05$). O= organic, FR= free-range.

A greater difference in antibiotic resistance might have been observed if the organic animals were compared to intensively produced sheep where the use of antibiotics is more common (Founou *et al*., 2016). Intensively reared food animals have more frequent contact with human activities, as well as greater herd/ flock densities; two causal factors which increase the possibility of transfer of antibiotic resistant bacteria (Anon., 2006). Additionally, intensively reared food animals are often given antibiotics for growth promotion or for metaphylactic purposes, which, in turn, increases the selection of antibiotic resistant bacteria (Founou *et al*., 2016). Conventional farming thus results in a higher antibiotic resistant and MDR profile amongst the animals than organic farming (Silbergeld *et al*., 2008). Numerous studies have reported that there are lower antibiotic resistance levels in organic farms than conventional farms, which can mainly be attributed to the absence of antibiotic use in organic practice (Alali *et al*., 2010; Li *et al.,* 2014; Österberg *et al*., 2016). Organic farming has therefore been suggested as one of the methods that can be used to reduce the burden of antibiotic resistance development and antibiotic use, although the effects of abolishing antibiotic usage on the production performance of the intensively reared livestock is still being debated.

4.5. Conclusion

Overall, fairly low ABR levels were recorded on all farms from which samples were taken and analysed. Moreover, the wildlife bacterial isolates were shown to be more resistant than the wildlife isolates towards

sulphonamide, streptomycin and methicillin (cefoxitin). On the other hand, the livestock isolates were significantly more resistant to ampicillin, tetracycline and vancomycin.

Significant differences in ABR were observed between the wildlife and livestock who do not co-graze together. But no significant differences were observed between the wildlife and livestock who do co-graze together. Moreover, the isolates from an organic and isolated farm were significantly less resistant than isolates from free-ranging farms where livestock and humans are in close proximity.

To prevent the transfer of antibiotic resistant and disease-causing bacteria between the livestock and wildlife interface, some control measures can be put in place on farms which practice co-grazing. This can include: separation using fences and proper disease control of domestic animals. Both steps are likely to reduce the risk of transfer occurring.

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CHAPTER 5

Escherichia coli and *Enterococcus faecalis* antibiotic resistance patterns of wildlife species subjected to supplementary feeding on various South African Farms

5.1. Summary

The practice of supplementary wildlife feeding is hypothesised to result in increased antibiotic resistance of bacteria harboured by the wildlife. This theory was tested using the Kirby-Bauer disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) 2018 guidelines. *Escherichia coli* (N= 135) and *Enterococcus faecalis* (N= 135) were isolated from faecal samples originating from impala, wildebeest and buffalo from various farms which do, or do not, practice wildlife supplementary feeding. The *E. coli* and *E. faecalis* isolates from the supplementary fed wildlife were overall significantly more antibiotic resistant than those which were not supplementary fed. More specifically, the *E. coli* isolates from the supplementary fed wildlife were significantly more resistant towards sulphafurazole and tetracycline and the *E. faecalis* isolates towards tetracycline. The average level of antibiotic resistance was below the intermediate level for all the wildlife species in this study. Overall, the *E. coli* isolates from the wildlife which were supplementary fed were on average 12% resistant to the range of antibiotics (tetracycline, ampicillin, sulphafurazole and streptomycin) whilst those which were not fed were 3% resistant. No *E. coli* isolates were resistant to ceftazadime, chloramphenicol and nalidixic acid. The *E. faecalis* isolates from the wildlife which were supplementary fed were on average 12% resistant to the range of antibiotics (tetracycline, erythromycin, vancomycin and penicillin) whilst those which were not fed were 2% resistant. The wildlife which were not fed only showed noteworthy resistance towards streptomycin and erythromycin and negligible resistance to all the other antibiotics. The antibiotic resistance patterns observed in the non- fed wildlife species could serve as a baseline for monitoring the influence of human activities on the development of antibiotic resistance in wildlife species originating from various environments.

5.2. Introduction

Supplementary feeding of wildlife is a common practice in Europe to alleviate winter mortalities to increase reproductivity and growth and to control the conservation of crops (Sorensen *et al.,* 2013; Selva *et al.,* 2014). Wildlife supplementary feeding is also practiced in some South African farms, predominantly by specialist game ranchers, especially in periods of severe drought (Bekker, 2011; Stoddard, 2011). Bekker (2011) found that 71% of game farms in South Africa practice supplementary feeding. Wildlife are supplemented with feed most commonly during the winter months, followed by 29% of farms in drought periods, 12.3% all year and 0.7% in the summer season (Bekker, 2011).

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Food and water sources could be a potential source of antibiotic resistant bacteria as well as act as a selection pressure for the development and spread of antibiotic resistance (ABR). The anthropogenic use of antibiotics can also create a selection pressure, such as in clinical use, chemical pollution and intensive farming (Silbergeld *et al*., 2008; Wellington *et al*., 2013). The waste of these practices is released into the natural environment at sub-inhibitory levels through agricultural and aquaculture run-offs, sewage effluents, hospital effluents, landfills and via physical vectors such as birds, to name but a few. Anthropogenic use of antibiotics has resulted in changes to the natural microbial ecosystem, due to adaptations made by the microbiota in response to a changing environment. This has resulted in selection of antibiotic resistant determinants to confer a selective advantage in order to adapt to the changing environment (Chadha, 2012).

The majority of emerging infectious diseases in humans originate from wildlife reservoirs. This suggests that certain factors, mainly anthropogenic, are to blame (Rhyan & Spraker, 2010; van Doorn, 2014; Cunningham *et al*., 2017). Anthropogenic factors include, human encroachment into wildlife habitats, increased transport of wildlife, development of wildlife captive industries and more intensive management of selected wildlife species (Rhyan & Spraker, 2010; van Doorn, 2014; Cunningham *et al*., 2017).

South African game farmers most commonly use lucerne/grass as a natural supplement feed for their wildlife. Mineral blocks, commercial feed and self-mixed feeds are also used. An example of the composition of a self-mixed feed, obtained from a local farmer, is listed in Table 5.1 (A. van Heerden, 2016, Student, Stellenbosch University, South Africa, personal communication, 5 April). It should be noted that it was found by Bekker (2011) that 12.3% of South African game farmers use feed that is not registered by the South African Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies Act 36 of 1947 and 4.5% do not know if the feed is registered or not.

Table 5.2 shows how frequently certain feed ingredients are used by South African game farmers in wildlife supplementary feeds, according to a survey completed by game farmers in South Africa (Bekker, 2011).

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Table 5.1 Typical composition for a self-mixed feed for wildlife supplementary feeding on South African farms

Ingredient	Percentage (%)
Brewer's grain	12.55
Wheat	6.27
Soya cake	7.32
Cotton seed cake	5.23
Maize	15.39
Lime	0.94
Kimtraphos	0.94
Salt	0.26
Micronutrient pack	0.34
Molasses	12.55
Lucerne	3.92
Grass	33.99

Table 5.2 The feed components most commonly used in supplementary feed by game farmers in South Africa

Component	Used by farmers
Internal parasite control agents	37%
Bone meal	19.6%
Antimicrobial growth promoters	13.3%
Carcass meal	13%
Poultry manure	8.7%
Ionophores	6.5%
Hormones	4.3%
Blood meal	2.2%
Fly control agents	2.2%

The most commonly used antimicrobials are sulphonamides and tetracyclines for growth promotion. Ionophores, corticosteroids and hormones are also used a growth promoting substances (Bekker, 2011). Indirect sources of antimicrobial compounds include bone meal, carcass meal and poultry manure. These feeds are given on a "free-choice" basis which leads to incorrect dosing levels of the medicated feeds, promoting the development of drug resistance (Love *et al*., 2011).

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With regards to water supplies to farmed game, Bekker (2011) found that water available to the wildlife are mainly sourced from boreholes (75%), dams (68%), rivers (38%) and water troughs (43%).

It is hypothesised that the bacteria from the wildlife which are supplementary fed on a regular basis will have a higher ABR profile than those who are more isolated from human activities and only feed on the land's natural resources.

5.3. Materials & Methods

5.3.1. Study Area

Faecal samples from wildlife species were collected from different farms in South Africa between 2016 and 2018. A summary of the sample species, farm location and type of wildlife feeding is shown in Table 5.3 whilst Figure 5.1 displays the location of the farm across South Africa where sample collections took place.

Table 5.3 Details of the wildlife samples used in this study

Wildlife species	Farm location	Feeding type	Number of samples
Buffalo	Ekuseni	Not fed	5
Buffalo	Wellington (farm 1)	Fed	5
Buffalo	Wellington (farm 2)	Fed	5
Wildebeest	Ekuseni	Not fed	5
Wildebeest	Modimolle (farm 1)	Fed	5
Wildebeest	Wellington (farm 2)	Fed	5
Impala	Ekuseni	Not fed	5
Impala	Modimolle (farm 2)	Fed	5
Impala	Modimolle (farm 3)	Fed	5

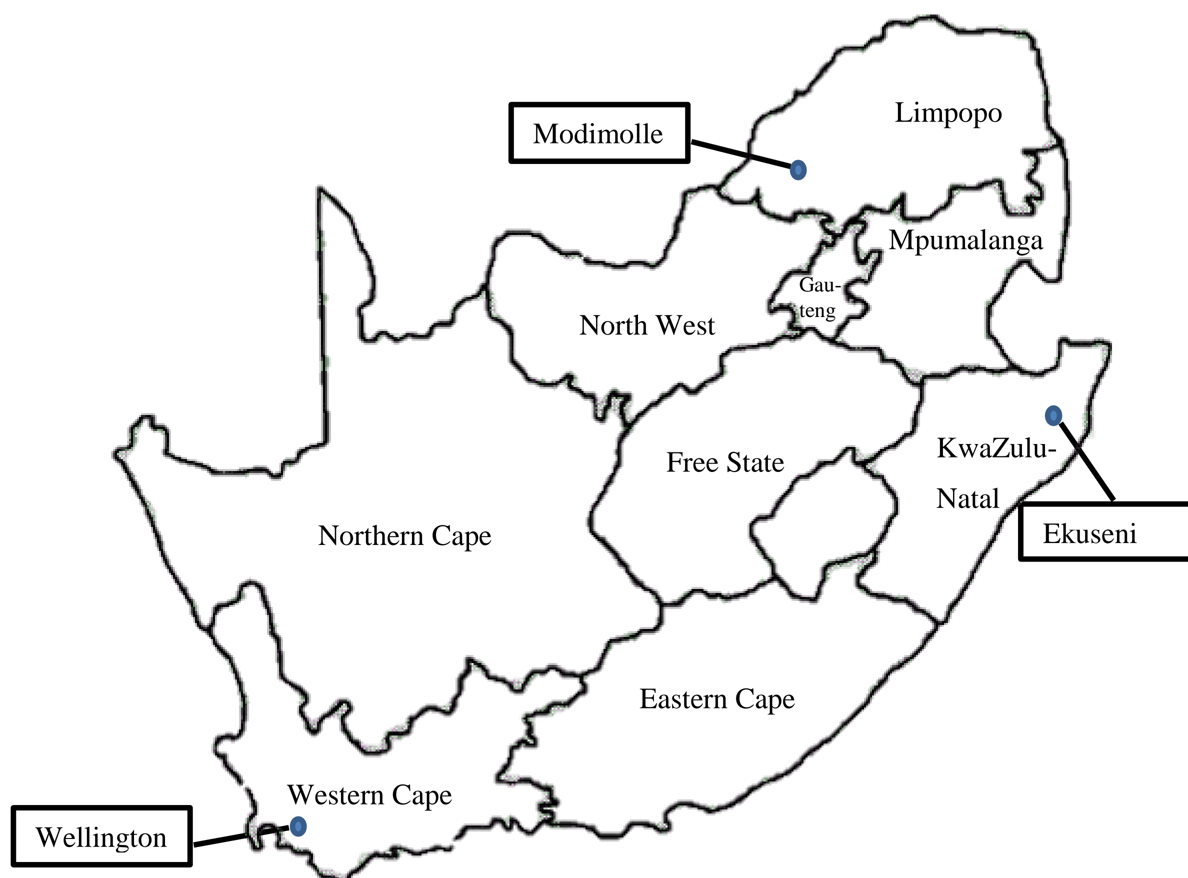

Figure 5.1 A map of South Africa which shows where sample collection took place.

Supplementary fed African Buffalo (*Syncerus caffer*) samples were collected in January 2018 from Wellington farm 1 and from another farm in close proximity, Wellington farm 2; both farms being in the Wellington district. On Wellington farm 1, the buffalo graze on grass and are occasionally fed hay in autumn months if the grass has become depleted. This farm was previously a sheep farm twenty years ago. The frequent application of antibiotics during the livestock farming period would have altered the soil microbial population and dynamics (Wegst-Uhrich *et al*., 2014). The farm which the buffalo roam on is fenced off from all other wildlife and livestock species on the farm, likewise with the buffalo on Wellington farm 2. African Buffalo (*Syncerus caffer*) samples which are not fed supplementary feed were collected from a game reserve in Ekuseni, Kwa-Zulu Natal. These buffalo are very wild and have never been supplementary fed or been in contact with others that have been fed and also do not receive any medical treatment. They are free to roam and graze on the open pastures of the reserve, along with the other wildlife species including predators.

Blue wildebeest (*Connochaetes taurinus*) samples were collected from farm 1 in Modimolle, Limpopo in April 2016. This farm hosts only blue wildebeest which are fed once a day at a single feeding point with multiple troughs containing a nutrient feed mix. The feeding point is rotated around the farm to avoid trampling of the vegetation. The wildebeest are also free to graze on the natural vegetation. All the wildebeest share the

same water points which are refilled when necessary. Blue wildebeest samples were also collected from farm 2 in Wellington. These wildebeest are also supplementary fed and are fenced off from all other wildlife species on the farm. The wildebeest samples that were classified as 'non-fed' were collected from the same game reserve in Ekuseni, Kwa-Zulu Natal as the buffalo samples.

Impala (*Aepyceros melampus*) samples which were supplementary fed were collected from farm 2 and farm 3 in Modimolle. The impala co-graze with other game species on the farm. The non-fed impala samples came from the same game reserve in Ekuseni, Kwa-Zulu Natal as the buffalo and wildebeest samples.

5.3.2 Sample collection

Approximately 20 g of fresh faecal matter was located in the small intestines after evisceration and collected in a sterile sample container using a clean, disinfected knife and a new set of gloves for each animal.

After collection, all samples were stored in a cooler box with ice at ~4°C and transported to the university's laboratory freezer and stored there at -20°C.

5.3.3. Enumeration of bacteria

Faecal samples were defrosted at 20°C for 2h before analysis commenced. A 10^{-1} dilution of the faecal samples were made by adding 10 g faecal matter to 90 mL Buffered Peptone Water (Biolab, South Africa). The 10^{-1} faecal dilutions were homogenised using a Stomacher (Interscience) for 2 min and incubated at 35°C for 12-14 h. This overnight incubation resuscitation step assists in recovery of the bacterial cells to allow for easier enumeration using selective agar media.

After incubation, 10^{-4} and 10^{-5} serial dilutions were prepared using Physiological Saline Solution (PSS) according to the South African National Standards method 6887-1 (SANS, 1999) in 9 mL units. The pour plate technique was used by pipetting 1 mL from the dilutions onto petri dishes (Willey *et al*., 2011a). After this step, selective agar was poured over and swirled in a "figure of 8" motion. Baird-Parker Agar (BPA) (Oxoid, South Africa) supplemented with Egg Yolk Tellurite Emulsion (Oxoid, South Africa) was used for enumeration of *E. faecalis*. Violet Red Bile Dextrose Agar (VRBDA) (Bioloab, South Africa) was used to select for *E. coli*. Once the petri dishes were set, they were inverted and incubated overnight at 35 °C.

Following incubation, the streak plate technique was used to streak three random colonies per animal onto three selective agar petri dishes. BPA was used for *E. faecalis* and Eosin Methylene Blue Agar (EMBA) (Oxoid, South Africa) was used for *E. coli.* Therefore, the antibiotic susceptibility test (AST) was performed in triplicate per animal sample. This step isolates the specific bacteria so that individual colonies can be selected. At the same time, one can presumptively identify the bacteria by the appearance of the colonies on the highly selective agar. The petri dishes were inverted and incubated overnight at 35°C.

One colony per plate was then streaked onto Nutrient Agar (NA) (Biolab, South Africa) plates to yield three pure cultures per animal. The plates were then inverted and incubated overnight at 35°C. These plates were stored at 4°C for further use for up to five days.

5.3.4. Bacterial species confirmation

Gram's stain (Lasec, South Africa) was performed on all colony isolates which showed characteristic growth, using the method described in the manufacturer's instruction manual. A drop of distilled water was placed on a microscope slide using a cooled sterile loop. The inoculation loop was sterilised through a flame and cooled and a small amount of bacterial colony from the NA plates was picked up. The bacteria were stirred into the water droplet to create a bacterial emulsion. The smear was left to air dry and then heat-fixed two to three times through a flame with the smear side facing up. The smear was flooded with crystal violet for 1 min and then rinsed with distilled water. The smear was then flooded with iodine for 1 min and then rinsed with distilled water. The smear was decolourised with alcohol until it ran clear and then was rinsed with distilled water. Lastly, the smear was flooded with safranin for 45 s and rinsed with distilled water. The smear was then gently blotted dry and observed under a microscope (Nikon YS100) on x1000 with immersion oil (Willey *et al.,* 2011b).

Eosin Methylene Blue Agar (Oxoid, South Africa) is a highly selective medium that produces characteristic colony growth specific to *E. coli*. Other lactose-fermenting gram negative rods that can also show the characteristic green metallic sheen are some species of *Citrobacter* and *Enterobacter*. Therefore, the citrate utilisation test was performed on presumptive *E. coli* isolates using Simmons Citrate agar (Oxoid). *E. coli* is citrate-negative, whereas *Citrobacter* and *Enterobacter* are both citrate-positive (Oxoid).

E. faecalis showed luxurious growth on Baird Parker Agar (BPA) but unlike *S. aureus*, there was no clear halo around the black, shiny colony. Colony identity was confirmed using Gram's stain, Staphylase test (Oxoid) (negative), catalase test (negative) as well as Matrix Assisted Laser Desorption/Ionization- Time of Flight Mass Spectrometry (MALDI-ToF MS).

After colony identification was confirmed, stock cultures were made and stored in the freezer at -20°C until further use. A loop full of bacterial colony was picked with a sterile loop from the NA plates and transferred into a sterile test tube containing 3 mL TSB. The bacterial suspension was vortexed and incubated overnight at 35°C. After incubation, each test tube was vortexed and 0.75 mL bacterial suspension was pipetted into a 2 mL microtube containing 0.75 mL sterile 50% glycerol (Fluka Analytical, Germany) (Gorman & Adley, 2004). This long-term preservation technique has been commonly used for the successful preservation of numerous bacterial species (Gorman & Adley, 2004).

5.3.5. Antibiotic susceptibility testing

The Kirby-Bauer disk diffusion method was used according to the Clinical and Laboratory Standards Institute (CLSI) 2018 guidelines using Mueller-Hinton agar (MHA) (Biolab, South Africa). The direct colony suspension method was used to prepare the inoculum suspension, using colonies from fresh NA plates. Three colonies per animal for each organism were selected randomly using an inoculating loop and suspended in 0.75 mL PSS in 2 mL Eppendorf tubes.

The antibiotic disks were applied within 15 min of inoculating the MHA plate. A 6-disk dispenser (Oxoid, South Africa) was used to place the disks onto the surface of the MHA plates. The disks were checked that they were firmly placed on the agar surface. Tables 5.4 and 5.5 shows the antibiotic disks applied for *E. coli* and *E. faecalis,* respectively. The plates were inverted and incubated at 35°C for 16-18 h and 24 h for vancomycin discs (CLSI, 2018).

Table 5.4 Selected antibiotic disks for *Escherichia coli*

Antimicrobial agent	**Disk content**	**Antibiotic class**
Ampicillin (AMP)	10 µg	Penicillin
Chloramphenicol (C)	30 µg	Macrolide
Streptomycin (ST)	10 µg	Aminoglycoside
Sulphafurazole (SF)	300 µg	Sulfonamide
Tetracycline (TE)	30 µg	Tetracycline
Ceftazadime (CAZ)	30 µg	β- Lactam

Table 5.5 Selection of antibiotic disks applied to *Enterococcus faecalis*

Antimicrobial agent	**Disk content**	**Antibiotic class**
Erythromycin (E)	15 µg	Macrolide
Penicillin (P)	10 U	Penicillin
Tetracycline (TE)	30 µg	Tetracycline
Vancomycin (VA)	30 µg	Glycolipid

Zone diameters were classified as either resistant (R), intermediate (I) or susceptible (S), according to the CLSI zone diameter interpretive standards for each microorganism (Tables 5.6 and 5.7) (CLSI, 2018). If a bacteria is classified as resistant, then it is able to grow in the presence of the antibiotic, rendering it ineffective. It is likely that these bacteria have developed specific resistance mechanisms. If a bacteria is classified as intermediate it means that a higher concentration is needed to inhibit growth and thus the response rate is lower than for susceptible isolates. Lastly, if a bacteria is classified as susceptible it means that it can't grow in the presence of the antibiotic, and thus the antibiotic is still effective (CLSI, 2018).

Table 5.6 Zone diameter interpretive standards for Enterobacteriaceae (CLSI, 2018)

Antimicrobial agent	Disk content	Zone diameter, nearest whole mm			Comments
		R	I	S	
Ampicillin (AMP)	10 µg	≤ 13	14- 16	≥ 17	Class representative for ampicillin and amoxicillin.
Chloramphenicol (C)	30 µg	≤ 12	13- 17	≥ 18	-
Streptomycin (ST)	10 µg	≤ 11	12- 14	≥ 15	-
Sulphafurazole (SF)	300 µg	≤ 12	13- 16	≥ 17	Represents any of the currently available sulfonamides.
Tetracycline (TE)	30 µg	≤ 11	12- 14	≥ 15	Organisms that are susceptible to tetracycline are also considered susceptible to doxycycline and minocycline.
Ceftazadime (CAZ)	30 µg	≤ 17	18-20	≥ 21	-

Table 5.7 Zone diameter interpretive standards for *Enterococcus* species (CLSI, 2018)

Antimicrobial agent	Disk content	Zone diameter, nearest whole mm			Comments
		R	I	S	
Erythromycin (E)	15 µg	≤ 13	14- 22	≥ 23	-
Penicillin (P)	10 U	≤ 14	-	≥ 15	Enterococci susceptible to penicillin are predictably susceptible to ampicillin, amoxicillin, ampicillin-sulbactam, amoxicillin-clavulanate, piperacillin and piperacillin-tazobactam.
Tetracycline (TE)	30 µg	≤ 14	15- 18	≥ 19	-
Vancomycin (VA)	30 µg	≤ 14	15-16	≥ 17	-

5.3.6. Statistical analysis

Statistical analysis was performed using Statistica 13.2 software (StatSoft, USA). The data was analysed using one-way analysis of variance (ANOVA). The main effect was the wildlife group (fed or non-fed) which was compared between each group of wildlife species separately (buffalo, impala and wildebeest). If the group

means were significantly different within each wildlife species group, post hoc tests were performed to determine where the differences occurred within each wildlife group. Levene's test was applied to determine homogeneity of variance. Significant results were identified by least significant means (LSM) by using a 95% confidence interval i.e. a 5% significance level ($p \leq 0.05$) as a guideline.

5.4. Results & Discussion

Table 5.8 gives a summary of the antibiotic susceptibility profiles of the *E. coli* isolates from the supplementary fed wildlife versus non-supplementary fed wildlife. The *E. coli* isolates from the supplementary fed wildlife were more resistant to ampicillin, streptomycin, sulphafurazole and tetracycline than the wildlife which were not fed.

Table 5.8 A summary of the disc diffusion antibiotic susceptibility test results for *E. coli* isolates

			Ampicillin	Ceftaza-dime	Chloram-phenicol	Strepto-mycin	Sulpha-furazole	Tetra-cycline
Fed wildlife	**(n=90)**	**Resistant**	7%	0%	0%	42%	26%	9%
		Intermediate	11%	0%	1%	54%	12%	24%
		Susceptible	82%	100%	99%	2%	62%	67%
Non-fed wildlife	**(n=45)**	**Resistant**	0%	0%	0%	12%	0%	0%
		Intermediate	5%	0%	0%	73%	2%	0%
		Susceptible	95%	100%	100%	15%	98%	100%

It should be noted, as also seen in Chapter 4, that the ABR profiles of the *E. coli* isolates towards chloramphenicol were not included in the analysis due to negligible resistant levels, leading to no variance in the data. Thus it can be hypothesised that there is no significant quinolone or chloramphenicol selective pressure present in the wildlife farming environments used in this study. This is consistent with other studies who have also found very low resistance or negligible levels towards chloramphenicol in wild animals (Rolland *et al.*, 1985; Lillehaug *et al.*, 2005; Costa *et al.*, 2008; Silva *et al.*, 2010).

Additionally, no *E. coli* isolates were resistant to ceftazadime and therefore these results were also excluded from the analysis due to no variance in the data between the two groups. Ceftazadime is a clinically-used third generation cephalosporin antibiotic and is used to screen for extended-spectrum β-lactamase (ESBL)-producing bacteria (Rawat & Nair, 2010). These bacteria have β-lactamase enzymes, encoded on plasmids, which are able to inactivate a wide spectrum of β-lactam antibiotics, thus displaying drug resistance to third generation antibiotics (Rawat & Nair, 2010). ESBL-producing bacteria are a great concern to human health as therapeutic treatment is largely compromised (Paterson & Bonomo, 2005). Thus it is suggested that

the *E. coli* isolates from the wildlife in this study are not exposed to nearby sources of ESBL-producing bacteria or third generation cephalosporins.

Table 5.9 gives a summary of the antibiotic susceptibility profiles of the *E. faecalis* isolates from the supplementary fed wildlife versus non-supplementary fed wildlife. The *E. faecalis* isolates from the supplementary fed wildlife were more resistant to tetracycline and vancomycin than the wildlife which were not fed.

Table 5.9 A summary of the disc diffusion antibiotic susceptibility test results for *E. faecalis* isolates

		Tetracycline	Erythromycin	Vancomycin	Penicillin
Fed wildlife (n=90)	**Resistant (R)**	18%	1%	14%	2%
	Intermediate (I)	30%	63%	-*	-*
	Susceptible (S)	52%	36%	86%	98%
Non-fed wildlife (n=45)	**Resistant (R)**	0%	0%	5%	3%
	Intermediate (I)	8%	70%	-*	-*
	Susceptible (S)	92%	30%	95%	97%

*No intermediate classification zone diameter guidelines available

It can be seen from Figures 5.2 and 5.3 that overall, both the *E. coli* and *E. faecalis* isolates from the fed wildlife were more ($p \leq 0.05$) resistant to the selection of antibiotics compared to the wildlife that were not fed; except for the *E. coli* from the impala which was the only group that did not fit into this trend. This confirms the theory hypothesised, that the practice of supplementary wildlife feeding does lead to increased ABR.

The insignificant differences in resistance between the different impala samples suggests that the farming activities and environment on the two farms in Modimolle where the fed impala samples came from, do not significantly encourage the development of ABR. Alternatively, impala are known to be a species that does not readily take to artificial/ supplementary feed (L.C. Hoffman, 2018, Distinguished Professor, Stellenbosch University, South Africa, personal communication, 18 August).

This implies that either the feed is a source of antibiotic resistant bacteria or determinants and/ or the practice of supplementary feed encourages ABR development and transfer. Rendered animal products, such as bone meal and fish meal have been shown to be sources of antibiotic resistant bacteria (Hofacre *et al*., 2001). These products are commonly added to animal feeds as a source of protein, as shown previously in Table 5.2. The inclusion of poultry manure, an inexpensive source of added nutrition, can also be a source of antibiotic resistant bacteria and/ or antimicrobial compounds due to the fact that many antibiotics pass directly through the animal into manure (Haapauro *et al*., 1997; Walker *et al*., 2005).

Alternatively, the feed given to the wildlife could contain antibiotic-based growth promoting agents, such as sulphonamides and tetracyclines, which would directly result in the development of ABR, exemplified by the act of 'free-choice' grazing, leading to exposure to sub-inhibitory antibiotic concentrations over

prolonged periods. Furthermore, the practice of supplementary feeding leads to crowding of animals at feeding and water sites, promoting the transfer of antibiotic resistant traits from one animal to another. The practice of supplementary feeding is also commonly associated with increased human contact, which could also further facilitate the transfer of ABR to and from the wildlife.

The differences in ABR observed between the two groups can suggest that increased human contact may also influence the ABR in bacterial communities, as humans could be regarded as a transfer vector of resistant elements (Vittecoq *et al*., 2016). The wildlife which were fed would be exposed to increased human contact on a daily basis when the feed is deposited into the feeding troughs and during treatment of any medical conditions. Other studies that investigated the differences between the ABR profiles of wild animals living in close proximity to human activity and those living in more remote areas found that increased human contact caused increased ABR (Allen & Donato, 2010; Guenthler *et al*., 2010; Dias *et al*., 2015). Skurnik *et al.* (2006) correlated human-wildlife contact intensity to ABR levels and found that wildlife with more contact with human activities showed a higher resistance profile.

When looking in more detail, Figure 5.4 shows the ABR profiles of *E. coli* toward each antibiotic, comparing the supplementary fed and non- supplementary fed wildlife species.

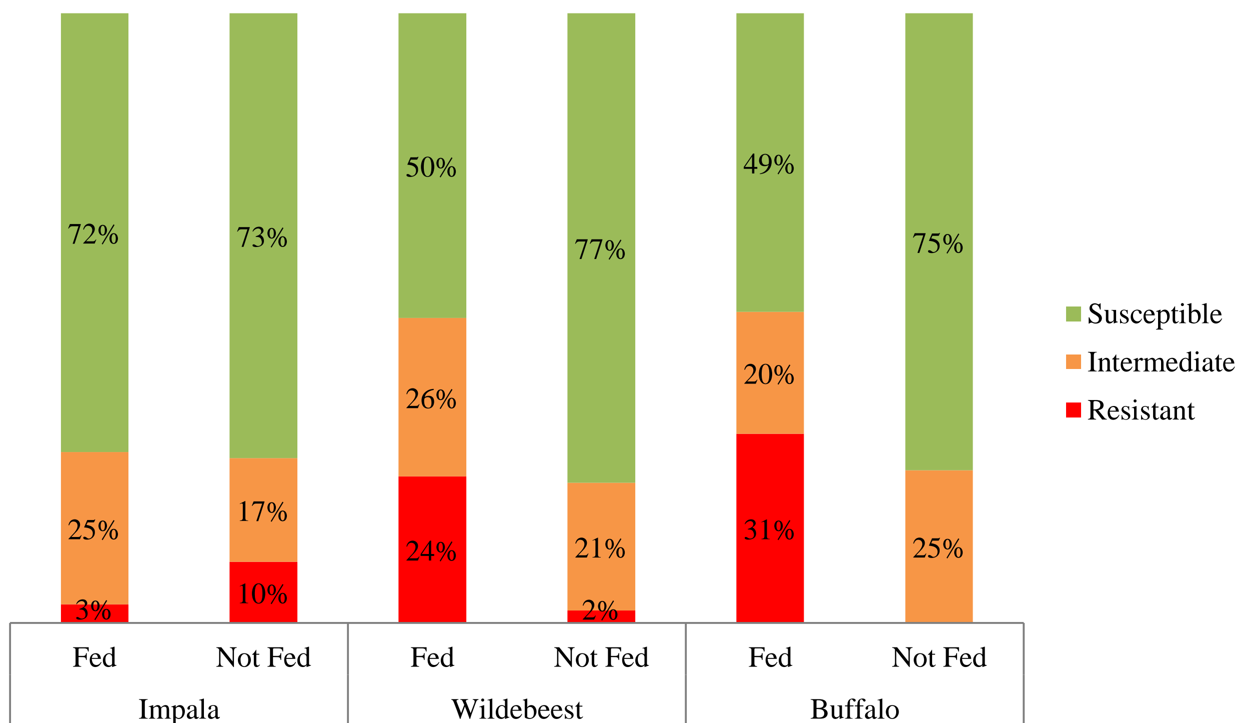

Figure 5.2 The averaged categorical antibiotic resistance levels of the *E. coli* isolates from the supplementary fed wildlife group vs. the non- fed wildlife group including impala ($p > 0.05$), wildebeest ($p \leq 0.05$) and buffalo ($p \leq 0.05$).

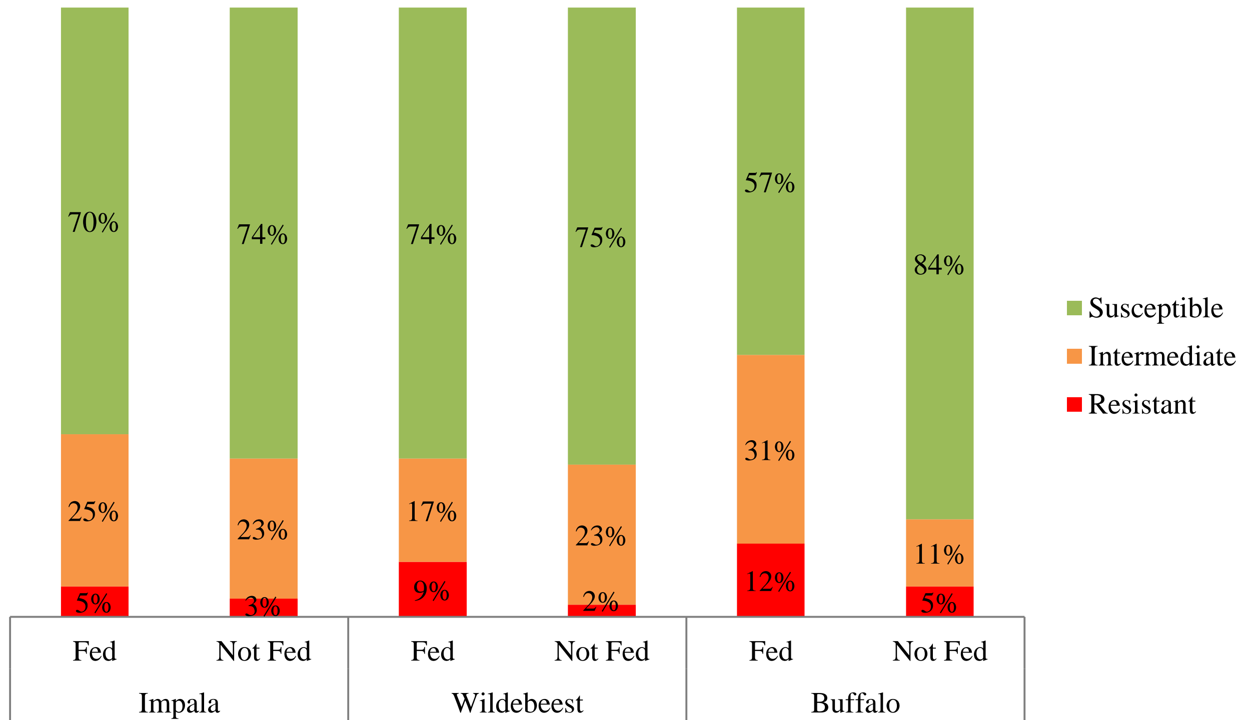

Figure 5.3 The averaged categorical antibiotic resistance levels of the *E. faecalis* isolates from supplementary fed wildlife vs. non- fed wildlife including impala ($p > 0.05$), wildebeest ($p \leq 0.05$) and buffalo ($p \leq 0.05$).

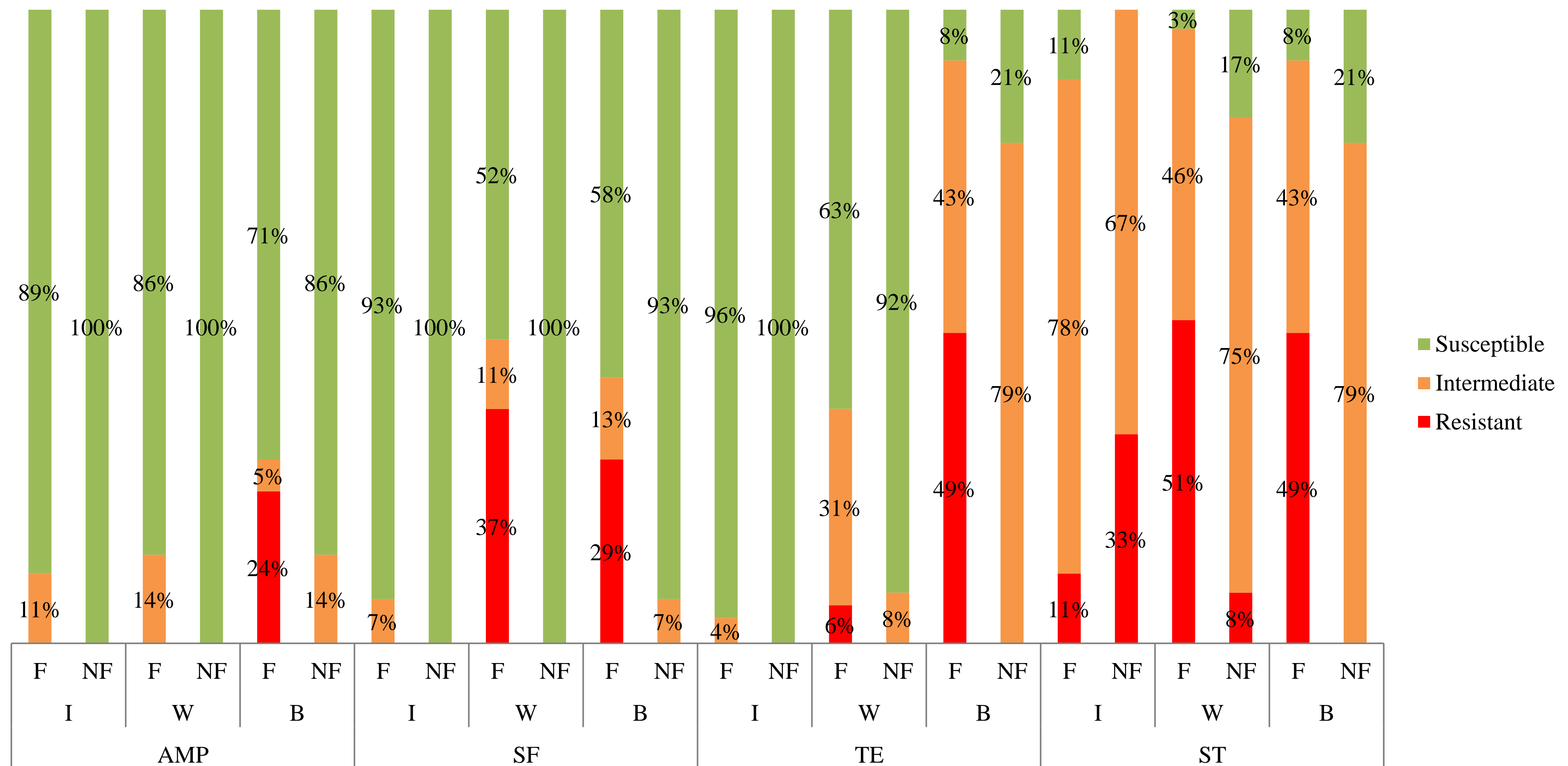

Figure 5.4 The averaged categorical antibiotic resistance levels of the *E. coli* isolates from supplementary fed (F) wildlife vs. non- fed (NF) wildlife against ampicillin (AMP, I: $p > 0.05$; W: $p > 0.05$; B: $p > 0.05$), sulphafurazole (SF, I: $p > 0.05$ W: $p \leq 0.05$; B: $p \leq 0.05$), tetracycline (TE, I: $p > 0.05$; W: $p \leq 0.05$; B: $p \leq 0.05$) and streptomycin (ST, I: $p > 0.05$; W: $p \leq 0.05$; B: $p \leq 0.05$). I= impala, W= wildebeest, B= buffalo.

The insignificant ABR differences seen between the two impala groups against all the antibiotics seems to be as a result of a lower ABR level of the fed impala compared to those from the fed buffalo and fed wildebeest groups, specifically towards sulphafurazole and tetracycline, as seen in Figure 5.4. This trend was also evident in the *E. faecalis* isolates in Figure 5.5.

What is more, Figure 5.4 shows that the fed buffalo samples were noticeably more (but not significantly more) resistant to ampicillin (a penicillin antibiotic) than the other wildlife species (buffalo - 24% vs. impala - 0% impala and wildebeest – 0%). Upon further analysis, the high ampicillin resistance recorded for the fed buffalo originated mostly from Wellington farm 1, where these buffalo were more ($p \leq 0.05$) resistant to ampicillin than the buffalo from the other two farms (Wellington farm 2 and Ekuseni). This farm, as mentioned previously, was once a sheep farm twenty years ago.

Penicillins are the most widely used antibiotics in sheep farming (Santman-Berends *et al*., 2014). The application of antibiotics during the farming period, would have altered the soil dynamics by creating an antibiotic selective pressure, encouraging the development of antibiotic resistance (Wegst-Uhrich *et al*., 2014). Twenty years later, these antibiotic resistant bacteria and/ or resistant determinants still remain in the soil and are transferred to the grazing wildlife, resulting in detection of antibiotic resistant bacteria in the gut of the wildlife grazing on this farm. It is likely that the buffalo on this farm would also be resistant to all other penicillin antibiotics, such as carbenicillin, as well as other antibiotics commonly used in livestock farming. These *E. coli* isolates were classified as multi-drug resistant; which showed resistance to ampicillin, sulphafurazole, tetracycline and streptomycin.

Higher ($p \leq 0.05$) ABR levels were observed for the *E. coli* from the fed wildlife towards sulphafurazole (fed – 22% vs. non-fed – 0%), tetracycline (fed - 18% vs. non-fed – 0%) and streptomycin (fed - 37% vs. non-fed – 14%). Tetracycline resistance was also commonly found in farm and food animals by Liu *et al*. (2012) and Daniel *et al.* (2017). Tetracycline and sulphafurazole are commonly used antibiotics for growth promotion in animal feed as it stimulates weight gain (Speer *et al*., 1992). Perhaps the animal feed contained tetracycline and/ or sulphafurazole, and hence the significant difference in resistance. Unfortunately, this information was not disclosed to the researchers by the farmers/ managers; it is postulated that they did not know what is in the pre-mix added to the feed as these are typically commercial products and the manufacturers' thereof are reluctant to disclose the exact composition of these due to propriety rights (as we saw in Chapter 4).

Due to its extensive use in both agricultural and clinical settings, tetracycline, sulphonamide and streptomycin resistance has become widespread and significant in food animals (Bryan *et al*., 2004; Wilkerson *et al*, 2004; Boerlin *et al*, 2005; Fan *et al*., 2006; Kozak *et al*., 2009). Walsh & Duffy (2013) and Kozak *et al.* (2009) noted that tetracycline, sulphafurazole and streptomycin resistance was higher in hospital/ farm areas than pristine/ natural areas.

Very low to negligible resistance was recorded for ampicillin, tetracycline and sulphafurazole in the non- fed wildlife isolates. The only notable resistance was towards streptomycin. Other studies have reported streptomycin resistant rates in wild animals of between 0% and 7% (Kozak *et al*., 2009).

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The significant resistance towards streptomycin in both groups, of which a large proportion is intermediate resistance, suggests that the bacteria found in the gut of the wild animals are intrinsically resistant to streptomycin, possibly due to the presence of streptomycin and its accompanying resistant determinants in the soil, produced by organisms such as *Streptomyces griseus*. Overbeek *et al*. (2002) found streptomycin-modifying genes in all the different environments that were tested, including pristine and polluted environments. A study on the ABR of soil bacteria revealed that most intrinsically resistant bacteria originate from the soil, where multidrug resistant bacteria are in abundance (Walsh & Duffy, 2013). This level of 'intrinsic' intermediate resistance against streptomycin and can be viewed as a potential reservoir of ABR, where if a selective pressure were applied to these environments, high levels of complete resistance could emerge.

Furthermore, Figure 5.5 gives a more detailed look at the ABR profiles of *E. faecalis* towards each antibiotic, comparing the supplementary fed and non- fed wildlife.

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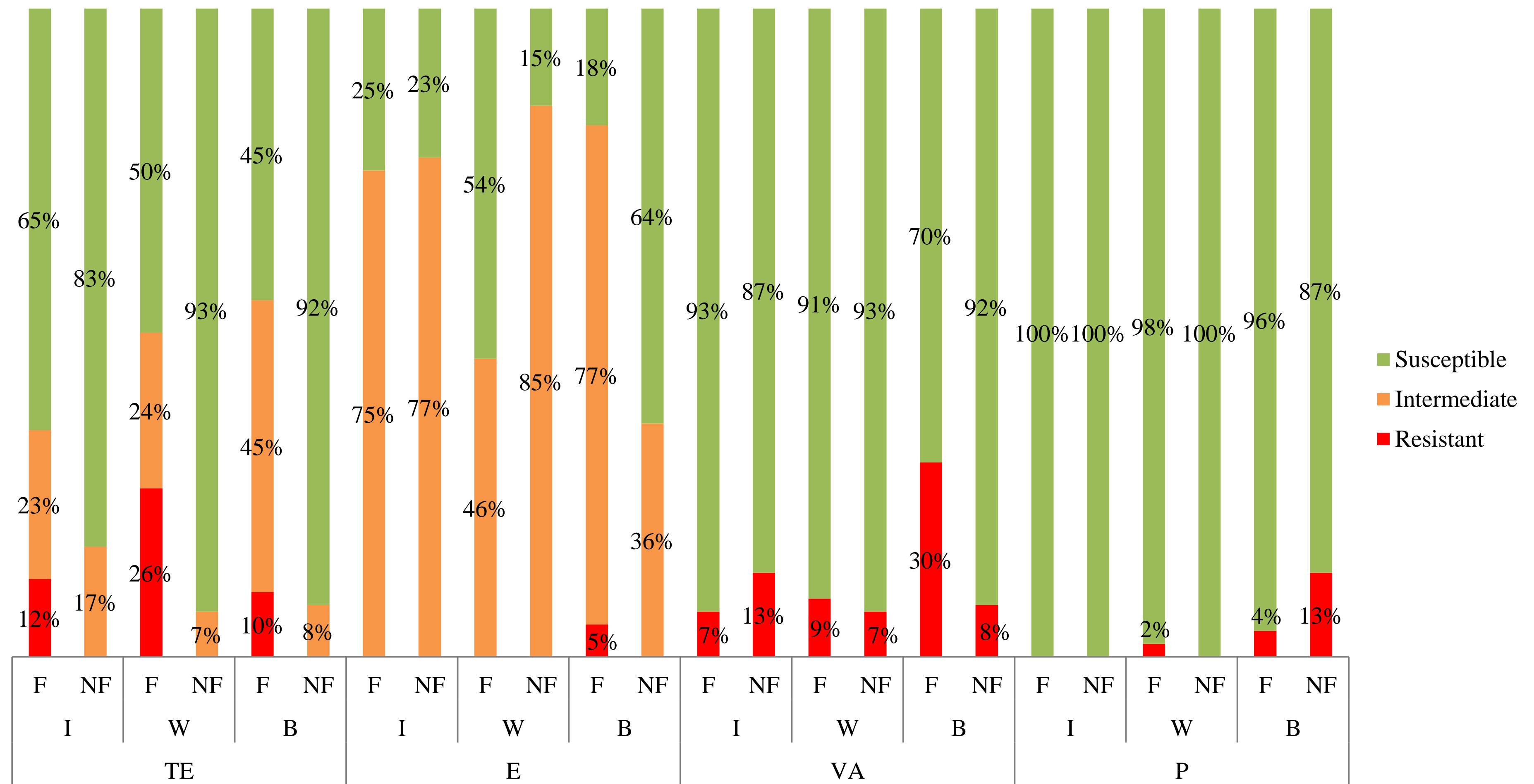

Figure 5.5 The averaged categorical antibiotic resistance levels of the *E. faecalis* isolates from supplementary fed (F) wildebeest vs. non- fed (NF) wildebeest against tetracycline (TE, I: p> 0.05; W: p≤ 0.05; B: p≤ 0.05), erythromycin (E, I: p> 0.05; W: p≤ 0.05; B: p≤ 0.05), vancomycin (VA, I: p> 0.05; W: p> 0.05; B: p> 0.05) and penicillin (P, I: p> 0.05; W: p> 0.05; B: p> 0.05). I= impala, W= wildebeest, B= buffalo

From Figure 5.5, it can be inferred that the most significant difference between the fed and non- fed wildlife was the level of tetracycline resistance, where the fed wildlife were notably more resistant (fed – 16% vs. non-fed - 0%). This trend was also seen for the *E. coli* isolates, for reasons previously explained.

Interestingly, erythromycin resistance gave contrary results between the two different wildlife groups. Macrolides (erythromycin) are one of the top most commonly used antimicrobial agents used in food-producing animals (Econoumou & Gousia, 2015). The fed buffalo (55% resistant) were more resistant ($p \leq 0.05$) towards erythromycin than the non-fed buffalo (0% resistance). This again, could be due to a carry over effect from the soil microbiota on Wellington farm 1 which was previously a sheep farm. But with the wildebeest group, the non-fed wildebeest (0% resistant, 85% intermediate) were more ($p \leq 0.05$) resistant than the fed wildebeest (0% resistant, 46% intermediate). Garrido *et al*. (2014) reported that many studies have found macrolide resistant in staphylococci, streptococci and Enterococci. Anderson *et al.* (2008) found erythromycin resistant Enterococci at a 4% prevalence in wildlife (American Bison). There were no significant differences between the impala groups (fed - 0% resistant, 75% intermediate; non-fed - 0% resistant; 77% intermediate). As previously seen with the *E. coli* isolates, the lower ABR observed in the fed impala isolates can be attributed to the fact that impala do not readily take to artificial/ supplementary feed.

A low vancomycin resistance level is expected as vancomycin resistant (VR) *E. faecalis* is not common. In this study, vancomycin resistance was low but still noteworthy (fed – 15% vs. non-fed – 9%) as vancomycin is the 'drug of last resort' to treat serious multi-drug gram positive infections (Boneca & Chiosis, 2005). The prevalence of VR *E. faecalis* is between 0% and 11% worldwide (Hayakawa *et al*., 2013; Jia *et al*., 2014; O'Driscoll & Crank, 2015). On the other hand, VR *E. faecium,* which is known to be more pathogenic and resistant than *E. faecalis*, is on the rise with occurrences of up to 80% (Kristich *et al.,* 2014; O'Driscoll & Crank, 2015).

Likewise, the overall resistance towards penicillin was also very low in both groups (fed – 2% vs. non-fed – 4%). β- lactam resistance varies between the different β- lactam antibiotics and enterococcal species: where penicillin and ampicillin have the highest activity against enterococci and *E. faecium* are generally more resistant than *E. faecalis* (Johnston & Jaykus, 2004; Kristich *et al.,* 2014; Miller *et al.,* 2015). Penicillin has the greatest activity against Enterococcus species than other gram positive cocci (Preston *et al*., 2017). Penicillin resistant *E. faecalis* is not commonly found because a moderate concentration of penicillin is sufficient to inhibit *E. faecalis* growth whereas a much higher dose is needed for inhibition of *E. faecium* isolates due to over production of the *pbp*-5 enzyme (Marothi *et al.,* 2005). Johnston & Jaykus (2004) and Jia *et al.,* 2014 also found a low prevalence of penicllin resistant *E. faecalis* isolates of between 0-5%.

5.5. Conclusion

Feeding of wildlife allows more opportunity for disease and mobile antibiotic resistant genes to be transferred directly between species due to increased herd density, more frequent direct contact at feeding and water points and increased human contact. The feed itself can also be a direct source of antibiotic resistant bacteria.

The non-fed wildlife had overall lower levels of ABR than those which were fed on a regular basis by humans. This suggests that the greater the intervention of human activities are on the animals, the greater the opportunity of ABR development and transfer of certain antibiotic resistance elements. Some intrinsic ABR was observed in the non-fed wildlife isolates but overall, the levels of ABR were low. Although wild animals are thought not to be exposed directly to antimicrobials, low concentrations of antibiotic compounds can reach the natural environment through a variety of natural sources and vectors.

Antibiotic resistance and its development is further enhanced by other vectors and sources, such as the wind, birds, rodents, contaminated water sources, transportation of animals, cross-contamination in the food production chain, which all have the potential to influence the movement of resistant genes from various reservoirs.

Thus, it is important to consider *all* possible transfer vectors and sources of ABR in order to control the transfer of resistance genes to pathogens, which would significantly affect the future of human health.

5.6. References

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CHAPTER 6

Antibiotic resistance profiles of *Escherichia coli*, *Enterococcus faecalis* and *Staphylococcus aureus* originating from the meat and faeces of game species

6.1. Summary

The rise in South African game meat exports over recent years indicates a growing trend of game meat consumption. A major aim of this study was to determine to what extent *Escherichia coli*, *Enterococcus faecalis* and *Staphylococcus aureus* found on the meat of game species are resistant to antibiotics, compared to the bacteria isolated from the faeces of the same animals. The Kirby-Bauer disk diffusion method was used according to the Clinical and Laboratory Standards Institute (CLSI) 2018 guidelines. Some of the *E. faecalis* meat isolates (N= 14) were significantly more resistant to tetracycline and erythromycin than the faecal samples (N= 45). These significant differences suggest that cross contamination of the meat occurred during slaughter by bacteria from the personnel or the equipment. There were no significant differences, except towards ceftazidime and sulphafurazole, in the antibiotic resistance profiles between the *E. coli* isolated from the meat (N= 23) and faeces (N= 45), suggesting that carcass faecal contamination and cross contamination from personnel occurred during slaughter. The *S. aureus* meat isolates (N= 26) showed high (75%) resistance towards penicillin, where 11.5% of meat samples were MRSA. This highlights the importance of food safety and hygiene procedures from farm to fork, to prevent cross-contamination of raw meat, leading to the transfer of antibiotic resistance bacteria and pathogens throughout the food chain.

6.2. Introduction

Recent studies have demonstrated that wild animals and their surrounding environments are important reservoirs of antibiotic resistant genes and bacteria (Costa *et al*., 2008; Karesh *et al*., 2012; Cantas *et al*., 2013). Studies have shown that antibiotic resistance (ABR) among wild animals is a growing public health issue, due to increased wildlife contact between humans, livestock and domestic animals, as well as increased co-habitation with other animals, as formerly addressed in chapters 4 and 5 (Martínez, 2008; Katakweba *et al.,* 2015). In addition, there is a rising trend of consumption of game meat (Sousa *et al*., 2014; Dias *et al*., 2015). Consumption of improperly cooked meat that is contaminated with bacteria that are resistant to antibiotics is a direct transmission route of antibiotic resistant bacteria to humans (Landers *et al.,* 2012).

Many antibiotic resistant genes are located on mobile genetic elements which can easily be transferred among bacteria, including pathogens, via a process known as horizontal gene transfer (HGT) (Allen & Donato, 2010). Horizontal gene transfer is one of the main ways in which resistance to antibiotics spreads in bacteria.

There is therefore a potential likelihood of antibiotic resistant traits entering the food chain and being found in food-producing animals (Aminov & Mackie, 2007).

To the author's knowledge, very few studies have been conducted in recent times on antibiotic susceptibilities of food animals in South Africa, and these have been mainly concentrated in the Gauteng region (Gelband & Duse, 2011). Filling this knowledge gap will enable a greater understanding of this complex phenomenon among the extensive farming community in South Africa, specifically wildlife farming. Additionally, this study will highlight whether natural, more remote environments are a potential source of antibiotic resistant bacteria, as found in some other studies (Jeters *et al.,* 2009; Kozak *et al*., 2009; Allen & Donato, 2010; Bhullar *et al.,* 2012; Sousa *et al*., 2014). If this is the case, antibiotic resistant microorganisms have the potential to be transferred to more commercialised environments, and become part of human food systems via numerous different vectors, of which one is the food chain itself (Nhung *et al*., 2015).

To fill this knowledge gap, a study was conducted to determine whether the meat of wild ungulates host antibiotic resistant bacteria, specifically *Escherichia coli*, *Enterococcus faecalis* and *Staphylococcus aureus* on various South African farms. Additionally, the ABR of the bacteria from the faeces of the same animals was analysed. If the ABR profiles of the bacteria on the meat and in the faeces of the same animal are similar, it can indicate that cross contamination of the animal itself occurred during the slaughter process. Whereas if the ABR profiles of the bacteria on the meat and in the faeces of the same animal are significantly different, it can indicate that cross contamination of the personnel or equipment occurred during evisceration; a phenomenon known to occur in South African abattoirs/ processing plants (Gouws *et al.,* 2017).

The three microorganisms which were analysed in this study are emerging antibiotic resistant commensal and pathogenic bacteria which are often present in the food chain and in the gastrointestinal tract of humans and animals (Wallmann, 2006; Oprea & Zervos, 2007). *Escherichia coli* and *Enterococcus faecalis* are commonly used as indicator organisms of ABR and faecal contamination in a given environment as they are found in a diverse range of hosts, are known to acquire ABR easily and are considered a good ABR indicator of other pathogenic bacteria (Catry *et al.,* 2003; Alexander *et al.*, 2008; Alm *et al*., 2014; Adefisoye & Okoh, 2016; Economou *et al*., 2017). Due to their intrinsic resistance to widely used antibiotics and the presence of virulence factors, they have been seen as potential reservoirs of ABR genes and a threat to the food chain (Kuhnert *et al*., 2000; Giraffa, 2002; Anderson *et al.,* 2016). Furthermore, *S. aureus*, specifically methicillin-resistant *S. aureus*, is a significant pathogen in human and food-producing animals which has been detected in retail meat worldwide (Hiroi *et al*., 2012).

It is hypothesised that the ABR levels detected would be fairly low, as all samples were taken from wild animals, where the use of antibiotic compounds is not evident (Mariano *et al*., 2009). The presence of *E. coli* and *E. faecalis* on the game meat would indicate that faecal cross-contamination occurred during the slaughter process, including evisceration and removal of the hide, as the meat of a healthy animal can be regarded as sterile (Gill, 2007). The presence of *S. aureus* on the game meat would indicate cross-contamination of the hide or that unhygienic practices occurred during the slaughter process, resulting in contamination of the meat from personnel (Gutiérrez *et al*., 2012).

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6.3. Materials and Methods

6.3.1. Study area

The study was conducted in South Africa, using meat and faecal samples from three wildlife species from three different farms across the country (Figure 6.1). Samples were collected between 2016 and 2018. Faecal and meat samples were collected from the same animal in order to compare the ABR profiles between each animal. A summary of the sample species, farm location and sample type is shown in Table 6.1. Impala (*Aepyceros melampus*) faecal and meat samples were collected from a farm in Modimolle, Limpopo, where supplementary feed is available to the wildlife. The impala on this farm co-graze with other wildlife species on the farm. Bontebok (*Damaliscus pygargus*) faecal and meat samples were collected from a farm in Wellington Nature Reserve, Western Cape. The bontebok graze on grass and are occasionally fed hay in autumn months if the grass has become depleted. The bontebok are free to roam with the other wildlife species on the farm. Springbok (*Antidorcas marsupialis*) faecal and meat samples were collected from a farm in Witsand, Western Cape. The springbok are free to co-graze with the other wildlife and livestock species on the farm. The wildlife are free-roaming and graze and drink on the farm's natural resources.

Table 6.1 Details of the wildlife samples used in this study

Wildlife species	**Farm location**	**Faecal samples**	**Meat samples**
Impala	Modimolle	5	5
Bontebok	Wellington	5	5
Springbok	Witsand	5	5

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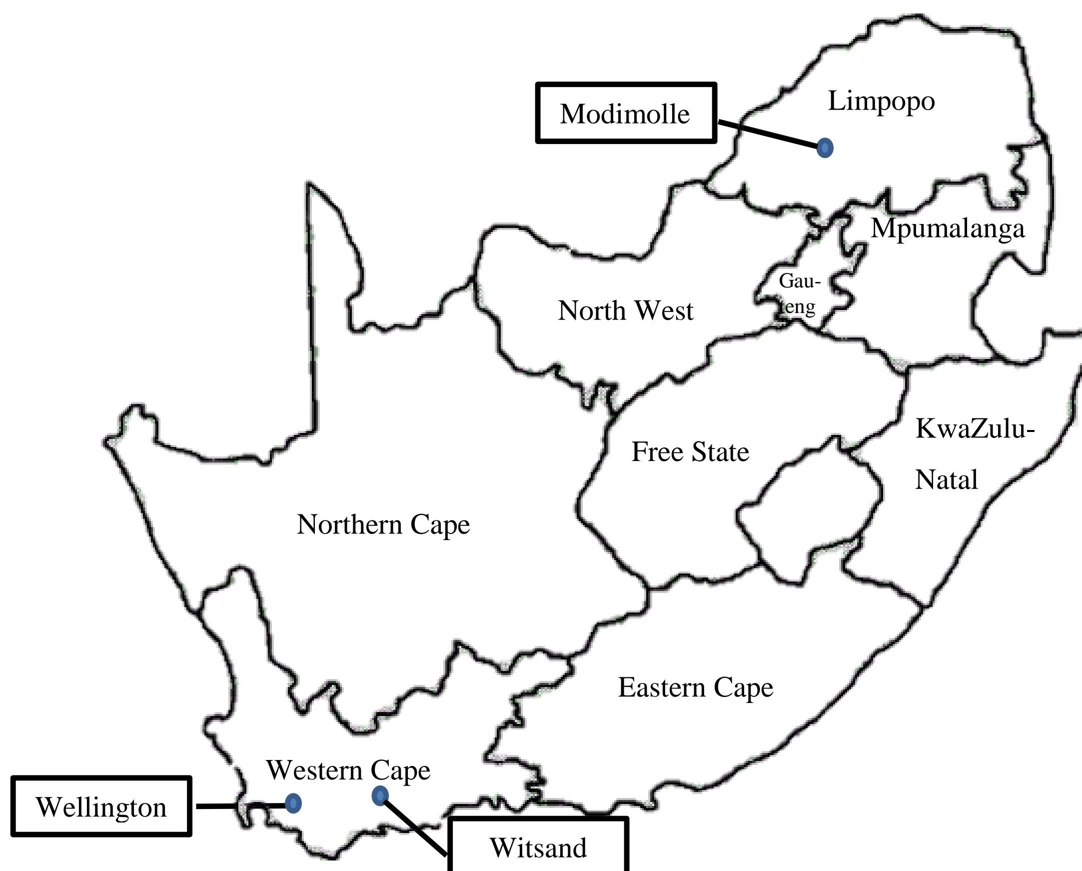

Figure 6.1 A map of South Africa which shows where sample collection took place.

6.3.2. Sample collection

Approximately 20 g of fresh faecal matter was located in the middle of the small intestines after evisceration and collected in a sterile sample container using a clean, disinfected knife and a new set of gloves for each animal. All faecal samples were transported at ~4°C to the university's laboratory.

The *infraspinatus* muscle was taken from deboned carcasses that have been stored overnight at 4°C. The *infraspinatus* muscle was sampled due to the high possibility of contamination during the slaughter process, to ensure isolation of bacteria from the meat samples. The muscles were vacuum packaged and transported at ~ 4°C to the university's laboratory. The meat samples were homogenised using a meat homogeniser and vacuum packaged.

After collection, all samples were stored in the university's laboratory freezer and stored there at -20°C.

6.3.3. Enumeration of bacteria

Escherichia coli and *Enterococcus faecalis* were isolated from the meat and faecal samples. *S. aureus* was only isolated from the meat samples. Faecal and homogenised meat samples were defrosted at room

temperature for 2 h before analysis commenced. A 10^{-1} dilution of the faecal and homogenised meat samples were made by adding 10 g faecal or meat to 90 mL Tryptone Soya Broth (TSB) (Biolab, South Africa). All 10^{-1} dilution samples were mixed using a Stomacher (Interscience) for 2 min and then incubated at 35°C for 12-14 h. This resuscitation step assists in recovery of the bacterial cells to allow for easier enumeration using selective agar media.

After incubation, 10^{-4} and 10^{-5} serial dilutions were prepared using Physiological Saline Solution (PSS) according to the South African National Standards method 6887-1 (SANS, 1999) in 9 mL units. The pour plate technique was used by pipetting 1 mL from the dilutions onto petri dishes (Willey *et al*., 2011a). After this step, selective agar was poured over and swirled in a "figure of 8" motion. Baird-Parker Agar (BPA) (Oxoid, South Africa) supplemented with Egg Yolk Tellurite Emulsion (Oxoid, South Africa) was used for enumeration of *S. aureus* and *E. faecalis*. Violet Red Bile Dextrose Agar (VRBDA) (Bioloab, South Africa) was used to select for *E. coli*. Once the petri dishes were set, they were inverted and incubated overnight at 35°C.

Following incubation, the streak plate technique was used to streak three random colonies per animal onto three selective agar petri dishes. Therefore, the antibiotic susceptibility test (AST) was performed in triplicate per animal sample. BPA was again used for *S. aureus* and *E. faecalis* and Eosin Methylene Blue Agar (EMBA) (Oxoid, South Africa) was used for *E. coli.* This step isolates the specific bacteria so that individual colonies can be selected. At the same time, one can presumptively identify the bacteria by the appearance of the colonies on the highly selective agar. The petri dishes were inverted and incubated overnight at 35°C.

One colony per plate was then streaked onto Nutrient Agar (NA) (Biolab, South Africa) plates to yield three pure cultures per animal. The plates were then inverted and incubated overnight at 35°C. These plates were stored at 4°C for further use for up to five days.

6.3.4. Bacterial species confirmation

Gram's stain (Lasec, South Africa) was performed on all colony isolates which showed characteristic growth, using the method described in the manufacturer's instruction manual. A drop of distilled water was placed on a microscope slide using a cooled sterile loop. The inoculation loop was sterilised through a flame and cooled and a small amount of bacterial colony from the NA plates was picked up. The bacteria were stirred into the water droplet to create a bacterial emulsion. The smear was left to air dry and then heat-fixed two to three times through a flame with the smear side facing up. The smear was flooded with crystal violet for 1 min and then rinsed with distilled water. The smear was then flooded with iodine for 1 min and then rinsed with distilled water. The smear was decolourised with alcohol until it ran clear and then was rinsed with distilled water. Lastly, the smear was flooded with safranin for 45 s and rinsed with distilled water. The smear was then gently blotted dry and observed under a microscope (Nikon YS100) on x1000 with immersion oil (Willey *et al.,* 2011b).

Eosin Methylene Blue Agar (Oxoid, South Africa) is a highly selective medium that produces characteristic colony growth specific to *E. coli*. Other lactose-fermenting gram negative rods that can also show the characteristic green metallic sheen are some species of *Citrobacter* and *Enterobacter*. Therefore, the citrate utilisation test was performed on presumptive *E. coli* isolates using Simmons Citrate agar (Oxoid). *E. coli* is citrate-negative, whereas *Citrobacter* and *Enterobacter* are both citrate-positive (Oxoid).

To confirm the presence of *S. aureus* isolated from the meat samples, the Staphylase test (Oxoid) and catalase test was performed according to the manufacture's instructions to ensure only colonies of *S. aureus* were selected from the BPA plates for antibiotic susceptibility testing. The Staphylase test identifies *S. aureus* by its unique ability to produce free and bound coagulase. *S. aureus* are catalase positive.

Enterococcus faecalis showed luxurious growth on Baird Parker Agar (BPA) but unlike *S. aureus*, there was no clear halo around the black, shiny colony. Colony identity was confirmed using Gram's stain, Staphylase test (Oxoid) (negative), catalase test (negative) as well as Matrix Assisted Laser Desorption/Ionization- Time of Flight Mass Spectrometry (MALDI-ToF MS).

After colony identification was confirmed, stock cultures were made and stored in the freezer at -20°C until further use. A loop full of bacterial colony was picked with a sterile loop from the NA plates and transferred into a sterile test tube containing 3 mL TSB. The bacterial suspension was vortexed and incubated overnight at 35°C. After incubation, each test tube was vortexed and 0.75 mL bacterial suspension was pipetted into a 2 mL microtube containing 0.75 mL sterile 50% glycerol (Fluka Analytical, Germany) (Gorman & Adley, 2004). This long-term preservation technique has been commonly used for the successful preservation of numerous bacterial species (Gorman & Adley, 2004).

6.3.5. Antibiotic susceptibility testing

The Kirby-Bauer disk diffusion method was used according to the Clinical and Laboratory Standards Institute (CLSI) 2018 guidelines using Mueller-Hinton agar (MHA) (Biolab, South Africa). The direct colony suspension method was used to prepare the inoculum suspension, using colonies from fresh NA plates. Three colonies per animal for each organism was selected randomly using an inoculating loop and suspended in 0.75 mL PSS in 2 mL Eppendorf tubes.

The antibiotic disks were applied within 15 min of inoculating the MHA plate. A 6-disk dispenser (Oxoid, South Africa) was used to place the disks onto the surface of the MHA plates. The disks were checked to ensure that they were firmly placed on the agar surface. Tables 6.2, 6.3 and 6.4 show the antibiotic disks applied for *S. aureus*, *E. faecalis* and *E. coli,* respectively. The plates were inverted and incubated at 35°C for 16-18 h and 24 h for vancomcyin discs (CLSI, 2018).

Table 6.2 Selection of antibiotic disks applied to *Staphylococcus aureus*

Antimicrobial agent	Disk content	Antibiotic class
Cefoxitin* (FOX)	30 µg	Penicillin
Erythromycin (E)	15 µg	Macrolide
Oxacillin (OX)	1 µg	Penicillin
Penicillin (P)	10 U	Penicillin
Tetracycline (TE)	30 µg	Tetracycline
Vancomycin (VA)	30 µg	Glycolipid

*Cefoxitin disc is used to confirm oxacillin resistance

Table 6.3 Selection of antibiotic disks applied to *Enterococcus faecalis*

Antimicrobial agent	Disk content	Antibiotic class
Erythromycin (E)	15 µg	Macrolide
Penicillin (P)	10 U	Penicillin
Tetracycline (TE)	30 µg	Tetracycline
Vancomycin (VA)	30 µg	Glycolipid

Table 6.4 Selected antibiotic disks for *Escherichia coli*

Antimicrobial agent	Disk content	Antibiotic class
Ampicillin (AMP)	10 µg	Penicillin
Chloramphenicol (C)	30 µg	Macrolide
Streptomycin (ST)	10 µg	Aminoglycoside
Sulphafurazole (SF)	300 µg	Sulfonamide
Tetracycline (TE)	30 µg	Tetracycline

After incubation of the MHA plates, the diameter of the zones was measured to the nearest millimetre using a digital calliper (0-150 mm). The zones, except for oxacillin and vancomycin, were measured using reflected light. This was achieved by measuring the zones from the back of the plate with light shining from above and looking directly above the plate. The oxacillin and vancomycin zones were measured using transmitted light by holding the plate up towards the light to measure the zone diameter (CLSI, 2018).

Zone diameters were classified as either resistant (R), intermediate (I) or susceptible (S), according to the CLSI zone diameter interpretive standards for each microorganism (Tables 6.5, 6.5 and 6.7) (CLSI, 2018).

Table 6.5 Zone diameter interpretive standards for *Staphylococcus* species (CLSI, 2016 & 2018)

Antimicrobial agent	Disk content	Zone diameter, nearest whole mm			Comments
		R	I	S	
Cefoxitin (FOX)	30 µg	≤ 24	-	≥ 25	The cefoxitin disk test is the preferred method of testing for prediction of mecA-mediated resistant to oxacillin for S. aureus.
Erythromycin (E)	15 µg	≤ 13	14- 22	≥ 23	-
Oxacillin (OX)	1 µg	≤ 10	11- 12	≥ 13	Based on the cefoxitin result, report oxacillin as susceptible or resistant.
Penicillin (P)	10 U	≤ 28	-	≥ 29	Penicillin-resistant, oxacillin-susceptible strains of S. aureus produce β–lactamase.
Tetracycline (TE)	30 µg	≤ 14	15- 18	≥ 19	Organisms that are susceptible to tetracycline are also considered susceptible to doxycycline and minocycline.
Vancomycin (VA)	30 µg	-	-	≥ 15	MIC tests should be performed to determine vancomycin susceptibility

Table 6.6 Zone diameter interpretive standards for *Enterococcus* species (CLSI, 2018)

Antimicrobial agent	Disk content	Zone diameter, nearest whole mm			Comments
		R	I	S	
Erythromycin (E)	15 µg	≤ 13	14- 22	≥ 23	-
Penicillin (P)	10 U	≤ 14	-	≥ 15	Enterococci susceptible to penicillin are predictably susceptible to ampicillin, amoxicillin, ampicillin-sulbactam, amoxicillin-clavulanate, piperacillin and piperacillin-tazobactam.
Tetracycline (TE)	30 µg	≤ 14	15- 18	≥ 19	-
Vancomycin (VA)	30 µg	≤ 14	15-16	≥ 17	-

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Table 6.7 Zone diameter interpretive standards for Enterobacteriaceae (CLSI, 2018)

Antimicrobial agent	Disk content	Zone diameter, nearest whole mm			Comments
		R	I	S	
Ampicillin (AMP)	10 µg	≤ 13	14- 16	≥ 17	Class representative for ampicillin and amoxicillin.
Chloramphenicol (C)	30 µg	≤ 12	13- 17	≥ 18	-
Ceftazadime (CAZ)	30 µg	≤ 17	18- 20	≥ 21	-
Streptomycin (ST)	10 µg	≤ 11	12- 14	≥ 15	-
Sulphafurazole (SF)	300 µg	≤ 12	13- 16	≥ 17	Represents any of the currently available sulfonamides.
Tetracycline (TE)	30 µg	≤ 11	12- 14	≥ 15	Organisms that are susceptible to tetracycline are also considered susceptible to doxycycline and minocycline.

6.3.6. Statistical analysis

Statistical analysis was performed using Statistica 13.2 software (StatSoft, USA). The data was analysed using one-way analysis of variance (ANOVA). Levene's test was applied to determine homogeneity of variance. The main effect was the sample type (faecal vs. meat). If the group means were significantly different within the wildlife group, post hoc tests were performed to determine where the differences occurred within each sample group. Significant results were identified by least significant means (LSM) by using a 95% confidence interval i.e. a 5% significance level ($p \leq 0.05$) as a guideline.

6.4. Results and Discussion

The intestines have a high microbial diversity and load where extensive gene exchange can occur, increasing the probability that resistance genes will be transferred between bacterial species (Acton *et al.,* 2009; Schjørring & Krogfelt, 2011; Carlet, 2012). The intestines, specifically the large intestine, consists of high densities of over 300-500 different bacterial species (Guarner & Malagelada, 2003; Schjørring & Krogfelt, 2011). The bacterial composition of the intestines consists mainly of anaerobic bacteria, and facultative anaerobes are the subdominant genera found in the intestines, consisting mainly of, *Escherichia, Enterobacter, Enterococcus, Klebsiella, Lactobacillus* as well as other bacteria (Sørum & Sunde, 2001; Guarner & Malagelada, 2003).

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Raw meat contains a variety of bacteria, including spoilage and some pathogenic bacteria. The microbial load of raw meat can further be increased by contamination during the slaughter process with bacteria from the carcass hide or faeces or from the surrounding environment, equipment and personnel (Gouws *et al*., 2017a).

Escherichia coli (N= 45) and *Enterococcus faecalis* (N= 45) were isolated from all faecal samples. *Enterococcus faecalis* (N= 14) was isolated from fourteen impala meat samples (93%) and none of the springbok meat samples (0%) or bontebok meat samples (0%). *Escherichia coli* (N=23) was detected on eleven impala meat samples (73%) and nine springbok meat samples (66%) and three bontebok meat samples (20%). The presence of these gut microorganisms on the meat indicates that faecal contamination from the carcass occurred on these animals during the slaughter process (Aslam *et al.,* 2003). The recovery rate of *E. coli* on raw meat has been between 20-100% in other studies (Gouws *et al.,* 2017a; Gouws *et al.,* 2017b; Messele *et al*., 2017).

As seen in Figure 6.2, there were no overall significant differences in the ABR profiles between the *E. coli* isolates from the meat and faecal samples from the three wildlife species. This suggests that the *E. coli* isolated from the meat samples originated from the carcass via faecal contamination during the slaughter process. There was however a significant difference in the ABR profiles between the *E. faecalis* isolates from the meat and faecal samples, where the meat samples were significantly more resistant than the faecal samples. This suggests that cross contamination of the meat occurred during slaughter by bacteria from the personnel or equipment. This point highlights a significant risk in the meat production chain which needs to be minimised.

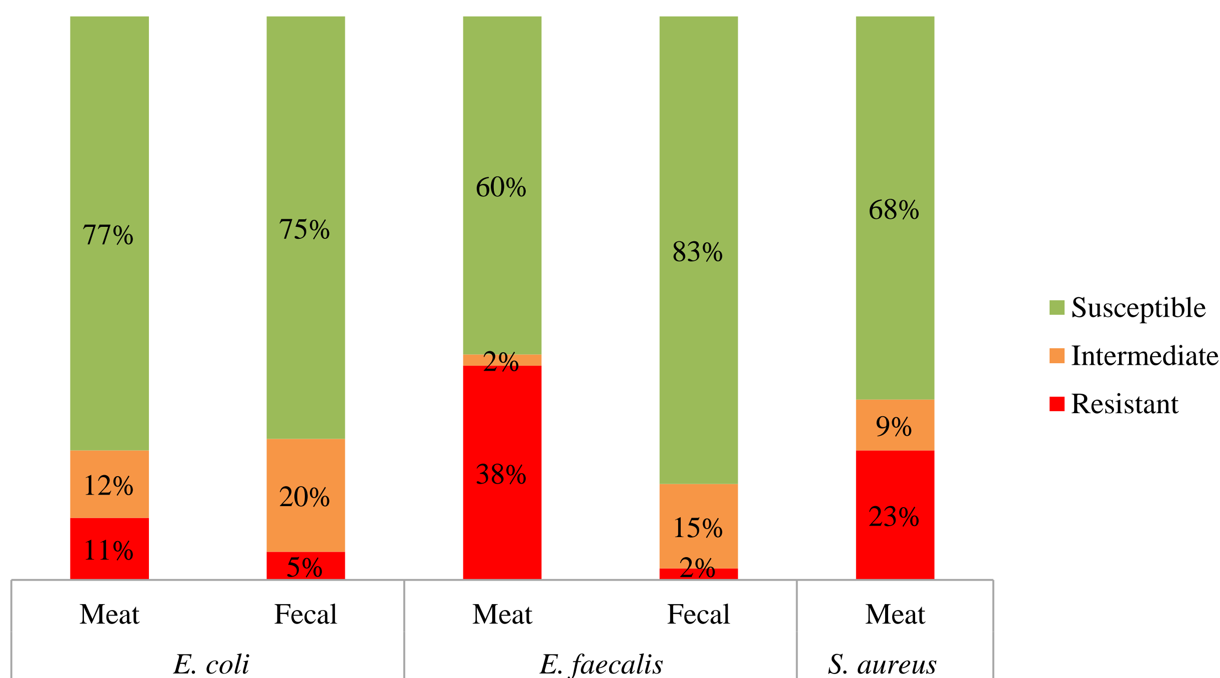

Figure 6.2 The averaged categorical antibiotic resistance levels of *E. coli* (SB: p> 0.05; I: p> 0.05; BB: p> 0.05), E. faecalis (I: p≤ 0.05) and S. aureus isolated from meat and faecal samples of springbok (SB), impala (I) and bontebok (BB).

The ABR profiles of *E. coli* against each of the individual antibiotics is shown in Figure 6.3. Chloramphenicol was not included in the analysis due to negligible resistant levels, leading to no variance in the data. This was also found in all other samples from Chapters 4 and 5. Thus it can be hypothesised that there is no significant quinolone or chloramphenicol selective pressure present in the wildlife farming environments used in this study. This is consistent with other studies which have also found very low resistance or negligible levels towards nalixidic acid and chloramphenicol in wild animals (Rolland *et al.,* 1985; Lillehaug *et al*., 2005; Costa *et al.,* 2008; Silva *et al.,* 2010).

Substantial resistance was observed towards ampicillin from the *E. coli* bontebok faecal and meat samples (meat - 100% vs. faecal - 73%). This is considerably higher than what was found in other studies which have reported ampicillin resistant *E. coli* in game meat in only 1.45- 3% of isolates (Li *et al*., 2007; Mateus- Vargas *et al.,* 2016). Low ampicillin resistance was detected for the other two wildlife species (springbok - 0% and impala - 9%). The bontebok originated from a farm in Wellington which was once a sheep farm. In Chapter 5, buffalo samples were analysed from this same farm (although the buffalo were maintained in camps that were not in the vicinity of the bontebok and the two wildlife species had never co-mingled) and it was found that 43% of the *E. coli* faecal samples were ampicillin resistant, significantly higher than the other wildlife species from the different farms. The high ampicillin resistance observed in the bontebok samples, as previously mentioned in Chapter 5, could once again, be due to a carry over effect from the soil bacteria. The

application of antibiotics (particularly penicillins which are widely used in livestock production) during the sheep farming period would have altered the soil dynamics by creating an antibiotic selective pressure, encouraging the development of antibiotic resistance (Santman-Berends *et al*., 2014; Wegst-Uhrich *et al*., 2014).

The only significant difference in ABR between the *E. coli* meat and faecal samples was to ceftazadime, from the springbok samples and to sulphafurazole from the impala samples. Of the springbok meat isolates, 22% were ceftazadime resistant and 0% of the faecal isolates and 22% of the impala meat isolates were sulphafurazole resistant and 0% of the faecal isolates. These differences ($p \leq 0.05$) could suggest that human cross-contamination of the meat occurred during the slaughter process, as none of the faecal samples were resistant.

The ceftazadime 30µg disc is an indicator of extended-spectrum β-lactamase (ESBL) producing bacteria, specifically the TEM and SHV variants which are related to hospital infections, suggesting that the resistance found in the springbok meat samples is of human origin (Rawat & Nair, 2010; Overdevest *et al*., 2011; Dahms *et al.,* 2015). The rise of ESBL-producing bacteria is a response to the increased use of antibiotics which has induced a continuous production and mutation of β-lactamases in these bacteria (Shaikh *et al*., 2015). Over the last decade, the prevalence of ESBL-producing *E. coli* from the faeces of wild animals has ranged from 0-10% (Literak *et al*., 2009; Silva *et al.,* 2010; Ho *et al*., 2011; Wallensten *et al*., 2011). Studies have speculated that environmental ESBL *E. coli* is as a result of human influence (Skurnik *et al.,* 2006; Guenther *et al*., 2011). To the author's knowledge, the prevalence of ESBL-producing *E. coli* from game meat has not been investigated. The prevalence of ESBL-producing *E. coli* found in the meat of the springbok samples is considerably lower than those found in broiler chickens, where the use of antibiotics is common. Other studies have documented a 60-80% prevalence of ESBL-carrying *E. coli* from raw retail chicken meat (Hiroi *et al*., 2011; Overdevest *et al.,* 2011; Geser *et al*., 2012).

Low levels of resistance were detected towards sulphafurazole and tetracycline. This is consistent with another study which reported similar resistance levels of *E. coli* isolates from game meat, with sulphafurazole resistance at 7.9% and tetracycline resistance at 13% (Li *et al*., 2007).

The highest averaged resistance over all three wildlife species was towards streptomycin, which was also found in other wildlife samples in Chapters 4 and 5. Other studies have reported that streptomycin resistance has become widespread and significant in food animals due to its extensive use in both agricultural and clinical settings (Bryan *et al*., 2004; Wilkerson *et al*, 2004; Boerlin *et al*, 2005; Fan *et al*., 2006; Kozak *et al*., 2009). In addition, streptomycin is present in the soil and natural environments and is produced by organisms such as *Streptomyces griseus* (Overbeek *et al*., 2002).

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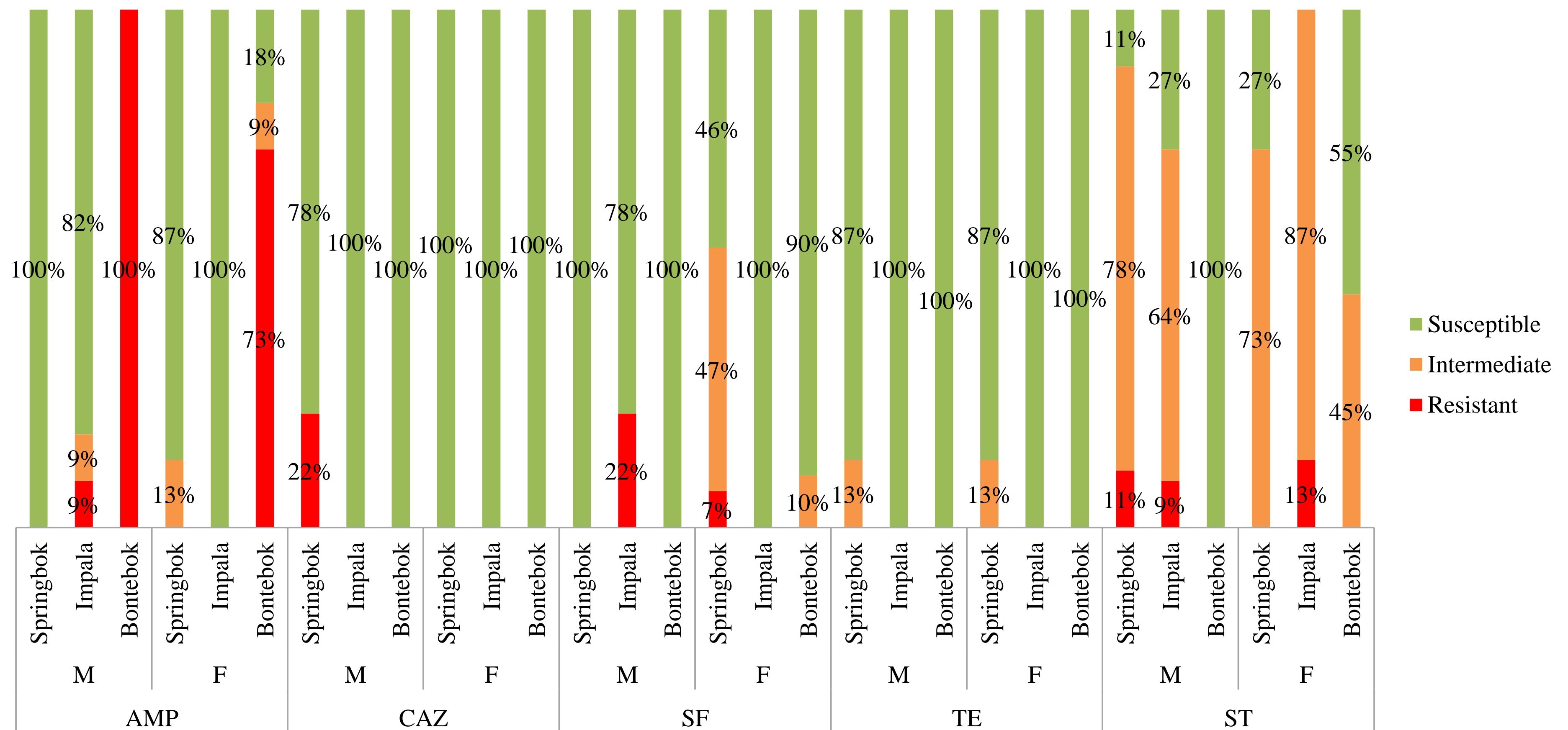

Figure 6.3 The averaged categorical antibiotic resistance levels of *E. coli* from meat vs. faecal samples against AMP (ampicillin: SB, p> 0.05; I, p> 0.05; BB, p> 0.05), CAZ (ceftazadime: SB, p≤ 0.05; I, p> 0.05; BB, p> 0.05); SF (sulphafurazole: SB, p≤ 0.05; I, p≤ 0.05; BB, p> 0.05), TE (tetracycline: SB, p> 0.05; I, p> 0.05; BB, p> 0.05) and ST (streptomycin: SB, p> 0.05; I, p> 0.05; BB, p> 0.05). SB= springbok, I= impala, BB= bontebok, M= meat and F= faecal.

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The ABR profiles of *E. faecalis* against each of the individual antibiotics is shown in Figure 6.4. Low resistance was found towards vancomycin and penicillin in both meat and faecal samples, leading to no significant differences between the meat and faecal samples. Low vancoymcin and penicillin *E. faecalis* resistance was also found with the wildlife isolates in Chapter 5 and by Johnston & Jaykus (2004). These results agree with other studies conducted on wild animals, who found very low (0-4%) numbers of vancomyin resistant and penicillin/ ampicillin resistant *E. faecalis* isolates (Silva *et al*., 2010; Gonçalves *et al.,* 2013). Therefore, vancomycin and penicillin/ampicillin resistant *E. faecalis* can be regarded as a rare occurrence (Kristich *et al.,* 2014).

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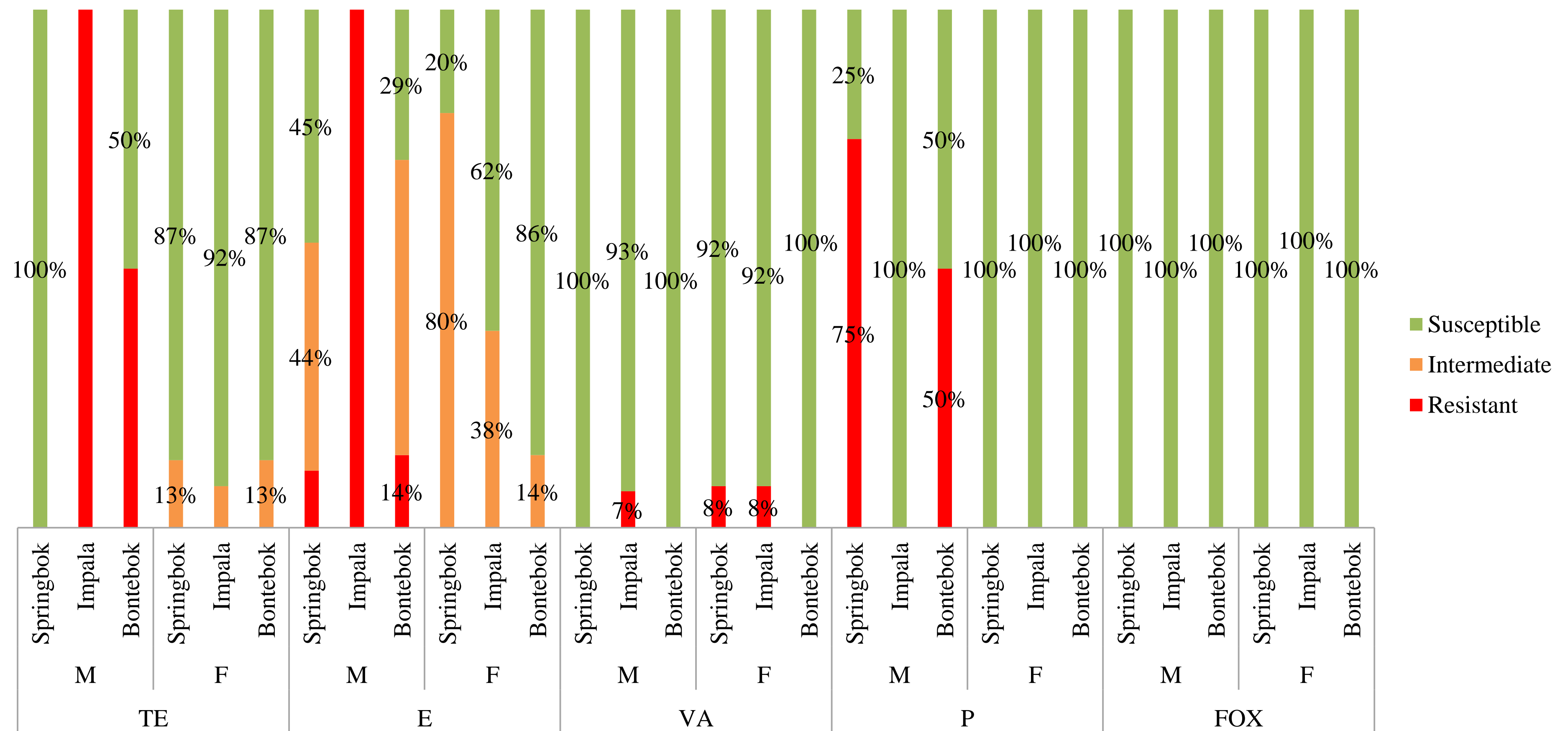

Figure 6.4 The averaged categorical antibiotic resistance levels of *E. faecalis* from meat vs. faecal samples against TE (tetracycline: I: p≤ 0.0), E (erythromycin: I: p≤ 0.05), VA (vancomycin: I: p> 0.05) and P (penicillin: I: p> 0.05).

Furthermore, the *E. faecalis* isolated from the meat samples were more ($p \leq 0.05$) resistant to tetracycline and erythromycin compared to those originating from the faeces. Moreover, the same *E. faecalis* isolates which were resistant to tetracycline were also resistant to erythromycin. The tetracycline-erythromycin cross-resistance observed is a common occurrence because the resistant genes for tetracycline are often found on the same mobile unit as those for erythromycin resistance (Culebras *et al*., 2002). For example, the *erm*B gene is often found together with the *tet*M gene (Culebras *et al*., 2002).

The differences in tetracycline and erythromycin ABR between the faecal and meat samples suggests that cross-contamination of bacteria occurred from the personnel or equipment, leading to significant differences in ABR levels between that of the meat and faecal samples (Madoroba *et al*., 2016). Other studies have also reported differences in ABR profiles of bacteria isolated from faeces versus raw meat, concluding that the meat processing steps could play a substantial part in introducing contamination with bacteria than the slaughter process itself (Hiroi *et al*., 2012; Madoroba *et al*., 2016; Karikari *et al.,* 2017).

The *E. faecalis* isolates from the meat samples were 100% resistant to tetracycline and erythromycin. The high level of resistance would suggest that the isolated bacteria originated from the personnel as it is thought that bacteria originating from humans would have higher ABR profiles than those from wildlife due to the more frequent exposure to antibiotics. This was also found in others' studies which investigated the ABR of *E. coli* originating from sources of humans and animals (Guan *et al.,* 2002; Vantarakis *et al,* 2005).

Staphylococcus aureus (N= 26) was isolated from fourteen springbok meat samples (93%), twelve bontebok meat samples (80%) and no impala meat samples (0%). The springbok were slaughtered by amateur slaughtermen, indicated by the high isolation rate of *S. aureus* on the meat of the springbok due to unhygienic practices and cross-contamination of the hide and personal onto the meat. Other studies have reported the prevalence of *S. aureus* on raw meat to be between 7% and 100% (Hanson *et al*., 2011; Hiroi *et al*., 2012; Gouws *et al.,* 2017b).

A summary of the AST results for the *S. aureus* isolates from the wildlife (springbok and bontebok) meat samples is shown in Figure 6.5. A comparison between the meat and faecal samples was not evaluated for *S. aureus* as this bacterium was only isolated from the meat samples.

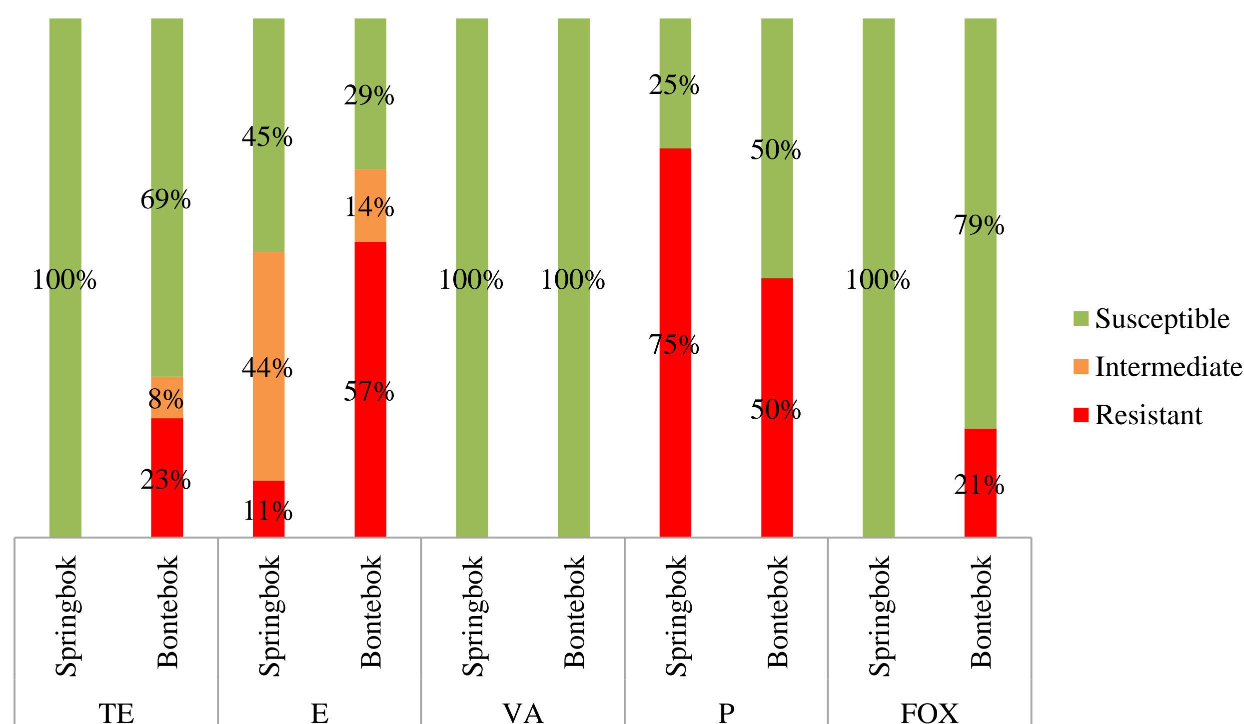

Figure 6.5 The averaged categorical antibiotic resistance levels of *S. aureus* from meat samples against tetracycline (TE), erythromcyin (E), vancomcyin (VA), penicillin (P) and cefoxitin (FOX) from springbok and bontebok.

Resistance to penicillin was high in both the springbok and bontebok meat samples (springbok - 75% and bontebok – 50%). Other studies have reported penicillin resistant *S. aureus* from commercial raw meat in 79-100% of samples (Hanson *et al*., 2011; Jackson *et al*., 2013; Osman *et al*., 2015). Resistance to penicillin is now widespread in humans and animals since the 1960s, in both community and hospital staphylococcal isolates (Lowy, 2003; Appelbaum, 2007; Chambers & DeLeo, 2009). Resistance to penicillin is achieved by an enzyme known as β-lactamase which hydrolyses the antibiotic (Lowy, 2003; Chambers & DeLeo, 2009). The gene which encodes this enzyme is located on a large plasmid, often with other resistant genes, such as those which are active against gentamicin and erythromycin (Lowy, 2003). Other studies have reported penicillin resistant *S. aureus* on raw meat of up to 100% (Jackson *et al.,* 2013).

A notable percentage of the *S. aureus* meat isolates were resistant to tetracycline (23%) and cefoxitin (21%) from the bontebok meat samples. No resistance to tetracycline and cefoxtin was reported in *S. aureus* from the springbok meat samples. Furthermore, no vancomcyin resistance was detected in any of the meat samples. These figures are considerably lower than the numbers of resistant *S. aureus* isolates recovered from retail raw meat of commercial beef, chicken and pork (Pesavento *et al*., 2007; Hanson *et al*., 2011; Hiroi *et al*., 2012; Das & Mazumder, 2016). Other studies have reported antibiotic resistant *S. aureus* with varying rates of resistance recorded from raw commercial meat ranges, with tetracycline resistance ranging from 19%-67%, vancomycin resistance from 0%-3% and MRSA 2%-35% (Pesavento *et al*., 2007; Hanson *et al*., 2011; Hiroi

et al., 2012; Das & Mazumder, 2016). These meat samples were purchased from supermarkets or meat markets where the use of antibiotics in animal production is probable but this was not specifically stated by the authors. This comparison demonstrates the already-proven theory that the use of antibiotics in food-producing animals leads to increased ABR, as the level of antibiotic resistance found on the raw game meat was lower than that which was found on commercial raw meat (Karikari *et al*., 2017).

The considerably higher resistance seen in *S. aureus* from the bontebok meat samples to tetracycline, erythromycin and cefoxitin compared to the springbok meat samples can again be attributed to the bontebok's farm history. As previously mentioned, the farm was a sheep farm 30 years ago. These three antibiotics are commonly used in veterinary farming (Table 6.8) and thus resistant determinants and bacteria could still be present in the soil microbiota and picked up by the wildlife species due to their grazing nature. It is important to take a farm's history into account in determining all the possible variables at play in the building up of ABR.

Moreover, from Figures 6.3, 6.4 and 6.5, it is postulated that the significantly high resistance levels detected against erythromycin (a macrolide), penicillin and ampicillin (a penicillin) from the meat samples is a result of human cross contamination, as the faecal samples did not show the same high resistance patterns. These three antibiotics fall into the top antibiotic classes commonly used in human medicine, as shown in table 6.8 (Moulin *et al*., 2008; Anon. 2015).

Table 6.8 A list in descending order of the top antibiotic classes used in human medicine which are also used in veterinary medicine (adapted from Moulin *et al*., 2008 and Anon., 2015)

Antibiotic class	Human medicine	Veterinary medicine
Penicillins	1	2
Macrolides	2	4
Tetracyclines	3	1
Sulfonamides	4	3

6.5. Conclusion

This study has revealed antibiotic resistant bacteria in the faecal content and meat of wild animals, inferring that the food chain can act as a vector of transmission to humans. Tetracycline, erythromycin and penicillin were the most common resistance observed in the selected meat samples, where the resistance level was high - contrary to what was hypothesised. These three antibiotics are some of the most commonly used antibiotics in human and veterinary medicine and thus their effectiveness can be greatly compromised due to the development of antibiotic resistant bacteria.

The ABR detected in the meat and faecal samples of the wildlife species highlights the importance of hygienic practices during the slaughter process, as well as the entire food production chain, to minimise the risk of cross-contamination of pathogenic and antibiotic resistant bacteria onto raw meat.

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In order to prevent cross-contamination of harmful bacteria onto raw meat, various precautionary steps can be put in place. For example, before skinning, a sanitising spray can be applied to the hide to kill any pathogens present and thus reduce the risk of cross-contamination onto the raw meat. Other precautionary steps include proper cleaning and disinfection of facilities and equipment; frequent washing of hangs; avoidance of stomach shots which would contaminate the carcass internally; proper chilling of the carcass after evisceration to hinder the growth of existing bacteria and prevention of human contact as best as possible throughout the slaughter process. Furthermore, processing facilities should follow strict hygiene and santitation protocols. Additionally, the consumer should ensure that raw meat is properly cooked and hygienic food preparations are followed to prevent cross-contamination between raw meat and foods that will not be further cooked. Consumer education on this point is important.

Further research on ABR in game meat should involve further investigation of ESBL-producing bacteria on game meat, as to the author's knowledge, this has not been investigated in South Africa. Confirmation of ESBL-producing bacteria can be achieved via the disc diffusion test using ceftazidime 30 µg, cefotaxime 30 µg and both of these in combination with clavulanic acid 30/10 µg. PCR can also be used to confirm the resistance patterns by detecting the common ESBL genes. It would also be interesting to investigate the source of the ESBL-producing bacteria found on the game meat, as this study suggested the resistant bacteria is of human origin because bacteria from the game meat were ceftazidime resistant but not the bacteria from the faeces.

It is also suggested to investigate the prevalence of antibiotic resistant bacteria from game meat throughout the meat production chain. This would highlight the keys areas which have the potential to introduce antibiotic resistant bacteria into the production chain.

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CHAPTER 7

Detection of antibiotic resistance genes in *Escherichia coli*, *Enterococcus faecalis* and *Staphylococcus aureus* isolates

7.1. Summary

Antibiotic resistant genes were detected in *Escherichia coli* (N= 44), *Enterococcus faecalis* (N= 22) and *Staphylococcus aureus* (N= 5) isolates originating from the faeces and meat of livestock and wildlife from farms located across South Africa. Polymerase chain reaction (PCR) was used to detect various antibiotic resistant genes in the phenotypically resistant isolates. The resistant genes were detected in frequencies of *sul*1 (40%), *sul*2 (80%), *sul*3 (0%), *bla*CMY (98%), *tet*A (63%), *tet*B (75%), *tet*C (0%) and *aad*A (89%) from the *E. coli* isolates and *tet*K (7%), *tet*L (100%), *tet*M (100%), *bla*Z (100%), *van*A (95%) and *van*B (10%) from the *S. aureus* and *E. faecalis* isolates. qPCR showed a 20 to 6.8 x 10^6 fold increase of the *aad*A1 gene relative to a bacterial housekeeping gene (16s rRNA) in streptomycin resistant *E. coli* isolates (N= 10). Resistant genes were detected in the bacteria of the wildlife and livestock from all the farm locations, including those of the free-ranging wildlife and organic livestock. Thus it is speculated that these resistant genes are picked up from the soil and the surrounding environment and are spread by the animals, as well as by other natural vectors like the wind and rivers. Thus wildlife species can be considered reservoirs of antibiotic resistant genes. Detection of antibiotic resistant genes is scientifically useful for confirming phenotypic resistance and for gaining more insight into knowledge of the type of resistance mechanisms the bacteria uses against the antibiotic.

7.2. Introduction

"DNA sharing", known as horizontal gene transfer (HGT) occurs in almost all bacterial genomes, assisting in spreading antibiotic resistance (Robinson & Hotopp, 2016). These transfers can be mediated by the conjugative movement of mobile genetic elements, such as plasmids and transposons (Salyers *et al.,* 2008). Due to the fast replication rate of bacteria, their genetic code is able to constantly adapt to the environment (Robinson & Hotopp, 2016).

The gastrointestinal tract of any animal is a complex ecosystem, containing hundreds of different bacterial species and this has potential to generate extensive genetic transfer (Allen & Donato, 2010; Schjørring & Krogfelt, 2011). Likewise, the natural environment, such as the soil, hosts a vast diversity of microorganisms, including bacteria which are antibiotic resistant and those which produce antibiotic compounds (Aminov, 2009; de Castro *et al.,* 2014).

The detection of certain resistant genes which have been commonly implicated to confer phenotypic resistance in bacterial isolates is the latest technique to allow for a better understanding of the antibiotic resistance phenomenon. Detection of resistant genes is also useful for confirming phenotypic antibiotic

resistance. In addition, it enables a better understanding of the mechanisms developed by certain bacteria which encode genes that confer specific antibiotic resistance mechanisms (Ledeboer & Hodinka, 2011). Although qualitative molecular antibiotic susceptibility testing has its advantages, such as increased sensitivity and speed, it can result in false-positive results compared to culture-based, phenotypic testing (Ledeboer & Hodinka, 2011). The accuracy of molecular testing is also dependent on the resistant genes selected for testing, the presence of unknown ABR genes can result in false-negatives. Thus, an integrated approach, combing phenotypic and genotypic methods, would yield the most reliable results.

There are numerous resistance genes that have been documented which give rise to a single resistance phenotype. Often, multiple resistance genes have been found to confer antibiotic resistance to a single antibiotic. This shows that there are a number of genes that can lead to a common resistant phenotype (Fluit, 2008).

Ideally, multiplex PCR may be used to detect multiple resistant genes in a single reaction. This allows detection of multiple resistant genes that have been associated with a resistant phenotype. Real-time PCR (RT-PCR) is a more advanced molecular detection method which allows for qualitative and quantitative detection of genes that is rapid and highly accurate. This method has application in the field of antibiotic resistance detection, where the relative concentration of an antibiotic resistant gene can be compared to a control using normalised data (Walsh *et al*., 2011).

Table 7.1 lists a few of the most common resistance genes detected in *Escherichia coli* for the selected antibiotics which are regularly used for genotypic resistance detection.

Table 7.2 lists a few of the most common resistance genes detected in *Enterococcus faecalis* and *Staphylococcus aureus* for the selected antibiotics which are typically used for genotypic resistant detection.

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Table 7.1 The most common resistant genes detected in *E. coli* which are associated with phenotypic resistance

Antimicrobial agent	Gene	Resistance mechanism	Reference
Tetracycline	*tet*A	Efflux pump	Boerlin *et al*., 2005; Gonçalves *et al*., 2013
	*tet*B	Efflux pump	
	*tet*C	Efflux pump	
	*sul*1	Target site modification	
Sulphonamide	*sul*2	Target site modification	Gonçalves *et al.,* 2013
	*sul*3	Target site modification	
Ampicillin	*bla*TEM	β-lactamase enzyme production	
	*bla*OXA	β-lactamase enzyme production	Briñas *et al.,* 2002; Kozak *et al.,* 2009
	*bla*CMY	β-lactamase enzyme production	
	*amp*C	β-lactamase enzyme production	
Streptomycin	*aad*A	Enzyme inactivation	Sunde & Norström, 2005; Gonçalves *et al*., 2013
	*str*A/*str*B	Enzyme inactivation	
Nalidixic acid	*gyr*A & *gyr*B	Target site modification	Sáenz *et al*., 2003
	*par*C & *par*E	Target site modification	
	*cat*1 & *cat*2	Enzyme inactivation	
Chloramphenicol	*flo*R	Efflux pump	Bischoff *et al*., 2002; Gonçalves *et al*., 2013
	*cml*A	Efflux pump	

Table 7.2 The most common resistance genes detected in *E. faecalis* and *S. aureus* associated with phenotypic resistance

Antimicrobial agent	**Gene**	**Resistance mechanism**	**Reference**
Tetracycline	*tet*K	Efflux pump	Gordon *et al*., 2014; Miller *et al*., 2014
	*tet*L	Efflux pump	
	*tet*M	Ribosomal protection protein	
Vancomycin	*van*A	Altered target site	Gold, 2001
	*van*B	Altered target site	
Penicillin	*bla*Z	Enzyme inactivation	Gordon *et al*., 2014; Miller *et al*., 2014
	*pbp*5	Altered target site	
Erythromycin	*erm*A	Altered target site	Gordon *et al*., 2014; Miller *et al*., 2014
	ermB	Altered target site	
Methicillin	*mec*A	Altered target site	Gordon *et al*., 2014
	*mec*C	Altered target site	

This study aimed to detect a selection of the most common antibiotic resistant genes in bacterial isolates which showed phenotypic resistance in order to correlate the genotypic and phenotypic resistance. It is hypothesised that there would be a high genotypic-phenotypic correlation rate (G±P±). However, this rate could be lowered due to correlations of 'phenotype, no genotype' (P+G-) because only a selection of antibiotic resistant genes was used in this analysis, even though there are many more genes that can confer the same resistant phenotype.

7.3. Materials and Methods

7.3.1 Study area

Faecal and meat samples were collected from various wildlife and livestock species from farms across South Africa (Table 7.3 and Figure 7.1).

Table 7.3 Details of the wildlife samples used in this study

Animal species	**Farm location**	**Number of samples (faecal)**	**Number of samples (meat)**
Black Wildebeest	Bredasdorp	5	-
Cattle	Bredasdorp	5	-
Eland	Bredasdorp	5	-
Sheep	Bredasdorp	5	-
Bontebok	Wellington 1	5	5
Buffalo	Wellington 1	5	-
Buffalo	Wellington 2	5	-
Blue wildebeest	Wellington 2	5	-
Buffalo	Ekuseni	5	-
Impala	Ekuseni	5	-
Blue wildebeest	Ekuseni	5	-
Impala	Modimolle 1	5	5
Blue wildebeest	Modimolle 2	5	-
Impala	Modimolle 3	5	-
Blue wildebeest	Modimolle 4	5	-
Sheep	Sutherland	5	-
Springbok	Sutherland	5	-
Cattle	Witsand	5	-
Deer	Witsand	5	-
Sheep	Witsand	5	-
Springbok	Witsand	5	5

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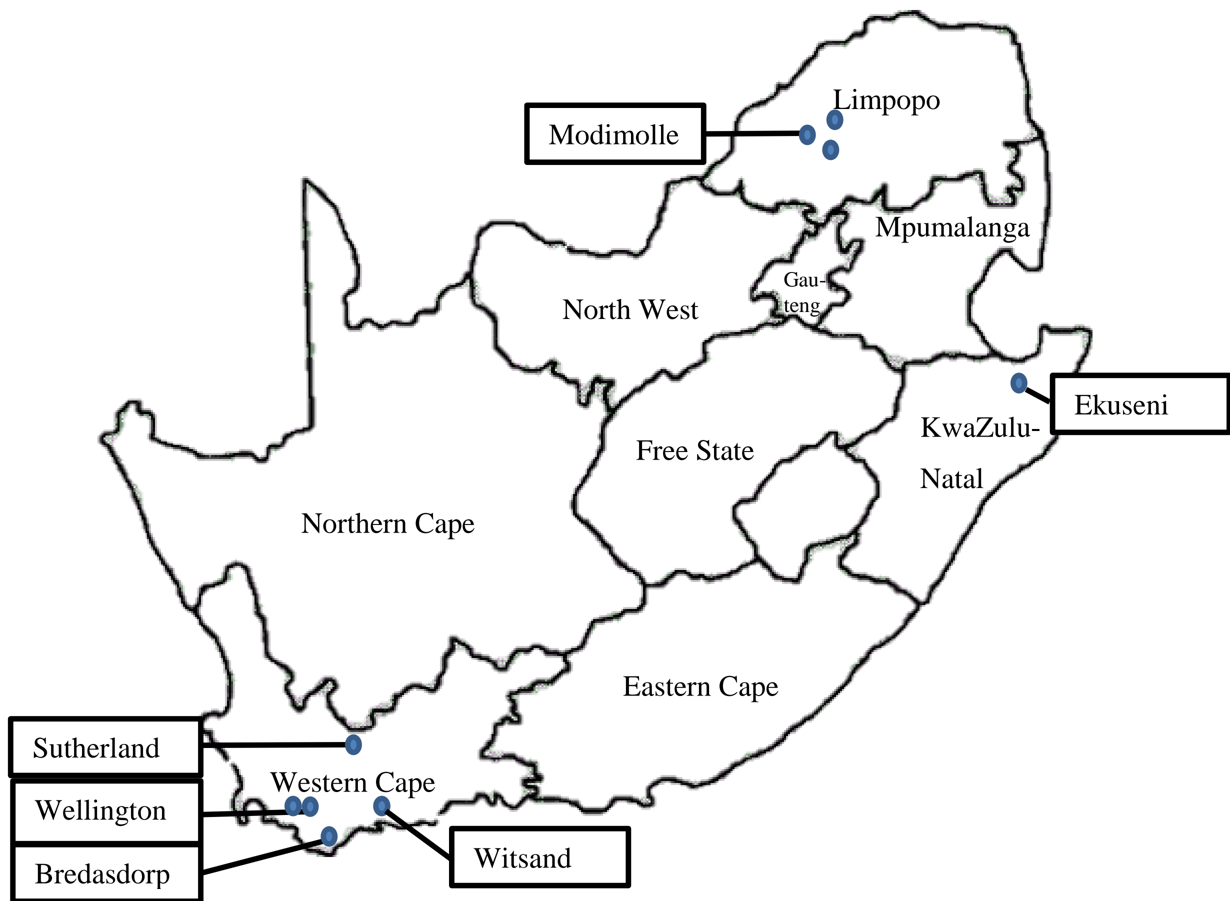

Figure 7.1 A map of South Africa which shows where sample collection took place.

Samples were collected from a farm in Bredasdorp which hosts livestock and wildlife species, namely, Merino sheep (*Ovis aries*) , Angus cattle (*Bos taurus*), eland (*Taurotragus oryx*) and black wildebeest (*Connochaetes gnou*). The wildlife graze on pastures and are fed supplementary feed in summer. The wildlife are separated from the livestock by a fenced off region but are occasionally allowed to co-graze with the cattle. The livestock are fed a premixed feed on a daily basis.

African buffalo (*Syncerus caffer*) and bontebok (*Damaliscus pygargus*) samples were collected from farm 1 in Wellington. The buffalo are fenced off from all other wildlife species on the farm. The buffalo and the bontebok graze on grass and are occasionally fed hay in autumn months if the grass has become depleted. This farm was previously a sheep farm twenty years ago. The frequent application of antibiotics during the livestock farming period would have altered the soil microbial population and dynamics (Wegst-Uhrich *et al*., 2014).

Samples of African buffalo (*Syncerus caffer*) and blue wildebeest (*Connochaetes taurinus*) were collected from a farm on Wellington farm 2. The buffalo and wildebeest are also fenced off from all other wildlife species on the farm and graze on grass and are occasionally fed hay in autumn months if the grass has become depleted.

Faecal samples of African buffalo (*Syncerus caffer*), impala (*Aepyceros melampus*) and blue wildebeest (*Connochaetes taurinus*) were collected from a game reserve in Ekuseni, Kwa-Zulu Natal. These animals are very wild and have never been supplementary fed or been in contact with others that have been fed and also do not receive any medical treatment. They are free to roam and graze on the open pastures of the reserve, along with the other wildlife species including predators.

Impala (*Aepyceros melampus*) samples which were supplementary fed was collected from farm 1 and farm 3 in Modimolle, Limpopo. The impala co-graze with other game species on the farms.

Blue wildebeest (*Connochaetes taurinus*) samples were collected from farm 2 in Modimolle. This farm hosts only blue wildebeest which are fed once a day at a single feeding point with multiple troughs containing a nutrient feed mix. The feeding point is rotated around the farm to avoid trampling of the vegetation. The wildebeest are also free to graze on the natural vegetation. All the wildebeest share the same water points which are refilled when necessary.

Samples from organic livestock and wildlife were collected from a farm in Sutherland, namely, springbok (*Antidorcas marsupialis*) and Merino sheep (*Ovis aries*). The sheep are kept in a fenced-off region but the springbok occasionally co-mingle with the sheep due to their nature of jumping fences. The sheep and springbok only graze and drink on the farm's natural resources and are not given any medication or supplementary feed.

More samples were collected from a farm in Witsand. Springbok (*Antidorcas marsupialis*) and fallow deer (*Dama dama*) are free to co-graze with the livestock species, namely Angus cattle (*Bos taurus*) and Merino sheep (*Ovis aries*), particularly the deer move between both the cattle and sheep whilst the springbok co-graze more often with the sheep. Both the livestock and wildlife graze and drink on the farm's natural resources, although in times of drought, the livestock are supplied with supplementary feed.

7.3.2. Sample collection

Approximately 20 g of faecal matter was collected from livestock from the ground shortly after deposition in sterile sample containers that were labelled with unique identifying codes for each animal. To avoid sampling from the same animal more than once, faecal samples were selected a distance apart (≥10 m) or immediately after deposition from the specific animal. Additionally, all samples taken from the same farm were collected on the same day during the same time period to avoid sampling the same animal more than once.

Wildlife faecal samples were either collected as described for the livestock or collected from the middle of the small intestine from recently slaughtered animals. Approximately 20 g of fresh faecal matter was located in the small intestines after evisceration and collected in a sterile sample container using a clean, disinfected knife and a new set of gloves for each animal. All faecal samples were transported at ~4°C to the university's laboratory.

The *infraspinatus* muscle was taken from deboned carcasses that have been stored overnight at 4°C. The *infraspinatus* muscle was sampled due to the high possibility of contamination during the slaughter

process, to ensure isolation of bacteria from the meat. The muscles were vacuum packaged and transported at ~ 4°C to the university's laboratory. The meat samples were homogenised used a meat homogeniser and vacuum packaged. After collection, all samples were stored in the university's laboratory freezer and stored there at -20°C.

7.3.3. Enumeration of bacteria

Escherichia coli and *Enterococcus faecalis* was isolated from the meat and faecal samples. *S. aureus* was only isolated from the meat samples. Faecal and meat samples were defrosted at room temperature for 2h before analysis commenced. A 10^{-1} dilution of the faecal and homogenised meat samples were made by adding 10 g faeces or meat to 90 mL Tryptone Soya Broth (TSB) (Biolab, South Africa). All 10^{-1} dilution samples were mixed using a Stomacher (Interscience) for 2 min. All 10^{-1} samples were incubated at 3 °C for 12-14 h. This overnight incubation resuscitation step assists in recovery of the bacterial cells to allow for more effective enumeration using selective agar media.

After incubation, 10^{-4} and 10^{-5} serial dilutions were prepared using Physiological Saline Solution (PSS) according to the South African National Standards method 6887-1 (SANS, 1999) in 9 mL units. The pour plate technique was used by pipetting 1 mL from the dilutions onto petri dishes (Willey *et al*., 2011a). After this step, selective agar was poured over and swirled in a "figure of 8" motion. Baird-Parker Agar (BPA) (Oxoid, South Africa) supplemented with Egg Yolk Tellurite Emulsion (Oxoid, South Africa) was used for enumeration of *S. aureus* and *E. faecalis*. Violet Red Bile Dextrose Agar (VRBDA) (Bioloab, South Africa) was used to select for *E. coli*. Once the petri dishes were set, they were inverted and incubated overnight at 35°C.

Following incubation, the streak plate technique was used to streak three random colonies per animal onto three selective agar petri dishes. Therefore, the antibiotic susceptibility test (AST) was performed in triplicate per animal sample. BPA was again used for *S. aureus* and *E. faecalis* and Eosin Methylene Blue Agar (EMBA) (Oxoid, South Africa) was used for *E. coli.* This step isolates the specific bacteria so that individual colonies can be selected. At the same time, one can presumptively identify the bacteria by the appearance of the colonies on the highly selective agar. The petri dishes were inverted and incubated overnight at 35°C.

One colony per plate was then streaked onto Nutrient Agar (NA) (Biolab, South Africa) plates to yield three pure cultures per animal. The plates were then inverted and incubated overnight at 35°C. These plates were stored at 4°C for further use for up to five days.

7.3.4. Bacterial species confirmation

Gram's stain (Lasec, South Africa) was performed on all colony isolates which showed characteristic growth, using the method described in the manufacturer's instruction manual. A drop of distilled water was placed on a microscope slide using a cooled sterile loop. The inoculation loop was sterilised through a flame and cooled and a small amount of bacterial colony from the NA plates was picked up. The bacteria were stirred into the

water droplet to create a bacterial emulsion. The smear was left to air dry and then heat-fixed two to three times through a flame with the smear side facing up. The smear was flooded with crystal violet for 1 min and then rinsed with distilled water. The smear was then flooded with iodine for 1 min and then rinsed with distilled water. The smear was decolourised with alcohol until it ran clear and then was rinsed with distilled water. Lastly, the smear was flooded with safranin for 45 s and rinsed with distilled water. The smear was then gently blotted dry and observed under a microscope (Nikon YS100) on x1000 with immersion oil (Willey *et al.,* 2011b).

Eosin Methylene Blue Agar (Oxoid, South Africa) is a highly selective medium that produces characteristic colony growth specific to *E. coli*. Other lactose-fermenting gram negative rods that can also show the characteristic green metallic sheen are some species of *Citrobacter* and *Enterobacter*. Therefore, the citrate utilisation test was performed on presumptive *E. coli* isolates using Simmons Citrate agar (Oxoid). *E. coli* is citrate-negative, whereas *Citrobacter* and *Enterobacter* are both citrate-positive (Oxoid).

To confirm the presence of *S. aureus*, the Staphylase test (Oxoid) and catalase test was performed according to the manufacture's instructions, to ensure only colonies of *S. aureus* were selected from the BPA plates for antibiotic susceptibility testing. The Staphylase test identifies *S. aureus* by its unique ability to produce free and bound coagulase. *S. aureus* are catalase positive.

E. faecalis showed luxurious growth on Baird Parker Agar (BPA) but unlike *S. aureus*, there was no clear halo around the black, shiny colony. Colony identity was confirmed using Gram's stain, Staphylase test (Oxoid) (negative), catalase test (negative) as well as Matrix Assisted Laser Desorption/Ionization- Time of Flight Mass Spectrometry (MALDI-ToF MS).

After colony identification was confirmed, stock cultures were made and stored in the freezer at -20°C until further use. A loop full of bacterial colony was picked with a sterile loop from the NA plates and transferred into a sterile test tube containing 3 mL TSB. The bacterial suspension was vortexed and incubated overnight at 35°C. After incubation, each test tube was vortexed and 0.75 mL bacterial suspension was pipetted into a 2 mL microtube containing 0.75 mL sterile 50% glycerol (Fluka Analytical, Germany) (Gorman & Adley, 2004). This long-term preservation technique has been commonly used for the successful preservation of numerous bacterial species (Gorman & Adley, 2004).

7.3.5. DNA extraction

Bacterial stock cultures that were kept at -20°C (0.75 mL suspension + 0.75 mL 50% glycerol) were defrosted at room temperature for 1h. The stock culture was centrifuged and 100 μL was suspended in 10 mL Tryptic Soya Broth (Oxoid) in a sterile centrifuge tube and incubated overnight at 37°C (Rip & Gouws, 2009). This resuscitation step was included to allow sufficient recovery of the bacteria cells.

7.3.5.1. Crude DNA extraction of Escherichia coli

The fresh overnight broth culture was centrifuged and 1 mL transferred into a sterile 1.5 mL Eppendorf tube. The overnight broth culture was then centrifuged at 1 000 g for 1 min to obtain a pellet. The supernatant was discarded. An additional 1 mL of the overnight broth culture was transferred to the Eppendorf tube containing the pellet and re-centrifuged. The supernatant was again discarded to be left with a pellet.

Three hundred microliters of lysis buffer (10 mM Tris-HCl, 1% Triton X-100, 0.5% TWEEN 20, 1 mM EDTA) was added to the 1.5 mL Eppendorf tube containing the culture pellet and centrifuged to ensure cells are properly suspended. The suspension was placed in a waterbath and boiled for 10 min at 100°C, centrifuged at 1 000 g for 2 min, where after 250 µL of the supernatant was transferred to a sterile 1.5 mL Eppendorf tube and 250 µL ice cold 99% ethanol added. The supernatant was then centrifuged at 13000 g for 1 min. The supernatant was again discarded. The Eppendorf tube was left to dry with the lid open for 1h. The dried pellet was then suspended in 100 µL TE buffer (10mM Tris-HCl, 1mM EDTA) and stored frozen at -20°C until further use.

7.3.5.2. Kit DNA extraction of Enterococcus faecalis and Staphylococcus aureus

The ZymoBiomics DNA kit (Inqaba Biotec) was used according to the manufacturer's instructions. Fresh overnight broth culture (1.5 mL) was added to a 1.5 mL Eppendorf tube and centrifuged for 5 min at 13000 g. The supernatant was discarded. An additional 1.5 mL of the overnight broth culture was transferred to the Eppendorf tube containing the pellet and re-centrifuged. The supernatant was again discarded to be left with a pellet only. Phosphate buffered saline (1 mL) was added to the pellet and vortexed to mix. 250 µL of the mixture was added to a BashingBead™ lysis tube. ZymoBIOMICS™ lysis solution (750 µL) was added to the tube. The tube was placed in a bead beater at maximum speed for 5 min and then centrifuged at 10 000 g for 1 min. The supernatant (400 µL) was added to a Zymo-Spin™ IV spin filter with a collection tube and centrifuged at 8 000 g for 1 min. ZymoBIOMICS™ DNA binding buffer (1200 µL) was added to the filtrate in the collection tube. Then 800 µL of the mixture was transferred to a Zymo-Spin™ IIIC-Z column with a collection tube and centrifuged at 10 000 g for 1 min. The filtrate was discarded. An additional 800 µL of the remaining mixture was transferred to a Zymo-Spin™ IIIC-Z coloumn with a collection tube and centrifuged at 10 000 g for 1 min. The filtrate was discarded. ZymoBIOMICS™ DNA wash buffer 1 (400 µL) was added to the Zymo-Spin™ IIIC-Z column with a new collection tube fitted and centrifuged at 10 000 g for 1 min. The filtrate was discarded. 700 µL of ZymoBIOMICS™ DNA wash buffer 2 was added to the Zymo-Spin™ IIIC-Z column and centrifuged at 10 000 g for 1 min. The filtrate was discarded. 200 µL of ZymoBIOMICS™ DNA wash buffer 2 was added to the Zymo-Spin™ IIIC-Z column and centrifuged at 10 000 g for 1 min. The filtrate was discarded and a 1.5 mL microcentrifuge tube fitted. ZymoBIOMICS™ DNase/RNase free water (100 µL) was added directly to the center of the membrane and incubated at room temperature for 1 min and then centrifuged at 10 000 g for 1 min to elute the DNA. The eluted DNA was transferred to a prepared Zymo-

Spin™ IV-HRC spin filter with a new 1.5 mL microcentrifuge tube fitted and centurfuged at 8 000 g for 1 min.

7.3.5.3. DNA extraction for Real-Time PCR

Microbial DNA extraction for RT-PCR was performed using the QIAamp UCP pathogen kit (Qiagen), according to the manufacturer's instructions (spin protocol). Fresh overnight broth culture (1.5 mL) was added to a 1.5 mL Eppendorf tube and centrifuged for 5 min at 13 000 g. The supernatant was discarded. An additional 1.5 mL of the overnight broth culture was transferred to the Eppendorf tube containing the pellet and re-centrifuged. The supernatant was again discarded to be left with a pellet. 40 µL proteinase K was added to the pellet and vortexed to mix for 10 s. The sample was then incubated at 56°C for 10 min. Then 200 µL of buffer APL2 was added and vortexed to mix for 30 s. The sample was then incubated at 70°C for 10 min. 300 µL of ethanol was then added and vortexed to mix for 30 s. 600 µL of the mixture was then transferred to a QIAamp UCP mini spin column with a 2 mL collection tube and centrifuged at 6 000 g for 1 min. The collection tube was discarded with the filtrate. 600 µL of buffer APW1 was added to the spin column with a new collection tube and centrifuged at 6 000 g for 1 min. The collection tube was discarded with the filtrate. Buffer APW2 (750 µL) was added to the spin column with a new collection tube and centrifuged at 13 000 g for 3 min. The collection tube was discarded with the filtrate. A new collection tube was fitted to the spin column and the sample was centrifuged again at 13 000 g for 1 min. The collection tube was discarded with the filtrate. A new collection tube was fitted to the spin column. The lid of the spin column was opened and left to dry at 56°C for 3 min until the membrane was dry. The collection tube was discarded and a 1.5 mL microcentrifuge tube was fitted to the spin column. Buffer AVE (50 µL) was added to directly to the center of the spin column membrane and incubated at room temperature for 1 min and then centrifuged at 13 000 g for 1 min to elute the DNA. An additional 50 µL of buffer AVE was added to the center of the spin column membrane and incubated at room temperature for 1 min and then centrifuged at 13 000 g for 1 min to elute the remaining DNA.

7.3.5.4. DNA concentration determination

Extracted DNA concentration and quality was determined using a spectrophotometer (Nanodrop-1000) according to the manufacturer's instructions, using the elution buffer as a blank.

7.3.6. Polymerase Chain Reaction

Polymerase chain reaction (PCR) was used to detect resistant genes in the bacterial isolates. The genes, primers and reaction conditions used in each reaction are listed in Tables 7.4 and 7.5. All reactions were performed in duplicate.

Polymerase chain reactions 3, 4 and 7 was performed in 25 µL volumes consisting of 1X One*Taq* Standard Reaction Buffer (New England BioLabs Inc), 0.2 µM each of forward and reverse primer (Inqaba

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Biotec), 1U One*Taq* Hot Start DNA Polymerase (New England BioLabs Inc), 200 µM dNTP Solution Mix (New England BioLabs Inc), 1 µL template DNA and the remaining volume distilled nuclease-free water.

Polymerase chain reactions 1, 2, 5 and 6 was performed in 25 µL volumes consisting of 1 unit of Ampliqon multiplex TEMPase 2x Master Mix (Lasec), 0.2 µM each of forward and reverse primer (Inqaba Biotec), 1 µL template DNA and the remaining volume distilled nuclease-free water (Inqaba Biotec).

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Table 7.4 PCR conditions for detection of resistant genes in *E. coli* isolates

PCR	Gene	Primers F: 5'-3' R: 5'-3'	bp	Reaction conditions	Reference	Positive control from this study
1	*tet*A	F: GGCGGTCTTCTTCATCATGC R: CGGCAGGCAGAGCAAGTAGA	502	15 min initial denaturation at 95°C followed by 35 cycles of 20s at 95°C, 40s at 66°C, and 40s at 72°C; and a final extension step of 4 min at 72°C.	Adapted from Boerlin *et al*., 2005	*E. coli* CA4c
1	*tet*B	F: CATTAATAGGCGCATCGCTG R: TGAAGGTCATCGATAGCAGG	930			*E. coli* CA4c
1	*tet*C	F: GCTGTAGGCATAGGCTTCCT R: GCCGGAAGCGAGAAGAATCA	888			-
2	*sul*1	F: CGGCGTGGGCTACCTGAACG R: GCCGATCGCGTGAAGTTCCG	433	15 min initial denaturation at 95°C followed by 30 cycles of 20s at 95°C, 40s at 66°C, and 40s at 72°C and a final extension step of 4 min at 72°C.	Adapted from Kozak *et al*., 2009	*E. coli* BB3a
2	*sul*2	F: CGGCATCGTCAACATAACCT R: TGTGCGGATGAAGTCAGCTC	721			*E. coli* BB3a
2	*sul*3	F: CAACGGAAGTGGGCGTTGTGGA R: GCTGCACCAATTCGCTGAACG	244			-
3	*bla* CMY	F: GACAGCCTCTTTCTCCACA R: TGGACACGAAGGCTACGTA	1000	15 min initial denaturation at 94°C followed by 30 cycles of 1 min at 94°C, 1min at 55 °C, and 1 min at 72°C and a final extension step of 10 min at 72°C.	Kozak *et al*., 2009	*E. coli* E1B2b
4	*aad*A	F: GTGGATGGCGGCCTGAAGCC R: AATGCCCAGTCGGCAGCG	525	15 min initial denaturation at 95°C followed by 35 cycles of 1 min at 94°C, and 1 min at 60°C and 1 min at 72°C and a final extension step of 7 min 72°C.	Adapted from Boerlin *et al*., 2005	*E. coli* E1B1b

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Table 7.5 PCR conditions for detection of resistant genes in *E. faecalis* and *S. aureus* isolates

PCR	Gene	Primers F: 5'-3' R: 5'-3'	bp	Reaction conditions	Reference	Positive control from this study
5	*tet*K	F: GATCAATTGTAGCTTTAGGTGAAGG R: TTTTGTTGATTTACCAGGTACCATT	1515	15 min initial denaturation at 95°C followed by 30 cycles of 95°C for 30sec, 62°C for 1min and 65°C for 1min and a final extension step of 72°C for 4min.	Adapted from Malhotra-Kumar *et al.*, 2005	*E. faecalis* I1aM
5	*tet*L	F: TGGTGGAATGATAGCCCATT R: CAGGAATGACAGCACGCTAA	229			*E. faecalis* I1aM
5	*tet*M	F: GTGGACAAAGGTACAACGAG R: CGGTAAAGTTCGTCACACAC	406			*E. faecalis* I1aM
6	*van*A	F: GGGAAAACGACAATTGC R: GTACAATGCGGCCGTTA	732	15min initial denaturation at 95°C followed by 30 cycles of 95°C for 30sec, 54°C for 1min and 72°C for 1min and a final extension step of 72°C for 4min.	Adapted from Depardieu *et al.*, 2004	*E. faecalis* S1d
6	*van*B	F: ACGGAATGGGAAGCCGA R: TGCACCCGATTTCGTTC	647			*E. faecalis* SB4c
7	*bla*Z	F: AAGAGATTTGCCTATGCTTC R: GCTTGACCACTTTTATCAGC	498	5min initial denaturation at 94°C followed by 35 cycles of 94°C for 30s, 55°C for 30s and 72°C for 10min and a final extension step of 72°C for 10min.	Russi *et al.*, 2015	*S. aureus* SB1aM

7.3.7. Gel Electrophoresis

Gel electrophoresis was performed using 1.2% agarose gel (SeaKem) stained with EZ-Vision® in-gel solution DNA dye (Amresco). Gels were run for 60-90 min at 85V. A 100 bp DNA ladder was used (New England BioLabs Inc). Gel visualisation was performed using the Bio-Rad Gel Doc XR+ System (Bio-Rad, South Africa) in combination with Image Lab Software V5.2.1.

7.3.8. Real-Time PCR analysis

Real-time PCR provides an added benefit over PCR as it yields not only qualitative data but also information about the quantity of the ABR gene (Luby *et al*., 2016). Real-time PCR was performed using the Rotor-Gene Q (Qiagen) according to the manufacturer's instructions on a selection of the *E. coli* extracted DNA samples and the Microbial DNA qPCR assay kit (Qiagen) for *aad*A1 and the Microbial DNA qPCR assay kit (Qiagen) for Pan Bacteria 1 to normalise the data (16s rRNA gene= housekeeping gene). The following equation was used to determine the difference in gene copy number between the phenotypically resistant, intermediately resistant and susceptible bacteria based on the relative fold gene expression formula ($2^{\Delta\Delta Ct}$) (Schmittgen & Livak, 2008; Qiagen, 2015):

$$2^{\Delta\Delta Ct} = \frac{2^{\Delta Ct_{group\ 2}}}{2^{\Delta Ct_{group\ 1}}}$$

Where: $\Delta Ct = Ct\ (gene\ of\ interest) - Ct\ (housekeeping\ gene)$

$group\ 2 =$ phenotypically resistant or intermediately resistant bacteria

$group\ 1 =$ phenotypically susceptible bacteria

7.4. Results and Discussion

7.4.1. *Enterococcus faecalis* and *Staphylococcus aureus*

The genotypic-phenotypic antibiotic resistance correlations of the *E. faecalis* and *S. aureus* isolates can be viewed in Table 7.6. The individual gel images can be viewed in Addendum A.

Ruppe *et al.* (2017) defines G+P- (positive genotypic, no phenotype) as a major error, G-P+ (no genotype, positive phenotype) as a very major error and G±P± (positive/ negative genotypic, positive/ negative phenotype) as a correct corresponding result. In this study, samples were categorised as G±P± if they were G+ or G- for intermediately resistant phenotypes.

Unexplained resistant phenotypes (G-P+) (provided all known resistant genes have been tested) can occur either (i) due to the presence of a novel resistance gene that has been unaccounted for, (ii) due to mutations in the promoter-attenuator regions of known resistant genes, resulting in a different sequence, and is thus not detected or (iii) insufficient PCR replication of each animal, resulting in false negative detection

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(Davies *et al.,* 2011; Luby *et al.,* 2016). In this study however, unexplained resistant phenotypes can also be due to the presence of a different ABR gene that was not detected.

Unexplained resistant genotypes (G+P-) can occur due to various reasons including; (i) the resistant gene is present in a dead cell or extracellular DNA, (ii) the gene is not expressed or (iii) the gene may have mutated to a non-functional form, effecting expression (Luby *et al.,* 2016). Davis *et al.* (2011) hypothesises that areas of low antibiotic use can sometimes lead to more G+P- strains, where the genes are inactive but are stable in the genome, or mutations have occurred in the genes that make it unable to express its resistance.

Table 7.6 Phenotypic- genotypic antibiotic resistant profiles of *E. faecalis* and *S. aureus** isolates

Location	Animal	Phenotypic resistance[a]	Genotypic resistance					
			*tet*L	*tet*K	*tet*M	*van*A	*van*B	*bla*Z
Witsand	Sheep 4	TE(S), VA(R), P (S)	-	-	-	+	-	-
Bredasdorp	Sheep 1	TE(I), VA(R), P (S)	-	-	-	+	-	-
Ekuseni	Buffalo 3	TE(S), VA(S), P (S)	-	-	-	-	-	-
Wellington 1	Buffalo 1	TE(I), VA(R), P (S)	-	-	-	+	-	+
Wellington 1	Buffalo 2	TE(I), VA(R), P (R)	-	-	-	+	-	+
Wellington 1	Buffalo 3	TE(I), VA(R), P (S)	-	-	-	+	-	-
Wellington 1	Buffalo 4	TE(R), VA(R), P (S)	-	-	+	+	-	-
Witsand	Deer 1	TE(R), VA(S), P (S)	-	-	-	-	+	-
Bredasdorp	Eland 3	TE(R), VA(S), P (S)	-	-	-	+	+	-
Modimolle 1	Impala 4	TE(S), VA(R), P (S)	-	-	-	+	-	-
Modimolle 3	Impala 3	TE(S), VA(R), P (S)	-	-	-	+	-	-
Modimolle 3	Impala 4	TE(S), VA(R), P (S)	-	-	-	+	-	-
Witsand	Springbok 1	TE(I), VA(R), P (R)	-	-	-	+	-	+
Witsand	Springbok 4	TE(S), VA(R), P (S)	-	-	-	-	+	-
Ekuseni	Wildebeest 4	TE(S), VA(R), P (S)	-	-	-	+	-	-
Modimolle 2	Wildebeest 2	TE(R), VA(S), P (S)	-	-	+	+	-	-
Modimolle 2	Wildebeest 3	TE(R), VA(R), P (S)	+	-	+	+	-	-
Wellington 1	Bontebok 4 (meat)*	TE(S), VA(S), P (R)	-	-	-	-	-	+
Modimolle 1	Impala 1 (meat)	TE(R), VA(S), P (S)	+	+	+	+	-	+
Modimolle 1	Impala 2 (meat)	TE(R), VA(S), P (S)	+	+	+	+	-	+
Modimolle 1	Impala 3 (meat)	TE(R), VA(S), P (S)	+	+	+	+	-	+
Modimolle 1	Impala 4 (meat)	TE(R), VA(S), P (S)	+	+	+	+	-	+
Modimolle 1	Impala 5 (meat)	TE(R), VA(S), P (S)	+	+	-	+	-	+
Witsand	Springbok 1 (meat)*	TE(S), VA(S), P (R)	+	-	-	+	-	+
Witsand	Springbok 2 (meat)*	TE(S), VA(S), P (R)	+	-	-	-	-	+
Witsand	Springbok 4 (meat)*	TE(S), VA(S), P (R)	-	-	-	-	-	+
Witsand	Springbok 3 (meat)*	TE(S), VA(S), P (R)	-	-	-	-	-	+

[a] TE: tetracycline; VA: vancomcyin; P: penicillin

S, susceptible; I, intermediate; R, resistant

**S. aureus* isolates (all others are *E. faecalis*)

From Table 7.6, it can be inferred that 7% were G+P-, 7% were G-P+ and 86% G±P± for tetracycline (TE). Furthermore, of the intermediately resistant phenotypes, 100% were G-. This implies that the tetracycline 30 μg disc diffusion AST is an accurate method for detection of tetracycline resistant *Enterococci* and *Staphylococci*.

There are two main mechanisms of tetracycline resistance that have been documented in *Enterococci* and *Staphylococci* (Huys *et al*., 2004; Fluit *et al.,* 2005). These are, efflux pumps, encoded by *tet*K and *tet*L and production of a ribosomal protection protein, encoded most commonly by *tet*M but also the *tet*O and *tet*S genes (Huys *et al*., 2004). Efflux pumps actively pump the antibiotic out of the cell, thereby decreasing the antibiotic concentration to a sub-lethal level, rendering it ineffective. Ribosomal protection proteins interact with the cells ribosome and promote release of bound antibiotic compounds (Emaneini *et al.,* 2013). Resistant strains most often carry the *tet*M gene, as also found in this study, and are known to be resistant to all tetracycline drugs (Malhotra- Kumar *et al*., 2005; Nishimoto *et al.,* 2005). In this study, *tet*K was detected in 26% of the G+ samples, *tet*L in 35% and *tet*M in 39%.

Resistance to vancomcyin (VA) was reported as 33% which were classified as G+P-, 0% as G-P+ and 67% as G±P±. Currently, there are nine vancomycin resistance clusters that have been found in enterococci, *van*A, *van*B, v*an*C, *van*D, *van*E, *van*G, *van*L, *van*M and v*an*N (Miller *et al*., 2014). These clusters differ by having different amino acids on the ligase enzyme. Of these clusters, the *van*A cluster has been the most common resistance gene in vancomycin resistant enterococci, followed by *van*B (Périchon & Courvalin, 2009; Miller *et al*., 2014). Also, *van*A confers the highest resistance to vancomycin as well as to another glycopeptide known as teicoplanin. Whereas *van*B has variable levels of resistance to only vancomycin (Courvalin, 2006). In this study, *van*A was more commonly detected (86%) than *van*B (14%). Moreover, research has shown that the *van*A gene, which is the most common vancomycin resistant gene, has been detected in nature, possibly due to the natural production of vancomycin in nature by Actinomycetes (Périchon & Courvalin, 2009; Lo Grasso *et al*., 2016). This could explain the origin of the small percentage of vancomycin resistant *Enterococci* found in the wildlife species.

The high minor error rate correlation (G+P-) of 33% can be attributed to the unreliability of the vancomycin disc diffusion method, as suggested by the Clinical and Laboratory Standards Institute (CLSI) of 2016 and European Committee on Antimicrobial Susceptibility Testing (EUCAST) of 2017. Thus, confirmation of vancomycin resistant isolates is required for official reporting and can be achieved using PCR for detection of the *van*A gene or by using the minimum inhibitory concentration (MIC) method. The high minor error rate (Table 7.5) indicates that the vancomycin disc diffusion test can often give false negative results.

In addition, the antibiotic resistance profiles towards penicillin (P) were 30% for G+P-, 0% G-P+ and 70% G±P± (Table 7.6). Resistance to β-lactams is achieved by the presence of β-lactamase enzymes, which inactivates the antibiotic through hydrolysis of the β-lactam ring rendering the drug inactive (Lowy, 2003; Jensen & Lyon, 2009). The most common gene encoding production of beta-lactamases to hydrolyse penicillin is the *bla*Z gene (Bagcigil *et al*., 2012).

The CLSI recommends that detection of the *bla*Z gene should be used in phenotypically penicillin susceptible strains in cases of serious infection that requires penicillin therapy (Pereira *et al*., 2014; Ruppe *et al*., 2017). This is because the phenotypic detection method has been shown to have a sensitivity rating of about 70%, explaining the 30% G+P- correlation found in this study (Table 7.5) which indicates that PCR is a more sensitive method than agar-based culture methods.

Antibiotic resistance to β-lactam antibiotics is the most common form of resistance documented in pathogenic microorganisms, which is likely to be acquired from the natural antibiotic producer microorganisms (Davies, 1994). As a result, the soil is known to be a reservoir of β- lactamase genes which could be the source of the *bla*Z gene detected in the wildlife species in this study (Cantas *et al.,* 2013). However, most of the *bla*Z positive isolates were of game meat origin and detected mostly in the *S. aureus* isolates, suggesting possible contamination of human DNA during the slaughter process, seeing as penicillin-susceptible *S. aureus* rates are as low as 5- 20% in human clinical isolates (Pereira *et al*., 2014).

7.4.2. *Escherichia coli*

7.4.2.1. Qualitative PCR

The genotypic-phenotypic antibiotic resistance correlations of the *E. coli* isolates can be viewed in Table 7.7. The individual gel images can be viewed in Addendum A.

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Table 7.7 Correlation between *E. coli* phenotypic antibiotic resistance and PCR results.

Location	**Animal**	**Phenotypic resistance**[b]	**Genotypic resistance**							
			***bla* CMY**	***sul* 1**	***sul* 2**	***sul* 3**	***tet* A**	***tet* B**	***tet* C**	***aad* A1**
Bredasdorp	Cattle 1	AMP(I), SF(S), TE(S), ST(R)	-	-	-	-	-	-	-	+
Bredasdorp	Cattle 10	AMP(I), SF(S), TE(S), ST(I)	-	-	-	-	-	-	-	+
Bredasdorp	Cattle 12	AMP(R), SF(S), TE(S), ST(I)	+	-	-	-	-	-	-	-
Witsand	Cattle 4	AMP(I), SF(S), TE(R), ST(I)	-	-	-	-	+	+	-	+
Witsand	Cattle 5	AMP(I), SF(R), TE(I), ST(R)	-	-	-	-	-	+	-	+
Bredasdorp	Sheep 4	AMP(S), SF(S), TE(I), ST(R)	-	-	-	-	-	-	-	+
Bredasdorp	Sheep 11	AMP(S), SF(S), TE(S), ST(I)	+	-	-	-	-	-	-	+
Witsand	Sheep 3	AMP(R), SF(S), TE(I), ST(I)	+	-	-	-	-	-	-	+
Witsand	Sheep 5	AMP(I), SF(S), TE(R), ST(I)	-	-	-	-	-	-	-	+
Sutherland	Sheep 2	AMP(S), SF(S), TE(S), ST(R)	-	-	-	-	-	-	-	+
Witsand	Blesbok 2	AMP(I), SF(R), TE(R), ST(R)	-	+	+	-	-	+	-	+
Witsand	Blesbok 3	AMP(S), SF(R), TE(R), ST(R)	-	+	+	-	-	+	-	+
Witsand	Deer 1	AMP(I), SF(S), TE(S), ST(R)	-	-	-	-	-	-	-	+
Wellington 1	Bontebok 1	AMP(R), SF(I), TE(I), ST(I)	+	-	-	-	-	-	-	+
Wellington 1	Bontebok 3*	AMP(R), SF(S), TE(S), ST(S)	+	-	-	-	-	-	-	-
Wellington 1	Bontebok 4	AMP(R), SF(S), TE(S), ST(I)	+	-	-	-	-	-	-	+
Wellington 1	Bontebok 5	AMP(R), SF(S), TE(S), ST(I)	+	-	-	-	-	-	-	+
Wellington 1	Buffalo 1	AMP(S), SF(S), TE(S), ST(R)	+	-	-	-	-	-	-	+
Wellington 1	Buffalo 2	AMP(R), SF(S), TE(R), ST(R)	+	-	-	-	+	-	-	+
Wellington 1	Buffalo 3	AMP(I), SF(I), TE(I), ST(R)	+	-	-	-	-	-	-	+
Wellington 1	Buffalo 4	AMP(R), SF(S), TE(R), ST(R)	+	-	-	-	-	+	-	+
Wellington 1	Buffalo 5	AMP(R), SF(R), TE(S), ST(R)	+	-	+	-	-	-	-	+
Wellington 2	Buffalo 1	AMP(S), SF(I), TE(I), ST(R)	+	-	-	-	-	-	-	+
Wellington 2	Buffalo 5	AMP(S), SF(S), TE(S), ST(I)	+	-	-	-	-	-	-	+
Bredasdorp	Eland 1	AMP(S), SF(S), TE(S), ST(R)	+	-	-	-	-	-	-	+
Bredasdorp	Eland 2	AMP(S), SF(R), TE(S), ST(I)	-	-	+	-	-	-	-	+
Bredasdorp	Eland 4	AMP(S), SF(S), TE(I), ST(R)	-	-	-	-	+	-	-	+
Bredasdorp	Impala 1	AMP(S), SF(S), TE(S), ST(I)	+	-	-	-	-	-	-	+
Ekuseni	Impala 2	AMP(S), SF(S), TE(S), ST(I)	+	-	-	-	-	-	-	-
Modimolle 1	Impala 1*	AMP(R), SF(S), TE(S), ST(S)	+	-	-	-	-	-	-	-
Modimolle 1	Impala 2*	AMP(S), SF(S), TE(S), ST(S)	-	-	-	-	-	-	-	-
Modimolle 1	Impala 4*	AMP(S), SF(S), TE(S), ST(I)	-	-	-	-	-	-	-	+
Modimolle 3	Impala 3	AMP(I), SF(I), TE(I), ST(R)	-	-	-	-	-	-	-	+
Witsand	Springbok 1*	AMP(S), SF(S), TE(S), ST(R)	+	-	-	-	-	-	-	+
Witsand	Springbok 4*	AMP(S), SF(S), TE(S), ST(I)	+	-	-	-	-	-	-	+
Sutherland	Springbok 2	AMP(S), SF(S), TE(I), ST(I)	-	-	-	-	-	-	-	+
Bredasdorp	Wildebeest 1	AMP(S), SF(S), TE(S), ST(R)	+	-	-	-	-	-	-	+
Bredasdorp	Wildebeest 2	AMP(S), SF(R), TE(I), ST(R)	-	-	+	-	-	-	-	+
Bredasdorp	Wildebeest 3	AMP(S), SF(R), TE(S), ST(I)	+	-	+	-	+	+	-	-
Bredasdorp	Wildebeest 5	AMP(S), SF(S), TE(I), ST(R)	+	-	-	-	-	-	-	+
Modimolle 2	Wildebeest 1	AMP(S), SF(S), TE(I), ST(R)	-	-	-	-	-	-	-	+
Modimolle 2	Wildebeest 4	AMP(I), SF(I), TE(I), ST(R)	+	-	-	-	-	-	-	+
Wellington 2	Wildebeest 2	AMP(S), SF(I), TE(I), ST(I)	-	-	-	-	-	-	-	+
Wellington 2	Wildebeest 3	AMP(S), SF(I), TE(S), ST(R)	+	-	-	-	-	-	-	+

[b] AMP, ampicillin; SF, sulphonamide; TE, tetracycline; ST, streptomycin

S, susceptible; I, intermediate; R, resistant

*Meat samples (all others are faecal samples)

The genotype-phenotype antibiotic resistance correlations shown in Table 7.7 for ampicillin (AMP) is 25% G+P-, 0% G-P+ and 75% G±P±. Beta- lactam antibiotic resistance in *E. coli* is primarily mediated by the production of β-lactamase enzymes which inactivate the antibiotic (Briñas *et al*., 2002). Over 200 β-lactamases have been identified, of which the TEM-1, TEM-2 (*bla*TEM gene), CTX-M (*bla*CTX-M gene), SHV-1 (*bla*SHV gene) and CMY-2 (*bla*CMY-2 gene) enzymes are the most common in *E. coli* (Briñas *et al*., 2002; Aslam *et al*., 2009; Touzain *et al*., 2018). Other studies have reported highly accurate (98.6-100%) prediction rates for ampicillin antibiotic susceptibility testing (Ruppe *et al*., 2017). Önen *et al*. (2015) and Shaheen *et al.* (2011) also found a high correlation between ampicillin resistance and the *bla*CMY-2 gene. The 2% major error rate is likely due to the presence of a different β-lactamase encoding gene which was not detected. Shaheen *et al.* (2011) found that the *bla*CTX-M was the most common β-lactamase detected in *E. coli*, followed by the *bla*CMY-2 gene and then *bla*TEM. Furthermore, of the intermediately resistant phenotypes, only 10% were G+. This highlights the importance of confirming phenotypically intermediately resistant isolates using an alternative method.

Sulphonamide (SF) resistant bacteria have been reported since the 1930s, when sulphonamide resistant *Streptococcus pyogenes* was first reported in clinical settings (Cantas *et al*., 2013; Berglund, 2015). Since then, sulphonamide resistance genes are widespread in the environment (Berglund, 2015). In this study however, a low number of sulphonamide resistant genes were detected. The antibiotic resistance profiles for sulfafurazole were 0% G+P-, 2% G-P+ and 98% G±P±. Furthermore, of the intermediately resistant phenotypes, 100% were G-. This implies that the sulfafurazole 300µg disc diffusion antibiotic susceptibility test (AST) is a reliable method for detection of sulphonamide resistant *E. coli.* Boerlin *et al*. (2005) also found a high genotype-phenotype correlation for sulphonamide resistance using the microdilution method and detection of *sul*1, *sul*2 and *sul*3.

The acquisition of altered target enzymes, which act as competitive inhibitors of dihydropteroate synthetase, known as dihydropteroate synthases, is the most common mechanism with which *E. coli* acquire resistance to sulphonamides (Frank *et al*., 2007; Geirgopapadakou, 2008). There are three genes which encode for three types of these enzymes that have been characterised in Gram negatives, namely *sul*1, *sul*2 and *sul*3. In this study, the *sul*2 gene was the most commonly detected *sul* gene and no *sul*3 genes were detected. Wang et *al*. (2014) and other studies detected *sul* genes in sulphonamide resistant *E. coli* in the same frequencies of *sul*2 > *sul*1 > *sul*3 (Blahna *et al*., 2006; Hoa *et al*., 2008). However, some other studies have found trends of *sul*1 > *sul*2 > *sul*3 (Arabi *et al.,* 2015).

For tetracycline (TE), 2% were G+P-, 2% were G-P+ and 96% were classified as G±P±. Boerlin *et al*. (2005) also found a high genotype-phenotype correlation for tetracycline resistance using the microdilution method and detection of *tet*A, *tet*B and *tet*C. Furthermore, of the intermediately resistant phenotypes, 86% were G-. Resistance to tetracycline in *E. coli* is usually acquired by genes *tet*A-E located on plasmids which encode for efflux pump proteins. Efflux pumps actively pump out the antibiotic compound which results in a lower intracellular concentration that is no longer bacteriocidal (Geirgopapadakou, 2008). However, other resistant genes have also been detected in tetracycline resistant *E. coli* isolates, although generally at much

lower frequencies, and include *tet*K and *tet*M. In this study, no *tet*C genes were detected, and the *tet*A and *tet*B genes were detected in equal frequencies. Bryan *et al*. (2004) found that 97% of tetracycline resistant *E. coli* harboured at least one *tet* gene from a selection of fourteen known *tet* genes; *tet*A and *tet*B have been the most frequently detected *tet* genes in other studies (Jurado-Rabadán *et al*., 2014; Mercat *et al*., 2016).

Lastly, Table 7.7 shows that 0% were G+P-, 0% were G-P+ and 100% G±P± for streptomycin (ST). Boerlin *et al*. (2005) on the hand, found a low (66%) genotype-phenotype correlation for streptomcyin resistance using the microdilution method and detection of *aad*A *and str*A/*str*B. This was mainly due to the presence of the *aad*A gene in streptomycin susceptible isolates, as also found in other studies (Kozak *et al.,* 2000; Boerlin *et al*., 2005). This finding explains why most isolates (86%) in this study that were classified as intermediately resistant to streptomycin had the *aad*A gene. Research has determined that the *str*A-*str*B gene pair and the closely related *aad*A gene cassette are the most common resistant determinates that give *E. coli* resistance to streptomycin (Sunde & Norström, 2005). The *aad*A gene cassette, detected in this study, encode for aminoglycoside adenyltransferases which are enzymes that inactivate streptomycin and spectomycin (Sunde & Norström, 2005). Davis *et al.* (2011) found that streptomycin G+P- isolates were due to nonsynonymous changes in the *aad*A gene, rendering it inactive.

The development and transfer mechanisms of antibiotic resistance has proven to be a complex occurrence (Wellington *et al*., 2013). This is especially true in natural environments, where microbial ecosystems are highly diverse. The increased prevalence of antibiotic resistance has been blamed by some on the overuse of antibiotics. However, the origin of most antimicrobial resistance genes seems to reside from naturally occurring antibiotic synthesising organisms; alternatively, they are native to organisms where the resistant gene has a physiological function but is "silent" in the sense of not showing a detectable form of resistance, since their function is to protect the hosts' own metabolism (Gilmore *et al*., 2008). This phenomenon has indeed been demonstrated in this study, where resistant genes were detected in the bacteria originating from the more isolated animals with little human contact, such as the free-roaming wildlife as well as the organic livestock. Numerous other studies have also detected ABR genes in a diverse range of environments not considered to be exposed to antimicrobials (Miteva *et al*., 2004; Costa *et al*., 2008; Allen *et al*., 2011; Bhullar *et al.,* 2012; Agga *et al*., 2015; Carroll *et al*., 2015).

7.4.2.1. Real- Time PCR

Relative quantification of the *aad*A1 gene (Qiagen Microbial DNA qPCR assay kit for *aad*A1) in a selection of the streptomycin non-susceptible *E. coli* isolates was determined against the streptomycin susceptible isolates, using the 16s rRNA gene (Qiagen Microbial DNA qPCR assay kit for Pan Bacteria 1) to normalise the data (Table 7.8). The *aad*A1 gene encodes aminoglycoside adenylyl transferase enzymes which causes streptomycin and spectinomycin resistance by modification of the antibiotic by adenylylation (Recchia & Hall, 1995).

Table 7.8 The fold gene expression level of the *aad*A1 gene relative to the bacterial housekeeping gene (16s rRNA) in *E. coli* compared to the streptomycin phenotypic resistance level

Location	**Animal**	**Sample type**	**Group**	**Phenotypic resistance**	***aad*A1 gene***	$2^{\Delta\Delta Ct}$
Bredasdorp	Sheep 11	Faecal	2	Intermediate	+	$1.3x10^5$
Bredasdorp	Eland 1	Faecal	2	Resistant	+	64
Bredasdorp	Impala 1	Faecal	1	Susceptible	+	1
Wellington 1	Buffalo 3	Faecal	2	Resistant	-	0.02
Wellington 1	Buffalo 4	Faecal	2	Resistant	+	20
Wellington 1	Buffalo 5	Faecal	2	Resistant	+	$6.8x10^6$
Wellington 2	Buffalo 1	Faecal	2	Intermediate	+	60
Wellington 2	Wildebeest 3	Faecal	2	Intermediate	+	739
Modimolle 2	Wildebeest 4	Faecal	2	Resistant	-	0.04
Witsand	Springbok 1	Meat	1	Susceptible	+	1

*Gene detected using qualitative PCR

Samples showing a relative fold change ($2^{\Delta\Delta Ct}$) of less than one indicates that gene expression is down regulated, while a relative fold change of greater than one indicates up regulation where at least a five-fold change is considered significant (Qiagen, 2015). Up-regulation occurs when a cell is triggered by a signal and results in increased expression of a gene and thus increased protein encoded by the gene/s. Down-regulation results in a decrease of gene expression.

Buffalo 3 (Wellington 1) and wildebeest 4 (Modimolle 2) samples (Table 7.8) had a relative fold change value of less than one, indicating that the expression of the *aad*A1 gene was down regulated. This is consistent with the qualitative PCR results, as the *aad*A1 gene was not detected in these samples. These samples were phenotypically streptomycin resistant but were not positive for the *aad*A1 gene. It is therefore likely that they harboured a different streptomycin resistant gene, such as the *str*A-*str*B gene pair, which is one of the most prevalent streptomycin resistant genes found in *E. coli* (Karczmarczyk *et al*., 2011). The remaining samples had relative fold changes greater than one, ranging from 20 to 68 x 10^6 fold increases and thus exhibited up regulation of the *aad*A1 gene. This also agrees with the qualitative PCR results, as all the samples tested positive for the *aad*A1 gene.

The expression of antibiotic resistance appears to be frequently regulated, as the acquisition of antibiotic resistant mechanisms usually involves a fitness cost to the bacterial host (Depardieu *et al*., 2007). Gene expression regulation allows the bacterium to respond to a changing environment and can involve mutations and/ or the movement of mobile genetic elements (Depardieu *et al*., 2007).

The *aad*A1 gene has been frequently found as part of a gene cassette on class I integrons in *E. coli* (Ponce-Rivas *et al.,* 2012). Integrons are genetic units that can express and mobilise genes and are therefore involved in the horizontal gene transfer and dissemination of antibiotic resistant genes (Lindstedt *et al*., 2003; Engelstädter *et al.,* 2016; Kheiri & Akhtari, 2016). The expression of a gene cassette is principally controlled by the P_c promoter which enables transcription of the gene cassettes. Gene expression is influenced by its proximity to the promoter, where expression decreases with distance from the P_c promoter (Recchia & Hall, 1995). A gene cassette can remain silent if there is no promoter available or if it is too distant from the promoter (Engelstädter *et al*., 2016). When a selective pressure, such as an increased antibiotic concentration, is exerted onto a population then the corresponding resistance gene cassette at the first position within the integron is selectively favoured (Engelstädter *et al*., 2016). This is an important mechanism of relevance to this study.

It has been hypothesised that numerous antibiotic resistant genes found in pathogens originate from the antibiotic producer organisms in the environment. More specifically, it is hypothesised that the aminoglycoside inactivating enzymes found in gram-negative pathogens originate from Actinobacteria (which produce aminoglycoside antibiotic compounds) through HGT of the genes which encode these enzymes (Jiang *et al*., 2017). In most cases, the gene clusters containing the biosynthesis of the antibiotic compounds also contain the accompanying resistant genes for self-protection and thus there is a large reservoir of antibiotic resistant genes which can be transferred to other bacteria. Thus it is postulated that the high abundance of the streptomycin resistant gene, *aad*A1 found in the *E. coli* of the wildlife and livestock species in this study is likely to have originated from the Actinobacteria in the soil environment due to the grazing nature of these animal species.

7.5. Conclusion

Minor inconsistencies in genotypic and phenotypic detection of antibiotic resistance and the different limitations of each method demonstrates that complementary methods involving both molecular and phenotypic approaches provide the best detection of antibiotic resistant bacteria in a given environment, where each method can be used to validate the other.

Most samples that were phenotypically intermediately resistant to an antibiotic, were found to be genotype negative, except for the streptomycin disc diffusion test where the opposite correlation was found. Most genes were detected with a correct phenotypic correlation (G±P±), with an average of 84%. Very major errors (G-P+) were detected on average at 2%. Major errors (G+P-) were detected on average at 14%, suggesting that PCR is a more sensitive method than the disc diffusion method for ABR detection. This could be due to the fact that the samples used in this study originated from environments of low antibiotic use, possibly resulting in the presence of inactive genes, which have the potential to become active if routinely exposed to an antibiotic selective pressure.

There was a fairly high major error rate for the wildlife species (27 of 60 wildlife species tested had atleast one major error result), where the resistant gene is present but there is no phenotypic expression. This

could suggest that bacteria from the wildlife species harbour a vast array of antibiotic resistant genes, which can be 'switched on' when a selective pressure is applied to the environment. Thus, the results from this study agree with the hypothesis that wildlife can be seen as a reservoir of antibiotic resistant genes.

The very major error rate (G-P+) could potentially be lowered by the inclusion of more antibiotic resistant genes during gene detection, as this study only included a limited number of resistant genes that have been shown to cause phenotypic resistance. This could be more effectively achieved by the use of Whole Genome Sequencing (WGS), where all known antibiotic resistant genes in the bacteria's genome can be detected simultaneously. Genotypic ABR could then be confirmed with phenotypic methods.

Quantitative PCR using the microbial DNA assays proved to be a reliable and rapid method for identifying and profiling antibiotic resistant genes and correlated well with the qualitative PCR results. Ideally, a microbial DNA array can be used to predict microbial antibiotic resistance as it covers a range of antibiotic resistant genes in one array. The quantitative microbial DNA array method (real-time PCR) is recommended for future research, as analysis of antibiotic resistance is achieved via detection of an array of resistant genes, as well as the expression thereof, which indicates phenotypic patterns.

Knowledge of the varied mechanisms of antimicrobial resistance as well as the vectors involved in spreading antimicrobial resistance and ways to circumvent them is essential for developing robust antibacterial therapies against potential resistant pathogens.

7.6. References

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CHAPTER 8

General Discussion & Conclusions

The fight against antimicrobial resistance is part of the global One Health initiative which aims to improve future human, animal and environmental health through interdisciplinary collaborations and communications (Anon., 2017a). The schematic in Figure 8.1 outlines the campaign's key variables for understanding where antimicrobial resistance (indicated with an arrow) fits into the plans for improving global health.

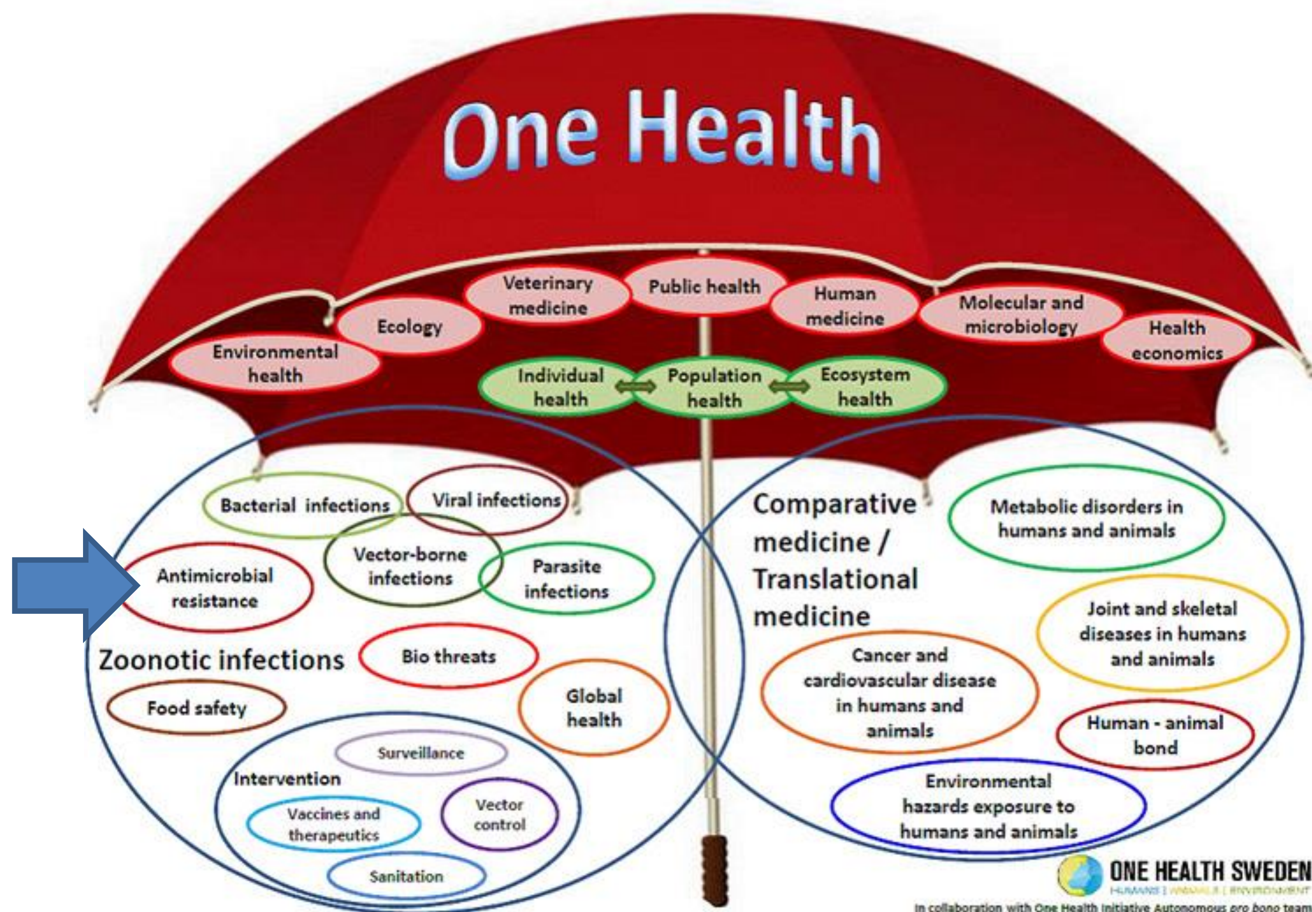

Figure 8.1 The One Health initiative schematic (Anon., 2017a).

Regarding improving global health levels and patterns, the World Health Organisation (WHO) has outlined the top ten pathogens that are of critical concern to human and animal health with regards to antibiotic resistance (ABR) development, namely, *Pseudomonas aeruginosa, Enterobacteriaceae, Acinetobacter baumannii, Enterococcus faecium, Staphylococcus aureus, Helicobacter pylori, Campylobacter, Salmonellae* and *Neisseria gonorrhoeae* (Anon., 2017b). In this study, the antibiotic resistance prevalence of *E. coli, Enterococcus* and *Staphylococcus* was investigated.

Antimicrobial resistance has increased dramatically in recent years (Bisht *et al.,* 2009). In addition, the future of human health is threatened by the anticipated growth of ABR. This scenario will have three main consequences, namely, higher rates of mortality in patients who have resistant infections, longer duration of infections and a reduced ability to perform major common procedures such as caesareans and organ transplants due to the increased risk of infection (Moellering, 1998; Bisht *et al.,* 2009; Laxminarayan *et al.,* 2013). Alternative therapies to antibiotics are often more expensive, less effective and more toxic (WHO, 2011). Ultimately, the rise in resistance, coupled with a decline in new antimicrobial therapy, could result in a post antibiotic era, where the use of antibiotics will no longer be effective (Laxminarayan *et al.,* 2013). This would be a global catastrophe for human health.

Antibiotic resistance and its development and transfer has been proved to be a complex occurrence (Laxminarayan *et al.,* 2013; Wellington *et al*., 2013). This is especially true in natural environments, where microbial ecosystems are highly diverse. Figure 8.2 illustrates that animals, humans, and animal products are interconnected through direct contact, the environment and the food chain.

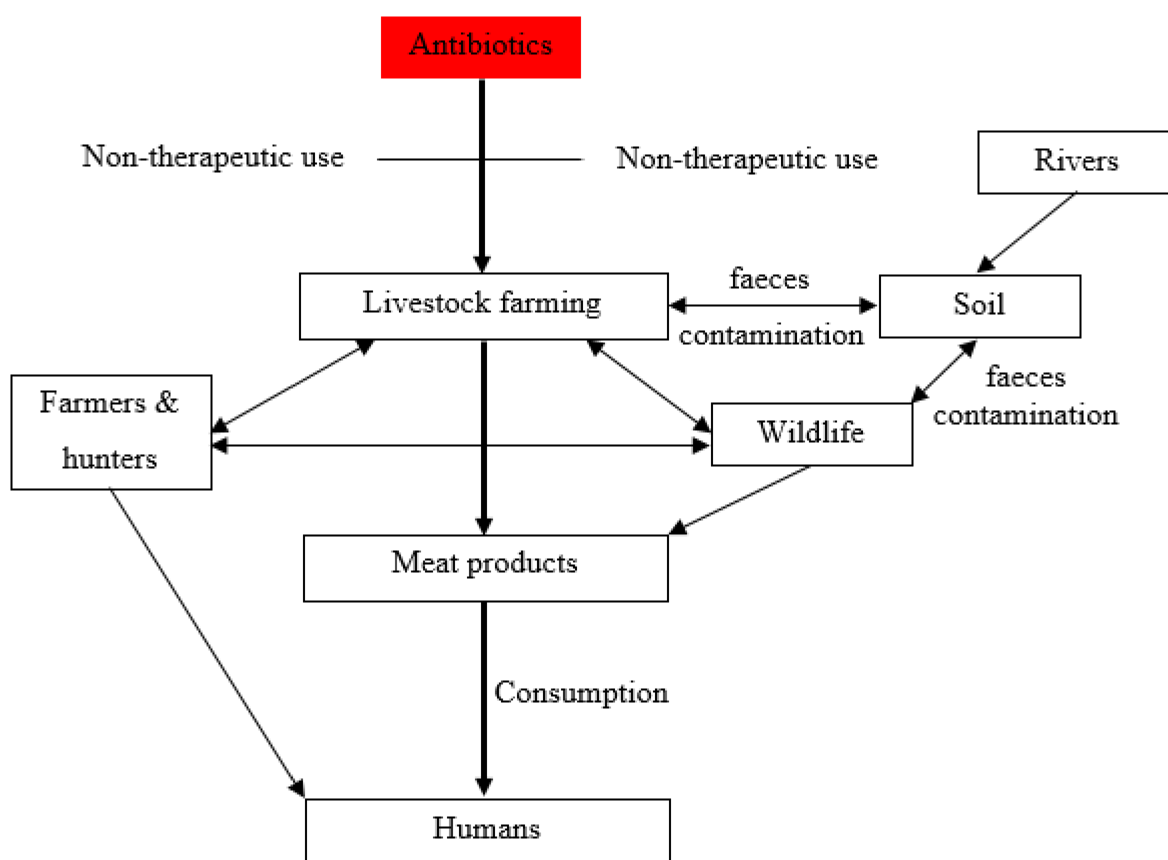

Figure 8.2 The epidemiology of antibiotic use with a focus on livestock and wildlife farming (adapted from WHO, 2011).

The development of antibiotic resistance has been blamed on the extensive use of antibiotics. However, the origin of most antimicrobial resistance genes is derived from naturally occurring antibiotic-synthesising organisms as well as in organisms where the resistant gene actually has a physiological function for protecting

the host's metabolism and so is generally undetectable (Gilmore *et al*., 2008). Antibiotic resistance genes have long been present in nature, and those residing on mobile genetic elements can be easily transferred via horizontal gene transfer (HGT) to commensal and pathogenic bacteria. This phenomenon is further enhanced by external activities that have the potential to influence the movement of resistant genes from various reservoirs.

Microbial ecosystems in nature are not isolated and thus extensive gene exchange occurs in natural systems. This was noted in Chapter 7, for example. The transfer and stimulation of resistant bacteria in nature is promoted by the presence of antibiotic concentrations greater than that which would normally occur in nature (Krümmerer, 2004; Modi *et al.,* 2014). Consequently, exposure to sub-inhibitory antibiotic concentrations, due to the release of antibiotic compounds into the environment, increases the speed at which resistant strains are selected (Krümmerer, 2004; Modi *et al.,* 2014). In particular, the creation of a selective pressure by the use of antibiotics can enter natural environments through waste water effluent, agricultural run-off, among others and result in a large pool of resistant genes (Zhang *et al*., 2006; Modi *et al.,* 2014; Yoneyama & Katsumata, 2014; Dias *et al.*, 2015). Just as climate change, for example, seems to have been exacerbated by human interventions and actions, so too in the field of ABR, studied in this research, have human and social factors come into play in the causation of spreading ABR. But this study has shown that the causation matrix of ABR is complex, with a combination of natural and man-made causes at play in the development and spread of ABR.

Whilst the majority of scientific research has focussed on the fate of antibiotics in clinical settings, research on the impacts of antibiotics in natural environments has been comparatively limited (Martínez, 2008). Additionally, surveillance of antibiotic resistant bacteria in the animal food chain is lacking in developing countries such as South Africa (Levy, 1998). Therefore, research in this area is key to assess the antimicrobial situation in South Africa in order to develop a strategy plan.

The ABR levels of the bacteria isolated from the wildlife and livestock species in this study had an overall fairly low resistance level to the antibiotics selected for testing (8% *E. coli*, 4% *E. faecalis* and 22% *S. aureus*) (Chapters 4, 5 and 6). It should be noted, however, that due to the complexity and diversity of the natural microbiome and intestines, the culturable bacteria only represent a small fraction of the complete microbiome (Krümmerer, 2004). Overall, very low to negligible resistance was detected towards the antibiotics, ceftazadime, chloramphenicol and nalidixic acid. Thus it can be hypothesised that there is no significant selective pressure of these antibiotics present in the farming environments used in this study. Other studies have also reported very low resistance levels towards these antibiotics in wild animals (Rolland *et al*., 1985; Lillehaug *et al*., 2005; Costa *et al*., 2008; Silva *et al*., 2010).

The bacteria isolated from the wildlife and livestock species in this study, were most commonly resistant to the β-lactam and streptomycin antibiotics (Chapters 4, 5 and 6). These antibiotics are produced naturally in the environment by fungi and bacteria in the soil. They are commonly used in agriculture and human medicine and therefore have shown widespread resistance worldwide (Allen & Donato, 2010; Laxminarayan *et al.,* 2013; Berglund, 2015). The accompanying antibiotic resistant genes serve many other purposes besides their resistance to antibiotics. Indeed, they play a part in the dynamics of the bacterial

population such as interspecies signalling (Krümmerer, 2004; Modi *et al.,* 2014). These resistant genes have been present for thousands, perhaps even millions of years, long before the use of antibiotics began (Modi *et al.,* 2014). Future work could involve testing the antibiotic susceptibility patterns of wildlife species using a more extensive range of different antibiotics. This would give a more comphrehensive overview of the antibiotic susceptibility patterns of the wildlife species which could give a better understanding on the various factors which could influence ABR in wildlife.

Although there are some naturally occurring antibiotic resistant bacteria in wildlife and nature, this study demonstrated that there are various farming practices and situations which can potentially increase the ABR of bacteria from wildlife species. For example, this study revealed that the practice of wildlife and livestock co-grazing creates a bi-directional transfer vector through the sharing of pastures and water points, where bacteria and their antibiotic resistant genes can be exchanged (Chapter 4). The *E. coli* isolates from the non-co-grazing livestock and wildlife had significant differences in their antibiotic susceptibility patterns, whilst the *E. coli* and *S. aureus* isolates from the wildlife and livestock which co-grazed together showed no significant differences in antibiotic resistance patterns.

However, it is recommended to confirm this theory by performing antibiotic susceptibility tests on samples from a controlled farm experiment where the separation and co-grazing of wildlife and livestock is strictly controlled. To prevent the transfer of antibiotic resistant and disease-causing bacteria between the livestock and wildlife interface, it is suggested to minimise contact between livestock and wildlife by using fences and proper disease control of domestic animals. Controlling diseases will help prevent the transfer and spread of pathogenic bacteria, which, in turn, helps prevent the spread of antibiotic resistant bacteria. Moreover, controlling diseases should also lessen the use of antibiotics, which will help prevent the development of antibiotic resistant bacteria. It is also suggested to minimise animal-human contact as much as possible. Furthermore, reducing herd density will help to prevent the spread of antibiotic resistant and disease-causing bacteria between animals.

Furthermore, it was found that the wildlife bacterial isolates were more multidrug resistant compared to the livestock isolates (Chapter 4). This ties in with previous studies, which suggest that wildlife can be considered a reservoir of antibiotic resistant genes (Cole *et al*., 2005; Jeters *et al.,* 2009; Guenthler *et al*., 2010; Wellington *et al.,* 2013; King & Schmidt, 2017). The higher multidrug resistance seen in the wildlife isolates compared to the livestock isolates can be caused by a variety of factors. Firstly, the wildlife species could pick up a vast array of naturally occurring bacteria from the soil, where antibiotic resistant bacteria and antibiotic resistant genes are present, although at low concentrations. The livestock, on the other hand, are kept in smaller areas and their feed is controlled. This possibly creates a more selective environment, which favours the development of resistance to the antibiotics commonly used in the livestock farming, which were tetracycline and ampicillin in this study. Secondly, wildlife species typically have a longer lifespan than livestock (however, this is dependent on the frequency of hunting and culling practised by the particular game farm) which could allow more time for antibiotic resistant bacteria to evolve and exchange genetic material in the intestines and thus develop into multidrug resistant bacteria.

More specifically, the *E. coli* isolates from the livestock species were significantly more resistant than the wildlife species against ampicillin and tetracycline - both antibiotics commonly used in livestock farming (Chapter 4). This agrees with the statement that the use of antibiotics in food animal farming selects for the development of antibiotic resistant bacteria (Luangtongkum *et al.,* 2006; Millman *et al.,* 2014; Koga *et al*., 2015). Thus discontinuing the use of antibiotics as growth promoters will reduce the development of antibiotic resistant bacteria, as found by the banning of avoparcin as a growth promoter in Europe (Woolhouse *et al.,* 2015). Also, increasing hygiene and good herd management as well as reducing animal density in livestock farming could reduce the need for antibiotics to treat diseases (Anon., 2006).

Moreover, wildlife in closer contact with human activities or which live in an environment that has been altered from its natural state, such as on farms where wildlife supplementary feeding is practiced (e.g. buffalo and eland), were revealed to have a higher resistance level compared to those in more isolated environments (Chapter 5). The *E. coli* and *E. faecalis* isolates from the supplementary fed wildlife were overall significantly more antibiotic resistant than those which were not supplementary fed. Interestingly, the bacteria from the supplementary fed wildlife were significantly more resistant towards sulphafurazole and tetracycline. Tetracycline and sulphafurazole are used extensively in both agricultural and clinical settings. They are commonly used antibiotics for growth promotion in animal feed as it stimulates weight gain (Speer *et al*., 1992; Bryan *et al*., 2004; Boerlin *et al*, 2005; Kozak *et al*., 2009). There is not much control on the contents of game feed in South Africa on the farm level, as Bekker (2011) found that 16.8% of South African game farmers use feed that is not registered or they are unsure of whether it is registered or not. It is suggested that game farmers become more knowledgeable of the ingredients that are used during the preparation of the feed that is used to feed both their livestock as well as their wildlife, as certain feed ingredients, such as antibiotics, can have a detrimental effect on health and food safety. Bekker (2011) showed that bone meal is added to some wildlife supplementary feeds, which is banned for use in food animal feeds but it is still available from horticulture sources and can be a potential source of antibiotic resistant bacteria. Additionally, it is advised that legislation governing the administration of antibiotics for food animals is updated to state that all antimicrobials should be administered and used only by licenced professionals, as suggested by WHO. This would help to limit the unnecessary use of antibiotics in agriculture, thus helping to preserve there efficacy.

Wildlife supplementary feeding increases herd density and creates more frequent contact at feed and water points which allows for more disease and ABR transfer between the intermingling species (Baquero *et al*., 2011; Laxminarayan *et al.,* 2013). Additionally, the inclusion of antimicrobial compounds as growth promoters, or other ingredients such as bone or blood meal originating from antibiotic resistant animals into wildlife supplementary feed, can further promote the development of ABR. This effect is further exacerbated by the 'free of choice' practice which typically occurs with wildlife supplementary feeding and can lead to uncontrolled consumption.

However, it is also recommended to confirm this theory by performing antibiotic susceptibility tests on samples from a controlled farm experiment where the feeding of wildlife is strictly controlled. Future work could involve a comparative study conducted on different types of wildlife supplementary feed ingredients.

This could give a clearer indication as to what the causative factor is of increased antibiotic resistance seen in supplementary fed wildlife.

Farm history can also impact the animals which graze on the pastures, as antibiotic resistant genes can persist in an environment for decades and can lie 'silent' until a selective pressure is applied. These antibiotic resistant genes can be transferred to the commensal or pathogenic bacteria via grazing on the pastures. This carry-over effect was observed in this study, where bacteria from game species grazing on a farm that was a sheep farm thirty years previously showed higher ABR than bacteria from game species from farms which did not have a history of intensive farming (Chapter 5). In addition, high ABR was observed in the bacteria isolated from these game species towards antibiotics which are commonly used in sheep farming, endorsing the carry-over effect.

The grazing/ browsing nature of the game species analysed in this study may also play a part in the transfer and development of ABR as grazing allows more direct contact with the soil bacteria which is said to contain naturally produced antimicrobial compounds and the accompanying antibiotic resistant genes. King & Schmidt (2017) revealed that the ABR levels of bacteria from wildebeest and zebra (grazers) were higher than those from giraffe (browser), indicating the influence of soil bacteria on ABR.

This study has indicated that various factors and farming practices can influence the antibiotic resistance of bacteria harboured by wildlife but there are also other factors that can be researched in future work. For example, other research has shown that antibiotic residues from medical and agricultural practices can pollute water sources (Swift *et al*., 2019). Future research could involve investigating the influence of water sources as a potential antibiotic resistance transfer vector to nearby wildlife.

The food chain has been characterised as one of the main transfer routes of ABR to humans, where resistant bacteria and pathogenic bacteria can reach humans by consumption of undercooked, contaminated meat or fresh produce, or by cross contamination of food preparation surfaces (Laxminarayan *et al.,* 2013) Thus ABR is a major food safety challenge.

In this study, the bacterial isolates from the game meat showed higher resistance towards tetracycline, erythromycin and penicillin than the bacteria from the faecal samples (Chapter 6). These three antibiotics are some of the most commonly used antibiotics in human and veterinary medicine and thus their effectiveness can be greatly compromised due to the development of antibiotic resistant bacteria. This also indicates that cross-contamination may have occurred from humans during processing. This highlights the importance of hygienic practises during slaughter and throughout the meat production chain. Perhaps those working with meat in abattoirs should be screened for ABR, in addition to testing whether they are Staphylococcal carriers.

To gain a more comprehensive study on the antibiotic resistance levels of bacteria isolated from game meat, it is recommended to increase the sample size of wildlife species and to include more food-borne pathogens in the antibiotic susceptibility analysis, such as *Campylobacter* and *Salmonella*.

Importantly, the same safety and hygiene procedures that are used to prevent and control the spread of pathogenic bacteria via food in the food industry will likewise help to control the spread of antibiotic-resistant bacteria in the food chain (Capita *et al.*, 2016). It is important to note that the control of antibiotic resistant

bacteria in a food production or processing environment could be more challenging than susceptible bacteria, as resistant microorganisms have evolved through antibiotic selection, making them more difficult to eliminate (Anon., 2010).

Therefore, steps to limit the creation of antibiotic selection pressures within the supply chain of game meat should be key preventative measures in the control of ABR development. In order to prevent cross-contamination of harmful bacteria onto raw meat, various precautionary steps can be put in place. For example, (i) proper cleaning and disinfection of facilities and equipment during processing, (ii) avoidance of stomach shots which would contaminate the carcass internally, (iii) proper chilling of the carcass after skinning to hinder the growth of existing bacteria and (iv) prevention of human contact as best as possible throughout the slaughter process and the entire food production chain. Additionally, the consumer should ensure that raw meat is properly cooked at the correct temperature and time and hygienic food preparations are followed to prevent cross-contamination between raw meat and foods that will not be further cooked.

Detection of antibiotic resistant genes is scientifically useful for confirming phenotypic resistance and for gaining more insight into knowledge of the type of resistance mechanisms the bacteria uses against the antibiotic. Knowledge of the varied mechanisms of antimicrobial resistance and ways to circumvent them will be essential as a preliminary step in the development of new antibacterial therapies to counteract the growing global health threat. Resistant genes detected in this study using PCR corresponded well with the phenotypic resistance patterns, with varying correct correlations ranging from 67% to 100% G±P± (positive/ negative genotypic, positive/ negative phenotype) (Chapter 7). The differences in phenotypic and genotypic results may be due to the presence of other resistant genes which confer the same phenotypic resistance but which were not detected in this study. Low or no gene expression could also result in these differences. Real-time PCR can be used to assess the level of gene expression in G-P+ or G+P- samples. Ideally, it is recommended to use Whole Genome Sequencing (WGS) to effectively assess the antibiotic susceptibility profile of a bacterium as all known antibiotic resistant genes in the bacteria's genome can be detected simultaneously, as opposed to just a handful using the PCR technique.

Resistant genes were detected in the bacteria of the wildlife and livestock from all the farm locations, including those of the free-ranging wildlife and organic livestock (Chapter 7). Thus it is speculated that these resistant genes are picked up from the soil and the surrounding environment and are spread by the animals, as well as by other natural vectors like the wind and rivers. Thus wildlife species can be considered reservoirs of antibiotic resistant genes.

In food animal production, focus should be put on preserving the beneficial use of antibiotics by eliminating the use of growth promoters and reducing the need for antibiotics for therapeutic use (WHO, 2011; Founou *et al*., 2016). This can be achieved by minimising infections by improving general animal health management and hygiene (WHO, 2011; Founou *et al*., 2016). In addition, other measures should be implemented, such as the use of vaccines, probiotics, prebiotics and competitive exclusion products (McEwen & Fedorka-Cray, 2002; Anon., 2006; WHO, 2011).

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With the game meat industry continuing to grow in South Africa, along with the rising of ABR, it is essential that the important stakeholders in the game meat production chain, as well as in all agricultural sectors, are educated about ABR in the food chain and the factors that can potentially influence antimicrobial resistance. This study aimed to highlight some of the influencing factors affecting ABR and to contribute surveillance data on the antibiotic resistance situation in the game meat production chain in South Africa.

To conclude, this study has highlighted that ABR in wildlife in South Africa does exist. The overall aim of this study was achieved, as it was found that livestock and wild ungulates do host antibiotic resistant bacteria. This study made a contribution to the greater global understanding of how ABR can increase in more natural, remote areas due to various agricultural practices, as mentioned previously. Futhermore, this study revealed that wildlife species have some intrinsic ABR, as wildlife from remote areas were found to be resistant to certain antibiotics.

Given the kind of complex causation processes discussed above and throughout this study, it is clear that multi-sectorial action is needed in order to control the development of antimicrobial resistance in South Africa and globally (Singh, 2017). Surveillance of the antimicrobial resistance situation on a worldwide scale is a vital feature that is needed in order to assist with the control of ABR development (Anon., 2010; WHO, 2011; Founou *et al*., 2016). In addition, better control and more restricted use of antibiotics in clinical and agricultural settings will help to curb the further development of ABR which could impair the future of human and animal health, disrupting the ecological balance between humanity and nature (Laxminarayan *et al.,* 2013).

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Addendum A

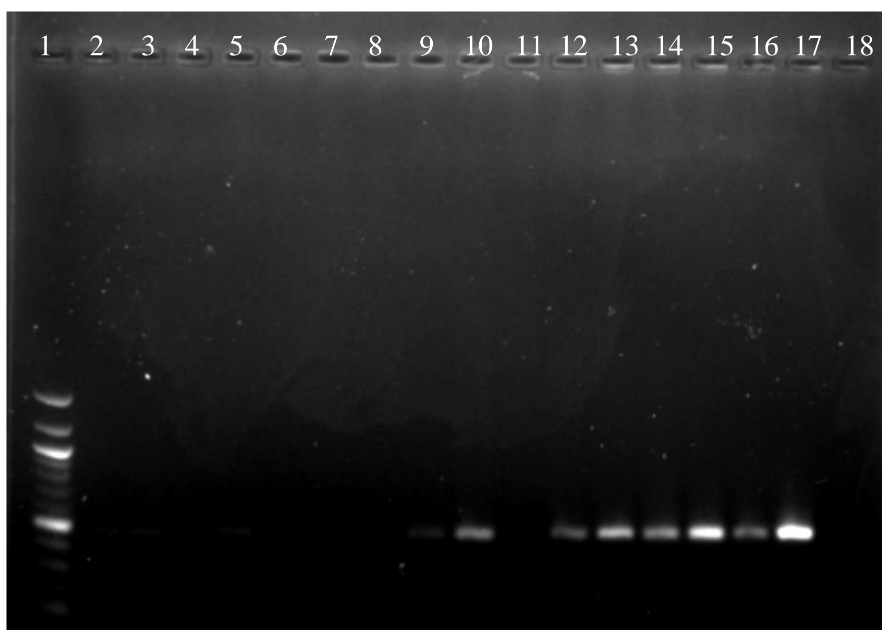

Figure A3.1 *bla*Z PCR gel image for *E. faecalis* isolates at 498bp (a).

Lane 1: 100bp ladder; lane 2: Deer 1 fecal (Witsand); lane 3: Wildebeest fed 2 fecal (Modimolle); lane 4: Wildebeest fed 2 fecal (Modimolle); lane 5: Wildebeest fed 3 fecal (Modimolle); lane 6: Wildebeest fed 3 fecal (Modimolle); lane 7: Wildebeest fed 3 fecal (Modimolle) ; lane 8: Buffalo 4 fecal (Elandsberg 1); lane 9: Springbok 1 fecal (Witsand); lane 10: Impala 1 meat (Modimolle); lane 11: Impala 1 meat (Modimolle); lane 12: Impala 1 meat (Modimolle); lane 13: Impala 2 meat (Modimolle); lane 14: Impala 2 meat (Modimolle); lane 15: Impala 2 meat (Modimolle); lane 16: Impala 3 meat (Modimolle); lane 17: positive control; lane 18: negative control.

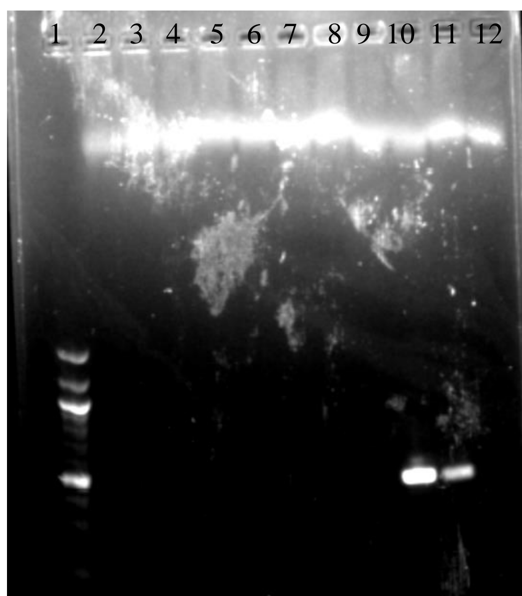

Figure A1.2 *bla*Z PCR gel image for *E. faecalis* (*S. aureus**) isolates at 498bp (b).

Lane 1: 100bp ladder; lane 2: Buffalo 2 fecal (Elandsberg 1); lane 3: Buffalo 3 fecal (Elandsberg 1); lane 4: Springbok 4 fecal (Witsand); lane 5: Buffalo 3 fecal (Ekuseni); lane 6: Impala 4 fecal (Modimolle); lane 7: Impala fed 3 fecal (Modimolle); lane 8: Impala fed 4 fecal (Modimolle); lane 9: Wildebeest 4 fecal (Ekuseni); lane 10: Bontebok 4 meat* (Elandsberg 1); lane 11: positive control; lane 12: negative control.

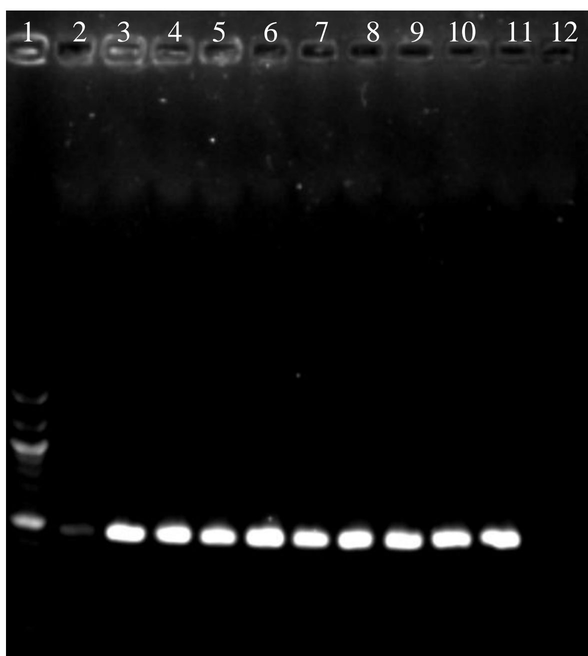

Figure A1.3 *bla*Z PCR gel image for *E. faecalis* (*S. aureus**) isolates at 498bp (c).

Lane 1: 100bp ladder; lane 2: Impala 5 meat (Modimolle); lane 3: positive control; lane 4: Springbok 1 meat* (Witsand); lane 5: Springbok 2 meat* (Witsand); lane 6: Springbok 2 meat* (Witsand); lane 7: Springbok 4 meat* (Witsand); lane 8: Springbok 4 meat* (Witsand); lane 9: Springbok 4 meat* (Witsand); lane 10: Springbok 9 meat* (Witsand); lane 11: Springbok 9 meat* (Witsand); lane 12: negative control.

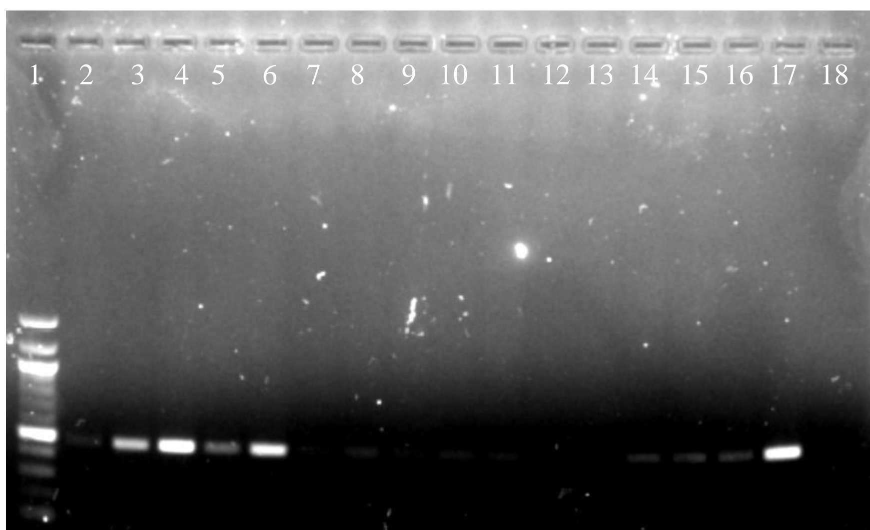

Figure A1.4 *bla*Z PCR gel image for *E. faecalis* isolates at 498bp (d).

Lane 1: 100bp ladder; lane 2: Impala 3 meat (Modimolle); lane 3: Impala 3 meat (Modimolle); lane 4: Impala 4 meat (Modimolle); lane 5: Impala 4 meat (Modimolle); lane 6: Impala 4 meat (Modimolle); lane 7: Impala 5 meat (Modimolle); lane 8: Eland 3 fecal (Bredasdorp); lane 9: Sheep 1 fecal (Bredasdorp); lane 10: Sheep 4 fecal (Witsand); lane 11: Wildebeest fed 3 (Modimolle); lane 12: Buffalo 1 fecal (Elandsberg 1); lane 13: Buffalo 1 fecal (Elandsberg 1); lane 14: Buffalo 1 fecal (Elandsberg 1); lane 15: Buffalo 2 fecal (Elandsberg 1); lane 16: Buffalo 2 fecal (Elandsberg 1); lane 17: positive control; lane 18: negative control.

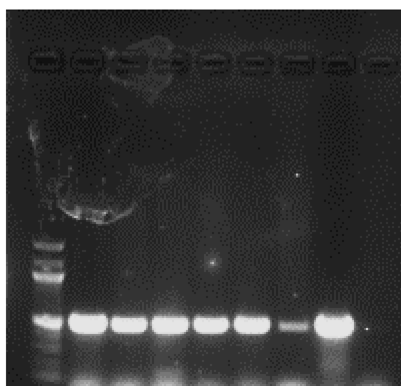

Figure A1.5 *bla*Z PCR gel image for *E. faecalis* isolates at 498bp (e).

Lane 1: 100bp ladder; lane 2: Impala 1 meat (Modimolle); lane 3: Impala 1 meat (Modimolle); lane 4: Impala 2 meat (Modimolle); lane 5: Impala 2 meat (Modimolle); lane 6: Impala 5 meat (Modimolle); lane 7: Buffalo 1 fecal (Elandsberg 1); lane 8: positive control; lane 9: negative control.

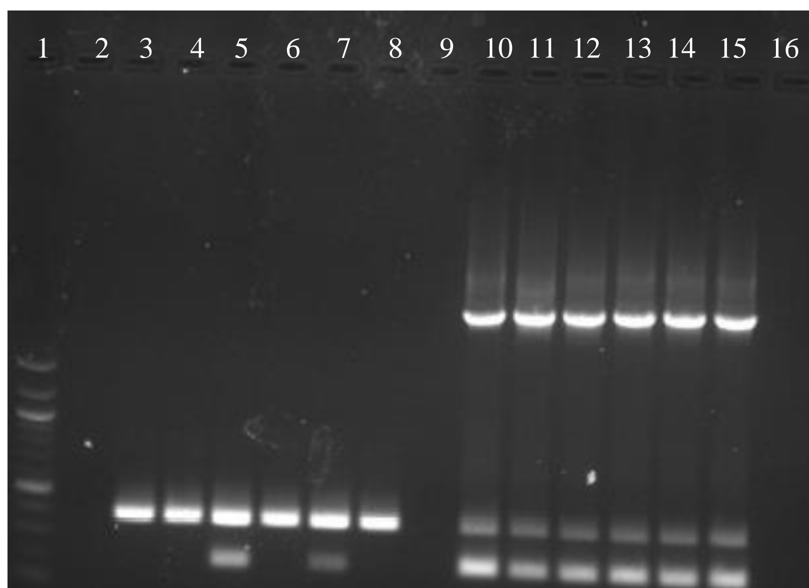

Figure A2.1 *tet*K, *tet*L and *tet*M multiplex PCR gel image for *E. faecalis* isolates at 1515bp, 267bp and 406bp (a).

Lane 1: 100bp ladder; lane 2: Deer 1 fecal (Witsand); lane 3: Wildebeest fed 2 fecal (Modimolle); lane 4: Wildebeest fed 2 fecal (Modimolle); lane 5: Wildebeest fed 3 fecal (Modimolle); lane 6: Wildebeest fed 3 fecal (Modimolle); lane 7: Wildebeest fed 3 fecal (Modimolle); lane 8: Buffalo 4 fecal (Elandsberg 1); lane 9: Springbok 1 fecal (Witsand); lane 10: Impala 1 meat (Witsand); lane 11: Impala 1 meat (Witsand); lane 12: Impala 2 meat (Witsand); lane 13: Impala 2 meat (Witsand); lane 14: Impala 3 meat (Witsand); lane 15: positive control; lane 16: negative control.

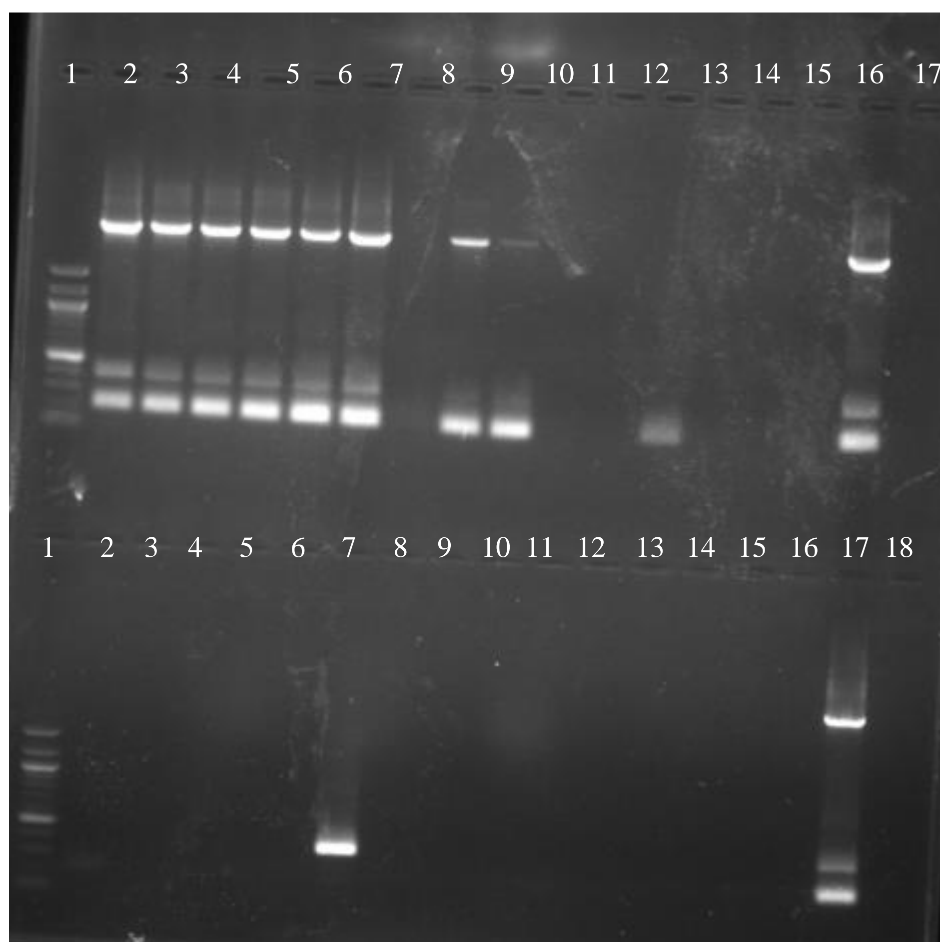

Figure A2.2 *tet*K, *tet*L and *tet*M multiplex PCR gel image for *E. faecalis* (**S. aureus*) isolates at 1515bp, 267bp and 406bp (b).

Row 1: Lane 1: 100bp ladder; lane 2: Impala 1 meat (Modimolle); lane 3: Impala 2 meat (Modimolle); lane 4: Impala 3 meat (Modimolle); lane 5: Impala 4 meat (Modimolle); lane 6: Impala 4 meat (Modimolle); lane 7: Impala 4 meat (Modimolle); lane 8: Impala 5 meat (Modimolle); lane 9: Impala 5 meat (Modimolle); lane 10: Springbok 1 meat* (Witsand); lane 11: Springbok 1 meat* (Witsand); lane 12: Springbok 2 meat* (Witsand); lane 13: Springbok 2 meat* (Witsand); lane 14: Springbok 4 meat* (Witsand); lane 15: Springbok 4 meat* (Witsand); lane 16: Buffalo 3 fecal (Ekuseni); lane 17: positive control; lane 18: negative control.

Row 2: Lane 1: 100bp ladder; lane 2: Springbok 9 meat* (Witsand); lane 3: Springbok 9 meat* (Witsand); lane 4: Eland 3 fecal (Bredasdorp); lane 5: Sheep 1 fecal (Bredasdorp); lane 6: Sheep 4 fecal (Witsand); lane 7: Wildebeest fed 3 fecal (Modimolle); lane 8: Buffalo 1 fecal (Elandsberg 1); lane 9: Buffalo 1 fecal (Elandsberg 1); lane 10: Buffalo 2 fecal (Elandsberg 1); lane 11: Buffalo 2 fecal (Elandsberg 1); lane 12: Buffalo 2 fecal (Elandsberg 1); lane 13: Buffalo 3 fecal (Elandsberg 1); lane 14: Springbok 4 fecal (Witsand); lane 15: Buffalo 3 fecal (Ekuseni); lane 16: blank; lane 17: positive control; lane 18: negative control.

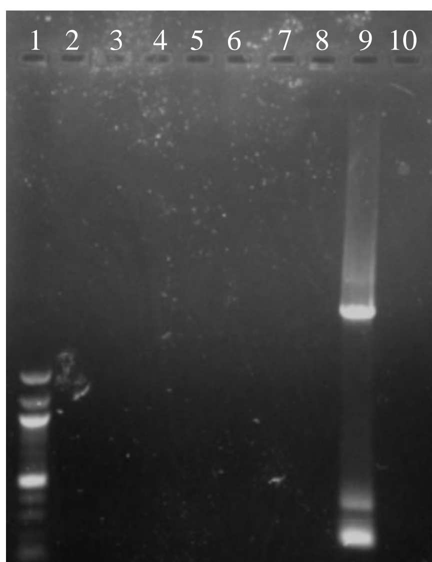

Figure A2.3 *tet*K, *tet*L and *tet*M multiplex PCR gel image for *E. faecalis* (**S. aureus*) isolates at 1515bp, 267bp and 406bp (c).

Lane 1: 100bp ladder; lane 2: Impala 4 fecal (Modimolle); lane 3: Impala fed 3 fecal (Modimolle); lane 4: Impala 4 fecal (Modimolle); lane 5: Wildebeest 4 fecal (Ekuseni); lane 6: Bontebok 4 meat* (Elandsberg 1); lane 7: Bontebok 4 meat* (Elandsberg 1); lane 8: blank; lane 9: positive control; lane 10: negative control.

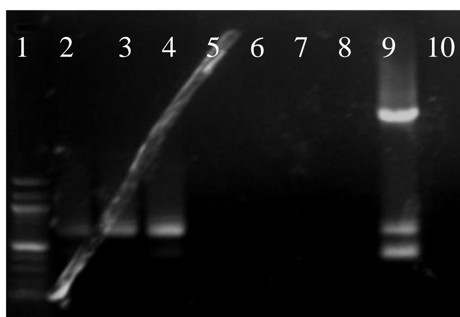

Figure A2.4 tetK, tetL and tetM multiplex PCR gel image for E. faecalis (*S. aureus) isolates at 1515bp, 267bp and 406bp (d).

Lane 1: 100bp ladder; lane 2: Wildebeest fed 3 fecal (Modimolle); lane 3: Wildebeest fed 3 fecal (Modimolle); lane 4: Springbok 1 meat* (Witsand); lane 5: Springbok 2 meat* (Witsand); lane 6: Eland 3 fecal (Bredasdorp); lane 7: Bontebok 4 meat* (Elandsberg 1); lane 8: Bontebok 4 meat* (Elandsberg 1); lane 9: positive control; lane 10: negative control.

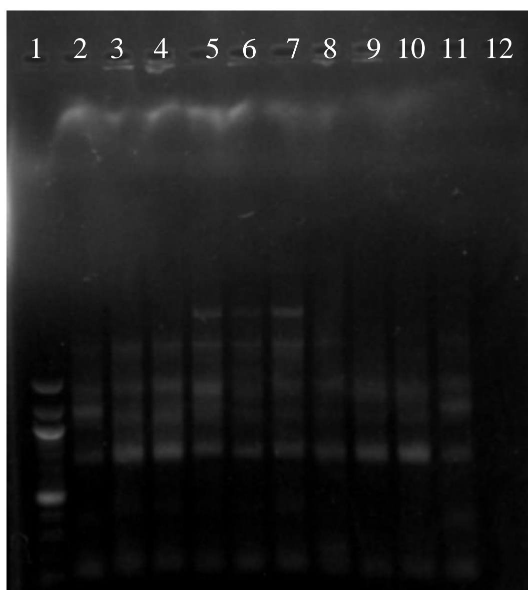

Figure A3.1 *van*A and *van*B multiplex PCR gel image for *E. faecalis* isolates at 732bp and 647bp (a).

Lane 1: 100bp ladder; lane 2: Sheep 1 fecal (Bredasdorp); lane 3: Sheep 4 fecal (Witsand); lane 4: Wildebeest fed 3 fecal (Modimolle); lane 5: Buffalo 1 fecal (Elandsberg 1); lane 6: Buffalo 1 fecal (Elandsberg 1); lane 7: Buffalo 1 fecal (Elandsberg 1); lane 8: Buffalo 2 fecal (Elandsberg 1); lane 9: Buffalo 2 fecal (Elandsberg1); lane 10: positive control; lane 11: Buffalo 3 fecal (Elandsberg 1); lane 12: negative control.

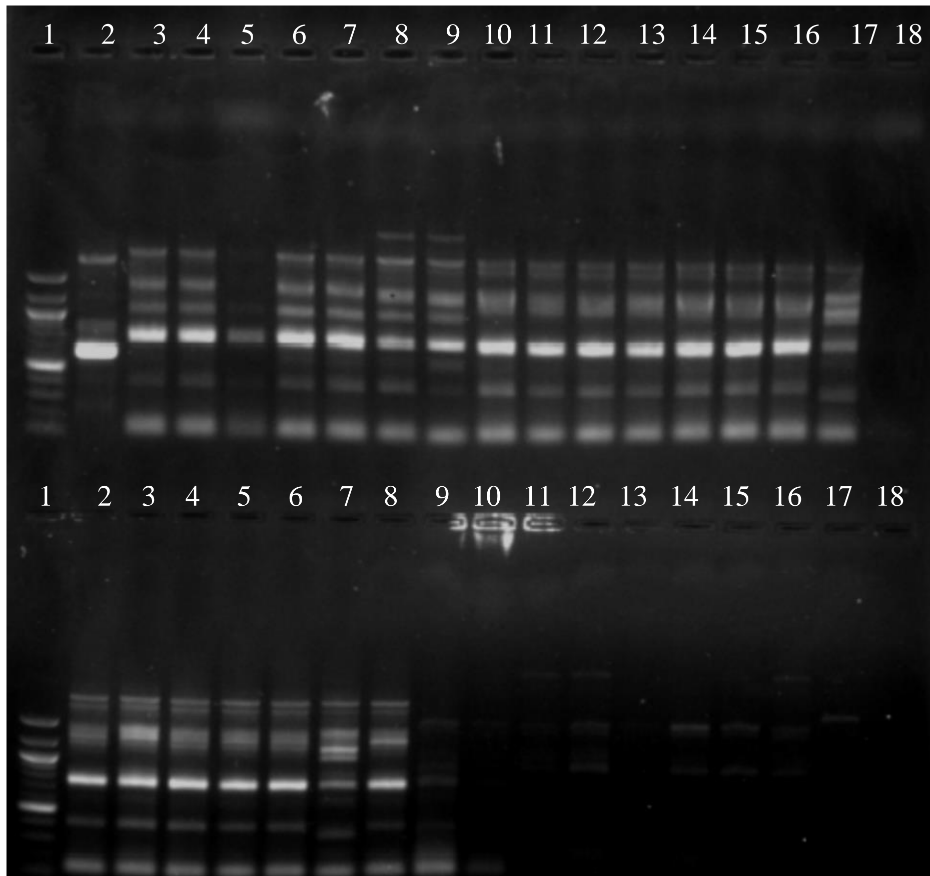

Figure A3.2 *van*A and *van*B multiplex PCR gel image for *E. faecalis* (**S.aureus*) isolates at 732bp and 647bp (b).

Row 1: Lane 1: 100bp ladder; lane 2: Deer 1 fecal (Witsand); lane 3: Wildebeest fed 2 fecal (Modimolle); lane 4: Wildebeest fed 2 fecal (Modimolle); lane 5: Wildebeest fed 3 fecal (Modimolle); lane 6: Wildebeest fed 3 fecal (Modimolle); lane 7: Wildebeest fed 3 fecal (Modimolle); lane 8: Buffalo 4 fecal (Elandsberg farm 1); lane 9: Springbok 1 fecal (Witsand); lane 10: Impala 1 meat (Modimolle); lane 11: Impala 1 meat (Modimolle); lane 12: Impala 1 meat (Modimolle); lane 13: Impala 2 meat (Modimolle); lane 14: Impala 2 meat (Modimolle); lane 15: Impala 3 meat (Modimolle); lane 16: positive control; lane 17: negative control.

Row 2: Lane 1: 100bp ladder; lane 2: Impala 3 meat (Modimolle); lane 3: Impala 3 meat (Modimolle); lane 4: Impala 4 meat (Modimolle); lane 5: Impala 4 meat (Modimolle); lane 6: Impala 4 meat (Modimolle); lane 7: Impala 5 meat (Modimolle); lane 8: Impala 5 meat (Modimolle); lane 9: Springbok 1 meat* (Witsand); lane 10: Springbok 1 meat* (Witsand); lane 11: Springbok 2 meat* (Witsand); lane 12: Springbok 2 meat* (Witsand); lane 13: Springbok 4 meat* (Witsand); lane 14: Springbok 4 meat* (Witsand); lane 15: Springbok 4 meat* (Witsand); lane 16: Springbok 9 meat* (Witsand); lane 17: Springbok 9 meat* (Witsand); lane 18: negative control.

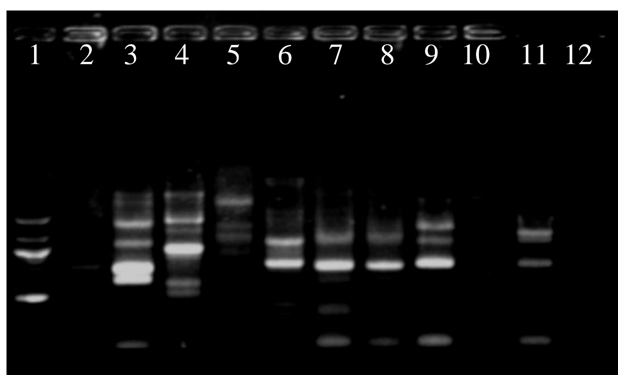

Figure A3.3 *van*A and *van*B multiplex PCR gel image for *E. faecalis* (**S.aureus*) isolates at 732bp and 647bp (c).

Lane 1: 100bp ladder; lane 2: Springbok 9 meat* (Witsand); lane 3: Eland 3 fecal (Bredasdorp); lane 4: Springbok 4 fecal (Witsand); lane 5: Buffalo 3 fecal (Ekuseni); lane 6: Impala fed 4 fecal (Modimolle); lane 7: Impala fed 3 fecal (Modimolle); lane 8: Impala fed 4 fecal (Modimolle); lane 9: Wildebeest 4 fecal (Ekuseni); lane 10: Bontebok 4 meat* (Elandsberg 1); lane 11: positive control; lane 12: negative control.

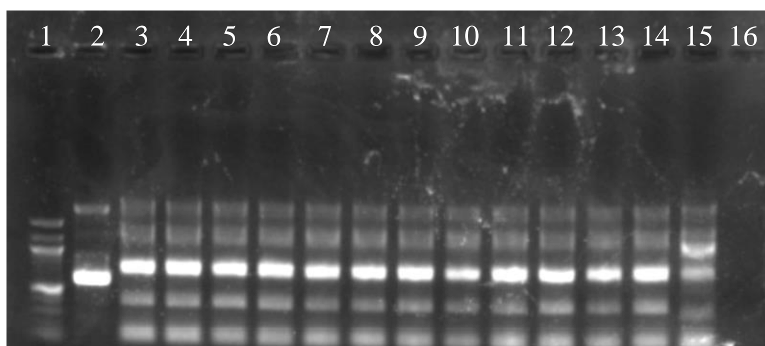

Figure A3.4 *van*A and *van*B multiplex PCR gel image for *E. faecalis* isolates at 732bp and 647bp (d).

Lane 1: 100bp ladder; lane 2: Deer 1 fecal (Witsand); lane 3: Impala 1 meat (Modimolle); lane 4: Impala 1 meat (Modimolle); lane 5: Impala 1 meat (Modimolle); lane 6: Impala 2 meat (Modimolle); lane 7: Impala 2 meat (Modimolle); lane 8: Impala 2 meat (Modimolle); lane 9: Impala 3 meat (Modimolle); lane 10: Impala 3 meat (Modimolle); lane 11: Impala 3 meat (Modimolle); lane 12: Impala 4 meat (Modimolle); lane 13: Impala 4 meat (Modimolle); lane 14: Impala 4 meat (Modimolle); lane 15: positive control; lane 16: negative control.

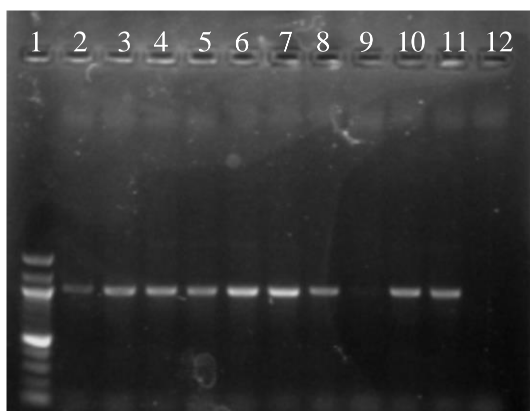

Figure A4.1 *bla*CMY PCR gel image for *E. coli* isolates at 1000bp (a).

Lane 1: 100bp ladder; lane 2: Wildebeest 5 fecal (Bredasdorp); lane 3: Wildebeest fed 4 fecal (Modimolle); lane 4: Buffalo 1 fecal (Elandsberg1); lane 5: Buffalo 3 fecal (Elandsberg 1); lane 6: Buffalo 3 fecal (Elandsberg1); lane 7: Buffalo 4 fecal (Elandsberg 1); lane 8: Buffalo 4 fecal (Elandsberg 1); lane 9: Buffalo 5 fecal (Elandsberg 1); lane 10: Buffalo 1 fecal (Elandsberg 2); lane 11: Buffalo 5 fecal (Elandsberg 2); lane 12: negative control.

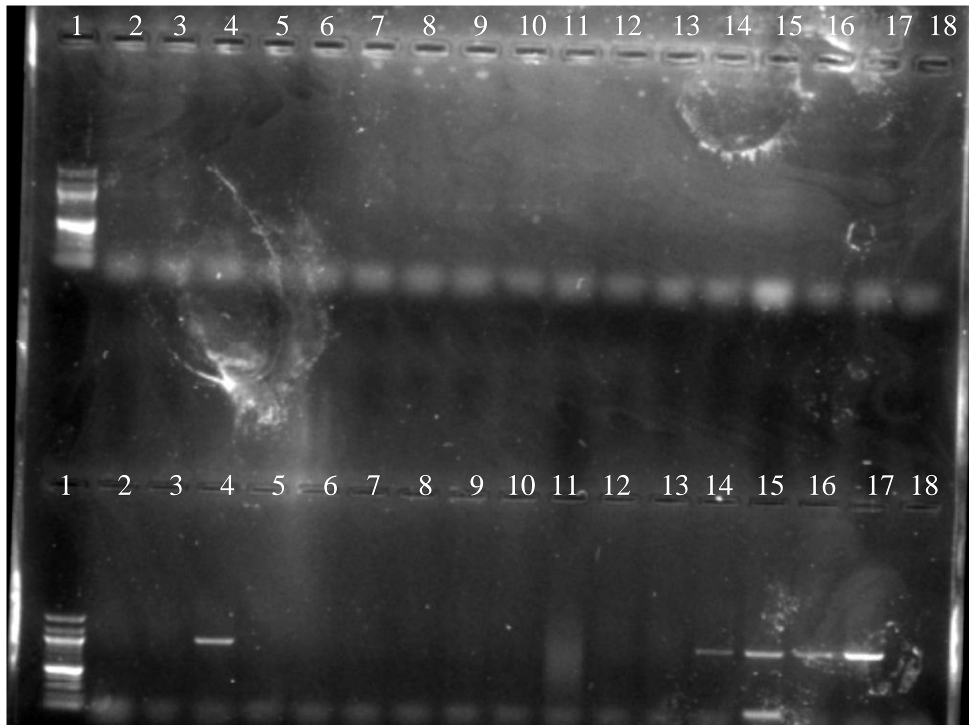

Figure A4.2 *bla*CMY PCR gel image for *E. coli* isolates at 1000bp (b).

Row 1: Lane 1: 100bp ladder; lane 2: Sheep 5 fecal (Witsand); lane 3: Cattle 4 fecal (Witsand); lane 4: Deer 1 fecal (Witsand); lane 5: Sheep 3 fecal (Witsand); lane 6: Cattle 1 fecal (Bredasdorp); lane 7: Wildebeest 13 fecal (Bredasdorp); lane 8: Wildebeest 13 fecal (Bredasdorp); lane 9: Cattle 10 fecal (Bredasdorp); lane 10: Wildebeest 13 fecal (Bredasdorp); lane 11: Eland 2 fecal (Bredasdorp); lane 12: Wildebeest FR 1 fecal (Modimolle); lane 13: Wildebeest FR 1 fecal (Modimolle); lane 14: Sheep 2 fecal (Sutherland); lane 15: Springbok 2 fecal (Sutherland); lane 16: Eland 4 fecal (Bredasdorp); lane 17: Wildebeest fed 4 fecal (Modimolle); lane 18: Buffalo 1 fecal (Elandsberg 1).

Row 2: Lane 1: 100bp ladder; lane 2: Buffalo 2 fecal (Elandsberg 1); lane 3: Buffalo 2 fecal (Elandsberg 1); lane 4: Buffalo 3 fecal (Elandsberg 1); lane 5: Buffalo 5 fecal (Elandsberg 1); lane 6: Buffalo 1 fecal (Elandsberg 2); lane 7: Wildebeest 2 fecal (Elandsberg 2); lane 8: Wildebeest 3 fecal (Elandsberg 2); lane 9: Blesbok 2 fecal (Witsand); lane 10: Blesbok 3 fecal (Witsand); lane 11: Cattle 5 fecal (Witsand); lane 12: Sheep 4 fecal (Bredasdorp); lane 13: Cattle 12 fecal (Bredasdorp); lane 14: Wildebeest 6 fecal (Bredasdorp); lane 15: Sheep 11 fecal (Bredasdorp); lane 16: Wildebeest 3 fecal (Bredasdorp); lane 17: Eland 1 fecal (Bredasdorp); lane 18: negative control.

Figure A4.3 *bla*CMY PCR gel image for *E. coli* isolates at 1000bp (c).

Row 1: Lane 1: 100bp ladder; lane 2: Eland 1 fecal (Bredasdorp); lane 3: Wildebeest 3 fecal (Elandsberg 2); lane 4: Impala 1 fecal (Bredasdorp); lane 5: Impala 1 fecal (Bredasdorp); lane 6: Impala 1 fecal (Bredasdorp); Impala fed 3 fecal (Modimolle); lane 7: Springbok 1 meat (Witsand); lane 8: Springbok 1 meat (Witsand); lane 9: Springbok 4 meat (Witsand); lane 10: Springbok 4 meat (Witsand); lane 11: Impala 1 fecal (Ekuseni); lane 12: Impala 2 fecal (Ekuseni);lane 13: Impala 1 meat (Modimolle); lane 14: Impala 1 meat (Modimolle); lane 15: Impala 2 meat (Modimolle); lane 16: Impala 4 meat (Modimolle); lane 17: positive control; lane 18: negative control.

Row 2: Lane 1: 100bp ladder; lane 2: Sheep 3 fecal (Witsand); lane 3: Buffalo 2 fecal (Elandsberg 1); lane 4: Buffalo 3 fecal (Elandsberg 1); lane 5: Buffalo 5 fecal (Elandsberg 1); lane 6: Wildebeest 6 fecal (Bredasdorp); lane 7: Sheep 11 fecal (Bredasdorp); lane 8: Wildebeest 3 fecal (Bredasdorp); lane 9: Wildebeest 5 fecal (Bredasdorp); lane 10: Wildebeest fed 4 fecal (Modimolle); lane 11: Buffalo 1 fecal (Elandsberg 1); lane 12: Buffalo 5 fecal (Elandsberg 1); lane 13: Buffalo 1 fecal (Elandsberg 2); lane 14: Buffalo 5 fecal (Elandsberg 2); lane 15: Bontebok 3 meat (Elandsberg 1); lane 16: Bontebok 3 meat (Elandsberg 1); lane 17: Impala 4 meat (Modimolle); lane 18: negative control.

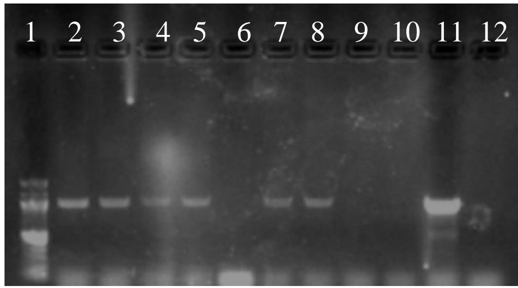

Figure A4.4 *bla*CMY PCR gel image for *E. coli* isolates at 1000bp (d).

Lane 1: 100bp ladder; lane 2: Springbok 1 meat (Witsand); lane 3: Springbok 1 meat (Witsand); lane 4: Springbok 4 meat (Witsand); lane 5: Springbok 4 meat (Witsand); lane 6: Impala 1 fecal (Ekuseni); lane 7: Impala 2 fecal (Ekuseni); lane 8: Impala 1 meat (Modimolle); lane 9: Impala 1 meat (Modimolle); lane 10: Impala 2 meat (Modimolle); lane 11: positive control; lane 12: negative control.

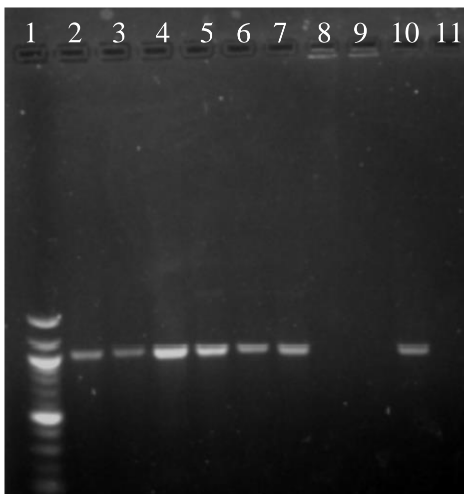

Figure A4.5 *bla*CMY PCR gel image for *E. coli* isolates at 1000bp (e).

Lane 1: 100bp ladder; lane 2: Cattle 12 fecal (Bredasdorp); lane 3: Wildebeest 3 fecal (Bredasdorp); lane 4: Eland 1 fecal (Bredasdorp); lane 5: Buffalo 1 fecal (Elandsberg 1); lane 6: Buffalo 1 fecal (Elandsberg 2); lane 7: Buffalo 5 fecal (Elandsberg 2); lane 8: Bontebok 3 meat (Elandsberg 1); lane 9: Bontebok 3 meat (Elandsberg 1); lane 10: positive control; lane 11: negative control.

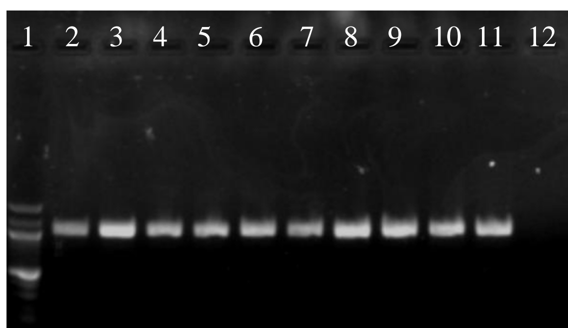

Figure A4.6 *bla*CMY PCR gel image for *E. coli* isolates at 1000bp (f).

Lane 1: 100bp ladder; lane 2: Sheep 3 fecal (Witsand); lane 3: Bontebok 1 fecal (Elandsberg 1); lane 4: Bontebok 1 fecal (Elandsberg 1); lane 5: Bontebok 4 fecal (Elandsberg 1); lane 6: Bontebok 4 fecal (Elandsberg 1); lane 7: Bontebok 4 fecal (Elandsberg 1); lane 8: Bontebok 5 fecal (Elandsberg 1); lane 9: Bontebok 5 fecal (Elandsberg 1); lane 10: Bontebok 5 fecal (Elandsberg 1); lane 11: positive control; lane 12: negative control

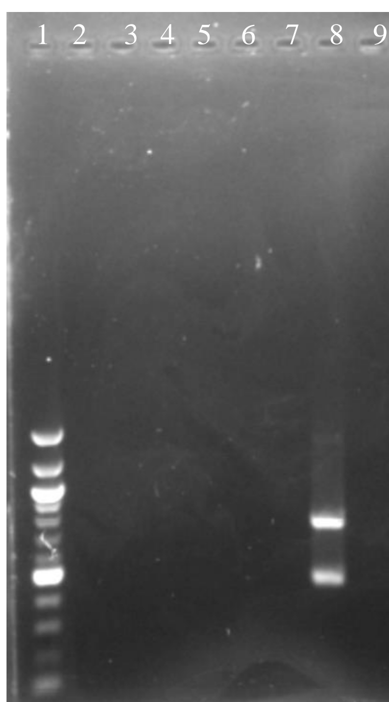

Figure A5.1 *sul*1, *sul*2 and *sul*3 multiplex PCR gel image for *E. coli* isolates at 433bp, 721bp and 244bp (a).

Lane 1: 100bp ladder; lane 2: Wildebeest fed 4 fecal (Modimolle); lane 3: Buffalo 2 fecal (Elandsberg 1); lane 4: Buffalo 4 fecal (Elandsberg 1); lane 5: Buffalo 4 fecal (Elandsberg 1); lane 6: Buffalo 1 fecal (Elandsberg2); lane 7: Wildebeest 3 fecal (Elandsberg 2); lane 8: positive control (Blesbok 3 fecal- Witsand); lane 9: negative control.

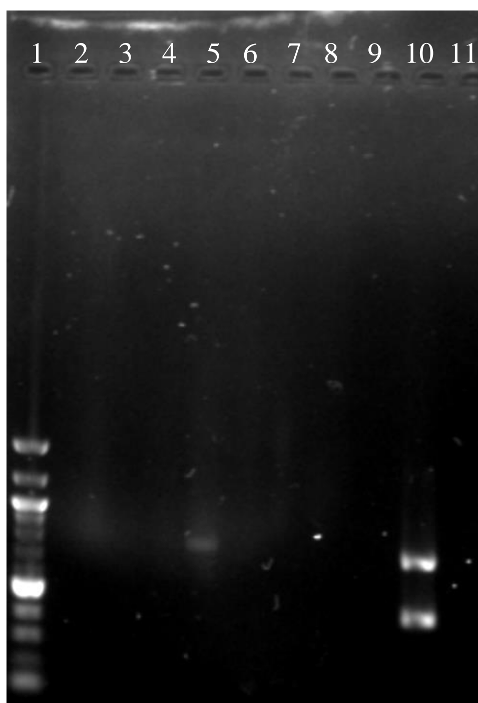

Figure A5.2 *sul*1, *sul*2 and *sul*3 multiplex PCR gel image for *E. coli* isolates at 433bp, 721bp and 244bp (b).

Lane 1: 100bp ladder; lane 2: Sheep 5 fecal (Witsand); lane 3: Deer 1 fecal (Witsand); lane 4: Cattle 1 fecal (Bredasdorp); lane 5: Wildebeest 13 fecal (Bredasdorp); lane 6: Cattle 10 fecal (Bredasdorp); lane 7: Wildebeest 13 fecal (Bredasdorp); lane 8: Eland 2 fecal (Bredasdorp); lane 9: Wildebeest FR 1 (Modimolle); lane 10: positive control; lane 11: negative control.

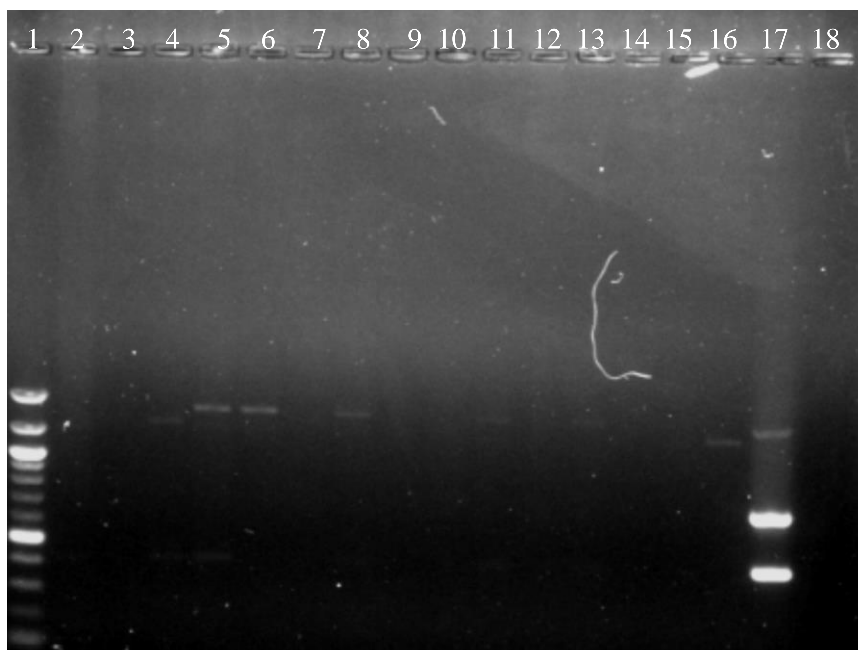

Figure A5.3 *sul*1, *sul*2 and *sul*3 multiplex PCR gel image for *E. coli* isolates at 433bp, 721bp and 244bp (c).

Lane 1: 100bp ladder; lane 2: Sheep 2 fecal (Sutherland); lane 3: Eland 4 fecal (Bredasdorp); lane 4: Wildebeest fed 4 fecal (Modimolle); lane 5: Buffalo 1 fecal (Elandsberg 1); lane 6: Buffalo 1 fecal (Elandsberg 1); lane 7: Buffalo 2 fecal (Elandsberg 1); lane 8: Bontebok 4 fecal (Elandsberg 1); lane 9: Buffalo 1 fecal (Elandsberg 2); lane 10: Wildebeest 2 fecal (Elandsberg 2); lane 11: Wildebeest 3 fecal (Elandsberg 2); lane 12: Cattle 5 fecal (Bredasdorp); lane 13: Cattle 12 fecal (Bredasdorp); lane 14: Wildebeest 3 fecal (Bredasdorp); lane 15: Eland 1 fecal (Bredasdorp); lane 16: Wildebeest 5 fecal (Bredasdorp); lane 17: positive control; lane 18: negative control.

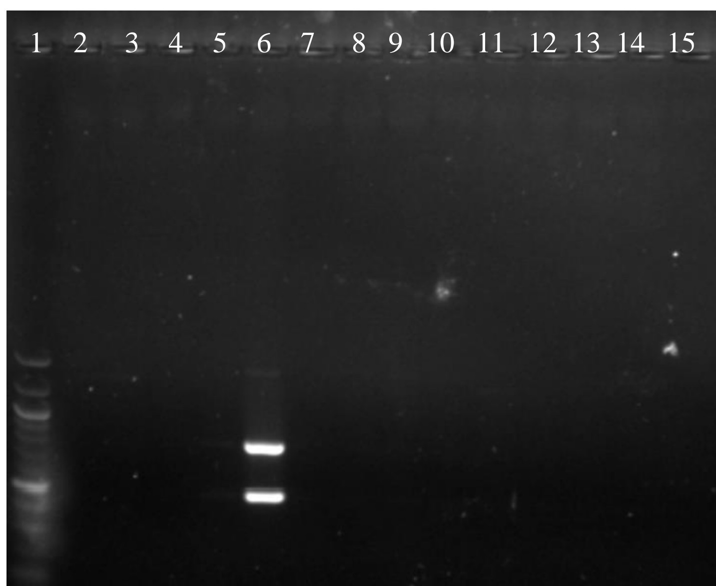

Figure A5.4 *sul*1, *sul*2 and *sul*3 multiplex PCR gel image for *E. coli* isolates at 433bp, 721bp and 244bp (d).

Lane 1: 100bp ladder; lane 2: Cattle 4 fecal (Witsand); lane 3: Cattle 1 fecal (Bredasdorp); lane 4: Springbok 2 fecal (Sutherland); lane 5: Blesbok 2 fecal (Witsand); lane 6: positive control; lane 7: Wildebeest 6 fecal (Bredasdorp); lane 8: Buffalo 1 fecal (Elandsberg 1); lane 9: Buffalo 3 fecal (Elandsberg 1); lane 10: Buffalo 3 fecal (Elandsberg 1); lane 11: Buffalo 4 fecal (Elandsberg 1); lane 12: Buffalo 5 fecal (Elandsberg 1); lane 13: Buffalo 5 fecal (Elandsberg 1); lane 14: Impala fed 3 fecal (Modimolle); lane 15: negative control.

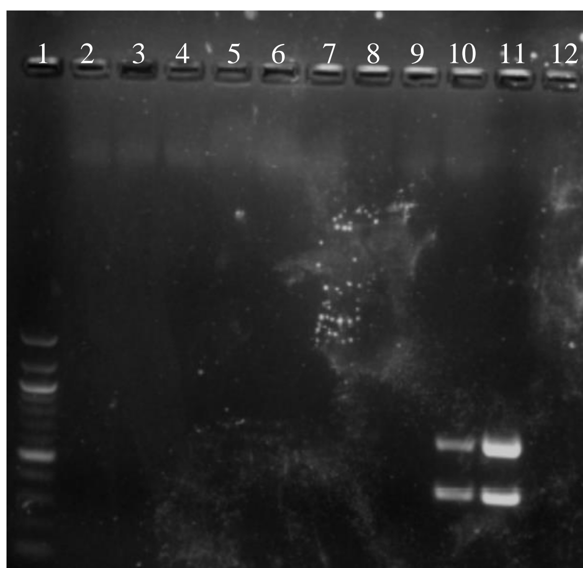

Figure A5.5 *sul*1, *sul*2 and *sul*3 multiplex PCR gel image for *E. coli* isolates at 433bp, 721bp and 244bp (e).

Lane 1: 100bp ladder; lane 2: Cattle 4 fecal (Witsand); lane 3: Sheep 3 fecal (Witsand); lane 4: Bontebok 1 fecal (Elandsberg farm 1); lane 5: Bontebok 4 fecal (Elandsberg 1); lane 6: Bontebok 5 fecal (Elandsberg 1); lane 7: Bontebok 5 fecal (Elandsberg 1); lane 8: Impala 1 fecal (Bredasdorp); lane 9: Impala 1 fecal (Bredasdorp); lane 10: positive control; lane 11: positive control; lane 12: negative control.

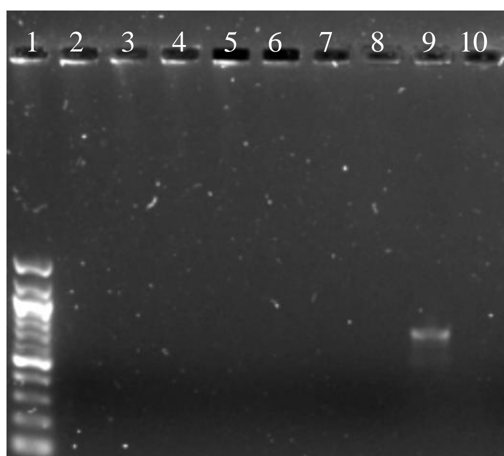

Figure A5.6 *sul*2 PCR gel image for *E. coli* isolates at 720bp (f).

Lane 1: 100bp ladder; lane 2: Cattle 9 fecal (Bredasdorp); lane 3: Sheep 3 fecal (Witsand); lane 4: Springbok 4 fecal (Witsand); lane 5: Wildebeest fed 5 fecal (Modimolle); lane 6: Blesbok 1 fecal (Witsand); lane 7: Cattle 1 fecal (Witsand); lane 8: Sheep 4 (Bredasdorp); lane 9: Wildebeest 13 fecal (Bredasdorp); lane 10: negative control.

Figure A5.7 *sul*2 PCR gel image for *E. coli* isolates at 720bp (g).

Lane 1: 100bp ladder; lane 2: Cattle 1 fecal (Bredasdorp); lane 3: Blesbok 4 fecal (Witsand); lane 4: Wildebeest fed 5 fecal (Modimolle); lane 5: Wildebeest fed 5 fecal (Modimolle); lane 6: blank; lane 7: blank; lane 8: Cattle 10 fecal (Bredasdorp); lane 9: Sheep 11 fecal (Bredasdorp); lane 10: Wildebeest 13 fecal (Bredasdorp); lane 11: Wildebeest 13 fecal (Bredasdorp) ; lane 12: Wildebeest 5 fecal (Bredasdorp); lane 13: Wildebeest 3 fecal (Bredasdorp); lane 14: Wildebeest 3 fecal (Bredasdorp); lane 15: Eland 1 fecal (Bredasdorp); lane 16: Eland 2 fecal (Bredasdorp); lane 17: positive control; lane 18: negative control.

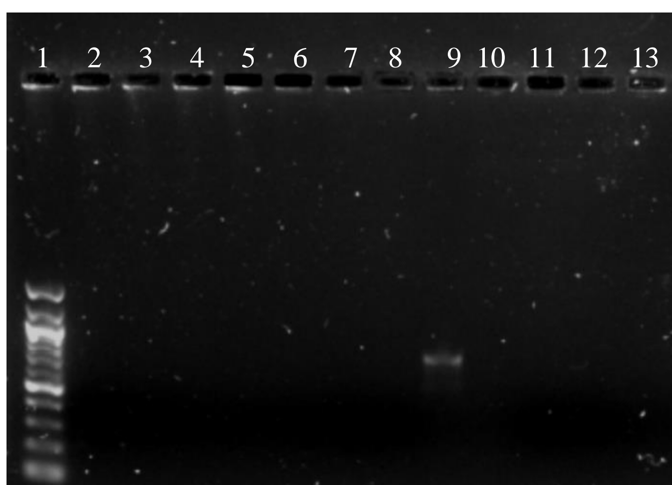

Figure A5.8 *sul*2 PCR gel image for *E. coli* isolates at 720bp (h).

Lane 1: 100bp ladder; lane 2: Cattle 12 fecal (Bredasdorp); lane 3: Sheep 3 fecal (Witsand); lane 4: Springbok 4 fecal (Witsand); lane 5: Wildebeest fed 5 fecal (Modimolle); lane 6: Blesbok 1 fecal (Witsand); lane 7: Sheep 4 fecal (Bredasdorp); lane 8: Wildebeest 13 fecal (Bredasdorp); lane 9: Eland 2 fecal (Bredasdorp); lane 10: Wildebeest FR 1 fecal (Modimolle); lane 11: Sheep 2 fecal (Sutherland); lane 12: Springbok 2 fecal (Sutherland); lane 13: negative control.

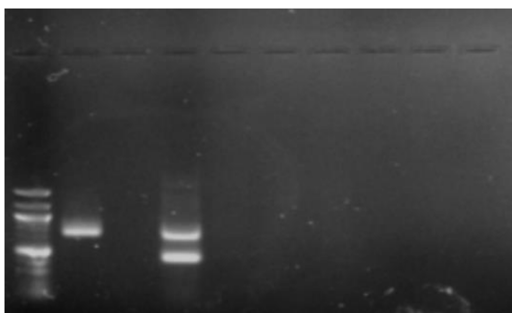

Figure A5.9 *sul*1, *sul*2 and *sul*3 multiplex PCR gel image for *E. coli* isolates at 433bp, 721bp and 244bp (i).

Lane 1: 100bp ladder; lane 2: Buffalo 5 fed (Elandsberg 1); lane 3: Wildebeest 3 fecal (Elandsberg 2); lane 4: Blesbok 2 fecal (Witsand); lane 5: Cattle 5 fecal (Witsand); lane 6: Buffalo 4 fecal (Elandsberg 1); lane 7: Buffalo 4 fecal (Elandsberg 1); lane 8: Buffalo 5 fecal (Elandsberg 1); lane 9: Wildebeest 3 fecal (Elandsberg 2); lane 10: negative control.

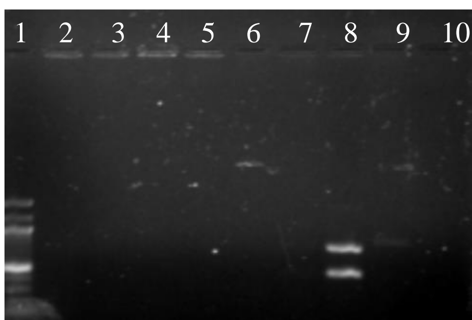

Figure A5.10 *sul*1, *sul*2 and *sul*3 multiplex PCR gel image for *E. coli* isolates at 433bp, 721bp and 244bp (j).

Lane 1: 100bp ladder; lane 2: Impala 1 fecal (Modimolle); lane 3: Impala 2 fecal (Modimolle); lane 4: Impala 4 fecal (Modimolle); lane 5: Bontebok 3 fecal (Elandsberg 1); lane 6: Bontebok 3 fecal (Elandsberg 1); lane 7: Blesbok 2 fecal (Bredasdorp); lane 8: positive control; lane 9: Wildebeest 13 fecal (Bredasdorp); lane 10: negative control.

Figure A5.11 *sul*1, *sul*2 and *sul*3 multiplex PCR gel image for *E. coli* isolates at 433bp, 721bp and 244bp (k).

Lane 1: 100bp ladder; lane 2: Springbok 1 meat (Witsand); lane 3: Springbok 1 meat (Witsand); lane 4: Springbok 4 meat (Witsand); lane 5: Springbok 4 meat (Witsand); lane 6: Impala 1 fecal (Ekuseni); lane 7: Impala 2 fecal (Ekuseni); lane 8: Impala 1 meat (Modimolle); lane 9: positive control; lane 10: negative control.

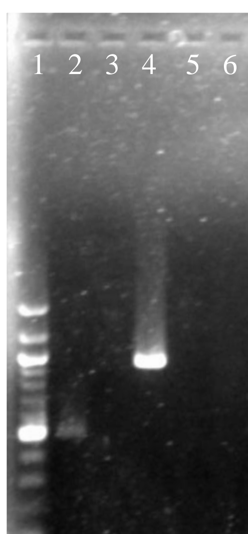

Figure A6.1 *tet*A, *tet*B *and tet*C *multiplex* PCR gel image for *E. coli* isolates at 562bp, 930bp and 888bp (a).

Lane 1: 100bp ladder; lane 2: Buffalo 2 fecal (Elandsberg 1); lane 3: Blesbok 2 fecal (Witsand); lane 4: Blesbok 3 fecal (Witsand); lane 5: Wildebeest fed 4 (Modimolle); lane 6: negative control.

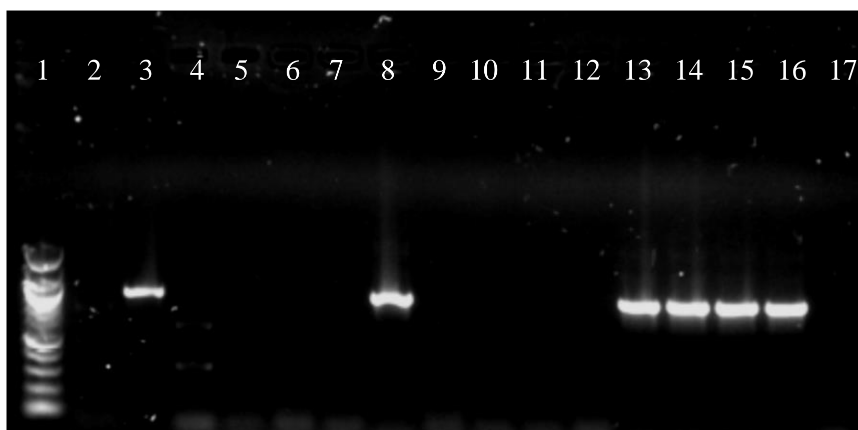

Figure A6.2 *tet*B PCR gel image for *E. coli* isolates at 930bp (b).

Lane 1: 100bp ladder; lane 2: Wildebeest 5 fecal (Bredasdorp); lane 3: Blesbok 2 fecal (Witsand); lane 4: Blesbok 3 fecal (Witsand); lane 5: Springbok 4 fecal (Witsand); lane 6: Blesbok 1 fecal (Witsand); lane 7: Blesbok 1 fecal (Witsand); lane 8: Blesbok 1 fecal (Witsand); lane 9: Blesbok 3 fecal (Witsand); lane 10: Wildebeest fed 4 fecal (Modimolle); lane 11: Blesbok 1 fecal (Witsand); lane 12: Blesbok 5 fecal (Witsand); lane 13: Blesbok 2 fecal (Witsand); lane 14: Blesbok 3 fecal (Witsand); lane 15: Blesbok 3 fecal (Witsand); lane 16: Blesbok 3 fecal (Witsand); lane 17: negative control.

Figure A6.3 *tet*B PCR gel image for *E. coli* isolates at 930bp (c).

Lane 1: Cattle 12 fecal (Bredasdorp); lane 2: Sheep 5 fecal (Bredasdorp); lane 3: Eland 3 fecal (Bredasdorp); lane 4: Wildebeest fed 4 fecal (Modimolle); lane 5: Blesbok 2 fecal (Witsand); lane 6: Blesbok 3 fecal (Witsand); lane 7: Springbok 4 fecal (Witsand); lane 8: Deer 1 fecal (Witsand); lane 9: Springbok 2 fecal (Sutherland); lane 10: Cattle 1 fecal (Bredasdorp); lane 11: Blesbok 4 fecal (Witsand); lane 12: Blesbok 4 fecal (Witsand); lane 13: Blesbok 5 fecal (Witsand); lane 14: Cattle 10 fecal (Bredasdorp); lane 15: Sheep 11 fecal (Bredasdorp); lane 16: Wildebeest 13 fecal (Bredasdorp); lane 17: negative control; lane 18: 100bp ladder.

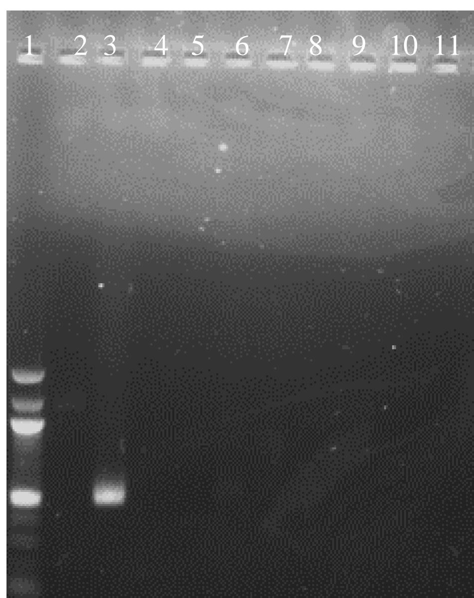

Figure A6.4 *tet*A, *tet*B and *tet*C multiplex PCR gel image for *E. coli* isolates at 562bp, 930bp and 888bp (d).

Lane 1: 100bp ladder; lane 2: Sheep 5 fecal (Witsand); lane 3: Cattle 4 fecal (Witsand); lane 4: Deer 1 fecal (Witsand); lane 5: Sheep 3 fecal (Witsand); lane 6: Cattle 1 fecal (Bredasdorp); lane 7: Wildebeest 13 fecal (Bredasdorp); lane 8: Buffalo 1 fecal (Elandsberg 2); lane 9: Cattle 10 fecal (Bredasdorp); lane 10: Wildebeest 13 fecal (Bredasdorp); lane 11: negative control.

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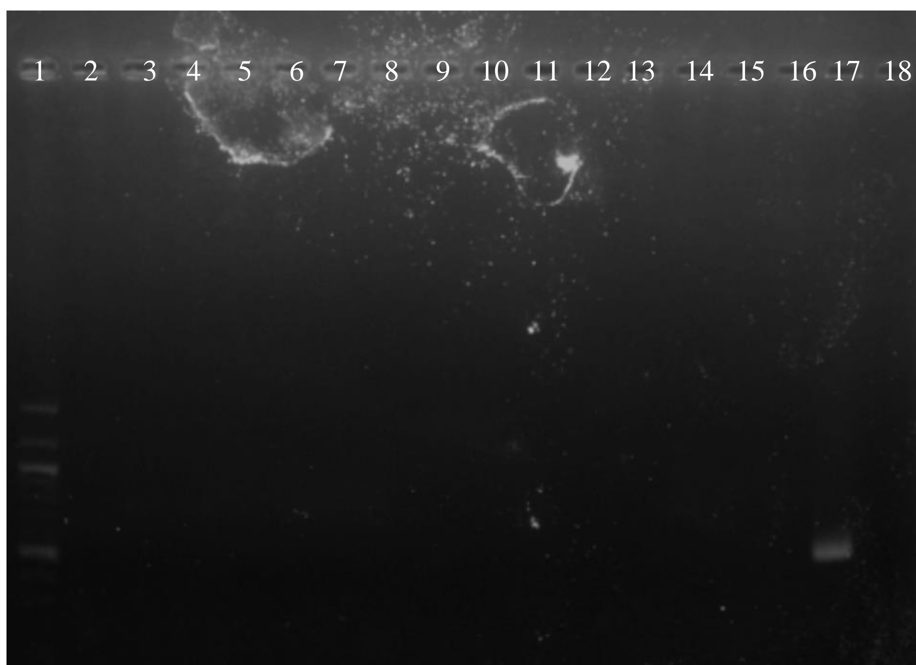

Figure A6.5 *tet*A, *tet*B and *tet*C multiplex PCR gel image for *E. coli* isolates at 562bp, 930bp and 888bp (e).

Lane 1: 100bp ladder; lane 2: Eland 2 fecal (Bredasdorp); lane 3: Wildebeest FR 1 fecal (Modimolle); lane 4: Buffalo 1 fecal (Elandsberg 2); lane 5: Sheep 2 fecal (Sutherland); lane 6: Springbok 2 fecal (Sutherland); lane 7: Wildebeest fed 4 fecal (Modimolle); lane 8: Buffalo 2 fecal (Elandsberg 1); lane 9: Buffalo 3 fecal (Elandsberg 1); lane 10: Buffalo 5 fecal (Elandsberg 2); lane 11: Buffalo 1 fecal (Elandsberg 2); lane 12: Wildebeest 2 fecal (Elandsberg 2); lane 13: Wildebeest 3 fecal (Elandsberg 2); lane 14: Bontebok 1 fecal (Elandsberg 1); lane 15: Bontebok 5 fecal (Elandsberg 1); lane 16: Bontebok 4 fecal (Elandsberg 1); lane 17: Cattle 4 fecal (Witsand); lane 18: negative control.

Figure A6.6 *tet*A, *tet*B and *tet*C multiplex PCR gel image for *E. coli* isolates at 562bp, 930bp and 888bp (f).

Lane 1: 100bp ladder; lane 2: Sheep 5 fecal (Witsand); lane 3: Cattle 4 fecal (Witsand); lane 4: Deer 1 fecal (Witsand); lane 5: Buffalo 5 fecal (Elandsberg 1); lane 6: Eland 1 fecal (Bredasdorp); lane 7: Buffalo 2 fecal (Elandsberg 1); lane 8: Buffalo 3 fecal (Elandsberg 1); lane 9: Buffalo 3 fecal (Elandsberg 1); lane 10: Buffalo 4 fecal (Elandsberg 1); lane 11: Buffalo 4 fecal (Elandsberg 1); lane 12: Buffalo 4 fecal (Elandsberg 1); lane 13: Buffalo 1 fecal (Elandsberg 1); lane 14: Impala 1 fecal (Bredasdorp); lane 15: Impala 1 fecal (Bredasdorp); lane 16: Impala fed 3 fecal (Modimolle); lane 17: positive control; lane 18: negative control.

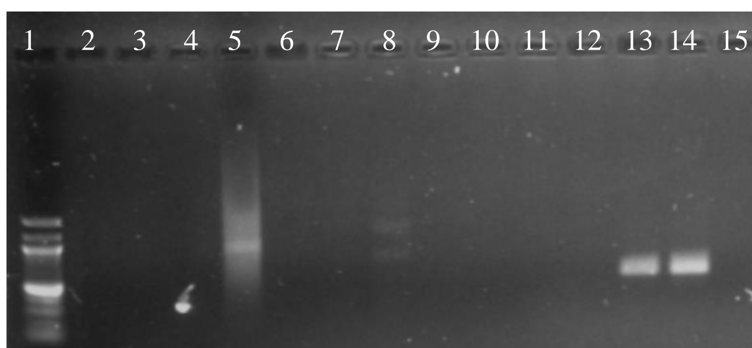

Figure A6.7 *tet*A, *tet*B and *tet*C multiplex PCR gel image for *E. coli* isolates at 562bp, 930bp and 888bp (g).

Lane 1: 100bp ladder; lane 2: Sheep 5 fecal (Witsand); lane 3: Eland 4 fecal (Bredasdorp); lane 4: Buffalo 5 fecal (Elandsberg 2); lane 5: Cattle 5 fecal (Witsand); lane 6: Wildebeest 3 fecal (Bredasdorp); lane 7: Buffalo 3 fecal (Elandsberg 1); lane 8: Impala 1 fecal (Bredasdorp); lane 9: Springbok 1 meat (Witsand); lane 10: Springbok 1 meat (Witsand); lane 11: Springbok 4 meat (Witsand); lane 12: Springbok 4 meat (Witsand); lane 13: Bontebok 3 meat (Elandsberg 1); lane 14: Bontebok 3 meat (Elandsberg 1); lane 15: negative control.

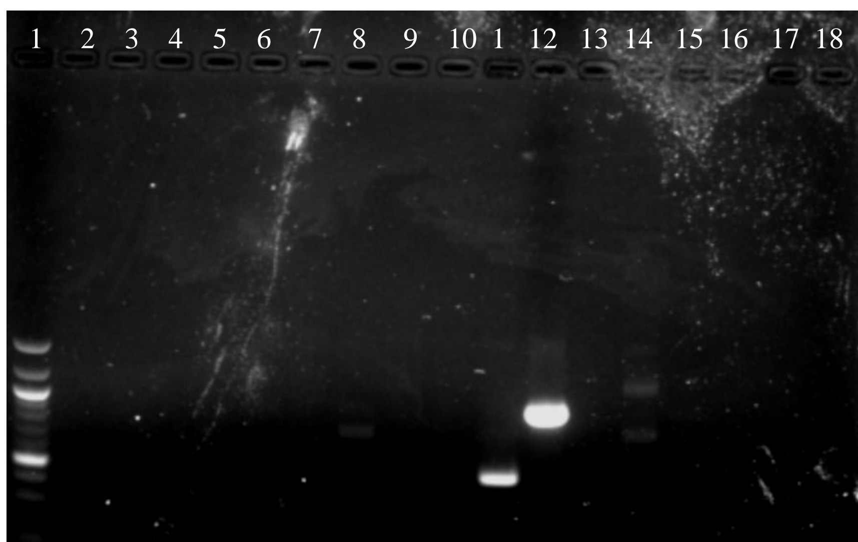

Figure A6.8 *tet*A, *tet*B and *tet*C multiplex PCR gel image for *E. coli* isolates at 562bp, 930bp and 888bp (h).

Lane 1: 100bp ladder; lane 2: Bontebok 4 fecal (Elandsberg 1); lane 3: Bontebok 5 fecal (Elandsberg 1); lane 4: Bontebok 5 fecal (Elandsberg 1); lane 5: Bontebok 5 fecal (Elandsberg 1); lane 6: Blesbok 3 fecal (Witsand); lane 7: Sheep 4 fecal (Bredasdorp); lane 8: Wildebeest 6 fecal (Bredasdorp); lane 9: Sheep 11 fecal (Bredasdorp); lane 10: Sheep 4 fecal (Bredasdorp); lane 11: Buffalo 2 fecal (Elandsberg 1); lane 12: Buffalo 2 fecal (Elandsberg 1); lane 13: Wildebeest 3 fecal (Elandsberg 2); lane 14: Wildebeest 2 fecal (Elandsberg 2); 15: negative control.

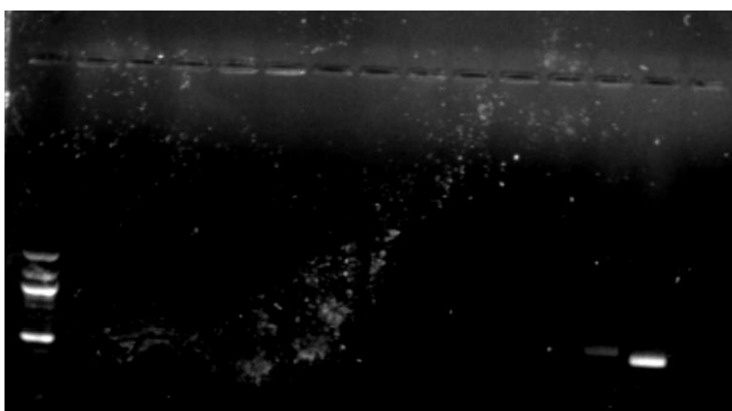

Figure A6.9 *tet*A, *tet*B and *tet*C multiplex PCR gel image for *E. coli* isolates at 562bp, 930bp and 888bp (i).

Lane 1: 100bp ladder; lane 2: Impala 1 fecal (Ekuseni); lane 3: Impala 2 fecal (Ekuseni); lane 4: Springbok 4 meat (Witsand); lane 5: Springbok 4 meat (Witsand); lane 6: Impala 1 fecal (Ekuseni); lane 7: Impala 2 fecal (Ekuseni); lane 8: Impala 1 meat (Modimolle); lane 9: Impala 1 meat (Modimolle); lane 10: Impala 2 meat (Modimolle); lane 11: Impala 4 meat (Modimolle); lane 12: Impala 4 meat (Modimolle); lane 13: Impala 2 meat (Modimolle); lane 14: positive control; lane 15: negative control.

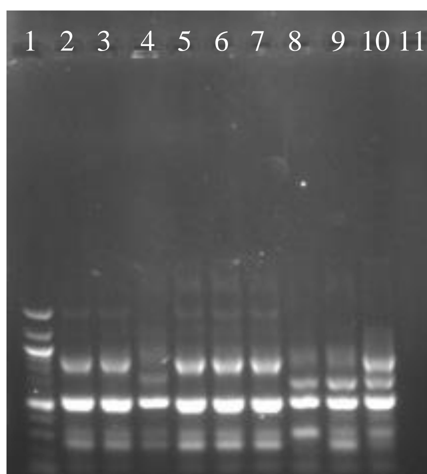

Figure A7.1 *aadA1* PCR gel image for *E. coli* isolates at 525bp (a).

Lane 1: 100bp ladder; lane 2: Buffalo 1 fecal (Elandsberg 1); lane 3: Buffalo 3 fecal (Elandsberg 1); lane 4: Buffalo 5 fecal (Elandsberg 1); lane 5: Buffalo 1 fecal (Elandsberg 1); lane 6: Buffalo 3 fecal (Elandsberg 1); lane 7: Buffalo 3 fecal (Elandsberg1); lane 8: Buffalo 4 fecal (Elandsberg 1); lane 9: Buffalo 4 fecal (Elandsberg 1); lane 10: Buffalo 5 fecal (Elandsberg 1); lane 11: negative control.

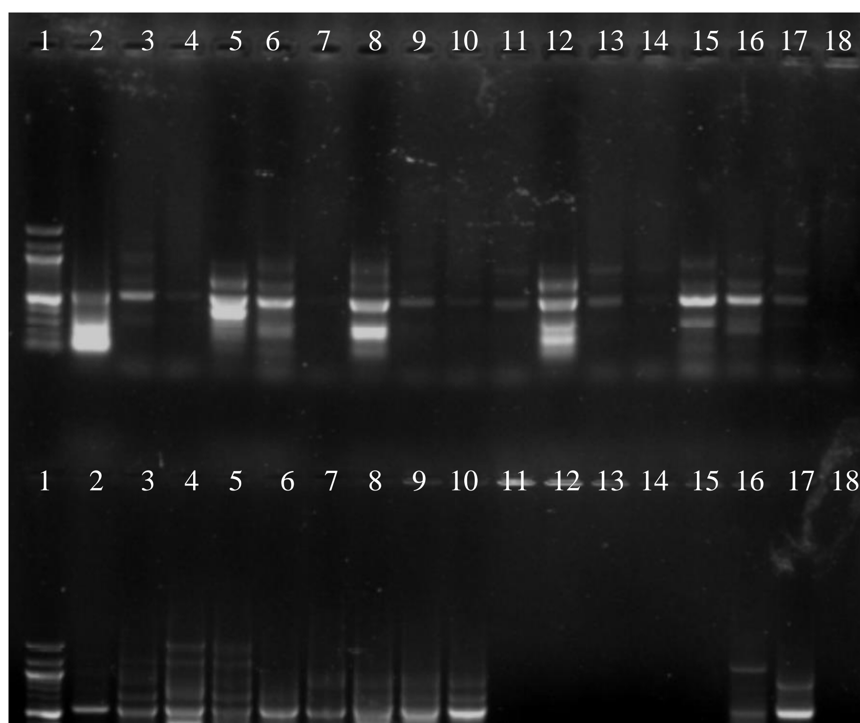

Figure A7.2 *aadA1* PCR gel image for *E. coli* isolates at 525bp (b).

Row 1: Lane 1: 100bp ladder; lane 2: Cattle 5 fecal (Witsand); lane 3: Sheep 4 fecal (Bredasdorp); lane 4: Cattle 12 fecal (Bredasdorp); lane 5: Wildebeest 6 fecal (Bredasdorp); lane 6: Sheep 11 fecal (Bredasdorp); lane 7: Wildebeest 3 fecal (Bredasdorp); lane 8: Eland 1 fecal (Bredasdorp); lane 9: Wildebeest 5 fecal (Bredasdorp); lane 10: Wildebeest fed 4 fecal (Modimolle); lane 11: Buffalo 1 fecal (Elandsberg 1); lane 12: Buffalo 5 fecal (Elandsberg 2); lane 13: Buffalo 3 fecal (Elandsberg 1); lane 14: Buffalo 3 fecal (Elandsberg 1); lane 15: Wildebeest 2 fecal (Elandsberg 2); lane 16: Buffalo 4 fecal (Elandsberg 1); lane 17: Buffalo 5 fecal (Elandsberg 1); lane 18: Buffalo 1 fecal (Elandsberg 2).

Row 2: Lane 1: 100bp ladder; lane 2: Buffalo 5 fecal (Elandsberg 2); lane 3: Wildebeest 3 fecal (Elandsberg 2); lane 4: Impala 1 fecal (Bredasdorp); lane 5: Impala 1 fecal (Bredasdorp); lane 6: Impala fed 3 fecal (Modimolle); lane 7: Springbok 1 meat (Witsand); lane 8: Springbok 1 meat (Witsand); lane 9: Springbok 4 meat (Witsand); lane 10: Springbok 4 meat (Witsand); lane 11: Impala 1 fecal (Ekuseni); lane 12: Impala 2 fecal (Ekuseni); lane 13: Impala 1 meat (Modimolle); lane 14: Impala 1 meat (Modimolle); lane 15: Impala 2 meat (Modimolle); lane 16: Impala 4 meat (Modimolle); lane 17: positive control; lane 18: negative control.

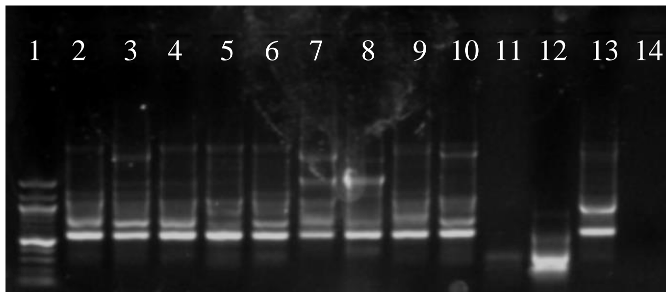

Figure A7.3 *aadA1* PCR gel image for *E. coli* isolates at 525bp (c).

Lane 1: 100bp ladder; lane 2: Sheep 5 fecal (Witsand); lane 3: Cattle 4 fecal (Witsand); lane 4: Deer 1 fecal (Witsand); lane 5: Sheep 3 fecal (Witsand); lane 6: Cattle 1 fecal (Bredasdorp); lane 7: Wildebeest 13 fecal (Bredasdorp); lane 8: Eland 4 fecal (Bredasdorp); lane 9: Cattle 10 fecal (Bredasdorp); lane 10: Wildebeest 13 (Bredasdorp); lane 11: Bontebok 3 meat (Elandsberg farm 1); lane 12: Bontebok 3 meat (Elandsberg 1); lane 13: positive control; lane 14: negative control.

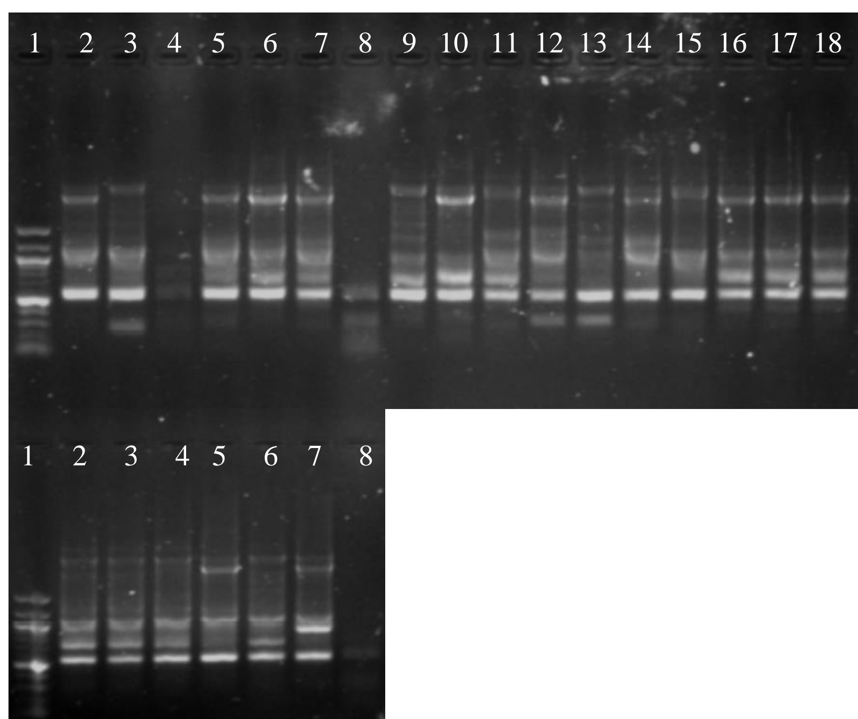

Figure A7.4 *aadA1* PCR gel image for *E. coli* isolates at 525bp (d).

Row 1: Lane 1: 100bp ladder; lane 2: Eland 2 fecal (Bredasdorp); lane 3: Wildebeest FR 1 fecal (Modimolle); lane 4: blank; lane 5: Sheep 2 fecal (Sutherland); lane 6: Springbok 2 fecal (Sutherland); lane 7: Wildebeest fed 4 fecal (Modimolle); lane 8: Buffalo 1 fecal (Elandsberg 1); lane 9: Buffalo 2 fecal (Elandsberg 1); lane 10: Buffalo 2 fecal (Elandsberg 1); lane 11: Buffalo 1 fecal (Elandsberg 2); lane 12: Wildebeest 2 fecal (Elandsberg 2); lane 13: Wildebeest 3 fecal (Elandsberg 2); lane 14: Bontebok 1 fecal (Elandsberg 1); lane 15: Bontebok 1 fecal (Elandsberg 1); lane 16: Bontebok 4 fecal (Elandsberg 1); lane 17: Bontebok 4 fecal (Elandsberg 1); lane 18: Bontebok 4 fecal (Elandsberg 1).

Row 2: Lane 1: 100bp ladder; lane 2: Bontebok 5 fecal (Elandsberg 1); lane 3: Bontebok 5 fecal (Elandsberg 1); lane 4: Bontebok 5 fecal (Elandsberg 1); lane 5: Blesbok 2 fecal (Witsand); lane 6: Blesbok 3 fecal (Witsand); lane 7: positive control; lane 8: negative control.