EVALUATION OF INHIBITORY EFFECTS OF *KHAYA SENEGALENSIS AND VERNONIA AMYGDALINA* EXTRACTS AGAINST SOME BACTERIA ISOLATED FROM POULTRY DROPPINGS

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P14SCMC8030

B.Sc. MICROBIOLOGY (BUK, 2003)

A DISSERTATION SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES,

AHMADU BELLO UNIVERSITY, ZARIA

IN PARTIAL FUFILMENT OF THE REQUIREMENTS FOR THE AWARD OF A

MASTER OF SCIENCE DEGREE IN MICROBIOLOGY, DEPARTMENT OF

MICROBIOLOGY, AHMADU BELLO UNIVERSITY, ZARIA, NIGERIA

DECLARATION

I declare that the work in this dissertation entitled EVALUATION OF INHIBITORY EFFECTS OF *KHAYA SENEGALENSIS AND VERNONIA AMYGDALINA* EXTRACTS AGAINST SOME BACTERIA ISOLATED FROM POULTRY DROPPINGS has been performed by me in the Department of Microbiology, Faculty of Sciences, Ahmadu Bello University, Zaria, Nigeria. The work of other investigators have been duly acknowledge and referenced. No part of this dissertation was previously presented for the award of another degree or diploma at this or any other institution.

AUWAL BELLO MUHAMMAD SIGNATURE…… DATE……

CERTIFICATION

This dissertation entitled **Evaluation of Inhibitory Effects of *Khaya senegalensis* and *Vernonia amygdalina* Extracts Against Some Bacteria Associated with Poultry Droppings** by Auwal Bello MUHAMMAD meets the regulations governing the award of degree of Master of Science of Ahmadu Bello University, Zaria and is approved for its contributions to knowledge and literary presentation.

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DEDICATION

This dissertation is dedicated to the Almighty Allah, the giver of life, Knowledge, wisdom and Understanding and to my lovely parents and family.

ACKNOWLEDGEMENT

I am indebted to Almighty Allah for his infinite mercies and faithfulness towards me. If not for his grace, this would not be possible.

My sincere appreciation goes to my supervisors Prof. O.S. Olonitola and Prof. E.D. Jatau for their untiring support, efforts, encouragement and guidance throughout the period of this work. God will never forget your labor of love and will continually bless and increase you (Ameen)

Special thanks to my parents, Alh Bello Muhammad Shehu and Haj. Z. B. Muhammad for teaching and helping me to be diligent in the pursuit of my dreams and to my wife for her untiring care and concern thanks for your endless love shown in different ways. My lovely brothers like Alh. Umaru Shehu and others, also not forgetting my lovely sisters all may Allah in his infinite mercy continues to bless you and meet you at the point of needs.

My appreciation goes to Mal. M.S. Aliyu, Mal. H.I Doko, DR. A.B. Suleman and DR.M B. Tijjani all of the Department of Microbiology A.B.U., Zaria for their positive criticisms and contributions that make the work a reality, thanks and may Allah bless you abundantly, Also My appreciation goes to the institution Ahmadu Bello University, Zaria and especially the Department of Microbiology for giving me this opportunity to fulfill my dreams. To the head of Department, my lecturers and other staff who have helped increase my knowledge in one way or the other, God bless you all.

My thanks go to my colleague Salisu Danyaro of Veterinary Pathology Department A.B.U., Zaria who assisted me during the course of this research, also not forgetting a laboratory technician of the same Department Mr. Dodo and Pastor Ezekiel Dangana of Pharmaceutical microbiology Department A.B.U Zaria, Alh. Adamu of the Department of Pharmacognosy all of faculty of pharmacy A.B.U., without you, this work would have been more stressful than it was. I say thanks and may Allah always send help whenever you are in need. Ameen.

Special thanks to my colleagues in service for their encouragement and prayers especially the Head of the Department, May Allah bless you all. How can I forget people like Adamu dagona, Muhammad dagona, Baraatu, Rabiatu, Bukky, Uncle Julius and Alh. L.Giwa, who made the time of this study one to remember, may all settle us in all aspects of our lives. Keep the flag flying.

To lovely friends and relatives who showed their care and concern in different ways: Aliyu Ibrahim(Bala), Auwal Inusa, Sambo B.M, Mairiga Alhaji, Bashir Abdullahi, Sani Uba (Dept of chemistry, A.B.U.), Aminu Ibrahim, Bashir mikail, Saidu, Hamza, Hassan, Jamilu, Isah to mention but a few. I am thankful to Allah for you and may Allah bless you and meet you at the point of your need. Amen.

Finally, to all who have contributed in different ways that space will not allowed me to mention, Allah who sees in secret will reward you openly. Thank you, thank you and thank you all.

ABSTRACT

Recen**t** studies have incriminated the use of antibiotics in poultry feeds to be associated with emergence of resistance among bacteria. The use of traditional plants remedies has not been found to be associated with resistance. This work determined the inhibitory effects of extracts of *Khaya senegalensis* and *Vernonia amygdalina* used in traditional poultry farming against *Salmonella* sp, *Escherichia coli, Proteus* sp, *Pseudomonas* sp and *Staphylococcus aureus.* The method of maceration was adopted to extract the plant materials with methanol (70%) and sterile water as solvents for the extraction and phytoconstituents were screened. One gram of each sample (poultry dropping) was inoculated in selenite F broth, peptone water and another one gram each on nutrient broth and incubated at 35^{o}C for 18-24hrs. These overnight cultures were inoculated directly into *Salmonella-Shigella* agar, Brilliant green agar, MacConkey agar, Manitol salt agar and Eosin methylene blue agar (EMB). After incubation at 37^{o}C for 18-24hrs, plates were read. Typical colonies were Gram stained using standard procedures, consequent upon that, the isolated Gram negative and Gram positive organisms were subjected to biochemical tests. Bioassay of the isolates was conducted and further subjected to the plant extracts, using agar well diffusion method and conventional antibiotics using disc diffusion method. Findings show the effect of the plant extracts with MICs/MBCs values of 6.25 for both *Khaya senegalensis* and *Vernonia amygdalina* against *S. aureus, E. coli*, and *salmonella* sp, while the MICs/MBCs values of 12.50mg/ml were obtained against *Proteus* sp, *Pseudomonas* sp. This indicated that it can serve as an alternative to conventional antibiotics as used in poultry production in Nigeria.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the Study

Most of the developing countries including Nigeria rely wholly or partly on traditional herbal medicine for treatment and control of animal and human diseases (Sofowora, 1993). In Nigeria, traditional veterinary practices play important roles in many areas (Kudi and Myint, 1999). Rural poultry farmers are aware of the need to keep birds in good health and when they are sick to source for prescription and procure medicaments for treating rural poultry diseases (Fernandez *et al.,* 2002). They often do so through various means and the use of traditional (indigenous) method of Medicare seems to be the main method of treatment. The use of traditional medicine may be due to its low cost, availability and ease of application compared to modern veterinary medicine (Alders and Spradbrow, 2001).

The unavailability and cost of veterinary drugs in most pharmaceutical shops and local market will have contributed to low patronage by farmers in sourcing drugs for treatment of sick chickens. A study by Mapiye and Sibanda (2005) has shown that large flock sizes were obtained by farmers that gave traditional medicine to their chickens and indicates that traditional medicines in some instances have potential to improve the health status of rural household flocks. Hence, there is a need for researchers to take inventory of common medicaments used by local farmers in treating rural household chickens and determine their chemical properties and routes of application. There has been very little research conducted on medicinal plants used for control although their use is wide spread in the treatment of other livestock diseases (Bywater and Casewel, 2000)). Diseases are the main constraints of

indigenous poultry production in northern Nigeria where Indigenous poultry farming is an integral part of mixed farming (Gibson and Feller, 2002). The birds are raised mostly on low input extensive free-range system of production. Farmers usually have to start with new flock after heavy losses incurred due to disease outbreaks (Fernandez *et al.,* 2002). Due to high cost of conventional medicines and vaccines coupled with the lack of knowledge on their use, these drugs are usually out of reach of the small-scale farmers. There is therefore need for cheap easy to use and sustainable local poultry disease control programs (Gibson and Fellers, 2002). Since ancient times, plants and plant parts have an indispensable source of medicine for indigenous poultry production systems **(**Mwale *et al.,* 2005).

Although modern medical science has developed to a great extent, many farmers in Northern Nigeria depend on plant parts and herbal remedies for indigenous poultry health management. Unfortunately, local medical traditions are being lost because they are communicated orally from generation to generation and are largely un-documented. Very little has been done to verify and validate information gathered. A major concern in poultry production today is the need for reduction in use of antibiotics and other allopathic medicinal products, largely due to concerns over bacterial resistance (Cavanzoni *et al.,* 1998). Alongside this trend, however, is an ever-growing demand for low-priced, high quality food, improved feed hygiene, greater poultry health and welfare and reduced environmental impact (Abdu and Faya, 2000). Many researches has focused on the development of alternative strategies to maintain poultry health and enhance performance within intensive systems, and numerous substances, commonly known as natural growth promoters (NGPs) have been identified as effective alternatives to antibiotics (Atawodi *et al.,* 2000). Photobiotic are NGPs that have been growing in popularity as feed additives, due to their beneficial effect on gut

health and immunity and growth performance (Allen *et al.*, 1998). Photobiotic can be defined as plant derived products added to feed in order to improve performance. They originate from leaves, roots, tubers or fruits of herbs, spices and other plants. They may be available in solid, dried, and ground forms, or as extracts (essential oils). In simple terms, Photobiotic are products of plant origin, and preparations such as thyme, oregano species, turmeric species and garlic are gaining interest among researchers and poultry producers (Abdu and Faya, 2000). The beneficial effects of Photobiotic in poultry may arise from the activation of feed intake and the secretion of digestive enzymes, immune stimulation, antibacterial, coccidiostatic, anthelmintic, antiviral or anti-inflammatory activities, or from antioxidant properties. Many plant secondary metabolites, such as isoprene derivatives, flavonoids and glucosinolates, may act as antibiotics or as antioxidants in vivo. As a result of these beneficial effects, they lead to an increase in performance. Many plants have beneficial multifunctional aspects which are derived from their specific bio-active components (Kamel, 2000). Biologically active constituents of plants are mostly secondary metabolites, such as terpenoids (mono-and sesquiterpenes), steroids, phenolics (tannins), glycosides and alkaloids (present as alcohols, aldehydes, ketones, esters, and lactones). There is a lot of variation in composition due to biological factors such as kind of plant, growing location, harvest conditions, manufacturing factors such as extraction/distillation, stabilization and storage conditions like light, temperature, oxygen tension and time (Atawodi *et al.*, 2000). The challenge is to identify and quantify the multitude of actions and claims, improving feed utilization, animal physiology and health status.

1.2 STATEMENT OF THE RESEARCH PROBLEM

Government programs such as poverty alleviation, in which government invest money in agriculture to increase production in poultry farming has lead to the use of antibiotic to improve poultry farming. This brings about problem of antibiotic resistance which is on the world-wide increase. Due to public concerns over the widespread use of antibiotics in poultry, some producers have started eliminating the use of antibiotics in order to produce and market chicken that may be labeled “antibiotic free”. In 2007, Perdue removed all human antibiotics from its feed and launched products labeled “antibiotic free” under the Harvest land brand. Consumer response was positive, and in 2014 Perdue also phased out ionophores from its hatchery and began using the “antibiotic free” labels on its Harvest land, Simply Smart and Perfect Portions products.

Moreover, herbal remedy in poultry farming has not been fully exploited, though some technical knowledge inherited from past generations have sustained local poultry production but it lacks scientific back up, Moreover, the existing indigenous technical knowledge inherited from past generations has sustained the local poultry production system. This knowledge is passed on verbally and is hardly documented. Although modern medical science has developed to a great extent, use of herbs and plant parts in poultry keeping is already widely practiced in western part of Kenya, on the contrary, such practices are not widely obtainable in Nigeria and therefore the need for research and documentation. Increase medical cost due to drug resistance support alternative approach.

1.3 JUSTIFICATION OF STUDY

The existing indigenous technical knowledge inherited from past generations has sustained the local poultry production. This knowledge is passed on verbally and is hardly documented.

Conventional disease prevention methods are geared towards birds in confinement and not free range in an indigenous poultry production. However, modern medical science has developed to a great extent, use of plants and plant parts in poultry farming is already widely practiced in western part of Kenya (Alder *et al.*, 2001), and there is need for such practices in Nigeria. In addition knowledge of traditional medical practices are being lost because they are communicated orally from generation to generation and are largely un-documented (Sofowora, 1993). There is high need to verify and validate information gathered. Due to high cost of conventional medicines and vaccines coupled with the lack of knowledge on their use, these drugs (Synthetic) are usually out of reach of the small-scale farmers (Bywater and Casewel, 2000). There is therefore need for cheap easy to use and sustainable local poultry farming programs. The above reasons are therefore enough to justify this research(h project.

1.4 AIM AND OBJECTIVES

1.4.1 Aim

The work was undertaken with the purpose of evaluating the inhibitory effects of *Khaya senegalensis*, and *Vernonia amygdalina* extracts against some bacteria associated with poultry droppings.

1.4.2 Objectives

1. To carry out extraction, phytochemicals screening of authenticated *Khaya senegalensis* and *Vernonia amygdalina*.

2. To isolate and characterize bacteria associated with poultry droppings.
3. To determine the susceptibility of the isolates to the plant extracts and conventional antibiotics
4. To assess the potency of the extracts on growth performances of birds raised on the test plant extracts *Khaya senegalensis* and *Vernonia amygdalina* and those raised on the conventional antibiotic (Ciprofloxacin)

CHAPTER TWO

2.0 LITERATURE REVIEW

A great interest has developed in searching for antimicrobial drugs from natural plant products and this interest arises from the belief that, drugs derived from plants are safe and dependable, compared to synthetic drugs.

2.1 **VERNONIA AMYGDALINA**

A member of the Asteraceae family, is a woody shrub or small tree of 2-5 m with petiolate leaf of about 6 mm in diameter and elliptic in shape. The leaves are green with characteristic odour and bitter taste (Bonsi *et al*., 1995a). *Vernonia amygdalina* is a valuable medicinal plant that is widespread in East and West Africa (Burkill, 1985), it is known as bitter leaf, due to its characteristic bitter taste and flavour, and may be used as an active anticancer (Izevbigie, 2003), antibacterial, anti-malaria and antiparasitic agent (Tadesse *et al.*, 1993). This plant contains complex active components that are pharmacologically useful. The roots and the leaves are used in ethno-medicine to treat fever, hiccups, kidney problems and stomach discomfort. The stem and root divested of the bark are used as chew sticks in many West African countries like Cameroon, Ghana and Nigeria (Burkill, 1985, Hamowia, 1994). It is also documented that *V. amygdalina* has been used traditionally in blood clotting and has elicited a significant reduction in blood glucose levels at post-prandial time point (Uchenna *et al*., 2008).

Fasola *et al*.(2010) reported that *V. amygdalina* has hypoglycemic activity. They observed a dose-dependent reduction in fasting blood sugar level in alloxan-induced diabetic rats after treatment with different concentrations of the aqueous leaf extracts. Yedjou *et al*. (2008) also demonstrated that *V. amygdalina* leaf extracts as a DNA - damaging, anticancer agent in the

management of breast cancer. However, much work has not been documented on the antimicrobial effectiveness of the stem extracts of this plant. This research is designed to screen the antibacterial effectiveness of *V. amygdalina* leaf extracts on some bacteria isolated from poultry droppings, *Vernonia amygdalina* belongs to the family Asteraceae and was named after William Vernon a 17th century botanist. It is particularly abundant in grasslands through the tropics with bitter taste. It is a popular leafy vegetable among the Ibos of Eastern Nigeria. The Yoruba name is Ewurojije, Olugbu in Igbo and Shuwaka in Hausa. The shrub is usually about 5m high. The leaves are simple and entire (5x15cm), finely glandular below and displaying few lateral nerves. The flowers occur in a panicle, white and fragrant. It is differentiated from its counterpart *V. colorata*, which grows wildly and with hairy leaves of the latter.(Roura *et al*., 1992; Martin *et al*., 1996; Rassow and Schaper,1996).

V*ernonia amygdalina* have been in many homes in the eastern and Western parts of Nigeria as food especially in the preparation of soups. The peeled stem is often used as chewing stick for cleaning the teeth and is very effective as anticaries. The bitterness of the leaves is also exploited by nursing mothers to assist in weaning their babies by rubbing the juice on their breast. It is also suggest being useful to nursing mother as it improves lactation. The plants leaves and other parts have been used solely or mixed with other plants for the treatment of various illnesses. Interaction with some traditional medicine healers in Ilorin metropolis revealed that the leaves can be extracted raw in aqueous solution and taken as anti-dysentery agent. It is also said to be useful for sugar level control in diabetes patients; a cup full daily is prescribed to patients in serious cases. Despite the usefulness and closeness of *Vernonia amygdalina* to homes, the modern agriculturists regard it as weed on their farmlands (Con Lindsay *et al*., 1987; Vazquez-Moreno *et al*., 1990). Little has been reported about the plant scientifically and almost no notice is given to its antimicrobial property, though its

metabolites are Vernodaline and Vernomygsline. Another of its chemical constituents is sesquiterpenelactone which is made up of vernodaline subunits. This accounts for the bitter taste and has been exploited to discover its antitumor and antimicrobial activities (Mudd, 1998 and Taljanski-Zygmunt *et al*., 1998).



Plate 1: Leaves and Plant of *Vernonia amygdalina*

2.2 KHAYA SENEGALENSIS

Khaya senegalensis (Desr.) A Juss (Family: Meliaceae) the dry-zone mahogany, originates from tropical Africa along a belt approximately parallel to the equator from Senegal, Gambia and Mali in the west through Nigeria and Cameroon to northern Uganda and southern Sudan in the east. It has been successfully planted in Southeast Asia, Australia and a few other places in the tropics. It prefers deep alluvial soils, and is often found growing near rivers and in hollow borders, in areas with annual rainfall between 700 and 1300 mm and a dry season of 4–7 months (Schneitz *et al*., 1998). The tree can grow up to 35 m high. The bark is brownish to grey and splintery; the slash is red and exudes red sap. The leaves are 12–25 cm long, feather-like with 2–6 pairs of glabrous leaflets. Like other species of the Meliaceae family, *K. senegalensis* produces hard, valuable timber that is widely used on a commercial scale, particularly in West Africa. The leaves serve as fodder. Oil can be extracted from the seeds and used for cooking. The bark is used in traditional medicine. The ashes are used as a protective agent for storing millet grains (Schneitzdell, 1998). The flowers are small (5 mm). The fruits are globular capsules, with a diameter of 5–10 cm, which split into four valves at maturity. Each of the four cells contains 6–18 brown, flat seeds with narrow wings. Flowering occurs shortly before or early in the rainy season and insects carry out pollination. The fruits mature when the colour changes from grey to black and they remain on the tree during most of the dry season. The species is becoming vulnerable in many countries due to over exploitation and is now listed in the International Union for Conservation of Nature (IUCN) Red List of Threatened Species (IUCN 2002).

In Burkina Faso, an ex situ conservation programme has therefore been set in motion (Barrow *et al.*, 1992) to support the planting programmes and to sustain the long-term

conservation of this species. However, very little is known about the biology of *K. senegalensis* seeds. According to Schneitzdell (1986), the seeds must be sown fresh after harvest. Similarly, two other species in the *Khaya* genus, i.e. *K. ivorensis* and *K. anthothec*a have been reported to have short lived seeds (Kelley *et al.*, 1994). Studies have been carried out on some Nigerian medicinal plants and these studies have emphasized the antimicrobial activities of *Azadiracta indica (*neem*), Magnifera indica (*mango*), Carica papaya (*paw paw*) and Khaya senegalensis* (Africa mahogany) and pointed out that they are used in various combinations for the treatment of upper respiratory tract infection, diarrhea, abdominal pains and malaria.



Plate 2: The tree and Stem bark of *Khaya senegalensis*

2.3 BACTERIA ASSOCIATED WITH POULTRY FARMS CONTAMINATION

2.3.1 Escherichia coli

Most strains of *E. coli* ferment lactose rapidly and produce Indole. These and other biochemical reactions are sufficient to separate it from the other species. There are over 150 distinct O antigens and a large number of K and H antigens, all of which are designated by number. The antigenic formula for serotypes is described by linking the letter (O, K, or H) and number of the antigens present (e.g. O111:K76:H7) (Abubakar *et al., 2007*).

2.3.1.1 *Pili*

Pili also called fimbriae are frequently present on the surface *of E. coli* strains. Research has shown that some of these structures play a role in virulence as mediators of attachment to human epithelial surfaces. Pili show marked tropism for different epithelial cell types, which is determined by the availability of their specific receptor on the host cell surface. Most *E. coli* express type 1 (common) pili. Type 1 pili bind to the D-mannose residues commonly present on epithelial cell surfaces and thus mediate binding to a wide variety of cell types. More specialized pili are found in subpopulations of *E. coli.* P pili (also called Pap or Gal–Gal) bind to digalactoside (Gal–Gal) moieties present on certain mammalian cells, including uroepithelial cells and erythrocytes of the P blood group. Other pili bind to intestinal cells and have their own set of specificities. Those binding to human enterocytes are called colonization factor antigens (CFAs) or bundle-forming Pili (BFP), depending on the pathogenic type of *E. coli* involved and possibly the cell type in the gastrointestinal tract. The specific binding receptors for the enterocyte binding Pili are not known. The genetics of Pili expression is complex. The genes are organized into multicistronic clusters that encode structural Pili subunits and regulatory functions. Pili of different types may coexist on the

same bacterium, and their expression may vary under different environmental conditions. Type 1 pili expression can be turned "on" or "off" by inversion of a chromosomal DNA sequence containing the promoter responsible for initiating transcription of the Pili gene (Abubakar *et al., 2007*).

2.3.1.2 *Toxins*

Escherichia coli can produce every kind of toxin found among the Enterobacteriaceae. These include a pore-forming cytotoxin, inhibitors of protein synthesis, and a number of toxins that alter messenger pathways in host cells. The β-haemolysin is a pore-forming cytotoxin that inserts into the plasma membrane of a wide range of host cells in a manner similar to streptolysin O and *Staphylococcus aureus* β-toxin. The toxin causes leakage of cytoplasmic contents and eventually cell death. Shiga toxin is named after the microbiologist who discovered *Shigella dysenteriae*, and this toxin was once believed to be limited to that species. It is now recognized to exist in at least two molecular forms released by multiple *E. coli* and *Shigella* strains on lysis of the bacteria. In the years following the discovery of this toxin, the term Shiga toxin was reserved for the original toxin, and others were called Shiga-like. The term Shiga toxin will be used for all the molecular variants that have the same mode of action. Shiga toxins are of the AB type. The B unit directs binding to a specific glycolipid receptor (Gb3) present on eukaryotic cells and is internalized in an endocytotic vacuole. Inside the cell, the A subunit crosses the vascular membrane in the trans-Golgi network, exits to the cytoplasm, and enzymatically modifies 28S-ribosomal RNA of the 60S-ribosomal subunit by removing an adenine base. This prevents the elongation-factor-1–dependent binding of amino acyl tRNA to the ribosome blocking protein syndissertation , leading to cell death. Labile toxin (LT) is also an AB toxin. Its name relates to the physical property of heat

lability, which was important in its discovery and contrasts with the heat-stable toxin. The B subunit binds to the cell membrane, and the A subunit catalyzes the ADP-ribosylation of a regulatory G protein located in the membrane of the intestinal epithelial cell. This inactivation of part of the G protein causes permanent activation of the membrane-associated adenylate cyclase system and a cascade of events, the net effect of which depends on the biological function of the stimulated cell. If the cell is an enterocyte, the result is the stimulation of chloride secretion out of the cell and the blockage of NaCl absorption. The net effect is the accumulation of water and electrolytes into the bowel lumen. The structure and biological effect of LT is very similar to cholera toxin. Stable toxin (ST) is small (17 to 18 amino acid) peptide that binds to a glycoprotein receptor, resulting in the activation of a membrane-bound guanylate cyclase. The subsequent increase in cyclic guanosine monophosphate (GMP) concentration causes an LT-like net secretion of fluid and electrolytes into the bowel lumen (Abubakar *et al., 2007*).

2.3.2 Salmonellae

Salmonella are gram-negative facultative intracellular anaerobes that cause a wide spectrum of disease. This spectrum can range from a gastroenteritis, enteric fever (caused by typhoid and paratyphoid serotypes), bacteremia, focal infections, to a convalescent lifetime carrier state. The type of infection depends on the serotype of *Salmonella* and host factors. It maintains a broad host range and, for unknown reasons, results in different diseases in different hosts. Although the taxonomy of *Salmonella* can be confusing, all salmonella serotypes are members of a single species, *Salmonella enterica*. More than 2500 serovars (Coburn *et al., 2007*)have been described, of which humans are almost exclusively infected by *Salmonella enterica* subspecies *enterica* serotypes Typhi, Typhimurium, and Choleraesuis

worldwide(Chambers *et al*., 2008).In the United States, *Salmonella* serotype Enteritidis (22%), *Salmonella* serotype Newport (14%), and *Salmonella* serotype Typhimurium (13%) account for nearly half of the human isolates (CDC, 2010)

Salmonellosis caused by *Salmonella* Enteritidis is the most common bacterial infections cause of food-borne disease in the United States (Linam *et al., 2007*). Ninety-five percent of cases of *Salmonella* infection are food-borne; however, the incidence of direct contact exposure with animal carriers is on the rise (Linam *et al., 2007*). Once infected, salmonellosis harbors a significant morbidity and mortality. One third of untreated patients experience complications and account for three fourths of deaths associated with salmonellosis (Chambers *et al*., 2008). *Campylobacter* and *Salmonella* are the most common bacterial pathogens found in stool cultures recovered from patients presenting with gastroenteritis or severe diarrhea (Peques *et al., 2009; CDC,* 2010) *Salmonella* has a widespread distribution in the environment and certain host factors make humans particularly susceptible to infection. Its increasing antimicrobial resistance, prevalence, virulence, and adaptability are a challenge worldwide.

2.3**.3 Proteus species**

The genus *Proteus* currently consists of five named species (*P. mirabilis*, *P. penneri*, *P. vulgaris*, *P. myxofaciens*, and *P. hauseri*) and three unnamed genomospecies (*Proteus* genomospecies 4, 5, and 6). The literature abounds with studies on the taxonomy of the genus *Proteus* since the original publication by Hauser, who first described the genus (Sule *et al.,* 2008). The term *Proteus* means "changeability of form, as personified in the Homeric poems in Proteus … and has the gift of endless transformation" (Wang *et al*., 2014).

The genus originally had two species: *P. mirabilis* and *P. vulgaris*, both first described by Hauser in 1885 (Sule *et al.,* 2008). He noted the swarming nature of the organisms and divided the strains into the two species based on the speed of their ability to liquify gelatin: *P. vulgaris* liquefies gelatin "rapidly," and *P. mirabilis* does so "more slowly". Hauser also described "*Proteus zenkeri*," which neither swarmed nor liquefied gelatin, but he rescinded this particular finding 7 years later (Tsai *et al.,* 2014).

In 1894, Theobald Smith reported the fermentative characteristics of the two species using glucose, sucrose, and lactose and their greatly diminished or complete loss of the ability to liquefy gelatin as the culture aged (Atata *et al.,* 2003). The previous year, he had demonstrated the unusually small amount of gas produced from glucose by *P. vulgaris* compared to other then-known enteric organisms.

In 1919, Wenner and Rettger in Connecticut were studying the biochemical characteristics of a larger group of *Proteus* strains. Consistent with Hauser's work of 1885, their strains also swarmed and liquefied gelatin. All of these strains also produced hydrogen sulfide and were lactose nonfermenters (Tsai *et al.,* 2014). The ability to ferment glucose, sucrose, and maltose served as a means to further subdivide the strains into two groups, as Hauser had done. *P. vulgaris* fermented glucose, sucrose, and maltose readily, while *P. mirabilis* fermented glucose readily and sucrose slowly and did not ferment maltose (Tsai *et al.,* 2014).

In 1966, Cosenza and Podgwaite described another new species, *Proteus myxofaciens*, that they isolated from the larvae of the gypsy moth (Wang *et al*., 2014). It produces slime and is not known to have been isolated from a human. The name derives from "myxo" (Greek for slime) and "faciens" (Latin for producing). Until the early 1960s, bacterial classification had been based primarily on cultural observations and phenotypic analysis. After the advent of

techniques such as DNA-DNA hybridization and guanine-plus-cytosine (G+C) determination, which enabled scientists to place new species into their correct genera, biochemical testing remained important but only secondarily to the results of experiments in genetic relationships. It has become clear after years of experience in determining the current taxonomic stature of human isolates that recognizing the correct classification is often a key to ascribing appropriate etiologic relationships to these strains. In a 1978 study of 122 strains, Brenner et al., utilizing this technology, showed for the first time the genetic heterogeneity of *P. vulgaris* (Wu *et al.,* 2015). One group of strains was indole, salicin, and esculin negative and was designated *P. vulgaris* biogroup 1. In 1982, Hickman et al. proposed that this group be renamed *Proteus penneri* in honor of John Penner, the Canadian microbiologist who made many contributions to studies of the three genera of *Proteeae* (Wu *et al.,* 2015).

The mid-1980s saw the advent of high-resolution polyacrylamide gel electrophoresis of proteins with computerized analysis of patterns as another taxonomic tool to identify and type bacteria (Atata *et al.,* 2003). In 1993, Costas et al. in England used this technique to further subdivide *P. vulgaris* into biogroups 2, 3a, and 3b, with the type strain (NCTC 4175/ATCC 13315) being included in biogroup 3a with only one other strain (Helmy and Wasfi 2014).

2.3.4 Pseudomonas species

There are a large number of *Pseudomonas* species, the most important of which is *P. aeruginosa.* The total number of infections produced by the other species is far lower than that produced by *P. aeruginosa* alone. *Pseudomonas species* are most frequently seen as colonizers and contaminants but are able to cause opportunistic infections. The assignment of species names has little clinical importance beyond differentiation from *P. aeruginosa.* Reports vary regarding the frequency of their isolation from cases of bacteremia, arthritis, abscesses, wounds, conjunctivitis, and urinary tract infections. In general, unless isolated in

pure culture from a high-quality (direct) specimen, it is difficult to attach pathogenic significance to any of the miscellaneous *Pseudomonas* species.

Pseudomonas aeruginosa is an aerobic, motile, Gram-negative rod that is slimmer and pale staining than members of the Enterobacteriaceae. Its most striking bacteriologic feature is the production of colorful water-soluble pigments. *P. aeruginosa* also demonstrated the most consistent resistance to antimicrobial of all the medically important bacteria. *P. aeruginosa* produces infection at a wide range of pulmonary, urinary and soft tissue sites, much like the opportunistic Enterobacteriaceae. The clinical manifestations of these infections reflect the organ system involved and are not unique for *Pseudomonas.* However, once established, infections are particularly virulent and difficult to treat. Affected patients almost always have some form of debilitation or compromise of immune defenses (Perez, 2001).

2.3.5 Staphylococcus aureus

Members of the genus *Staphylococcus* are Gram-positive cocci that tend to be arranged in grape-like clusters (Tong *et al.,* 2015). *Staphylococcus aureus* is one of the most common and virulent causes of acute purulent infections worldwide. Other species are common in the skin flora but produce lower grade disease, typically in association with some abridgment of the host defenses such as an indwelling catheter. Characteristic of the group, although *staphylococci* have a marked tendency to form clusters (from the Greek staphyle, bunch of grapes), some single cells, pairs, and short chains are also seen (Waters *et al.,* 2011). *Staphylococci* have a typical Gram-positive cell wall structure. Like all medically important cocci, they are non-flagellate, non-motile, and non-spore-forming. *Staphylococci* grow best aerobically but are facultatively anaerobic. In contrast to *Streptococci*, *Staphylococci* produce catalase. More than one dozen species of *Staphylococci* colonize humans; of these, three are

of major medical importance: *S. aureus, S. epidermidis,* and *S. saprophyticus.* The ability of *S. aureus* to form coagulase separates it from the other less virulent species (Perez, 2001).

2.3.5.1 Morphology and structure

In growing cultures, the cells of *S. aureus* are uniformly Gram-positive and regular in size, fitting together in clusters with the precision of pool balls. In older cultures, in resolving lesions, and in the presence of some antibiotics, the cells often become more variable in size, and many lose their Gram positivity (Waters *et al.,* 2011). The cell wall of *S. aureus* consists of a typical Gram-positive peptidoglycan interspersed with molecules of a ribitol-teichoic acid, which is antigenic and relatively specific for *S. aureus*. In most strains, the peptidoglycan of the cell wall is overlaid with surface proteins, protein A, is unique in that it binds to the Fc portion of IgG molecules, leaving the antigen-reacting portion directed externally. This phenomenon has been exploited in test systems for detecting free antigens. It probably contributes to the virulence of *S. aureus* by interfering with opsonization (Tong *et al.,* 2015).

2.4 CLASSES OF ANTIBIOTICS USED AGAINST COMMOM BACTERIA IN POULTRY KEEPING

2.4.1 Beta-Lactams

Beta-lactams are a wide range of antibiotics, the first of which to be discovered was penicillin, which Alexander Fleming identified in 1928. All beta-lactam antibiotics contain a beta-lactam ring; they include penicillins, such as amoxicillin, and cephalosporins. They work by interfering with the synthesis of peptidoglycan, an important component of the bacterial cell wall, and are mostly used against gram-positive bacteria. Bacteria can, however, develop resistance to beta-lactams via several routes, including the production of enzymes that break down the beta-lactam ring. In the NHS,

penicillins are the most commonly prescribed antibiotics, with amoxicillin being the most common in the class (Hu, *et al*., 2010).

2.4.2 Sulfonamides

Prontosil, a sulfonamide, was the first commercially available antibiotic, developed in 1932. A significant number of sulfonamide antibiotics were subsequently developed defined as broad-spectrum antibiotics capable of acting on both Gram-positive and Gram-negative bacteria. Unlike the beta-lactams, they do not act by directly killing the bacteria, but by inhibiting bacterial synthesis of the B vitamin folate, thus preventing growth and reproduction of the bacteria. In the present day, sulfonamides are rarely used, partially due to the development of bacterial resistance, but also due to concern about unwanted effects such as hepatotoxicity (Hu, *et al*., 2010).

2.4.3 Aminoglycosides

Aminoglycosides inhibit the synthesis of proteins in bacteria, eventually leading to cell death. They are only effective against certain Gram-negative bacteria, as well as some Gram-positive bacteria, but are not absorbed during digestion, so must be injected. In the treatment of tuberculosis, streptomycin was the first drug found to be effective; however, due to issues with toxicity of aminoglycosides, their present day use is limited (Helmy and Wasfi 2014).

2.4.4 Tetracyclines

Tetracyclines are broad-spectrum antibiotics, active against both Gram-positive and Gram-negative bacteria. Like the sulfonamides, they inhibit protein synthesis, inhibiting growth and reproduction of bacteria. Their use is decreasing to increasing instances of bacterial resistance; however, they still find use in treatment of acne, urinary tract, and respiratory tract infections, as well as chlamydia infections. They must be taken in isolation, often two hours before or

after eating, as they can easily bind with food, reducing their absorption (Helmy and Wasfi 2014).

2.4.5 Chloramphenicol

Another broad-spectrum antibiotic, chloramphenicol also acts by inhibiting protein synthesis, and thus growth and reproduction of bacteria. However, it is also bactericidal against a limited number of bacteria. Due to the possibility of serious toxic effects, in developed countries it is generally only used in cases where infections are deemed to be life-threatening, although it is also occasionally used in the treatment of eye infections. Despite this, it is a much more common antibiotic in developing countries due to its low cost and availability, and is recommended by the World Health Organisation as an effective first line treatment for meningitis in those countries with a low income (Helmy and Wasfi 2014).

2.4.6 Macrolides

Much like the beta-lactams, the macrolides are mainly effective against Gram-positive bacteria; however, they act in a bacteriostatic manner, preventing growth and reproduction by inhibiting protein synthesis. Their effectiveness is marginally broader than that of penicillins, and they have been shown to be effective against several species of bacteria that penicillins are not. Whilst some bacterial species have developed resistance to macrolides, they are still the second most commonly prescribed antibiotics in the NHS, with erythromycin being the most commonly prescribed in the class (Helmy and Wasfi 2014).

2.4.7 Glycopeptides

Glycopeptides include the drug vancomycin – commonly used as a ‘drug of last resort’, when other antibiotics have failed. Whilst this used to be the last line of defence against infections, particularly MRSA, the more recent development of newer antibiotics in other classes has provided other options. Nonetheless, there remain strict guidelines on the circumstances in

which vancomycin can be used to treat infections, in order to delay the development of resistance. The bacteria against which glycopeptides are active are otherwise somewhat limited, and in most they inhibit growth and reproduction rather than killing bacteria directly (Helmy and Wasfi 2014).

2.4.8 Oxazolidinones

Oxazolidinones are active against Gram-positive bacteria, and act by inhibiting protein synthesis, and hence growth and reproduction. Linezolid, approved for use in 2000, was the first marketed antibiotic in the class, although the compound cycloserine has been used as a second line tuberculosis treatment since 1956. Whilst linezolid is expensive, resistance seems to be developing relatively slowly since its introduction (Helmy and Wasfi 2014).

2.4.9 Ansamycins

This class of antibiotics is effective against Gram-positive bacteria, as well as some Gram-negative bacteria. They inhibit the production of RNA, which has important biological roles inside the cells of the bacteria, and as such leads to the death of the bacterial cells. A subclass of antibiotics, rifamycins,is used to treat tuberculosis and leprosy. Uncommonly, ansamycins can also demonstrate anti-viral activity (Hu, *et al*., 2010).

2.4.10 Quinolones

Quinolones are bactericidal compounds that interfere with the replication and transcription of DNA in bacteria cells. They are broad-spectrum antibiotics, and are widely used for urinary tract infections, as well as other hospital-acquired infections where resistance to older classes of antibiotics is suspected. Additionally, their use for veterinary purposes is widespread; a use that has been criticised in some quarters for hastening the development of resistance. Resistance to quinolones can be particularly rapid in its development; in the US, they were the most commonly prescribed antibiotics in 2002, and their prescription for unrecommended

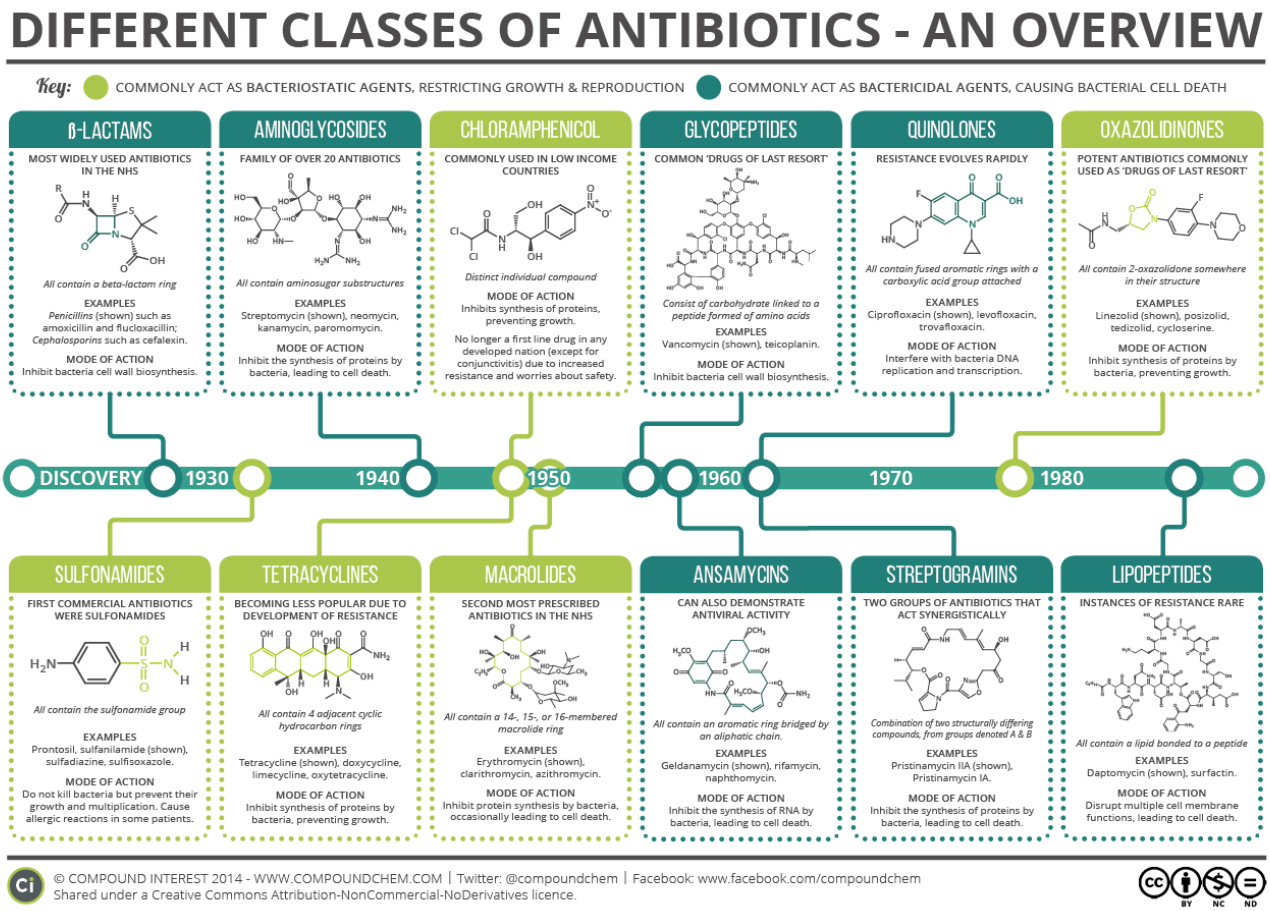
conditions or viral infections is also thought to be a significant contributor to the development of resistance (Hu, *et al*., 2010).

2.4.11 Streptogramins

Streptogramins are unusual in that they are usually administered as a combination of two antibiotic drugs from the different groups within the class: streptogramin A and streptogramin B. On their own, these compounds only show growth-inhibiting activity, but combined they have a synergistic effect and are capable of directly killing bacteria cells, by inhibiting the synthesis of proteins. They are often used to treat resistant infections, although resistance to the streptogramins themselves has also developed (Hu, *et al*., 2010).

2.4.12 Lipopeptides

Discovered in 1987, lipopeptides are the most recent class of antibiotics, and are bactericidal against Gram-positive bacteria. Daptomycin is the most commonly used member of the class; it has a unique mechanism of action, disrupting several aspects of cell membrane function in bacteria. This unique mechanism of action also seems to be advantageous in that, currently, incidences of resistance to the drug seem to be rare – though they have been reported. It is given via injection, and commonly used to treat infections in the skin (Hu, *et al*., 2010).



Source http://www.journals.uchicago.edu/doi/full/10.1086/652237 Compound interest 2014

FIGURE 1: The structures and mode of action of different classes of antibiotics.

2.5 ANTIMICROBIAL RESISTANCE

The continuing success of antimicrobial therapy depends on keeping ahead of the ability of the microorganisms to develop resistance to antimicrobials. At times, resistance seems to occur at a rate equal to that of the development of new antimicrobials.

2.5.1 Risks of Antibiotics in Animal Feed

After animals have been fed antibiotics over a period of time, they retain the strains of bacteria which are resistant to antibiotics. These bacteria proliferate in the animal. Through interaction, the resistant bacteria are transmitted to the other animals, thus forming a colonization of antibiotic resistant bacteria. The bacteria flourish in the intestinal flora of the animal, as well as, in the muscle. As a result, the feces of the animal often contain the resistant bacteria. Transfer of the bacteria from animal to human is possible through many practices (McDonald *et al,* 1997). The primary exposure of humans to resistant bacteria occurs in farms and slaughterhouses. Humans clean the feces, which contain the bacteria, of the animals on farms. During the cleaning process, humans may get bacteria on their body and hands. If the body or hands are not properly cleaned, the bacteria could be ingested by the person. Likewise, in slaughterhouses, during slaughter, the intestine is severed. Resistant bacteria are exposed to slaughterhouse workers, which could get the bacteria on their bodies and hands. Transmission occurs when the bacteria is ingested. Along with the previous sources of contamination, humans can get infected by eating meat from animals with resistant bacteria. Even though cooking reduces the survival of the bacteria, some may still survive and infect the human. For example, 1983, 18 people in four midwestern states developed multi-drug resistant Salmonella food poisoning after eating beef from cows fed antibiotics (McDonald *et al,*1997). After initial transmission and infection to humans, the transmission to other humans

has a couple paths. Transmission can take place through the many mediums (aerosol, physical contact, and bodily fluids) of human contact in the community. An infected individual may also be admitted to a hospital for treatment. Treatment may not work in drug resistant bacteria, therefore, identifying a drug resistant infection. Bacteria is transmitted to other patients via the hospital environment or health care worker=s hands. After transmission, the bacteria will colonize in several of the patients. Colonization in other patients with other resistant bacteria can produce bacteria with multi-drug resistance. Once the patients recover, they are discharged into the community. These patients could potentially infect several community members. Multiple infections could potentially produce a super germ which is resistant to many drugs due to resistance sharing between bacteria (FDA 1995).

2.6 SUSCEPTIBILITY AND RESISTANCE

Deciding whether any bacterium should be considered susceptible or resistant to any antimicrobial involves an integrated assessment of in vitro activity, pharmacologic characteristics, and clinical evaluation. Any agent approved for clinical use has demonstrated in vitro its potential to inhibit the growth of some target group of bacteria at concentrations that can be achieved with acceptable risks of toxicity. That is, the minimal inhibitory concentration (MIC) can be comfortably exceeded by doses tolerated by the patient. Use of the antimicrobial in animal models and then human infections must have also demonstrated a therapeutic response. The influence of antimicrobials on the natural history of different infections (e.g. pneumonia, meningitis, and diarrhea) varies; extensive clinical trials must include both a range of bacterial species and infection sites. With regard to organisms, use of the term susceptible (sensitive) implies that their MIC is at a concentration attainable in the blood or other appropriate body fluids (e.g. urine) using the usually recommended doses (Parks *et al*., 2001).

Resistant which is the converse of susceptible implies that the MIC is not exceeded by normally attainable levels. As in all biological systems, the MIC of some organisms lies in between the susceptible and resistant levels. Borderline strains are called intermediate, moderately sensitive, or moderately resistant, depending on the exact values and conventions of the reporting system. Antimicrobials may be used to treat these organisms but at increased doses, perhaps to reach body compartments where pathogens are concentrated. For example, nontoxic antimicrobials such as the penicillins and cephalosporins can be administered in massive doses and may thereby inhibit some pathogens that would normally be considered resistant in vitro (Novick *et al*., 1981).

2.6.1 Mechanisms of Resistance

The major mechanisms of bacterial resistance are (1) accumulation barriers to antimicrobials due to impermeability or active efflux; (2) alterations of an antimicrobial target, which render it insusceptible and (3) inactivation of an antimicrobial by an enzyme produced by the microorganism. Changes in metabolic pathways can also translate into resistance in a few antimicrobial combinations (Novick *et al*., 1981).

2.6.2 Genetics of Resistance

2.6.2.1 *Intrinsic Resistance*

For any antimicrobial, there are bacterial species that are typically within its spectrum and those which are not. The resistance of the latter group is referred to as intrinsic or chromosomal to reflect its inherent nature. The resistant species have features such as permeability barriers, a lack of susceptibility of the cell wall, or ribosomal targets that make them inherently insusceptible. Some species constitutively produce low levels of inactivating enzymes, particularly the β-lactamases of Gram-negative bacteria. The chromosomal genes

encoding these β-lactamases may be under repressor control and subject to induction by certain β-lactam antimicrobics. This leads to increased production of β-lactamase, which usually results in resistance not only to the inducer but other β-lactams to which the organism would otherwise be susceptible (Novick *et al*., 1981)

2.6.2.2 *Acquired Resistance*

When an initially susceptible species develops resistance, such is called acquired resistance and can be mutational or derived from another organism by the acquisition of new genes using one of the mechanisms of genetic exchange. Of these, conjugation and transposition are the most important and often work in tandem (Novick *et al*., 1981).

Mutational resistance is an acquired resistance which may occur when there is a crucial mutation in the target of the antimicrobial or in proteins related to access to the target (i.e. permeability). Mutations in regulatory proteins can also lead to resistance. Mutations take place at a regular but low frequency and are expressed only if they are not associated with other effects that are disadvantageous to the bacterial cell. Mutational resistance can emerge in a single step or evolve slowly requiring multiple mutations before clinically significant resistance is achieved. Single-step mutational resistance is most likely when the antimicrobial binds to a single site on its target. Resistance can also emerge rapidly when it is related to gene regulation, such as mutational derepression of a chromosomally encoded cephalosporinase. A slow, progressive resistance evolving over years, even decades, is typical for β-lactam resistance related to alter PBPs (Parks *et al*., 2001).

Plasmids and Conjugation the transfer of plasmids by conjugation was the best discovered mechanism for acquisition of new resistance genes, and it continues to be the most important. Resistance genes on plasmids (R plasmids) can determine resistance to one antimicrobial or

to several that acts by different mechanisms. After conjugation, the resistance genes may remain on a recircularized plasmid or less often become integrated into the chromosome by recombination. Of course, resistance is not the only concern of plasmids. A single cell may contain more than one distinct plasmid and or multiple copies of the same plasmid. Although most resistance mechanisms have been linked to plasmids in one species or another, plasmid distribution among the bacterial pathogens is by no means uniform. The compatibility systems that maintain plasmids from one bacteria cell generation to the next are complex. Some species of bacteria are more likely than others to contain plasmids at all. For example, *N. gonorrhea* typically has multiple plasmids, whereas closely related *N. meningitides* rarely has any. Plasmids are most likely to be transferred to another strain if they are conjugative, that is, if the resistance plasmid also contains the genes mediating conjugation. Another factor in the spread of plasmids is their host range. Some plasmids can be transferred only to closely related strains; others can be transferred to a broad range of species in and beyond their own genus. A conjugative plasmid with a broad host range has great potential to spread any resistance genes it carries (Parks *et al*., 2001).

Transposons and Transposition: Transposons containing resistance genes can move from plasmid to plasmid or between plasmid and chromosome. Many of the resistance genes carried on plasmids are transposing insertions which can be carried along with the rest of the plasmid genome to another strain by conjugation. Once there, the transposing is free to remain in the original plasmid, insert in a new plasmid, insert in the chromosome, or any combination of these. Theoretically, plasmids can accomplish the same events by recombination, but the nature of the transposition process is such that it is much more likely to result in the transfer of an intact gene. Transposons also have a variable host range which in general is even

broader than plasmids. Together, conjugation and transposition provide extremely efficient means for spreading resistance genes (Akortha *et al.,* 2002).

Other genetic mechanisms although the transfer of resistance genes by transduction has been demonstrated in the laboratory, its association to clinically significant resistance has been uncommon. Transduction of impanel resistance by wild-type bacteriophages carried by *P. aeruginosa t*o other strains of the same bacteria is a recent example. Because of the high specificity of bacteriophages, transduction is typically limited to bacteria of the same species. Transformation is the most common way genes are manipulated in the laboratory, but detecting its occurrence in the clinical situation is particularly difficult. Plasmids are readily isolated and characterized, and transposes have banking insertion sequences to bag their presence, but there is little to mark the uptake of naked DNA. Molecular epidemiologic studies suggest that the spread of PBP mutations in *Streptococcus pneumonia* is due to transformation, and there may be many more examples awaiting discovery (Wegener *et al*., 1997).

2.6.3 Epidemiology of Resistance

The laws of evolution dictate that sooner or later microorganisms will develop resistance to any antimicrobial to which they are exposed. Since the start of the "antibiotic era," each new antimicrobial has tended to go through a remarkably similar sequence. When an agent is first introduced, its spectrum of activity seems almost completely predictable; some species are naturally resistant, and others are susceptible, with few exceptions. With clinical use, resistant strains of previously susceptible species begin to appear and become increasingly common (Parks *et al*., 2001).

In some situations, resistance develops rapidly, in other cases it takes years, even decades. For example, when penicillin was first introduced in 1944, all strains of *S. aureus* appeared to be

fully susceptible to this antimicrobial. By 1950, less than one third of isolates remained susceptible. Enteric Gram-negative rods rapidly developed resistance to antimicrobial such as Ampicillin, Cephalosporin, tetracycline, Chloramphenicol, and aminoglycosides, with many strains becoming resistant to as many as 15 agents. Fortunately, these developments have not been universal. The spirochete of syphilis and the group A Streptococcus have thus far retained their susceptibility to penicillin. Since ancient times, plants and plant parts have an indispensable source of medicine for indigenous poultry production systems. Although modern medical science has developed to a great extent, many farmers in western Kenya depend on plant parts and herbal remedies for indigenous poultry health management. Unfortunately, local medical traditions are being lost because they are communicated orally from generation to generation and are largely un-documented (Bywater and Casewell, 2000). Very little has been done to verify and validate information gathered. Many antibiotics are used in animal production, especially in the intensive husbandry. Although some of the antibiotics are used therapeutically to improve the health and well-being of animals, a large portion is used for prophylactic purpose as antibacterial performance enhancers (APE), such as improvements in growth rate and feed conversion efficiency. Antibiotic use and antibiotic resistance are clearly connected. Antibiotic resistant bacteria were already identified several decades ago, but while new antibiotics were being discovered at a steady rate, the consequences of this phenomenon were slow to be appreciated (Bezoen *et al*. 1999).

The excessive use of antibiotics compounded by the paucity of new agents has meant the problem of antibiotic resistance is fast escalating into a global health crisis. There is no doubt that the excessive prescription of these drugs in human beings has contributed to the major rates of resistance such as MRSA (Bywater and Casewell, 2000), but recently the use of antibiotics in food animals and its consequence on resistance levels in people has also come

under scrutiny. More controversially, antimicrobials are also used as growth promoters to improve the ability of the animal to convert feed into body mass. It is well recognized that pathogens, such as some zoonotic bacteria (*Salmonella* and *Campylobacter*) and other bacteria are transmitted from animals to human beings via the food chain. These bacteria may possess antibiotic-resistance genes or may transfer their resistance genes to existing susceptible normal human flora (Wegener *et al*., 1997). The main problem is the limited understanding of the nature of the antibiotic resistance and the background levels and distribution of specific antibiotic-resistance genes. Many questions will be difficult to resolve, such as how you distinguish the fraction of resistance in human beings that originated from animals. There is a need for strategies to minimize the risk of spreading antibiotic resistance among the entire ecosystem. However, risk-management solutions are often based on a perceived benefit rather than on actual science. Because of the growing concern over the transmission and the proliferation of resistant bacteria via the food chain, the European Union (EU) decided in 1999 to ban four commonly used growth promoters (virginiamycin, spiramycin, tylosin, and zinc bacitracin). This political solution was taken by invoking the precautionary principle where there are threats of serious or irreversible damage; lack of full scientific certainty shall not be used as a reason for postponing cost-effective measures to prevent environmental degradation. The EU has also signalized its intention to remove the other so-called growth-promoting antibiotics by 2006 (flavomycin and avilamycin/monensin and salinomycin) (Bezoen *et al*. 1999).

Nevertheless, any benefit in antibiotic resistance moderation in animals from the removal of growth promoting antibiotics may be in part offset by resulting disease problems and consequent use of therapeutic antibiotics. The impact of phasing out of animal growth promoters could be minimized provided that adequate attention is given to implement

alternative disease-prevention strategies and management factors, such as alternative husbandry practices in food animal production. The essential role of the animal husbandry is to maintain a consistent supply of healthy animals entering the food chain. Foods borne bacterial infections have a major and perhaps increasing effect on the public health and economy of industrialized countries. It is difficult to determine the exact mortality associated with bacterial infections that are usually food borne. Pathogen specific surveillance systems rarely collect systematic information on outcomes of illness and outcome specific surveillance systems might under-report many pathogen specific conditions. So, there are good reasons to limit agricultural use of antimicrobials, as well as to explore alternatives for the growth promoters that will limit disease status of the animals and food borne transmission of resistant and other pathogens. A wide variety of antibiotics are routinely added to animal feed in sub therapeutic doses for growth promotion of animals produced for human consumption (Siegel *et al*., 1974; Feighner and Dashkevicz, 1987). This practice may lead to a selection of resistant microbial populations including pathogens in the animal's native micro biota and the local environment due to shedding in the feces (Siegel *et al*., 1974, Novick, 1981, Mamber and Katz, 1985; Mashoor *et al*., 1987; Gast and Stephens, 1988; Bastianello *et al*., 1995; Sundin *et al*., 1995).

Normally, in a wild type bacterial population, approximately 2% are resistant to any gave antibiotic (Novick, 1981). However, in bacterial populations isolated from animals exposed to antibiotics on a regular basis, up to 10% have been found to be resistant (Novick, 1981).These resistant bacteria are shed in feces, where they can share extra chromosomal antibiotic resistance plasmids (r-plasmids) with native bacteria and may be disseminated to other animals. Also, antibiotics may accumulate in the tissues of animals and be ingested by consumers whose own resident micro flora may become antibiotic-resistant (Siegel *et al*.,

1974; Novick, 1981; Kobe *et al*., 1995; Richter *et al*., 1996). This antibiotic resistance may lead to problems with antibiotic therapy in humans and other animals (Cooke *et al*., 1971; Novick, 1981, Koenraad *et al*., 1995; Corpet, 1996; Kolawole and Shittu, 1997).

The potential for transmission of agents of food borne illness like *Salmonella* spp., *Campylobacter* species and other enteric pathogens through feed, meat, and wastes of animals is a recognized problem (Johnston,1990; Shane, 1991; Koenraad *et al*., 1995; Koenraad *et al*.,1995).Many other genera have also been implicated in this problem, including *Staphylococcus, Streptococcus, Clostridium, Aeromonas, Pseudomonas,* and *Yersinia* (Goyal and Hoadley, 1979; Dodd *et al*., 1988; Barnhart and Pancorbo,1992; Bongers *et al*., 1995; Corpet, 1996; Kolawole and Shittu, 1997).

Escherichia coli has been frequently implicated in this problem (Mamber and Katz, 1985; Kobe *et al*., 1995), including isolation of resistant species from hospital patients, veterinarians, and the general public (Cooke *et al*., 1971; Bonger *et al*., 1995; Koenraad *et al*., 1995). *Salmonella* species are particularly important because of their prevalence as agents of food borne enteritis (Gast *et al*., 1988). *Salmonella* spp. and their r-plasmids have been studied in poultry processing wastes (Goyal and Hoadley, 1979), and were found to be capable of interspecies r-plasmid transmission (Gast *et al*., 1988), including transfer of antibiotic-resistance to *E. coli*. .*Staphylococcus*, an important agent of enteritis and wound infections in humans (Kolawole and Shittu, 1997), has also been studied in poultry processing plant environments (Dodd *et al*., 1988). Antibiotic resistance of *Pseudomonas aeruginosa* isolated from internal organs of poultry was determined in a study by Koncicki and Szubstarska (1988). *Salmonella* species, *E. coli*, and *P. aeruginosa* were also isolated from broiler litter in a study by Mashoor *et al*. (1987). *Yersinia enterocolitica* was isolated from

poultry litter during litter storage and re-utilization (Kelley *et al*., 1994 and Kelley *et al*. 1995).

Aeromonas hydrophila has been isolated from poultry processing plants, and the antibiotic resistance of these isolates was examined in a study by Barnhart and Pancorbo (1992). *Campylobacter jejuni* has also been isolated from poultry litter and is an emerging agent of food borne enteritis in humans (Shane, 1991; Kelley *et al*., 1994, Koenraad *et al*., 1995). Multiple-antibiotic resistance (MAR) was linked with metal tolerance in a study by Calomiris *et al*., (1984). Their results implied that a simultaneous selection for metal and antibiotic resistance may be occurring in some microorganisms. They reported positive correlations found between copper, lead, and zinc tolerance. Previous research has shown accumulation of copper, zinc and other metals in broiler litter during exposure to multiple broiler flocks (Feighner *et al*., 1987, Kelley *et al*., 1996).

Many antibiotics are used in animal production, especially in the intensive husbandry. Although some of the antibiotics are used therapeutically to improve the health and well-being of animals, a large portion is used for prophylactic purpose as antibacterial performance enhancers (APE), such as improvements in growth rate and feed conversion efficiency. Antibiotic use and antibiotic resistance are clearly connected. Antibiotic resistant bacteria were already identified several decades ago, but while new antibiotics were being discovered at a steady rate, the consequences of this phenomenon were slow to be appreciated. Today, the excessive use of antibiotics compounded by the paucity of new agents has meant the problem of antibiotic resistance is fast escalating into a global health crisis (Barnhart and Pancorbo 1992). The essential role of the animal husbandry is to maintain a consistent supply of healthy animals entering the food chain. Food borne bacterial infections has a major and perhaps increasing effect on the public health and economy of industrialised countries. It is difficult to

determine the exact mortality associated with bacterial infections that are usually food borne. Pathogen specific surveillance systems rarely collect systematic information on outcomes of illness and outcome specific surveillance systems might under-report many pathogen specific conditions (Barnhart and Pancorbo 1992).

2.7 MODE OF ACTION OF ANTIBIOTIC GROWTH PROMOTERS

Antibiotic growth promoters have undoubtedly improved animal performance and health status. Comprehensive reviews of Thomke and Elwinger, (1998) and Rosen, (1995) go into greater detail on the mechanism of antibiotic growth promotion and the resulting animal responses observed. Antibiotic growth promoters exert no benefits on the performance of germ-free animals as an aspect which clearly points to their effect being one of antimicrobial activity rather than being caused by direct interaction with physiology of the animal (Muramatsu *et al*., 1994). The lack of a microbial challenge will obviously limit the response to growth promoters. This challenge is made up of the background environmental micro flora. The nearly sterile intestine of the newly hatched chicken is gradually challenged by several bacterial species attended by colonization and mutual competition. The microbial population depends clearly on the physico-chemical conditions throughout the respective gastro-intestinal segments (Spring, 1998). Poultry in particular has a very rapid nutrient transit through the gastro-intestinal tract. Because of the very low pH in the gizzard and proventriculus, feed entering the duodenum is significantly depopulated of bacteria. The array of digestive enzymes, the high oxygen tension and the presence of high concentrations of antimicrobial compounds such as bile salts in the duodenum further limit bacterial growth in this region of the gut. Further along the small intestine the environment changes and becomes more favourable for bacterial growth because of the lower oxygen tension and the lower

concentrations of enzymes and bile salts partly as a result of the reabsorption and partly from the microbial driven deconjugation of bile salts (Tannock *et al*., 1989).

The development of the microbial population in the more distal part of small intestine depends to a great extent on the availability of nutrients escaping from the endogenous digestion and absorption process; in this respect, the micro-morphology and physiology of the intestinal tract have a predominant impact. Feeding diets with higher concentrations in viscous non-starch polysaccharides (e.g. in wheat, barley and corn) inhibits partly digestion and absorption resulting in a proliferation of the micro flora in the latter part of the ileum (Danicke *et al*., 1999).

On the other hand, the microbial population in the caecum and colon are depending more on the non-digestible but fermentable fraction of the diet (Tannock *et al*., 1989). Of much greater significance in the present context, however, is the knowledge that the microbial population in the gastro-intestinal tract comprises of two distinct populations, one which exists in intimate association with the epithelium of the tract (e.g. lactobacilli species.) and one which occurs free or attached to digest particles within the lumen of the tract. The respective populations have different impacts on performance and health status of the host animal. So, the natural bacterial challenge will interact with the activity spectrum and effective dose rate of the various growth promoters. The response to such products, however, is variable and may, to a large extent, be dependent upon several factors, such as the environment and type of diet depending on viscosity-enhancing components (Manner and Wang, 1991).

The growth promoters are largely unabsorbed in general because of their relatively high molecular weight from the intestine at the dietary concentrations usually used; exception for the ionophore antibiotics whereby a withdrawal period of 3-5 days has to be taken into

account. The main site of antibiotic activity is within the gastro-intestinal tract. A number of antibiotics have the characteristic of growth promoting despite differences in their mechanism in acting on microorganisms (e.g. differing in inhibitory concentrations). It is apparent that performance promotion functions by modifying mainly the intestinal "Gram positive" species because of their much less complex cell wall structure (mainly consisting of peptidoglycan and both teichoic acid and teichuronic acid) than of the Gram negative species with e.g. lipopolysaccharides. The growth promoters are balancing the different microbial species within the alimentary tract and are compensating for the adverse effects of the intestinal micro flora on animal efficiency through the following mechanisms: modifying the gut wall structure with considerable changes in ultra structure, limiting the turn-over of the intestinal epithelial layer with sparing effects on the metabolic energy costs to maintain the gut, sparing nutrients by inhibition of fermentative losses and competition with the host for nutrients in the intestinal tract, enhancing absorption and metabolic ability (Huyghebaert, 1996; Huyghebaert and De Groote, 1997), up-regulating the immune response (Roura *et al*., 1992; Devrieze *et al*., 1993), reducing secondary diseases by e.g. *E. coli* (polyserositis) and *Clostridium perfringens* (necrotic enteritis).

Avilamycin and flavomycin are the two remaining growth promoters on the EU-feedstuffs market; avilamycin has a more pronounced inhibiting effect on *Clostridium perfringens* (Manner and Wang, 1991). Furthermore, some of these antibiotics such as Zn-bacitracin appear to improve the post absorptive metabolism in terms of heat tolerance (Manner and Wang, 1991). Estimates by (Rosen, 1995) and (Thomke and Elwinger (1998) suggest that the average benefit of antibiotic growth promoters is an improvement of approximately 3-4%, with a quite wide range of 0-8%.There are many possible ways to influence the intestinal micro flora population once the antibiotic growth promoters are removed. The obvious

method is the use of therapeutic doses of antibiotics under prescription, a practise which will undoubtedly increase and ironically probably increase the likelihood of the emergence of resistant human pathogens (Abubakar *et al.*, 2007).

As far as non-antibiotic measures are concerned, however, not one of the suggestions offered below will fully compensate for their removal. It must be emphasised that some strategies are only measures that will help to compensate partially for, but not replace, antibiotic growth promoters, and will work through indirect mechanisms. The need is, however, by no means exhaustive and there are also other products claiming to be of value in antibiotic growth promoter-free diets. Non-starch polysaccharides (NSP) in animal feedstuffs are a complex group of components differing widely in chemical composition, physical properties and physiological activity (Armstrong, 1986 and Boorman, 1987).

The NSP includes (hemi) celluloses, pectines and oligosaccharides and furthermore arabinoxylans and β-glucans consisting of either a more soluble or a non-soluble fraction. The main low Oxygen-carotenoid level cereals, wheat and barley contain variable NSP-levels with concomitant differences in chemical composition such as arabinoxylans and β-glucans. The mechanism by which NSP exert their anti-nutritive effects is complex. There is a direct effect by either encapsulating nutrients and or depressing digestion through gastrointestinal modifications. The viscous nature of the NSP in the primary cause for their anti-nutritive effect in poultry, because of the increased bulk and viscosity of the intestinal contents decrease the rate of diffusion of substrates and digestive enzymes with a more specific effect on lipids and hinder their effective interaction at the mucosal surface (Choct *et al*., 1996).

In addition to the direct effect of viscous NSP on gut physiology and morphology, there appear to be some indirect effects that could have important implications for the efficient utilisation of nutrients by the chicken (Danicke *et al*., 1999). One such indirect effect may be

related to the gut micro flora, their activity can be measured by the intestinal concentration of Volatile fatty acid (VFA) e.g. acetic acid, propionic acid, butyric acid). Under normal circumstances with low NSP-diets, facultative anaerobes dominate in the small intestine and nearly strict anaerobes such as *Clostridium perfringens*; make up the entire caecal microflora of the chicken (Salanitro *et al*., 1978). Bacteria colonize the epithelium of the small intestine as well as the caeca. On a NSP-rich diet, the VFA-concentration increased only in the ileal lumen due to an excess fermentation combined with a proliferation of the fermentative micro flora, without any effect on the activity of the hindgut micro flora. On the other hand, supplementing the NSP-rich diet with enzymes results in both a reduction of the ilea VFA-concentration and an elevation of the caecal VFA-concentration (Choct *et al*., 1996); more 'low molecular weight fermentable material is entering the caecum. In poultry, the energy contribution from these VFA is not high. The favourable mechanisms of dietary NSP-enzymes can be described as follow: By reducing the viscosity of the digest in the small intestine, digest passage and nutrient digestion rate increases, thereby giving less substrate and less time for the fermentative organisms to proliferate. This in turn may restore the normal and efficient endogenous enzymatic digestion of nutrients in the small intestine. Enzymes are counterbalancing partially the adverse effects of soluble NSP on zoo technical performance (Bedford and Classen, 1992; Huyghebaert, 1995).

There is also an inverse correlation between the nutritive value of wheat and the corresponding bio-efficacy of the NSP-enzymes (Huyghebaert and Lippens, 2002). Moreover, the bio-efficacy of the NSP-enzymes is correlated with the fat-level and degree of saturation of the wheat based-diets (Huyghebaert, 1997and Danicke *et al*., 1999). It is however, not possible to estimate the relative contribution of the improved nutrient utilisation as well as the

selective reduction in microbial population. But there is evidence that the consequence of a NSP mediated reduced rate of digestion is much more radical in the presence of intestinal micro flora than in their absence, being caused by the degradation of both digestive enzymes and bile salts and colonisation of the absorptive surface area (Smits and Annison, 1996). In the EU, in the absence of antibiotic growth promoters, there will be a greater response to enzymes, particularly in less well-digested diets (Elwinger and Shane, 1991). Furthermore, the ultimate effect of a more balanced intestinal flora might reduce the proliferation of some pathogenic bacteria such as *Clostridium perfringens* and *E. coli*. The VFA mainly propionic acid reduce the caecal carriage of *Salmonella*, *Campylobacter* and *Clostridium* (Apajalahti and Bedford, 1999; Bedford, 2000).

2.8 ORGANIC ACIDS USE IN POULTRY FEED MANUFACTURING FACTORIES

Organic acids are widely distributed in nature as normal constituents of plants or animal tissues. They are also formed through microbial fermentation of carbohydrates predominantly in the large intestine of poultry. Many of them are also available as sodium, potassium or calcium salts. The advantage of salts over acids is that they are generally odourless and easier to handle in the feed manufacturing process owing to their solid and less volatile form (Annison, 1991). They are also less corrosive and may be more soluble in water. Rather than dietary acidifiers, organic acids are better known as effective preservatives. Their primary antimicrobial action (strain-selective growth inhibition or delay) is through pH depression of the diet and the drinking water as a rather in-vitro activity the pH-reduction the optimal pH-range of the microbes the direct in cell-effect on the microbial population and a reduction of the buffering capacity of the feed with subsequent effect on the physiology of the proventriculus (Annison, 1991).

The in-vivo action is mainly based on the same mechanisms acidification of the stomach with favourable effects on pepsinogen to pepsin conversion and de-loading the chymus from micro-organisms depending on pH-range and type of micro-organism. However, more importantly is the ability of organic acids to change from undissociated to dissociated form, depending on the environmental pH, makes them ineffective antimicrobial agents. When acid is in the un-dissociated form it can be freely diffuse through the semi-permeable membrane of the micro-organisms into their cell cytoplasm (Adams and Hall, 1988; Shane, 1991). Once in the cell, where the pH is maintained near 7, the acid will dissociate and suppress cell enzymes (e.g decarboxylases and catalases) and nutrient transport systems. The efficacy of an acid in inhibiting microbes is dependent on its pKa value, which is the pH at which 50% of the acid is dissociated. Organic acids with higher pKa values are more effective preservatives and their antimicrobial efficacy is generally improved with increasing chain length and degree of un-saturation. The input factors are: pKa-value of the acid, organic or inorganic form, chemical form (acid, salt, coated or not), molecular weight as indication for its equivalence, MIC-value of the acid (0.1-0.15% for formic acid, 0.15-0.20% for propionic acid, 0.25-0.40% for lactic acid), kind of micro-organism (bacteria, moulds, yeasts with their specific pH-range), animal species, site and location in the gastro-intestinal tract with typical microbial activity and buffering capacity of the feed (Corpet *et al.,* 1996).

Micro-encapsulating the fatty acids with a special slow release matrix are essential for their anti-microbial activity throughout the distal part of the gastro-intestinal tract. Furthermore, there are indications that the medium chain fatty acids with a chain length between 6 and 12 carbon atoms, e.g. caproic acid with a lower absorption rate by the host may improve the efficacy of the short fatty acids (Simon and Jadamus, 2002). The effects of the acid are higher than the salt, because the acid has acidification in combination with the anion effects. Formic

acid is an effective acidulant by inhibiting microbial enzymes. Overall, the antimicrobial activity of formic acid is primarily against yeasts and some bacteria (e.g. *E. coli* and *Salmonella*), whereas lactic acid (LA) bacteria and moulds are relatively resistant to its effects. As a preservative, acetic acid inhibits the growth of many species of bacteria, and to a lesser extent of yeasts and moulds. The action of propionic acid is primarily against moulds (Patten and Maczulak, 1988; Thompson and Hinton, 1997; Ratcliff, 2000).

It has only a poor activity against bacteria and none against yeasts, since the latter can metabolize it. The MIC of formic acid against *E. coli* is five times higher than of acetic and propionic acid. The antimicrobial action of lactic acid is directly primarily against bacteria, whereas many moulds and yeasts can metabolize it. Citric acid is a generally less effective antimicrobial agent than the other organic acids, partly because many micro-organisms can metabolize it and also because of the low pKa. Malic acid is active against some bacteria and yeasts. Ascorbic acid inhibits yeasts, moulds and some bacteria. Normally, mixtures of organic acids are used, which makes their spectrum broader and combines the good qualities of the different acids. The ultimate effect of acids might induce a more balanced intestinal flora by reducing the proliferation of some pathogenic bacteria. Huyghebaert *et al*. (1999) demonstrated that a mixture of organic acids added to a negative control diet in comparison with a positive control diet on Zn-bacitracin at 50 mg/kg could only partially compensate for the higher polyserositis (pathogenic *E. coli*) related mortality in broilers.

On the other hand, Huyghebaert (2002) observed under more optimal environmental conditions a similar nutritive value for a coated acid mixture (a blend of inorganic acids with a protection matrix of fatty acids, silicate and phosphorglucopeptides) as for flavomycin (with a dosage of 15 mg/kg). It might be concluded that in comparison with antibiotics organic acids have a more limited and variable impact on the microbial population. Furthermore, the

possibility that the provision of additional organic acids in the feed may act as a rapidly absorbed energy source cannot yet be ruled out. It is worthwhile noting that organic acids reduce the shedding of *Salmonella* and *Campylobacter* ((Simon and Jadamus, 2002).

2.9 PREBIOTICS USED IN POULTRY FEEDS

Prebiotics can be defined as non-digestible feed ingredients with selective effects on the intestinal microflora. The oligosaccharides (OS) are the main components belonging to Prebiotics. The range of oligosaccharides used is diverse and may be based on any of the hexose monosaccharides including glucose, fructose, galactose and mannose (Durst, 1996) with a polymerisation degree between 2 and 20 monosaccharides. Grain legumes are the most common natural sources of oligosaccharides, being present as raffinose, stachyose and verbascose. Synthetic oligosaccharides are derived from the direct polymerisation of dissaccharides or from the fractionation of both vegetable and microbial cells (Barrow. 1992). OS such as arabinogalactoside, arabinoxylan and rhamnogalacturonose are derived from polysaccharides of respectively soybean (with about 3-5% galacto-oligosaccharides), wheat and fruit. Prebiotics are non-digestible feed ingredients, which beneficially affect the host by selectively stimulating the growth or metabolic activity of a limited number of intestinal microflora, e.g. Bifidobacteria and Lactobacillus sp. (Gibson and Roberfroid, 1995). Substrates like inulin, fructo-oligosaccharides (FOS) and mannan-oligosaccharides (MOS) (derived from yeast bacteria cells), which are neither hydrolysed by the endogenous digestive enzymes nor absorbed by the host have been identified as prebiotic agents (Newman, 1986; Gibson and Fuller, 2000; Amagase *et al*., 2001).

The possible mechanisms are: decrease in pH (production of short chain fatty acids), secretion of bacteriocin-type substances and immune-stimulation. The lack of a beneficial effect might be related to the non-specificity of the process of hindgut fermentation. When ingested they stimulate the growth and/or the metabolic activity of different bacterial species, including species that are both potentially harmful and beneficial (Maczulak *et al*., 1993). Results obtained from synthetic materials tend to suggest some benefit. Inulin and FOS act as substrates for 'desired' micro-organisms (Amagase *et al*., 1993; Iji and Tivey, 1998), whereas MOS have receptor properties for fimbriae of *E. coli* sensitive to mannose and *Salmonella* sp. which leads to elimination of these bacteria with the digesta flow instead of binding to mucosal receptor (Maczulak *et al*., 1993; Spring *et al*., 2000; Fernandez *et al*., 2002). In contrast with these latter species Clostridia do not express type-1-fimbriae and as a consequence there are other mechanisms such as: aggregation/'cleaning-up by attaching' and modulation of the immune responses. Simon and Jadamus (2002), accentuated that the fermentation of inulin is a characteristic of Bifido bacteria, but also of both Enterobacteriaceae and Streptococcaceae. Baily, *et la.,* (1991) concluded that there exists an interaction between dosage and chemical structure of the oligosaccharides and bacterial species concerning the ultimate colonisation as a so-called structure-function relationship. The response in zoo technical performance is quite variable (Sims *et al*., 1998; Simon, and Jadamus, 2002).

In poultry lactose can also be considered as a prebiotic because of the absence of the endogenous lactase; the microbial fermentation in the hindgut can convert lactose into lactic acid, which results in a decrease of the intestinal pH, which in turn results in a reduction of the concentration of both *Clostridium* and *Salmonella typhimurium* (Maczulak *et al*., 1993). One approach that may be encouraged for future research is the combination of both probiotics and

prebiotics as symbiotic, which may defined as a mixture of Probiotics and Prebiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the gastro-intestinal tract, by selectively stimulating the growth and/or by activating the metabolism of one or a limited number of health-promoting bacteria, and thus improving host welfare'. Another approach that may be encouraged for future research is the combination of both Prebiotics or Probiotics and organic acids (as pH conditioner) as symbiotic (Parks *et al*., 2001).

2.10 HERBS AND ETHERIC OILS USED IN POULTRY FEEDS

Many plants have beneficial multifunctional aspects which are derived from their specific bio active components (Kamel, 2000). Biologically active constituents of plants are mostly secondary metabolites, such as terpenoids (mono-and sesquiterpenes, steroids), phenolics (tannins), glycosides and alkaloids present as alcohols, aldehydes, ketones, esters, ethers, lactones,. There is a lot of variation in composition due to: biological factors (kind of plant, growing location, and harvest conditions), manufacturing factors (extraction, distillation, and stabilisation) and storage conditions (light, temperature, oxygen tension and time). There is a lot of experimental data showing the in-vitro anti-microbial activity with respective MIC-values and specificities; e.g. Garlic is inhibitory against *E. coli, Salmonella* sp., *Campylobacter* sp., *Staphylococcus aureus* and *Clostridium perfringens* than against lactic acid bacteria. Even though most suppliers of feed grade products based on plant extracts are careful not to use this evidence due to the legislative constraints, their effects cannot be ignored. According to Adams (1999), the antimicrobial activity is rather weak for ginger and pepper, medium for cumin (p-cymene), coriander (lialol), oregano(carvacrol), rosemary (cineol), sage (cineol) and thyme (thymol) and strong for clove (eugenol), mustard

(allylisothiocyanate), cinnamon (cinnamaldehyde) and garlic (allicin). The main components in oregano-oil are carvacrol (60-80%) and thymol (2%) patented as Ropadiar, carvacrol inhibits the metabolism of the microbial cell wall (Langhout, 2000).

The anti-microbial activity in garlic is mainly mediated through the activity of allicin, which inhibits the growth of many bacteria, yeasts and fungi (Amagase *et al*., 2001). The broad spectrum mechanism of these components (as a thiosulphonate) is based on the inactivation of the Containing microbial enzymes. Cinnamon also has an anti-microbial an antifungal activity through the activity of cinnamaldehyde. The products of Enter guard are combining garlic and cinnamon substances, whereby Enterguard-starter has a high allicin or cinnamaldehyde-ratio while Enteroguard-finisher has a high cinnamaldehyde/allicin-ratio between. According to Sarma and Sapcota (2000) a polyherbal compound is very efficient in improving the zoo technical performance of broilers. The dietary supplementation with Crina (patented etheric oil) had favourable effects in broilers in terms of digesta viscosity, %-sticky droppings, intestinal concentrations of *Clostridium perfringens* (Langhout *et al.*, 2000).

The results of a balance trial with broilers shows that both flavomycin and Crina had a very significant effect on the dietary content being mainly related to an increased digestibility of the dietary fat, the higher fat digestibility might be explained by a lower ileal microflora mediated deconjugation of the bile salts (Feighner and Dashkevicz, 1987; Tannock *at al*., 1989; Huyghebaert, 1999). Langhout (2000) suggested combining organic acids (with activity in the feed and in the crop and stomach) and etheric oils (with activity in the more distal part of the intestinal tract).The main botanical additives are encompassed by the EU-legislation Directive 91/248, in group C. All natural products may be included in animal feeds without any limitations. Regarding the botanical additives there is consequently no well defined

legislation. In fact, herbal products should undergo the same procedure for definite standards, efficacy and safety testing as other additives before being allowed to enter the feed chain. The ultimate quality criterion of herbs, however, is the presence of chemical constituents that confer a health benefit (Langhout 2000). For most herbs, a range of compounds has been ascribed with biological activity with often-unresolved synergistic effects between individual compounds (Huyghebaert, 1999).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 COLLECTION AND IDENTIFICATION OF THE PLANT MATERIALS

Leaves of *Vernonia amygdalina* Del (bitter leaf) and stem bark of *Khaya senegalensis* (Desr.) A Juss (Family: Meliaceae) (African mahogany) were collected at three different locations in Makarfi local government. Identification of the plant was aided by local people in the area and confirmed by Botanist in the Department of Biological Sciences Ahmadu Bello University, Zaria. The voucher numbers of 675 and 900181 were assigned to the leave and stem bark of *Vernonia amygdalina* and *Khaya senegalensis respectively* and they were deposited at the reference catalogue of herbarium plants section of the Department of Biological sciences, Ahmadu Bello University, Zaria. The plant materials were dried under shade and ground to powder and dispensed into different containers and labeled appropriately ready for extraction at the Department of Pharmacognosy, Ahmadu Bello University, Zaria.

3.2 EXTRACTION AND PREPARATION OF PLANT MATERIALS *KHAYA SENEGALENSIS* AND *VERNONIA AMYGDALINA*

Maceration method was employed for the extraction of plant active constituents, and methanol (70%) and water were used as solvents for the extraction of the plant materials. The method of maceration was adopted (Salisu *et al.,* 2008). Twenty (20) grams each of ground samples of leave/stem bark extract of *Vernonia amygdalina* and *Khaya senegalensis* plants were suspended separately into distilling flask, 100ml of sterile distilled water was added to rinse and 100ml of 70% methanol, was added as solvent for extraction for a period of about 72hours on a retort stand and the extracts were collected into a beaker under the flask. The extracts were collected and filtered separately and the filtrates were evaporated over a boiling

water bath at 45^{O}C. The crude extracts were then scraped off from the bottom of the evaporating dish and dispensed into separate sterile capped containers, labeled and stored as reserved stock concentrate (*Salisu et al., 2008*).

3.3 PHYTOCHEMICAL SCREENING OF THE PLANT EXTRACTS.

Phytochemical screening using simple chemical tests as described by Silva *et al.,* (2004) were performed to detect the presence of carbohydrates, flavonoids, Glycosides, saponins, tannins, alkaloids and steroids in the plant extracts. The tests were carried out as follows:

Test for carbohydrates (Molisch test); A few drops of Molisch reagent were added to two cubic centimeters of test extract in a test tube. One milliliter of concentrated tetraoxosulphate VI acid was allowed to run down the side of the inclined test tube so that the acid forms a layer beneath the aqueous solution without mixing it. The appearance of a reddish brown colour indicates a positive test.

Test for Flavonoids: To the alcoholic solution of the extracts, magnesium powder and few drops of concentrated HCL were added. Formation of orange or pink colours indicated positive while absence indicated negative results.

Test for Glycosides: To 50mg of the methanolic extracts, 2mls of chloroform was added and few drops of conc. H_2SO4 were also gradually added by the side of the test-tube. A brown ring at the inter phase indicated positive while absence indicated negative results.

Test for saponins; to about 0.5g of the powder in a test tube, 5mi of distilled water was added and shaken vigorously. A persistent froth that lasted for at least 15minutes indicated the presence of saponins.

Test for tannins; A few drops of $FeCl_3$ (5%w/v) solution was added to the extract in a test tube and shaken. A dark-blue color indicated a positive result.

Test for alkaloids; A small portion of the acidified extract solution was subjected to reactions with Wagner's reagent, a reddish brown precipitate indicated the presence of alkaloids.

Test for steroids; one cubic centimeter of concentrated H2SO4 was added to one cubic centimeter of test extract. A red colour indicates the presence of a steroidal glycoside.

Thin layer chromatography of the most active components

Thin layer chromatography (TLC) was carried out on the most active components of the *Vernonia amygdalina* and *Khaya senegalensis* extracts in accordance with procedure adopted by Abalaka *et al.* (2010). About 1 gram of the extract was subjected to TLC over silica gel F254 using different gradient solvent system to obtain the best solvent. The extract was spotted on the silica precoated TLC plate of thickness of about 0.2mm. The plates were developed in saturated chromatography tank and spots detected using detecting reagent (P Anisaldahyde) following development and drying of the solvent. Bands of separated fractions were detected under Ultraviolet light.

3.4 ISOLATION OF BACTERIA FROM POULTRY DROPPINGS

100 Samples of Poultry droppings were collected at 3 points in each poultry farm located within Makarfi local government (Na aka farm and kwalegi farm) once in a week. This was done to have a good representation and distribution of the organisms in question. The droppings were collected from litter at random points and transported to the laboratory where they were processed within one hour from the time of collection as adopted by Okwori *et al*., 2013.

The GPS coordinate of the farm located in Makarfi local government along Zaria-Kano road is on Latitude=11.71309149 and Longitude=7.95339111.

3.4.1 Isolation and Identification of *Staphylococcus aureus*

Enrichment of the bacteria was done by adding one gram (1g) of the sample into peptone water in sterile MacCartney bottle and incubated at 35^{o}C for 18hrs. Isolation of the *Staphylococcus aureus* was achieved by streaking the pre-enriched culture from the peptone water on to a selective differential agar plate of Mannitol Salt Agar. The plates were then incubated at 37^{o}C for 24hrs under aerobic conditions. Colonies showing golden yellow with yellow zones in the medium were considered to be presumptive *Staphylococcus aureus* and further subjected to biochemical tests such as catalase and coagulase.

3.4.2 Isolation and Identification of *Escherichia coli*

Enrichment of the bacteria was achieved by adding one gram (1g) of the sample into a sterile McCartney bottle containing nutrient broth and incubated at 35^{o}C for 18hrs. The pre-enriched culture was then streaked on to surfaces of an Eosin Methylene Blue (EMB) agar and incubated at 37^{o}C for 18- 24hrs. Dark colonies with metallic sheen indicated the presence of lactose fermenters and were to be considered as presumptive *E. coli.* The culture was streaked on nutrient agar slant and kept for further biochemical identification.

3.4.3 Isolation and Identification of *Salmonella* sp

Enrichment was done by transferring 1g of the sample into test tube containing selenite F broth and incubated at 37^{o}C for 18hrs.The enriched culture after incubation was then streaked on to the surfaces of Salmonella-Shegella Agar (SSA) and incubated at 37^{o}C for 18hrs. After incubation; typical black colonies were regarded as presumptive *Salmonella.* The colonies were then inoculated on nutrient agar slants and kept for biochemical identification.

3.4.4 Isolation and Identification of *Pseudomonas* sp

Enrichment of the bacteria was achieved by adding one gram (1g) of the sample into a sterile McCartney bottle containing nutrient broth and incubated at 35^{o}C for 18hrs.The pre-enriched culture was then streaked on to surfaces of blood agar and incubated at 37^{o}C for 24hrs. Colonies that appears large, flat, spreading which are haemolytic and usually pigment producing (dark greenish-blue color) which is an indication of *Pseudomonas* characteristic. The colonies were inoculated on nutrient agar slants and kept for further biochemical identification.

3.4.5 Isolation and Identification of *Proteus* sp

Enrichment of the bacteria was achieved by adding one gram (1g) of the sample into a sterile McCartney bottle containing nutrient broth and incubated at 35^{o}C for 18hrs. The pre-enriched culture was then streaked on to surfaces of MacConkey agar and Blue Green Agar (BGA) and incubated at 37^{o}C for 24hrs.On BGA a pinkish coloration indicate non-lactose fermenting organism which could be either *Proteus* or *Salmonella*, but on MacConkey agar, colonies appeared as colorless with swarming characteristic. The colonies were inoculated on nutrient agar slant as presumptive *Proteus* sp and kept for further biochemical identifications as described by Okwori *et al*., 2013.

3.4.6 Gram staining

From the colonies on the culture plates, smear were made each on a clean, grease-free slide and allowed to dry and fixed. It was then stained with gram's stain using the following procedure:

The fixed smear was flooded with crystal violet and allowed to stand for 30 seconds, after which it was washed off with distilled water.

It was flooded with Lugol's iodine for 30 seconds and again washed off with distilled water.

It was decolorized rapidly with acetone and then washed thoroughly with distilled water.

It was counterstained with neutral red for 30 seconds after which it was washed, air-dried and observed microscopically under × 100 objective (oil immersion) (Cheesbrough, 2004).

The Gram reaction for each of the isolates was recorded accordingly.

3.4.7 Biochemical characterization of the isolates

Suspected colonies were subjected to biochemical tests which are motility, indole, urease, citrate, methyl red, voges-proskauer (VP), triple sugar iron (TSI) agar, citrate utilization test, for *Escherichia coli, Salmonella species, Proteus species, Pseudomonas species,* while catalase and coagulase tests for *Staphylococcus aureus* as described by Sule *et al.*,2008.

Motility Test

From the nutrient agar slant, a colony was inoculated into 5ml peptone water in test-tubes and incubated at 37°C.For 24 hours. If the organism is motile, the peptone water change cloudy and if not there would be no change in appearance

Indole Test

Colonies from the nutrient agar slant were grown in 5ml of peptone water and incubated for 24 hours at 37°C. After 24 hours of incubation, 3 drops of Kovac's indole reagent was added and shaken gently. A positive result was indicated by the development of a red colour in the reagent layer above the medium within one minute. In a negative reaction the Indole reagent still retained its yellow color.

Urease Test

Urea agar slants in test-tubes were inoculated with isolates from the nutrient agar slant and incubated for 24 hours at 37°C. The development of a bright pink color or red color indicates a positive reaction. In negative reactions the urea agar slants do retain their brownish colour.

Citrate Utilization Test

Simon citrate agar slants in test-tubes were inoculated with isolates from the nutrient agar slant and incubated at 37°C for 24 hours. The development of a deep blue color indicated positive reaction. In a negative reaction the Simon citrate agar slant retained its green color.

Methyl Red Test

About 5ml of methyl red broth were inoculated with the test organisms from the nutrient agar slant and incubated for 48 to 72 hours. After the period of incubation, 1ml of the broth was transferred to a small serological test tube and 2 – 3 drops of methyl red indicator was added. A red color on the addition of the indicator signified a positive methyl red test. A yellow color signified a negative test.

Voges-Proskauer test

A test tubes containing glucose phosphate medium was prepared and inoculated with the test organisms from the nutrient agar slant and incubated at 37^0C for 48hrs. Creatine was added followed by 1ml of KOH and mixed thoroughly. Appearance of pink colour after 2hrs indicated a positive result.

Catalase Test

About 2 – 3 ml of hydrogen peroxide solution was poured into a test tube. Using a sterile glass rod, a little growth of the test organisms was removed and immersed in hydrogen peroxide solution. Observation was made for immediate bubbling.

Coagulase Test

A drop of physiological saline was dropped on two separate slides (one as test and one as control). A colony of the test organism was emulsified in each of the drops to make two thick suspensions. A drop of rabbit plasma was added to one of the suspensions, and mixed gently. Observation was made for clumping of the organisms within 10 seconds.

Preparation of McFarland Standard Scale No 1

McFarland standard (0.5) was prepared, by combining 0.05ml of 1% barium chloride dehydrate($Bacl_2.2H_2O$) with 9.95ml of 1% Sulfuric acid(H_2SO_4) to yield 1.0%w/v barium sulphate suspension. The turbid solution (McFarland standard scale No 1) formed was transferred into a test tube for comparison with bacterial inoculums suspension.

Standardization of the Bacterial Cell Suspension

Five colonies of each test organism were picked into sterile test tube containing sterile nutrient broth and incubated at 37^0c for 18-24 hours. The turbidity produced by this organism was adjusted and used to match the turbidity (opacity) Standard prepared above as described by Sule *et al.,* 2008.

Preparation of different concentrations of plant extracts

A concentration of 2.0mg/ml was prepared for each of the plant extracts; by weighing approximately 2mg in 1ml of distilled water for each extract as stock solutions. A double fold serial dilution of the plant extracts was carried out from their stock solutions to obtain varying concentrations. The varying concentrations obtained were 1.00, 0.50 and 0.25 mg/ml respectively.

3.5 SUSCEPTIBILITY OF THE BACTERIAL ISOLATES TO PLANT EXTRACTS

Agar well diffusion technique was used as described by Sule *et al.*, (2008), to determine the antibacterial activity of plant extracts. A 20ml Mueller Hinton agar plates was prepared. One

ml aliquot of each test organism suspension (standardized) was transferred onto the well-dried Mueller Hinton agar plates in duplicate and was spread evenly following slow rotation of the plates and excess was decanted into disinfectant container. The plates were allowed to dry; a Standard sterile cork borer of 4mm diameter was used to cut uniform wells on the Mueller Hinton agar plates. Each of the wells was appropriately labeled on the reverse side of the plates and the base of the wells was sealed with one to two drop of the molten agar. A sterile syringe (2ml) was used to transfer 0.5 ml of the 1.00mg/ml,0.5mg/ml and 0.25mg/ml of the plant extracts into respective wells on the Mueller Hinton agar plates. Water was used as control in centre wells. The plates were allowed to stand for 30 min at room temperature to allow proper diffusion of the extract to occur. All the plates were incubated at 37°C for 18-24hrs.The zones of inhibition were measured using Vernier calipper to the nearest millimeter.

3.6 SUSCEPTIBILITY OF THE BACTERIAL ISOLATES TO ANTIBIOTICS

Clinical Laboratory Institute Standard (CLIS, 2005) disk diffusion method was adopted: About five (5) colonies of the test bacteria each from Nutrient agar slant were inoculated into Mueller Hinton broth and incubated at 37ºC for 18- 24hrs. Using sterile syringe and needle 0.5ml each of the isolate was transferred on to the surfaces of Mueller Hinton agar and spread evenly by gently rotating the plates. Using sterile forceps, the multiple antibiotic discs were placed onto the inoculated plates appropriately. The plates were incubated at 37°C for 18-24hrs. After incubation, the diameter of the zones of inhibition was measured using Vernier calipper to the nearest millimeters (CLIS, 2005).

The following commercially prepared antibiotic discs were used for susceptibility testing.

Cotrimoxazole(SXT)30µg,Amoxicillin(AM)30µg,Chloranphenicol(CH)30µg,Gentamicin(CN)10µg,Ciprofloxacin(CPX)10µg, Pefloxacin(PEF)30µg, Augmentin(AU)30µg, Ofloxacin (OFX) 10µg, Streptomycin(S)30µg, and Sparfloxacin (SP) 10µg.

3.7 DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC) OF PLANT EXTRACTS

The broth dilution method was used. A stock solution of 200mg /ml was prepared for each plant extract separately. One ml of Mueller Hinton broth was dispensed into three test tubes and sterilized by autoclaving at 121°C after 15 min. The different extracts were serially diluted from the solutions of 0.25mg/ml of the stock solution to obtain varying concentrations. The concentrations were; 12.5, 6.25 and 3.125mg/ml. About 0.1 ml each of standard inoculums was inoculated into the various test tubes containing varying concentrations and then, a set of test tubes containing only nutrient broth were used as negative control, another set of test tubes containing nutrient broth and test organisms were used as positive control. All the test tubes and control were then incubated at 37°C for18- 24hrs. After incubation, the presence or absence of growth in each tube was compared by turbidity. The least concentration of plant extract in the test tube with no turbidity was considered as the minimum inhibitory concentration (MIC) (Andrews, 2004).

3.8 DETERMINATION OF MINIMUM BACTERICIDAL CONCENTRATION (MBC) OF THE PLANT EXTRACTS

This was an offshoot of the previously determined MIC. The least concentration of plant extracts in the test tubes with no turbidity were taken as the Minimum Inhibitory Concentration (MIC). Subsequently, those tubes that showed no turbidity were plated out on sterile Mueller Hinton agar plates and incubated at 37°C for 18-24hrs; absence of growth after incubation period of 48hrs was considered to be the Minimum Bactericidal Concentration (MBC) (Andrews, 2004).

3.9 COMPARATIVE EFFECTS OF PLANT EXTRACTS AND CONVENTIONAL ANTIBIOTICS ON BIRDS PERFORMANCE

This was carried out by purchasing 20 day old chicks, from National Animal Production Research Institute (NAPRI). They were all weighted, match and grouped into Four (4) Groups. Group A was raised with *Khaya senegalensis* extract, group B was raised on *Vernonia amygdalina* extract and group C on antibiotics (Ciprofloxacin) while group D served as control (i.e. raised neither on antibiotic nor on plant extracts) for the period of eight weeks. The body weights were measured at intervals. The supplements (plant extracts and antibiotic) were administered in water and as well as mixed with feeds prepared locally so as to avoid other incorporated antibiotics in commercial feeds. The addition of extracts was made taking into consideration the body mass on weekly bases. Adopted with some modifications from Audu *et al*., 2000

3.10 STATISTICAL ANALYSIS

Statistical analysis to compare the individual activity of the plant extracts variables on the test bacteria and also the susceptibility of the isolates to conventional antibiotics were compared statistically. The values obtained were also compared with that obtained for the plants extracts against the test organisms. With the help of Computer software Graphpad Prism® 4 (GraphPad Software Inc., San Diego,USA). Individual differences between the formulations were performed using the Dunn's multiple range comparison tests. At 95 % confidence interval, *p* values less than or equal to 0.05 were considered significant.

CHAPTER FOUR

4.0 RESULTS

Table 4.1 Shows the reference number for identification of the plants used as obtained from the herbarium section of the Department Biological Sciences Ahmadu Bello University, Zaria, Nigeria. *Khaya senegalensis* (Desr.) has reference number of V/No 675 and *Vernonia amygdalina* has V/No 900181. Table 4.2 showed the result of phytochemicals constituents of the plant extracts, where all the constituents screened for *Khaya senegalensis* were found presence with the exception of glycocides which was found to be absence, while *Vernonia amygdalina* showed the presence of all the tests carried out. Table 4.3 showed the frequencies and percentages of the bacteria isolated from poultry dropping, five different bacterial isolates were identified from the cultured samples of poultry droppings. The isolates were Gram stained and biochemically characterized and they are; *Staphylococcus aureus*, *E.coli*, *Proteus* sp, *Pseudomonas* sp, *and Salmonella* sp. Table 4.4: also shows the susceptibility of the bacterial isolates to *Khaya senegalensis* extract at 0.25mg/ml, there was no significant difference statistically. At 0.50mg/ml *S. aureus* and *E. coli* were more susceptible, while at 1.00mg/ml also *S.aureus* and *E.coli* were most sensitive followed by *Pseudomonas* sp, with significant difference at $p \leq 0.01$ and $p \leq 0.05$ respectively.

Table 4.1: Reference number for identification of the plants used obtained from the catalogue section of the Department of Biological sciences, Ahmadu Bello University, Zaria.

Family	Latin name	English name	Common name (Hausa)	Reference number
Meliaceae	*Khaya senegalensis*	Mahogani	{Maddaci)	V/NO 675
Asteraceae	*Vernonia amygdalina*	Bitter leaf	(Shuwaka)	V/no 900181

Key: V/No= Voucher number

Table 4.2: Phytochemical constituents of the plant extracts

TEST	KSE	VAE
Carbohydrate		
Molisch test	+	+
Glycosides		
Fehlings test	_	+
Tannins		
Fe_3Cl_2	+	+
Alkaloid		
Wagner's test	+	+
Dragendoff's test	+	+
Saponins		
Frothing test	+	+
Flavonoids		
Shinoda test	+	+
Steroids		
Salkwoski test	+	+

KEY: **+ =**presence - = Absence

KSE=*Khaya senegalensis* extract VAE=*Vernonia amygdalina* extract

Table 4.3: Distribution of Bacteria isolates from poultry droppings.

S/no	Isolates	Frequency	Percentage (%)
1	*S. aureus*	7	9.5
2	*E. coli*	30	40.5
3	*Proteus* sp	12	16.2
4	*Pseudomonas* sp	10	13.5
5	*Salmonella* sp	15	20.3
7	Total No. of samples	100	
8	No. of sample positive	74	

Key: No=number

Table 4.4; Inhibitory activity of *Khaya senegalensis* extract against the bacterial isolates.

Bacteria	Mean ± SEM (mm)		
	25mg/ml	50mg/ml	100mg/ml
S. aureus	15.50 ± 0.50	18.00 ± 0.00[a]	19.75 ± 0.75[a]
E. coli	16.50 ± 0.50	18.00 ± 0.00[a]	20.25 ± 0.25[a]
Proteus sp	14.50 ± 0.50	14.75 ± 0.25[b]	16.00 ± 0.00[b]
Pseudomonas sp	14.75 ± 0.75	15.5 ± 0.50[b]	17.25 ± 0.25[b]
Salmonella sp	14.50 ± 0.50	14.50 ± 0.50[b]	16.00 ± 0.00[b]
Statistics:	F = 2.360; p = 0.186	F =26.556; p= 0.001*	F= 30.136;p= 0.001**

NOTE: Values are expressed as Mean ± SEM (standard error of means). Mean having different superscripted alphabets along the columns are different at $p \leq 0.05$.

Table 4.5: Shows susceptibility of the bacterial isolates to *Vernonia amygdalina* extract, at 0.25mg/ml *Salmonella* sp was most susceptible, followed by *S .aureus* and *Pseudomonas* sp. At 0.50mg/ml *Salmonella* sp had the highest susceptibility, followed by *S. aureus, E.coli, Pseudomonas* sp. At 1.00mg/ml *salmonella* sp, had the highest zone, while *E.coli*, and *S.aureus* having similar activity with significance difference at $p \leq 0.001$. Table 4.6 shows the result of susceptibility of the bacterial isolates to the conventional antibiotics, the result showed that Ciprofloxacin (CPX) had the highest zones of inhibition across the isolates (most effective), followed by argumentin (AU), *Pseudomonas sp* showed higher resistance, but with little activity by Argumentin and Gentamycin. Table 4.7 Showed comparison of susceptibility of the bacterial isolates to the plant extracts and standard antibiotics CPX, CN and OFX, at 250µg/ml *Khaya senegalensis* appear to have higher activity against the test isolates than *Vernonia amygdalina* extracts, with only exception of *Salmonella* sp. which was found to be more sensitive to *V. amygdalina*, with significance difference at $p \leq 0.05$. Table 4.8 showed comparison of susceptibility pattern of bacterial isolates to plant extracts and standard antibiotics CPX, CN and OFX at 500µg/ml, *Khaya senegalensis* was more effective against *S.aureus* than *Vernonia amygdalina* extracts while *E.coli* was more sensitive to *Vernonia amygdalina* than *Khaya senegalensis*. *Proteus and Pseudomonas* had similar activity on both extracts. *Salmonella* sp was more susceptible to *V. amygdalina*. The antibiotic Ciprofloxacin was effective across the test isolates but 100% resisted by *Pseudomonas*, also Ofloxacin was effective against the test bacteria but 100% resisted by two organisms (*Pseudomonas and Salmonella*).

Table4.5: Inhibitory activity of *Vernonia amygdalina* extract against the bacterial isolates.

	Mean ± SEM (mm)		
	0.25 mg/ml	0.50 mg/ml	1.00 mg/ml
Bacteria			
S. aureus	14.75 ± 0.75^{ab}	17.00 ± 0.00^{b}	18.00 ± 0.00^{b}
E. coli	14.00 ± 0.00^{b}	16.00 ± 0.00^{c}	18.00 ± 0.00^{b}
Proteus sp	13.00 ± 0.00^{b}	14.25 ± 0.25^{d}	15.00 ± 0.00^{d}
Pseudomonas spp	14.75 ± 0.75^{ab}	15.50 ± 0.50^{c}	16.50 ± 0.50^{c}
Salmonella sp	16.25 ± 0.25^{a}	18.00 ± 0.00^{a}	19.00 ± 0.00^{a}
Statistics:	F = 5.974; p = 0.038*	F = 32.800; p = 0.001**	F = 49.000; p = 0.001**

NOTE: Values are expressed as Mean ± SEM (standard error of means).Means having different superscripted alphabets along the columns are different at $p<0.05$.

Table 4.6: Sensitivity of the test bacterial isolates to commercially available antibiotic discs

	Mean ± SEM (mm)								
	SXT(30µg)	**CH(**30µg**)**	**SP(**10µg**)**	**CPX(**10µg**)**	**AU(**30µg**)**	**CN(**10µg**)**	S(30µg)	OFX(10µg)	PEF(30µg)
Isolate									
S. aureus	20.25 ± 0.25^{b}	20.25 ± 0.25^{a}	18.00 ± 0.00^{b}	40.25 ± 0.25^{a}	30.25 ± 0.25^{a}	20.25 ± 0.25^{a}	20.00 ± 0.00^{b}	24.00 ± 0.00^{c}	25.00 ± 1.00^{a}
E. coli	14.00 ± 0.00^{c}	16.25 ± 0.25^{c}	0.00 ± 0.00^{d}	20.00 ± 0.00^{d}	18.00 ± 0.00^{b}	20.00 ± 0.00^{a}	14.50 ± 0.50^{c}	28.00 ± 0.00^{b}	15.00 ± 1.00^{c}
Proteus sp	30.25 ± 0.25^{a}	18.25 ± 0.50^{b}	20.25 ± 0.25^{a}	37.50 ± 0.50^{b}	18.00 ± 0.00^{b}	20.25 ± 0.25^{a}	30.50 ± 0.00^{a}	30.25 ± 0.25^{a}	20.50 ± 0.50^{b}
Pseudomonas **sp**	0.00 ± 0.00^{d}	0.00 ± 0.00^{e}	0.00 ± 0.00^{d}	0.00 ± 0.00^{e}	18.25 ± 0.25^{b}	14.50 ± 0.50^{b}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}
Salmonella sp	14.25 ± 0.25^{c}	10.50 ± 0.50^{d}	14.25 ± 0.25^{c}	24.50 ± 0.50^{c}	10.50 ± 0.50^{c}	20.00 ± 0.00^{a}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	14.00 ± 0.00^{c}
Statistics F = 3225.833, <0.001**	F=537.150	F= 3858.750	F=2305.667	F = 671.250	F = 84.583	F=3391.000	F = 18441.000	F = 197.889	

NOTE: Values are expressed as Mean ± SEM (standard error of means).Means having different superscripted alphabets along the columns are different at $p<0.05$.

KEY:Septrin(SXT)30µg,amoxicillin(AM)30µg,chloranphenicol(CH)30µg,gentamicin(CN)10µg,Ciprofloxacin(CPX)10µg,pefloxacin(PEF)30µg,Augmentin(AU)30µg,Ofloxacin(OFX)10µg,Streptomycin(S)30µg,and Sparfloxacin(SP)10µg.

Table 4.7: Comparison of Sensitivity of bacterial isolates to plant extracts and standard antibiotic CPX, CN and OFX at 250µg/ml

Bacteria	Mean inhibition Zone Diameter(mm)				
	KSE	VAE	CPX(10µg)	CN(10µg)	OFX(10µg)
S. aureus	15.50 ± 0.50^{d}	14.75 ± 0.75^{d}	40.25 ± 0.25^{a}	20.25±0.25^{c}	24.00±0.00^{b}
E. coli	16.50 ± 0.50^{c}	14.00 ± 0.00^{d}	20.00 ± 0.00^{b}	20.00±0.00^{b}	28.00±0.00^{a}
Proteus	14.50 ± 0.50^{d}	13.00 ± 0.00^{e}	37.50 ± 0.50^{a}	20.25±0.25^{c}	30.25±0.25^{b}
Pseudomonas	14.75 ± 0.75^{a}	14.75 ± 0.75^{a}	0.00 ± 0.00^{b}	14.50±0.50^{a}	0.00±0.00^{b}
Salmonella	14.50 ± 0.50^{d}	16.25 ± 0.25^{c}	24.50 ± 0.50^{a}	20.00±0.00^{b}	0.00±0.00^{e}

NOTE: Values are expressed as Mean ± SEM (standard error of means).Means having different superscripted alphabets across the columns are different at $p<0.05$.

Key: KSE=*Khaya senegalensis* extract, VAE= *Vernonia amygdalina* extract, CPX= Ciprofloxacin, CN=Gentamicin, OFX= Ofloxacin.

Table 4.8: Comparison of Sensitivity of bacterial isolates to plant extracts and standard antibiotic CPX, CN and OFX at 500µg/ml

Bacteria	Mean inhibition Zone Diameter(mm)				
	KSE	VAE	CPX(10µg)	CN(10µg)	OFX(10µg)
S. aureus	18.00±0.00^{d}	17.00±0.00^{e}	40.25 ± 0.25^{a}	20.25±0.25^{c}	24.00±0.00^{b}
E. coli	18.00±0.00^{c}	16.00±0.00^{d}	20.00 ± 0.00^{b}	20.00±0.00^{b}	28.00±0.00a
Proteus	14.75±0.25^{d}	14.25±0.25^{d}	37.50 ± 0.50^{a}	20.25±0.25^{c}	30.25±0.25^{b}
Pseudomonas	15.50±0.50^{a}	15.50±0.50^{a}	0.00 ± 0.00^{b}	14.50±0.50^{a}	0.00±0.00^{b}
Salmonella	14.50±0.50^{d}	18.00±0.00^{c}	24.50 ± 0.50^{a}	20.00±0.00^{b}	0.00±0.00^{e}

NOTE: Values are expressed as Mean ± SEM (standard error of means).Means having different superscripted alphabets across the columns are different at $p<0.05$.

Key: KSE=*Khaya senegalensis* extract, VAE= *Vanonia amygdalina* extract, CPX= Ciprofloxacin, CN=Gentamycin, OFX= Ofloxacin.

Similarly, Table 4.9; Shows comparison of susceptibility of bacterial isolates to plant extracts and standard antibiotics (CPX, CN and OFX) at 1000µg/ml, *Khaya senegalensis* were most effective against the test isolates than *V. amygdalina* except with *Salmonella* sp.that was more susceptible to *V. amygdalina*. The antibiotic was also effective against the test isolates, but 100% resisted by *Pseudomonas* sp. There exist significance differences at $p \leq 0.05$. Table 4.10; showed the results of Minimum Inhibitory concentration (MIC) and Minimum Bactericidal concentrations (MBC) of the test isolates in the presence of plant extracts. The *Khaya senegalensis* showed MIC against *S.aureus* and *E.coli* at 6.25mg/ml, while *Proteus* sp, *Pseudomonas* sp and *Salmonella* sp, appeared at 12.5mg/ml. *Vernonia amygdalina* showed MIC against *Salmonella* sp at 6.25mg/ml while *S.aureus, E.coli, Proteus* sp, and *Pseudomonas* sp appeared at 12.5mg/ml. The *Khaya senegalensis* extracts showed MBC against *S.aureus* and *E.coli* at 6.25mg/ml, while *Proteus* sp, *Pseudomonas* sp and *Salmonella* sp appeared at 12.5mg/ml. *Vernonia amygdalina* showed MBC against *Salmonella* sp at 6.25mg/ml, but as for the rest isolates appeared at 12.5mg/ml. Figure 1; showed the weight changes of birds raised on feeds supplemented with *Khaya senegalensis* and *Vernonia amygdalina* plant extracts and those raised on conventional antibiotics. *Khaya senegalensis* extract was more effective than *V. amygdalina* extract in vivo and the mean weights were interestingly increasing during the period of eight weeks as observed. (Figure 2) Showed Death occurrence across the groups with the control group showed the least survival rates at the end of the period of eight weeks.

Table 4.9: Comparison of Sensitivity of bacterial isolates to plant extracts and standard antibiotic CPX, CN and OFX at 1000µg/ml

Bacteria	Mean inhibition Zone Diameter(mm)				
	KSE	VAE	CPX**(**10µg**)**	CN**(**10µg**)**	OFX**(**10µg**)**
S. aureus	19.75 ± 0.75^{c}	18.00 ± 0.00^{d}	40.25 ± 0.25$^{\mathbf{a}}$	20.25±0.25$^{\mathbf{c}}$	24.00±0.00$^{\mathbf{b}}$
E. coli	20.25 ± 0.25^{b}	18.00 ± 0.00^{c}	20.00 ± 0.00$^{\mathbf{b}}$	20.00±0.00$^{\mathbf{b}}$	28.00±0.00a
Proteus	16.00 ± 0.00^{d}	15.00 ± 0.00^{e}	37.50 ± 0.50$^{\mathbf{a}}$	20.25±0.25$^{\mathbf{c}}$	30.25±0.25$^{\mathbf{b}}$
Pseudomonas	17.25 ± 0.25^{a}	16.50 ± 0.50^{a}	0.00 ± 0.00$^{\mathbf{b}}$	14.50±0.50$^{\mathbf{a}}$	0.00±0.00$^{\mathbf{b}}$
Salmonella	16.00 ± 0.00^{d}	19.00 ± 0.00^{c}	24.50 ± 0.50$^{\mathbf{a}}$	20.00±0.00$^{\mathbf{b}}$	0.00±0.00$^{\mathbf{e}}$

NOTE: Values are expressed as Mean ± SEM (standard error of means).Means having different superscripted alphabets across the columns are different at $p<0.05$.

Key: KSE=*Khaya senegalensis* extract, VAE= *Vanonia amygdalina* extract, CPX= Ciprofloxacin, CN=Gentamycin, OFX= Ofloxacin.

Table 4.10; MIC and MBC values of the plant extract against the test isolates at various concentrations.

Isolates	MIC (mg/ml)		MBC (mg/ml)	
	K SE	V AE	K SE	V AE
S. aureus	6.25	12.50	6.25	12.50
E. coli	6.25	12.50	6.25	12.50
Proteus sp	12.50	12.50	12.50	12.50
Pseudomonas sp	12.50	12.50	12.50	12.50
Salmonella sp	12.50	6.25	12.50	6.25

Key: KSE= *Khaya senegalensis Extract* VAE= *Vernonia amygdalina Extract*

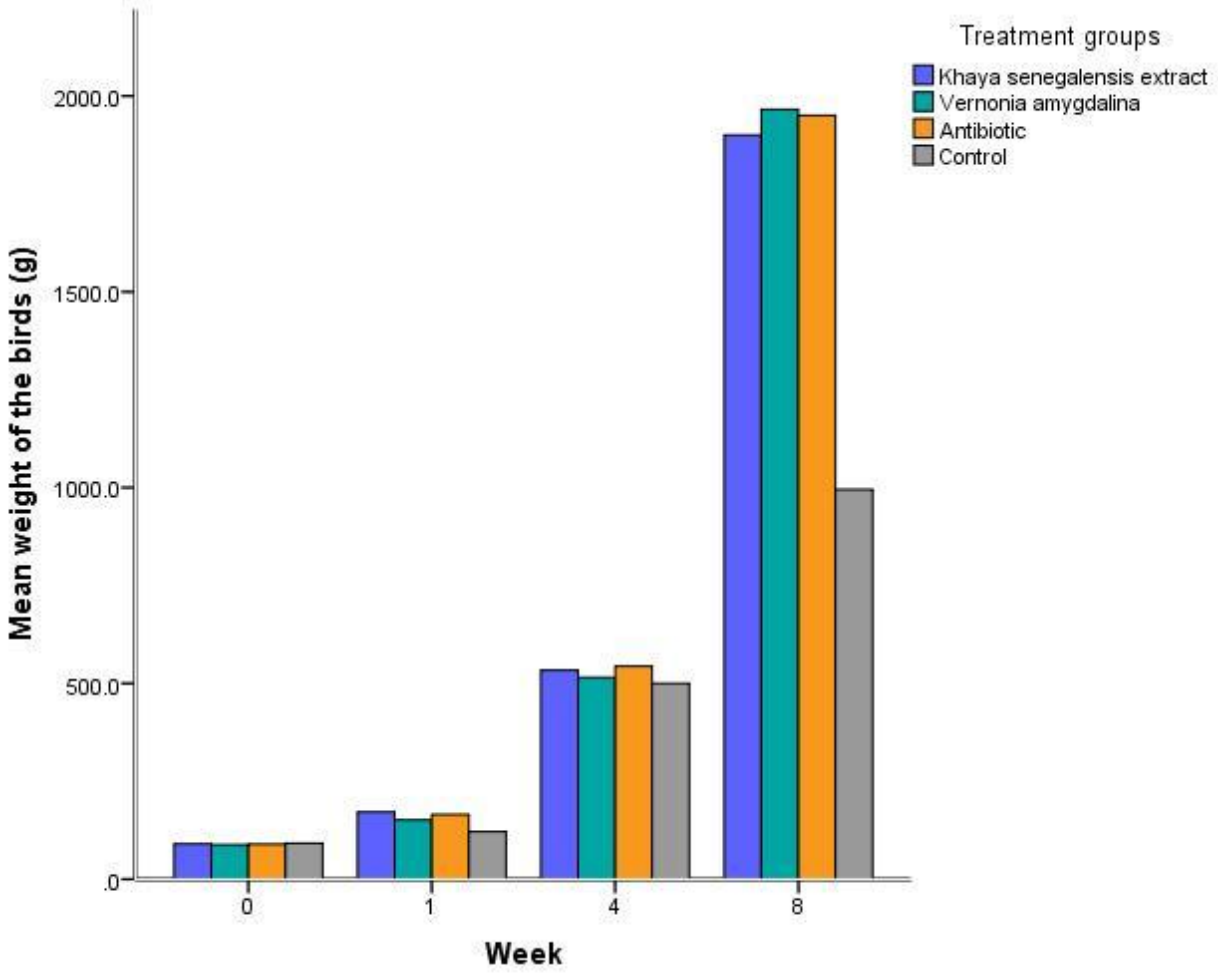


Figure1: Weights changes of birds (in grams) raised on feeds supplemented with *Khaya senegalensis* and Vernonia *amygdalina* plant extracts and antibiotics (Ciprofloxacin).

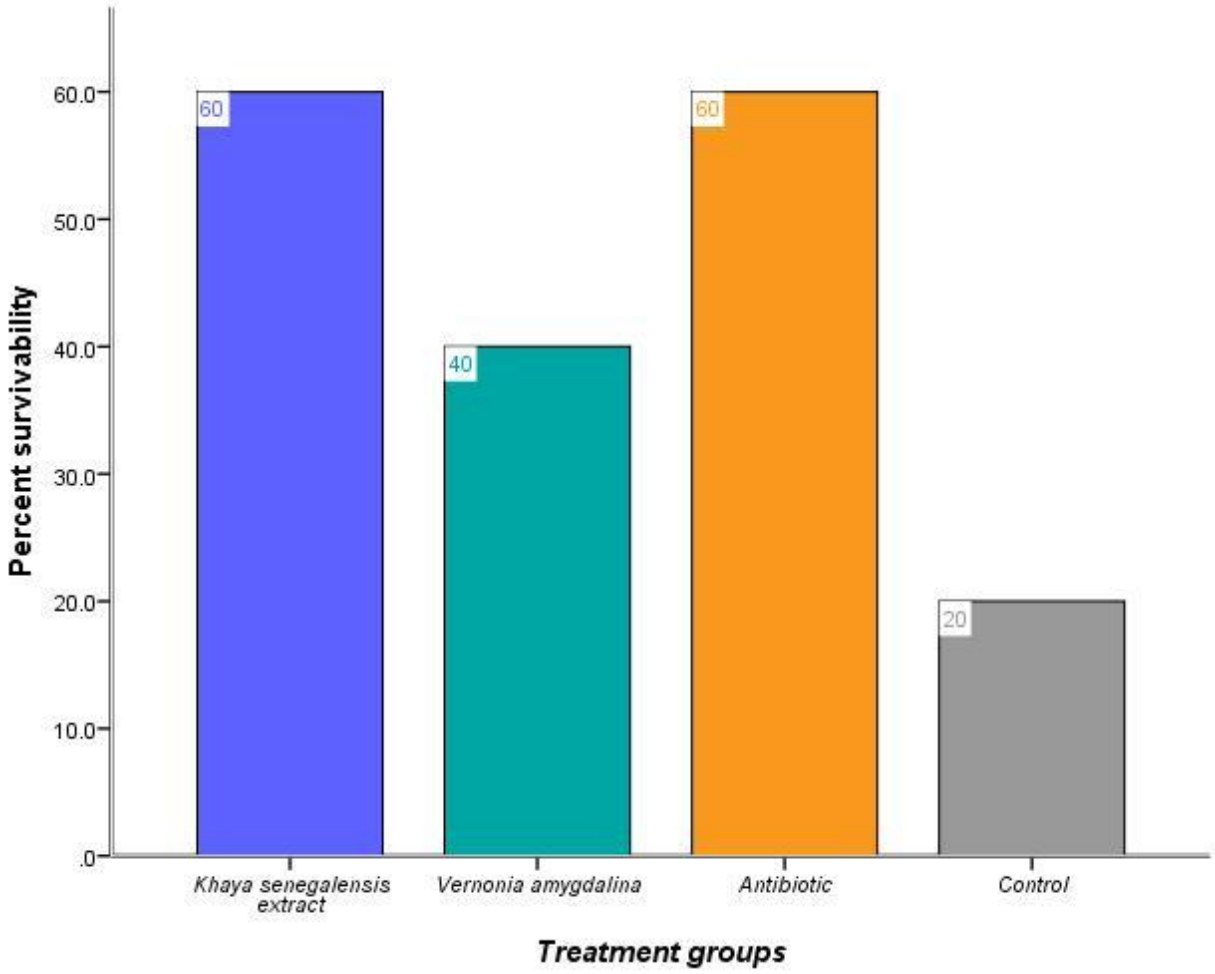


Figure 2 survival rates of the birds raised across the treatment groups

NOTE; *K. senegalensis* extract MIC (6.25mg/ml), *V. amygdalina* extract MIC (12.50mg/ml), Ciprofloxacin (CPX) 10µg, Control (feed alone)

CHAPTER FIVE

5.0 DISCUSSION

The selection of these two plants was base on the fact that, usage of these plants locally in the management of poultry has sustained the local poultry production system, the knowledge was in heritage from past generations and is passed on verbally without documentation. The plants are valuable medicinal plant that is widespread in East and West Africa (Burkill 1985). Overdependence on modern antibiotic which bring about resistance development, couple with increase medical cost support alternative approach. Identification of the stated plants becomes necessary. The reference number for identification of the plants used was obtained from herbarium section, Department of Biological sciences Ahmadu Bello University Zaria thus *Khaya senegalensis* has reference number of V/No 675 and *Vernonia amygdalina* has V/No 900181.The search for antimicrobial agents of plants origin began long time ago since during Ehrlich, 1902 and to date (Ahmad *et al.*, 2014).

The results of phytochemical screening of *Khaya senegalensis* showed the presence of carbohydrates, flavonoids, saponins, tannins, alkaloids and steroids but glycosides were found to be absent, while *Vernonia amygdalina* showed the presence of carbohydrates, glycosides, flavonoids, saponins, tannins, steroids and alkaloids. This is in line with the findings of Sani *et al.*, 2012. The presence of these organic substances may have been responsible for antimicrobial activity of these plant extracts. Tannins are polyphenols obtained from various parts of different plants (Gajendiran and Mahadevan, 1990). In addition to use in leather processing industries, tannins have shown potential antiviral activity (Ibrahim *et al.*, 2004), and antibacterial activity (Akiyama *et al.*, 2001). In the past few years, tannins have also been studied for their effects against cancer through different mechanisms and have been found to form proline-rich-protein

which leads to inhibition of cell wall synthesis, a property that may explain the mode of action of these extracts. Alkaloid is a member of a large group of chemicals that are made by plants and have nitrogen in them. Many alkaloids possess potent pharmacological effects. The alkaloids include cocaine, nicotine**,** strychnine, caffeine, morphine, pilocarpine, atropine, methamphetamine, mescaline, ephedrine and tryptamine (Sani *et al.,* 2012). According to Danladi *et al*., (2008), *V. amygdalina* extract contained the aforementioned phytochemicals which are responsible for the observed antibacterial activities. This corroborates the findings in the present study. According to Clinical and laboratory standard institute CLSI (2005), any plant material should be considered an effective therapeutic agent if it extract produces zones of inhibition of between 15-22mm on the target pathogenic organism. Activity of plant extracts to test bacteria is normally expressed *in-vitro* as zones of inhibition in millimeter (greater than or equals to 7mm) around the organism on test (Gislene *et al., 2000).*

The zones of inhibition of *K. senegalensis* extract at 0.25mg/ml were higher against *E. coli* followed by *S. aureus*. Lower zones of inhibition were seen against *Pseudomonas* sp, *Proteus* sp, and *Salmonella* sp. There was no significant difference in the zones of inhibition across the test bacteria (P=0.186). At 1.00mg/ml however, there was significant difference ($P \leq 0.01$) in the zones of inhibition against the test organisms. This indicated that the antibacterial efficacy of the extract is dependent on the concentration (Abdu *et al*., 2000; Cos *et al*. 2002). Also, *V. amygdalina* extract at 0.25mg/ml was more effective against *Salmonella* sp, *S. aureus* and *Pseudomonas* sp than *Proteus* sp and *E. coli*. Statistical analysis revealed significant difference ($P \leq 0.05$) in the zones of inhibition at all the concentrations tested. Antibacterial activities observed in this study might be due to the presence of phytoconstituents such as flavonoids and tannins in these plants. This further buttresses the well established fact that antibacterial

substances are present in the higher plants. This is in agreement with the findings of (Sani *et al.*, 2012).

The test bacteria showed different levels of susceptibility to the different antibiotics used in the study as shown by the zones of inhibition. The highest activity (40.25mm) was recorded for Ciprofloxacin against *S.aureus*, followed by *Proteus* sp, *Salmonella* sp, and *E coli; Pseudomonas* sp was however found to be resistant to Ciprofloxacin. This could be due to Gram positive bacteria are often more susceptible to antibiotics than the Gram negative bacteria due to the fact that Gram negative organisms have an additional outer phospholipids membrane and porines that makes the cell wall impermeable to lipophilic and hydrophilic solutes respectively (Akortha *et al*., 2002; Isyaku *et al.,* 2008). Gentamycin were also active against *S. aureus, E.coli, Salmonella* sp, and *Proteus* sp with the least activity on *Pseudomonas* sp. This might be possible because among the aminoglycosides, gentamycin have an extended spectrum of activity on most of the enterobacteriaceae family (Sherris *et al*., 1966).

The comparisons between the antibacterial activities of methanolic extracts of *K.senegalensis* and *V. amygdalina* with that of standard antibiotics (Ciprofloxacin, Gentamycin and Ofloxacin) showed that the extracts had broader spectrum than the antibiotics even though the antibiotics in some instances had better zones of inhibition against some organisms. The differences were statistically significant ($P \leq 0.05$). The better activity of the standard antibiotics over the extracts could be due to the fact that organic extracts are in crude and not in pure form as the synthetic antibiotics; hence the secondary active metabolites could be present in low concentration or even be masked in the extracts (Ahmad *et al.*, 2014).

As for Minimum Inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), the general trend is that Minimum Inhibitory concentration (MIC) inhibited the organisms while minimum bactericidal concentration (MBC) had cidal effects on the organisms.

The MIC value of 0.0625mg/ml was obtained for the methanolic extract of *K. senegalensis* against *S. aureus and E. coli*; 0.125mg/ml for *Proteus* sp, *Pseudomonas* sp *and Salmonella* sp. The MBC values obtained were however the same as the MIC values for *K. senegalensis* extract. The MIC and MBC values for *V. amygdalina* were 0.0625mg/ml against *Salmonella* sp, while for *S. aureus*, *E.coli*, *Proteus* sp and *Pseudomonas* sp, the MIC and MBC were observed at 0.125mg/ml. The lower the MIC and MBC of a given extract the higher the efficacy of such extract against the test organisms (Ahmad *et al.,* 2014).The order of ease of susceptibility to the *K. senegalensis* extract was; *S. aureus, E. coli, Proteus sp, Pseudomonas sp and Salmonella* sp, while for *V. amygdalina*, it was; *Salmonella sp, S. aureus, E. coli, Pseudomonas sp and Proteus* sp. Antimicrobial agents with low activity against an organism had a high MIC while a highly active Antimicrobial agent gave a low MIC. The antibacterial effectiveness of the extracts with the increasing concentration of phytoconstituents observed in the present study is in agreement with the earlier investigations of (Kurosaki and Nishi, 1983; Banso and Adeyemo, 2007). This has also shown that the concentration required to achieve bactericidal activity as observed in this study varied. This is in line with the finding of Abalaka *et al*., (2010).

The results for the mean weights of the birds raised on the plant extracts and those on standard antibiotics showed an increase in the performance of the birds raised on the extracts and the antibiotics as compared to the controls (i.e. neither raised on antibiotics nor on the extracts). However, those raised on the antibiotics only performed slightly better than those raised on the extracts only. This finding suggests that the two plants could be considered an effective alternative to conventional antibiotics.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1: CONCLUSION

The study provides an impetus in the use of *Khaya senegalensis* and *Vernonia amygdalina* which can be used both as diet and antibiotic in animal feeds. The study presents plants with stable and biologically active antibacterial components with low drug resistance against common bacterial strains infecting both human and livestock. Hence the work contributes to the exploration of an alternative source of antibiotics with view to reducing overdependence on commercial antibiotics associated with the emergence of drug resistant bacteria strain.

6.2: RECOMMENDATIONS

In view of the aforesaid findings, the following recommendations were made.

1. These local ethno medical preparations and prescriptions of plant sources to poultry should be scientifically evaluated and disseminated properly.
2. The knowledge of the botanical preparation of traditional sources of medicinal plants can be extended for future Investigation into the field of pharmacology, phytochemistry, ethno botany and other biological actions for drug recovery.
3. The possible exploration of *V. amygdalina and K. senegalensis* as sources of natural products for future use in the management of multi-drug resistant infections especially caused by *Pseudomonas* sp.

4. Birds should be supported with plant supplements so as to reduce over dependence on conventional antibiotics, as such reduce the cost implication and the likelihood of resistance development.

5. The research also enhances the prescription of these plants to local poultry by farmers, with a view to verify and scientifically document information about the antibacterial activity of these plants.

6. Efforts should be made to further investigate the antibacterial as well as other antimicrobial activities of these plants using other solvents of extraction.

7. The Government should provide a ministry for traditional medicine at local, state and Federal Government levels. This is with a view of encouraging the use of plants such as the plants used in this study as alternative to conventional antibiotics in poultry farming.

8. In Government programmes such as that of poverty alleviation, where Government funds are used in agriculture (poultry farming), focus should be geared toward the use of plants remedy. This will surely reduce resistant strains in our communities.

9. Bioactive compounds with antibacterial effects need to be explored from local natural resources. Being new, such compounds may not have the problem of microbial resistance, and with some structural modification their activity could be diversified. Seeking remedies for human ailments from the environment has formed the basis for therapeutics.

10. African plants constitute a rich untapped pool of natural products. Therefore plants such as *Khaya senegalensis* and *Vernonia amygdalina* are used in many African countries by traditional medical practitioners for the treatment of various ailments including bacterial diseases. The prescriptions and doses of these plants should been enhanced.

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APPENDICES

Appendix I: Table Showing: List of organisms isolated

Organisms	Gram Reactions
Staphylococcus aureus	+ve
Salmonella sp	- ve
Escherichia coli	-ve
Proteus sp	-ve
Pseudomonas sp	-ve

Appendix II: Table showing frequencies of organisms isolated

S/no	*Bacteria*	No. Isolated (positive)	Percentage (%)
1	*S. aureus*	7	9.5
2	*E. coli*	30	40.5
3	*Proteus* sp	12	16.2
4	*Pseudomonas* sp	10	13.5
5	*Salmonella* sp	15	20.3

Appendix III Table showing Biochemical screening tests

	Biochemical tests							
Isolates	OF	TSI	UREA	CITRATE	INDOLE	MOTILITY	M.R	V.P
E. coli	Fermenter	Acid/Acid + gas	Negative	Negative	Positive	Positive	Positive	negative
Proteus sp	Fermenter	Alkaline/ Acid +heavy H_2S	Positive	Positive	Positive	Positive	Positive	negative
Salmonella sp	Fermenter	Alkaline/ Acid + H_2S	Negative	Positive	negative	Positive	Positive	negative
Pseudomonas sp	Oxidizer	Alkaline/ No change	Negative	Positive	negative	Positive	Positive	negative

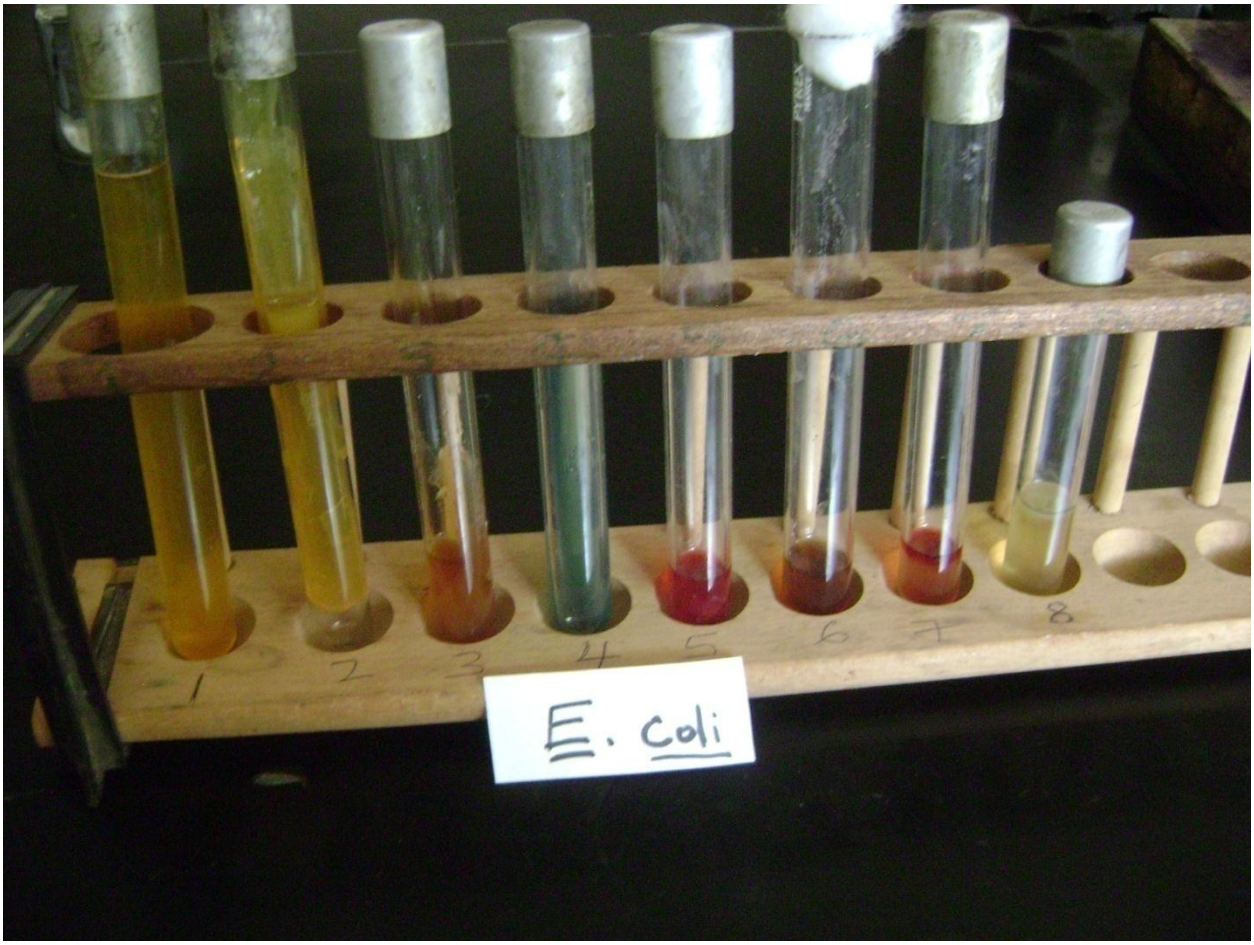
Appendix IV: Zone Diameter Interpretive chart Stds (mm)

ANTIBIOTIC AGENT	CODE	DISK POTENCY	R	I	S
Cotrimoxazole	SXT-30	30µg	≤10	11-15	≥16
Amoxicillin	AM-30	30µg	≤13	14-17	≥18
Chloramphenicol	CH-30	30µg	≤12	13-17	≥18
Gentamicin	CN-10	10µg	≤12	13-14	≥15
Ciprofloxacin	CPX-10	10µg	≤15	16-20	≥21
Pefloxacin	PEF-30	30µg	≤14	15-17	≥18
Augmentin	AU-30	30µg	≤13	14-22	≥23
Ofloxacin	OFX-10	10µg	≤12	13-15	≥16
Streptomycin	S-30	30µg	≤13	14-17	≥18
Sparfloxacin	SP-10	10µg	≤10	11-15	≥16

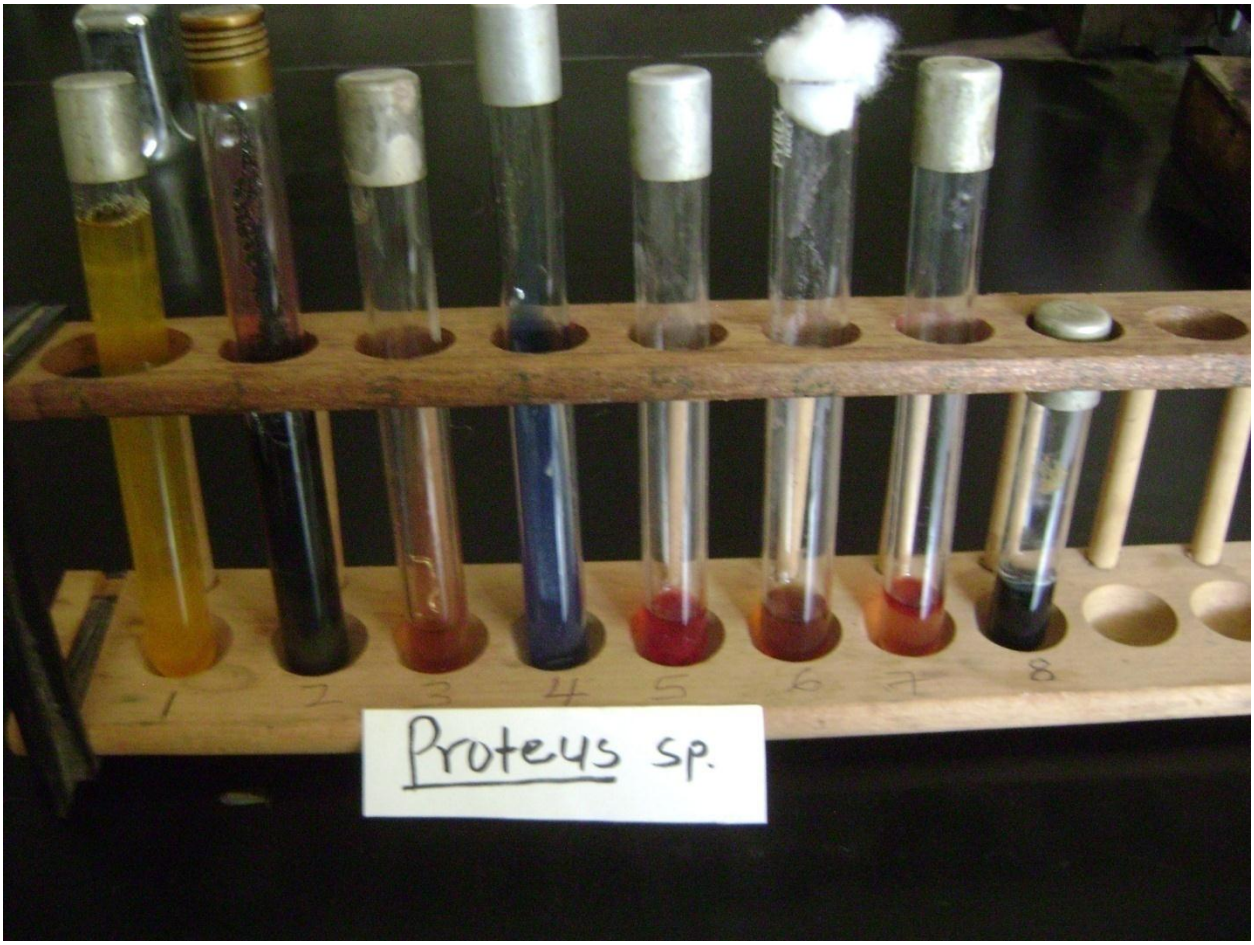
KEY; R=Resistant I= Intermediate S= Sensitive

(CLIS, 2005)0

Appendix V: Plate showing biochemical screening for identification of *E coli*



Appendix VI: Plate showing biochemical screening for identification of *Proteus* sp



Appendix VII: Plate showing biochemical screening for identification of *Salmonella* sp



Appendix VIII: Plate showing thin layer chromatography (TLC) for separation

