AMERICAN UNIVERSITY OF BEIRUT

UPTAKE OF GENTAMICIN, TYLOSIN AND OXYTETRACYCLINE BY LETTUCE AND RADISH PLANTS

by

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AN ABSTRACT OF THE THESIS OF

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Antibiotics are extensively being administered to livestock to promote growth and reduce illness. Studies have shown that antibiotics may be present in manure as the parent compound or their metabolites. Manure is used as plant fertilizer and a source of quality enhancement consequently affecting the environment. Research articles point out the potential human health risks and increased microbial resistance associated with the consumption of fresh vegetables grown in soil amended with antibiotic rich manure. In Lebanon, gentamicin, oxytetracycline, and tylosin are widely used in animal production. The aim of this study is to examine the ability of plant (lettuce and radish) in absorbing these antibiotics at four concentrations (0, 2.5, 5, and 10 mg/kg) from two growth media (manure amended soils and soil without manure) and investigate in their accumulation sites. A factorial pot experiment was conducted at the greenhouse of the American University of Beirut. The antibiotic analysis was accomplished using the enzyme linked immunosorbent assay (ELISA). The results showed that gentamicin accumulated in lettuce and radish leaves and roots. Tylosin accumulated in lettuce and radish roots but not in the lettuce leaves. Moreover, oxytetracycline was not absorbed by lettuce but it accumulated in radish roots. Among the three antibiotics gentamicin was the only antibiotic that was translocated to the lettuce leaves whereas the three tested antibiotics accumulated in the radish roots (edible part). In addition, manure enhanced the uptake of the three antibiotics by lettuce and radish. The obtained results indicated also, that increasing the concentration of the antibiotic in the growing media did not lead to a significant increase in the accumulation levels of antibiotics in plant tissues.

Keywords: Antibiotics, gentamicin, tylosin, oxytetracycline, greenhouse, pot experiment, radish, lettuce, Lebanon,

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ABBREVIATIONS

°C	Degree Celsius
µg	Microgram
Al^{3+}	Aluminum
AREC	American Research And Educational Center
AUB	American University of Beirut
Ca^{2+}	Calcium
CTC	Chlortetracycline
d	Day
ELISA	Enzyme Linked Immune Sorbent Assay
EU	The European Union
FDA	Food and Drug Administration
Fe^{3+}	Ferric
K_d	Distribution coefficient
kg	Kilogram
L	Liter
mg	Milligram
mg/Kg	Milligram per Kilogram
Mg^{2+}	Magnesium
ml	Milliliters

ng/g	Nanogram per gram
ppm	Parts per million
spp.	Species
Trt.	Treatment
UK	The United Kingdom
USA	United States Of America
WHO	World Health Organization
wk	Week
Zn^{2+}	Zinc

CHAPTER 1

INTRODUCTION

Infections are a common phenomenon accountable for a broad spectrum of diseases harmfully affecting human and animal health. Mostly, bacteria are the reason behind infectious diseases. Despite their risky effect on humans and animals, bacteria can be prohibited, managed and treated through antibacterial group of compounds known as antibiotics (Aminov, 2010).

Antibiotics were revealed in 1920's by the chemist Alexander Fleming. He perceived that some organisms, such as *Penicillium*, restrain the progression of bacteria, and that was the beginning of antibiotic age.

Their prevalent use as feed additives in livestock production has raised several apprehensions about not only the development of antibiotic resistant bacteria in the environment but also the appearance of antibiotics in food and water supplies. According to Kang et al. (2013) the main cause behind these effects is when manure holding antibiotic is applied to land.

In many countries around the world, including Lebanon, veterinary antibiotics are not only used to treat or prevent diseases but also to promote growth of animals.

The antibiotics used in human medicine belong to the same general classes as those used in animals, and in many cases even if they are not exactly the same compounds their mode of action is the similar (Phillips et al., 2004). It has been stated that once agricultural lands are fertilized by manure, crops become exposed to antibiotics because antibiotics tend to persist in soils from a few to several hundred days depending on the antibiotic compound, sorption interactions with soil, and environmental conditions (Dolliver et al., 2007). Thus, the major problem that would burst the occurrence of antibiotics in plants would be the probable consequence on the human health.

Several studies have confirmed that antibiotics can be excreted into the environment as parent compounds and/or their metabolites, since most antibiotics fed to animals are not fully absorbed in the animal's gut and consequently considerable amount of these substances are defecated in urine and feces, which in turn ends up in manure. It has been suggested that up to 90% of an administered dose of antibiotics may be excreted through urine and feces (Phillips et al., 2004; kumar et al., 2005).

In addition of their potential ecological effects, they can expand the generation and spread of drug resistance bacterial stocks (Baquero et al., 2011). The antibiotic dose varies from 3 to 220 grams per ton of feed depending on the type and size of the animal and the type of antibiotic (Kumar et al., 2005).

The continuous use of antibiotics in food animals selects for bacteria resistant to antibiotics used in humans, and these might transfer from food to humans and eventually cause human contamination. The hazard in all of this might seem minor but it might also lead to disadvantages to human and animal health. It is significant to be attentive of the fate of these antimicrobials and comprehend their toxicity to plants and their uptake and transport into plants. The accumulation may or not disrupt the growth and development of plants; however the uptake into plants may indicate a notable exposure pathway of these compounds to humans and other biota. Thus, as stated by Kong et al. (2006) there is a potential risk that plants are capable of spreading antibiotics from the soil into the food chain. Therefore, it is important to understand the potential impact of veterinary antibiotics in the environment and their uptake and accumulation in different plant tissues.

Thus, the objectives of this research were to evaluate:

1. The uptake of gentamicin, tylosin and oxytetracycline, three different antibiotics widely used in Lebanon, mainly in animal and poultry production, by lettuce and radish plants and their accumulation sites.
2. The effect of the level of the three antibiotics in soil on accumulation in lettuce and radish tissues.
3. The effect of manure on plant uptake of the three antibiotics gentamicin, tylosin and oxytetracycline from soil.

The work described herein was performed at the Department of Agricultural Sciences of the American University of Beirut (AUB)

Controlled greenhouse pot experiments were conducted by planting lettuce and radish in soils spiked with different levels of antibiotic. The measurement of antibiotic concentrations in shoots and roots of lettuce and radish was done by ELISA technique.

CHAPTER 2

LITERATURE REVIEW

A. Antibiotics

An antibiotic is a chemical produced by one microorganism and has the ability to harm other microorganisms. Antibiotics are one class of antimicrobials, a larger group which also includes anti-viral, anti-fungal, and anti-parasitic drugs.

According to Khan et al. (2008) antibiotics can be naturally occurring (from soil microorganisms such as bacteria or fungi), semi-synthetic, or synthetic chemical compounds. They may be classified as either broad spectrum or narrow spectrum, and bactericidal or bacteriostatic. Antibiotics may also be categorized according to their modes of action.

There are two types of antibiotics; bactericidal and bacteriostatic. Antibiotics that kill bacteria are called “bactericidal”. Whereas, antibiotics that stop the growth of bacteria i.e., keeps them in a stationary phase of growth are called “bacteriostatic” such as tetracycline group of antibiotic, sulfonamides, and macrolides. Bactericidal drugs are those that kill target organisms, generally by either interfering with the formation of the bacterium’s cell wall or its cell contents, examples include aminoglycosides, beta-Lactams, and fluoroquinolones. Pankey & Sabath (2004) stated that some antibiotics can be both bacteriostatic and bactericidal, depending on the dosage, period of exposure and the state of the invading bacteria. For example, aminoglycosides and fluoroquinolones exert concentration-dependent killing characteristics; their rate of killing increases as the drug concentration increases. Nevertheless, bacteriostatic and bactericidal drugs should not be mixed together or used at the same time since their properties cancel each other.

Most antibiotics are derived from natural sources and were then further chemically improved to generate better properties of the drug. They include different classes of antibiotics such as beta-lactam, tetracyclines, aminoglycosides, streptogramins, macrolides, glycopeptides, and lincosamides. Other antibiotics are man-made, originating completely from synthetic chemical practices such as sulfonamides, trimethoprim, and fluoroquinolones (Coates et al., 2011).

1. Classification of antibiotic according to spectrum of activity

The range of bacteria that an antibiotic upsets can be separated into two categories; narrow spectrum and broad spectrum (Walsh, 2003).

Broad spectrum antibiotics refer to an antibiotic that function against a wide variety of disease-causing bacteria. In other words, it acts against both Gram-negative and Gram-positive bacteria. Examples of broad spectrum antibiotics include tetracycline, aminoglycosides (not effective against anaerobic bacteria), 3^{rd} generation fluoroquinolones, Beta-lactams (2^{nd}, 3^{rd} and 4^{th} generation cephalosporins), and sulfonamides.

Narrow spectrum antibiotics tend to have a limited action against bacteria. They are effective against selective families of bacteria. In other words, they are either effective against Gram-negative or Gram-positive bacteria. Examples include; beta-lactams (penicillin, 1^{st} generation cephalosporins, monobactams), other fluoroquinolones, glycopeptides (against gram positive bacteria), macrolides, polymixin (effective against gram negative bacteria), and streptogramins (mainly Gram positive bacteria).

2. Classification of antibiotics according to mechanism of action

Antibiotics can also be separated according to their mechanism of action and their target sites in the bacterium (Kohanski et al., 2010). Table 1 lists some of the main antibiotics with different mode of action.

Table 1. List of some antibiotics with different mechanism of action

Antibiotic	**Mechanism of action**
Tetracycline, Macrolides, Aminoglycosides	Protein synthesis inhibitors
Beta-lactams	Cell wall synthesis inhibitors
Fluoroquinolones	Nucleic acid inhibitors
Isoniazid	Mycolic acid synthesis inhibitors
Sulfonamides	Folic acid synthesis inhibitors

Source: Kohanski et al., 2010

B. Veterinary Antibiotics

Veterinary antibiotics are antibiotics having disease-fighting and growth-promoting capabilities. Approximately half of all antibiotics manufactured are for human consumption and the other 50% are administered to livestock either to treat sick animals or used as growth promoters. In general, antimicrobials are used in everything from apples to aquaculture. As mentioned by Henderson & Coats (2010) veterinary antibiotics are feed additives of poultry, swine, cattle, equine, and aquaculture. The problem ascends when such practice results in the development of bacterial resistance in or near livestock.

Chemically speaking, most veterinary antibiotics are amphiphilic or amphoteric, ionizable organic compounds consisting of a nonpolar core and multiple polar functional groups (Thiele-Bruhn, 2003). These chemicals are generally divided into five groups; anthelmintic (de-wormers), tranquilizers, antibiotics, hormones and agonists.

Veterinary antibiotics serve a wide purpose of administration. They may be used therapeutically in animals for treating bacterial diseases, or used non-therapeutically as growth promoters, prophylaxis, and metaphylaxis treatments (Song & Guo, 2014). The non-therapeutic practice takes in long-term, low dose treatment through feed and water to whole flock or herds. Prophylactic practice of antibiotics is admitted to healthy animals in advance of expected exposure to disease whereas the metaphylactic practice is administered after an exposure to an infectious agent to prevent symptoms. Moreover, according to McEwen and Fedroka-Cray (2002) growth promoting antibiotics are often administered in relatively low concentrations, ranging from 2.5-125 mg/kg (ppm), depending on the drug and species treated.

1. Antibiotic use in livestock production

As mentioned previously antibiotics are used to treat diseases and promote growth in animals. It is understood that sub-therapeutic levels of antibiotics in feed (3-220 gram/ton feed) help animal nurture faster and decrease their vulnerability to stress-related diseases (Kumar et al., 2005b). However, animal feed hold antibiotics at concentrations higher than the recommended value leading to the occurrence of bacterial resistance. Kumar et al. (2005b) explained the mechanism of antibiotics in enhancing growth;

i) inhibition of subclinical infections,

ii) reduction in growth-depressing microbial metabolites,

iii) reduction in microbial use of nutrients,

iv) Enhancing uptake of nutrients through the thinner intestinal wall

Tables 2 and 3 are adopted from Regassa et al. (2009) showing a partial list of FDA- approved antibiotics used in the production of beef and cow-calf and poultry. The tables provide the name of antibiotic, use level, and treatment objectives.

Table 2. FDA-Approved commonly used antibiotics for therapeutic and sub-therapeutic purposes in beef and cow-calf production.

Drug	**level in feed (mg/head/day)**	**Treatment objective**
Bacitracin Zinc	35-70	Feed efficiency and growth
Bambermycin	1-5	Feed efficiency and growth
Chlortetracycline	350	Disease control
Monensin	25-400	Intensive feeding and weight gain
Oxytetracycline	75	Feed efficiency and growth
Oxytetracycline	75	Disease control
Oxytetracycline	0.1-5 mg/lb of body weight	Disease control
Tylosin	8-10	Disease control

Source: Modified and adopted from (Regassa et al., 2009)

Table 3. FDA-approved commonly used antibiotics for the therapeutic and sub-therapeutic use in poultry production

Drug	level in feed (g/ton)	Treatment objective
Arsanilic acid	75-120	Feed efficiency, growth, and pigmentation
Bacitracin	4-50	Feed efficiency and growth
Bambermycin	1-20	Feed efficiency and growth
Chlortetracycline	10-100	Feed efficiency, growth, and disease control
Oxytetracycline	5-50	Feed efficiency, growth, and disease control
Tylosin (Banned in EU)	4-50	Feed efficiency and growth

Source: Modified and adopted from (Regassa et al., 2009)

2. Antibiotic usage in the world

Antibiotic usage was previously used to treat infections causing death for humans and animals. Nowadays, they serve a much wider purpose in the livestock production, mainly as a feed additive enhancing growth. More than 400 active chemical ingredients have been produced into approximately 2,000 veterinary pharmaceutical products to treat various species of animals including pig, cattle, horses, sheep, goats, birds, fish, deer, cats and dogs (FDA, 2015).

Aquaculture is a booming industry around the world where large amount of antibiotics are being administered. Besides, Marshall and Levy (2011) clarified that these antibiotics not only encourage resistant bacteria in the farmed fish but also transmit resistance to wild fish populations and the broader environment.

Intensive animal farming implies considerable drug use. It is vital to stress on the fact that most antibiotics used in animal production are more or less comparable to those used in humans. The World Health Organization (2011) estimated that the top three classes by global sales for animal use in 2009 were macrolides ($600 million), penicillins ($600 million) and tetracyclines ($500 million) all of which are considered as critically important in human medicine.

Nonetheless, as stated by Van Boeckel et al. (2015), worldwide in 2010, at least 63,200 tons of antibiotics were mainly consumed by livestock, an amount much likely to be matched by human consumption. This figure is expected to increase by two-third reaching 105,600 tons to be able to meet the demand of a projected 8.5 billion human population in year 2030. The two-third increase is contributed to the increase in the number of food producing animals and to the shift from small scale to industrial scale production system to meet population demand.

One of the complications in assessing the use and effects of antibiotics in livestock is the lack of consistent data on global use. Due to the difficulty in collecting information on the total amount of veterinary antibiotic used in individual stock farms, most countries simply provided the amount sold as an estimate of the amount used. For example, at 11,148 tons/year, the USA was the biggest consumer of veterinary antibiotic followed by China at approximately 6,000 tons annually (Zhao et al., 2010).

In the year 2000, it has been stated that 897 tons of antibiotics were applied to animal production in the United Kingdom (Thiele-Bruhn, 2003). In Turkey; antimicrobial usage has been reported to be 33% of the total veterinary pharmaceutical consumptions (Karcı & Balcıoğlu, 2009). Kumar et al. (2005) stated that by the year 2005 the annual EU consumption of veterinary antibiotics was approximately 5,000 tons; this number is estimated to decrease after the EU ban of antibiotics usage as feed supplement in 2006.

The most commonly used antibiotics are tetracycline, tylosin, sulfonamides and fluoroquinolones (Xu et al., 2007). In EU, the mostly used antibiotics are tetracyclines followed by sulfonamides, beta-lactams, macrolides, aminoglycosides, fluoroquinolones and others. On the other hand, in UK, sulfonamides are the second mostly used antibiotic accounting for nearly 21% of total sales (Sarmah et al., 2006).

It is evident that the uncontrolled consumption and usage of veterinary antibiotics disturb not only the environment and ecosystems but also the human health. In Lebanon, unfortunately, there is no adequate data or statistical analysis displaying an assessment of the total amount of veterinary pharmaceuticals utilized and this is because livestock production is barely monitored or surveyed and farmers tend to unreasonably use more than the recommended dosage. On the other hand, a survey done to assess antibiotic usage across several Lebanese farms stated that the top five mostly used antibiotics by dairy farms are streptomycin, gentamicin, penicillin, oxytetracycline and tylosin. (Choueiri, 2008).

C. Antibiotics in the Environment

The presence of antibiotics in the environment is caused by unmonitored excretion done by humans and animals. Antimicrobials can be present in the environment through several different routes, these include, the drug manufacturing process, disposal of unused drug containers, medical waste and through the use and application of waste material containing the drugs. Unfortunately, as mentioned by Pruden et al. (2013) many countries do not have a well-established take back programs for such situations and tend to neglect the matter of unused medicines.

Animal agriculture is only one source of entry of drug residues in the environment. Animals do not utilize all the antibiotics in the feed and a large quantity of the added antibiotics are excreted in urine or manure. Once excreted, these antibiotics can enter the environment through land application of manure and potentially alter the soil microbial system. (Kumar et al., 2005b). The problem is that livestock manure holds elevated levels of veterinary antibiotics that stay active even after normal digestive procedure (Kim et al., 2010). Once in the environment, antibiotics can be transported either in dissolved phase or adsorbed to colloids or soil particles into surface and groundwater (Chee-Sanford et al., 2009).

Consequently, the persistence of antibiotic in the environment will lead to microbial resistance. They have lipophilicity, which permits them to pervade bio-membranes and stability, which stops their inactivation before therapeutic effect. Therefore, drugs have the properties they need to accumulate in organisms and cause change in water and soil ecosystems (Lillenberg et al., 2010).

1. Antibiotic residue

Medicines used by humans or animals are excreted in an unchanged form or as metabolites. Manure containing antibiotic residue is being used as a source of fertilizer to enhance soil quality, consequently affection the soil flora and accumulates in plants. Drugs and their metabolites found in soil are either mineralized by soil organisms or enter the groundwater unaltered (Lillenberg et al., 2010).

The excretion rate of antibiotic says a lot about the antibiotic’s residual fate and behavior in the environment. Most antibiotics fed to animals are poorly absorbed in the animal’s gut as much as 90% of them can be excreted as their parent compounds. Boxall et al. (2002) and Kumar et al. (2005) illustrated further in the topic. They explained that the excretory organs eliminate polar compounds such as tetracycline and tylosin more efficiently than compounds that have high lipid solubility. Lipid soluble antibiotics are often not eliminated until they are metabolized to more polar compounds.

Sulfonamides a synthetic antibiotic used against gram-negative and gram-positive bacteria, has a 90% recovery rate after excretion (Choueiri, 2008). A field study in Germany showed that a concentration of 15µg/kg of sulfamethazine, member of the sulfonamide group, was measured in the soil after seven month of manure fertilization on fields (Accinelli et al., 2006). In 2001, Halling-sorensen showed that 90 % of the water soluble antibiotics can be found in urine and 75% in animal feces. Sarmah et al., (2006) stated that about 95% of the excreted antibiotics enter the environment in active forms. Namely, out of a dose of 70 mg/head/day of chlortetracycline which is a growth promoter administered to cattle to treat enteritis and leptospirosis, 14µg/g was found in fresh manure (Sarmah et al., 2006).

Many researchers suggested different behavior and fate of drug residues in the soil. For example, in his article, Lillenberg (2010) , suggested that the substance in the soil can either be easily degraded and changes into carbon dioxide and water or if it is lipophilic may take time to degrade or it may be metabolized into a more hydrophilic matter but doesn't decompose at all affecting the environment.

The destiny and persistence of an antibiotic in the environment rest upon several aspects such as binding to soil, biodegradation, chemical complexation or chelation, hydrolysis and photolysis. Kumar et al., (2005a) noted that the process of chemical complexation or chelation of antibiotics with organic or inorganic compounds or ions may render the antibiotic inactive in soil or manure. For example, tetracyclines chelate with divalent and trivalent metal ions such as Mg^{2+} , Ca^{2+} , Fe^{3+} , Zn^{2+} , and Al^{3+} (Halling-Sørensen et al., 2002). This implies that the levels of metals in the soil will affect the potency of antibiotic and their degradation. According to Halling-Sorensen, the degradation product of tetracycline, chlortetracycline and oxytetracycline represent a similar potency as their parent compounds but different mode of action against bacteria in the soil and manure. Furthermore, it is important to note that antibiotics such as oxytetracycline remained potent for as long as 100 days (Kumar et al., 2005a).

Hu et al., (2010) in their article about the "occurrence and source analysis of typical veterinary antibiotics in manure, soil, vegetables and groundwater from organic vegetable bases, northern china" stated that antibiotic residues from manure in summer were significantly lower than those in winter and the latter was more 1-20 folds than the former due to the enhanced biodegradation of antibiotics at the high temperature and strong activity of bacteria in the summer.

Consequently, the antibiotic residues will lead to serious environmental problems including ecological risk and human health damage.

2. Antibiotic levels in Manure

Manure contributes to the fertility of the soil by adding organic matter and nutrients. Globally, farmers use manure to fertilize their soil prior to planting especially in organic and sustainable agriculture. However, before applying manure to agricultural lands, soil nutrient level and crop need should be evaluated and tested so that the manure level added is adequate. However, in many cases, farmers disregard this recommendation and apply manure irrationally without complying with the suggested rate.

Song & Guo (2014) mentioned that the worldwide heavy use of veterinary pharmaceuticals in confined animal feeding operations has resulted in annual discharge of 3,000-27,000 tons of drug chemicals through livestock manure into the environment. Baguer et al. (2000) claimed that land application of antibiotic-laced manure seems to be the ruling pathway for the release of antibiotics in terrestrial environment and in fact is the main source of resistance.

With the advance of analytical techniques, many researchers estimated the level of antibiotics in manure. For example, antibiotics such as tetracycline, tylosin, monensin, sulfadimidine and sulfathiazole have been detected in swine slurry, cattle manure, poultry litter and fish farm sediment from different countries at a wide concentration ranging from traces to 200 mg/kg (Kumar et al., 2005b). In addition, a study done to estimate the level of antibiotics in manure confirmed that the concentrations of tetracycline and chlortetracycline were 4.0 and 0.1 mg/kg respectively (Hamscher et al., 2002). Song & Guo (2014) reported that more than 50 major antibiotics have been detected in poultry, swine, cattle, and horse manures at 0.01-765 mg/kg dry manure mass. Table 4 shows a number of reported concentrations of residual veterinary antibiotics in animal manures.

In animal manures, most veterinary pharmaceuticals degrade rapidly via biochemical reactions, demonstrating a half-life of 2-30 days. Heuer et al. (2011) indicated that the macrolide class of antibiotics such as tylosin once excreted along with manure degrades quickly during storage with half-life in the order of days, yet many other antibiotic compounds in are transferred to soil.

Many research suggested that storing or composting of animal manure helps eliminate the residual antibiotic by biodegradation. Liguoro et al (2003) reported that the concentrations of oxytetracycline and tylosin in cattle manure decreased from 366.8 to 2.1 mg/kg and from 32.8 to < 0.1 mg/kg, respectively, after stacking the manure outdoor for 135 days.

Table 4. Reported concentrations of residual veterinary antibiotics in animal manures

Manure type	**Antibiotic**	**Concentration (mg/kg)**	**Country**	**References**
Swine manure	Tetracycline	0.3-56.8	China	Li et al., (2013)
	Tylosin	0.2-1.9		
Poultry Manure	Tetracycline	0.5-13.4	China	Li et al.,(2013)
	Tylosin	0.2-0.4		
Poultry Manure	Tetracycline	0.05-0.5	Turkey	Karci and Balcioglu (2009)
	Enrofloxacin	0.01-0.08		
Dairy Cow manure	Tetracycline	0.2-10.4	China	Li et al., (2013)
	Tylosin	0.2-0.3		
Fresh cattle manure	Oxytetracycline	872	Italy	De Liguoro et al., (2003)
	Tylosin	116		
Newly removed cattle bedding	Oxytetracycline	367	Italy	De Liguoro et al., (2003)
	Tylosin	32.8		
Aged cattle manure	Tetracycline	0.05-0.4	Turkey	Karci and Balcioglu (2009)
Cattle (matured-5m)	Oxytetracycline	0.82	Italy	De Liguoro et al., (2003)
	Tylosin	0.1		
Cattle (day 30-day 135)	Oxytetracycline	2-19	Italy	De Liguoro et al., (2003)
	Tylosin	0.001-0.1		
Poultry	Chlortetracycline	23	Canada	Warman and Thomas (1981)
Liquid	Tetracycline	20	Germany	Winckler and Grafe (2000)
	Sulfadimidine	40		

Source: (Song & Guo, 2014) & (Kumar et al., 2005a)

3. Antibiotic in soil

Land application of antibiotic-polluted manure is an agricultural practice all over the world. Thereby antibiotics are transferred to agricultural soils.

Several literatures found that discovery level of antibiotic compounds in soil were noticeably lesser than in manure and the reason behind such outcome is the fact that detection levels are strongly affected by the binding of the antibiotic compounds to soil matrix (aging) and the extraction procedure resulting in an underestimated detection level of veterinary pharmaceuticals in soil (Heuer et al., 2011).

It has been clear in considerable research the presence of antibiotics and their residues in soil. For example, the concentrations of tetracycline and chlortetracycline were up to 86.2-198.7 and 4.6-7.3μg/kg respectively in the soil in Germany (Hamscher et al., 2002). A substantial amount of oxytetracycline was bound to soil regardless of soil type (Thiele-Bruhn, 2003). Also, the antibiotic level in the soil varies with soil depth. For instance, from a field study where soil had been fertilized with liquid manure, Hamscher et al. (2002) reported the presence of 4.0 and 0.1 mg/kg of tetracycline and chlortetracycline in liquid manure, while in the soil samples the concentrations of these compounds varied from an average 86.2μg/kg in the top soil (0-10 cm) to as high as 171.7μg/kg in the 20-30 cm layer. Hamscher et al. (2002) provided a possible explanation of higher concentration of antibiotics at greater depths, it has been attributed to the additional release of bound residues in the form of 4-epi-tetracycline, a metabolite of tetracycline, and the authors concluded that 4-epi tetracycline is transferred from the liquid manure into the soil (Sarmah et al., 2006).

Kemper (2008) elucidated in her article details on antibiotic adsorption to soils. The physical and chemical properties, such as molecular structure, size, shape, solubility and hydrophobicity of antibiotic vary with the compound and thus, the sorption and fixation of these substances in soils vary significantly. Some antibiotics seem to persist a long time in the environment, especially in soil, while others degrade very fast.

Many aspects can possibly affect the distribution of antibiotics in soils. The dilution with soil, degradation, leaching, and uptake by plants are main reasons why the residue of antibiotic in soil was much lower than that in manure (Hu et al., 2010). The degree of antibiotic adsorption to soils depend on the antibiotic species existing and soil properties including pH , organic matter content, and the concentration and type of divalent cations present (Rabølle & Spliid, 2000).

The persistence of antibiotic in soil poses an environmental, animal and human risk; making it a controversial research topic. To date, however, there is no clear evidence for interrupted ecosystem services in soil communities due to antibiotic exposure given the prevailing exposure levels documented in the field (Larsson, 2014).

a. Sorption of veterinary antibiotics in soils

In order to clearly comprehend the consequence of antibiotic residues in the environment we must first understand the biochemical processes of the antibiotic persistence and its interaction with the terrestrial environment. Table 5 provides a list of some veterinary antibiotics along with their fate and mobility in soil.

Table 5. Fate and mobility of selected veterinary antibiotics in soil

Antibiotics	Solubility in water (g/L)	Chemical degradation	Mobility
Tylosin	5	Stable at pH 4 to 9,	Low to immobile
Tetracycline	1.7	-	Immobile
Chlortetracycline	0.6	Half-time in manure 1wk at 37^{o}C & >20 d at 4^{o}C or 28^{o}C	Immobile
Oxytetracycline	1	Stable compared to CTC	Immobile

Source: Modified from Chee-Sanford et al., 2009

Many factors come into play when speaking of antibiotic persistence such as antibiotic property, soil characteristics and weather conditions. Nevertheless, sorption, binding and fixation of the chemical on soil matrix occur when veterinary antibiotics interact with clay minerals and organic matter. The most important antibiotic properties are binding and adsorption to soil solids, photo-stability, biodegradation, and water solubility.

Antimicrobial activities of antibiotics are associated with different functional groups of the molecular structure (Thiele-Bruhn, 2003). The antibiotics classified by different structural classes can be ionized, amphiphilic or amphoteric. For example, the tetracycline group exhibit amphoteric compounds that are stable in acids but not in bases. These compounds form chelate complexes with divalent metal ions and beta-diketones, strongly bind to proteins and silanolic groups and are prone to photo-degradation. Aminoglycosides (such as gentamicin) are polar compounds, highly soluble in water and prone to photo-degradation. Most macrolides (such as tylosin) are composed of lactone structure with more than 10 C-atoms and are weak bases and thus unstable in acids. Penicillin belongs to beta-lactam class of antibiotics. The antibiotic effect of penicillin is connected to the beta-lactam ring, which is not stable in acidic or basic conditions. Fluoroquinolones, on the other hand, are highly stable and resist hydrolysis but degrade under UV light (Thiele-Bruhn, 2003).

b. Binding of antibiotics to soil

A parameter known as distribution coefficient (K_d) is a parameter used to predict the transport and behavior of organic contaminants in the soil. By definition, it is the ratio of concentrations of a compound in a mixture of two immiscible phases at equilibrium. Moreover, distribution coefficient (K_d) is commonly used to measure the sorption of a solute to soil. The sorption of organic contaminants in soil is maintained through the interaction with soil organic matter.

The mobility of antibiotics further increases if these compounds are bound to dissolved organic carbon in manure or soil (Tolls, 2001). However, the soil distribution coefficient (K_d) values of animal antibiotics vary dramatically with the chemical species , from 0.3 to 6,300 L/Kg (Song & Guo, 2014). Usually sorption of veterinary pharmaceuticals with high K_d values happens naturally and ubiquitously. For instance, in antibiotics-spiked (400-12,000 mg/kg) soil slurry systems under agitation, more than 95% of the chlortetracycline adsorption to a sandy loam and a clay soil occurred within 10 min and 95 % of the tylosin adsorption occurred within 3h (Allaire et al., 2006). Table 6 presents the distribution coefficient of some of the veterinary antibiotics. Compounds with high K_d values are strongly bound to soil particles and less mobile. Compounds with less K_d value are less strongly bound and more mobile in the soil. The latter group of antibiotics can be easily transported to contaminate the ground as well as surface waters. Strongly bound antibiotics can however, be transported mainly to surface waters with the sediments during run off losses of soil (NAAS, 2010). Chen et al. (2011) clarified that antibiotics that are weakly bound to soil components having a small K_d value are likely to drift out of the fields through runoff or be leached down in the soil profile by percolation water, while those strongly bound to soil solids with high K_d value can simply move with the soil particles to other areas by runoff water.

Table 6. Distribution coefficient of several antibiotics

Antibiotic	**$K_{d, solid}$ (L/kg)**
Tetracycline	400-1620
Oxytetracycline	420-1030
Enrofloxacin	260-6310
Tylosin	8.3-128
Sulfamethazine	0.6-31

Source: Modified from (Tolls, 2001 & Kumar et al., 2005a)

Clay minerals are also central in binding veterinary pharmaceuticals. The binding mechanism includes van der walls interactions, electrostatic attraction, and cation bridging and anion exchange (JEON et al., 2014). It is assumed that antibiotics can be divided into four groups depending on the reaction with the clay minerals, table 7.

Table 7. Grouping antibiotics according to their reaction with clay minerals

Category	**Antibiotics**
Strongly basic	Streptomycin, Neomycin, Kanamycin
Amphoteric	Bacitracin, Aureomycin, Tetramycin
Acidic	Penicillin
Neutral	Chloromycetin, Cyclohexamide

Source: (JEON et al., 2014)

Cationic antibiotics bind to the soil particles through ionic interaction, while acidic and amphoteric antibiotics may bind to the soil through non-ionic interaction (NAAS, 2010). The pH plays a role in the interaction between the antibiotic and the soil by altering the charges of the pharmaceuticals and the cation exchange capacity of the soil. For example, at pH 5 , oxytetracycline has zero charges and interacts with organic matter mainly through hydrophobic partitioning; at lower and higher pH, the chemical becomes positively and negatively charged, respectively, and was sorbed to soil minerals mainly through cation exchange and cation bridging , respectively (Kulshrestha et al., 2004).

c. Transformation of veterinary antibiotics in soils

Once manure is applied to soil residual antibiotics are subject to abiotic and biotic transformation and degradation. Many aspects influence the degradation process, such as ; variation of veterinary chemicals , transformation rate , soil type, soil conditions , manure type , soil-manure ratio , pH , light , temperature , moisture and oxygen status (Lin & Gan, 2011)

Degradation of veterinary pharmaceuticals in agricultural soils is a comprehensive result of microbial decomposition, organic transformation, oxidation, photolysis and hydrolysis.

Chee-Sanford et al. (2009) studied the probable degradation pathways of antibiotics in manured soils. As water is at all times present in animal waste and natural soils, hydrolysis may be an important mechanism for animal pharmaceuticals to disperse in the environment. It is known that the antibiotics beta-lactams, macrolides, and sulfonamides are susceptible to hydrolysis (Huang et al., 2001). Higher soil moisture content allows more chemicals in the solution phase, boosting the accessibility to microorganisms. Wang et al. (2006) stated that the half-life time of sulfadimethoxine in a silt loam decreased from

10.4 days to 6.9 days and further to 4.9 days as the soil moisture content was elevated from 15 % to 20 % and additionally to 25 %, respectively.

Moreover, by exposure to daylight, antibiotics may experience photolysis at the soil-atmosphere interface. Quinolones and tetracyclines are particularly sensitive to photo irradiation, and photo degradation of oxytetracycline was three times more rapid under light then dark conditions (Doi & Stoskopf, 2000). Nevertheless, sulfonamides tend not to be readily photodegradable (Boxall et al., 2004). Compared to other reactions, however, photo-degradation of antibiotics may be insignificant under field conditions due to limited light exposure (Beausse, 2004). Furthermore, biodegradation depends upon the temperature; lower temperatures reduce the degradation rate. When manure is applied in late fall or during winter where temperatures are low and soils may be frozen, antibiotics in manure or soil will persist longer and accordingly provides greater opportunities for spread in the environment through snow-melt runoff (Kumar et al., 2005a).

Adding to biodegradation, chemical processes other than hydrolysis and photolysis are similarly important for antibiotic transformation in soil. Temperature impacts degradation of veterinary pharmaceuticals in soils. Li et al. (2011) observed that ceftiofur hydrolyzed to desfuroylceftiofur in deionized water, with a half-life time of 289 days at 15^{o}C. The half-life time was shortened to 96, 21, and 5 days, respectively as the hydrolysis temperature increased to 25, 35, and 45^{o}C. Degradation of veterinary pharmaceuticals is also altered by soil oxygen availability. Dissipation of sulfamethoxazole and trimethoprim from two mineral soils under anaerobic conditions was substantially slower than under aerobic conditions (Lin & Gan, 2011). Sorption to soil minerals and soil organic matter preserves veterinary antibiotics and enhances their persistence in soils (Zitnick et al., 2011).

Reported studies on degradation of veterinary antibiotics in soils are summarized in Table 8.

Table 8. Degradation of some antibiotic in soils

Antibiotic	**Conditions**	**Degradation (%)**	**Half-life $t_{1/2}$ (day)**	**References**
Tylosin	Spiked a 12 % moisture sandy loam at 2mg/kg and incubated the soil at 20ºC in the dark for 120 days	100	8	Sclusener and Bester (2006)
Oxytetracycline	200L of liquid swine manure were fortified with 7.08g oxytetracycline and surface applied to a 120-m^2 sandy loam field plot. 127 days	83	21-23	Blackwell et al. (2007)
Tylosin	Incubated 50mg/kg tylosin spiked sandy loam (field capacity) at 20ºC in the dark for 30 days	93	7-8	Hu and Coats (2007)

Source: (Song & Guo, 2014)

Kumar et al., (2005) stated that half-life varies between a few days to as high as 300. As the half-life of several antibiotics increases at low temperatures and in the dark, this suggests that antibiotics may persist longer in deeper soil layers and in deep waters. Quinolones and tetracycline were the most persistent with half-lives approaching 100 days (Boxall et al., 2004). The half life span of some antibiotics in manure is reported in table 9.

Table 9. Half-life of some antibiotics in manure

Antibiotic class	Half-life (days)
Aminoglycosides (Gentamicin)	30
Beta-lactams	5
Macrolides (Tylosin)	<2-21
Quinolones	100
Sulfonamides	<8-30
Tetracyclines (Oxytetracycline)	100

Source: Modified by Chee-Sanford et al., 2009

4. Antibiotic in water

The worry concerning environmental exposure to antibiotics has been growing, ever since numerous antibiotics were spotted in river water in UK more than two decades ago (Watts et al., 1982). Recently, many countries have been investigating the occurrence and fate of antibiotics in the aquatic environment. In the USA, a nationwide survey of pharmaceuticals compounds discovered that a number of antibiotics were detected in 27% of 139 rivers at concentrations up to 0.7μg/L (Kolpin et al., 2002).

According to Kemper (2008) veterinary antibiotics and their metabolites or their degradation products reach the aquatic environment through surface runoff, driftage or leaching. Thus, soil act as an antibiotic reservoir gathering antibiotic contaminating the aquatic environment (Thiele-Bruhn, 2003). Lillenberg et al. (2010) clarified that significant volume of drugs reaching the surface water can end up in drinking water.

In wastewater and sewage treatment plants, resistant and multi-resistant bacteria have been detected, possibly entering the food chain directly via sewage sludge used as fertilizer or wastewater serving for irrigation (Kümmerer, 2004).

The movement of pharmaceuticals into the aquatic environment varies with the antibiotic compound and its physiochemical properties. For example, Penicillin and tetracycline are not usually expected to be found in aquatic environment. This is due to the easy hydrolysation of penicillin and the precipitation and accumulation of tetracycline (Myllyniemi et al., 2000). This coincides with Hamscher et al. (2002) where neither tetracycline nor tylosin was detected in any water sample.

A study was conducted, in northwest Germany, sampling a series of surface waters detected a wide range of antibiotics in all samples, such as macrolides, sulphonamides and lincosamides were examined regularly, but no traces of beta-lactams antibiotics were found. Moreover, tetracyclines were also not detected due to their strong adsorption to organic matter of the soil (Christian et al., 2003). On the other hand, in Germany, Hamscher et al. (2002) collected soil water samples and found concentrations of chlortetracycline, oxytetracycline, tetracycline, tylosin ranging from 0.1-0.3μg/L. Also, in another study residual oxytetracycline at concentrations ranging from 500 to 4000μg/ kg were observed in marine sediment following chemotherapy treatment in fish farms in the US (Capone et al., 1996).

The transport of antibiotics to ground and surface water poses a risk of some antibiotics to enter the drinking water supply especially those that are highly mobile and do not easily degrade during water treatment process. On the other hand, less mobile antibiotics are potentially toxic to plants and soil organisms and provide an environment for antibiotic resistance to emerge in native soil bacteria (Tolls, 2001).

5. Antibiotics in plants

Streptomycin and oxytetracycline are two antibiotics registered by the United States Environmental Protection agency (USEPA) for use in plant agriculture to control bacterial infections in plants. Up to 53,000 Ha of fruit and vegetable plants are sprayed annually with antibiotics. In 2009, in the United States, 16,465 kg (active ingredient) was applied to orchards, which is 0.12% of the total antibiotics used in animal agriculture (Stockwell & Duffy, 2012).

Furthermore, many researchers studied the accumulation and uptake of veterinary antibiotics by various plants and its potential health risks. It is important to note that on a daily basis, an adult consumes 0.512 kg of plant material from crops grown above ground and 0.333 kg of plant material from crops grown below ground (Boxall et al., 2006).

A plant uptake study of ten antibiotics to lettuce and carrot from a sandy soil spiked at a soil antibiotic concentration of 1 mg/kg detected florfenicol, levamisole and trimethoprim in lettuce leaves at concentrations ranging 6-170μg/kg, whereas enrofloxacin, florfenicol and trimethoprim were detected in carrot at concentrations ranging from 2.8-13μg/kg fresh weight (Boxall et al., 2006). Moreover, Lillenberg et al. (2010) suggests that when the vegetation period is longer, antibiotics accumulate in plants; it was highest in lettuce and lowest in cucumber.

Kumar et al. (2005b) conducted a greenhouse study to test whether or not plants take up antibiotics from manure amended soil. The tested crops were corn, green onion and cabbage. The study concluded that the three crops absorbed chlortetracycline at a rate of 2-17 ng/g fresh weight but did not absorb tylosin and that the more antibiotic present in the manure the higher the concentration of it in the plant tissue (Kumar et al., 2005b). To justify his results, he deduced that macrolides class of antibiotics , to which tylosin belongs, are less soluble in water (0.45-15 mg/L) compared to tetracycline class antibiotics (230-52000 mg/L) and have a higher log K_{ow} values (octanol-water partition coefficient) as compared to tetracycline class antibiotics and has a larger molecular weight (Kumar et al., 2005b). Sulfamethazine, which has a low molecular weight and is not strongly adsorbed to soil particles, was also taken up by plants such as corn, lettuce and potato (Dolliver et al., 2007). A study made by Hu et al., (2010) reported that antibiotics in vegetables were apparent, and the range of antibiotics was 0.1-532μg/kg in vegetables. Moreover, it has been stated that antibiotics from manure reach up plants by passive absorption (Hu et al., 2010). In a study conducted in Lebanon, Basil et al. (2013) reported that carrot, lettuce and radish absorbed relatively higher amounts of gentamicin than streptomycin. They also mentioned that, the levels of antibiotics in plant tissue increased with increasing the antibiotic concentration in the manure (1 mg/kg > 0.5 mg/kg). Willow and maize grown in greenhouse potting soils spiked with 10mg/kg sulfadiazine for 40 days showed presence of the chemical in the roots at 333 and 26.5 mg/kg dry weight, respectively, but not in the above ground tissues (Michelini et al., 2012).

In another experiment done by Kong et al. (2007) results showed that oxytetracycline had a significant inhibitory effect on alfalfa growth. The effect was more obvious on root growth than on shoot which is supported by previous findings that showed that the roots are the main accumulation site for antibiotics. As concentration of oxytetracycline increased, the leaves turned from light green to yellow. Oxytetracycline inhibited alfalfa shoot and root growth by up to 61% and 85% respectively (Kong et al., 2007). Moreover, in a study on pinto beans grown in aerated nutrient media with chlortetracycline and oxytetracycline at 160 mg/L, top and root dry matter were reduced by 71 to 87% and 66 to 94%, respectively (Patten et al., 1980).

In order to clearly evaluate the danger of antibiotics in vegetables, different tissues of vegetable samples need to be analyzed. Migliore et al. (1998) in his article proved that the absorption of antibiotics in the roots was higher than that in the leaves of wheat and corn. The types and growth stages of vegetables would affect the distribution of antibiotics in vegetables (Hu et al., 2010).

Finally, the bioaccumulation of veterinary antibiotics in food crops may be insignificant since the concentrations of residual antibiotics in soils receiving manure is much lower compared with the levels of antibiotics tested in laboratory of greenhouse research. Hence, it is still unclear whether or not the bioaccumulation of antibiotics in field crops poses health risks to consumers.

D. Antibiotic resistance and impact on human health

The misuse and overuse of antibiotics in food animals contribute to the emergence of resistant form of disease-causing bacteria. Such resistant bacteria can be communicated from food animals to humans, mainly through the food (WHO, 2000). The rise in antibiotic resistance is now acknowledged worldwide as one of the greatest possible threat to human and animal health. The public has become increasingly alarmed about the connection between the overuse of antibiotics in both medicine and the agriculture agro-food industry and the emergence and spread of antibiotic resistant bacteria.

Resistance refers to the conditions where antibiotics that customarily inhibit certain types of bacteria no longer have the anticipated result. Any kind of antibiotic use in people, animals or plants can encourage the development and spread of antibiotic resistance.

Marshall and Levy, (2011) explained that the low-dose and prolonged courses of antibiotics among food animals create ideal selective pressures for the propagation of resistant strains. Therefore, without suitable regulations, it is thought that a large diverse reservoir for resistant bacteria and resistance genes could facilitate the emergence and spread of resistant pathogens to humans, and even the ongoing transmission of such resistant organisms within the human population (Chang et al., 2015).

In many cases, antimicrobial agents of the same class are used in both humans and animals. Similarly, *E.coli, Salmonella enterica*, *Campylobacter spp. and S.aureus* are important bacterium species that cause disease in both humans and animals. Therefore, the transmission of these species is very common between humans, animals and the environment. Nevertheless, it is very critical to justify the class concept because antimicrobials belonging to the same class normally act on the same target area in the bacteria. Having said that, if bacteria emerged resistance to one member of the class, then those bacteria will most likely exhibit resistance to some or all antibiotics belonging to the same class (Anderson et al., 2002; WHO, 2011b).

Many researchers provided evidence for animal to human spread of antibiotic resistance. The latter was either through direct acquisition from animal to human or through resistance transmission along the food chain. Resistance genes travel from a resistant bacterium in animals to a bacterium pathogenic to people. Resistance genes can willingly be transferred between bacteria from terrestrial animals, fish and people. Further, such transfers can take place in various environments, such as kitchens, barns and water sources (WHO, 2011). Farm workers are directly at risk of acquiring resistance since they are always in close contact with colonized or infected animals. Thus this might provide a channel of spread of resistance genes into the environment wherever possible (Marshall and Levy, 2011). Levy et al. (1976) reported the very first incidence of acquisition of resistance in human from direct contact with infected animals. It was proved with a study where they reported the existence of the same tetracycline-resistant *E.coli* strains in the gut flora of the chicken workers as in the chicken receiving tetracycline rich feed. Gentamicin is an antibiotic mostly used in poultry as growth promoting agent, it prevent early poultry mortality. A revelatory 2007 study established that the threat for carrying gentamicin-resistant *E.coli* was 32 times higher in poultry workers than in other members of the community: half of all poultry workers were colonized with gentamicin-resistant *E.coli*, compared to only 3% of non-poultry workers were colonized (Price, et al., 2007). Several studies documented the transmission of resistance to humans through contact with infected animals. Marshall and Levy (2011) demonstrated several examples of bacterial species (*E.coli, salmonella, Enterococcus faecalis, E.Faecium and MRSA*) and antibiotic resistance including poultry, pigs and cattle and even resistance in humans to a range of antibiotics only used in animals (example: Apramycin). Table 10 display major classes of antibiotics shared by animals and humans.

Table 10. Major classes of antibiotic shared by animals and humans

Antibiotic Class	Antibiotic
β-lactams	Penicillin , Amoxicillin , Ceftiofur
Macrolides & Lincosamides	Tylosin, Tilmicosin, Lincomycin
Aminoglycosides	Gentamicin, Neomycin
Fluroquinolones	Enrofloxacin, Danofloxacin
Tetracyclines	Oxytetracycline, Chlortetracycline
Sulfonamides	Various
Streptogramins	Virginiamycin
Polypeptides	Bacitracin
Phenicols	Florfenicol
Pleuromultilin	Tiamulin

Source: (NIAA, 2011)

The hypothesis is that the food chain is the main mean of transmission. But data on antibiotic resistance is limited and mainly gathered through research papers. For instance, Marshall & Levy, (2011) stated that resistant *E.coli* have been found in beef carcasses that were stored for 24hrs in a cooler and later made into ground beef in North American Feedlot. Work-related transmission of *MRSA* from food animals to humans is well documented and transfer of *MRSA* through the food chain has also been documented (Hanselman, et al., 2006; Lewis, et al., 2008).

Manure amendment of agricultural soils typically adds a considerable amount of bacteria carrying antibiotic resistance genes. Resistant bacteria attach to crops and are exposed to humans through antibiotic uptake by plants. Despite the fact that the daily (per-capita) risk of antibiotic resistance uptake is low, the impact may even be greater than hospitals transmission, as modeled for uptake of resistance with food. Quantitatively the massive input of resistance genes and selective agents with manure could well contribute to the resistance problem in human antibiotic therapy (Heuer et al., 2011). Moreover, fish farming involves the use of antibiotics and fish as food may be contaminated with resistant bacteria (Phillips et al., 2004).

Alternatives to growth-promoting and prophylactic use of antimicrobials in agriculture include improved management practices, wider use of vaccines, and introduction of probiotics. Monitoring programs, prudent use guidelines, and educational campaigns provide approaches to minimize the further development of antimicrobial resistance. The existing information concerning the insinuations of veterinary antibiotics on the terrestrial environment and impacts on human health is still limited. Thus, a wide range of investigations to interpret the impact of antibiotics on humans and the environment is essential to launch safe management protocols for antibiotic usage and treatments.

E. Methodological Approach to Analysis of Antibiotics

Analysis of antibiotics, their occurrence and degradation in the environment, has been studied since 1998 and analytical methods have been used for their detection (Hirsch et al., 1999; Golet et al., 2001).

The mostly used methods are high-performance liquid chromatography along with Ultra Violet (HPLC-UV) and diode-array detection (HPLC-UV, -DAD) or liquid chromatography with mass-spectrometry (LC-MS) or tandem mass-spectrometry (LS-MS/MS) (Oka et al., 2000; Hamscher et al., 2002).

Also, Enzyme-linked Immunosorbent Assay (ELISA) tests are used especially in milk, meat, fish, eggs, honey, plasma, urine and tissue and are optimized to analyze environmental samples for the measurement of antibiotics (Franek et al., 1999; Christian, et al., 2003). For example, concentrations of gentamicin, neomycin, and streptomycin were measured in milk and kidney samples. The limits of detection for all three were below the maximum residue levels (MRL) 0f 100, 500 and 200 μg/L in milk and 1000, 5000, and 1000 μg/L in kidney as allowed by the EU (Stead, 2000). Irwin et al. (2001) used ELISA to measure the amount of the hormone, estradiol, in ponds receiving runoff from USA beef cattle farms and found concentration ranging between 0.05 to 1.80 ng/L. Algal toxins, cyanotoxins, were also detected by Billiam et al. (2006) in lakes in the USA using ELISA and had a maximum concentration of 0.15 μg/L.

Kumar et al. (2005b) used ELISA as a method of detection for analyzing tylosin and tetracycline; the recoveries of both were close to 100%. These results show that these kits can be adapted to quantify tylosin and tetracycline concentrations in water and manure in various water quality samples. In another study, comparing ELISA with HPLC, using ELISA approach offers a rapid, low-cost assessment of antibiotic presence and concentration in plant while HPLC analysis is more costly and time consuming and requires optimization and extensive sample processing to minimize background noise and enhance signal (Dolliver et al., 2007).

F. Drug description

In my research I have studied gentamicin, oxytetracycline and tylosin uptake by lettuce and radish plants. These three antibiotics are widely used in the Lebanese farms.

1. Oxytetracycline

a. <u>Chemical structure</u>

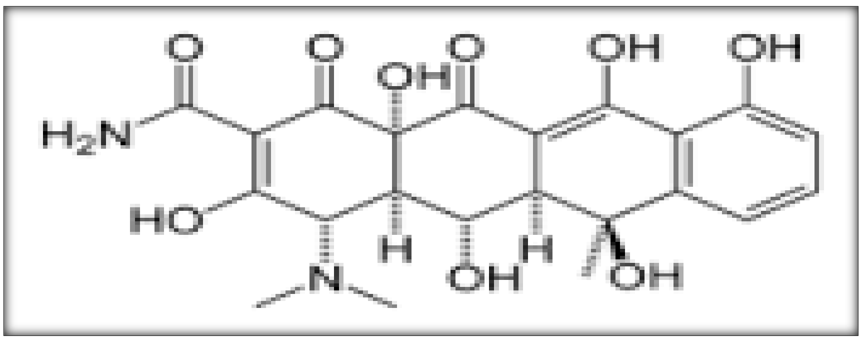

Figure 1. Chemical structure of oxytetracycline (Molecular formula: $C_{22}H_{24}N_2O_9$ & molecular weight: 460.43396 g/mol)

b. <u>Overview</u>

To start with, all of the tetracycline derivatives are crystalline, yellowish, amphoteric substances that, in aqueous solution, form salts with both acids and bases.

Tetracyclines are broad-spectrum agents, active against a wide range of gram-positive (*Cocci, Corynebacterium, Clostridia, Erysipelothrix, Actinomycetes,* etc) and gram-negative bacteria (*Escherichia coli, Salmonella, Pasteurella, Haemophilus, Brucella, Pseudomonas, Bordetella,* etc.), chlamydia, mycoplasmas, rickettsia and protozoan parasites. Chlortetracycline and oxytetracycline were the first members of the tetracycline group to be described (Chopra & Roberts, 2001). They were formed by *Streptomyces* *aureofaciens* and *S. rimosus*, respectively. They exhibit bacteriostatic activity by interacting with bacterial ribosomes and blocking of the protein synthesis. Consequently, oxytetracycline stops the spread of the infection and the lasting bacteria are destroyed by the immune system or eventually die.

In veterinary medicine, they are well absorbed, exhibit low toxicity and are relatively inexpensive. Consumption of tetracycline antibiotics is fairly high as compared with other classes of antibiotics. They are widely used mostly for the treatment of gastrointestinal, respiratory and skin bacterial infections, infectious diseases of locomotive organs and of genito-urinary tract as well as systemic infections and sepsis (Castillo, 2013).Target animal species for the application of these preparations are beef cattle, pig, sheep, goat, horse, dog, cat, poultry, rabbit and fish.

In plant agriculture, oxytetracycline is either formulated as oxytetracycline-calcium complex or oxytetracycline hydrochloride. In the USA, it is registered on pear for control of *E. amylovora* and on peach and nectarine for control of *Xanthomonas arbricola*, which causes bacterial spot. It is also used to control *E. amylovora* on apple in Mexico and *Pseudomonas* spp. and *Xanthomonas* spp. on several vegetable crops in Latin American countries. Moreover, it is rarely used as an injection into the trunk of palm and elm trees to mitigate symptoms of lethal yellows diseases caused by phytoplasmas.

Oxytetracycline can also be used to correct breathing disorders in livestock. It is administered in a powder or through an intramuscular injection.Rapid intravenous administration may result in animal collapse. Hence, it should be administered intravenously slowly over a period of at least 5 minutes.

The oxytetracycline injection used in the experiment contains 200 mg/ml where each 1 ml contains 200mg of oxytetracycline base as amphoteric. Regarding withdrawal times; oxytetracycline treatment should be ceased at least 21 days prior to slaughter for cattle and swine whereas for milk its 6 days and 5 days for poultry.

2. *Gentamicin*

a. Chemical structure

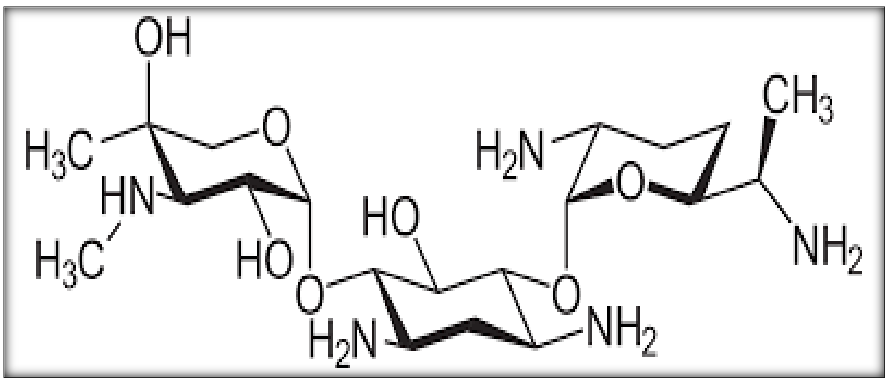

Figure 2. Chemical structure of gentamicin (Molecular formula: $C_{21}H_{43}N_5O_7$ & molecular weight: 477.59542 g/mol)

b. Overview

Gentamicin is an aminoglycoside antibiotic with polar organic base and bactericidal activity mostly against aerobic gram-negative bacteria (Gehring et al., 2005). After oral administration, gentamicin is essentially not absorbed. However, it is well absorbed after intramuscular injection and is excreted unchanged via the kidneys (Brown & Riviere, 1991). Generally the aminoglycosides are combined with other antibiotics when both gram-negative and gram-positive bacteria are present.

In plant agriculture, gentamicin is formulated as gentamicin sulfate and it is sometimes mixed with oxytetracycline. It is mainly used in Mexico to control fire blight of apple and pear and in several Latin American countries to control various bacterial diseases of vegetable crops caused by species of *Erwinia, Pectobacterium, Pseudomonas, Ralstonia*, and *Xanthomonas* (McManus et al., 2002).

It is mainly used for the treatment of respiratory, gastrointestinal and urogenital infections (bronchitis, pneumonia, pyelonephritis, cystitis, urethritis, endometritis, metritis, colibacillosis, salmonellosis, infected wounds, pyodermia sepsis, etc.), caused by gentamicin-sensitive microorganisms. It is produced by the fermentation of *Micromonospora Purpurea*. It works by inhibiting bacterial ability to synthesize protein and proliferate by irreversibly binding to 30S ribosomal subunits.

In veterinary medicine, gentamicin is mainly found as a solution for injection for pigs, cattle and horses and as an oral solution for poultry. It is also used in human medicine, intramuscularly every 8 hours to provide a total daily dose of 3mg/kg body weight/day (EMEA, 2001). It is administered to animals intramuscularly, intravenously or orally. It is rapidly absorbed and excreted unchanged with urine. The dosage of administration varies with the animal species, for example, the gentamicin 10% solution is given to ruminants at 2-4 mg/kg body weight, 5mg/kg body weight for pigs, and 0.2 ml/L (distilled water) for day old chickens (EMEA, 2001).

Gentamicin is normally available in a 100mg/ml injectable solution. Regarding the withdrawal period for meat its 7 days after the last administration (Tan et al., 2009). Moreover, for chicken a withdrawal period of 5 weeks has been established (ANADA, 2014). The persistence of aminoglycoside residues appear to be variable and dependent on numerous factors such as the formulation used, the dose administered, the dosage interval, and the health and physiological features of the animal (Gehring, et al., 2005).

3. *Tylosin*

a. Chemical structure

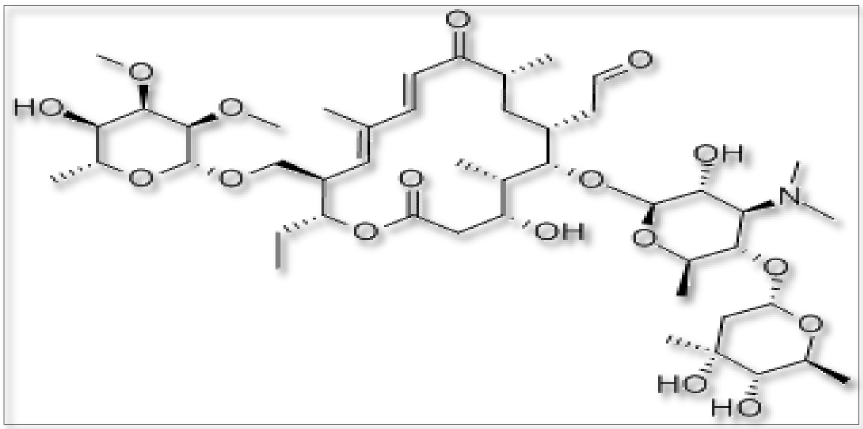

Figure 3. Chemical structure of tylosin (Molecular Formula: $C_{46}H_{77}NO_{17}$ & Molecular weight: 916.10 g/mol)

b. Overview

Tylosin is a bacteriostatic feed additive used in veterinary medicine. It has a broad spectrum of activity against gram positive organisms such as (*Staphylococcus, Streptococcus*, *Corynebacterium, and Erysipelothrix*) and a limited range of gram negative organisms against *Campylobacter coli*. It is found naturally as a fermentation product of *Streptomyces fradiae.* It belongs to the macrolide class of antibiotics, they tend to be weak bases and unstable in acids. Tylosin inhibits protein synthesis through binding to the 50S subunit of the bacterial ribosome.

In veterinary medicine, tylosin is one of the most broadly used antibiotics in modern animal production (Hu & Coats, 2009). It is widely used for therapeutics and growth promotion in swine, beef cattle and poultry production (Hu et al., 2008). It is administered for use in the chronic respiratory disease complex in chickens. Tylosin is also used to treat bovine respiratory and swine dysentery diseases. It may be administered to calves , orally in the milk replacer at a dose of 40 mg/kg body weight and to cattle by intramascular injection at a dose of 4-10 mg/kg body weight. In pigs, it may be administered in the drinking water at a dose of 25 mg/kg body weight , in the feed at a dose of 3-7 mg/kg body weight or by intramascular injection at a dose of 2-10 mg/kg for the prevention and control of swine dysentery and enzootic pneumonia. Moreover , it is given to poultry in the drinking water at a dose equivalent to 35 mg/kg body weight. As a feed additive, the substance may be incorportated in pig feed at concentrations in the range of 5-40 mg/kg feed depending on the age.

CHAPTER 3

MATERIALS AND METHODS

In this chapter the materials and methods used are presented in three parts. The first part will illustrate the methods for soil analysis. The second part will describe in details the pot experiment and antibiotic analysis. And the third part will explain the methods of statistical analysis.

A. Soil Analysis

Soil, free from antibiotics, was purchased from and supplied by AUB purchasing office from the supplier KAWTHARANI Co for Agriculture. The physical and chemical properties of the soil were analyzed according to the procedure outlined by Bashour and Sayegh (2007). All the tests were performed on triplicates and the average values of the results are reported.

The soil sample was spread on a tray and left to air-dry for two days then it was sieved using a 2 mm-sieve (mesh = 20) and was placed in a labeled air-tight clean plastic container for analysis.

1. Physical Analysis

Soil texture, moisture content, and color were the physical parameters measured on the soil sample.

a. Soil texture

Soil texture is a basic soil characteristic which affects the physical and chemical performances of the soil. It is influenced by the size and type of particles that makes up the soil matrix. Thus soil texture refers to the weight proportions (relative proportion by weight percentage of sand, silt and clay) of the mineral soil separates for particles less than two millimeters. Different methods can be used to test soil texture however the method used in this experiment was the Bouyoucos Hydrometer method.

Using the Bouyoucos method, soil particles are freely settling down in a water column and are sorted according to particle size. The sample is treated with dispersing agent sodium hexametaphospate ($Na_6O_{18}P_6$) to complex Ca^{+2} , Al^{+3}, Fe^{+3}, and other cations that bind clay and silt particles into aggregates. The density of the soil suspension is determined using a calibrated hydrometer at 20^0C to read the amount (grams) of solid particles remaining in suspension. The hydrometer is used twice, once after 40 seconds (after the sand particles settles) and out again after 2 hours (after the silt particles settles).

A volume of 50 ml of dispersing agent (1 N Sodium Hexametaphospate solution) was added to 50 g of soil into a baffled stirring cup and filled to its half using distilled water. The cup was placed on a stirrer and stirred until soil aggregates are broken (5 minutes). Later, the suspension was transferred to the settling cylinder by washing the cup with distilled water and filling the cylinder to the mark. The hydrometer was placed in the suspension (at 40 seconds and at 2 hours) and measurements were obtained. The temperature of the suspension was also measured and corrections were made for the density and temperature of the dispersing solution. Texture class of the soil sample was obtained using the soil textural triangle.

b. Soil Moisture Content

By definition, soil moisture content, is the amount of water held associated with a given volume or mass of soil. Generally, soil moisture is expressed on oven-dry basis. Thus, for this experiment, the moisture percentage of the soil sample was conducted by oven-drying the soil sample (50 g) at 105^0C for 24 hours. The percent moisture content is then computed on oven-dry basis by subtracting the oven-dry weight from the air-dry weight (initial weight).

c. Soil Color

Soil color can state many things about the morphological properties of a soil. Soil color was obtained using the Munsell Soil Color Charts, 1954 edition. Soil color was taken on wet and dry soil sample.

2. Chemical Analysis

The chemical analyses conducted on the soil samples were as follows: soil pH, Soil Salinity (Electric Conductivity), available sodium and potassium, available phosphorus, available micronutrients (Fe, Cu, Mn, and Zn), and the total free calcium carbonate.

a. Soil pH

In order to get a better understanding of the soil acidity and alkalinity, the soil sample was mixed with distilled water at a ratio of 1:2 by weight (Soil:distilled water) in a falcon tube. The mixture was shaken and left to settle for 24hrs and then the pH value of the soil supernatant was measured using a pH-meter (Oakton instruments, Model # WD-35613-24).

b. Soil Salinity (Electric Conductivity)

Electric conductivity (EC) of a soil extract shows the concentration of total soluble salts in solution, thus reflecting the degree of soil salinity. The apparatus used in this analysis is the electrical conductivity meter (Eutech instruments, CyberScan CON 11) and the unit of measurement was millisiemen per centimeter. Soil sample was mixed with distilled water at a ratio of 1:2 by weight (Soil:distilled water) in a falcon tube and was left to settle. After 24hrs measurements were taken.

c. Available Sodium and Potassium(NH4OAC-K+Na)

The apparatus used in this experiment is the flame photometer. Three grams of soil was mixed with 30 ml of 1 M ammonium acetate solution pH 7. The concentrations of Na and K in the extractant were measured by a calibrated flame photometer (BWB Technologies, XP 2011) to estimate the amounts of NH_4OAC-K and Na present in the soil.

d. Available Phosphorous (Olsen modified methods)

Since our Lebanese soils are mostly calcareous, Olsen's method (Watanabe and Olsen, 1965) is the most commonly used and acceptable technique in quantifying available phosphorous in the soil. In this test 5 grams of soil sample were extracted by100 ml of 0.5

M sodium bicarbonate solution ($NaHCO_3$). The mixture was shaken on the mechanical shaker for 30 minutes and later filtered using Whatman No. 40 filter paper. Ten milliliters (10 ml) of filtrate were transferred to a 50 ml volumetric flask where 8 ml of ascorbic acid solution were applied (reducing agent) and made to volume giving a blue color. The sample absorbance was measured on spectrophotometer (OPTIMA INC. model Sp-300) at 882 nm. The extent of available phosphorous in the soil was then calculated by comparing to a series of standard solutions of known concentrations.

e. DTPA-Extractable Micronutrients

Available Iron, Zinc, Copper, and manganese in soil were extracted by DTPA (Diethylenetriaminepentaacetic acid) solution following the DTPA extraction method. In this experiment 5 grams of soil were mixed with 20 ml DTPA solution. The suspension was shaken on a mechanical shaker for 30 minutes. The concentrations of the micronutrients in the extract (Fe, Zn, Cu, Mn) were measured on atomic absorption spectrophotometer (Shimadzu AA-630) by comparing to already adjusted standards.

f. Total Free Calcium Carbonate

Calcite a key component of lime stone and calcareous soils supplies calcium and magnesium for plants. The $CaCO_3$ content ranges from few percent in slightly calcareous soils to more than 80 percent in extremely calcareous soils. In this test, the reagents used were hydrochloric acid (1M HCL), sodium hydroxide (0.5M NAOH), and phenolphthalein indicator. Five grams of soil were mixed with 100 ml of HCL in a 250 ml Erlenmeyer flask. The flask was covered with aluminum foil and left to settle overnight. After filtering the mixture, 10 ml of filtrate were titrated back by standard 0.5N NaOH and were used to estimate the calcium carbonate content in the soil using phenolphthalein as an indicator.

B. Pot Experiment

The pot experiment took place in the green house of the American university of Beirut, Beirut Campus. This pot test has a formal experimental design, with replication and defined controls and treatments. The soil was collected from KAWTHARANI Co supplier ; the antibiotic free manure was collected from AREC (the American Research and Education Center) in Beqaa valley and was left to dry for more than 6 months.

This section includes crop description, antibiotics used and the tested concentrations, soil mix (pot preparation) and finally the tissue analysis (harvesting stage) for the antibiotic contents in roots and leaves.

1. Crop description

To understand the uptake and accumulation of antibiotics in plants, two freshly consumed vegetables were selected to be used in the experiment. The crops used were lettuce and radish. Lettuce represents leafy green crops and radish represents root crops. Radish started from seeds (5 radish seeds per pot) whereas the lettuce, young seedlings were obtained from an agricultural nursery in Sidon (Hiba Nursery). The seedlings were transplanted (1 lettuce per pot) and crops were grown in a greenhouse and watered as needed. The antibiotics concentrations were later (after harvest) measured in the leaves and roots of both crops.

2. Antibiotics included in the study and their Concentrations

Three antibiotics widely used in livestock and poultry production in Lebanon having the same mode of action (inhibition of protein synthesis), were used in this experiment. The antibiotics are gentamicin, oxytetracycline, and tylosin (figures 4, 5 and 6 respectively). They were purchased from a vet store in Al-Beqaa. The chemical properties of each are shown in table 11.

Figure 4. Front and Back view of gentamicin bottles

Gentamycin 10% (injection)

Each ml contains: Gentamycin sulphate equivalent to Gentamycin base 100 mg.

Manufactured by: ADWIA Co. S.A.E

Made in: Egypt

Figure 5. Front and Back view of Oxytetracycline bottles

Oxytetracycline 20% (injection)

Each ml contains: oxytetracycline hydroxide equivalent to 200 mg oxytetracycline.

Manufactured by: KELA LABORATORIA N.V

Made in: Belgium

Figure 6. Front and Back view of Tylosin bottles

Tylosin 20 % (Tylokel 20) (injection)

Each ml contains: Tylosin tartrate equivalent to 200 mg base.

Manufactured by: KELA LABORATORIA N.V

Made in: Belgium

Table 11. The chemical properties of the three antibiotics included in the study

Antibiotic	Family	Derived from	Mode of action	Molecular formula	Molecular mass (g/mole)
Gentamicin	Aminoglycoside	Micromonospora purpurea	Inhibit protein synthesis	$C_{21}H_{43}N_5O_7$	477.596
Oxytetracycline	polyketide	Streptomyces genus	Inhibit Protein synthesis	$C_{22}H_{24}N_2O_9$	460.434
Tylosin	Macrolide	Streptomyces fradiae	Inhibit protein synthesis	$C_{50}H_{83}NO_{23}$	916.1

Four levels of each of the antibiotics were tested (0, 2.5, 5, 10 mg/kg) and each treatment was replicated three times.

3. *Soil Mix*

This section will explain the experimental scheme along with the technique used in pot preparation, measurement of antibiotics, procedure adopted for tissue analysis, and finally ELISA kit description and the applied procedure for estimating the antibiotics concentrations in plant tissues (roots and leaves) using specific ELISA-kits for each antibiotics.

a. Experimental Scheme

The design used in this study was Factorial + One-way ANOVA with defined replicates.

Crops used: 2 crops (Lettuce and Radish)

Antibiotics used: 3 antibiotics (two having small molecular mass gentamicin and oxytetracycline, and one large molecular mass tylosin)

Growing media: 2 soil mixes (soil alone and soil + 5 % manure)

Antibiotic concentration used: 4 levels (0, 2.5, 5, 10 mg/kg)

Replicates: 3 replicates per treatment (3 pots)

The total number of pots in the experiment is 2 crops x 2 soil medias x 3 antibiotics x 4 levels each x 3 replicates = 144 pots

b. Pot Preparation

- Growing Media: 2 growing media (Soil + 5 % manure mix and Soil alone without manure).
- Total number of pots per antibiotic: 2 crops x 4 levels x 2 growing media x 3 replicates = 48 pots.
  - Soil with manure treatment: pots with 5 kg capacity were filled by 5 kg of soil mixed with 0.25 kg manure making 5.25kg/pot. The manure used was free from antibiotics and brought from AREC and collected from cows that haven't been treated with antibiotics for more than 6 months)
  - Soil without manure treatment: pots with 5 kg capacity were filled by 5 kg of soil after being sieved by a 10mm sieve.
- The required amount of antibiotics was diluted with 100 ml of water
- Fertilizer rate used: 0.5g / 5 kg soil (20-20-20 + TE)

- Both mixtures were placed in plastic bags and then the bags were placed in the pots to prevent leaching (Figure 7).

Figure 7. Plants grown in pots with a lining of plastic bags

- The pots were labeled clearly (antibiotics used, concentration level, crop type, pot number)

c. Antibiotic treatments

Each crop was subjected to three antibiotics at four different levels under two different soil growth media (with and without manure) replicated three times. Therefore for each antibiotic per soil mix treatment 48 pots were prepared.

d. Tissue Analysis

After 45 days, the plants (Radish and Lettuce) were harvested as whole (roots and leaves) and placed in labeled paper bags. The subsequent procedure was followed to all treatment:

- Whole plant was washed thoroughly in water
- leaves and roots were separated
- Plotted using tissue papers.
- Weights were directly recorded for shoots and roots parts separately
- The plant material (roots and leaves) were finely chopped and blended fresh. (Uniform grinding and mixing are critical in obtaining representative samples and accurate analytical results)
- The mixture was vortexed for at least 2 minutes (to ensure homogenization of sample)
- The sample is then filtered using (F40 Whatman) filter papers
- The filtrates were stored in new air-tight plastic tube and place in the refrigerator at a temperature of 5^{o}C.

As recommended by the manufacturer of the ELSA-Kit , for the gentamicin and tylosin treatments; roots and leaves were extracted using distilled water at a ratio of 1:3 (1 gram plant tissue to 3 ml distilled water).

For the oxytetracycline treatment; roots and leaves were extracted using Mcllvain buffer provided by the ELISA kit manufacturer (0.2M sodium dibasic solution, 0.1M citric acid, pH adjusted to 7.0 using 6N sodium hydroxide, and finally diluted 1:1 with methanol before measurement) at a ratio of 1:3 (1 gram plant tissue to 3 ml distilled water).

e. Antibiotic Analysis (ELISA Procedure)

Antibiotic analyses were done using commercially available Enzyme-Linked Immunosorbent Assay (ELISA) kits. The commercial vendors for the three kits was (Abraxis, Warminster PA, United States), which can measure small quantities of antibiotics in vegetable samples. The tetracycline ELISA kit was highly specific to oxytetracycline at 95% cross-reactivity.

The protocol described by the kit manual was followed without introducing any modifications.

i. Test principle for gentamicin, oxytetracyline, & tylosin

According to the gentamicin ELISA kit (product no. 5111GEN1A), Tetracycline ELISA kit (product no. 52254BA) and Tylosin ELISA kit (product no. 52256B) the following is the implemented test principle.

"This test is a direct competitive ELISA based on the recognition of antibiotic by specific antibodies. Antibiotic once present in a sample and an antibiotic-enzyme conjugate compete for the binding sites of anti-antibiotic antibodies which are immobilized on the wells of the microtiter plate. After washing and addition of the substrate solution, a color signal is produced. The intensity of the color is inversely proportional to the concentration of the used antibiotic present in the sample. The color is later stopped and evaluated using ELISA reader. The concentrations of the samples are determined by interpolation using the standard curve constructed with each run." The list of standards used is presented in table 12.

Table 12. List of standards provided by manufacturer for gentamicin, oxytetracycline and tylosin

Gentamicin	**Oxytetracycline**	**Tylosin**
Concentration (ng/mL)		
0	0	0
0.25	0.10	0.1
0.50	0.20	0.25
1.0	0.30	0.50
2.5	0.40	1
5.0	0.60	2.5
-	0.80	5

The ELISA kits were received about 2-4 weeks before using and were kept in the refrigerator at (6 ^{o}C), and allowed to warm up to reach room temperature (24-25^{o} C) prior to usage.

ii. Reagents Provided

The reagents specific for each antibiotic to run the test were provided by the manufacturer for the three specific antibiotics (Table 13). These reagents were kept in a cool dark place. Gentamicin, oxytetracycline and tylosin's ELISA kits include the same reagent but differ in volume.

Table 13. List of reagents used in ELISA test for measurement of gentamicin, oxytetracyline, and tylosin in plant tissue extract as provided by the manufacturer.

Gentamicin	Oxytetracycline	Tylosin
Assay Buffer (6ml)	Assay Buffer (6ml)	-
Sample Diluent (10X) concentrate, 25ml , to be diluted before use	Sample Diluent (10X) concentrate, 2 x 25ml , to be diluted before use	Stabilizer/Sample Diluent (10X concentrated), to be diluted before use
Gentamicin-HRP Conjugate solution (12ml)	tetracycline-HRP Conjugate 2 vials (lyophilized)	Tylosin-HRP Enzyme Conjugate (6ml)
-	-	Anti-Tylosin Antibody (6ml)
Wash Solution (5X) concentrate, (100ml)	Wash Solution (5X) concentrate, (100ml)	Wash Buffer Solution (5X) concentrate, (100ml) need to be further diluted
Color Substrate Solution TMB, (12ml)	Color Substrate Solution TMB, (16ml)	Color Substrate Solution TMB, (12ml)
Stop Solution (12ml)	Stop Solution (12ml)	Stop Solution (12ml)

iii. ELISA Assay Procedure

The ELISA assay procedure, as provided by manufacturer, was followed. The sequence of analysis for the three antibiotics was the same. However, they differ in the period of incubation and volume of reagents used. The procedure may be summarized as follows:

- Add (25µL of gentamicin and 50µL for oxytetracycline) of assay buffer solution to the individual wells successively. This step is excluded in the tylosin assay procedure.
- Add (25µL of gentamicin , 100µL of oxytetracycline and 50µl of tylosin) of the standard solutions and plant tissue sample extract (1g sample + 3g water = 4x) into the wells in duplicates
- Add (100µL of gentamicin , 50µL of oxytetracycline and 50µL of tylosin) enzyme conjugate solution to the individual wells successively
- Dispense 50µL of antibody solution into each test well (only for tylosin)
- Cover the wells with parafilm and mix the content by moving the strip holder in a circular motion on the benchtop for 30 seconds
- Incubate the strips for (30 minutes for gentamicin and 60 minutes for both oxytetracycline and tylosin) at room temperature
- After incubation, remove the covering and vigorously shake the contents of these wells into sink. Wash the strips three times using 1X washing buffer solution. Use 250µL of washing buffer for each well in each washing step.
- Remaining buffer in the wells should be removed by patting the plate dry on a stack of paper towels.
- Add (100µL of gentamicin, 100µL of tylosin and 150µL of oxytetracycline) substrate color solution to the wells , cover , shake and incubate for 20-30 minutes

- Add 100μL of stop solution to the gentamicin, oxytetracyline and tylosin plates separately
- Read the absorbance at 450 nm using a microplate ELISA photometer within 15 minutes after the addition of stop solution

C. Statistical Analysis

A 3 x 2 x 4 factorial arrangment of treatments with interactions in a complete randomized desgin was used to analyze the lettuce data. The factors being : 3 antibiotics (gentamicin , oxytetracycline and tylosin) 2 growth media (soil with 5% manure and soil without manure) and 4 concentration levels of antibiotics (0, 2.5, 5, 10 mg/kg). Treatments were replicated three times.

In the radish experiment, we had a 2 x 2 x 4 factorial arrangement of treatments with interactions in a complete randomized design. The factors were two antibiotics (gentamicin, oxytetracycline), two growth media (soil with 5% manure and soil without manure) and 4 antibiotic concentrations (0, 2.5, 5, 10 mg/kg). Again in the radish trial a factorial (3 antibiotics x 4 concentration levels for each) arrangement of treatement in a complete randomized design was used with 3 replicates per treatement. Whenever, interactions were significant, data were analyzed as One-way ANOVA.

The Statistical Analysis System (SAS) version 9 was used using the General Linear Models (GLM) procedure and means were compared with Student-Newman-Keuls Test (SNK) whenever applicable.

CHAPTER 4

RESULTS AND DISCUSSION

This chapter provides the overall analysis and results attained and it is divided into two parts. The first part will present the soil analysis results, whereas the second part will present the overall results of the gentamicin, tylosin and oxytetracycline uptake by lettuce and radish plants subjected to four different antibiotic levels (0 , 2.5, 5, 10 mg/kg) planted in two growth media (soil with 5 % manure and soil alone without manure).

A. Soil Type

Soil, free from antibiotics, was supplied by Kawtharani Co for Agriculture and analyzed for its physical and chemical properties according to accepted international procedures.

Table 14. Physical & chemical characteristics of soil sample used in the experiment

Characteristic	**Value**
Texture	Sandy Loam
pH (1:2)	7.47
EC (1:2)	0.34 dS/m
Free $CaCO_3$	50 %
$NaHCO_3$-P	6.83 mg/kg
NH_4OAC-Na	165 mg/kg
NH_4OAC-K	250 mg/kg
DTPA-Fe	0.764 mg/kg
DTPA-Zn	0.248 mg/kg
DTPA-Cu	0.38 mg/kg
DTPA-Mn	0.028 mg/kg

The results of the soil analysis presented in table 14 show that the soil is Sandy Loam (75% Sand + 20% Clay + 5% silt), highly calcareous, slightly alkaline and non-saline. The color is dark red (dry 7.5R 3/6), dusky red (wet 10R ¾) , which indicates that the soil is mainly under aerobic condition and belongs to the Aridisol soil order. The avaialbe nutrients levels in the orignial soil were low in (P, Fe, Zn, and Mn) and medium in (K and Cu). Therefore, 0.5g of (20-20-20 + TE) fertilizer per 5 kg soil were applied to all treatments to provide sufficient nutrients for the plants to grow.

B. Antibiotic Uptake by Lettuce and Radish Plants

This section is divided into three parts. The first part includes the results of the lettuce pot experiment. The second part includes the results of the radish experiment. Whereas the third part includes a comparison for gentamicin, oxytetracycline, and tylosin uptake by lettuce and radish plants grown in different soil treatments.

1. Antibiotic uptake by lettuce

In the first experiment, the uptake of gentamicin, tylosin and oxytetracycline by lettuce crop was tested at four antibiotic concentration levels (0, 2.5, 5, 10 mg/kg). The antibiotics were added to two different growth media; soil mixed with 5 % manure and soil without manure. The results of the statistical analysis are plotted on a bar graph, where means with the same superscripts are not significantly different ($P > 0.05$) and means with different superscripts are significantly different ($P < 0.05$).

a. <u>Gentamicin accumulation in lettuce leaves and roots</u>

The concentrations of gentamicin in lettuce leaves and roots are shown in figures 8 and 9 respectively.

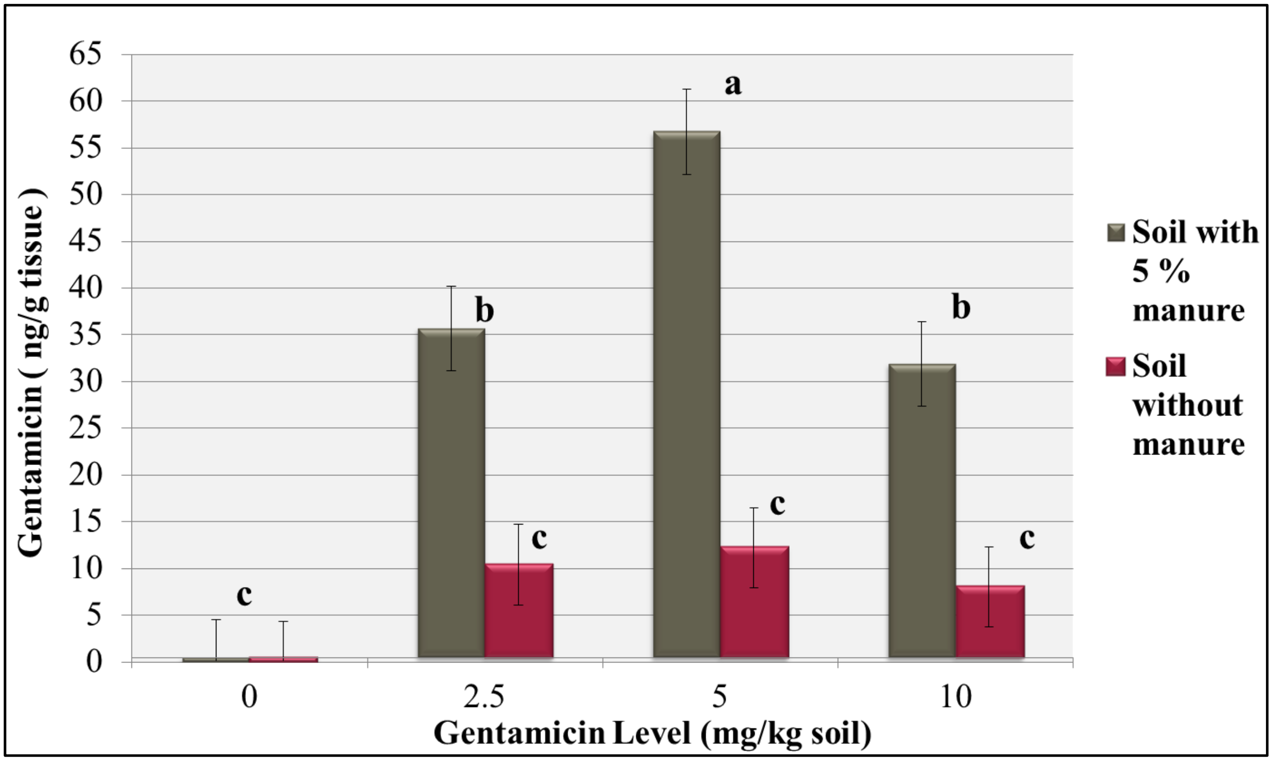

Figure 8. Concentration of gentamicin in lettuce leaves

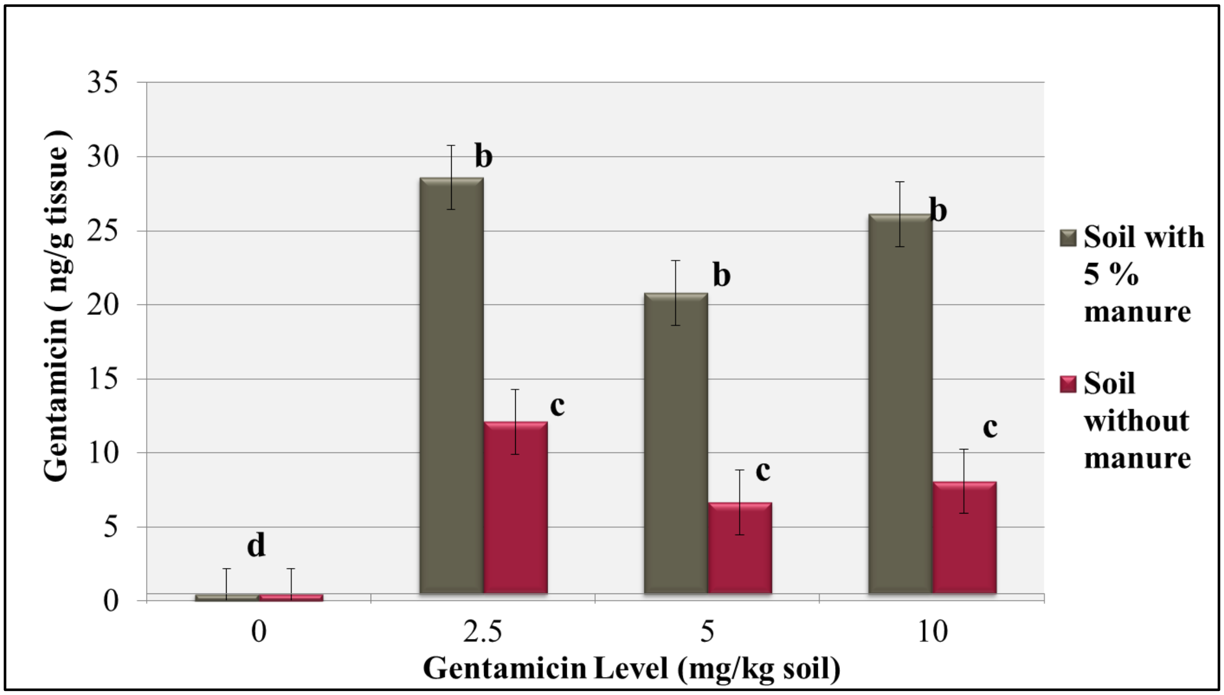

Figure 9. Concentration of gentamicin in lettuce roots

The result of analysis of lettuce leaves (Fig. 8) indicates that there is a significant difference between the control and the three other levels of gentamicin (2.5, 5, 10 mg/kg) in the soil with 5% manure treatment. On the other hand, no significant difference existed between the control and the other three gentamicin levels in the soil without manure. This indicates that manuring the soil enhanced gentamicin absorption by lettuce. Soil amended with 5% manure showed significantly higher accumulation rate of gentamicin in lettuce leaves than soil without manure. The result agrees with Sukul et al. (2008) stating that the presence of manure increased the sorption tendency of antibiotics significantly and with Dolliver et al. (2006) stating that sulfamethazine concentrations in plant tissue increased with corresponding increase of sulfamethazine in manure.

The data showed that increasing the gentamicin level in the two growing media did not lead to an increase in gentamicin accumulation in lettuce leaves. This result disagrees with Ahmed et al. (2015) stating that the total amount of antibiotics accumulated in the plant tissues were significantly increased as the level of their additions increased. However, our results match with the findings of Azanue et al. (2016) in their study "Uptake of antibiotics from irrigation water by plants" that plants have a limited sorption capacity for organic contaminant, which becomes saturated at higher soil concentrations.

The results of analysis in lettuce roots (Fig.9) indicate that there is a significant difference between the control and all other treatments. However, no significant differences among the three gentamicin levels (2.5, 5, 10 mg/kg) in each media alone. This indicates that the lettuce root accumulated gentamicin to a limit before it translocated it to the leaves.

Again similar to the leaves, the addition of manure facilitated the accumulation of gentamicin in the lettuce roots. This result is in agreement with the reported findings of Sukul et al. (2008) and Dolliver et al. (2006).

The data indicates that irrespective of the gentamicin level, lettuce's leaves and roots accumulated gentamicin. This agrees with the results of Basil et al. (2013) stating that gentamicin uptake in lettuce took place when manure amended soil was spiked with low rates of gentamicin (0.5 and 1.0 mg/kg), However, the differences in concentrations were not significant.

b. <u>Oxytetracycline accumulation in Lettuce Leaves and Roots</u>

The concentrations of oxytetracycline in lettuce leaves and roots are shown in figures 10 and 11 respectively.

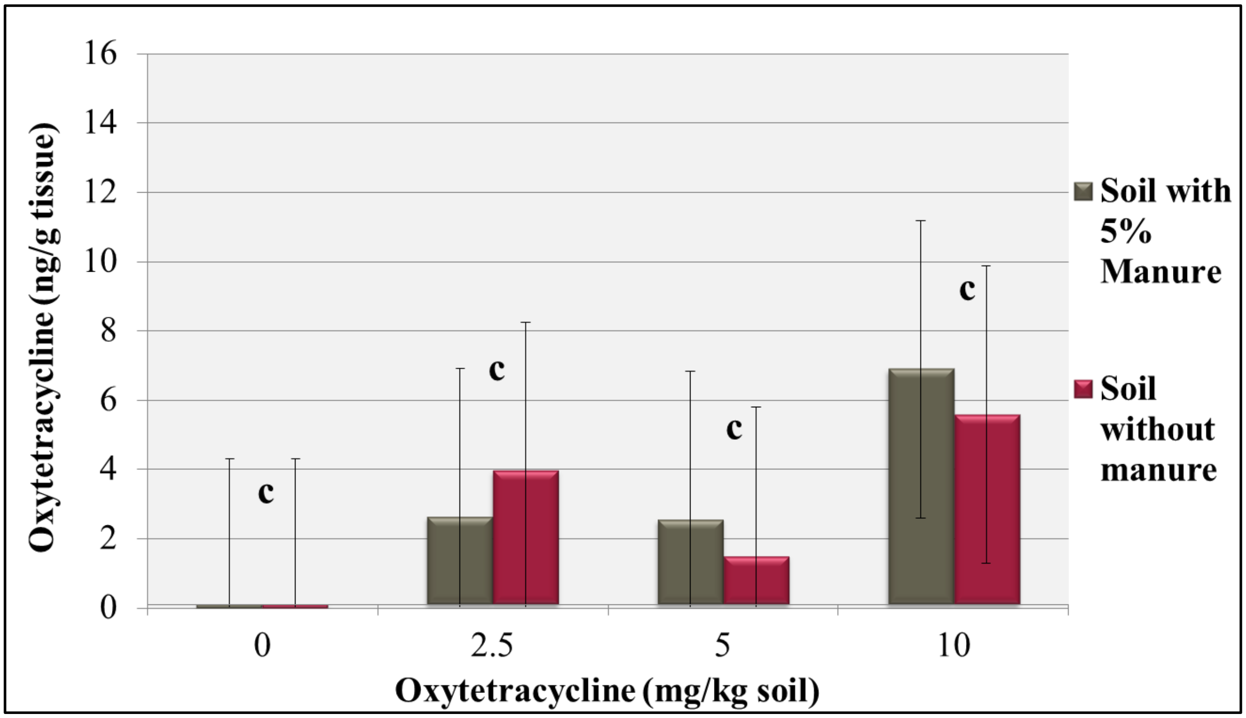

Figure 10. Concentration of oxytetracycline in lettuce leaves

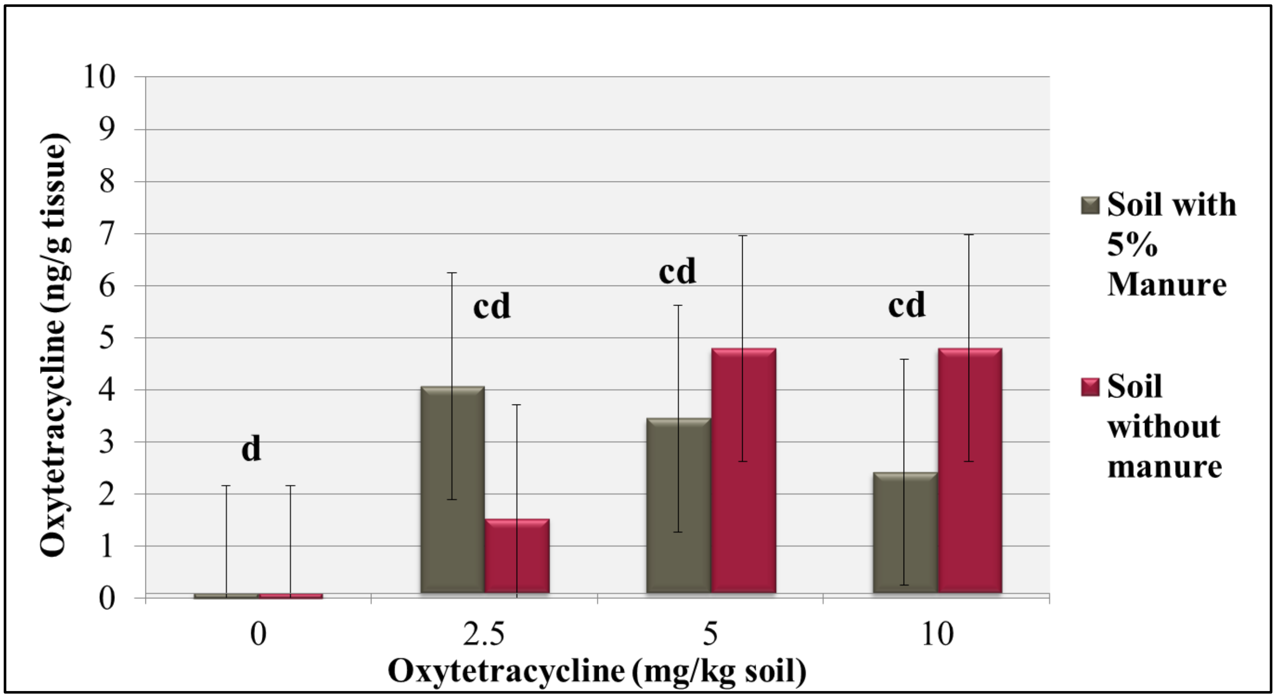

Figure 11. Concentrations of oxytetracycline in lettuce roots

The results of the analysis (Fig. 10 & 11) indicate that there is no significant difference in the accumulation of oxytetracycline in leaves between the control and any of the treatments, regardless of the nature of the media or the concentration of oxytetracycline. Therefore, it can be speculated that lettuce plants did not absorb oxytetracycline.

The uptake of sulfamethoxazole, oxytetracycline and ketoconazole by two types of plants (grass and watercress) was studied at concentrations of 5 and 10 mg/kg in the soil. From the results, it was concluded that the plant materials used for this study were able to take up sulfamethoxazole and ketoconazole when the soil was contaminated with these compounds at a concentration ranging from 5 to 10 mg/kg. Sulfamethoxazole was detected in all samples, at levels ranging from 7 to 21 mg/kg for grass and 4 to 7.5 mg/kg for watercress. For ketoconazole, the results showed low absorption. Oxytetracycline was not detected in any sample (Chitescu et al., 2013).

Although oxytetracycline is known to persist in the soil for a long time , more than 150 days (Boxall et al., 2006). It can be tightly adsorbed onto the soil particles and hardly be desorbed (Rabolle and Spliid, 2006) into the soil solution. Thus making it not available for plant uptake.

c. Tylosin accumulation in lettuce leaves and roots

The concentration of tylosin in lettuce leaves and root are present in figures 12 and 13 respectively.

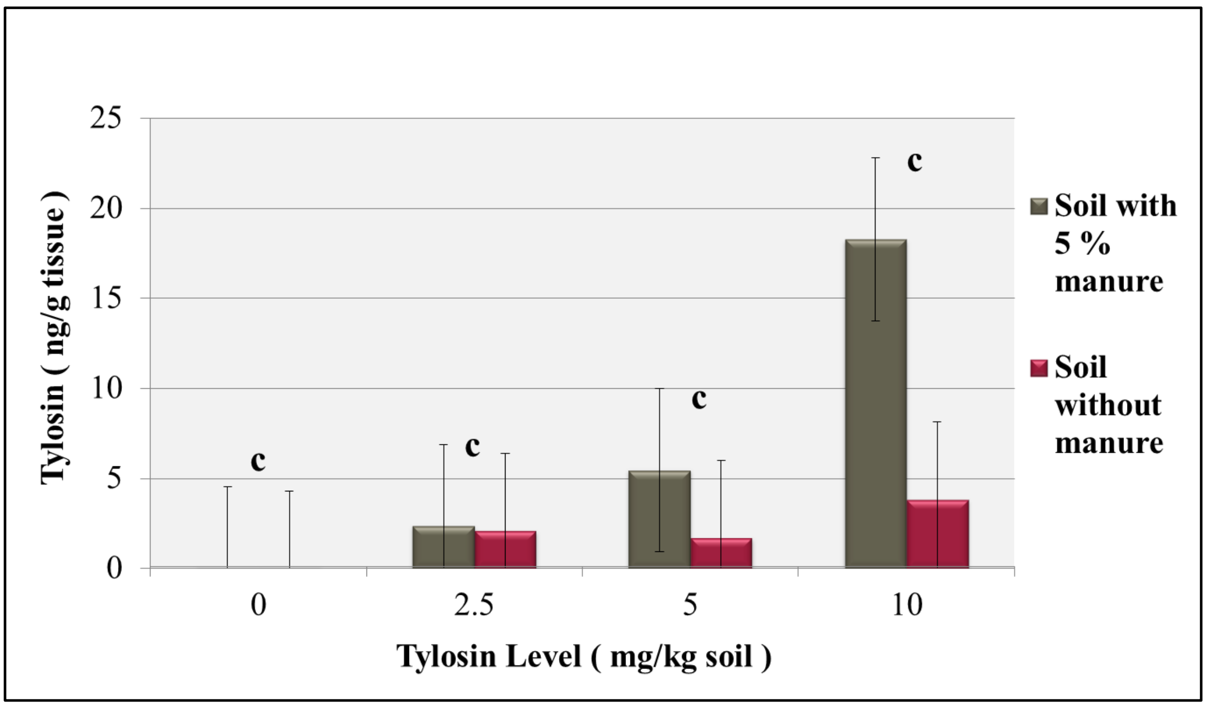

Figure 12. Concentration of tylosin in lettuce leaves

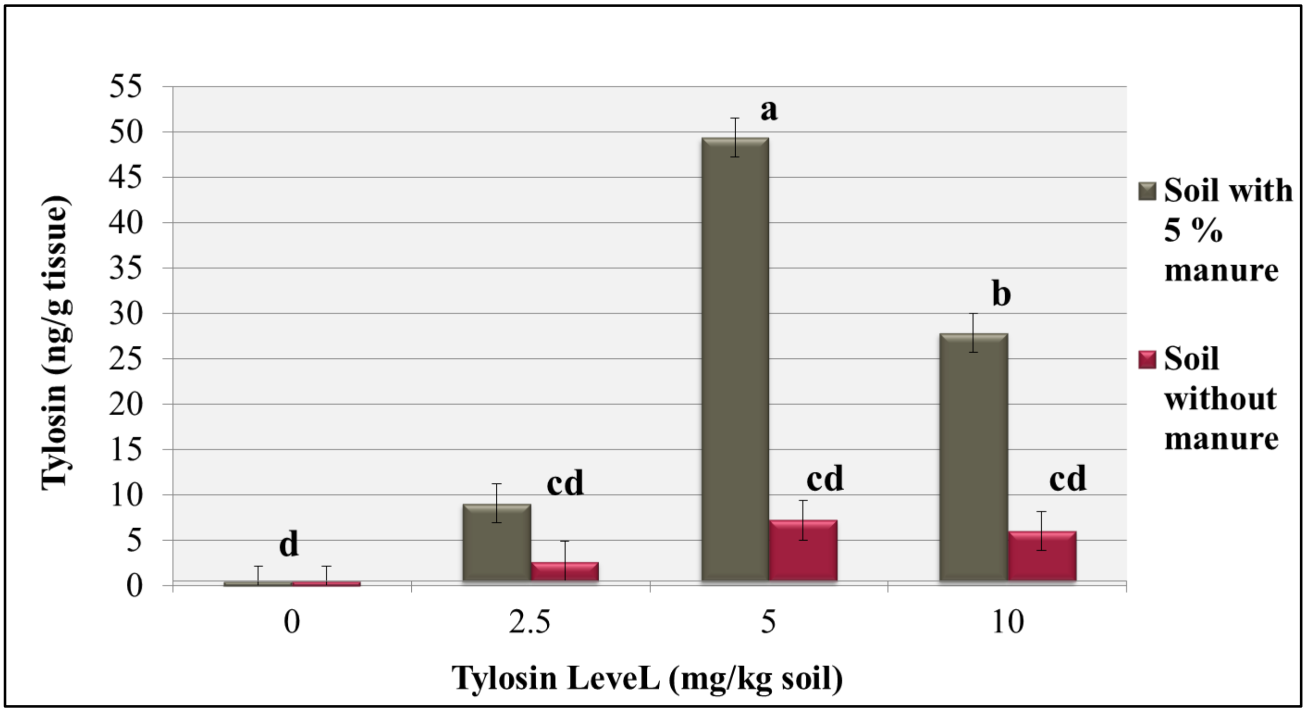

Figure 13. Concentrations of tylosin in lettuce roots

The results of the analysis (Fig. 12) indicate that there is no significant difference in the accumulation of tylosin in leaves between the control and any of the treatments, regardless of the nature of the media or the concentration of tylosin. However, the concentration of tylosin in lettuce roots at 5 and 10 mg/kg were significantly different from the control in the manure amended soil treatment. The accumulation of tylosin in the roots (Fig. 13) was not significant in the soil without manure treatment.

Again, this indicates that the manure facilitated the tylosin absorption by lettuce roots but the translocated amounts to the leaves were insignificant at the tested levels. This implies that manure once applied to soil enhances the uptake of tylosin by lettuce roots. Many studies argued the same point, such as Kang (2013) who mentioned that almost all vegetables took some antibiotics from manure treatment. Also, Kumar et al. (2005) stated that the more antibiotic present in manure the higher its concentration in plant tissue.

d. <u>Comparison of gentamicin, tylosin, and oxytetracycline in lettuce</u>

The average concentrations of the three antibiotics (gentamicin, tylosin, and oxytetracycline) in lettuce leaves and roots are present in figure 14.

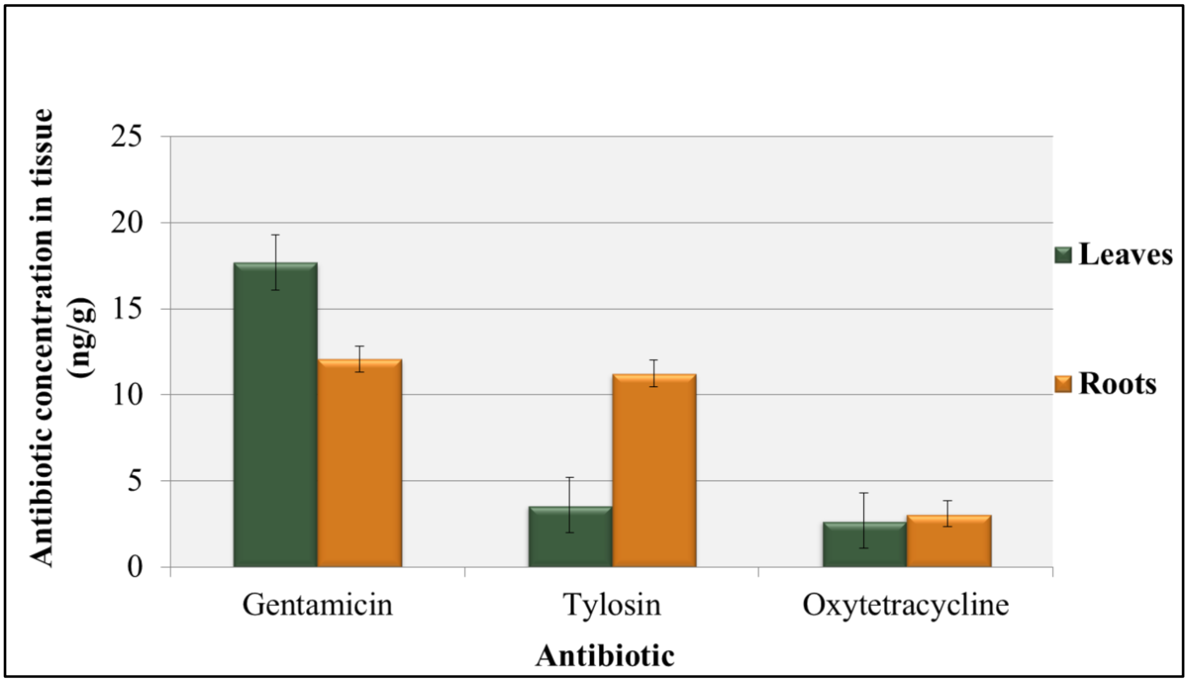

Figure 14. Average concentrations of gentamicin, tylosin, and oxytetracycline in lettuce

The results of analysis (Fig. 14) indicate that the concentrations of gentamicin in leaves and roots were higher than tylosin and oxytetracycline irrespective of the treatment. The distribution sequence of accumulation for the three antibiotics is: gentamicin > tylosin > oxytetracycline.

Gentamicin was absorbed by the roots of lettuce and later translocated to the leaves at an average value of 17.7 ng/g. Tylosin accumulated mostly in the roots and no significant translocation to the leaves took place. Finally, oxytetracycline was not absorbed by lettuce roots nor translocated to the leaves.

2. *Antibiotic uptake by radish plant*

This section is divided into two parts. The first part will show the statistical results of two antibiotics (gentamicin and oxytetracycline) experimented under two different growth media (soil with 5% manure and soil without manure) treated under three antibiotic levels (0, 2.5, and 10 mg/kg) . The second part will show the overall statistical results for tylosin accumulation in radish leaves and roots grown in manure amended soil by which the growing media was spiked with four levels of tylosin (0, 2.5, 5 and 10 mg/kg).

a. <u>Gentamicin accumulation in radish roots and leaves</u>

The concentration of gentamicin in radish roots and leaves are present in figures 15 and 16 respectively.

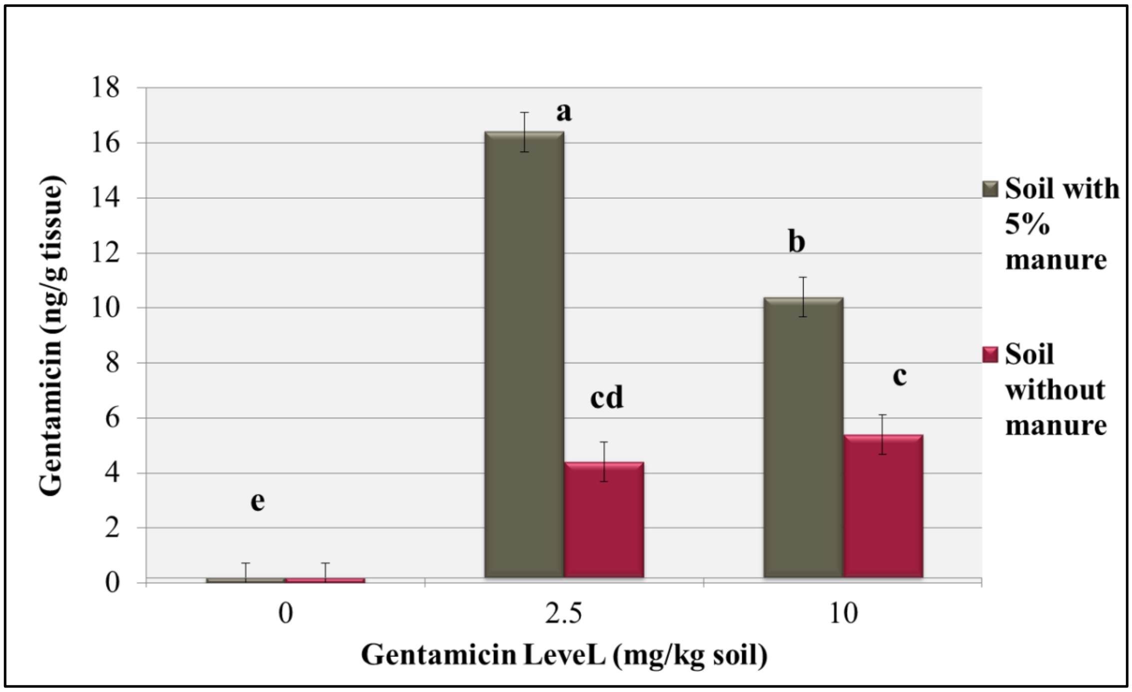

Figure 15. Concentrations of gentamicin in radish roots

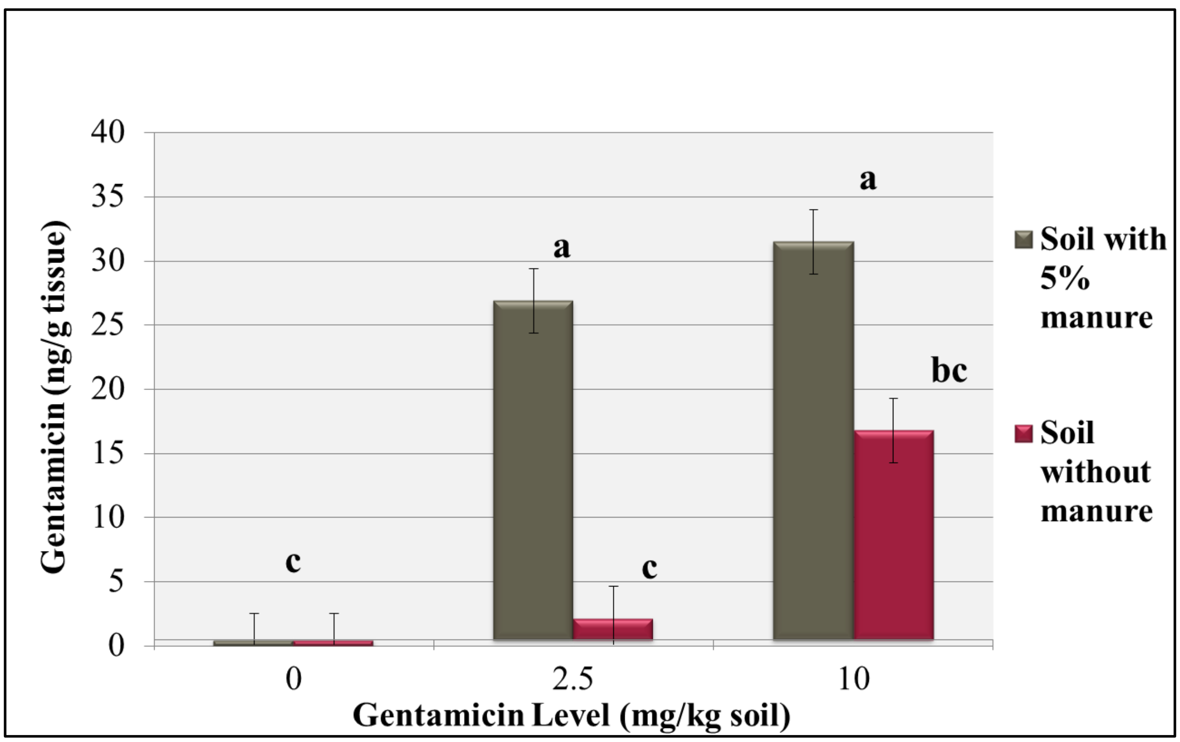

Figure 16. Concentrations of gentamicin in radish leaves

The result of the analysis (Fig. 15) shows that there are significant differences among the concentration of gentamicin in radish root between the control and all the other treatments, regardless of the concentration of gentamicin spiked to the soil and the nature of the growing media. This implies that radish root absorbs gentamicin. However, the absorbance and accumulation in the radish roots were significantly higher from the manure amended soil treatment versus the soil without manure treatment. This matches the finding of Basil et al. (2013) but using lower gentamicin concentrations, stating that in radish, the increase in the concentration of gentamicin was significant between the 0 and both of 0.5 and 1.0 mg/kg treatments, but not significant between the 0.5 and 1.0 mg/kg.

Overall, the concentration of gentamicin in radish root from manure amended soil was significantly higher than that of soil without manure treatments. This proves again that manure boosted the uptake of gentamicin by radish. This is generally in agreement with Basil et al. (2013).

The result (Fig 16) shows that given the two growing media, soil with 5% manure and soil without manure, gentamicin concentration in radish leaves was significantly higher in the manure amended soil. However, there was no significant difference in the gentamicin concentration in radish leaves between the control and the treatments (2.5, 10 mg/kg) grown in soil without manure.

b. Oxytetracycline accumulation in radish roots and leaves

The concentration of oxytetracycline in radish roots and leaves are shown in Figures 17 and 18.

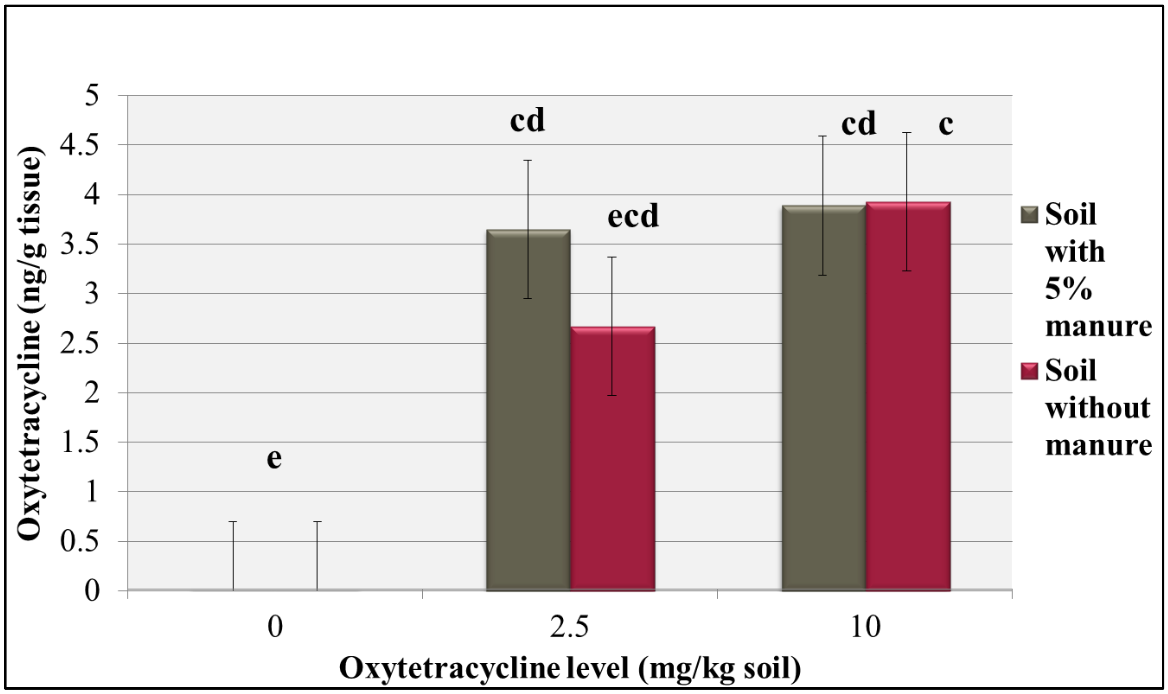

Figure 17. Concentrations of oxytetracycline in radish roots

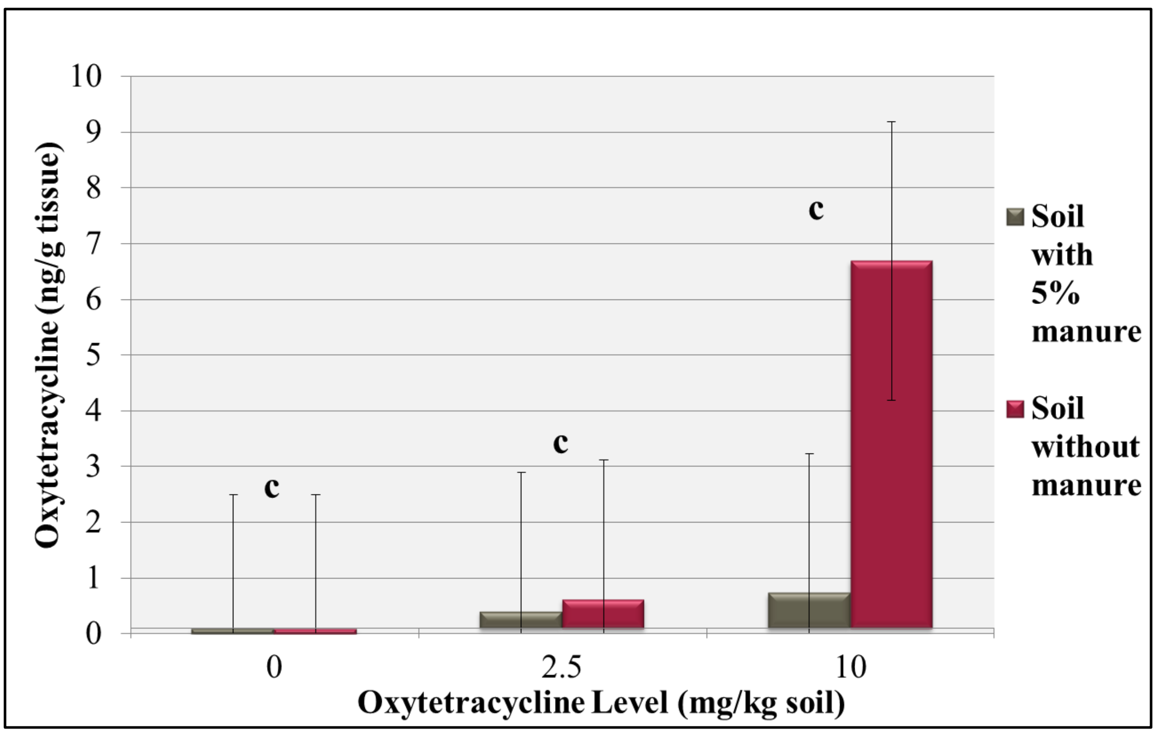

Figure 18. Concentrations of oxytetracycline in radish leaves

The result of the analysis (Fig. 17 & 18) show that there is a significant difference between the control and the other oxytetracycline levels (2.5 and 10 mg/kg) in radish roots grown in manure amended soils. However, there is no significant difference between the control and the oxytetracycline level 2.5 mg/kg in radish roots grown in soil without manure treatment. However, the highest level of oxytetracycline 10 mg/kg leads to a significant increase in the oxytetracycline concentration in radish roots grown in soil without manure.

Nevertheless, the concentration of oxytetracycline in radish leaves was insignificant in all treatments irrespective of the oxytetracycline level or the growing media. Hence, this implies that translocation of oxytetracycline from the radish roots to the leaves didn't not take place at the tested levels. Therefore, all the absorbed oxytetracycline from the growing media was accumulated in the root (edible part).

Overall, oxytetracycline once applied to soil whether with manure or not radish is capable of accumulating it in the roots. This agrees with Xu & Zhang (2014) in their study where they stated that the radish plant can absorb oxytetracycline from the soil (amended with pig manure and spiked with six levels 0, 2, 5, 10, 25, and 50 mg/kg of soil). Moreover, they found that the amount of oxytetracycline absorbed by plants increased with increasing the antibiotic present in the soil. This, by far , is consistent with our results where radish absorbed oxytetracycline at the highest rate.

c. <u>Tylosin accumulation in radish roots and leaves</u>

The concentrations of tylosin in radish plant grown in manure amended soil are shown in figure 19.

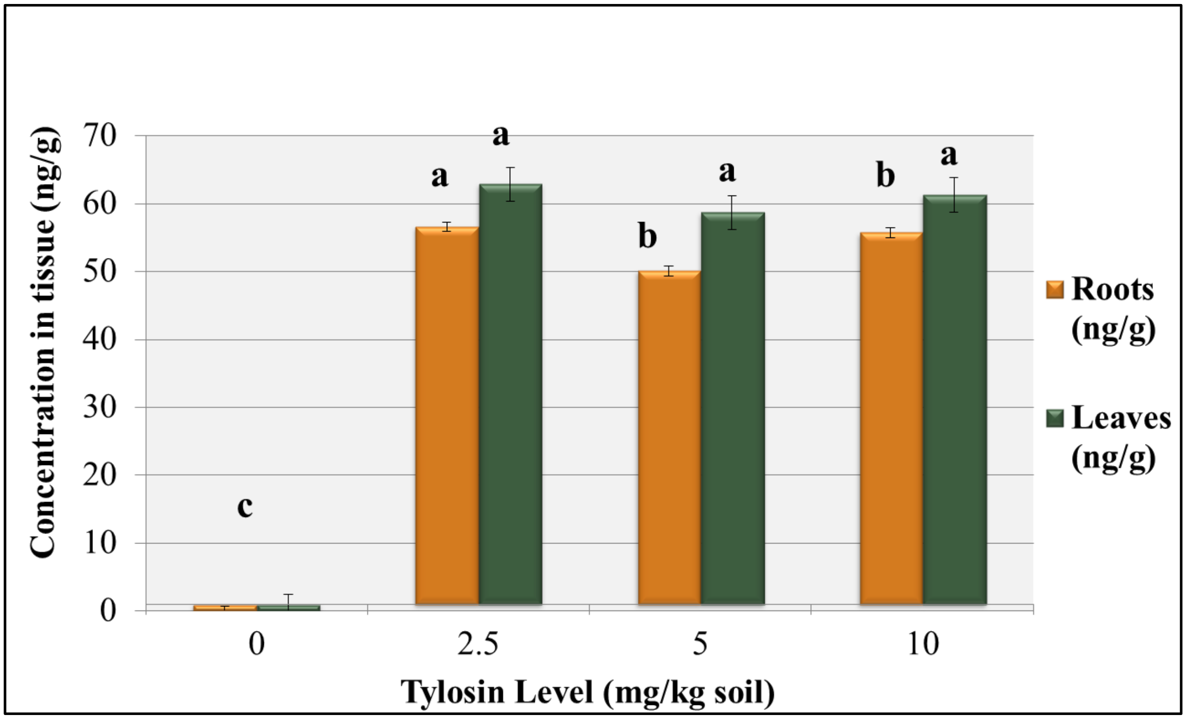

Figure 19. Concentration of tylosin in radish roots and leaves

The result of analysis (Fig.19) indicates that there was a significant difference in the concentration of tylosin in radish roots and leaves between the control and all other treatments.

Increasing the rate of tylosin in the growing media (soil with 5% manure) did not lead to an increase in the concentration of tylosin in radish roots or leaves.

Contrary to oxytetracycline and similar to gentamicin, the translocation of tylosin from the roots to the leaves readily took place at all tested levels.

d. Comparison of tylosin, gentamicin, and oxytetracycline by radish plant

The concentration of tylosin, gentamicin, and oxytetracycline in radish roots and leaves from manure amended soils are shown in figures 20.

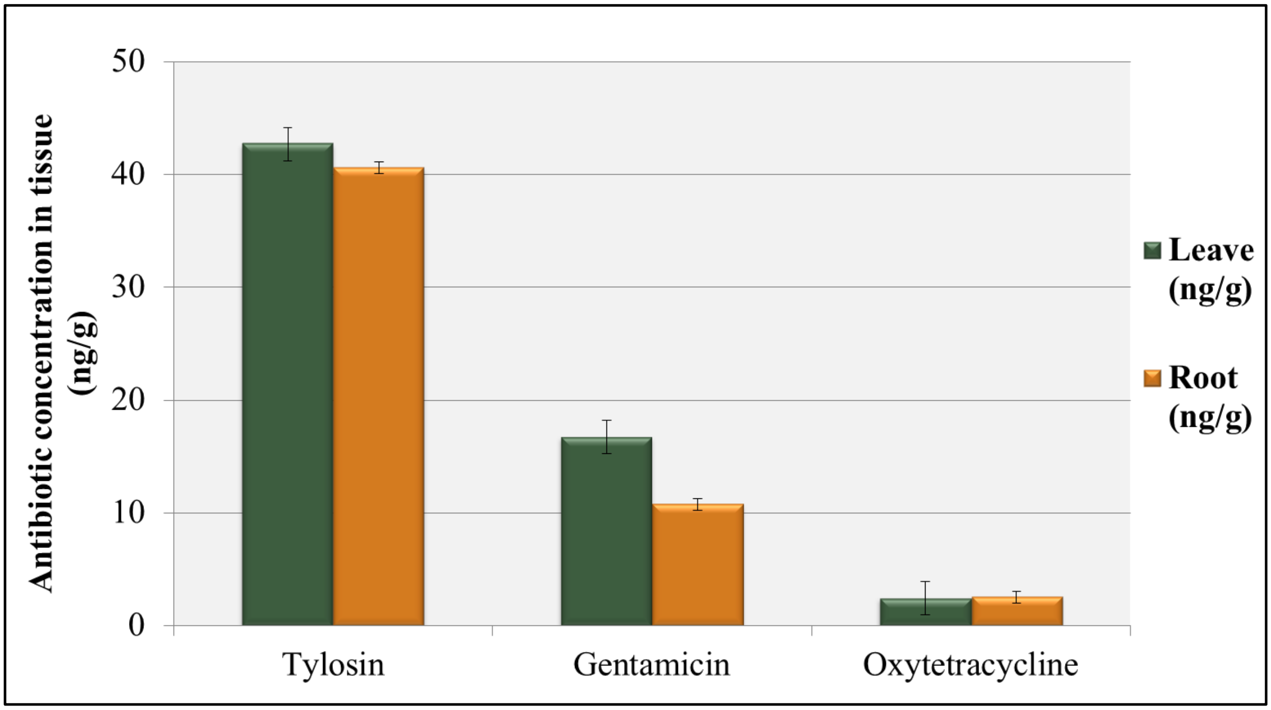

Figure 20. Tylosin, gentamicin and oxytetracycline concentration in radish leaves and roots

The results of analysis (Fig. 20) indicate that the concentrations of tylosin in leaves and roots were higher than gentamicin and oxytetracycline in radish plant irrespective of the treatment. The distribution sequence of accumulation for the three antibiotics is: tylosin > gentamicin > oxytetracycline.

Tylosin was absorbed by the roots of radish and accumulated at a rate of (40.59 ng/g) and translocated into the leaves. Gentamicin was absorbed by the roots of radish at a rate of (10.78 ng/g) and later translocated to the leaves. Basically, oxytetracycline was not absorbed by radish roots nor translocated to the leaves.

Lastly, the concentration of tylosin in radish root tissue was significantly higher than that of gentamicin and oxytetracycline. This might disagree with Kumar et al. (2005) stating that corn, green onions and cabbage took up chlortetracycline but not tylosin.

CHAPTER 5

SUMMARY, CONCLUSION, AND RECOMMENDATIONS

In many countries including Lebanon, veterinary antibiotics are used by farmers not only to prevent diseases but to promote growth as well. These antibiotics eventually find their way to contaminate the food chain by applying antibiotic rich manures to agricultural land. Thus, there is a need to investigate what antibiotics are absorbed by plants and their accumulation in the edible parts.

A pot experiment was carried in the greenhouse to study the absorption of gentamicin, oxytetracycline and tylosin by lettuce and radish plants grown in two media (soil with 5% manure and soil without manure)

The results of the experiment can be summarized in the following points:

For the lettuce plant,

- Gentamicin is absorbed by the roots and translocated to the leaves at an average concentration range of 31.9-56.7 ng/g
- Tylosin is absorbed by the roots of lettuce and partial translocation to the leaves took place at an average concentration range of 2.38-18.27 ng/g
- Oxytetracycline was not taken up by the lettuce plant
- The amount of gentamicin and tylosin absorbed by the lettuce plant increased significantly with the addition of 5% manure to soil

- The accumulation of gentamicin and tylosin in the lettuce leaves did not significantly increase with increasing the antibiotic concentration in the growing media.

For the radish plants,

- Tylosin was significantly accumulated in radish roots with translocation to the leaves at a value of 55.7 ng/g
- Gentamicin was significantly absorbed by the radish roots with translocation to the leaves at an accumulation range of 10.4-16.4 ng/g
- Oxytetracycline was accumulated in radish root with no significant translocation to the leaves.
- Applying manure to the soil results in higher antibiotic accumulation in radish roots compared to soil without manure..

The variation in uptake among the different antibiotics needs to be further explained and studied. Inconsistency in antibiotics concentration levels in lettuce and radish can be a result of the physical and chemical properties of the growing media, the antibiotics, and the plant.

More research should be conducted on the subject covering other antibiotics used in livestock production. Also, it is vital to understand the fate of antibiotic residues in manure, soil, water, milk, meat, etc. that may all lead to ecological risks and health problems. Proper regulation for the use of antibiotics in agriculture should be developed and implemented.

APPENDIX

Table 1: Concentrations of gentamicin in lettuce leaves and roots

	Soil with 5 % manure			**Soil without manure**	
Gentamicin (mg/kg)	**Leaves (ng/g)**	**Roots (ng/g)**		**Leaves (ng/g)**	**Roots (ng/g)**
0	0^{c}	0^{d}		0^{c}	0^{d}
2.5	35.7^{b}	28.6^{b}		10.4^{c}	12.1^{c}
5	56.7^{a}	20.8^{b}		12.2^{c}	6.67^{c}
10	31.9^{b}	26.1^{b}		8.03^{c}	8.08^{c}
SER*	**4.5319**	**2.1777**		**4.3292**	**2.1777**
P value**	**<0.05**				

*SER: Standard Error of Regression

**P value: significant at 5%

Table 2: Concentration of oxytetracycline in lettuce leaves and roots

	Soil with 5 % manure			**Soil without manure**	
Oxytetracycline (mg/kg)	**Leaves (ng/g)**	**Roots (ng/g)**		**Leaves (ng/g)**	**Roots (ng/g)**
0	0^{c}	0^{d}		0^{c}	0^{d}
2.5	2.63^{c}	4.07^{cd}		3.96^{c}	1.54^{cd}
5	2.54^{c}	3.45^{cd}		1.49^{c}	4.79^{cd}
10	6.89^{c}	2.42^{cd}		5.58^{c}	12.3^{c}
SER*	**4.5319**	**2.1777**		**4.3292**	**2.1777**
P values**	**<0.05**				

*SER: Standard Error of Regression

**P value: significant at 5%

Table 3: Concentrations of tylosin in lettuce leaves and roots

	Soil with 5 % manure			**Soil without manure**	
Tylosin (mg/kg)	**Leaves (ng/g)**	**Roots (ng/g)**		**Leaves (ng/g)**	**Roots (ng/g)**
0	0^{c}	0^{d}		0^{c}	0^{d}
2.5	2.38^{c}	9.13^{cd}		2.08^{c}	2.73^{cd}
5	5.48^{c}	49.4^{a}		1.69^{c}	7.25^{cd}
10	18.27^{c}	27.9^{b}		3.83^{c}	6.06^{cd}
SEM	**4.5319**	**2.1777**		**4.3292**	**2.1777**
P values	**P <0.05**				

Table 4: Uptake of gentamicin, tylosin, and oxytetracycline by lettuce's leaves and roots against two different soil treatments (with and without manure) at four different antibiotic concentration levels (0, 2.5, 5, 10 mg/kg)

Lettuce		
Treatment	**Leaves Concentration (ng/g)**	**Roots Concentration (ng/g)**
Antibiotic		
Gentamicin	17.70^{a}	12.07^{a}
Tylosin	3.58^{b}	11.23^{a}
Oxytetracycline	2.71^{b}	3.10^{b}
SEM	**1.608**	**0.773**
Growth Media		
Soil amended with 5% manure	12.83^{a}	13.00^{a}
Soil without manure	4.11^{b}	4.64^{b}
SEM	**1.312**	**0.632**
Antibiotic Level (mg/kg)		
0	0.00^{b}	0.00^{c}
2.5	9.95^{a}	9.56^{b}
5	11.14^{a}	14.76^{a}

10	12.40[a]	13.15[a]
SEM	**1.856**	**0.891**
P values		
Antibiotic	0.0001	0.0001
Manure	0.0001	0.0001
Antibiotic Level	0.0001	0.0001
Antibiotic x Manure	0.0001	0.0001
Antibiotic x Level	0.0001	0.0001
Manure x Level	0.0159	0.0001
Antibiotic x Manure x Level	0.0259	0.0001

Table 5: Concentrations of gentamicin in radish leaves and roots

	Soil with 5% manure			Soil without manure	
Gentamicin (mg/kg)	**Leaves (ng/g)**	**Roots (ng/g)**		**Leaves (ng/g)**	**Roots (ng/g)**
0	0[c]	0[e]		0[c]	0[e]
2.5	26.9[a]	16.4[a]		2.16[c]	4.41[cd]
5	4.34[c]	16.2[a]		10.51[b]	4.92[cd]
10	31.51[a]	10.4[b]		16.8[bc]	5.40[c]
SEM	**2.5093**	**0.7222**		**2.5093**	**0.7222**
P value	**P <0.05**				

Table 6: Concentrations of oxytetracycline in radish leaves and roots

	Soil with 5% manure			Soil without manure	
Oxytetracycline (mg/kg)	**Leaves (ng/g)**	**Roots (ng/g)**		**Leaves (ng/g)**	**Roots (ng/g)**
0	0[c]	0[e]		0[c]	0[e]
2.5	0.40[c]	3.65[cd]		0.62[c]	2.67[ecd]
5	5.49[c]	1.83[ed]		4.85[c]	3.98[cd]
10	0.73[c]	3.89[cd]		6.69[c]	3.93[cd]
SEM	**2.5093**	**0.7222**		**2.5093**	**0.7222**
P value	**P <0.05**				

Table 7: Concentrations of gentamicin, oxytetracycline and tylosin in radish leaves and roots from manure amended soil

	Leaves (ng/g)	Roots (ng/g)
Antibiotic Level (mg/kg)		
Gentamicin (ng/g)		
0	0^{c}	0^{e}
2.5	26.90^{b}	16.4^{c}
5	4.34^{c}	16.2^{c}
10	31.51^{b}	10.4^{d}
Oxytetracycline (ng/g)		
0	0^{c}	0^{e}
2.5	0.40^{c}	3.65^{e}
5	7.85^{c}	2.75^{e}
10	1.09^{c}	3.89^{e}
Tylosin (ng/g)		
0	0^{c}	0^{e}
2.5	62.9^{a}	56.6^{a}
5	58.7^{a}	50.1^{b}
10	61.3^{a}	55.7^{b}
SEM	**2.969**	**1.028**
P Values	**P < 0.05**	

Table 8: accumulation of antibiotic by radish plant from manure amended soil

Treatment	Leave concentration (ng/g)	Root concentration (ng/g)
Antibiotic		
Tylosin	42.70^{a}	40.59^{a}
Gentamicin	16.72^{b}	10.78^{b}
Oxytetracycline	2.45^{c}	2.56^{c}
SEM	**1.491**	**0.515**
Antibiotic Level (mg/kg)		

0	0.00^{c}	0.00^{b}
2.5	25.97^{b}	25.53^{a}
5	21.38^{b}	25.54^{a}
10	35.06^{a}	24.95^{a}
SEM	**1.721**	**0.595**
P Values		
Antibiotic	0.0001	0.0001
Antibiotic Level	0.0001	0.0001
Antibiotic x Antibiotic Level	0.0001	0.0001

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