Determination of antibiotic residues in fish sold in the supermarkets around Mafikeng, North West Province

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Dissertation accepted in fulfilment of the requirements for the degree Masters of Science in Agriculture in Animal Health at the North West University

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DECLARATION

I, NEOYAME GLORIA SEKHOTO, declare that the dissertation entitled “Determination of antibiotic residues in fish sold in the supermarkets around Mafikeng, North West Province”, hereby accepted in fulfilment of the requirements for the degree of Master of Science in Agriculture in Animal Health at the North-West University, is my own work in design and execution and has not previously been submitted to this or any other university. I further declare that all the materials contained herein, have been duly acknowledged.

.....

Signature

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ABSTRACT

Aquaculture production has grown in the past decade, leading to a concurrent substantial growth in the use of antibiotics by the industry. The primary aim of this study was to determine antimicrobial residue levels of tetracycline, chloramphenicol, sulphonamide (sulfadiazine), quinolone (ciprofloxacin), nitrofuran, doxycycline, and penicillin to identify bacterial species in fish samples and observe their sensitivity to different antibiotics. This investigation was performed by collecting fish samples from supermarkets in Mafikeng, North West Province (n=50). Five samples of each fish type were collected during the study. Determination and detection of antibiotic residues from fish samples were conducted by using the Enzyme Linked Immuno-Sorbent Assay (ELISA), Thin Layer Chromatography (TLC) and High-Pressure Liquid Chromatography (HPLC). The results obtained revealed the presence of antimicrobial residues to be within the following ranges: 84; 84; 96; 12 and 20% of samples with concentrations ranging between 0-2240 (398 µg/kg); 0-120 (22.19 µg/kg); 0.3-9.7 (40.44 µg/kg); 0-30 (3.92 µg/kg) and 0-4840 (259.96 µg/kg) respectively for tetracycline, chloramphenicol, sulphonamide, quinolone and nitrofuran. It was also observed that among the positive samples, 54%, 84%, 6%, 4% and 10% respectively for tetracycline, chloramphenicol, sulphonamide, quinolone and nitrofuran were found to be above the Codex Alimentarius/ Republic of South African Maximum residue limits (Codex/ RSA MRL) using ELISA. The results for TLC showed a high prevalence rate of antimicrobials (88%, 76%, 74%, 74% and 64%) for sulphonamide, ciprofloxacin, tetracycline, doxycycline and chloramphenicol respectively. HPLC detected 28%, 74%, 14%, 21% and 0% with concentrations ranging between 0-0.69 (0.23 µg/kg), 0-279.8% (49.47 µg/kg), 0.68-8 (2.79 µg/kg), 0.68-7% (0.24 µg/kg) and 0% (0 µg/kg) respectively for tetracycline, chloramphenicol, sulphonamide and doxycycline. However, no penicillin residues were detected in samples analyzed using HPLC. Among the positive samples, chloramphenicol was found to be above the Codex/ South African MRL (72% of samples were detected with the same antibiotic).

The presence of antimicrobial is regarded as a public health concern as they may cause allergic reactions, intestinal disruptions, soft tissue damage and nervous disorders in humans. Significant correlations ($P \geq 0.05$) between the different methods used (ELISA, TLC and HPLC) were used to show the regularity, repeatability and quality in the methods used. The calibration curves of each antibiotic and process show the reliability of results obtained in this study. Although this study was limited in size, all samples were subjected to conventional

methods as well as molecular techniques of 16rDNA species specific gene amplification by PCR.

Furthermore, the bacterial isolates revealed the presence of *Bacillus cereus* (11.11%), *Clostridium sordeli* (5.56%), *Enterococcus faecium* (19.44%) and *Enterococcus* species (13.89%). Other micro-organisms had only 2.78% of the 20 samples. The isolates were evaluated for their antibiotic resistance pattern against some antibiotics using Kirby-Bauer antibiotic discs' diffusion method. In this study, most of the strains were susceptible to tetracycline (42%), followed by resistant strains (33%) and those that were intermediate (25%). In addition, the results obtained revealed that most of the strains (47%) had an intermediate resistance reaction to chloramphenicol, followed by strains that were susceptible (39%) and those that were resistant (25%). Majority of the strains (97%) were susceptible to ciprofloxacin, with only 3% were resistant while none was intermediate. Three quarters (75%) of the strains were susceptible to sulphonamide, followed by strains that were resistant (17%) and those that were intermediate (8%). Furthermore, 39% of strains were resistant to norfloxacin, followed by strains that were susceptible (36%), while only a quarter were intermediate (25%). Generally, ciprofloxacin is the best antibiotic among the five used in this study since more than 90% of the bacterial strains were susceptible to it.

Nonetheless, the study revealed low levels of antimicrobial residues and MRL; their presence in fish might be of risk to consumers. There is, therefore, a need for proper monitoring of the quality of fish sold in the country as well as training on antibiotic monitoring. This would enable farmers to be able to adhere to withdrawal periods of antibiotics and maintain healthy standards.

Keywords: Antibiotics, Tetracycline, Sulphonamide (sulfadiazine), Nitrofuran, Quinolone-Ciprofloxacin, Chloramphenicol, Doxycycline, fish, ELISA, TLC, HPLC, antimicrobial resistance, biochemical methods, PCR, 16SrDNA

LIST OF ABBREVIATIONS AND ACRONYMS

ABR	Antibiotic Resistance
ADI	Acceptable Daily Intake
AGP	Antibiotic Growth Promoters
API	Analytic Profile Index
API	Analytical Profile Index
CDC	Centre for Diseases Control and Prevention
CLIS	Clinical Laboratory Institute Standards
DAFF	Department of Agriculture, Forestry and Fisheries
DNA	Deoxyribonucleic Acid
EIP	Emerging Infectious Pathogens
ELISA	Enzyme-Linked Immuno-Sorbent Assay
et al	(et alii) and others
EU	European Union
FAO	Food and Agriculture Organization
HPLC	High Performance Liquid Chromatography
HPLC-PDA	HPLC method equipped with a photodiode array detector
HPLC-UV	HPLC method equipped with Ultraviolet Detection
I	Intermediate
LOD	Limit of Detection
LOQ	Limit of Quantification
Min	Minutes
MRL	Maximum Residues Level
PCR	Polymerase Chain Reaction
PCR	Polymerase Chain Reaction
pH	Logarithm for the reciprocal of hydrogen ion concentration in grams' atom per liter, used to express the acidity or alkalinity of a solution on a scale of 0 to14
PPM	Parts Per Million
R	Resistant
Rpm	Rounds per minute
RPM	Rate per Minute
RSA	Republic of South Africa
RTE	Ready to Eat
S	Susceptible
Sec	Seconds

TAC	Total Allowed Catch
TLC	Thin Layer Chromatography
UK	United Kingdom
USA	United States of America
VGT	Vertical Gene Transfer
WHO	World Health Organization

LIST OF UNITS

<	Less than
-	Negative
%	Percentage
/	Per
:	Is to
+	Positive
>	Greater than
°C	Degree Celsius
bw	body weight
g	Gram
L	Liter
Mg	Milligram
mL	Milliliter
mm	Millimeter
Mol	Mole
n	Number of samples
nm	Nanometer
TM	Trade Mark
v/v	volume/volume
µ/g	Microgram/gram
µ/mL	Microgram/milliliter
µg	Micro gram
µL	Micro liter
µm	Micro meter

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CHAPTER ONE

1.1 INTRODUCTION

Antimicrobials, especially antibiotics, are added to feedstuff and drinking water of food-producing animals in order to decrease exposure of infections in animals, prevent and treat diseases as well as act as growth promoters in both veterinary medicine and aquaculture (Conti *et al.*, 2015). Such antibiotics are usually the same as those used in animal husbandry, the treatment of household pets and humans. Regardless of the positive effects of these antibiotics in the treatment of infectious diseases, antibiotics in fish, milk, eggs, meat and other products may have serious side-effects on humans such as bacterial resistance, allergic reactions, toxicity, carcinogenic effects and a change of the intestines (Gould *et al.*, 2013).

Due to the increase in the world's population, the demand for fish and meat products, and the need to cater for their nutritional needs through high food demand (Olatoye & Basiru, 2013), has been on the rise in almost all regions of the globe, especially in developing countries. As a result, aquaculture production has tripled in the last two decades, leading to an increase in the usage of antibiotics by this industry (Done & Halden, 2015). Antibiotics in food consumed for long periods could result in the spread of drug resistant microorganisms (Wen *et al.*, 2006). Misuse of antibiotics in aquaculture production without veterinary prescription and control, coupled with lack of awareness of food safety, are contributing factors for high levels of residues.

According to the Food and Agriculture Organization (FAO), fish contributes about 60% of the world's nutritious protein content. In addition, 60% of the population in developing countries derive more than 30% of their annual protein requirement from fish (Olatoye & Basiru, 2013). The benefit of eating fish regularly is associated with decreased incidence of chronic conditions such as heart diseases, type 2 diabetes, obesity and certain forms of cancers (Conti *et al.*, 2015).

South Africa is one of the important fishing nations in Africa in terms of both fish production and trade (Cawthorn *et al.*, 2011). The total marine population of fish in South Africa between 2005 and 2008 stood at 689 681 tons (live weight) per annum, significantly greater than the numbers derived in the same period in other countries in the continents (Namibia, ca. 466 930 tons per annum and Angola, ca. 264 440 tons per annum) (Cawthorn *et al.*, 2011). In 2008, almost 21% of South Africa's catch was exported (Cawthorn *et al.*, 2011).

Fish farming in South Africa is practiced in the four coastal fishing provinces of the country (KwaZulu-Natal, Western Cape and Eastern Cape where most fishing activity is located and the Northern Cape where only 1% of South Africa's total allowed catch (TAC) is landed) (DAFF, 2012). In South Africa, fish farming is divided into fresh water and marine aquaculture. The fresh water fish nation is strictly limited by the supply of suitable water. The main areas to produce fresh water species are Limpopo, Mpumalanga Low-veld and Northern KwaZulu-Natal. Trout is farmed along the high mountain in the Lynden-burg area, KwaZulu-Natal Drakensburg and Western Cape and other fresh water species cultivated on a small-scale are catfish, crayfish and tilapia species (DAFF, 2013).

South Africa is a member of BRICS (Brazil, Russia, India, China and South Africa), where fish products are sold, both locally and internationally (DAFF, 2012). The abalone industry markets the bulk of its stock in Asia. South Africa receives most of its fish from the different countries that form part of the BRICS group of nations (Brazil, Russia, India and China) without a thorough understanding of the levels of antibiotics in the fish (DAFF, 2012).

To protect humans from potentially harmful antibiotic residues, the Republic of South Africa where the Minister of National Health has made regulations in term of section of the food stuff, cosmetic and disinfectant Act 1972 (Act No.54 of 1972) (DAFF, 2012); the European Union; Codex Alimentarius and China have established maximum residues levels for substances authorized for use as veterinary medication in food-producing animals. The maximum residue levels (MRL) are expressed as microgram per kilogram (µg/kg) and the limit should be less than the estimated MRL (Wen *et al.*, 2006).

Thus, sensitive and reliable analytical methods for the detection of veterinary medications and pharmaceutical residues in food of animal origin are needed to ensure consumer safety (Dasenaki & Thomaidis, 2015). Residue monitoring plays a significant part in ensuring the safety of food. Hence, the need to compare the different methods to understand which method is the most suitable for the detection of antibiotic residues.

An increase in fish farming in South Africa would improve production; however, sustainability of such form of farming involves the use of antibiotics for the control of diseases which leads to an increase in the prevalence antibiotic resistance in humans, coupled with lack of awareness of the consequences of food safety. Furthermore, the usage of antimicrobials is free and undocumented, so unacceptable residues could be found in feed and fish all over the world, resulting in the exposure of consumers to residues and resistance to bacteria (Conti *et al.*, 2015).

In SA, medicines are scheduled from 1-6, where schedule 1-2 in bought without prescriptions and schedule 3-6 are bought with prescriptions but somehow farmers get them even without prescriptions (Clay, 2014).

Another issue is the fact that the levels and type of antibiotics in fish are not known and their effect is only estimated. Most of the fish consumed in South Africa are imported from China, Chile, France, Taiwan, United States, and Mozambique (e.g. oysters). Major exporting countries are New Zealand with 110 tons, followed by China with 68 tons, then Chile with 42 tons and Denmark and United Kingdom for mussels and Norwegian, Chilean and Scottish (e.g. Atlantic salmon) (DAFF, 2012); and these nations do not have strict control measures for the detection of antibiotic residues in fish that is commercially distributed in South Africa. Furthermore, the reliability of detection methods remains questionable. There is, therefore, a need to compare different methods to decide which is the most suitable for the detection of qualitative antibiotic residues.

1.2 Research questions

The monitoring of antibiotic residues is important to ensure food safety and the occurrence of food-borne pathogens in fish products. This is largely related to the harvesting environment, processing environment and practices with equipment and personnel in the processing surroundings. The following research questions were asked in this study: Do farmers respect the withdrawal period of antibiotics? If South Africa were to implement control measures within the country, what would be the microbial quality of fish sold around Mafikeng? In addition, what are the levels of antibiotics in fish sold in supermarkets around Mafikeng?

1.3 Aim and objectives of the study

The main aims of this study were to:
Determine antimicrobial residues and identify antibiotic resistance of selected veterinary drugs in fish sold in different supermarkets in Mafikeng; and to compare the results using different analytical methods used to detect antibiotic residues.

1.4 Specific objective of the study

The specific objectives of this study were to:

- Collect fish samples sold in supermarkets in Mafikeng;
- Evaluate possible risks for consumers;
- Screen the level of antimicrobial residues in fish sold in Mafikeng using ELISA, TLC and HPLC;
- Determine the prevalence of different microorganisms from samples confirmed positive for antibiotic residues;
- Identify isolates using preliminary biochemical tests (catalase, oxidase & API 20E);
- Confirm isolates using PCR methods; and
- Determine the antibiotics resistance profile of isolates from positive samples.

1.5 Significance of the study

This study provides an overview of antibiotic residues and bacterial contamination of fish sold in supermarkets as well as feedback on which method is appropriate for the detection of antibiotic residues among the three methods tested below. In addition, the study also provides information on fish sold in supermarkets and their microbiological profile as well as the different bacterial isolates in fish sold in supermarkets in Mafikeng.

CHAPTER TWO

2.0 LITERATIRE REVIEW

2.1 ANTIBIOTICS

The word antibiotic is derived from Greek Word Anti' (Against) and 'Bios' (Life) (Chardon & Brugere, 2014). The noun antibiotic was suggested in 1942 by Dr Selman A. Waksman (a soil microbiologist). Antibiotics are normal manmade substances that disrupt the growth of or kill microorganisms and are used to treat and prevent infections in human and animals (Founou *et al.*, 2016). Antibiotics are the most commonly used class of antimicrobials that was first discovered in1928 by Alexander Fleming, who noticed the killing effect of mold accidentally blown onto his agar plate, after an attempt at isolation of the compound responsible, judged to be too unstable for use as antibiotic (Jagessar & Gomathinayagam, 2012).

They are a type of antimicrobial drug used in the treatment and prevention of bacterial infections; they do not work against any infections caused by viruses and their mode of action is bactericidal and bacteriostatic. In addition, soil bacteria, which look like fungi in nature, could produce antibiotics. This gives microbes an advantage when competing for food, water and other inadequate resources in a habitat, since they kill off their competition (WHO, 2015).

2.1.1 Origin of antibiotics

Originally, antibiotics were derived from natural sources, and were then further chemically modified to give better therapeutic effects. The primary classes of antibiotics include the following: B-lactam antibiotics; Tetracycline's; amino glycosides; Macrolides; Sulphur antibiotics; Quinolones; and Oxazolidinones (Davies & Davies, 2010).

2.1.2 Classification of antibiotics

Antibiotics are classified according to their spectrum of activity. Narrow-spectrum antibacterial antibiotics target precise types of bacteria, such as Gram-positive and Gram-negative bacteria, while broad-spectrum antibiotics affect a wide range of bacteria, usually both gram-positive and gram-negative cells (WHO, 2015).

Table 2.1: Summary of mechanism of action of antibiotics

Antibiotic group	Mode of action	Examples
Bacteriostatic	Inhibits bacterial growth	Tetracyclines, Sulphonamides, Amphenicols, Macrolides, Lincosamides
Bactericidal	Kills by disrupting the process of bacterial survival	Flouroquinlone, Nitrofurantoin, Daptomycin, Metronidazole

Source: (WHO, 2015)

Table 2.2: Classification of main antibiotics used in veterinary medicine

Main antibiotic families used in veterinary medicine	**Sub-families of antibiotic**	**Mode of action**	**Example of active ingredients used in veterinary medicine**
Beta-Lactam	Penicillin Cephalosporin	Inhibits cell wall production by affecting the firmness of the structure and shape of the bacteria. In addition, it weakens the outermost part of the bacteria making it sensitive to osmotic pressure, temperature, mechanical stress and triggers cell lyses.	Penicillin G, M & C
Polymyxins	/	Interrupt the structure of plasma membrane by entering the outer phospholipids. Metabolites and ions exit the cell and kill the bacteria.	Colistin Polymim B
Aminoglycosides	/	Prevent protein synthesis by acting on ribosome thus blocking protein production. Also works by preventing the formation of new protein and bacterial reproduction, and even activates destruction by causing abnormal protein synthesis.	Gentamicin Apramycin
Macrolides & similar	Macrolides Lincosamides Pleuromutilins		Erythromycin Spiramycin Didamycin Tiamycin
Cyclines	/		Chlortetracycline Doxycycline
Amphenicols	/		Florfenicol Thiamphenicol
Quinolones	Quinolones Flouroquinolone	Interrupt DNA structure by attaching to the major regulatory enzymes; topoisomerase and DNA gyrase	Flumequine Enroflaxacin Marboflaxacin
Sulphonamides	/	Works by preventing the synthesis of base pair DNA. In addition, they stop bacterial growth.	Sulfadiazine Sulfadiamethoxine Sulfamethoxazole + Trimethoprim

NB: It is worthy to note that there are other antibiotics in veterinary medicine used that belong to other families not described above (Source: Chardon & Brugere, 2014).

2.1.3 The use of antibiotics in food-producing animals

In food-producing animals, antibiotics are administered not only for treating, but also as preventing bacterial infections, where Sub-therapeutic doses are administered to counteract adverse stress effects that generally lead to infectious diseases (Sneeringer *et al.*, 2017). Additionally, they are used as growth promoters for the rapid growth of food-producing animals and fish (Sneeringer *et al.*, 2017). Antimicrobial consumption by both animal and human is expected to rise by 67% by 2030, and to nearly double the number in Brazil, Russia, India, China and South Africa if no additional restrictions on their use are taken. The prophylactic use of antibiotics and their application as a growth promoter are currently under review in many countries. As an example the European Commission decided to ban all antimicrobial growth promoters (AGP) in 2006 (den Hartog *et al.*, 2016).

2.1.4 Antimicrobial usage in animal feed

Antimicrobials are added to the feed and drinking water of food-producing animals to reduce susceptibility to infections and as a growth promoter, to speed up weight gain. Even though some antimicrobials have been banned for food safety reasons, the US Food and Drug Administration (FDA) is implementing a plan with industry to phase out a number of antibiotics (Conti *et al.*, 2015). Data show that aquaculture feed and fish contain some banned antimicrobials. The intake of farmed fish may involve risk for consumers besides contributing to the growth of antibacterial resistance. Furthermore, assessments of larger feed and fish samples are needed to achieve a more reliable assessment of consumer risk.

2.1.5 Use of antibiotics in aquaculture

In aquaculture, antibiotics are used to control diseases and infections as well as growth promoters (Van Huis, 2013). Such antibiotics are normally the same as those used in animal husbandry (for the treatment of household pets and humans). An increase in the density of antibiotics in water results in the vulnerability of fish to infections and exposure of the population to diseases. Stressed individuals often have immune systems that show reduced functionality (Dhama *et al.*, 2013). Pesticide contamination in farmed fish is used to assess risks and reduce contamination (Van Huis, 2013). In aquaculture, the use of antimicrobials is mostly unregulated (especially in Asia and American countries) and undocumented, as a result, unacceptable residues may be found in feed and fish.

2.2 ANTIBIOTICS USED IN VETERINARY MEDICINE INVESTIGATED IN THIS STUDY

2.2.1 Tetracycline

Tetracycline (TC) is a broad antibacterial spectrum antibiotic (Pena *et al.*, 2007). Tetracycline works by inhibiting bacterial protein synthesis. Tetracycline's are among the main antimicrobials used in aquaculture, and present a difficulty for extraction, due to the complex structure and high interaction with components of the biological matrix (Orlando *et al.*, 2013). Different classes of are as follows: tetracycline (TC); oxtetracycline (OTC), chlortetracycline (CTC); and doxycycline (DCT) (Wen *et al.*, 2006). Tetracycline has four structure rings and is derived from species of Streptomyces bacteria. Even though Tetracycline's are not regulated in Brazil for use in aquaculture, they are commercially available for use in other livestock, and due to their economic advantage and easy accessibility, they are commonly used in both veterinary medicine and in aquaculture for the prevention treatment of diseases (Wen *et al.*, 2006); and are used in microbial control during the creation and management of fish (Orlando *et al.*, 2013). The European Union and China have both set a maximum residue limit of 100ng/g in muscle for all species (Wen *et al.*, 2006).

2.2.2 Nitrofuran

Nitrofurans (NFs) are broad-spectrum antibiotics made up of 5-nitrofuran rings. They are quickly metabolized and are difficult to detect. Their presence is established by seeking their main metabolites (Gea *et al.*, 2015). The history of application of NFs as pharmacologically active substances began in 1944 (Zhang *et al.*, 2016). Its primary use as a veterinary drug is to prevent and control diseases. They are often applied to animal feed to stimulate growth of animals such as swine, poultry and bovine (Wang & Zhang, 2006). Nitrofurans have been banned in veterinary medicine since no safe levels for human can be set (Gea *et al.*, 2015).

2.2.3 Chloramphenicol

Chloramphenicol (CAP) is a broad-spectrum antibiotic that prevents the growth of a variety of aerobic and anaerobic microorganisms. It works by interfering with the production of proteins (Takino *et al.*, 2003). However, increased exposure of CAP could cause aplasia or hypoplasia, which could lead to aplastic anemia, which is often fatal. Due to these health concerns, the Food and Agriculture Organization (FAO) and the World Health Organization (WHO), declared that CAP residues are unacceptable for human food supply (Takino *et al.*, 2003). The

use of CAP in food products has been banned in the EU and USA (Takino *et al.,* 2003). However, it's still being used in other countries due to its availability and low costs. It has also been noted that, whenever CAP is accessible, there is a possibility for its illegal use. In fact, the presence of CAP has been detected in shrimps imported from China and Vietnam, intended for human consumption (Takino *et al.,* 2003).

2.2.4 Quinolones

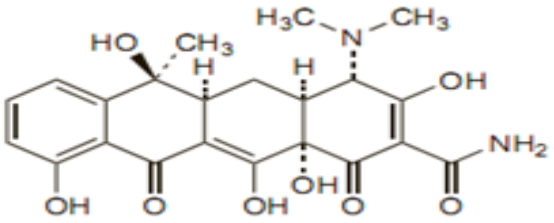
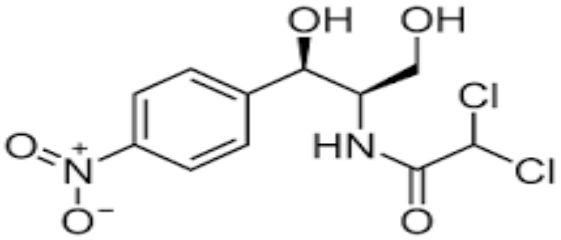
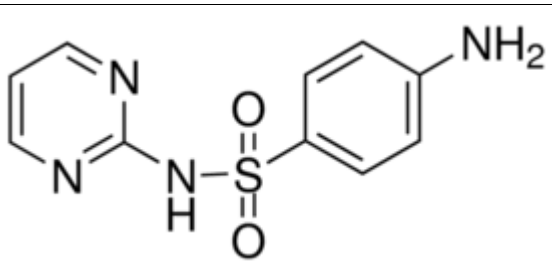
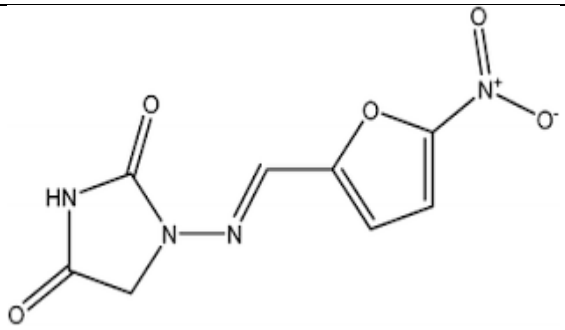
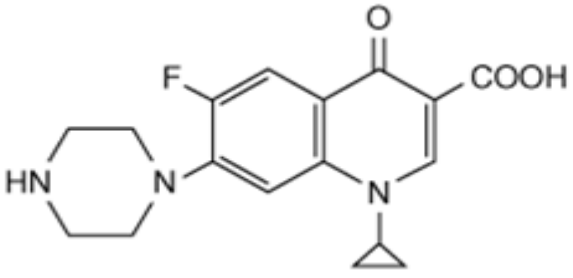
Quinolones are among the most important antibacterial agents used in both veterinary and human medicine, and work actively against both Gram-positive and Gram-negative bacteria through the inhibition of their DNA gyrase. Examples of Quinolones include Ciprofloxacin and Levofloxacin, which are used to treat bronchitis and pneumonia (Aldred, 2014). Ciprofloxacin and Ofloxacin are the most common quinolones used in hospitals and livestock, while Enrofloxacin is mostly used in veterinary medicine (Dorival-García *et al.,* 2015). Their main uses are in the treatment of human and veterinary diseases, as well as the prevention and therapy for many infections in fish farms (Rambla-Alegre *et al.,* 2010).

Quinolones are properly absorbed after oral administration and distributed extensively in tissues (Rambla-Alegre *et al.,* 2010). Administered Quinolones are excreted in urine and discharged into sewage (Dorival-Garcia *et al.,* 2015). Thus, humans could be exposed to residues of drugs in the environment through several routes, including the consumption of crops that have accumulated these substances from fertilizers (Dorival-García *et al.,* 2015). Accordingly, their residues need to be controlled since there is a growing concern about the possibility of their contamination (Rambla-Alegre *et al.,* 2010).

2.2.5 Sulphonamides

Sulphonamides are synthetic bacteriostatic antibiotics. They were the first antibiotics to be used systematically and paved the way for the antibiotic revolution in medicine (Hanifullah & Ayub, 2013). Most sulphonamides of them are administered absorbed orally, topically applied to burns and distributed all over the body. Its metabolism takes place in the liver and excreted through the kidneys. They are active against the broad-spectrum of gram-positive and gram-negative bacteria such as *Plasmodium* and *Toxoplasma spp*. Though resistance is extensive, resistance to one Sulphonamide indicates resistance to all (Hanifullah & Ayub, 2013), thus competitively inhibiting the synthesis of base pairs of DNA.

Table 2.3: Chemical structures of antibiotics

Antibiotics	Chemical formula	Chemical structures
Tetracycline	$C_{22}H_{24}N_2O_8$	
Chloramphenicol	$C_{11}H_{12}Cl_2N_2O_5$	
Sulphonamide (sulphodiazine)	$C_{10}H_9AgN_4O_2S$	
Nitrofuran	$C_8H_6N_4O_5$	
Ciprofloxacin	$C_{17}H_{18}FN_3O_3$	

Source: (Freitas, 2015)

2.3 POSSIBLE RISKS TO ANIMAL AND HUMAN HEALTH OF ANTIBIOTIC USE IN ANIMAL HUSBANDRY

2.3.1 Emergence of antibiotic resistance

Antibiotic resistance (ABR) occurs when an antibiotic loses its ability to successfully control or kill bacterial growth (Gutierrez D *et al.*, 2012). Whenever an antibiotic is consumed, it eliminates susceptible bacterial cells, leaving behind resistant strains that continue to grow (Founou *et al.*, 2016). Such resistant strains multiply, becoming the predominant bacterial population, and transmit the genetic resistance to offspring (Holmes *et al.*, 2012). A phenomenon, as explained above, occurs in pathogenic bacteria in humans, animals and in the environment (Founou *et al.*, 2016).

The more antibiotics are used, the greater the chance that a bacterial species may become resistant to them. The emergence and spread of antibacterial resistant bacteria continue to grow due to both the over-use and misuse of antibiotics. This is known as selective pressure or using antibiotics when they are not needed. Not taking them at the doses and time that a doctor prescribed, allows time for bacteria in the system to become resistant or even eating food with high levels of antibiotics (Prescott & Dowling, 2013).

For animals, especially fish and vegetables are considered large reservoirs of antibiotic resistant bacteria, since the food production chain is an ecosystem composed of different ecological niches, where large quantities are used and numerous bacteria co-exist (Founou *et al.*, 2016). ABR occurs in two pathways; the vertical gene transfer (VGT) and horizontal gene transfer (HGT). VGT refers to resistance that is mediated by a pre-existing phenotype (between bacterial populations that accumulate genetic errors) and involves genetic exchange within species (Holmes *et al.*, 2012), while HGT implies the acquisition of new resistant genes hidden in genetic elements (Founou *et al.*, 2016).

2.3.2 Cases of antimicrobial resistance in South Africa

In South Africa, only a few rational surveys and reports on antibiotic resistance in food have been conducted (most of them in Gauteng Province) (Apalata *et al.*, 2011). Clinical and environmental data suggests that the rate of antimicrobial resistance is high in South Africa. A report by Nyasulu *et al.*, (2012) revealed that surveillance of the pathogen *S. aureus* was resistant to cloxacillin at 29% and to Erythromycin at 38%; *Klebseilla pneunoniae* was resistant to Ciprofloxacin at 35% and 99% resistant to Ampicillin; *Pseudomonas aeruginosa* was

resistant at 43% and 35% to Amikacin. Ateba & Mbewe (2011) also reported antibiotic resistance in dairy and poultry products. Additionally, Penicillin was reported to remain intermediate in level, with a low prevalence of fully resistant isolates in South Africa (Nyasulu *et al.*, 2012).

2.3.3 Effects of antimicrobial residues

Regardless of the beneficial effects of antibiotics in the treatment of infectious diseases, antibiotic residues in fish, meat, milk, eggs and other products could have serious side-effects on human health (IFT, 2006), such as bacterial resistance, allergic reactions, toxicity, carcinogenic effects and change of natural micro flora of the intestine in consumers (Virolainen *et al.*, 2008). The risk of living in a post-antibiotic era cannot be avoided. There is need to revise current practices in the use of antibiotics in animal husbandry, including aquaculture. Proper monitoring of antibiotics residues in seafood is particularly critical since many antibiotics used in aquaculture are also used in human medicine (Done & Halden, 2015).

2.3.4 Pathological effects produced by antibiotic residues in food

Antibiotic residues in food could lead to different pathological effects in humans such as: immune-pathological effects; cancer causing (sulphamethazine and furazolidone), mutagenicity and paralysis of the nephron (gentamicin) and change in the abdominal flora (tetracycline), hepatotoxicity; reproductive disorders; bone marrow depression; (Chloramphenicol) and allergy (Penicillin) (Abjean, 1996). Antibiotics reported to affect the endocrine system of fish could be toxic to algae and invertebrates (Dorival-García *et al.*, 2015).

2.4 ANTIMICROBIAL EFFECTS ON BOTH ANIMALS AND HUMANS

2.4.1 Negative effects of antibiotics on food-producing animals

Antibiotic residues in edible animal products are of a great concern to regulatory agencies and consumers (Cháfer-Pericás *et al*., 2010). The extensive use of antibiotics triggers the development of bacterial resistance (Cháfer-Pericás *et al*., 2010), which in turn may continue to infect both animals and humans (Nyasulu *et al.*, 2012). Raw foods also contribute to the spread of resistant bacterial genes to humans through food chain (Moyane *et al.*, 2013). Data on antibiotics used in livestock production is scarce in South Africa, and there is limited information on the patterns of antibiotic consumption in food (Henton *et al.*, 2011). The use of antibiotics as growth promoters is said to be phasing out and is supported by the meat industry (Danese *et al.*, 2014).

2.4.2 Negative effects of antibiotic on humans

In humans, antibiotics are known to save lives; they are very effective in the treatment of illnesses caused by bacterial infections. However, they have side-effects. Even though most of these antibiotics are not dangerous, they could make life miserable during their intake (Xu *et al.*, 2017). Numerous studies have discovered that regular fish intake helps prevent chronic condition such as heart diseases and obesity. Seafood is essential for physical growth and development of newborn babies (Hunter & Roberts, 2000). These important effects are encouraged by the World Health Organization (WHO) (Conti *et al.*, 2015). The common side-effects that occur from antibiotics are diarrhea, nausea, vomiting, fungal infections of the mouth, intestinal tract infection, vaginal cancer and allergic reactions such as inflammation of the face, itchy skin and dyspnea in severe cases (Wu *et al*., 2012; Xu et al., 2017).

2.5 The occurance antibiotic residues

Antibiotic residues are a portion of antibiotics that remains in the body after has been discontinued (Looft & Allen, 2012). Many countries have implemented veterinary drug analysis programs to minimize residues and public health threats. The demand for routine analysis, has increased due to the increased amount of products traded in national and international markets, mainly to ensure that products are compliant with safety and quality criteria required by consumers (Hoff *et al.*, 2015). The stability of metabolites during the storage and cooking did not have significant effects on the residual concentration of antibiotics or drugs in incurred muscles (Vass *et al.*, 2008). The authors determined that between 67 and 100% of residues remained present in the tissue even after cooking, frying, grilling, roasting and microwaving (Hoff *et al*., 2015).

2.6 The development of residues in food-producing animals

Veterinary drugs usually build up in the liver or kidney rather than other tissues. However, it is common knowledge that different residue levels could be found in other tissues (Doyle, 2006). If veterinary drugs are used according to recommended label directions drug residues should not result in residues at slaughter. However, not following suggested label directions and dosage; not obeying withdrawal periods (overdose (long acting drugs) and under dosing) results into residue build up (Beyene & Tesega, 2014). Cross contaminations of feed and feeding stuff with accidentally applied drugs, environment and animal-animal transfer of drugs; inadequate sanitary care for animals and transportation of products (Beyene, 2016).

2.7 Aquaculture

Aquaculture, also known as fish farming (FDA, 2012). It involves human intervention in the rearing process to enhance production may include breeding, regular stocking, feeding and protection from predators; and to improve production and for corporate ownership, recreational and commercial or subsistence purposes (DAFF, 3013). Aquaculture is growing worldwide because of its nutritional benefit of proteins to humans (Orlando & Simionato, 2013). The main challenge in the aquaculture industry is the loss caused by diseases (Pallapothu, 2012), that results to an increase use of antimicrobials to control diseases and infections in order to improve the quality of aquatic environments and maintain the health status of aquaculture products (Pallapothu, 2012). The use of these antimicrobials require proper usage as improper usage may produce residues in animal tissues as well as the development of bacterial resistance transferred to humans (Orlando & Simionato, 2013).

2.7.1 Structure and characteristics of the fish industry in South Africa

In South Africa, fish farming started in the late 1890s and has increased thereafter (DAFF, 2013). It consists of about 2 798 km coastline of marine fisheries, which extends from the Orange River in the west, on the border with Namibia to Ponta do Ouro in the east near Mozambique. The western coastal ridge is greatly productive and incorporated with other flow ecosystems around the world, while the east coast is less productive but has high species of diversity, including both local and Indo-Pacific species. At present, there is a constant rights distribution process aimed at renewing fishing rights in most fishing sectors from 10 to 15 years (DAFF, 2013). However, fisheries constitute quite a small sector within the national economy of South Africa. The fishing industry was estimated to have generated about R2.63 billion in 2003 for wholesale revenue per annum to South Africa's Gross Domestic Products (GDP), thus resulting to the sector's overall contribution of about 1% to national GDP.

2.7.2 Global aquaculture segments

Due to growth in human population, the demand for fish products has increased in the world. It is estimated that about 40 million tons of fish products will be required by 2030 to maintain the current per capita consumption (FDA, 2012). Out of the 59.4 million tons produced in 2004, 70% was produced in China, 22% in Asia and the Pacific Region while the rest of the world produced only 8%. There was a significant growth of aquaculture segment between 2000 and 2009 in fish production (fresh water and marine), at a regular rate of 8.2%. Three hundred and thirty six species were produced in 2000, with Cyprinids (carps) dominating commercial

production species in Asia and the Pacific Region, while Salmons were dominant species in Europe, North and South America (FDA, 2012).

In 2004, about 22.2 million tons of fresh water species were produced. In addition, a 53% increase in marine fish production (about 1.95 million tons) were recorded in 2009. According to FDA (2012), it is suggested that future fish exports should mainly depend on supply constraints; a compliance with food safety standards in the form of sanitary and phyto-sanitary (SPS) measures as well as standards defined by the Technical Barriers to the Trade Agreement. Sanitary and phyto-sanitary implementation for fisheries products has largely shifted from product inspection to Hazard Analysis and Critical Control Point (HACCP) certification of harvest, post-harvest and processing standards, which are expected in exports (Pallapothu, 2012).

2.7.3 The impact of antibiotics on the environment and water systems

Antimicrobials are used in the aquatic environment for the improvement of water quality, and prevention and treatment of infections in water. Although these drugs are beneficial to the aquatic life, it is a concern as it poses health risks to human who depend on the water sources for drinking and cooking (Kümmerer, 2009; Kummerer, 2003), thus the need for water sources to be monitored. Aquatic ecosystems provide an ideal setting for the acquisition and spread of antibiotic resistance genes (ARGs), largely due to the continuous pollution by antimicrobial compounds derived from anthropogenic actions (Rodriguez-Mozaz *et al.*, 2015). Other ways through which antibiotics reach the environment are as follows: excreta from functional sewage sludge; and agricultural fields (Boxall *et al.*, 2004). Some antibiotics are considered to be resistant to the interruption and deprivation of conversion products under natural conditions (Thong & Modarressi, 2011). These promote long-term persistence of antibiotics at low levels, thus promoting the production of resistant bacteria in river bases or groundwater, which could cause serious environmental hazards (Pruden *et al.*, 2006).

Antibiotics are frequently detected in aquatic environments, comprising surface water, ground water and drinking water (Kümmerer, 2009). These antibiotic composites are not completely removed by conventional wastewater treatment plants and drinking water treatment systems. Hospital sewages are the most important source of residual drugs and other classes of pharmaceuticals in aquatic environments (Brown, 2004). In hospitals, wastewater is often discharged into public sewer systems, collected at wastewater treatment plants and co-treated with urban wastewater without any specific pre-treatment. Hospital sewages have been highlighted as exhibiting toxicity towards other aquatic organisms (Kümmerer, 2009).

2.7.4 Economic impact of aquaculture in South Africa

Aquacultures have the potential to contribute to food security, job creation and economic development (DAFF, 2013). About 1607 people have been employed on permanent basis and just a few on temporary basis (DAFF, 2013). Most of the jobs are created by the Abalone sub-sector, accounting for 1 219 employees, followed by the Oyster sub-sector, with 157 people, the Finfish sub-sector, with 152 employees and the Mussel sub-sector, with 79 job opportunities. South Africa's fishing products are marketed locally and internationally. The bulk of Abalone is marketed in Asia, whereas Trout is marketed locally (DAFF, 2013). Marine production per year in 2012 stood as follows: Western Cape (88%); Eastern Cape (12%); Northern Cape (0%); and Kwa-Zulu Natal (0%), (DAFF, 2013).

2.8 Legislation and control of antibiotics residues

Governments all over the world are increasing their efforts to improve food safety due to alarming food safety problems and rising consumer concerns (FDA, 2011). Divisions such as the Commission Decision 2002/657/EC, Food and Drug Administration (FDA) and International Conference on Harmonization (ICH), has implemented regulations to develop, validate and accredit methods for analysis of antimicrobial and non-antimicrobial residues in edible tissues of different animal species (Hoff *et al*., 2015).

In South Africa, aquaculture is governed by the following policies: Republic of South Africa Act (No 110 of 1983); the National Water Act (No 36 of 1998); National Environment Management Act (no 107 of 1998); Marine Living Resources Act (No 18 of 1998); Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies Act, 1947 (Act No. 36 of 1947), the Medicines and Related Substances Control Act, 1965 (Act No. 101of 1965), Animal Improvement Act, 1998 (Act No. 62 of 1998), Animal Identification Act, 2002 (Act No. 6 of 2002), Agricultural Product Standards Act, 1990 (Act No. 119 of 1990), National Environmental Management Act, 1998 (Act No. 107 of 1998) and the National Environmental Management Amendment Act, 2008 (Act No. 62 of 2008) and Food production is also regulated under the Foodstuffs, Cosmetics and Disinfectants Act (Act 54 of 1972) (DAFF, 2016), and the Health Act (Act 61 of 2003) by the Department of Health, National policies and Strategies; the National Development Plan 2030; Policy for the Small Scale Fishing Sector; DAFF Integrated Growth and Development Plan; Department of Water and Sanitation Resource Management Plans for State Dams; National Aquaculture Policy Framework; and

the Department of Trade and Industry's SMME and Small Business Development Strategies (DAFF, 2012)**.** Even though there are specific measures established for the control of antibiotic residues in foods, fraudulent or improper uses of veterinary drugs is still an on-going challenge that cannot be ruled out (Dorival-García *et al.*, 2015).

2.9 Maximum Residue Limits

Maximum Residue Limits (MRLs) are the levels of drug that are legally permitted for concentration and recognized as acceptable in food, set by law in many countries according to the European Union (EU) regulations (Lopez-Fernandez *et al.*, 2012). They are expressed as micro gram per kilogram (µg/kg) in food, but to compare them to the limits of detection (LODs), they are expressed in ng/100 mg or ng/g. Analysis of antimicrobial drug residues are sampled on animal-derived food produce such as muscle, liver, kidney, fat, milk, eggs and other tissues. A residue at or below MRL is considered safe because it minimizes the risk of the consumers to antibiotic resistance bacteria (Okocha, 2018), when food at that level is consumed daily for a lifetime (Beyene, 2016). In South Africa, the maximum residue limits for veterinary medicine and stock remedy are governed under the Foodstuffs, Cosmetic and Disinfectants Act No. 54 of 1972;). The drug specific MRLs are them applied by the Fertilizer, Farm feeds, Agriculture Remedy; and Stock Remedy Act 1947 and the Medicine and Related Substances Act No. 101 of 1965 to set the acceptable withdrawal periods (Chanda *et al.*, 2014). However, data on antibiotics used in livestock production is limited in South Africa (Henton *et al.*, 2011).

2.10 Fish and its importance

The word fish comes from the old English word *fisci*. It is referred to as cold-blooded, gill-bearing aquatic animals that lack limbs with digits (www.wikipedia.com). Fish is an important source of food worldwide and is known to produce about 60% of the world's protein, vitamin D and omega-3 fatty acids, which plays an important role in the development of the body and brain (Leech, 2016).

2.10.1 Fish vaccination

Like any other animal, fish is also susceptible to infections and diseases. There are different application routes for the vaccination of fish (Allen *et al.*, 2013). Oral vaccination is known to be the most effective than the usual injection method. There are other procedures used such as spray or bath immersion methods, and are known to pose less stress on fish (Nakao, 2014).

Vaccines and medicated feeds are the mainstay of bacterial infection management and control. Most countries in the world, such as Norway, Canada, Thailand, Iceland, Faroe Islands, France, Turkey, UK and the United States of America, have also adopted the use of vaccines as an alternative to antibiotics (Hoff *et al.*, 2015). Fish vaccination holds a promise for the rest of the world where vaccines are not currently being used (Pallapothu, 2012).

2.10.2 Fish diseases

Disease prevention plays an important role in aquaculture in order to promote animal health and reduce the susceptibility of a wide variety of infections. Fish diseases depend on the type of fish species, for example, Nitra disease is found in Salmon, and is caused by *Vibrio salmonicida*. Fishes are vaccinated against "enteric red-mouth" caused by a bacterium *Yersinia ruikeri* in trout. *Furunculosis* is mostly seen on salmon and is believed to be caused by *A. salmonicida* (Nakao, 2014). *A. hydrophila* causes serious problems in fish. Some inactivated viral vaccines are available for white spot diseases in catfish and carp spring viremia caused by *R. carpio* (Nakao, 2014).

Some viruses cause viral haemorrhagic septicaemia (VHS) and infectious hematopoietic necrosis (HN) in rainbow trout. Viral diseases are associated with several environmental factors and caused an intense reduction in the production of marine shrimps between 1990 and 1996. Stress is another factor associated with the development of diseases and is largely caused by opportunistic bacteria of the genus *Vibrio* pathogens of bacterial origin registered in aquatic organisms. V*ibriose**s*** and necrotizing hepatic pancreases (NHP), *Vibrio harveyii, V. vulnificus, V. parahaemolyticus, V. anguillarum* and *V. Alginolyticus* and less frequently, *V. damsela* and *V. fluvialis, vibrioses, Rickettsia, Mycobacterium fortuitum* and *Mycobacterium marinum,* are common diseases found in fish (Nakao, 2014).

2.10.3 Immune-stimulations

Even though bacterial and viral vaccines have been developed, many vaccines are based on the recumbent antigen and some are not very effective. Immune-stimulants are used to increase the efficiency of vaccines. These immune-stimulants are known as traditional adjuvant mineral oils such as ligands for toll receptors or cytokines; the B-glucans and certain plant extracts that are incorporated in food. In addition, probiotics yeast strains are administered to promote health and elevate phagocytic, lysozyme, complement and respiratory burst activity or influence cytokines production (Nakao, 2014).

2.11 ANTIMICROBIAL DETERMINATION METHODS

Analytical methods used to detect antibiotic residues in foods play an important role in ensuring food safety (Cawthorn *et al.*, 2011). They are classified into two groups as follows: confirmatory; and screening methods. Screening methods are those that do not require skills because they are easy to perform, flexible and enable large sample throughput (Cháfer-Pericás *et al.*, 2010). At present, there is an increase in commercial fraud in the export and import industry of fish species in the world thus, the need for foodstuffs to be investigated and managed correctly to protect the endangered species and the public (Cawthorn *et al.*, 2011).

2.11.1 Enzyme Linked-Immuno-Sorbent Assay (ELISA) method

Enzyme-Linked-Immunosorbent Assay is the most popular screening method used for the detection of veterinary residues due to its high sensitivity, specificity and simplicity to screen a large number at the same time and in a shorter period, although it requires precise sample preparation and extraction (Zhang *et al.*, 2009). It works by bounding the antibody in wells against the antibiotic and an enzyme.

2.11.2 Thin Layer Chromatography (TLC)

Thin Layer Chromatography is also widely used to determine antibiotic residues in foods. It is a screening test due to its sensitivity and specificity for monitoring even low amounts of different biological and chemical residues in animal tissues. Generally, it is not expensive and is less time-consuming than other quantitative methods. In a broader sense, they do not require very laborious sample pre-treatment steps (Mickey, 2014).

2.11.3 High Performance Liquid Chromatography (HPLC)

High Pressure Liquid Chromatography is a commonly used confirmatory test for the detection of antibiotic residues in foodstuff (Snyder *et al.*, 2009; Wang & Zhang, 2006). It is regarded as a qualitative and quantitative technique, commonly used for the estimation of pharmaceutical and biological samples (Wilson & Walker, 2001). It is time-consuming and requires skill for sample preparation and extraction (Malviya *et al.*, 2010). HPLC also involves the use of high pressure to drive the analyte into the solution through a packed chromatographic column, thus causing separation of the analyte under test, which could be detected using ultra violet, diode array detector or mass spectrometry (Wilson & Walker, 2001).

2.12 MICROBIAL QUALITY AND ITS CHALLENGES

Food-borne illnesses are defined as diseases, which are either infectious or toxic in nature, and occur following the consumption of contaminated food (Nsoesie *et al.*, 2014). The burden of unsafe food results from the use of chemical and parasitic contaminants (WHO, 2015). Food-borne diseases are a global concern and have been reported worldwide. In addition, they are known to have caused high morbidity and mortality both in developed and developing countries; and have always been an issue for all societies since the beginning of humankind. The global burden of illness and deaths caused by food-borne diseases has never been quantified even up to date (WHO, 2015).

Food-borne illnesses are associated with conditions such as diarrhea, vomiting, and abdominal pains and fever (Nsoesie *et al.*, 2014). Diarrhea contributes about 3% of mortality globally (Mutalib, 2015). Many incidences are rising in developed and developing countries (Redmond and Griffith, 2003), but only a few incidences are reported (Soon *et al.*, 2011). Everyone could be affected with food-borne illnesses, however, infants, young children, the elderly and people whose immune systems are compromised are most likely to be affected (Fleury *et* al., 2008). In addition, lack of clean water, cross contamination during food preparation, inappropriate transportation and storage of food, lack of awareness regarding safe and hygiene food practices are other factors that contribute to the occurrence of food-borne illnesses (Havelaar *et al.*, 2015).

The World Health Organization, in collaboration with its partners, launched initiatives to estimate the Global Burden of Food-borne Diseases in 2006; and also established a Food-borne Disease Burden Epidemiology Reference Group (FERG) to fill the data vacuum (WHO, 2015).

Table 2.4: Common causes of food-borne diseases

Antimicrobial	Example	Mode of action
Bacteria	Listeria	Usually results from blood poisoning and meningitis and spread by consuming contaminated raw vegetables, ready-to-eat food, processed meat, smoked fish and soft cheese.
	Brucella	Commonly found in pasteurized milk and cheese and could cause fever, muscle pain or arthritis, chronic fatigue, neurological symptoms and depression.
	Cholera	Caused through the consumption of contaminated food with vibrio cholera. It causes watery diarrhea that could result in death if left untreated.
Virus	Hepatitis A	A liver disease transmitted through food by stools of infected persons; could result in jaundice, nausea, anorexia, fever, malaise and abdominal pains.
Parasites	Toxoplasma	It is caused by Toxoplasma Gondi, spread through undercooked or raw meat and fresh produces; could result in impaired vision and neurological conditions.
	Taenia solium (pork-tapeworm)	Caused by the development of cysts in the brain and may lead to epilepsy.
	Echinococcus tapeworm	Humans get infected by being in direct contact with infected stool of dogs or fox. It could cause tumors in the liver, lungs and brain.
	Live fluke (Clonorchis sinensis)	It is normally contracted through raw and incorrectly processed or cooked fish. It could cause inflammation of the bile ducts and cancer.
Chemicals and toxins	Aflatoxin	It is a toxin produced by mould that grows on grain that has been stored inappropriately and could cause liver cancer.
	Cyanide	A poisoning that occurs when incorrectly processed cassava is ingested.

Source: (WHO, 2015)

2.12.1 Food-borne Diseases Active Surveillance Network

The Food-borne Disease Active Surveillance is an organization that tracks important food-borne illnesses (Food-Net). It generates information that provides a foundation for food safety policies that contributes to food safety by estimating the number of food-borne illnesses; and distributes information leading to improvements in both public and private health and development (Scallan & Mahon, 2012).

2.12.2 The impact of transportation on food

Transportation of food to food stores and food services has a huge impact in the distribution of pathogens in foods. The length of time spent during food delivery and the quality of packaging of different foods enables bacterial growth on foods. The type of vehicles and environments during food delivery also contribute to the development of pathogens (Scallan & Mahon, 2012).

2.12.3 Food handling practices of consumers

Consumers use different practices to handle their food, which explain differences in the rate of food-borne illnesses in the consumption of raw food, poor hygiene and cross-contamination. It has been found that individuals with high income consume rawer food, with less knowledge of hygiene. However, lower cross-contamination practice was reported in African-Americans, who are less likely than Caucasians, to eat potentially risky foods, except for raw clams. Additionally, research has showed that compared to women, men are less knowledgeable about food safety and have riskier hygiene and cooking practices whereas in lower-income households, people aged 65 years and older, and non-college graduates, practice safer food handling methods. It has also been reported that individuals with higher levels of education and high income are more likely to eat raw clams, raw oysters, raw fish, raw sprouts and pink hamburger, besides having unsafe hands and cutting board-washing practices (Scallan & Mahon, 2012).

2.12.4 Ready-to-eat foods at the retail level

Ready-to-eat (RTE) foods refer to foods that are in a form that is edible without washing, cooking or heating by consumers. Fruits and vegetables that are washed and cut are considered RTE foods, and any other foods that are accessible for consumption for which further washing, or cooking is not required and from which coats, peels, rinds, or shells are removed. Ready-to-eat foods are considered high-risk foods because they do not require any heating or process

prior to ingestion (Yapp & Fairman, 2004). Insufficient refrigeration or sanitation in the preparation of RTE foods might create conditions under which any existing bacteria may develop further, especially if lack of proper handling practices occurs. Many retail outlets do not have sufficient resources available to train employees on safe food handling and guarantee safest food supply to consumers (Yapp & Fairman, 2004). Therefore, a good understanding of hygiene and proper handling practices are required.

2.12.5 Food quality/safety indicators

A higher bacterial number will cause food to spoil faster and result in food quality loss. Grocery stores and corner markets in low socioeconomic and minority racial/ethnic areas may have insufficient workers training, and may lack resources needed for best sanitation practices and education on food safety (Yapp & Fairman, 2004). Such outlets may also have poor infrastructure and the condition of the machinery and refrigerators may cause insufficient refrigeration temperatures, allowing the growth of bacteria in food products stored at abusive temperatures. Food products contaminated with pathogens that are not safely handled during preparation (such that they cause cross-contamination) may lead to food-borne illnesses. Furthermore, food contamination could occur from improper handling of food at supplies (Yapp & Fairman, 2004).

CHAPTER 3

3.0 RESEARCH METHODS

The aim of this study was to determine levels of antibiotic residues in fish sold in supermarkets within Mafikeng. Enzyme Linked Immuno-Sorbent Assay (ELISA), Thin Layer Chromatography (TLC) and High Pressure Liquid Chromatography (HPLC) were used in this study to determine antimicrobial residues in fish samples namely: tetracycline; nitrofuran; penicillin; doxycycline; sulphonamide and quinolone-ciprofloxacin. ELISA and TLC were used as a screening methods, and HPLC was used as confirmatory method. Fish samples were cultured for bacterial isolation and tested for antibiotic resistance.

3.1 STUDY AREA

The study was conducted in Mafikeng, North West Province, South Africa. The city lies between 25°C and 28°C South of the Equator and 22°C and 28°C longitude east of the Greenwich Meridian. Temperatures range from 17°C to 31°C in the summer and from 3°C to 21°C in the winter. Mafikeng is located close to South Africa's border with Botswana and Northeast of Cape Town (Wikipedia.org). No study has been conducted so far to determine antimicrobial residues in fish sold in supermarkets around South Africa. In addition, these supermarkets are countrywide, and it is assumed that they receive their supplies from the same supplier.

Figure 3.1: Map of Mafikeng sampling area (www.google.com)

3.2 SAMPLING

In this study, 50 fish samples were purchased from five randomly identified supermarkets in Mafikeng, North West Province. Sampling of different fish species was done once every month for five months (from May to September 2015). Supermarkets selected for the study are among the major suppliers of fish in the area. All samples were purchased within the recommended date of consumption. After collection, samples were packed in a cooler box and every detail of the sample type, collection date and place of purchase recorded.

Table 3.1: Categorization of fish samples into two groups

Salt/sea water fish	Fresh water fish
Oysters (*Grassotrea gigas)*	Trout (*Oncorhynchu mykiss*)
Medditerian mussels (*Mytilus galloprovicials*)	Tilapia (*Oreochromis massambicus*)
Finfish (*Argyrosomus japonies*)	Catfish (*Clarias ganepinus*)
Abdole (*Haliotis midae*)	Carp (*Crpriuas carpio*)
Black mussels (*Choromytilus meridionalis*)	Atlantic salmon (*Salmo salar*)

*NB – For each fish type, 5 fishes were collected for analysis (Source: DAFF, 2012)

3.3 SAMPLE PREPARATION

To avoid contamination, the skin and bones of each raw fish were removed using a sterile knife. 1 kg of each type of fish was later chopped on a chopping board and homogenized with a stomacher for two minutes. 50 g of homogenized sample was used for analysis of antibiotic residues. This study was conducted in the Microbiology Laboratory, Department of Animal Health, North-West University (Mafikeng Campus), North West Province, South Africa. Raw samples were kept in a deep freezer until further analysis.

3.4 DETERMINATION OF ANTIMICROBIAL RESIDUES IN FISH (METHODS)

3.4.1 Enzyme-Linked Immuno-Sorbent Assay (ELISA)

In this study, ELISA was used as a screening method for tetracycline, quinolone, chloramphenicol, nitrofuran and sulphonamide in fish samples. The samples were prepared and analyzed using competitive enzyme immunoassay (ELISA kit RADISCREEN) to conduct the quantitative analysis of samples (Bio-pharma, AG Darmstadt, Germany). All samples were done in duplicates.

Each kit contained a sufficient material of 96 measurements and 12 strips with 8 removable wells. The kits also contained 1.3 ml of 6 standard concentrations for each antibiotic; tetracycline (0, 0.5, 1.5, 3, 6, 18 ppb), quinolone (0, 0.5, 1.5, 3, 6, 18 ppb), chloramphenicol (0, 25, 50, 100, 250, 750 ppt), nitrofuran (0, 100, 300, 900, 2700, 8100 ppt) and sulphonamide (0, 1, 3, 10, 30, 100 ppb) in aqueous solutions), conjugate (0.7 red cap), anti-antibiotic antibody, substrate (10 ml brown cap), chromogenic, stop solution (14 ml yellow cap), washing buffer (salt) and a buffer (100 ml) (sample and standard buffer). All ELISA kits were stored at 2-8°C.

Unused micro wells were returned to their original foil bags, resealed together with the desiccant and further stored at 2-8°C. The reddish substrate/chromogenic solution is light sensitive; therefore, exposure to direct light was avoided. Samples were stored in a cool place and protected against light. A different ELISA kit and method were used for each antibiotic tested, according to the manufactures instructions.

a) Equipments used for the elisa test

The following equipment was used to perform the ELISA test: A Micro-titer plate spectrophotometer (450 nm); ELISA kit's Centrifuge; Vortex shaker and mixer; equipment for

evaporation; Pasteur pipettes; Graduated pipettes; variable 20 µl – 200 µl; and 200 – 1000 µl micropipettes.

b) Prepartion and extraction of samples

In this study, nitrofuran RADISCREEN® Art No. R3715 was performed according to the manufacturer's instructions for the determination of antibiotics in fish samples. Extraction of samples was done as follows: a 1 g of homogenized sample was mixed with 3.9 ml distilled water; 0.5 ml of 1M HCl; and 200 µl 10mM 2– Nitro Benz-aldehyde (shaken vigorously). Samples were then incubated at 50°C for 3 hours, 5 ml of 0.1M K_2HPO_4; 0.4 ml 1NaOH and 5 ml ethyl acetate were added and then shaken vigorously for 30 seconds. Samples were then centrifuged for 10 minutes at 3000 rpm. After centrifuging, 2.5 ml of the ethyl acetate (upper layer) was transferred into a new vial and evaporated. Residues were dissolved in 1 ml n–hexane and mixed properly with 1 ml sample buffer. Furthermore, samples were centrifuged for 10 minutes at 3000 rpm then 100 µl of the lower aqueous phase were used per well in the assay.

For sulphonamide, RADISCREEN® Art No. R3004 was performed according to the manufacturer's instructions. One gram of homogenized sample was mixed with 2 ml methanol and vortex for 30 seconds. Centrifuging was performed for 10 minutes at 4000 rpm. Then 1.5 ml of methanol solution was transferred into a new centrifugal vial and allowed to evaporate using N_2. Thereafter, 0.5 ml sample dilution buffer was mixed with 1 ml of n-hexane (for degreasing) and vortex for 10 seconds. Centrifuging was performed for 10 minutes at 4000 rpm. A 50 µl of the lower phase per well was used in the test.

For chloramphenicol, RADISCREEN® Art No. R1505 was used according to manufacturer's instructions. Three-gram of homogenized samples were mixed with 3 ml distilled water and added into a 6 ml ethyl acetate and mixed intensively for 10 minutes. The solution was later centrifuged for separation for 10 minutes at 3000 rpm. The supernatant was later transferred into 4 ml ethyl acetate into new vials and evaporated for dryness by nitrogen. The evaporated vials were then dissolved into 1ml hexane. Later, 0.5 ml buffer was added to the solution and mixed intensively for 1 minute in a vortex mixer. They were later centrifuged for separation for 10 minutes at room temperature. A 50 µl of the aqueous lower phase was used per well in the assay.

For quinolone, RADISCREEN® Art No. R3113 was used and the extraction procedure performed according to the manufacturer's instructions. One gram of homogenized sample was used and mixed with 4 ml methanol/water (70; 30 v/v) and mixed vigorously for 10 minutes with a vortex mixer. The solution was centrifuged for 10 minutes at 4000 rpm and the supernatant diluted with 1:2 washing buffer. Then, 50 µl was used in the well in the test.

For tetracycline, RADISCREEN® Art No. R3505 was used. The method was performed as follows: one gram of homogenized sample was transferred into a centrifugal vial cap and 9 ml of 20 Nm PBS buffer added and vortex for 10 minutes and centrifuging on the sample was done after for 10 minutes at 4000 rpm. Then, 50 µl of the upper aqueous phase was used per well in the assay.

c) Analytical procedures

A sufficient number of wells were inserted into a micro well holder for all the standards and samples to be run. The sample position and standards were later numbered. Thereafter, 50 µl of each standard solution and prepared sample were added to separate duplicate wells (1 pipette tip was used for each standard and sample). Anti-microbial antibody solution (50 µl) was added to each well. The solution was gently mixed by shaking the plates manually and incubated for 1 hour at room temperature (20-25 ºC). Later, the liquid was poured out of the wells by tapping the micro well holder upside down vigorously three times, against an absorbent paper for complete removal of the liquid from the wells. A 250 µl of washing buffer was added and the liquid poured out again (repeated twice). 100ul Substrate/ chromogenic (100 µl) was added to the wells and mixed gently by shaking manually and incubated for 15 minutes at room temperature (20-25 ºC). Finally, 100 µl of the stop solution (yellow cap) was added to each well, mixed gently and shaken manually and the absorbance measured at 450 nm. The results were read within 30 minutes after adding the stop solution.

d) Calculation of results and interpretation

The results were calculated by obtaining the O.D (optical densities) values and calculating the percentages (%) of absorbance using a formula:

$$\frac{absorbance\ standard\ or\ sample}{absorbance\ zero}\ X\ 100 = \%\ absorbance$$

The calibration curves were plotted between the standard concentration and the O.D value. Then the values calculated for standards are entered in a system of coordinates on semi

logarithmic graph paper against the concentration. In order to obtain concentration contained in a sample, the concentration read from calibration curve must be further multiplied by the corresponding dilution factor. Dilution factors are enclosed in test kit.

Figure 3.2: Image of an ELISA machine (Model MB-580)

3.4.2 Detection of antimicrobial residues using Thin Layer Chromatography Test (TLC)

In this study, all samples analysed using ELISA were further screened using TLC. The analysis was performed according to the procedure described for each antibiotic tested with modification of the mobile phase. Antibiotic standards were used for the confirmation of results. For TLC and HPLC, the same methods of extraction were used. To run TLC, solvents were transferred into 15 ml centrifuge tubes and centrifuged at 7000 rpm for 10 minutes. The clear supernatant was then transferred into the fresh glass tubes and evaporated with N_2 stream. After complete drying, the deposits were dissolved in 0.2 ml methanol (Tajick & Shohreh, 2006) and ready for analysis using 20x10 silica gel plates (TLC A1 foil, St Louis, Germany).

a) Reagents used for Thin Layer Chromatography (TLC)

To run TLC method on fish samples, the following chemicals and reagents were used: Methanol; Dichloromethane; Acetone and distilled water; and deionized water obtained from the Animal Health Laboratory of the North-West University.

b) Instrumentation

To ensure proper running of TLC, the following consumables were used: antibiotic standards (tetracycline, chloramphenicol, sulphonamide, doxycycline and ciprofloxacin); 15 ml centrifuge tubes; nitrogen stream; silica gel plates; TLC chamber glass; micro-syringe TLC

spotter; UV light (Spectro line model CM-10A Westbury, New York, USA), (short wavelength 254 nm and long wavelength 365 nm); vortex; a pencil; a ruler; and a scale.

c) Preparation of standards

In this study, TLC was performed to compare extracted residues with five raw antibiotics that are commonly used (tetracycline, doxycycline, ciprofloxacin sulphonamide and chloramphenicol) obtained from Sigma Chemical Co., St Louis MO, USA. Each antibiotic was prepared by dissolving 0.1 g of each powder in 4 ml methanol according to Thangadu *et al.* (2002). Serial dilution was prepared for five standards ranging from 5, 2.5, 1.25, 0.625 and 0.3215 mg/ml. For fish samples, products were extracted as described in HPLC sample extractions according to each antibiotic. Prior the test, the products were stored at 4ºC until analysis.

d) Sample preparation

For sample preparation, penicillin extracts were used according to Rambla-Alegre *et al.* (2010) and performed as follows: 5 g of homogenized sample was mixed with 50 ml of 0, 5 MSDS buffered at pH 3, shaken for 1 hour, then the supernatant was filtered with a vacuum pump through 0.45 mm nylon membrane with a diameter of 47 mm and placed into auto sample vials.

For tetracycline and doxycycline, the extraction method according to Orlando & Simionato (2013) was performed as follows: a 2.5 g of homogenized fish sample was poured into 50 ml centrifuge tubes and 0.25 g of NaCl added, together with 5 ml n-hexane and vortex for 3minutes followed by ultrasound for 5 minutes. The products were then centrifuged at 6000 rpm for 10 minutes and 2.5 ml of the lower phase of the aliquot transferred into new centrifuge vials and 2.5 ml of acetonitrile added to the solution. Extraction was repeated. The extracts were let to evaporate for dryness with flow of N_2. The supernatants were suspended into 2 ml aqueous mobile phase and analysed.

Extraction of chloramphenicol was done according to Takino *et al.* (2003). A 5 g of homogenized fish sample was mixed with 5 g anhydrous Sodium Sulphate ($NaSO_{4)}$ and 10 ml Ethyl Acetate added and centrifuged for 5 minutes at 600 rpm. The supernatant was then removed and put into a round flask and extraction repeated twice and later evaporated for dryness with N_2. Later, 1 ml acetonitrile and 1 ml n-hexane were added to dissolve the solution and transferred into graduated glass stopper reagent bottles and shaken (repeated twice),

allowed to dry and then dissolved in 50 ml of 10% acetonitrile in 10 mM Ammonia acetate. The solution was filtered using a nylon centrifuge filter (0.22 µm).

Sulphonamide was extracted according to Rezk *et al.,* (2015) as follows: a gram of blank fish sample was inserted into a 20 ml plastic centrifuge tube fortified with a number of working standards CPX, TMP, SDM, and FLOR and allowed to stand for 15 minutes. Aqueous Formic Acid (1 %; 0.25 ml), 0.5 ml acetonitrile and 0.5 ml methanol were added and mixed for 30 seconds. The solution was then shaken manually for 10 minutes and centrifuged for 10 minutes at 6000 rpm. Supernatants were transferred into 15 ml centrifuge tubes (repeated 3x for the clean-up). The products were evaporated with nitrogen. Re-constitution was done by adding 1 ml mobile phase and 2 ml n- hexane and then vortexed. Lastly, centrifuging was done at 1000 rpm for 5 minutes and 500 µl of bottom layer filtered through a thin 0.22 µm nylon membrane filter and run.

e) Antibiotic detection

For the detection of antibiotic in fish samples using TLC, the method according to Shafqat *et al.* (2012) was used with modifications as follows: TLC glass chambers were saturated with mobile phases for 30 minutes. Standards and samples (20 µl each) were spotted on silica gel plates using micro-syringe TLC spotter and allowed to dry; after drying, the TLC plates were inserted at the bottom of the TLC glass chamber, mobile phase were then allowed to run to the end of TLC plates and removed immediately; since over running could cause the spot to diffuse). Thereafter, the TLC plates were air dried and viewed under both short (254 nm) and long (365 nm) ultraviolent light (Spectro-line model CM-10A West bury, New York, USA). The 'O' code was drawn around some fluorescing or absorbing spots and marked with a pencil and compared with standards.

Table 3.2: Summary of antimicrobial extraction chemicals and reagents used for TLC chamber

Contents	**Tetracycline**	**Chloramphenicol**	**Doxycycline**	**Sulphonamide**	**Ciprofloxacin**
Mobile phase	water/ methanol dichloromethane	SDM/TMP/ FLOR/methanol	water/ methanol dichloromethane	acetone and methanol	water/ methanol dichloromethane
Volume ratio	(6/35/59) (V/v).	1mg/ml in 50ml	(6/35/59) (V/v).	(50:50) (V/v)	(6/35/59) (V/v).
Mobile phase ascended point	7cm above the initial spot	7cm above the initial spot	7cm above the initial spot	7cm above the initial spot	7cm above the initial spot
Wavelength detector@	254 & 365nm UV	254 & 365nm UV	254 & 365nm UV	254 & 365nm UV	254 & 365nm UV
Plate drying	✓	✓	✓	✓	✓
Reference	(Dong *et al.*, 1999)	(Rezk *et al.*, 2015)	(Dong *et al.*, 1999)	(Thangadu *et al.*, 2002)	(Dong *et al.*, 1999)

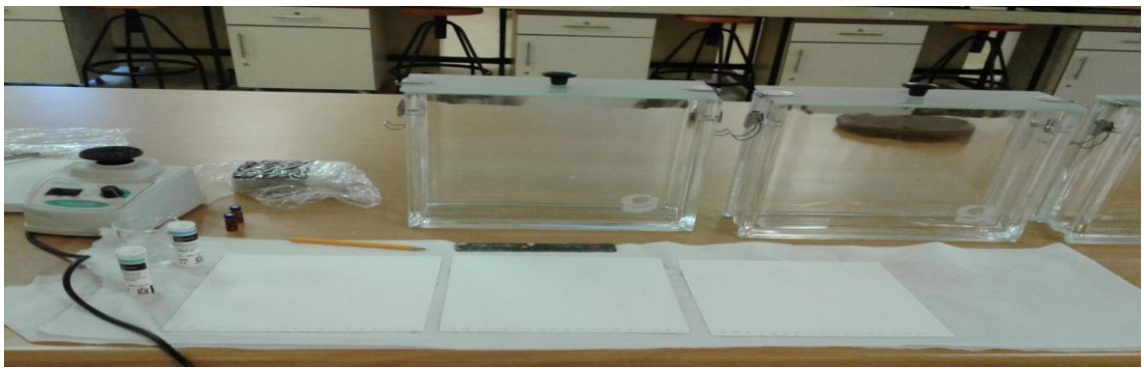


Figure 3.3: Image of standards and samples preparation on silica gel plates

Figure 3.4: Image of standards and samples in a TLC chamber with mobile phases

3.4.3 Detection of antimicrobial residues in fish samples using High Pressure Liquid Chromatography (HPLC)

a) Chemicals and reagents

HPLC was run for the confirmation of results. For HPLC to be run on all fish samples and antimicrobials, the following chemicals and reagents were used: methanol (CH_3OH) n-hexane (CH_3 $(CH_2)4CH_4$) and Oxalic Acid obtained from Merck (Merck, Germany); Acetonitrile (CH_3CN); Sodium sulphate ($NaSO_4$); Ammonium acetate; Formic acid; and Ethyl acetate obtained from Merck (Merck, Germany). Buffer (PBS) was obtained from Sigma Aldrich (Sigma Chemical Co, St. Louis MO. USA). Solutions prepared for HPLC were passed through a 0.45 μm nylon membrane filter (a single 110 mm filter paper, Germany) prior to usage. Stock solutions of sulphonamides, ciprofloxacin, tetracycline, doxycycline and penicillin were obtained from Sigma Chemical Co., St. Louis MO. USA. All solvents used were of HPLC standards.

b) Preparation of standard solutions and validation of HPLC

HPLC standards used were for individual compounds. Stock standard solutions of sulphonamide (sulphonamide S2151000), chloramphenicol (SLBM4187V), tetracycline and doxycycline (PHR1041-500), as well as penicillin (SZBD003XV) obtained from Sigma Chemical Co., St. Louis MO were prepared by dissolving 10 mg of each compound into 10 ml of methanol to obtain a final concentration. Each antibiotic standard was serial diluted (10, 5, 2.5, 1.25, 0.625, 0.3125, 0.15625, 0.078125, 0.0390625 and 0.01953125 mg/ml). Data obtained was used to test the linearity of the method.

Validation of HPLC was done by injecting ten different known concentrations of standards and repeating the injection of the same standard at least 3 times in HPLC, recording the areas, calculating the R^2 and checking the repeatability of the retention time and the area. The method was validated if the R^2 was between 0.9-1 and if the retention time variation was $\leq$ 2%.

c) Fortification of samples

Repeatability of the recovery assay was determined by analysing the triplicate of each of the matrice (fish muscle) spiked at three concentration levels. Fortified samples could stand for 20 minutes before analysis. The solutions were used in the preparation of the calibration curve. For doxycycline and tetracycline, the spiking concentration levels were 3, 6 and 18

μg/kg, for sulphonamides, the levels were 4.5, 13, 5 and 40, 5 μg/kg, for chloramphenicol, the levels were 10, 30 and 100 μg/kg and for penicillin, the levels were 3, 6 and 18 μg/kg. Data from these analyses was used to test linearity.

d) Stability

Stability of chloramphenicol, tetracycline, sulphonamide, doxycycline and penicillin were first measured at ambient temperature for 48 hours using both standard and all fish samples. The stability of all five antimicrobials during the freeze-thaw (to keep the condition of the sample stable in analyte level) procedure needed for sample analyses was further assessed at two different sample concentrations (9.20 μm and 33.6 μm). The samples were removed from the freezer and allowed to liquefy at ambient temperature, then frozen again overnight. This process was repeated three times prior to the final stability determination performed in this study.

e) Sample preparation

Sample preparation, as explained in 3.4.2D, was performed for both Thin Layer Chromatography (TLC) and High-Performance Liquid Chromatography (HPLC).

f) Analysis of High Performance Liquid Chromatography (HPLC)

A summary of the method used for the detection of antibiotics in fish using HPLC is shown in Table 3.3. Analysis of sulphonamides was done using Fluorometric detection according to Shareef *et al.,* (2009), with minor modifications. The mobile phase consisted of 0.2 % formic acid and water. The flow rate was 1.0 mL/minutes with temperature set at 35ºC. The injection volume of analyte and standard was 100 μL. Sulphonamide antibiotic was detected at 275 nm emission wavelengths, analysis of HPLC was done for 10 minutes and the antibiotic detected at approximately 1 minute.

Analysis for chloramphenicol was performed using Fluorometric detection method according to Tajick & Shohreh (2006), with minor modifications. The emission wavelengths were optimised at 275 nm respectively. The mobile phase used was methanol and water. The injection volume per analyte and standard used was 100 μL. The flow rate was 1.0 mL/minutes with temperature set at 35ºC. Analysis of HPLC was done for 10 minutes and the antibiotic detected at approximately 2.6 minutes.

Analysis of doxycycline and tetracycline was done using Photodiode detector (PDA) according to Abbasi *et al.* (2012), with modifications. Separation was done on Nucleosyl C_{18}

(5µm, 150 mm x 4.6 mm Shimadzu) column using methanol and acetonitrile as mobile phase at a flow rate of 1 mL/minute with temperature set at 35ºC. Moreover, 210 to 365 nm were used to analyse the data. Analysis of HPLC was done for 10 minutes and tetracycline antibiotic detected at approximately 1.7 minutes while Doxycycline was detected at 1.1 minutes.

Analysis of penicillin residues was done using Photodiode Array Detector (PDA) according to Pyun *et al.,* (2008), with modifications. Separation was done on Nucleosyl C_{18} (5µm, 150 mm x 4.6 mm Shimadzu) column with a mobile phase methanol and acetonitrile at a flow rate of 1.0 mL/minute with temperatures set at 35ºC. About 100 µL of the sample was injected and detection done at 350 nm emission wavelengths. Analysis was done for 20 minutes and antibiotics detected at approximately 15.15 minutes.

Table 3.3: Method used for the detection of antimicrobials in fish through HPLC

ANTIBIOTICS					
	Tetracycline	**Doxycycline**	**Sulphonamide**	**Chloramphenicol**	**Penicillin**
Method of extraction	Solid phase extraction (SPE)	SPE	UV-Florescence	PDA-wavelength 280nm	PDA
Samples amount	2.5g	2.5g	1g	5g	5g
Standards	10, 5, 2.5, 1.25, 0.625, 0.312,0.156, 0.078,0.039, 0.019	10, 5, 2.5, 1.25, 0.625, 0.312,0.156, 0.078,0.039, 0.019	10, 5, 2.5, 1.25, 0.625, 0.312,0.156, 0.078,0.039, 0.019	10, 5, 2.5, 1.25, 0.625, 0.312,0.156, 0.078,0.039, 0.019	10, 5, 2.5, 1.25, 0.625, 0.312,0.156, 0.078,0.039, 0.019
Injection volume	100µl	100µl	100µl	100µl	100µl
Mobile phase	Methanol/ Acetonitrile	Methanol/ Acetonitrile	Formic Acid/ water	Methanol/water	Methanol/ Acetonitrile
HPLC detection	365nm	210nm	270nm	275nm	350nm
Antibiotic detection time	1.1min	1.7min	1min	2.6min	15.15min

g) Method of verification by determining the amount of antimicrobial recoveries

HPLC recoveries were obtained by using samples with known concentrations. Antimicrobial standards in triplicates were inoculated (100 mL) with sulphonamides, chloramphenicol, tetracycline, doxycycline and penicillin. Recoveries were obtained using the identical extraction method as for the samples and the same techniques on HPLC, as summarized in Table 3.3.3a. The mean recoveries obtained were 76.67% for all fish samples for Chloramphenicol, 73% for fish samples respectively for sulphonamide, 80.67%,

respectively for penicillin, 67.64% for doxycycline and 64.33% for tetracycline. The results obtained in each case were deducted from the ones obtained from the spiked ones in order to obtain the recovery. Quantification of antimicrobial residues in fish samples were obtained and calculated from the peak heights extrapolated from the calibration curves of the standards using the following formula:

$$\frac{amount\ of\ residue\ obtained\ after\ spiking\ sample}{spiking\ concentration}\ X100 = \%\ recovery$$

3.5 MICROBIAL ANALYSIS

The risks of food-borne illnesses have increased over the years and continue to be one of the major public health challenges. Qualitative and quantitative approaches were used to determine the presence of food-borne pathogens in fish sold in Mafikeng, North West Province using conventional biochemical tests and molecular examination (DNA extraction, PCR and sequencing).

3.5.1 Bacterial culture, isolation and identification

a) Bacterial culture

Twenty grams of each fish sample were chopped using a sterile knife on a cutting board and transferred to 225 mL of Nutrient Broth (NB) and properly mixed for 5 minutes. The samples were enriched overnight and incubated at 37°C (Ruhe & Menon, 2006). After 24 hours, a sterile wire loop full of broth was streaked onto different media such as Mannitol Salt Agar, MacConkey and Nutrient Agar and then incubated for 24 hours at 37°C. Isolates obtained were purified by further sub-culturing and observed for presumptive identification based on their morphological characteristics and various biochemical tests. Bacterial colonies with dissimilar morphology were selected and purified on nutrient agar and biochemical confirmation. The routine laboratory (Biological) method of Cruickshank *et al.* (1975) was used to characterize different isolates.

Figure 3.5: Plates showing isolated *Enterococcus spp.* on a nutrient agar after streaking

3.5.2 BIOCHEMICAL TESTS

a) Gram staining

For cellular morphology, gram staining was used according to Haque *et al.* (2014) as follows: a small colony was spiked up using a sterile wire loop, smeared on a glass slide and fixed by softly heating. A crystal violet solution was then applied on the smear to stain for 2 minutes

and later washed with running water. Lugol's iodine was then added to act as mordant for 1 minute and washed with running water. Acetone alcohol was then added to act as a decolorizer for 5 seconds. After washing with distilled water, Safranin was added as a counter stain and allowed to stain for 2 minutes. The slide was then washed with water, blotted, dried in air and examined under a microscope with high power objectives (X100) using immersion oil.

Figure 3.6: Slide showing the purple stain colour before being observed under the microscope

b) Catalase test

The catalase method was performed according to Montso & Ateba (2014). A pure colony was transferred onto the surface of a microscopic slide using a sterile inoculating needle. A drop of 3% hydrogen peroxide was added and the slide observed for the presence of bubbles. All the results were recorded on the data sheet. A sample was deemed catalase positive if any bubbling was observed in the liquid after 10 seconds.

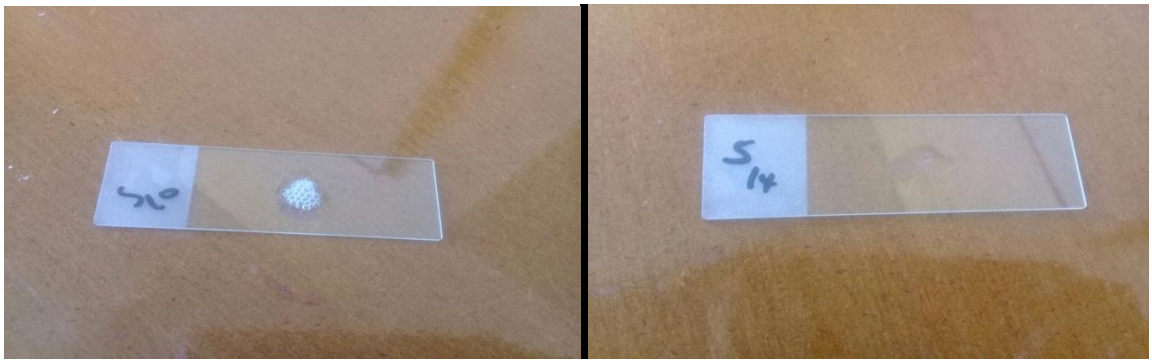


Figure 3.7: Images showing difference in results of the catalase test: catalase positive reactions evident by immediate bubbles (Figure A) formation whereas catalase negative reaction shows no bubble formation (Figure B) (meaning there is no catalase enzyme to hydrolyze the hydrogen peroxide) (Cheesbrough, 2006).

c) Oxidase test

An oxidase test was performed according to Ateba & Setona (2011). Pure isolated colonies were singled out using a sterile wire loop and positioned on a Whitman's filter paper and a drop of the Oxidase™ reagent added to make a smear. After 30 seconds, the formation of a purple or blue colour indicated an oxidase positive result and vice versa.

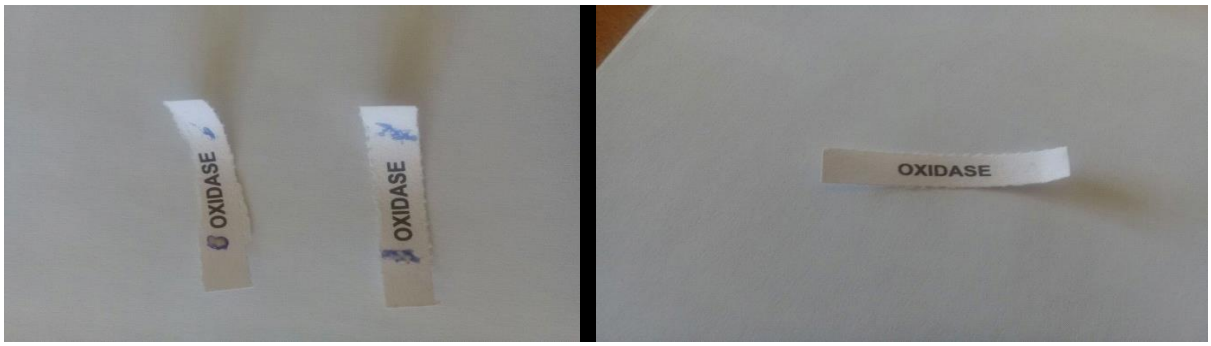


Figure 3.8: Images showing different results of the oxidase test: positive results are shown by the development of dark purple colour (indophenols) (Figure A) within 10 seconds and negative results (Figure B) indicate the absence of colour

d) Bacterial identification using Analytic Profile Index (API)

Bacterial isolates that were subjected to conventional biochemical tests were further re-examined using API-20E (BioMérieux, France). API-20E was used to classify species such as *Bacillus cereus, Clostridium spp., Enterococcus spp., Enterococeae bacterium, Lynisibacillus spp., Lynisibacillus fusiform, Bacillus spp.* and *Enterococcus faecium*. The test was performed according to the manufacturer's instructions (BioMérieux, France). Fresh colonies from Nutrient Agar were used to make bacterial suspensions and mixed with API 20E. Microtubules were inoculated with the suspensions as instructed. The strips were placed into trays hydrated with 5 ml distilled water to create a humid atmosphere. The strips were incubated using an anaerobic incubator for 24 hours. Results were read with or without the addition of reagents. Indices were generated for the different isolates and used to determine their identities using the API Web^{TM} identification software.

3.5.3 MOLECULAR IDENTIFICATION OF BACTERIAL ISOLATES

Molecular identification has provided powerful tools to investigate microbial communities at the level of species. This technique provides informative insights about bacteria, possible types of bioactive compounds, and if they are different or not (Donate-Correa *et al.*, 2005). The

identification of bacterial isolates in the present study was based on the analysis of 16S-rDNA gene sequence. Numerous methodologies were developed in this study to examine isolated bacteria and microbial diversity (Kirk *et al.*, 2004; Fakruddin & Mannan, 2013). These techniques involved DNA extraction, PCR amplification, Agarose gel electrophoresis and phylogenetic investigation.

a) Extraction of genomic DNA

The extraction of genomic DNA was performed as described by Ngoma *et al.*, (2013). A pure isolated bacterium from nutrient agar (after sub-culture for 24 hours aerobically) were inoculated into 5 mL of nutrient broth and incubated aerobically at 37˚C for 24 hours. The inoculum product was then transferred into 15 mL conical tube and centrifuged at 15000 rpm for 10 minutes. The supernatants (pellets) were collected and used for the extraction of DNA using Zymo Research kit (Zymo-Research fungal/Bacterial Soil Microbe DNA, D6005 USA supplied by Bio lab, South Africa) as follows: pellets were suspended in 750 µL lysis solution, and disrupted with disruptor gene (Inqaba biotech, mode SID258, USA) and vortex at 14.000 rpm for 14 minutes followed by centrifugation at 10000 rpm for 1 minute. Four hundred microlitre of the upper aqueous phase were aliquoted into a new Zymo-spin IV^{TM} and centrifuged at 7000 rpm for 1 minute. Buffer (12000 µl) was added to the filtrate and 800 µL of the mixture transferred to a new collection tube (Zymo-spin IIC^{TM}) and centrifuged at 10.000 rpm for 1 minute. The filtered DNA was pre-washed by adding 200 µL of DNA pre-wash buffer and centrifuged at 10.000 rpm for 1 minute. 500 µL of DNA wash buffer was added to the new collection tube (Zymo-spin IIC^{TM}) and centrifuged at 10000 rpm for 1 minute. Finally, 100 µL of DNA elution buffer was added to elute the DNA in a sterile 1.5 mL micro-centrifuge tube.

b) Amplification of 16S rDNA

Amplification of 16S rDNA gene was performed using polymerase chain reaction with the help of an Engine DYAD Peltier thermal cycler (Bio Rad. USA). A reaction volume of 25 µL containing 12 µL PCR Master Mix, 1 µL template DNA, 10 µL nuclease free water and 1µL of each oligonucleotide primer were prepared and mixed in PCR tubes (Ngoma *et al.*, 2013; Ngoma *et al.*, 2014). For the amplification of 16S rDNA gene, the procedure was performed according to Ngoma *et al.* (2013). PCR was conducted using Universal primers, forward 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and the Reverse 1492R (5'-TGA CTG ACT GAG ACG TTG CGA-3'). These primers were commercially synthesized by Inqaba Biotechnical Industrial (Pty) Ltd (Pretoria, South Africa). The thermos cycling (Bio-Rad T100^{TM} thermal

cycler, Singapore) conditions consisted of an initial denaturation step at 95˚C for 5 minutes followed by 35 cycles of denaturation at 94˚C for 30 seconds, annealing at 61˚C for 30 seconds and extension at 72˚C for 5 minutes, followed by a single and final extension step at 72˚C for 7 minutes in Peltier thermal cycler (DNA engine DYAD^{TM}, Bio-Rad, South Africa).

c) Agarose gel electrophoresis

The electrophoresis of PCR products was performed according to the methods and procedure described by Ngoma *et al.* (2013). The extraction of genomic DNA product was determined through analysis for the presence of DNA by 1% of Agarose gel. The Agarose gel was prepared as follows: 100g of Agarose gel was weighed and mixed with 100 ml of sterile water plus 10 ml of TE buffer; Agarose was then dissolved using a microwave for 5 minutes; the gel could cool to about 40˚C and Ethidium Bromide 0,5 ml added for staining; the gel was cast and allowed to set. After the gel had set inside the electrophoresis chamber, 5 µl DNA and 5 µl of loading buffer were mixed and transferred to one of the walls in the gel electrophoresis tank. Electrophoresis conditions were set for 90 minutes at 80 voltages and 250 MA. Gel was then visualized under UV light at 420 mm wavelength using a Bio-Rad chemi DOC^{TM}MP imaging system (Bio-Rad. USA). The imaging system (Bio-Rad Chemi Doc^{TM} MP imaging system, UK) was used to capture the presence of DNA bands (version 6.00.22) software. The presence of a single bright band (DNA bands) for each sample indicated a successful amplification. After, the PCR products were sent to Inqaba biotechnology in Pretoria, for sequencing.

d) DNA sequencing

DNA sequencing was performed according to the method described by Ngoma *et al.* (2013). Purified PCR fragment of 16S rDNA of the isolated bacteria was sent to Inqaba biotechnology, Pretoria, South Africa for sequencing. Sequences and chromatograms were observed with Bio-systems; forward and reverse sequences were compared and corrected for conformity. Blast program tools were applied to search for sequences according to Altschul *et al.* (1997) in order to find the closest match for each in the Gen-Bank. The closest sequences were then downloaded and aligned with the original sequences using claustral (Thompson *et al.*, 1994) and edited *via* Bio-edit for all to have the same length. The edited sequences were compared using maximum parsimony in the program DNA pair. Results were then showed as bootstrapped (1000 boot strap).

e) DNA quantification

Nanodrop 2000c (Thermo scientific) was used to determine the amount of each sample to use in downstream applications, such as reverse transcription. The absorbance of UV-Visible light was with usable concentration ranges of 4–15,000 Nano g/µl. It was used to detect contaminants using spectral data and purity ratios, indicating sample purities used for 5 seconds.

Figure 3.9: Image of Nanodrop 2000c (Thermo scientific) used to quantify DNA

3.6 ANTIBIOTIC SUSCEPTIBILITY TESTING

In this study, sensitivity testing (also referred to as susceptibility testing) was performed. The antibiotic resistance profiles of bacterial isolates (using the disc diffusion method) are described in this chapter. All fish samples collected in supermarkets in Mafikeng were subjected to antimicrobial sensitivity testing. Sensitivity of the isolates to antibiotics was examined against some common antibiotics, including Tetracycline, Chloramphenicol, Sulphonamide, Quinolones - Ciprofloxacin and Norfloxacin. This test was performed using the Kirby Bauer disc diffusion technique described by Bauer *et al.,* (1966).

A pure colony of isolates from a fresh culture was used to prepare a bacterial suspension. Aliquots of 100 µl from the suspensions were spread-plated on Mueller Hinton agar (MH), using a sterile cotton swab through the entire surface of Muller Hinton agar plates. After the inoculum was dried for about 5 minutes, four standard antibiotic disks, each containing a specific concentration of antibiotics, was applied per plate. The antibiotics used in this study were selected because of their usage in veterinary practices. Antibiotic discs were gently pressed onto the inoculated Mueller Hinton agar to ensure intimate contact with the surface and the plates incubated aerobically at 37°C for 18−24 hours. All isolates were subjected to Streptomycin (300 µg), Tetracycline (30 µg), Sulphonamide (300 µg) and Ciprofloxacin (5 µg). The diameter of the inhibition zone (clear area around discs) indicates the sensitivity of bacteria to that antibiotic. After incubation at 37°C for 24 hours, the diameters of the inhibition zone were measured in millimeters to interpret sensitive, intermediate or resistance in accordance with the guidelines of the Clinical Laboratory Institute Standards (Clinical and Laboratory Standards Institute (CLSI), 2012).

3.7 STATISTICAL ANALYSIS

The Statistical Analysis System (SAS® software) (general linear models program) was used to detect the presence of antimicrobial residues in fish samples detected by ELISA and HPLC. A probability of $P \leq 0.05$ was required for statistical significance. One-way analysis of variance (ANOVA) tests were used to correlate methods in different antimicrobials. Moreover, antimicrobials were compared using chi-square test. Data was analysed using the Statistical Package for the Social Sciences software (version 16; SPSS Inc., USA) and values of $p < 0.05$ considered statistically significant. Correlations between antimicrobial residues and antimicrobial resistance were also analyzed statistically using SPSS software (version 16; SPSS Inc., USA) and values of $P < 0.05$ were considered correlated. Also, the MRL obtained were

compared with the published MRL according to Codex/ RSA for investigated antimicrobials: tetracycline (100 µg/kg), sulphonamide (100 µg/kg), nitrofuran (1 µg/kg), chloramphenicol (10 µg/kg) and quinolone (1 µg/kg) (EU-Codex Allimentaris commission).

3.8 ETHICAL CLEARANCE

Ethical number NWU-00246-18A5 was obtained from the Ethical Committee of the Faculty of Natural and Agricultural Sciences, North-West University, Mafikeng Campus to carry out the study.

CHAPTER FOUR

4.0 RESULTS

The aim of this study was to determine antimicrobial residues and to compare methods used as well as determine the risks of consuming fish sold in supermarkets around Mafikeng, North West Province, South Africa. The results obtained are presented below.

4.1 Screening of antimicrobial residues using Enzyme-Linked Immunosorbent Assay (ELISA)

In this study, the concentration of antibiotics using ELISA as presented in Tables 4.1, 4.2 and 4.3 were calculated using the calibration curves plotted using standard concentrations as shown in Figures 4.1, 4.2, 4.3, 4.4 and 4.5 respectively for tetracycline, chloramphenicol, sulphonamide, quinolone and nitrofuran.

Results obtained in this study showed the presence of antimicrobial residues as follows: 84; 84; 96; 12 and 20% of samples with concentrations ranging between 0-2240 (398 µg/kg); 0-120 (22.19 µg/kg); 0.3-9.7 (40.44 µg/kg); 0-30 (3.92 µg/kg) and 0-4840 (259.96 µg/kg) respectively for tetracycline, chloramphenicol, sulphonamide, quinolone and nitrofuran (Table 4.1). It was also revealed that among the positive samples, 54%, 84%, 6%, 4% and 10% respectively for Tetracycline, Chloramphenicol, Sulphonamide, Quinolone and Nitrofuran were found to be above Codex/ RSAMRL.

Table 4.1: Overall summary of the detection of antimicrobial residues in all fish samples analysed using ELISA compared with the Maximum Residue Limits (MRLs) in µg/kg according to Codex/ RSA MRL.

All fish samples							
Antibiotics	N	Positive %	Mean (µg/kg)	STD Dev	Range (µg/kg)	Detection > MRL (%)	Codex/RSA-MRL (µg/kg)
Tetracycline	50	42(84)	398	669.65	0-2240	27(54)	100
Chloramphenicol	50	42(84)	22.19	26.46	0-120	42(84)	10
Sulphonamide	50	48(96)	40.442	36.30	0.3-9.7	3(6)	100
Quinolone	50	6(12)	3.92	9.96	0-30	2(4)	100
Nitrofuran	50	10(20)	259.76	877.95	0-4840	5(10)	1

The results obtained based on the type of fish, showed the presence of tetracycline (51%), chloramphenicol (100%), sulphonamide (6%) and nitrofuran (83%) to be above MRL while quinolone was detected but was not above MRL (Table 4.2) in sea/salt fish. However, in fresh water fish samples, the results showed the prevalence of 92%, 100%, 6%, and 100% respectively for tetracycline, chloramphenicol, sulphonamide and quinolone. All these antibiotics were above the Codex/ RSA MRL while only nitrofuran was not observed in any sample (Table 4.3).

Table 4.2: Summary of the detection of salt/sea water fish using ELISA

Salt/sea water fish							
Antibiotics	N	Positive %	Mean (µg/kg)	STD Deviation	Range (µg/kg)	Detection > MRL (%)	Codex/RSA-MRL(µg/kg)
Tetracycline	34	29 (85)	305.00	587.11	0 – 2240	15 (51)	100
Chloramphenicol	34	29 (85)	21.94	28.53	0-118.75	29 (100)	10
Sulphonamide	34	32 (94)	40.00	37.57	0.3 – 107	2 (6)	100
Quinolone	34	8 (24)	3.35	9.33	0 – 30	-	100
Nitrofuran	34	6 (18)	382.00	1036.88	0 – 4840	5 (83)	1

Table 4.3: Summary of fresh water fish samples using ELISA

Fresh water fish							
Antibiotics	n	Positive %	Mean (µg/kg)	STD Deviation	Range (µg/kg)	Detection > MRL (%)	Codex/RSA-MRL (µg/kg)
Tetracycline	16	13 (81)	595.63	21.21	0 – 2000	12 (92)	100
Chloramphenicol	16	13 (81)	22.72	7.95	0 – 82.5	13 (100)	10
Sulphonamide	16	16 (100)	41.38	55.86	6 – 110	1 (6)	100
Quinolone	16	2 (12)	5.13	11.15	0 – 30	2 (100)	100
Nitrofuran	16	-	0	0	0 – 0	-	1

Analysis of the different types of fish revealed that the range of tetracycline in the current study was 0 - 2240 µg/kg with a mean of 305.00 µg/kg for salt water fish and ranged between 0 and 2000 µg/kg with a mean of 595.65 µg/kg for fresh water fish respectively.

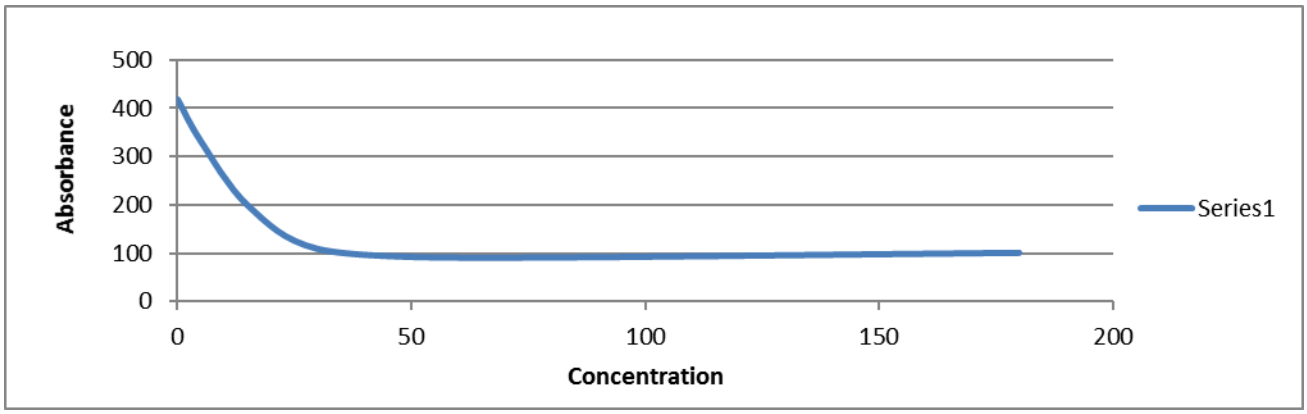


Figure 4.1: Calibration curve of tetracycline standards at 0, 5,15,30,60,180 µg/kg using ELISA

The ranges and mean of chloramphenicol were found to be 0 -118.75 and 21.94 µg/kg respectively for salt/sea water fish and 0-825 µg/kg and 22.72 µg/kg for fresh water fish.

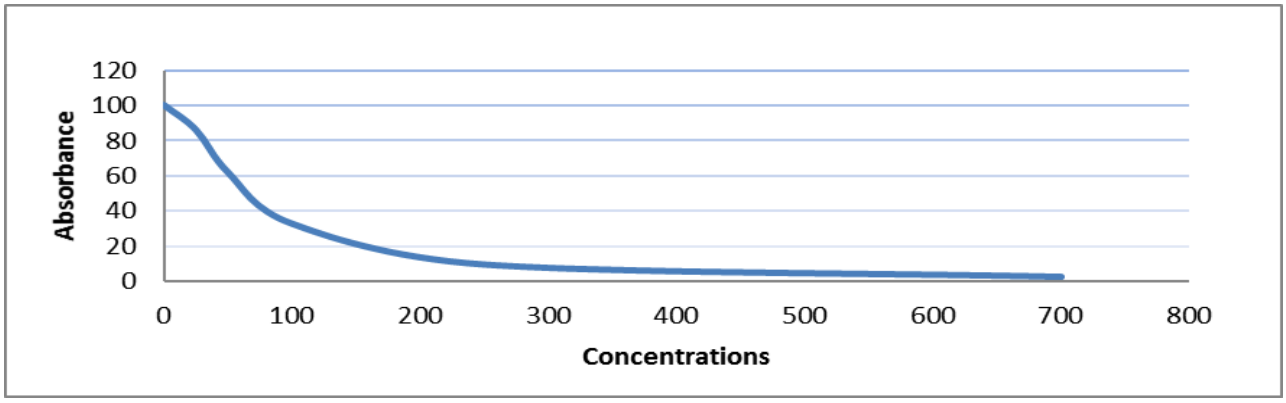


Figure 4.2: Calibration curve for chloramphenicol standards at 0, 25, 50, 100, 250, 700 µg/kg using ELISA

The levels of sulphonamide in the current study ranged between 0.3 and 107 µg/kg with a mean of 40.00 µg/kg for salt water fish. For fresh water fish, the mean was calculated to be 42.38 µg/kg ranging between 6 and 110µg/kg respectively. In addition, 6% of both salt and fresh water fish samples were detected above the Codex/RSA MRL as shown in Tables 4.2 and 4.3.

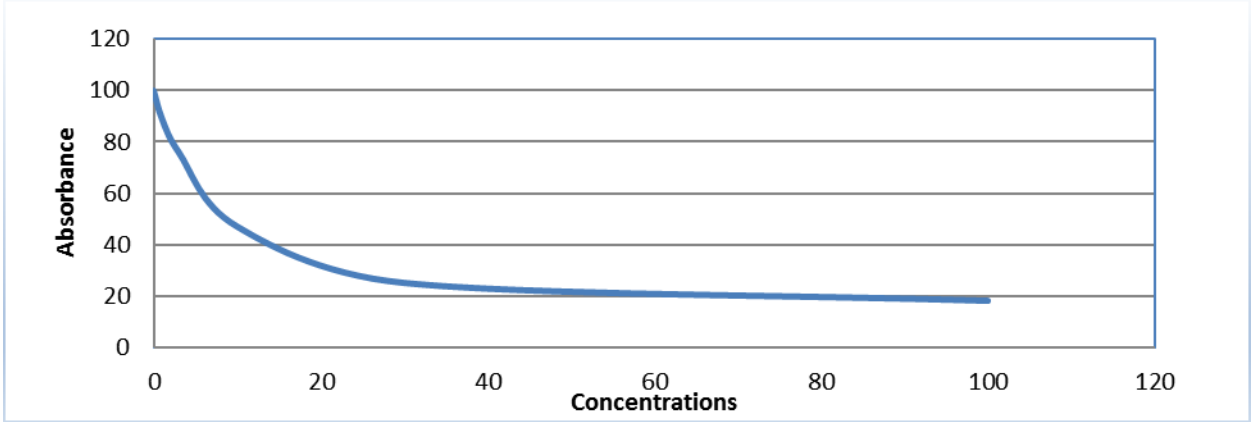


Figure 4.3: Calibration curve of sulphonamide standards at 0, 1, 3, 10, 30, 100 µg/kg using ELISA

For the levels of quinolone in the present study, all samples were detected to be positive and showed 100% above MRL in fish. For both salt and fresh water fish, quinolone ranged between 0 and 30 µg/kg, with a mean of 5.15 and 3.35 µg/kg respectively.

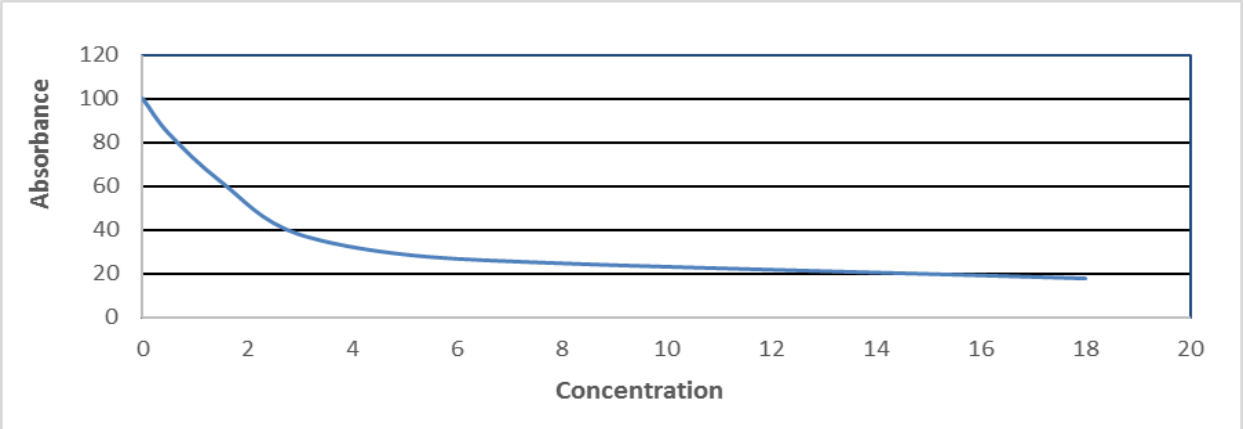


Figure 4.4: Calibration curve for quinolone standards at 0, 0.5, 1.5, 3, 6 and 18 µg/kg using ELISA

The levels of nitrofuran ranged between 0 and 4840 µg/kg, with a mean of 38.00 µg/kg for salt water fish while for fresh water fish, samples ranged between 0 and 0 µg/kg with a 0 µg/kg mean respectively.

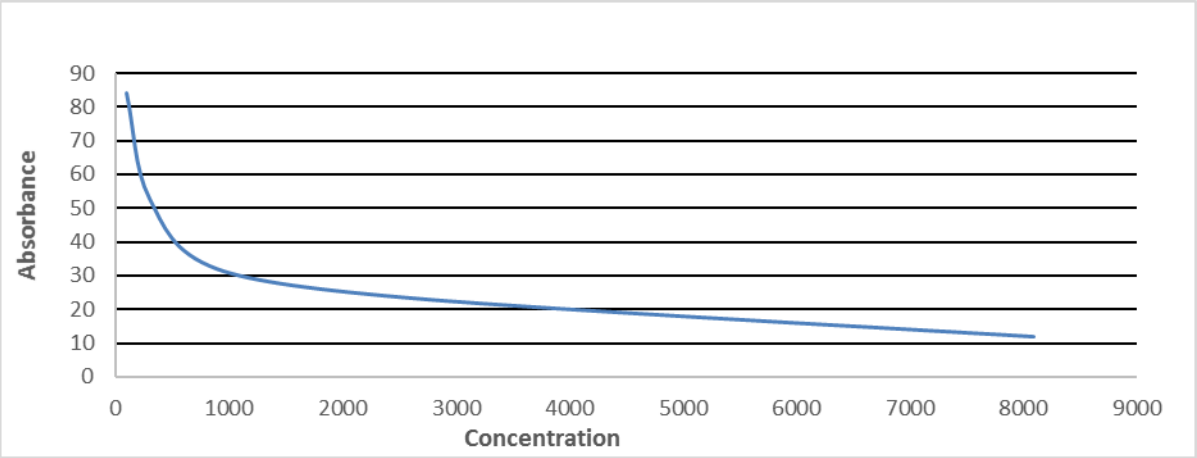


Figure 4.5: Calibration curves for nitrofuran standards at 0, 100, 300, 900, 2700 and 8100 µg/kg using ELISA

4.2 Screening of antimicrobial residues using results of Thin Layer Chromatography (TLC)

In this study, TLC identification was done by comparing the R_f values of antibiotic standards and the samples as illustrated in Table 4.4.

The results for TLC showed a high prevalence rate of antimicrobials with 88%, 76%, 74%, 74% and 64% for sulphonamide, ciprofloxacin, tetracycline, doxycycline and chloramphenicol respectively. Also, the results show a retention factor of 0.92, 0.5, 0.32, 0.28 and 0.2 for sulphonamide, chloramphenicol, tetracycline, doxycycline and ciprofloxacin (Table 4.4) respectively.

Table 4.4: Summary of the detection of antimicrobials in all fish samples using TLC

Samples Type	Tetracycline		Chloramphenicol		Ciprofloxacin		Doxycycline		Sulphonamide	
	n	**+ (%)**	**n**	**+ (%)**	**n**	**+ (%)**	**n**	**+ (%)**	**n**	**+ (%)**
All fish samples	50	(74)	50	(64)	50	(76)	50	(74)	50	(88)
R_f value										
Compounds	50	(0.32)	50	(0.5)	50	(0.28)	50	(0.2)	50	(0.92)
All fish samples	50	(0.76)	50	(0.53)	50	(0.68)	50	(0.6)	50	(0.92)

n = number of samples, R_f = Retention factor

Figures 4.6 (A, B, C, D and E) show that antibiotic residues were detected in fish samples using TLC seen under the UV light showing the presence of A = tetracycline, B = chloramphenicol, C = sulphonamide, D = doxycycline and E = ciprofloxacin.

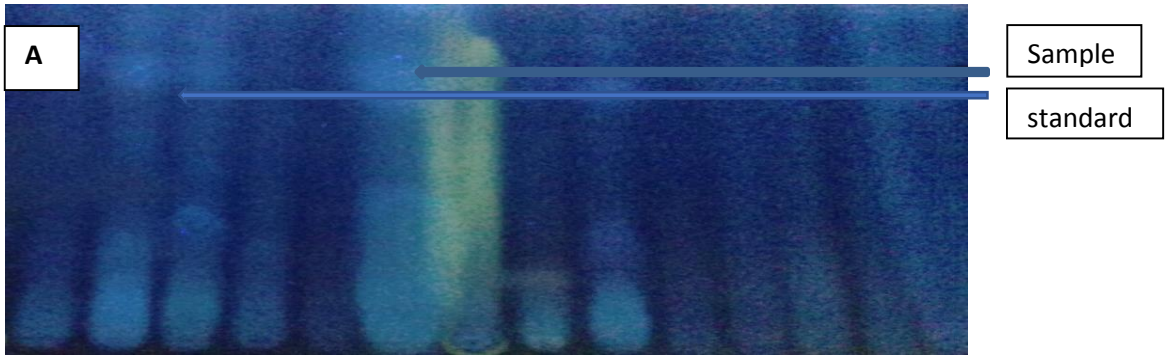


Figure A: TLC plate showing tetracycline seen under UV light

Figure B: Chloramphenicol TLC image of antibiotic standard and some samples under UV light

Figure C: Fluorescence excitation wavelength of sulphonamide at 250 nm to emission wavelength of 366 nm with absorbance of less than 1

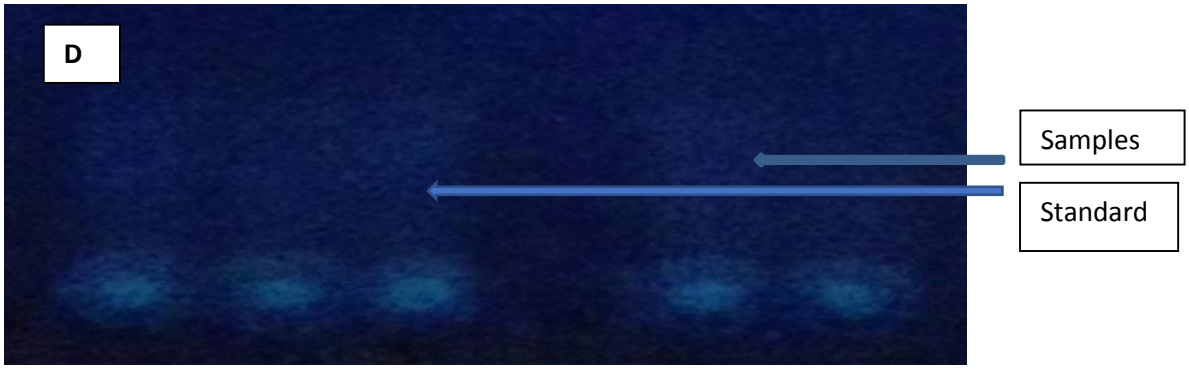


Figure D: Fluorescence excitation wavelength of doxycycline

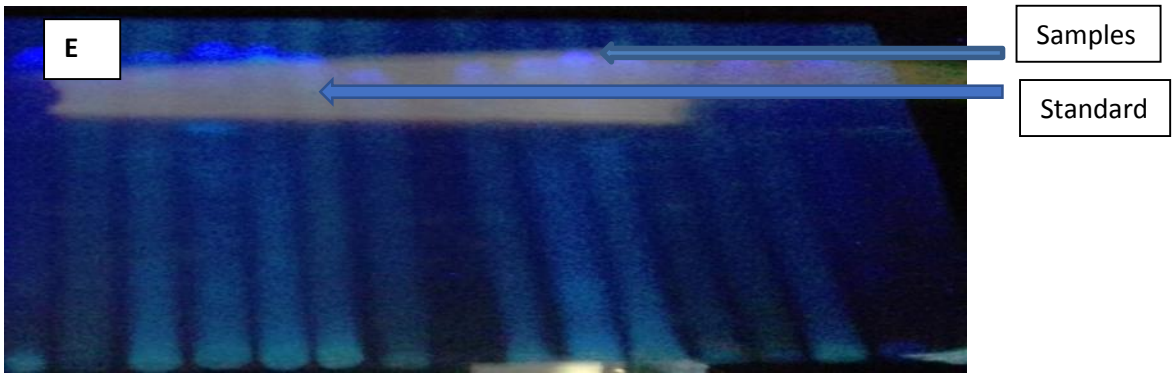


Figure E: TLC plates showing ciprofloxacin under UV light

4.3 Confirmation of antimicrobial residues using High Pressure Liquid Chromatography (HPLC)

Results of HPLC obtained in this study showed the presence of antimicrobial residues in 28%; 74; 14; 21 and 0% of samples with concentrations ranging between 0 and 0.69 (0.23 µg/kg), 0 and 279.8% (49.47 µg/kg), 0.68 and 8 (2.79 µg/kg), 0.68 and 7% (0.24 µg/kg) and 0% (0 µg/kg) respectively for tetracycline, chloramphenicol, sulphonamide and doxycycline. However, no penicillin residue was detected in samples analysed using HPLC (Table 4.5). Among the positive samples, chloramphenicol was found to be above the Codex/ RSA MRL.

Table 4.5: Overall summary of all antimicrobial residues detected in fish samples confirmed using HPLC

All fish samples							
Antibiotics	N	Positive %	Mean (µg/kg)	STD Deviation	Range (µg/kg)	Detection > MRL (%)	Codex/RSA-MRL(µg/kg)
Tetracycline	50	14(28)	0.23	0.72	0-0.69	-	100
Chloramphenicol	50	37(74)	49.47	56.42	0 –279.8	36(72)	10
Sulphonamide	50	7(14)	2.79	10.97	0.68-8	-	100
Doxycycline	50	21(42)	0.24	0.73	0.68-7	-	100
Penicillin	50	-	0	0	0-0	-	1

Recoveries of HPLC were obtained using samples with known concentrations. Antimicrobial standards in triplicates were inoculated (100 µg/kg) with sulphonamides, chloramphenicol, tetracycline, doxycycline and penicillin. Recoveries were obtained using an

identical extraction method as for the samples and the same technique for HPLC, as summarised in Table 4.6. The mean recoveries obtained were 76.67% for all fish samples for Chloramphenicol, 73% for fish samples respectively for sulphonamide, 80.67% respectively for penicillin, 67.64% for doxycycline and 64.33% for tetracycline. The results obtained in each case were deducted from the ones obtained from the spiked ones to obtain the recovery. Quantification of antimicrobial residues in fish samples were obtained and calculated from the peak heights extrapolated from the calibration curves of the standards using the following formula:

$$\frac{amount\ of\ residue\ after\ spiking}{spiked\ concentration}\ X\ 100 = recovery$$

Table 4.6: A resume of mean of recoveries obtained from fish spiked with antimicrobial standards on Performance Liquid Chromatography (HPLC)

Antibiotic and type of sample	**Fortification level (µg/kg)**	**Recovery (%)**	**Mean recovery (%)**	**LOD (µg/ml)**	**LOQ (µg/ml)**
Tetracycline	3, 6 and 18	62, 64 and 67	64.33	0.000059	0.00020
Chloramphenicol	10, 30 and 100	71, 74 and 75	76.67	0.204	0.681
Doxycycline	3, 6 and 18	61, 65 and 68	67.64	0.000059	0.000020
Sulphonamide	4.5, 13.5 and 40.5	74, 77 and 79	73	0.0064	0.0212
Penicillin	3, 6 and 18	77, 81 and 84	80.67	0	0

After several trials, the recovery rates for all antibiotics were considered as the best method.

4.3.1 Confirmation of Tetracycline residues in salt and fresh water fish samples

The results obtained (Table 4.7) showed the presence of tetracycline in different fish samples with a mean concentration of 0.29 µg/kg ranging between 0 and 3.75 µg/kg for salt water fish samples, while for fresh water fish samples, it ranged from 0-0.47 µg/kg with a mean concentration of 0–10 µg/kg. None of these samples were found to be above the Codex/RSA MRL.

Table 4.7: Summary of tetracycline for fresh and salt water fish samples using HPLC

Tetracycline							
Fish type	**N**	**+ (%)**	**Mean (µg/kg)**	**STD Deviation**	**Range (µg/kg)**	**Detected > MRL**	**Codex/RSA MRL (µg/kg)**
Salt/sea water fish	34	12 (35)	0.29	0.88	0-3.75	-	100
Fresh water fish	16	8 (50)	0.10	0.15	0-0.47	-	100

n = number of samples, + = positive, STD Dev = standard deviation, MRL= maximum residue limit, RSA = Republic of South Africa

Data analysis and quantification

To assess the repeatability and efficiency of the HPLC detection method for tetracycline, a calibration curve concentration for Tetracycline was plotted using results of the standards (concentration vs. area), which gave a trend line and the equation y= 36608 + 225.49, a good linearity of R^2= 0.9979 and a good dispersion of the standards.

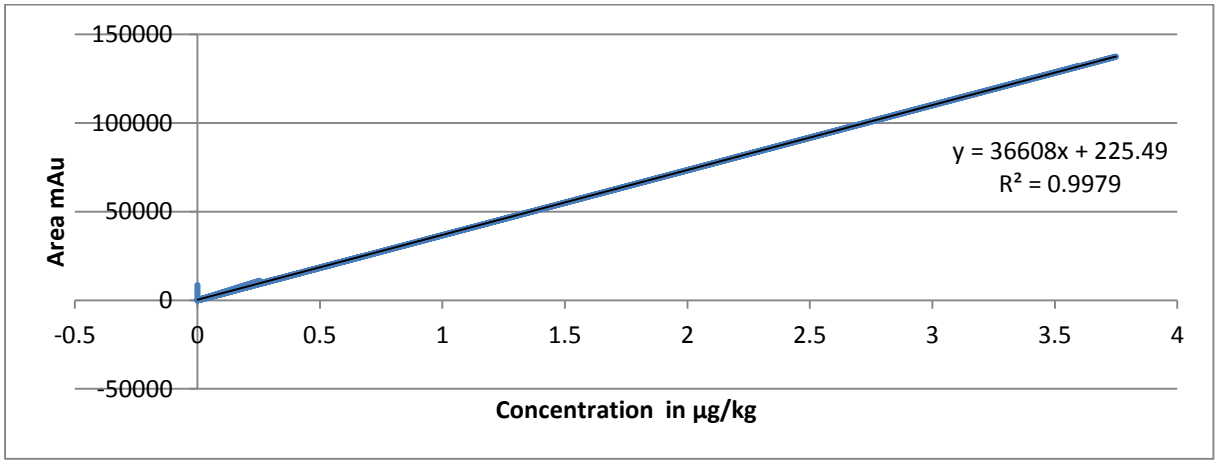


Figure 4.7: Calibration curve for tetracycline standards (10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039 & 0.019 µg/kg) run on HPLC.

HPLC chromatogram of Tetracycline standards

According to Figure 4.8, typical HPLC chromatogram of standard mixture for tetracycline (100 µg/ml) and doxycycline (100 µg/kg) were run simultaneously and obtained from HPLC chromatograms for fish samples were run at a flow rate of 1 ml/minute to the RF detector with emission wavelength, at a temperature of 35ºC and tetracycline and doxycycline detected at approximately ≥ 1.7 minutes. Peaks, 1 = TC (retention time, Rt =1.7 min); 2= DOX (Rt = 1.1 min).

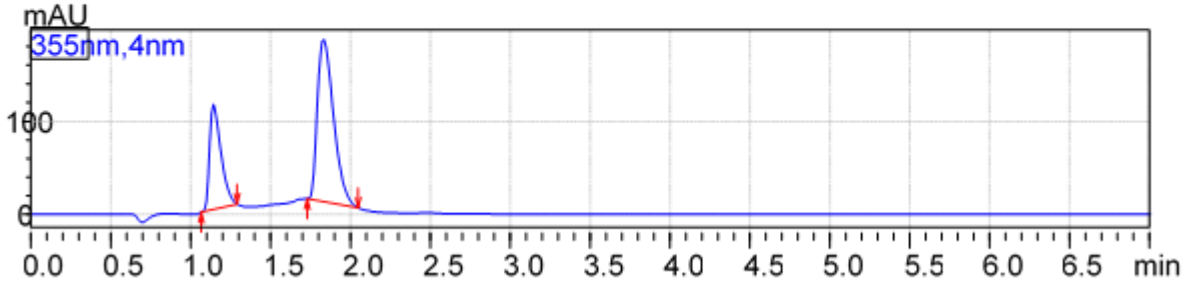


Figure 4.8: Typical HPLC chromatogram of standard mixture for tetracycline and doxycycline

4.3.2 Confirmation of chloramphenicol in salt and fresh water fish samples

Results presented in Table 4.8 show the presence of chloramphenicol in different fish samples with a mean concentration of 49.49 µg/kg, ranging between 0 and 279.8 µg/kg for salt water fish samples, while for fresh water fish samples, concentrations ranged between 0 and 143 µg/kg with a mean of 49.42 µg/kg. All samples analysed had concentrations above the Codex/RSA- MRL.

Table 4.8: Summary results of detection of chloramphenicol using HPLC in salt and fresh water fish samples

Chloramphenicol							
Fish type	**n**	**+ (%)**	**Mean (µg/kg)**	**STD Deviation**	**Range (µg/kg)**	**Detected > MRL**	**Codex/RSA-MRL (µg/kg)**
Salt/sea water fish	34	22 (65)	49.49	63.68	0-279.8	20 (91)	10
Fresh water fish	16	13 (81)	49.43	38.45	0-143	13 (100)	10

n = number of samples, + = positive, MRL= maximum residue limit, RSA= Republic of South Africa

The calibration curve for chloramphenicol was plotted and it showed a good linearity with an R^2 of 1, with the mean value of 49.49 µg/kg for salt water fish samples and 49.42 µg/kg for fresh water fish samples. When the standard was prepared for Chloramphenicol, a good dispersion of the standards on the curve was obtained.

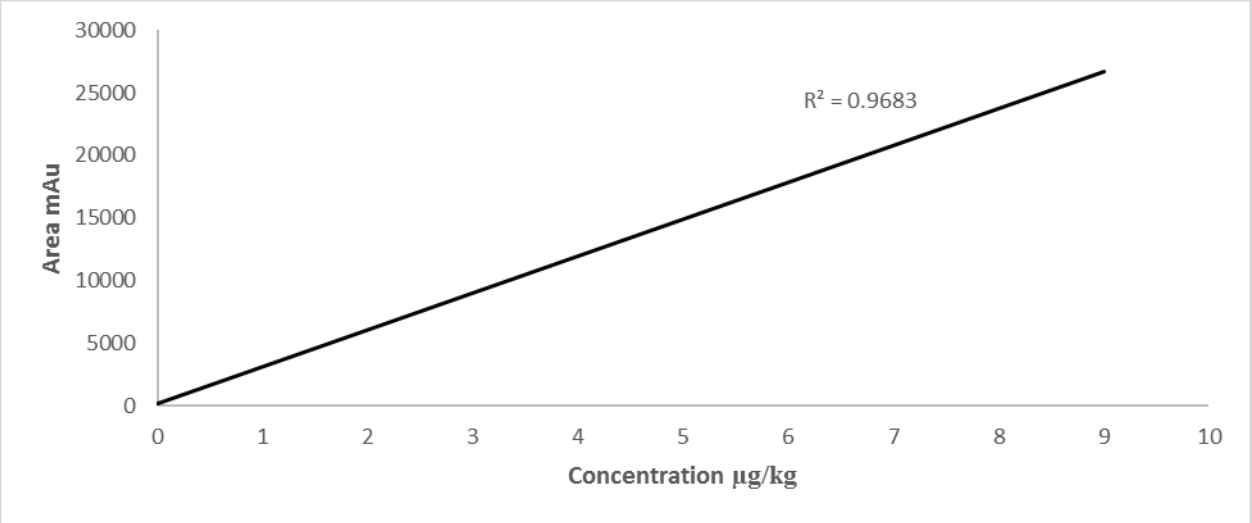


Figure 4.9: A calibration curve for chloramphenicol standards (10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039, 0.019 µg/kg) was run on HPLC

Peaks were obtained by the reversed phase column C18 (100 x 4 mm) at a temperature of 35ºC and at a flow rate of 1ml/minute (Figure 4.10). The UV wavelength of 255 nm to 365 nm and injection volume of 100µl was used. A good peak was obtained at a retention time of 2.6 minutes.

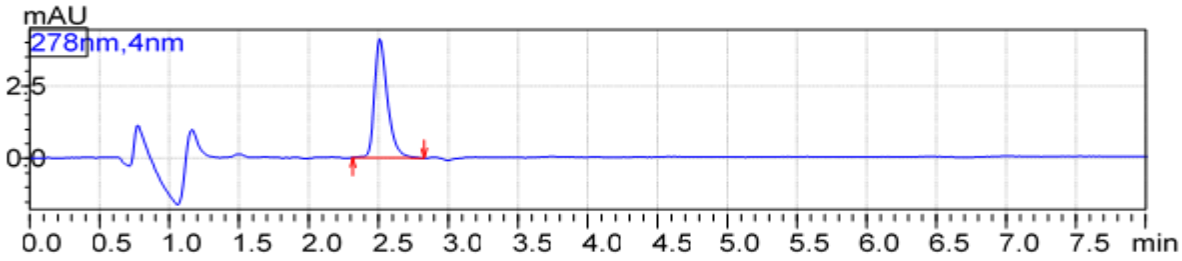


Figure 4.10: Illustration of Chromatogram of a fish sample with the presence of chloramphenicol on HPLC using the UV detector

4.3.3 Confirmation of penicillin salt and fresh water fish samples

In this study, penicillin was analysed in fish samples using HPLC and the results obtained are presented in Table 4.3.5. Residues of penicillin were found in different fish samples and the mean concentration was 3.83 µg/kg, ranging between 0 and 68.7 µg/kg for salt water fish samples, while for fresh water fish samples, concentration of samples ranged between 0 and 8.6 µg/kg with a mean of 0.58 µg/kg. None of the concentrations obtained were found to be above the Codex/ RSA MRL for penicillin.

Table 4.9: Summary of penicillin detected in fish samples using HPLC

Penicillin							
Fish type	**n**	**+ (%)**	**Mean (µg/kg)**	**STD Deviation**	**Range (µg/kg)**	**Detected > MRL**	**Codex/RSA -MRL (µg/kg)**
Salt/sea water fish	34	-	3.83*	13.16	0- 68.7	-	50
Fresh water fish	16	-	0.58	2.15	0-8.6	-	50

n= number of samples, + = positive, MRL= maximum residue limit, RSA= Republic of South Africa

For quantification of results and to check the repeatability and accuracy of HPLC, a calibration concentration curve of penicillin standards was plotted and showed linearity of 0, with a mean value of 3.83 µg/kg for salt water fish samples and 0.58 µg/kg for fresh water fish samples. The calibration curve was plotted using the prepared standards.

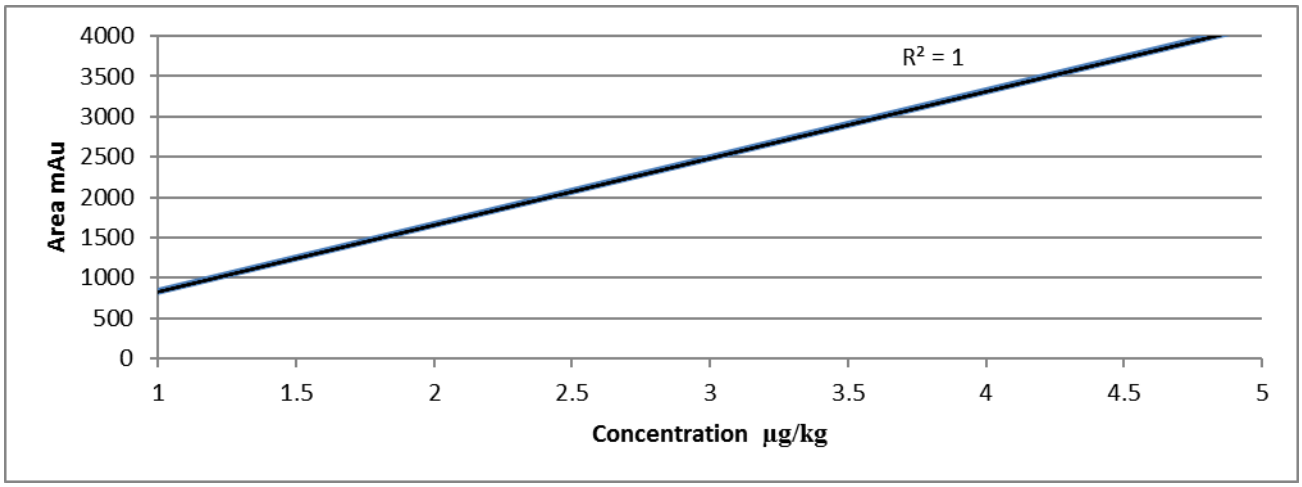


Figure 4.11: Calibration curve for Penicillin standards ppb injected at 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039, 0.019 µg/kg on HPLC coupled with a Diode Array detector.

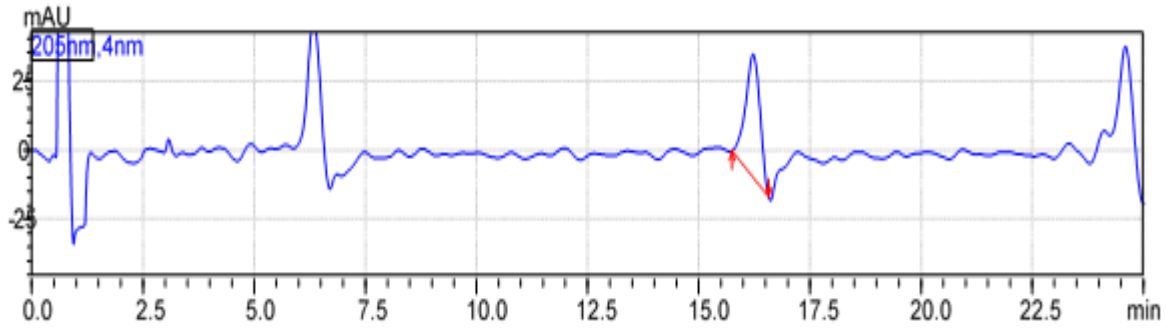


Figure 4.12: Illustration of an HPLC chromatogram of a fish sample (0.68) positive to penicillin.

4.3.4 Confirmation of summary of doxycycline antimicrobials in salt and fresh water fish samples using HPLC

In this study, doxycycline was analysed in fish and the results presented in Table 4.10 showed the presence of Doxycycline at different concentrations with a mean of 0.29 µg/kg and concentrations ranging between 0 and 3.75 µg/kg for salt water fish samples, and for fresh water fish, samples ranged between 0 and 0.47 µg/kg with a mean concentration of 0.10 µg/kg. None of these fish samples were found to be above the Codex/ RSA MRL.

Table 4.10: Summary of results of HPLC for the detection of doxycycline in salt and fresh water fish samples

Doxycycline							
Fish type	**n**	**+ (%)**	**Mean (µg/kg)**	**STD Deviation**	**Range (µg/kg)**	**Detected > MRL**	**Codex/RSA-MRL (µg/kg)**
Salt/sea water fish	34	12 (35)	0.29	0.88	0-3.75	-	100
Fresh water fish	16	8 (50)	0.10	0.15	0-0.47	-	100

n= number of samples, + = positive, MRL= maximum residue limit, RSA= Republic of South Africa

A calibration curve (Figure 4.16) was plotted using standards to confirm the repeatability of the method, get the equation to calculate the results. A good linearity with R^2 of 1 was obtained. When the standards were prepared, Doxycycline with a good dispersion of standards on the curve was also obtained.

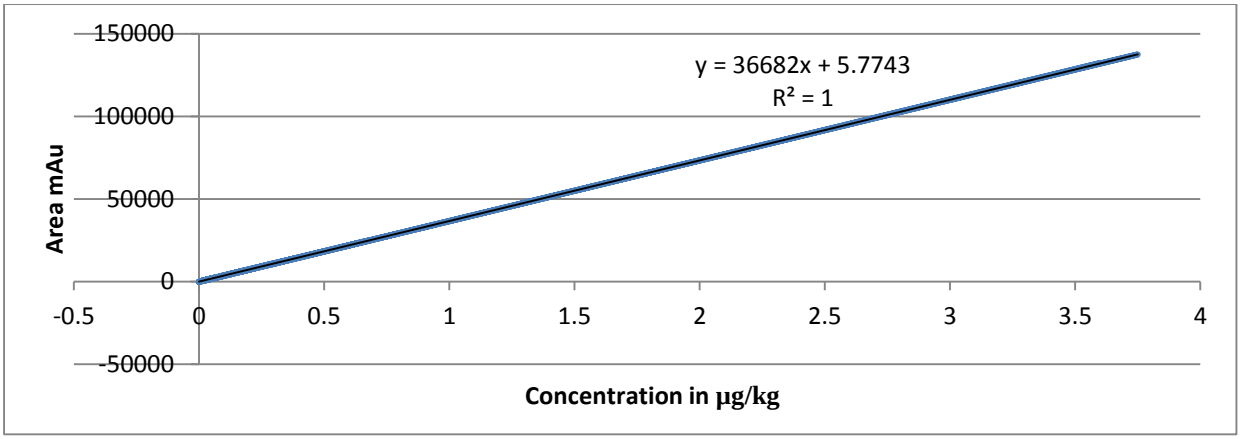


Figure 4.13: HPLC calibration curve for Doxycycline standards at 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039, 0.019 µg/kg

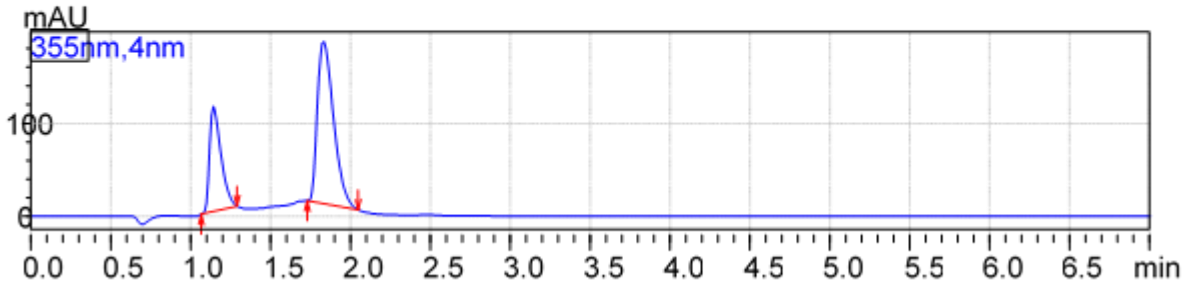


Figure 4.14: HPLC chromatogram of a standard mixture of doxycycline at 10 µg/ml and tetracycline at 10 µg/ml was obtained respectively at 1.7 and 1.1 minutes

4.3.5 Confirmation of sulphonamide in salt and fresh water fish samples using HPLC

The results of sulphonamides (Table 4.11) revealed contaminations in some fish samples analysed, with mean concentrations of 3.83 µg/kg (0 - 86.7 µg/kg) for salt water fish and 0.58 µg/kg (0 - 8.6 µg/kg) in fresh water fish. A significant statistical difference of ($P<0.05$) between data was obtained in fresh and salt water fish samples. None of these fish samples were found to be above the Codex/RSA- MRL.

Table 4.11: Summary of results of HPLC for the detection of sulphonamide in salt and fresh water fish samples

Sulphonamide							
Fish type	**n**	**+ (%)**	**Mean (µg/kg)**	**STD Deviation**	**Range (µg/kg)**	**Detected > MRL**	**Codex/ RSA-MRL (ppb)**
Salt/sea water fish	34	5 (15)	3.83	13.16	0- 86.7	-	100
Fresh water fish	16	2 (13)	0.58	2.15	0- 8.6	-	100

n= number of samples, += positive, MRL= maximum residue limit, RSA= Republic of South Africa

A calibration curve was plotted using sulphonamide standards and linearity was obtained and R^2 of 1.

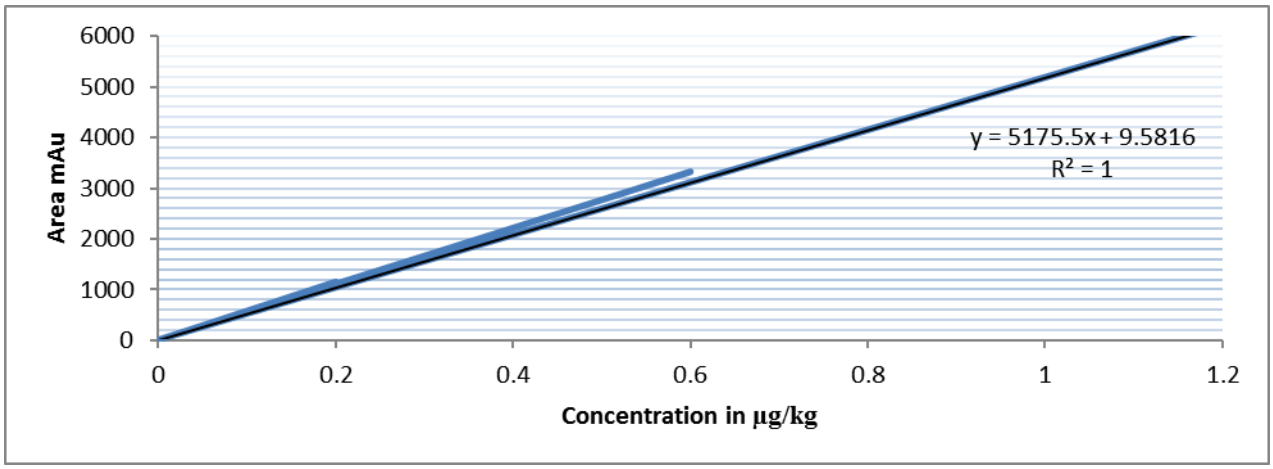


Figure 4.15: Calibration curve for sulphonamide standards at 1, 0.5, 0.25, 0.125 and 0.0625 µg/kg run on HPLC under the RF detector

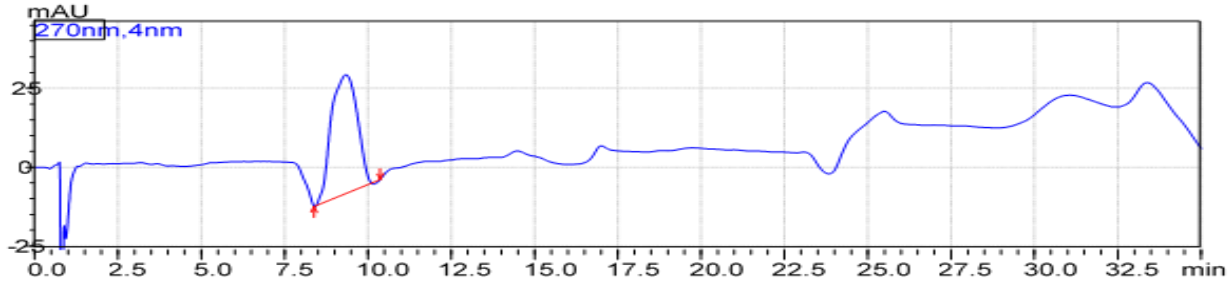


Figure 4.16**:** Sulphonamide HPLC chromatograms of a standard and fish samples obtained at a retention time of 9.2 minutes

Table 4.12: Summary of all results (multi-residues, statistics and antimicrobial residues patterns of antimicrobials)

Antibiotics	N	ELISA %	>MRL	TLC %	>MRL	HPLC %	>MRL
Tetracycline	50	42(84)	27(54)	74	-	14(28)	-
Chloramphenicol	50	42(84)	42(84)	64	74	37(74)	36(72)
Sulphonamide	50	48(96)	3(6)	88	-	7 (14)	-
Quinolone	50	6(12)	2(4)	76	-	-	-
Nitrofuran	50	10(20)	5(10)	-	-	-	-
Penicillin	-	-	-	-	-	50	-
Doxycycline	-	-	-	50	-	50	-

In this study, the results obtained (Table 4.13) showed that samples had more than one antimicrobial residue. Tetracycline, chloramphenicol and doxycycline were dominant in both salt and fresh water fish samples.

Table 4.13: Summary of fish samples with more than one antimicrobial residue in %

Fish type	TC	S3	PENI	CAP	DOX
Salt water fish	35	15	-	65	35
Fresh water fish	50	13	-	81	50

TC= Tetracycline, S3= Sulphonamide, PENI= Penicillin, CAP= Chloramphenicol, DOX= Doxycycline

Table 4.14: Fitness for five antimicrobial calibrations

Antimicrobials	R^2	Variation Retention Time
Tetracycline	0.9979	≤ 1.1
Chloramphenicol	1	≤ 2.6
Penicillin	1	≤ 15.15
Doxycycline	0.9979	≤ 1.7
Sulphonamide	1	≤ 8.5

The correlation coefficient was between 0.9979 and 1 as shown in Table 4.14. A correlation closer to 1 or that is 1, show that the better the calibration curve is, meaning the amount of detected compound is found to be the real amount.

4.3.6 Validation parameters of antibiotics in fish

Validation is based on the requirements of the EU regulation and validation parameters were evaluated during the validation process. Thus, calibration curves were assembled with the ten concentrations of standards. Validation parameters are important to demonstrate the good performance of the method, which can contribute positively to the analysis of food safety.

Table 4.15: Summary of validation parameters of antibiotics in fish according to Andrea Alexandra Ribeiro Freiras (2015)

Antibiotics	**Detection Limit CC_{α}**	**Detection capacity CC_{β}**	**Relative StD**	**Pre-cursor MPRL(ppb)**	**Production (m/z)**
Tetracycline	116.5	133.1	8	445.5	410.3
Chloramphenicol	0.1	0.2	15	320.9	151.9
Doxycycline	7.8	13.2	14	445.5	428.2
Penicillin	65.7	81.4	11	335.1	176.0/160.0
Sulphonamide	125.5	151.0	15	173.0	93.0

There is limited data on the detection of residues in food-producing animals and many conclusions cannot be made from such data. The use of an extreme value of the distribution (the MRL) is not realistic in a scenario describing chronic exposure. All concentrations of the distribution of residues should be considered (FAO, JECFA, 2015).

The results below show that among all the antibiotics described, Chloramphenicol is the only antibiotic found to be higher than the recommended ADI described by JECFA (2015), while the other antibiotics are lower than the recommended values for daily consumption by consumers.

Table 4.16: Summary of average daily intake of antibiotics (JECFA, 2015)

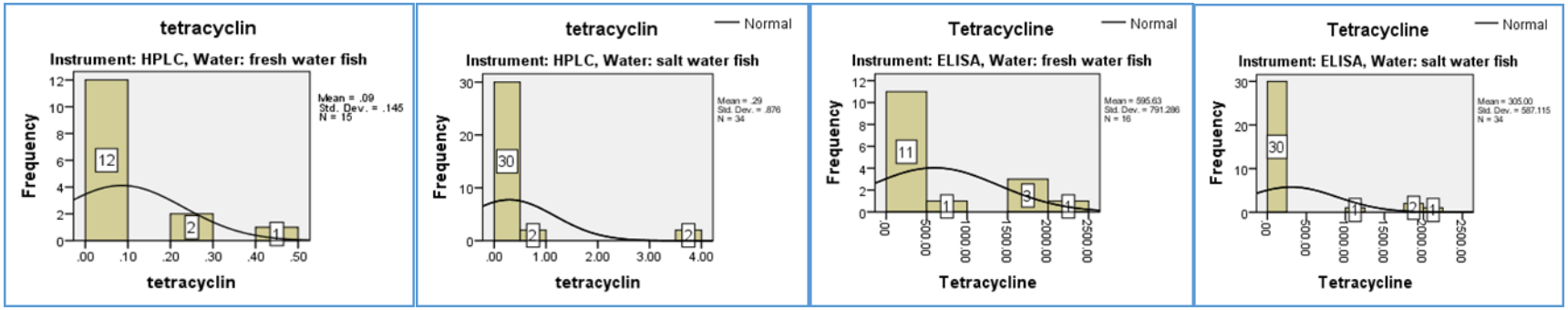
Antibiotics	**ADI (samples) in ppb/bw/day**	**JECFA (ppb/bw/day)**
Tetracycline	24.2	0–30
Chloramphenicol	114.5	0
Doxycycline	24.2	0 – 30
Penicillin	5.3	0 -31
Sulphonamide	7.8	0– 50

Table 4.17: Correlation between ELISA and HPLC detectable concentrations of Tetracycline, Penicillin, Sulphonamide, Chloramphenicol, Quinolone, Nitrofuran and Doxycycline in fresh and salt water fish samples

Descriptive statistics								
Method	Water		N	Minimum	Maximum	Mean	Std. Deviation	Variance
HPLC	Fresh water fish	Tetracycline	15	0.00	0.47	0.0853	0.14530	0.021
		Penicillin	15	0.00	0.00	0.0000	0.00000	0.000
		Sulphonamide	15	0.00	8.60	0.6133	2.21484	4.906
		Chloramphenicol	15	0.00	143.00	48.6933	39.68225	1574.681
		Doxycycline	15	0.00	0.47	0.0853	0.14530	0.021
	Salt water fish	Tetracycline	34	0.00	3.75	0.2938	0.87632	0.768
		Penicillin	34	0.00	0.00	0.0000	0.00000	0.000
		Sulphonamide	34	0.00	68.70	3.8294	13.15950	173.172
		Chloramphenicol	34	0.00	279.80	49.4941	63.67898	4055.012
		Doxycycline	34	0.00	3.75	0.2938	0.87632	0.768
ELISA	Fresh water fish	Chloramphenicol	16	0.00	82.50	22.7188	21.99884	483.949
		Tetracycline	16	0.00	2000.00	595.6250	791.28561	626132.917
		Quinolone	16	0.00	30.00	5.1250	11.14675	124.250
		Nitro furan	16	0.00	0.00	0.0000	0.00000	0.000
	Salt water fish	Sulphonamide	34	0.30	107.00	40.0029	37.57313	1411.740
		Chloramphenicol	34	0.00	120.00	21.9412	28.52637	813.754
		Tetracycline	34	0.00	2240.00	305.0000	587.11544	344704.545
		Quinolone	34	0.00	30.00	3.3529	9.33512	87.144
		Nitro furan	34	0.00	4840.00	382.0000	1036.88181	1075123.879

` The descriptive statistics differed across the tests (HPLC and ELISA) as well as between the types of water (Fresh vs Salty). No tetracycline, quinolone and nitrofuran were recorded under HPLC. Also, there were no results for tetracycline, penicillin and doxycycline recorded under ELISA. The differences in the distribution of residues are presented in the histograms below and the statistical significance of these differences was confirmed using t-tests.

Figure 4.17: HPLC and ELISA tetracycline concentration distribution of fresh and salt water fish samples



Results of HPLC showed that there was a slight difference in the distribution of the concentration of Tetracycline residues between fish from fresh water and that from salt water (both distributions are rightly skewed but the one for salt water is relatively more platykurtic/ flat). However, results of ELISA revealed that there was a slight difference in the distribution of the concentration of tetracycline residues between fish from fresh water and that from salt water (both distributions were rightly skewed but the one for salt water was relatively more platykurtic/ flat). Compared to results of HPLC, results of ELISA for fish from fresh water did not differ significantly in terms of the concentration of tetracycline residues, and similar results were observed for fish from salt water. The next analysis confirms the statistical significance of results shown by the histograms above.

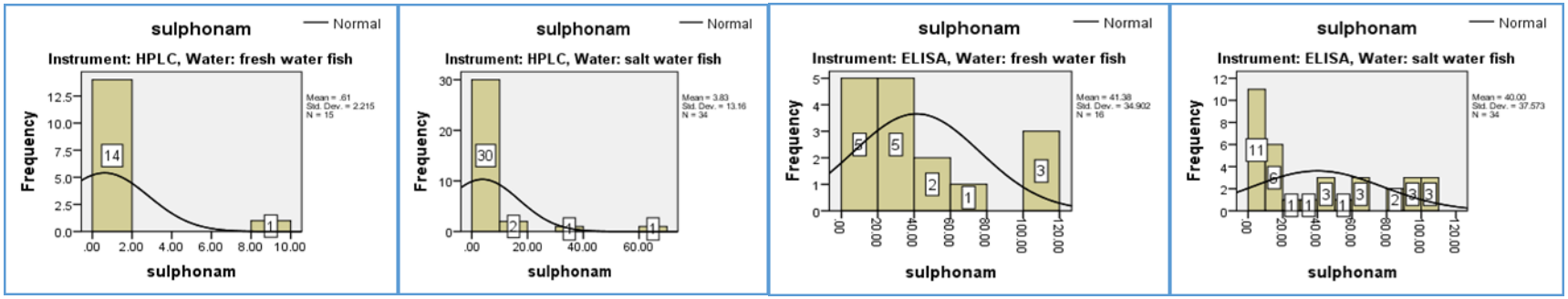
Table 4.18: Results of the t-test showed a statistically significant difference in the mean concentration of tetracycline between HPLC and ELISA, with the latter exceeding the former by 397.770 (p-value < 0.05).

	df	Sig.(2-tailed)	Mean Difference	Std. Error Difference
Tetracycline	49.000	0.000	-397.770	94.083

Table 4.19: Results of the t-test revealed that there was no statistically significant difference in mean concentration of tetracycline residues between fish from fresh water and those from salt water for both HPLC and ELISA (p-value > 0.05).

		t-test for Equality of Means		
		T	df	Sig. (2-tailed)
HPLC	Tetracycline	-0.911	47.000	0.367
ELISA	Tetracycline	1.309	23.075	0.203

Figure 4.18: HPLC and ELISA sulphonamide concentration distribution of fresh and salt water fish samples



Results of HPLC revealed that the distribution of the concentration of sulphonamide residues between fish from fresh water and that from salt water did not differ significantly (both distributions were rightly skewed). However, results of ELISA showed that there was a difference in the distribution of concentration of sulphonamide residues between fish from fresh water and those from salt water (both distributions were rightly skewed but that of salt water was relatively more platykurtic/ flat). Compared to the results for HPLC, those obtained using ELISA (for fish from fresh water) differed noticeably in terms of the concentration residues, and similar results were observed for fish from salt water. The next analysis confirms the statistical significance of results shown by the histograms above.

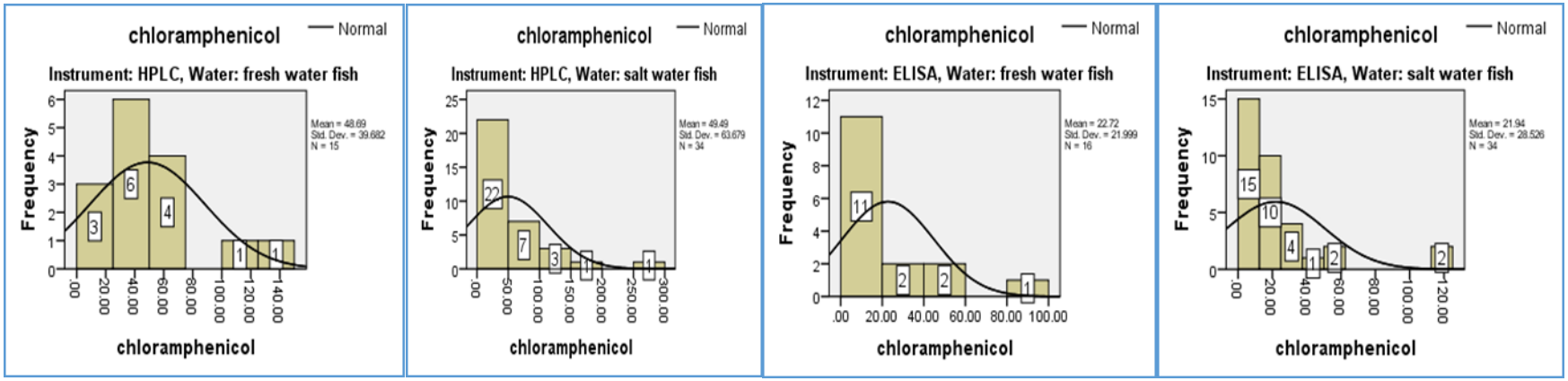
Table 4.20**:** Results of the t-test results showed a statistically significant difference in the mean concentration of sulphonamide between HPLC and ELISA with the latter exceeding the former by 37.597 (P-value < 0.05).

	t-test for Equality of Means				
	T	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference
Sulphonamide	-6.983	58.176	0.000	-37.597	5.384

Table 4.21: Results of the t-test showed that there was no statistically significant difference in the mean concentration of sulphonamide residues between fish from fresh water and those from salt water for both HPLC and ELISA (P> 0.05).

		Independent Samples Test		
		t-test for Equality of Means		
		t	**df**	**Sig. (2-tailed)**
HPLC	Sulphonamide	-0.935	47	0..354
ELISA	Sulphonamide	0..123	48	0..903

Figure 4.19: HPLC and ELISA chloramphenicol concentration distribution of fresh and salt water fish samples



Results of HPLC showed that the distribution of the concentration of chloramphenicol residues between fish from fresh water and those from salt water were different (both distributions were rightly skewed; fish from salt water was relatively more platykurtic/ flat). In addition, results of ELISA show that there was difference in the distribution of the concentration of chloramphenicol residues between fish from fresh water and those from salt water (both distributions were rightly skewed, however, fish salt water was relatively more platykurtic/ flat). Compared to results of HPLC, results of ELISA for fish from fresh water differed slightly in terms of the concentration of chloramphenicol residues, however, with regard to fish from salt water, the distributions were almost similar between HPLC and ELISA. The next analysis confirms the statistical significance of results shown by the histograms above.

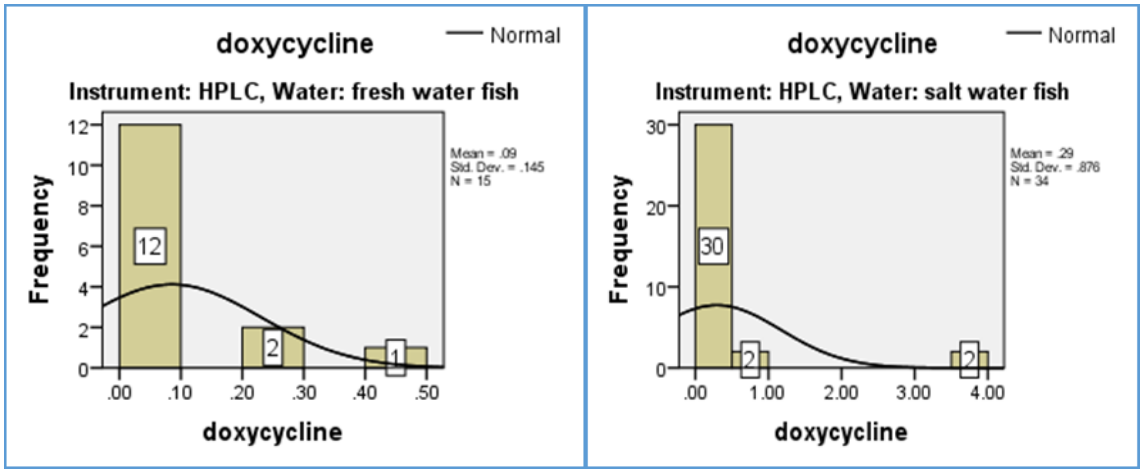
Table 4.22: Results for the t-test above show a statistically significant difference in the mean concentration of chloramphenicol residues between HPLC and ELISA, with the former exceeding the latter by 27.059 (P-value < 0.05).

	t-test for Equality of Means				
	t	**Df**	**Sig. (2-tailed)**	**Mean Difference**	**Std. Error Difference**
Chloramphenicol	3.022	67.379	0.004	27.059	8.955

Table 4.23: Results of the t-test showed that there was no statistically significant difference in the mean concentration of chloramphenicol residues between fish from fresh water and those from salt water for both HPLC and ELISA (P-value > 0.05).

Method		t-test for Equality of Means				
		T	**df**	**Sig. (2-tailed)**	**Mean Difference**	**Std. Error Difference**
HPLC	Chloramphenicol	-0.045	47	0.964	-0.801	17.850
ELISA	Chloramphenicol	0.096	48	0.924	0.778	8.082

Figure 4.20: HPLC doxycycline concentration distribution of fresh and salt water fish

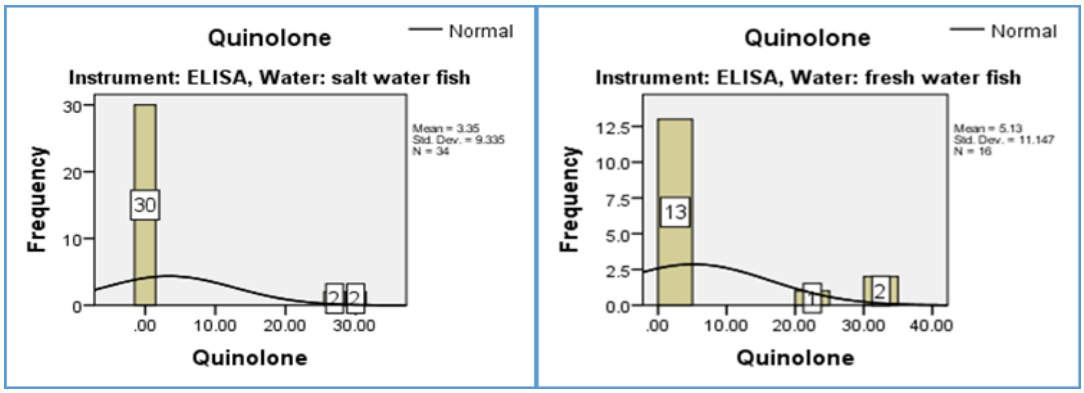


Results of HPLC revealed that the distribution of the concentration of doxycycline residues between fish from fresh water and those from salt water were different (both distributions were rightly skewed, however, fish from salt water was relatively more platykurtic/ flat). No results were recorded for doxycycline residues using ELISA.

Table 4.24: Results of the t-test showed that there was no statistically significant difference in the mean concentration of doxycycline residues between fish from fresh water and those from salt water for HPLC (p-value > 0.05).

Instrument		t-test for Equality of Means		
		t	Df	Sig. (2-tailed)
HPLC	Doxycycline	-0.911	47	0.367

Figure 4.21: ELISA quinolone concentration distribution of fresh and salt water fish samples

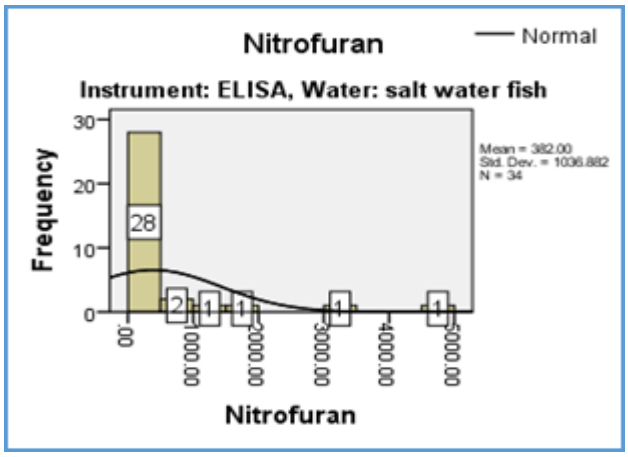


Results of ELISA showed that the distribution of quinolone residues in fish from fresh water did not differ significantly from those observed in fish from salt water. The next analysis confirms the statistical significance of these results.

Table 4.25: Results of the t-test revealed that there was no statistically significant difference in the mean concentration of quinolone residues between fish from fresh water and those from salt water for ELISA (P-value > 0.05).

Method		t-test for Equality of Means		
		t	Df	Sig. (2-tailed)
ELISA	Quinolone	0.588	48	0.559

Figure 4.22: ELISA nitrofuran concentration distribution of fresh and salt water fish samples



Results of ELISA revealed that there were nitrofuran residues in fish from salt water only. No results were recorded for fish from fresh water while no results were recorded from the HPLC test.

4.4 MICROBIOLOGICAL ANALYSIS OF FISH SAMPLES

4.4.1 The prevalence of micro-organisms per colony

Table 4.26 shows that microorganisms were found to be of the ***Cocci*** (staphy) shape (52.7%), followed by the *Bacillus* (strepto) shape (38.40%). Microorganisms of the shapes *Cocci* (strepto) and *Bacillus* (staphy) each formed only 4.00% of the detected microorganisms.

Table 4.26: Summary of Morphology and percentage according to each bacterium

			Cocci (staphy)		**Bacillus (strepto)**		**Cocci (strepto)**		**Bacillus (staphy)**	
			Count	**Percentage**	**Count**	**Percentage**	**Count**	**Percentage**	**Count**	**Percentage**
Clostridium sp.	MEDIUM	PINK	1	2.00%	0	0.00%	0	0.00%	0	0.00%
		PURPLE	0	0.00%	1	2.00%	0	0.00%	0	0.00%
Bacillus cereus	LARGE	PURPLE	3	6.10%	0	0.00%	0	0.00%	0	0.00%
		PURPLE	0	0.00%	0	0.00%	1	2.00%	0	0.00%
Enterococcus sp.	SMALL	PINK	1	2.00%	0	0.00%	0	0.00%	0	0.00%
		PURPLE	4	8.20%	0	0.00%	0	0.00%	0	0.00%
	MEDIUM	PURPLE	3	6.10%	1	2.00%	0	0.00%	0	0.00%
		RED/ PINK	0	0.00%	1	2.00%	0	0.00%	0	0.00%
Enterococceae bacterium	SMALL	PINK	0	0.00%	1	2.00%	0	0.00%	0	0.00%
	MEDIUM	PINK	1	2.00%	3	6.10%	0	0.00%	0	0.00%
	LARGE	PINK	2	4.10%	2	4.10%	0	0.00%	0	0.00%
		RED/ PINK	1	2.00%	0	0.00%	0	0.00%	0	0.00%
Lynisibacillus sp.	SMALL	PINK	1	2.00%	0	0.00%	0	0.00%	0	0.00%
	MEDIUM	PINK	1	2.00%	1	2.00%	0	0.00%	0	0.00%
	LARGE	PINK	0	0.00%	2	4.10%	0	0.00%	0	0.00%
Lynisibacillus fusiform	SMALL	PINK	0	0.00%	1	2.00%	0	0.00%	0	0.00%
		RED/ PINK	0	0.00%	1	2.00%	0	0.00%	0	0.00%
	MEDIUM	PINK	1	2.00%	1	2.00%	0	0.00%	0	0.00%
		RED/ PINK	2	4.10%	0	0.00%	0	0.00%	0	0.00%
	LARGE	PINK	0	0.00%	3	6.10%	1	2.00%	1	2.00%
		RED/ PINK	2	4.10%	0	0.00%	0	0.00%	0	0.00%
Bacillus sp.		PURPLE	1	2.00%	0	0.00%	0	0.00%	0	0.00%
Enterococcus faecium	SMALL	PINK	1	2.00%	0	0.00%	0	0.00%	0	0.00%
	MEDIUM	PINK	1	2.00%	0	0.00%	0	0.00%	1	2.00%
	LARGE	PINK	0	0.00%	1	2.00%	0	0.00%	0	0.00%
TOTAL			**26**	**52.70%**	**19**	**38.40%**	**2**	**4.00%**	**2**	**4.00%**

Staphylococcus= staphy and Streptococcus= strepto

4.4.2 Morphology and biochemical character of bacterial isolates

Bacterial isolates were identified according to the morphology (size, shape and colour), biochemical reactions (oxidase, catalase and gram stain) and molecular techniques. Preliminary results based on morphology and biochemical characterisation for samples collected in supermarkets around Mahikeng are presented in Tables 4.26 and 4.27. The results of biochemical tests showed the presence of different strains of bacteria such as *Bacillus cereus, Clostridium spp., Enterococcus spp., Enterococeae bacterium, Lynisibacillus spp., Lynisibacillus fusiform, Bacillus spp.* and *Enterococcus faecium*. The results were cautiously identified before being subjected to molecular identification using PCR, for further investigation.

Table 4.27**:** Preliminary results based on biochemical tests done on isolated strains

Sample identification	**Gram-stain**	**Catalyst Test**	**Oxidase test**	**Presumption micro organism**
Fish1	-	+	+	*Clostridium sp.*
Fish 2	+	+	-	*Bacillus cereus*
Fish 3	-	-	+	*Enterococcus sp.*
Fish4	-	+	-	*Enterococeae bacterium*
Fish5	-	+	+	*Lynisibacillus sp.*
Fish 6	-	+	-	*Enterococeae bacterium*
Fish7	+	+	+	*Enterococcus sp.*
Fish 8	-	+	+	*Lynisibacillus fusiform*
Fish 9	-	+	+	*Lynisibacillus fusiform*
Fish 10	-	-	+	*Enterococeae bacterium*
Fish 11	-	+	-	*Enterococeae sp.*
Fish 12	+	+	-	*Bacillus cereus*
Fish 13	+	+	+	*Enterococcus sp.*
Fish 14	+	+	+	*Enterococcus sp.*
Fish 15	-	+	-	*Enterococeae bacterium*
Fish 16	-	+	+	*Lynisisbacillus fusiform*
Fish 17	-	+	+	*Lynisibacillus sp.*
Fish 18	-	+	-	*Enterococaea bacterium*
Fish 19	-	+	+	*Lynisibacillus fusiform*
Fish 20	-	+	+	*Lynisibacillus sp.*
Fish 21	-	+	+	*Lynisibacillus sp.*

Fish 22	+	+	-	*Bacillus cereus*
Fish 23	-	+	+	*Lynisibacillus sp.*
Fish 24	-	+	-	*Enterococaea bacterium*
Fish 25	+	+	+	*Enterococcus sp.*
Fish 26	-	+	-	*Enterococaea bacterium*
Fish 27	+	+	+	*Enterococcus sp.*
Fish 28	+	+	-	*Bacillus sp.*
Fish 29	+	+	-	*Bacillus cereus*
Fish 30	-	+	+	*Lynisibacillus fusiform*
Fish 31	-	+	-	*Enterococaea bacterium*
Fish 32	+	+	+	*Enterococcus sp.*
Fish 33	-	+	+	*Lynisibacillus fusiform*
Fish 34	-	+	+	*Lynisibacillus fusiform*
Fish 35	+	+	+	*Enterococcus sp.*
Fish 36	+	-	+	*Clostridium sp.*
Fish 37	-	+	+	*Lynisibacillus fusiform*
Fish 38	-	+	+	*Lynisibacillus fusiform*
Fish 39	-	+	-	*Enterococaea bacterium*
Fish 40	-	+	+	*Lynisibacillus fusiform*
Fish 41	-	+	+	*Lynisibacillus fusiform*
Fish 42	-	+	-	*Enterococaea bacterium*
Fish 43	-	-	-	*Enterococcus faecium*
Fish 44	-	+	+	*Lynisibacillus fusiform*
Fish 45	-	-	+	*Enterococcus faecalis*
Fish 46	-	+	-	*Enterococaea bacterium*
Fish 47	-	+	+	*Lynisibacillus fusiform*
Fish 48	-	-	-	*Enterococcus faecium*
Fish 49	+	+	+	*Enterococcus sp.*
Fish 50	+	+	+	*Enterococcus faecium*

4.4.3 Results obtained using API-20E

The results obtained revealed that 100% of bacterial isolates analysed were *Enterococcus* species isolated from different fish samples. The diagram below shows that *Enterococcus Faecium* was the most detected micro-organism from the 20, amounting to 19.44%, *Enterococcus sp.* with 13.89% and *Bacillus Cereus* representing 11.11%. *Clostridium Sordeli* had only 5.56% of the detected micro-organisms, while the other micro-organisms each had only 2.78% of the 20.

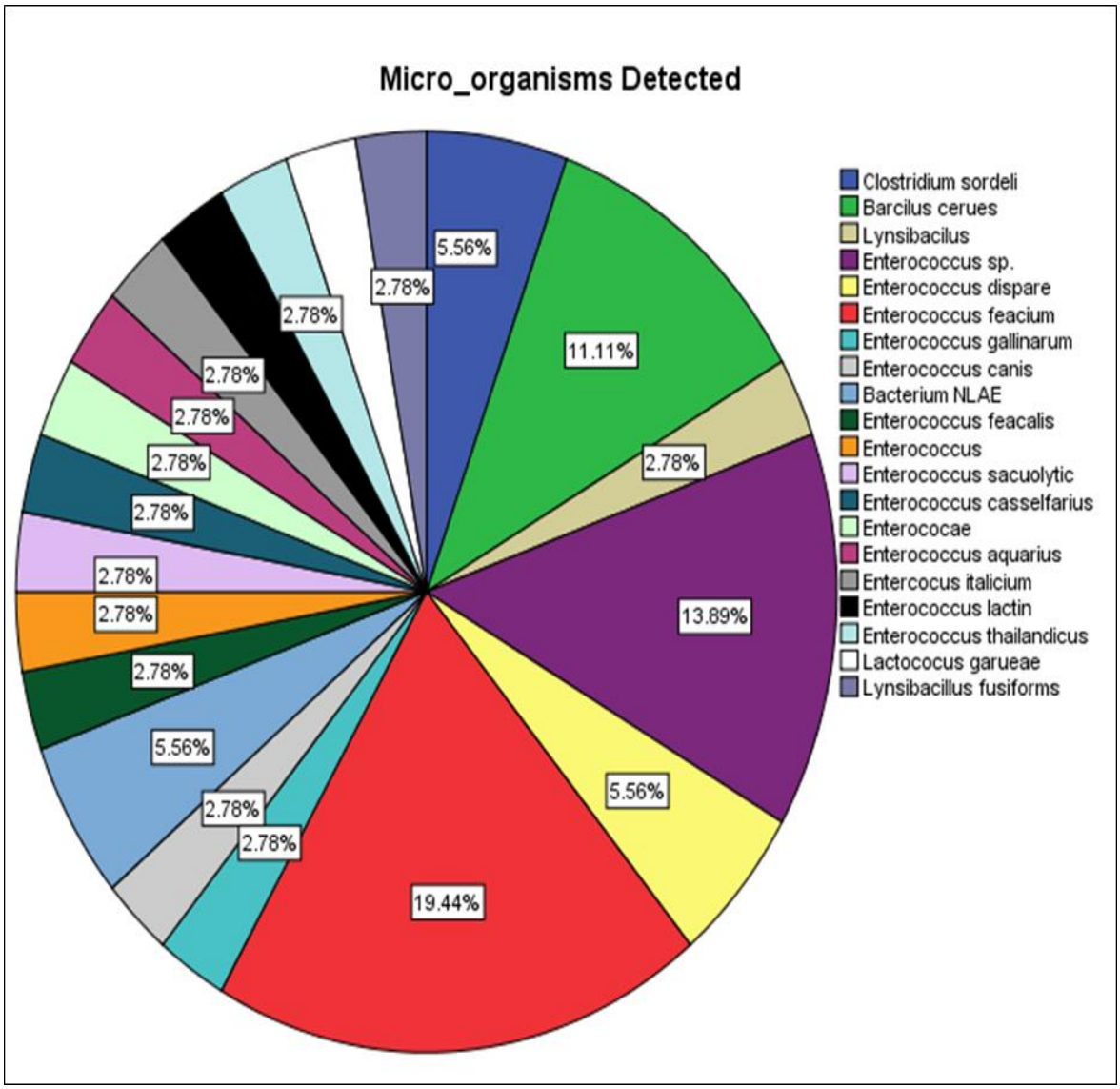


Figure 4.23: An overall summary of the pre-assumptive microorganism according to API 20E test

Figure 4.24: Pictures of Analytical Profile Index (API 20E) of bacterial isolates

4.4.4 Molecular identification of isolated bacteria

Fifty bacterial isolates were selected and subjected to PCR analysis. The results revealed a 1% (w/v) agarose gel, representing 16SrDNA gene fragment. Figure 4.31 was obtained by running the gel on electrophoresis for 80 minutes. After running the gel, an automatic UV Tran illuminator (UV tech system Germany) was used to view the bands of the genomic DNA and photographed using Bio profile gel documentation system to check for the presence of DNA bands and to confirm successful extraction.

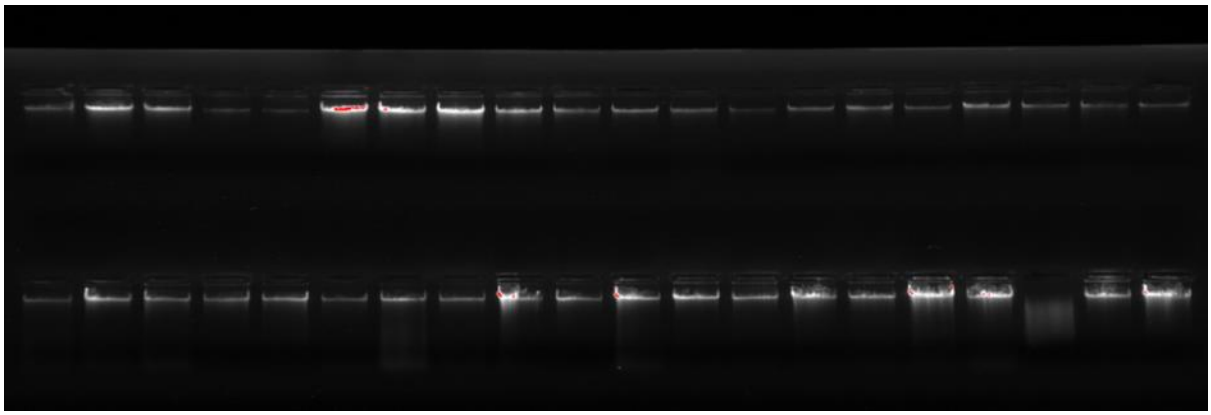


Figure 4.25: Agarose gel 1% (w/v) electrophoresis showing 16 rRNA gene fragments amplified from DNA extracted from isolated bacteria

4.4.5 Confirmatory results of bacterial isolates based on PCR and sequencing

Fifty (50) bacterial isolates were selected and subjected to PCR analysis. The results revealed a 1% (w/v) agarose gel, representing 16S rDNA gene fragments (Figure 4.31). The desired 1kb base pairs fragments were obtained after running the gel on electrophoresis for 65 minutes. After running the gel, an automatic UV trans-illuminator (UV Tec, Sigma, Germany) was used to view the bands of the genomic DNA and photographed using a Bio profile gel documentation system. The purpose was to check the presence or absence of DNA bands and confirm successful extraction.

Table 4.28: Summary of bacterial isolates based on PCR products, sequence analysis and their accession number

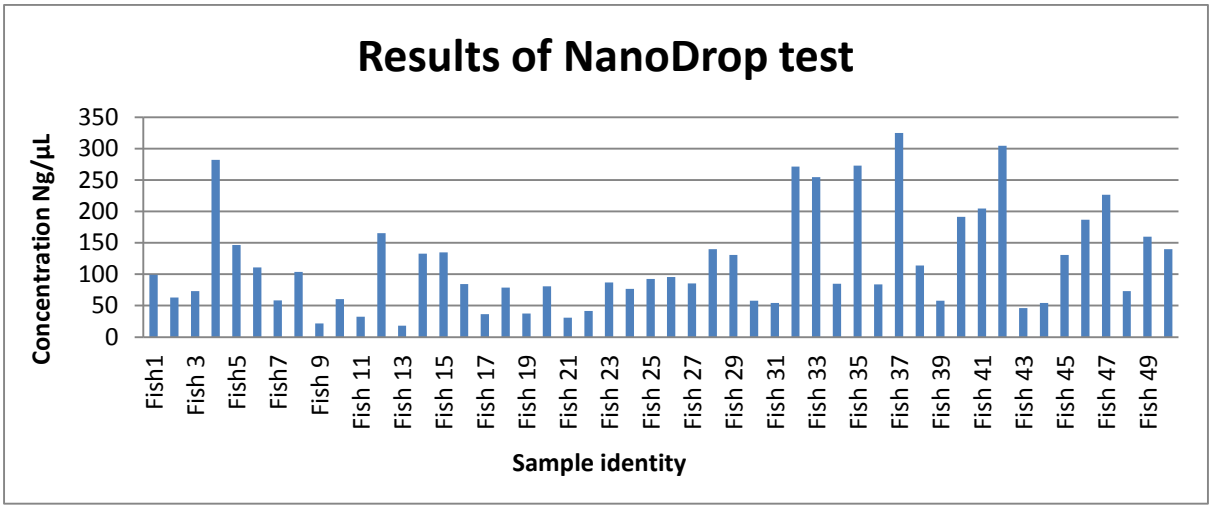
Sample identification	**Accession number**	**16SrDNAgene sequence based identity in %**	**Confirmatory organism**
1	KX073810.1	94%	*Clostridium sordeli strain*
2	HM480354.1	90%	*Bacillus cereus strain*
3	KU057028.1	90%	*Lysinibacillus sp.*
4	KX051379.1	90%	*Enterococcus sp. Strain*
5	KF286616.1	90%	*Enterococcus sp.*
6	KF183512.1	95%	*Enterococcus dispar*
7	KF286616.1	91%	*Enterococcus sp.*
8	KM257680.1	90%	*Enterococcus faecium strain*
9	KJ803882.1	86%	*Enterococcus gallinarum*
10	NR113931.1	94%	*Enterococcus canis*
11	KC663436.1	90%	*Bacillus cereus strain*
13	HQ418499.1	90%	*Bacillus sp.*
14	JX006617.1	91%	*Bacterium NLAE*
16	HG798520.1	96%	*Enterococcus faecalis*
17	KP823032.1	96%	*Enterococcaceae*
18	HG798520.1	93%	*Enterococcus faecalis*
19	KT260659.1	94%	*Enterococcus saccharolyticus*
20	KF826012.1	91%	*Enterococcus casseliflavus strain*
21	KF183512.1	90%	*Enterococcus dispar*
22	KP823032.1	90%	*Enterococcaceae bacterium*
25	KX267891.1	91%	*Enterococcus faecium strain*
26	GQ337015.1	94%	*Enterococcus aquimarinus strain*
27	JX006554.1	92%	*Bacterium NLAE-zl*
28	KX051379.1	94%	*Enterococcus sp. strain*
30	KF286616.1	94%	*Enterococcus sp.*
31	KM257680.1	93%	*Enterococcus faecium strain*

32	KM276808.1	90%	*Enterococcus italicus strain*
33	KU315086.1	98%	*Enterococcus lactis strain*
34	KM257680.1	95%	*Enterococcus faecium strain*
35	GU250442.1	90%	*Bacillus cereus strain*
36	KP406361.1	91%	*Enterococcus sp. clone*
37	FJ915708.1	92%	*Enterococcus faecium strain*
38	KM257680.1	91%	*Enterococcus faecium strain*
40	KF286616.1	91%	*Enterococcus sp.*
41	NR_044160.1	95%	*Enterococcus thailandicus strain*
42	KT260280.1	90%	*Lactococcus garvieae*
43	KX267939.1	91%	*Enterococcus faecium strain*
44	GU597972.1	90%	*Lysinibacillus fusiform*
45	JQ411244.1	90%	*Enterococcus faecalis*
46	KF286616.1	90%	*Enterococcus sp.*
47	JQ739690.1	93%	*Enterococcus sp.*
48	HG798520.1	92%	*Enterococcus*
49	KM257680.1	84%	*Enterococcus faecium strain*

4.4.6 Quantification of DNA using Nanodrop 2000c (Thermo scientific)

The results obtained showed that the samples were accurately measured for DNA without dilution. The software automatically utilises the optical path length to measure the absorbance of each sample. The level of concentration and the quantity were checked. Different concentrations of each sample were observed ranging from 18 Ng/µL to the highest value of 324.9 Ng/µL.

Figure 4.26: Results of Nanodrop according to their concentration (Ng/µl)



4.5 ANALYSIS OF ANTIMICROBIAL ANTIBIOTIC SUSCEPTIBILITY TESTING

In this study, all fish samples were subjected to antibiotic susceptibility testing. The results obtained (see Appendix 1 in mm) were used to classify isolates as being resistant, intermediate or susceptible to a particular antibiotic, using standard reference values followed by recommendations of the Clinical Laboratory Institute Standards (Wayne, 2009; Wikler, 2006; Wikler, 2007). Bacteria tested were represented as R (resistant), I (intermediate) and S (Susceptible). Different bacteria tested in this study were *Enterococcus faecium*, *Bacillus aureus*, *Staphylocum*, *Enterococcus faecalis*, *Bacillus cereus* and *Lysinibacillus spp.*

4.5.1 Isolated bacteria tested with five different antibiotics susceptibility

Table 4.29: Resistant, susceptible and intermediate resistance patterns of isolated bacteria

SI	Bacterium found	TC	CAP	CIP	S3	Nor
1	*Clostridium sordeli*	S	S	S	R	I
2	*Bacillus cereus*	I	S	S	S	I
3	*Lynsibacilus*	I	R	S	S	S
4	*Enterococcus sp.*	R	I	S	S	I
5	*Enterococcus sp.*	I	S	S	I	S
6	*Enterococcus dispare*	R	I	S	R	R
8	*Enterococcus feaciun*	R	S	S	R	R
9	*Enterococcus gallinarum*	R	I	S	S	R
10	*Enterococcus canis*	S	I	S	S	S
11	*Bacillus cereus*	I	I	S	S	S
12	*Clostridium sordeli*	S	S	S	I	I
13	*Bacillus cereus*	R	I	S	S	I
14	*Bacterium NLAE*	R	R	S	S	I
16	*Enterococcus faecalis*	S	S	S	S	S
17	*Enterococcus*	S	I	S	S	I
19	*Enterococcus sacuolytic*	S	R	S	S	S
20	*Enterococcus casselfarius*	S	I	S	S	S
21	*Enterococcus dispar*	S	S	S	S	I
22	*Enterococae*	S	S	S	S	R
24	*Enterococcus feacium*	I	S	S	S	R
25	*Enterococcus aquarius*	S	S	S	R	R
26	*Bacterium NLAE*	R	S	S	S	S
30	*Enterococcus faecium*	S	I	S	S	R
31	*Enterococcus italicium*	S	R	R	I	R
32	*Enterococcus lactin*	R	S	S	S	S
33	*Enterococcus faecium*	R	R	S	S	R
34	*Bacillus cereus*	I	S	S	S	I
35	*Enterococcus faecium*	I	I	S	S	R
37	*Enterococcus faecium*	R	I	S	S	R
40	*Enterococcus sp*	I	I	S	S	R
41	*Enterococcus thailandicus*	S	I	S	S	S
42	*Lactococcus garueae*	I	I	S	S	R
43	*Enterococcus faecium*	S	I	S	R	R
44	*Lysinibacillus fusiform*	S	I	S	S	S
46	*Enterococcus sp*	R	I	S	R	S
48	*Enterococcus sp*	R	S	S	S	S

SI = Sample Identification, TC = Tetracycline, CAP = Chloramphenicol, CIP = Ciprofloxacin, S3 = Sulphonamide Nor = Norfloxacin, R = Resistant, S = Susceptible and I = Intermediate

The results obtained (Figure 4.27) show susceptibility per antibiotic Most of the strains were susceptible (42%) to tetracycline, followed by strains that were resistant (33%) and those that were intermediate (25%). Figure 4.27 also shows that most of the strains were intermediate (47%) to chloramphenicol, followed by strains that were susceptible (39%) and those that were resistant (25%). Most of the strains (97%) were susceptible to ciprofloxacin, only 3% were resistant while none were intermediate. Three quarters (75%) of the strains were susceptible to sulphonamide, followed by strains that were resistant (17%) and those that were intermediate (8%). Most of the strains were resistant (39%) to norflaxacin, followed by strains that were susceptible (36%) and only a quarter were intermediate (25%). generally, ciprofloxacin is the best antibiotic from the five since more than 90% of the strains were susceptible to it.

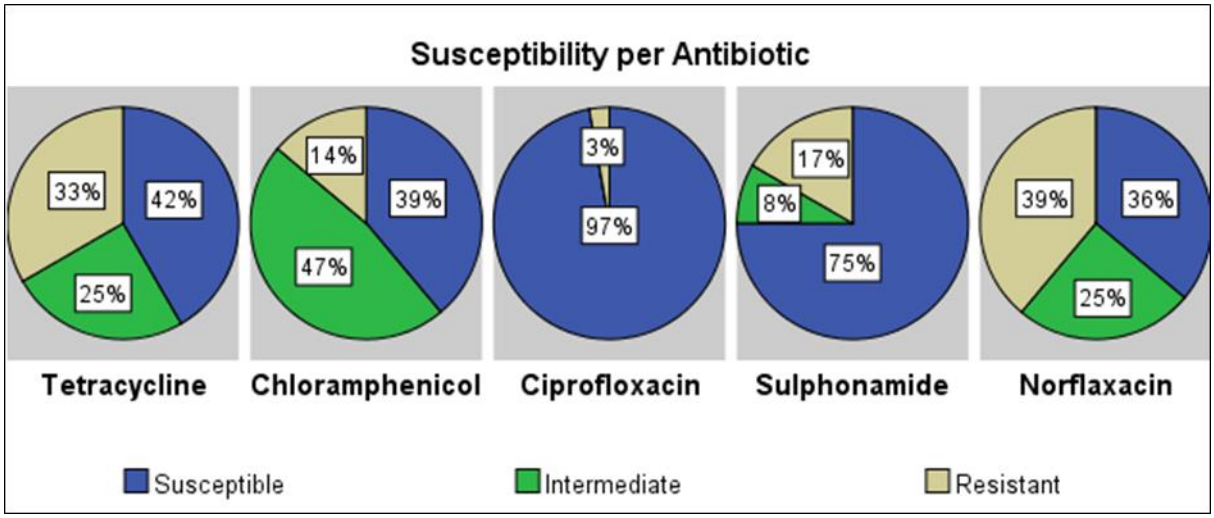


Figure 4.27: Summary of each antibiotic with susceptibility to organisms in percentages (%)

4.5.2 Summary of antibiotics in fish samples subjected to susceptibility testing in %

The results also revealed that slightly more than half (57.78%) of the strains of micro-organisms were susceptible to antibiotics while the remaining strains were either intermediate (21.11%) or resistant (21.11%) (Figure 4.28).

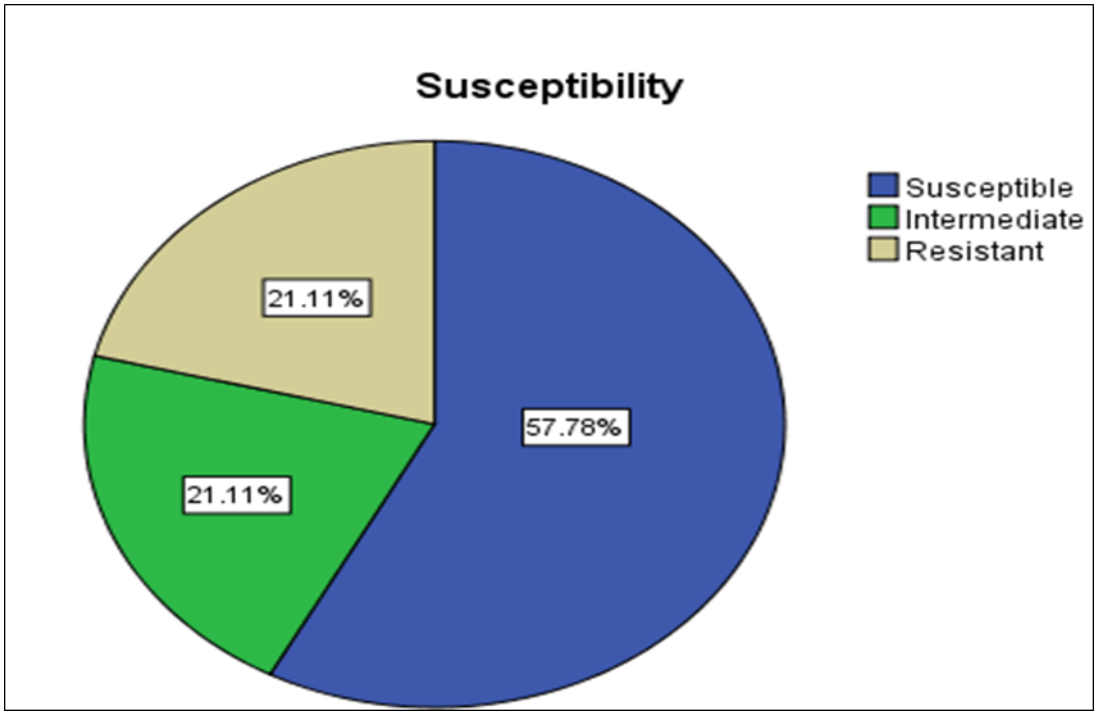


Figure 4.28: Overall summary of antibiotics in fish samples subjected to susceptibility testing

4.5.3 Multi-drug resistance

4.5.3.1 Overall bacteria with susceptibility profile

The results revealed that most bacteria were susceptible to 3 or 4 antibiotics except for *Enterococcus gallinarum* (which was susceptible to only 2 antibiotics), Enterococcus *faecalis* (susceptible to 5 antibiotics), *Enterococcus italicium* (susceptible to only one antibiotic) and *Lactococcus garueae* (susceptible to 2 antibiotics) (Table 4.30).

Table 4.30**:** Overall bacteria with susceptibility profile to selected antibiotics

	Bacterium									
	Clostridium sordeli	***Bacillus cereus***	***Lynsibacilus***	***Enterococcus dispare***	***Enterococcus feacium***	***Enterococcus gallinarum***	***Enterococcus canis***	***Bacterium NLAE***	***Enterococcus faecalis***	***Enterococcus***
Antibiotics that the bacteria are susceptible to:	Tetracycline, Chloramphenicol Ciprofloxacin	Chloramphenicol Ciprofloxacin Sulphonamide Norfloxacin	Ciprofloxacin Sulphonamide Norfloxacin	Tetracycline Chloramphenicol Ciprofloxacin Sulphonamide	Tetracycline Chloramphenicol Ciprofloxacin Sulphonamide	Ciprofloxacin Sulphonamide	Tetracycline Ciprofloxacin Sulphonamide Norfloxacin	Chloramphenicol Ciprofloxacin Sulphonamide Norfloxacin	Tetracycline Chloramphenicol Ciprofloxacin Sulphonamide Norfloxacin	Tetracycline Ciprofloxacin Sulphonamide

Table 4.30: Overall bacteria with susceptible profile continue…

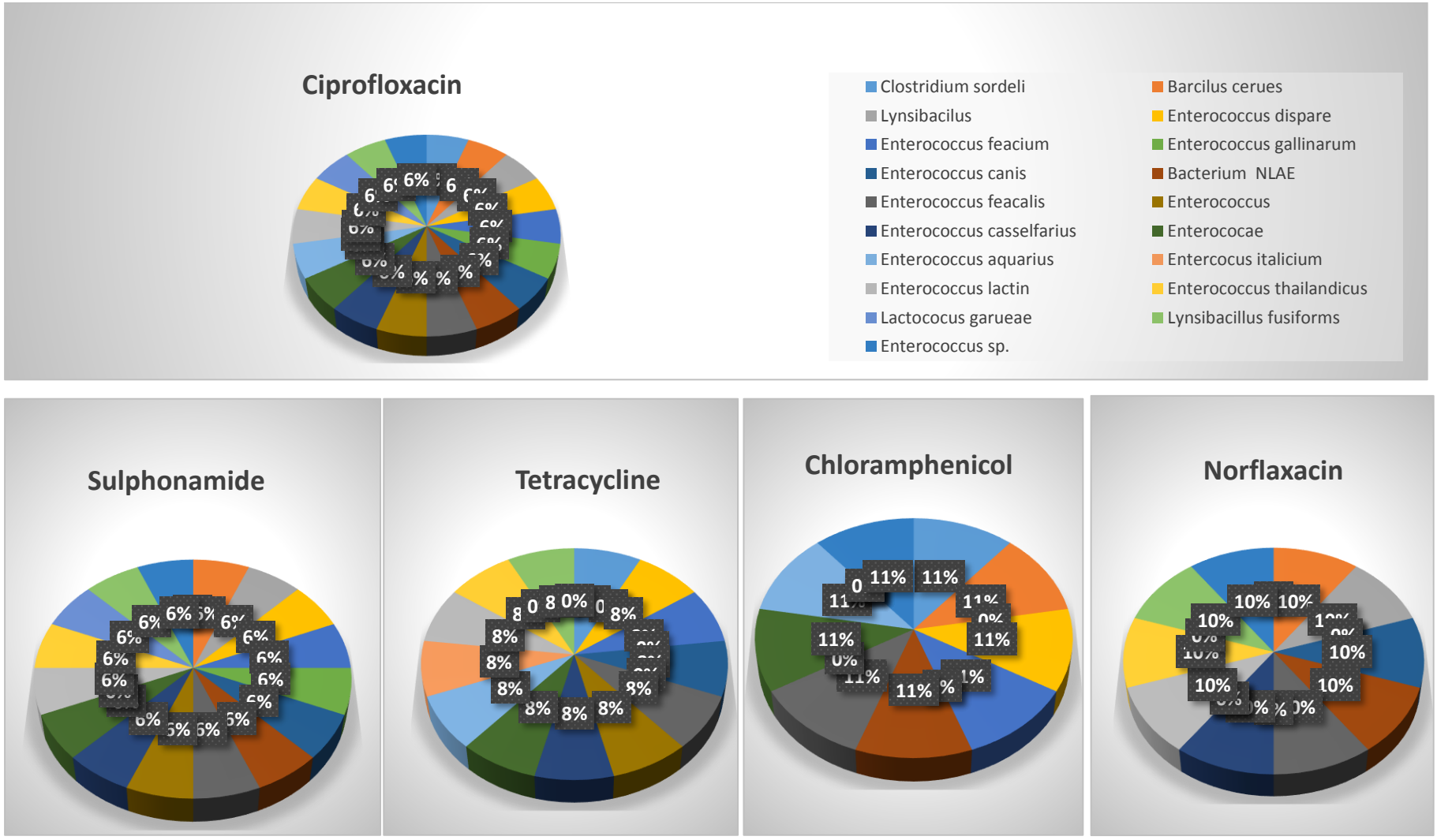
	Bacterium									
	Enterococcus sacuolytic	***Enterococcus casselfarius***	***Enterococae***	***Enterococcus aquarius***	***Enterococcus italicium***	***Enterococcus lactin***	***Enterococcus thailandicus***	***Lactococcus garueae***	***Lynsibacillus fusiform***	***Enterococcus sp.***
Antibiotics that the bacteria are susceptible:	Tetracycline Ciprofloxacin Sulphonamide Norfloxacin	Tetracycline Ciprofloxacin Sulphonamide Norfloxacin	Tetracycline Chloramphenicol Ciprofloxacin Sulphonamide	Tetracycline Chloramphenicol Ciprofloxacin	Tetracycline	Tetracycline Ciprofloxacin Sulphonamide Norfloxacin	Tetracycline Ciprofloxacin Sulphonamide Norfloxacin	Ciprofloxacin Sulphonamide	Tetracycline Ciprofloxacin Sulphonamide Norfloxacin	Chloramphenicol Ciprofloxacin Sulphonamide Norfloxacin

Table 4.31: Resistance profile of different bacteria to different antibiotics

	Clostridium sordeli	***Bacillus cereus***	***Lynsibacilus***	***Enterococcus dispare***	***Enterococcus feacium***	***Enterococcus gallinarum***	***Enterococcus canis***	***Bacterium NLAE***	***Enterococcus faecalis***	***Enterococcus***
Tetracycline	1	0	0	1	1	0	1	0	1	1
Chloramphenicol	1	1	0	1	1	0	0	1	1	0
Ciprofloxacin	1	1	1	1	1	1	1	1	1	1
Sulphonamide	0	1	1	1	1	1	1	1	1	1
Norfloxacin	0	1	1	0	0	0	1	1	1	0

Table 4.31: Resistance profile of bacteria to different antibiotics: the counts differ because there were different types of bacteria (numbers represent the number of bacteria found to be susceptible)

Figure 4.29: Percentage of bacterial isolates susceptible in each type of antibiotic



4.5.3.2 Overall bacteria showing resistance profile

The results show that bacteria were resistant to 1 or 2 antibiotics except for *Enterococcus sp*., *Enterococcus dispare* and *Enterococcus italicium* which were resistant to 3 antibiotics. *Enterococcus feacium* was resistant to 4 antibiotics while the other bacteria were not resistant (*Enterococcus canis Enterococcus faecalis, Enterococcus, Enterococcus casselfarius, Enterococcus thailandicus* and *Lynsibacillus fusiform).*

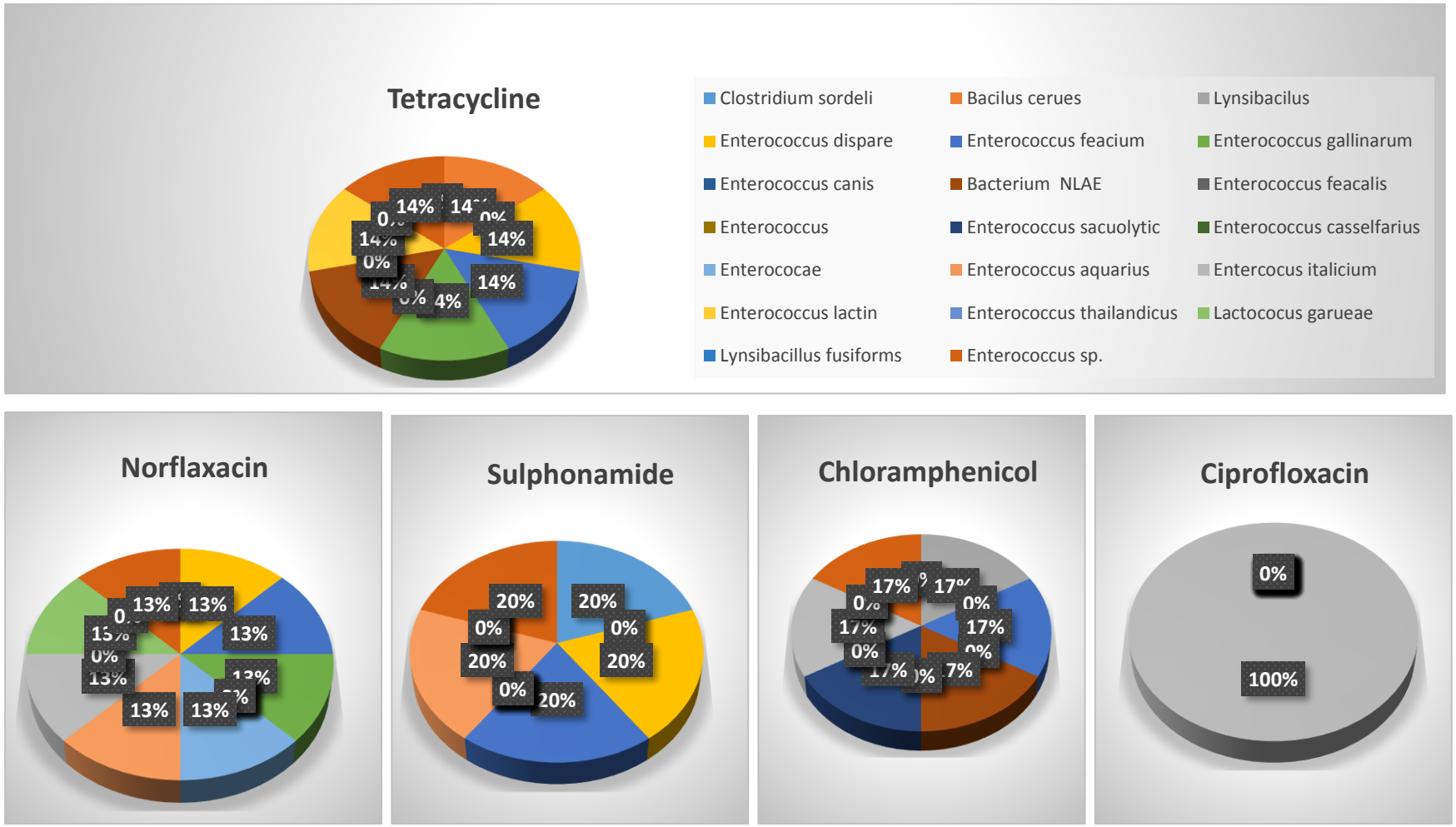
Table 4.32 shows that most of the bacteria were resistant.

	Bacterium									
	Clostridium sordeli	***Bacillus cereus***	***Lynsibacilus***	***Enterococcus dispare***	***Enterococcus feacium***	***Enterococcus gallinarum***	***Enterococcus canis***	***Bacterium***	***Clostridium sordeli***	***Bacillus cereus***
Antibiotics resistant to each isolate	Sulphonamide	Tetracycline	Chloramphenicol	Tetracycline	Sulphonamide	Tetracycline	Chloramphenicol	Tetracycline	Sulphonamide	Tetracycline
	Bacterium									
	Enterococcus sacuolytic	***Enterococcus casselfarius***	***Enterococae***	***Enterococcus aquarius***	***Enterococcus italicium***	***Enterococcus lactin***	***Enterococcus thailandicus***	***Lactococcus garueae***	***Lynsibacillus fusiform***	***Enterococcus sp.***
Antibiotics resistant to each isolate	Chloramphenicol	-	Norfloxacin	Sulphonamide			Chloramphenicol	-	Norfloxacin	Sulphonamide

Table 4.33 presents the count of occurrences of instances where the bacteria had resistance profiles to antibiotics. The counts differ because they were different types of bacteria and were from different species.

	Clostridium sordeli	*Bacilus cerues*	*Lynsibacilus*	*Enterococcus dispare*	*Enterococcus feacium*	*Enterococcus gallinarum*	*Enterococcus canis*	*Bacterium NLAE*	*Enterococcus feacalis*	*Enterococcus*	*Enterococcus sacuolytic*	*Enterococcus casseliflavus*	*Enterococcae*	*Enterococcus aquarius*	*Entercocus italicium*	*Enterococcus lactin*	*Enterococcus thailandicus*	*Lactococus garueae*	*Lynsibacillus fusiforms*	*Enterococcus sp.*
Tetracycline	0	1	0	1	1	1	0	1	0	0	0	0	0	0	0	1	0	0	0	1
Chloramphenicol	0	0	1	0	1	0	0	1	0	0	1	0	0	0	1	0	0	0	0	1
Ciprofloxacin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Sulphonamide	1	0	0	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1
Norflaxacin	0	0	0	1	1	1	0	0	0	0	0	0	1	1	1	0	0	1	0	1

Figure 4.30: Percentage of bacterial isolates resistant in each type of antibiotic



4.5.3.3 Summary of bacteria intermediate to antibiotics

The results in Table 4.35 show that most of the bacteria were intermediate to 1 or 2 antibiotics except for *Bacillus cereus* (moderate to 3 antibiotics), *Enterococcus spp.* (moderate to 4 antibiotics) while the following were not intermediate to any antibiotic: *Enterococcus; Enterococcus casselfarius; Enterococae; Enterococcus aquarius; and Enterococcus lactin.*

Table 4.34: Overall profile of intermediate isolates

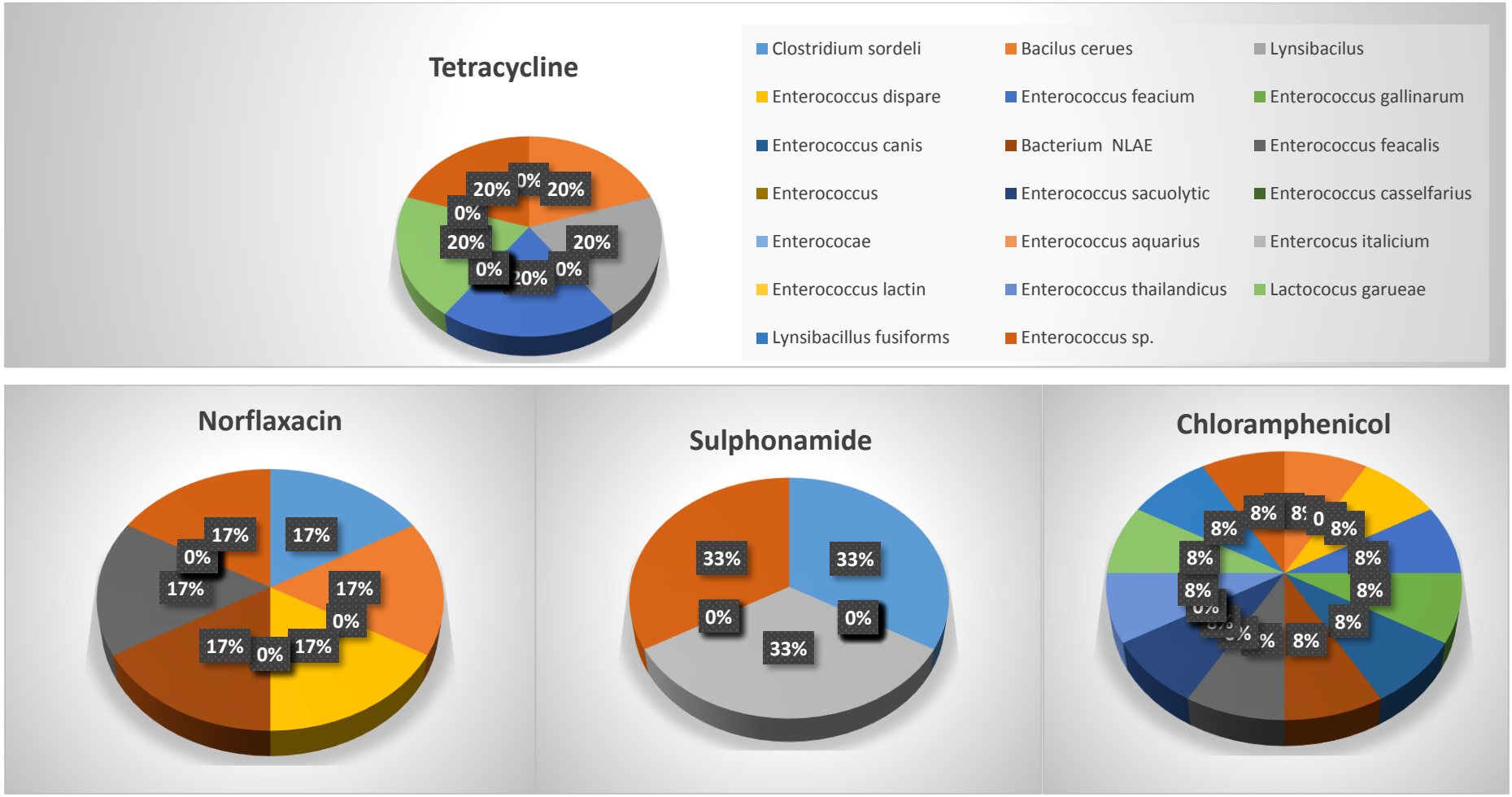
	Bacterium									
	Clostridium sordeli	***Barcilus cerues***	***Lynsibacilus***	***Enterococcus dispare***	***Enterococcus feacium***	***Enterococcus gallinarum***	***Enterococcus canis***	***Bacterium NLAE***	***Enterococcus feacalis***	***Enterococcus***
Antibiotics that the bacteria are susceptible to:	Sulphonamide Norflaxacin	Tetracycline Chloramphenicol Norflaxacin	Tetracycline	Chloramphenicol Norflaxacin	Tetracycline Chloramphenicol	Chloramphenicol	Chloramphenicol	Norflaxacin Chloramphenicol	Chloramphenicol Norflaxacin	-

	Bacterium									
	Enterococcus sacuolytic	***Enterococcus casselfarius***	***Enterococae***	***Enterococcus aquarius***	***Entercocus italicium***	***Enterococcus lactin***	***Enterococcus thailandicus***	***Lactococus garueae***	***Lynsibacillus fusiforms***	***Enterococcus sp.***
Antibiotics that the bacteria are susceptible to:	Chloramphenicol	-	-	-	Sulphonamide	-	Chloramphenicol	Tetracycline Chloramphenicol	Chloramphenicol	Tetracycline, Chloramphenicol Sulphonamide Norflaxacin

Table 4.35 presents the count of occurrences of instances where the bacteria were intermediate to antibiotics.

	Clostridium sordeli	***Bacilus cerues***	***Lysinbacilus***	***Enterococcus dispare***	***Enterococcus feacium***	***Enterococcus gallinarum***	***Enterococcus canis***	***Bacterium NLAE***	***Enterococcus feacalis***	***Enterococcus***	***Enterococcus sacuolytic***	***Enterococcus casselfarius***	***Enterococcae***	***Enterococcus aquarius***	***Entercocus italicium***	***Enterococcus lactin***	***Enterococcus thailandicus***	***Lactococcus aarueae***	***Lynsibacillus fusiforms***	***Enterococcus sp.***
Tetracycline	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1
Chloramphenicol	0	1	0	1	1	1	1	1	1	0	1	0	0	0	0	0	1	1	1	1
Ciprofloxacin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sulphonamide	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1
Norflaxacin	1	1	0	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1

Figure 4.31: Percentage of bacterial isolates intermediate in each type of antibiotic



CHAPTER FIVE

DISCUSSION

The aim of this study was to assess antibiotic residues in fresh fish sold in different outlets and identify possible health risks for consumers. To achieve this, three methods (ELISA, TLC and HPLC) were used to analyze antibiotic residues in fish samples. In addition, microbial contamination and antibiotic resistance were assessed using biochemical and molecular methods (PCR).

5.1 ANALYSIS OF ANTIMICROBIAL RESIDUES

ELISA test was performed as a qualitative screening test for tetracycline, chloramphenicol, nitrofuran, quinolone and sulphonamide in fish samples. All the samples collected were observed through ELISA, TLC and HPLC for quantification. The data in this study showed that all antimicrobials investigated (tetracycline, chloramphenicol, nitrofuran, quinolone and sulphonamide), were detectable in all fish samples sold in supermarkets around Mafikeng, North West Province. HPLC analysis showed antimicrobials in all the samples except chloramphenicol which was above the MRL set by EU/ RSA regulation as well as Codex for human consumption. However, all samples from HPLC were below the MRLs, the reason might be that the test is not equally sensitive to all types of antibiotics and some antibiotics are better detected than others hence antimicrobial Chloramphenicol was detected in higher level as compared to the MRL set by the European Union, South Africa's regulations governing the maximum limits for veterinary medicine and stock remedy residues as well as Codex Alimentarius Commission for human consumption safety.

In the current study, different types of fish samples (salt/sea and fresh water fish) were examined for antimicrobial residues. Overall, the results revealed that 84, 84, 96, 12 and 20% of samples with concentrations ranging between 0 and 2240 (398 µg/kg), 0 and 120 (22.19 µg/kg), 0.3 and 9.7 (40.44 µg/kg), 0 and 30 (3.92 µg/kg) and 0 and 4840 (259.96 µg/kg) respectively for tetracycline, chloramphenicol, sulphonamide, quinolone and nitrofuran were detectable in all fish samples as shown in Table 4.1. It was also noted that among the positive samples, 54%, 84%, 6%, 4% and 10% respectively for tetracycline, chloramphenicol, sulphonamide, quinolone and nitrofuran, were found to be above the Codex/ RSA-MRL, except for Nitrofuran that was not detected using ELISA. The results in this study are in agreement with the findings obtained by Rezk *et al.* (2015). In their study, they detected quinolones in fish tissues (±5.67, ±0.24, ± 20.34

and ±0.92 ng/g) and the samples were below the MRL. Quinolone (ciprofloxacin) is a broad-spectrum antibiotic known to be every effective in fighting several diseases in animal husbandry and aquaculture and are used extensively worldwide (Tsai *et al.*, 2012).

With regard to salt/sea water fish samples, the results revealed that 29 (85%), 29 (85%), 32 (94) %, 8 (24%), 6 (18%) contained tetracycline, chloramphenicol, nitrofuran, quinolone and sulphonamide as shown in Table 4.2 respectively. Moreover, with fresh water fish samples; 13 (81%), 13 (81%), 16 (100%), 2 (12%) was detected for tetracycline, chloramphenicol, quinolone and sulphonamide, except for nitrofuran, which was not detected using ELISA respectively (Table 4.3).

In this study, 60% of chloramphenicol was detected in all the 50 samples as shown in Table 4.4. Ciprofloxacin residues were detected while 76% of samples were positive. Doxycycline and tetracycline were both 74% positive, using TLC methods. Meanwhile, sulphonamide residues were detected in 88% of samples using TLC in all fish samples collected.

Despite the detection of all antimicrobial residues in salt/sea and fresh water fish samples, Tetracycline was among the antimicrobials detected and was not found to be above the RSA-MRL. The findings of this study agree with the report of a study conducted in South Africa by Eager Saran & Van Vuuren (2012), who found that tetracycline is among the largest group of antimicrobials used in animals. Tetracycline's are commonly used in animals due to their broad spectrum of activity as well as their low cost compared to other antibiotics (Granados-Chinchilla1 & Rodríguez2, 2016) for swine, poultry, and livestock and in aquaculture. Several factors that contribute to the usage of antibiotic residues in animals are due to the additives in foods fed to animals in order to promote growth and for the prevention of diseases to improve their health status (Granados-Chinchilla1 & Rodríguez2, 2016).

Buschmann *et al.* (2012) also maintain that antimicrobial agents are administered to fish with food and that undigested fish faeces containing antimicrobials are secreted in water and sediments of the environment, remaining in the aquatic environments for a variable period depending on their initial concentration, biodegradability, physical and chemical characteristics. In addition, Dasenaki & Thomaidis (2015) detected various antimicrobials in a study on multi-residues determination of 115 veterinary drugs and pharmaceutical residues in milk powder, butter, fish tissues and eggs using liquid chromatography-tandem mass spectrometry. This agrees with the statement reported by Ge *et al.* (2010) who confirmed that 75% of veterinary medicine used in

aquaculture could be lost to the surrounding environment due to some loss mechanism, which include dispersal of non-ingested pellets, gill and renal excretion of unprocessed drugs, renal and faecal excretion of drug metabolites.

The results of this study are not in agreement with those obtained by Babu & Ozbay (2013). These researchers conducted a study on the screening of imported Tilapia fillets for heavy metals and veterinary drug residues in the mid-Atlantic region, USA (which is the most consumed seafood in USA). Chloramphenicol residues were not detected in their study that made use of a comparative ELISA test. In the current study, chloramphenicol was detected using ELISA, as shown in Table 4.1. The reasons for high levels of chloramphenicol observed in this study might be due to reason that it was given in high doses, the withdrawal periods were not adhered to and / or the methods were light sensitive for detection and quantification. Linearity was also evaluated through the correlation coefficient close to one as shown in Figure 4.2. The best results in this study were achieved with the simplest extraction protocol as previously described in the methodology.

HPLC is one of the commonly used techniques for determining antibiotic residues in fish (Snyder *et al.*, 2009). In previous studies, different wavelengths ranges were used to detect antimicrobial residues.

In the current study, analysis of quinolone and ciprofloxacin residues in food animals is very essential for public health implication even though their use in animals is authorized (Dorival-García *et al.*, 2015). In this study, Quinolone was not detected in high percentages using ELISA as shown in Tables 4.1, 4.2 and 4.3. It was found to be prevalent when analyzed using TLC, with detection percentage at 76% as shown in Table 4.4.

Aquaculture is a growing sector in South Africa. The import and export of aquaculture products are combined with capture production and traded as fish and aquatic invertebrates (DAFF, 2013). Most of South Africa's fish and aquatic invertebrates sold in supermarkets are imported from India, which commands the greatest shares (17%) of South Africa's fish and aquatic invertebrate's imports market, followed by New Zealand with 15%, China with 10% and Norway with 9%. It is also important to mention that the four countries constitute 51% shares of South Africa's imports while the other countries share the remaining 49% of South Africa's fish and aquatic invertebrates imports (DAFF, 2013). Furthermore, South Africa's fish and aquatic invertebrates are exported to Italy (18%), followed by Spain (16%), Hong Kong (11%), Israel, Australia and Japan with a share of 9%, 7% and 6% respectively (DAFF, 2013).

Ciprofloxacin and enrofloxacin are the mostly used quinolones in hospitals and livestock, while enrofloxacin is the most used drug in veterinary medicine (Dorival-García *et al.*, 2015). Their main use is in the treatment of human and veterinary diseases, and the prevention of diseases producing animals, it has resulted in the development of ciprofloxacin-resistant *Salmonella, Campylobacter* and *E. coli,* which have caused human infections that prove difficult to treat (WHO, 2011).

In this study, doxycycline was below the MRL, and it was also detected at 42% with a mean of 0.24 µg/kg at a range of 0.68 – 7 µg/kg (Table 4.5). The presence of this antibiotic in fish might be explained by the fact that it is commonly used in cattle, pigs, poultry, turkey, fish and pets for the treatment of bacterial infections but not used in lactating animals and layers (EC, 2010). The use of several antibiotics such as sulphonamides, quinolones, some macrolides and certain tetracycline's are widely used in the treatment of animals for food readily used for human consumption (Dorival-García et al., 2015). In South Africa, several agencies have launched different programs for antibiotic resistance surveillance to assess the actual risk for public health associated with the consumption of antimicrobial residues (Suleman & Meyer, 2012). Even though there are specific measures established for the control of antibiotic residues in foods, fraudulent or improper uses of veterinary drugs is still an on-going challenge that cannot be ruled out (Dorival-García *et al.*, 2015). Governments all over the world are intensifying their efforts to improve food safety in response to an increasing number of food safety problems and rising consumer concerns (WHO, 2011).

Every country in the world is required to test for the presence of antibiotics and other veterinary drug residues in foods of animal origin as stated in the literature (Suleman & Meyer, 2012). Regardless of poor health status in the country, South Africa has had surveillance programs for antibiotic resistance than any African country (Gelband & Duse, 2011). However, it has not yet fully translated available antimicrobial resistance surveillance data into policy (Suleman & Meyer, 2012). The current study is in agreement with the research conducted by Leilei *et al.* (2014) on the simultaneous determination of florfenicol, chloramphenicol and diclazuril in compound powder by RP-HPLC-UV method widely used in chicken, turkey, pigs and cattle for the treatment of *coccidiosis*. In fact, the presence of chloramphenicol has also been detected in shrimps imported from China and Vietnam, intended for human consumption (Takino *et al.*, 2003).

In this study, antibiotic residue levels were confirmed to be low for tetracycline, sulphonamide, quinolone-ciprofloxacin, nitrofuran and penicillin compared to the maximum residue levels described by Codex/RSA-MRLs, except for Chloramphenicol, which was detected above the Codex/RSA-MRL at concentration of 49.47 µg/kg (Table 4.5). The antimicrobial pattern for tetracycline, chloramphenicol, sulphonamide, quinolone, doxycycline, penicillin and nitrofuran are shown in Table 4.12. The findings of these antimicrobials in fish are in correlation with the study conducted by Samanidou *et al.* (2016), who also detected the same antimicrobials in shrimps and other fish at an LOD 0.5-0.8 µg/kg, LOQ 1-10 µg/kg, 0 µg/kg and 0 µg/kg for nitrofuran, sulphonamide and quinolone, tetracycline and chloramphenicol respectively.

There are a number of reasons why antimicrobials may occur in fish species as follows: not following recommended label directions or dosage (extra-label usage); not adhering to recommended withdrawal periods; excessively administering a large volume at a single injection site; use of drug-contaminated equipment, or failure to properly clean equipment used to mix or administer drugs; dosing, measuring, or mixing errors; allowing animals access to spilled chemicals or medicated feeds; animal effects - age, pregnancy, congenital, illness, allergies; chemical interactions between drugs; variations in water temperature for fish species; environmental contamination; and improper use of agricultural chemicals such as pesticides (CFIA, 2014).

Nitrofuran was detected in fish in this study, even though it has been banned for usage in livestock by different countries due to its relationship with the production of carcinogenic metabolites (César *et al.*, 2015). The researchers also detected nitrofuran in a study of chromatographic detection of nitrofuran in shrimps, poultry, eggs, milk, honey, fish and other meats using LC-MS/MS, while in this study, it was detected using an ELISA screening method.

In the current study, results obtained using ELISA (Table 4.1) revealed that some samples had tetracycline, chloramphenicol, sulphonamide and nitrofuran concentrations above the MRL set by international standards. This could be explained by the fact that some antibodies cannot distinguish structurally similar compounds and some kits can detect even as little as a g/kg per body weight of the analyte. Furthermore, these tests are sensitive enough, cheap and fast, though sometimes, they lack specificity resulting to false negative or false positive results (Granados-Chinchilla1 & Rodríguez, 2016). In addition, the determination of antimicrobial residues in aquatic products is done through sample treatment due to high protein and fat contents in the matrix, which

can interfere with analytic procedures (Bai *et al.*, 2012). Also, the use of different mobile phases can also contribute to differences in results obtained. The other reason could be that veterinary drugs or residues usually build up in the liver or kidney rather than other tissues. It has been noted that different residue levels can be found in different tissue positions such as site and route of administration (Doyle, 2006). Early slaughter can also result in the occurrence of residues in animal tissues (FDA, 2014).

Sulphonamide and quinolones in foods of animal origin are of major concern because they are harmful to the health of the consumer (Hanwen *et al.*, 2012). The results in this study are in agreement with those obtained by Tsai *et al.* (2012), who detected sample positive for sulphonamide and quinolone - ciprofloxacin in fish with recovery rates ranging between 80 and 172% in all fish samples using HPLC-Tandem mass spectrophotometer. Pietroń *et al.* (2013) also detected Sulphonamide in medicated feeding stuff using HPLC-UV. The most likely reason for unacceptable residues in fish might result from human management, such as improper usage, including extra-label or illegal drug applications (Beyene & Tesega, 2014). Yet, the most noticeable reason for unacceptable residues might be due to failure to keep to the withdrawal period, including using overdose and long acting drugs (Beyene & Tesega, 2014). Humans are usually exposed to methyl mercury through the consumption of fish such as stark, swordfish and tuna (Babu & Ozbay, 2013).

In this study, there was a correlation between the three methods used (ELISA, TLC and HPLC). The differences observed could be explained by the limit of detection (ELISA is sensitive, while HPLC is sensitive and specific). In the current study, with regard to Thin Layer Chromatography (TLC), samples were detected to be positive for antimicrobials and most of the samples were found to be above the MRLs compared to ELISA and HPLC. The differences obtained in this study and other research could probably be due to factors such as dilution, where the methods would only require dilution of extracts prior to its analysis. While other techniques are direct analysis, others require gas phase of compounds that are achieved by electro spray, implying more variation between samples (Gavilán *et al.*, 2015). It might have happened that the mobile phases were too low hence, they were not detected with UV. The other reason might be due to antimicrobial dispersions differences in solubility in organic solvents such as acetonitrile and methanol. Also, the usage of mobile phase reagents could also provide minimal impurities and better separation (Gavilán *et al.*, 2015).

Furthermore, the differences obtained in this study as well as other research revealed that they could be partially due to detection methods used in those studies and sample preparation, which are very crucial in food analysis (Granados-Chinchilla & Rodriguez2, 2016). The presence of antimicrobial residues in fish might be justified by close proximity to the coast of South Africa (Some fish consumed in SA originates) and somehow the presence of pharmaceutical in marine environments induced by sewage effluent, which is recognized as a major source of multiple pharmaceutical, including metabolites entering aquatic environments (Gaw *et al.*, 2017). Sewage can also be discharged from hospitals and some waste water from pharmaceutical manufacturing companies (Gaw *et al.*, 2017). Also, boats, ships, including cruise liners, are a source of water contamination (Gaw *et al.*, 2017). The increase in the use of antimicrobial drugs to keep fish production as aquaculture continues to grow worldwide and the demand for fish keeps on increasing, thus a justification of the presence of these fish (Leilei *et al.*, 2014). In Asia, production of seafood has increased at about 90% (Leilei *et al.*, 2014). The use of antibiotics in aquaculture environments varies greatly between countries. In addition, the contamination could be explained by the fact that some fish farms are pond-based, located in coastal waters through leaks and discharges of waste water containing high concentration of pharmaceuticals (Leilei *et al.*, 2014).

Administration of several drugs in a short period can play a role in the elimination of the drug from the body, due to the reserve of hepatic enzymes essential for drug metabolism (Hou & Poole, 1971). Also, in intensive farming systems, where antibiotics are administered in drinking water or medicated feed, carry-over can result in the presence of residues in fishing products (FDA, 2014).

The difference obtained in this study between salt/sea water and fresh water fish is because in salt water fish, there is an increased concentration of antibiotics due to proximity to waste water treatment plants and high effluent outflow (Gaw *et al.*, 2017). The size of fish kept in areas, the population therewith and the number of rivers discharging into coastal waters, the type of waste water treatment and the type, scale and density of animal husbandry, proximity to aquaculture, hydrodynamic flushing and residence time for confined water bodies and the re-suspension of sediments during weather events, including monsoons and during incoming tides, differ with those of fresh water fish, which accumulate methyl mercury that live in contaminated lakes (Leilei *et al.*, 2014). The difference in insufficient good sanitary care during animal or product transportation, including the cross contamination of animal feeding stuffs with inadvertently

applied drugs, environmental and animal-to-animal transfer of drugs may also have an impact on how residues occur between sea and fresh water fish (Beyene & Tesega, 2014).

Difference in physio-chemical conditions between fresh and seawater fish includes salinity, pH and organic matter, which affect the environmental fate of pharmaceuticals. Ionized pharmaceuticals may be altered by increased pH of seawater. Photo degradation may be a less important removal mechanism in coastal water compared with shallower freshwater environments due to light attenuation. Indirect photo-degradation differs due to differences in water composition (Ge *et al.,* 2010) and some pharmaceuticals are more stable in seawater than fresh water (Choong *et al.,* 2006). Also, the differences come in transpiration behaviors.

Worldwide, the growing consumption of fish and its by-products, due to its nutritional value, has led to an increase in reports of adverse reactions to fish. These reactions to fish are not only refereed by the immune system causing allergies, but also caused by various toxins and parasites (Michael *et al.,* 2014). Fish allergy varies according to geographical eating habits, type of fish processing, and fish species exposure. The main fish allergen characterized is parvalbumin in addition to several less well-known allergens (Michael *et al.,* 2014). Consumption of fast foods and street foods were influenced by several socio-demographic factors, including ownership of major home appliances (Steyn *et al*., 2011).

Boothe & Reevers (2012) state that the disease status of an animal can affect the pharmacokinetics of drugs administered, which can influence the potential for residues. Pharmacokinetics is defined as the movement of drug into, through and out of the body: the time course of its absorption, bioavailability, distribution, metabolism and excretion. Drug absorption plays an important role in the passage of a compound from its site of administration into the bloodstream (Boothe & Reevers, 2012). It is influenced by many factors such as the properties of the cell membrane, drug properties and route of administration as well as physio-pathological state of the animal. The results in this study revealed that among the fish samples analysed, some were positive to antimicrobial residues while others had concentrations. The ELISA test showed the highest values compared to TLC and HPLC. Even so, the levels of antimicrobials detected by TLC and HPLC were lower than those of ELISA. This is because ELISA is used for screening purposes for single signal for first identification and detection of the presence of several analytes in large number of samples. Samples detected positive for TLC were also detected positive using HPLC but not above the MRL set by the Codex/ RSA and the European Union.

5.2 MICROBIOLOGICAL ANALYSIS OF FISH SAMPLES

Molecular analysis based on 16rDNA revealed a high frequency of occurrence of pathogenic bacteria such as *E. faecium* (19.44%), *Enterococcus* (13.89%), *Bacillus cereus* (11.11%), *Clostridium sordeli* (5.36%), while another bacterium such as *Lynisibacillus, E. dispare, E gallinarum, E. canis, Bacterium NLAE, E. faecalis, E sacrolytic, E. casselfarius, E. aquarius, E. italicium, E. lactin, E. thailandicus, E. gaiueae and Lynisibacillus fusiform* consist only of 2.78 % as shown in Figure 4.23.

The results in the current study correlate with the findings of a study conducted in Saudi Arabia, Eastern Province by Elhadi *et al.* (2016), who detected *Bacillus cereus* at 9.4% in imported fish samples. Sandra *et al.* (2012) also state that milk, dairy products, fatty foods, bread, cakes and sea foods can easily be contaminated with *B. cereus*. It has also been reported that food-borne pathogens in food plays a significant role in the prevention of food-borne pathogens and their transmission (Elhadi *et al.*, 2016).

The presence of the isolated *Enterococcus* species in investigated fish samples in the current study could be explained by the report of Prichula *et al.* (2016), who found *Enterococcus spp.* isolated from faecal samples of wild marine species in the Southern coast of Brazil. *Enterococcus spp*. being found in faecal and during cross contamination when human is handling food without proper hyiene measures during food processing (Okocha, 2018). Their isolation in fish can be explained by the exposure to sewage waters as said before or by farmers or faecal materials as source of feed for pond production systems (Cabral, 2010). These food-borne pathogens are an important cause of morbidity and mortality, and a significant obstruction to socio-economic development worldwide. However, the full extent and burden of unsafe food, and especially the burden arising from chemical and parasitic contaminants, is unknown (WHO, 2015).

In this study, *Enterococcus feacium* was isolated in different fish samples purchased in different supermarkets at varying percentages. High occurrence of *Enterococcus feacium, Bacillus cereus* and *Clostridium sordeli* in this study revealed that isolates are toxigenic and can cause food poisoning (Sandra *et al.*, 2012). These microorganisms could be due to improper food preparations, transportation and handling as well as exposure to temperatures of 30°C. *Clostridium sordeli* strains are known to produce exotoxins and broad spectrum of human diseases and may also cause lethal infections in several animals (Reddy *et al.*, 2013). Consumers use different practices to

handle their food to potentially explain differences in food-borne illnesses in different consumption of raw food, poor hygiene and cross-contamination (Henley *et al.*, 2012).

In this study, *E. coli*, *salmonella spp.*, *Listeria* and *Vibrio spp.* pathogens were not isolated even though they are known to cause the highest pathogenic incidences in fishery products (Elhadi *et al.*, 2016). Other studies (Carvalho *et al.*, 2016; Sajee & Karim, 2016) have isolated bacteria such as *Vibrio spp.* in fish. However, this study correlates with results reported by Herera *et al.* (2006), who did not detect any *Vibrio spp.* strains in fish samples. Stress in fish species is associated with the development of diseases, often found to be caused by opportunistic bacteria of the genus *Vibrio* (Nogueira-Lima *et al.*, 2006), pathogens of bacterial origin have been registered in aquatic organisms.

Salmonella is one of the pathogenic bacteria of the family *Enterobacteriaceae* (Herera *et al.*, 2006). The incidence of *salmonella* infections due to the consumption of sea food is still low compared to *salmonellosi*s associated with other foods. However, the occurrence of *salmonella spp.* in seafood cannot be skipped as it is responsible for most food-borne diseases or gastro enteritis characterized by diarrheal, abdominal pains, vomiting, nausea and fever. According to the Centre for Diseases Control and Prevention, approximately 14 million illnesses, 15000 hospitals and 400 deaths were discovered in the USA (Elhadi *et al.*, 2016).

Bacillus cereus isolates were also isolated in some ready-to-eat cooked rice in Malaysia (Sandra *et al.*, 2012). The presence of *Enterococcus spp.* and *Bacillus cereus* is an indication that contamination may result from inappropriate handling and processing practices. The primary responsibility for proper setting out of the necessary hygiene condition for production of foods that are safe and suitable for consumption was always directed by legislation accepted by manufacturers. *Bacillus cereus* strains are known to be widely distributed in the environment and their spores are resistant to drying and could easily spread with dust (Kasozi *et al.*, 2016). Researchers have found that individuals with higher levels of education, who have a strong positive correlation with high income (Merkin *et al.*, 2007, Younus *et al.*, 2007), are more likely to eat raw clams, raw oysters, raw fish, raw sprouts and pink hamburger, besides having unsafe hand and cutting board washing practices (Henley *et al.*, 2012). Additionally, available fish processing practices expose the fish to different kinds of microbial and chemical degradation (Kasozi *et al.*, 2016).

In this study, isolated *E. feacium and E. faecalis* strains agree with research conducted by Byppanahalli *et al.* (2012), who found that *E. feacium and E. faecalis* are commensal bacteria that are potentially helpful in digestion and other gut metabolic pathways. Also, they are used in probiotics to treat diarrhoea and improve host immunity. They are important causes of nosocomial infections, including urinary tract infections, endocarditis, bacteremia, abdominal and pelvic infections (Byappanahalli *et al.*, 2012).

Food contamination by different microorganisms isolated in this study could be due to fish processing materials (storage, transportation and water). Like many food items, fish has the ability to cause diseases from bacterial pathogens under certain circumstances (Sandra *et al.*, 2012). The presence of *Enterococcus* and *E. coli* in fish may result from the deposition of human and animal excreta as well as other environmental wastes into ponds and rivers that harbor fish or through washing of land surfaces into water bodies during the rainy season (Cabral, 2010b). In addition, it is important to indicate that *Enterococcus* contamination could lead to fish diseases depending on the type of fish species. The presence of multiple bacteria from fish and fish handlers poses not only a risk of disease to the fish but public health hazard to fish handlers and consumers in general (Grema *et al.*, 2015).

Seafood safety, which varies according to products, is influenced by a number of factors such as fish origin, product characteristics, handling and processing and preparation before consumption (Herera *et al.*, 2006). Moreover, other factors include poor environmental conditions, secondary infections resulting from fish having another disease, poor nutrition status of fish species, injury where there is in an open wound, stress associated factors, weaknesses, poor water parameters such as salt, pH and acid, salinity, hardness, nitrite-mercury and unbalanced lead and introduction into aquarium by means of a contaminated water source (fish eating flesh of another infected fish). In some instances, when fish skin or tissue gets damaged, whether from shipping, wetting, or any other circumstances, bacterial pathogens seize the opportunity to infect the fish. This could be caused by release of bacteria from sewage, biofilms that pass a high number of antibiotics resistance bacteria and some factors that contribute to bacteria in fish (DAFF, 2016).

5.3 ANALYSIS OF ANTIBIOTIC SUSCEPTIBILITY

In this study, fish samples examined were purchased from different supermarkets around Mafikeng, North West Province. Several bacteria were isolated. The susceptibility of these bacteria was studied to assess the risk for consumers as well as for fish. The aim of the study was also to assess if antibiotics used have any effects on isolated bacteria. Resistance, intermediate and susceptible categories were identified for bacterium towards an antibiotic.

The results obtained (Figure 4.27) in the current study revealed that most isolates were resistant to tetracycline (33%), norfloxacin (39%), followed by chloramphenicol (14%) and sulphonamide (17%), while only a few isolates were resistant to ciprofloxacin (3%). The results obtained in this study are in agreement with the findings of a study conducted by Hedayatianfard *et al.* (2014), who also identified high resistance bacteria to tetracycline. Also, Ghosh & Mandal (2010) found isolates resistant to chloramphenicol antibiotic in consumable fish from Dighi coast, West Bengal, India.

It has been reported in other studies that resistance to antibiotics is acquired into two ways as follows: chromosol mutation; or acquisition of plasmid. Chromosol mutation is not transferred very rapidly, producing high percentages of pathogenic bacteria that develop mediated plasmid resistance in a short period of time. There is some evidence which show that antibiotic resistance bacteria present in seabirds are of human origin (Gaw *et al.*, 2017).

In this study, some bacteria showed multiple resistance to different antibiotics (Tables 4.22, 4.24 and 4.26). Results obtained are in agreement with those of Cruz *et al.* (2012). In a study conducted in Ecuador, the researchers observed that multidrug resistance occurred in Ecuador during a cholera epizootic that happened between 1991 and 1994 from shrimp farm workers, where the use on probiotics in aquaculture microorganisms was beneficial to the host. The results in this study are in agreement with those reported by Grema *et al.* (2015), who also found that most bacteria isolated from fish samples showed resistance to tetracycline and norfloxacin antibiotics. Tetracycline is an antibiotic known to be active against mycoplasma, chlamydia, pasteurella, ornithodorus bacterium rhinotracheala and some protozoa (Granados-Chinchilla1 & Rodríguez2, 2016), rickettsia, spirochete and some large virus such as the lymph granuloma group (Samanidou & Evaggelopoullu, 2007).

It is also important to indicate that antibiotic resistance, as noted in this study, could be explained by the continuous and incorrect use of these antibiotics. This has been confirmed by

Granados-Chinchilla1 & Rodríguez, (2016), who noted that the continuous use of Tetracycline promotes bacterial resistance, allergic reactions in human and animals and also changes in bacterial population among other detrimental effects.

The presence of *Bacillus* bacterium in the fish samples analyzed in the current study is in agreement with the study by Grema *et al.* (2015) on multidrug resistant bacteria isolated from fish and fish handlers in Maiduguri, Nigeria. The above findings further contribute to the theory that resistant gene profiles can be a further characteristic of different regions of the world (Chen, 2012).

In this study, isolates were multidrug resistant, which means that most isolated strains of bacteria were resistant to at least 4 antimicrobial agents. Resistance to even as low as 2 antibiotics of different classes be regarded as multidrug resistant (Harnish *et al*., 2015). Widespread antibiotic resistance has also been reported in fish marine mammals and seabirds living in coastal waters, including North Eastern United States (Rose *et al.,* 2009).

In the present study, isolates such as *D. aureus, streptococcus, E. coli, Klebsiella, proteus* and *brucella* which are known to be the most prevalent in food of animal origins. These strains can easily be carried on the skin, skin glands, and mucus membrane of humans and animals as normal micro flora (Grema *et al.,* 2015), causing infections such as rashes, inflammation of bones and the mingles as well as septicemia not isolated in this study. *E. coli, S. aureus* and *Klebsiella* have been found to survive and multiply in the gut and tissues of fish, which render fish a potential source of human disease over a long period (Udeze *et al.*, 2012).

However, from the 20 isolated strains of bacterium, *E. canis, E. faecalis, enterococcus, Enterococcus cassaflavis, E. thailandicus* and *Lysinibacillus fusiform* (Figure 4.23) were not resistant to any of the antibiotics analysed. However, the presence of isolates in fish samples could result from indiscriminate deposition of human and animal excreta as well as other environmental wastes into ponds and rivers that harbor fish or through washing of land surfaces into water bodies during rainy seasons (Cabral, 2010a).

In addition, these results provide evidence that there is an increase in the emergence of antibiotic resistance from bacterial isolates of fish, which is indeed in agreement with a report by Albuquerque *et al.* (2007). Although the use of antibiotics in human medicine has influenced the emergence of resistant bacteria, the use of antibiotics in animals has contributed to the problem of resistance and complicate the choice of treatment in human diseases (Grema *et al.*, 2015),

especially due to the fact that the transfer of resistant bacteria between aquatic animals and humans through food production line has been documented and could pose a threat to public health.

A multidrug resistance bacterium in this study is also in agreement with the study conducted by Smaldone *et al.* (2014), on the occurrence of antibiotic resistance bacteria isolated from sea water organisms caught in Campaign region. This shows that the presence of multidrug bacteria in fish does not only pose a risk of diseases to fish but is a public health hazard to consumers in general.

CHAPTER SIX

CONCLUSSION

The primary objective of this study was to sample fish from retail supermarkets in Mafikeng, capital city of the North West Province, to provide baseline data on the incidence and prevalence of various drug residues, including tetracycline, sulphonamide, chloramphenicol, nitro-furan, quinolones, ciprofloxacin, doxycycline and penicillin, and to identify which method is the best suited for detecting antimicrobial residues among ELISA, TLC and HPLC.

As expected, ELISA assay used for detecting antimicrobials, possesses the virtues of high specificity and sensitivity. Simply put, rapid extraction methods of samples were obtained for determination according to each antibiotic. The accuracy and precision of ELISA was validated through HPLC and revealed its reliability with good correlations with linearity that is close to one value.

The HPLC is a rapid, precise and sensitive method for multi-residue determination for fish. All samples confirmed using HPLC for tetracycline, doxycycline, chloramphenicol and penicillin. Sulphonamide detected in fish samples was lower than international MRL standards. No samples were above MRL as prescribed by South Africa and the European Union in terms of the maximum limits for veterinary medicine and stock remedy residues and the Codex Alimentations Commission. However, there is an increasing incidence that pharmaceuticals are present and are impacting on marine and coastal environments (Gaw *et al.*, 2017). Marine risk assessments for pharmaceuticals are also relevant to veterinary medicine used in aquaculture. These results are, therefore, valid for the samples obtained in this study. Proper antibiotic monitoring and assessment guarantees that the correct doses and antibiotics are administered. Hence, guidelines based on antibiotics help in reducing antibiotic consumption in terms of its residues and bacterial resistance. Accurate and reliable analytic methods must be implemented to collect exact and precise qualitative data.

A conventional screening method is divided into analytical methods, microbial inhibitory assay-enzyme tests and immunological tests. Analytic methods are usually superior in specificity, selectivity and sensitivity. However, they tend to use expensive equipment that are not always available in every laboratory. Inhibition assays are relatively fast and cost-effective and may be

utilized as a screening test for first identification of analytes but a complete confirmation has to be performed thereafter.

The focus in this study was on safety consideration of fish for consumption by local citizens purchasing them at local supermarkets and to serve as good baseline information for the public. It was also to demonstrate the need to thoroughly monitor the quality of seafood in Mafikeng.

The presence of antibiotics in fish is explained by an increased aquaculture practice and has resulted in increased levels of infections among fish species. Various classes of antibiotics, including quinolone, tetracycline, b-lactams, sulphonamide and chloramphenicol, exhibit activity against both gram negative and gram-positive bacteria. Thus, they are widely used in aquaculture for treatments and prevention of diseases, which necessitate the demand for developing sensitive methods (Samanidou *et al.*, 2016).

The presence of antimicrobial and food pathogens in this study may indicate that some populations are exposed to poorer microbiological quality/safety in foods in Mafikeng area but also in the country as most of the fish is supplied country-wide. These findings are essential to identify one significant control point from fish production to fork range that may lead to different rates of illnesses. The usage of illegal antimicrobials in aquaculture is largely unregulated and undocumented all over the world, although it results in consumer exposure to residues and contributes to selection of resistant bacteria. The current study provides an assessment of antimicrobial residues detected in aquaculture fish. Surveys of larger samples of fish are needed to achieve more reliable consumer risk assessments. Consumer protection policies aimed at reducing drug residues in aquaculture products must, therefore, involve the entire supply chain through wider and more frequent monitoring of these antimicrobials. Secure, effective observation is thus, essential.

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Appendix 1

Guideline of results of susceptibility test

Table 36: Guideline of antibiotic resistances according to the Clinical Laboratory Institute CLSI (2011)

Antibiotics	Abbreviation	Antibiotic concentration (Disc/ mg)	Zone Diameter, Breakpoints Nearest whole Mm		
			Susceptible S	Intermediate I	Resistant R
Ampicillin	AMP10	(10 mg)	≥ 29	-	≤ 28
Gentamicin	CN10	(10 mg)	≥ 15	13-14	≤ 12
Tetracycline	TE30	(30 mg)	≥ 19	15-18	≤ 14
Sulphonamides	S3 300	(300 mg)	≥ 17	13-16	≤ 12
Streptomycin	S 300	(10 mg)	≥ 15	12-14	≤ 11
Ciprofloxacin	CIP5	(5 mg)	≥ 21	16-20	≤ 15
Chloramphenicol	C30	(30 mg)	≥ 18	13-17	≤ 12
Erythromycin	E5	(5 mg)	≥ 23	14-22	≤ 13

Table 37: Results of anti-bio gram for fish samples in mm

SI	Bacterium found	Tetracycline	Chloramphenicol	Ciprofloxacin	Nitrofuran	Sulphonamide
1	*Clostridium sordeli*	19mm	22mm	28mm	20mm	0mm
2	*Bacillus cereus*	17mm	22mm	23mm	19mm	18mm
3	*Lynsibacilus*	18mm	6mm	24mm	43mm	28mm
4	*Enterococcus spp*	12mm	18mm	25mm	19mm	23mm
5	*Enterococcus sp.*	17mm	24mm	21mm	30mm	13mm
6	*Enterococcus dispare*	12mm	16mm	24mm	15mm	10mm
8	*Enterococcus feaciun*	14mm	30mm	29mm	0mm	0mm
9	*Enterococcus gallinarum*	12mm	17mm	29mm	13mm	19mm
10	*Enterococcus canis*	21mm	17mm	30mm	23mm	24mm
11	*Bacillus cereus*	17mm	19mm	20mm	25mm	27mm
12	*Clostridium sordeli*	21mm	21mm	28mm	17mm	15mm
13	*Bacillus cereus*	14mm	19mm	26mm	19mm	21mm

14	*Bacterium NLAE*	13mm	14mm	22mm	20mm	17mm
16	*Enterococcus faecalis*	22mm	22mm	22mm	24mm	25mm
17	*Enterococcus*	21mm	20mm	23mm	17mm	19mm
19	*Enterococcus sacuolytic*	24mm	10mm	25mm	40mm	22mm
20	*Enterococcus casselfarius*	20mm	19mm	25mm	23mm	36mm
21	*Enterococcus dispar*	19mm	21mm	27mm	17mm	27mm
22	*Enterococcae*	20mm	22mm	34mm	14mm	14mm
24	*Enterococcus feacium*	15mm	25mm	29mm	13mm	14mm
25	*Enterococcus aquarius*	22mm	21mm	26mm	10mm	9mm
26	*Bacterium NLAE*	13mm	23mm	29mm	21mm	22mm
30	*Enterococcus faecium*	20mm	20mm	27mm	12mm	18mm
31	*Enterococcus italicium*	23mm	0mm	0mm	13mm	13mm
32	*Enterococcus lactin*	11mm	25mm	35mm	22mm	20mm
33	*Enterococcus faecium*	13mm	0mm	29mm	10mm	19mm
34	*Bacillus cereus*	17mm	21mm	24mm	20mm	14mm
35	*Enterococcus faecium*	19mm	16mm	23mm	0mm	26mm
37	*Enterococcus faecium*	10mm	18mm	30mm	10mm	30mm
40	*Enterococcus spp*	17mm	17mm	33mm	14mm	27mm
41	*Enterococcus thailandicus*	21mm	18mm	22mm	39mm	36mm
42	*Lactococcus garueae*	17mm	17mm	29mm	11mm	19mm
43	*Enterococcus faecium*	20mm	19mm	24mm	0mm	12mm
44	*Lynsibacillus fusiform*	28mm	20mm	24mm	17mm	29mm
46	*Enterococcus spp*	2mm	18mm	25mm	13mm	9mm
48	*Enterococcus spp*	12mm	21mm	34mm	15mm	23mm

SI = Sample identification