CHAPTER ONE

INTRODUCTION

Antibiotic resistance has been a major threat to disease treatment over the years. The inability of bacteria to respond positively to a wide range of antibiotics has brought about the need to search for further natural alternatives to bacteriosis prevention and treatment, hence the interest in probiotics and prebiotics.

Probiotics are microorganisms that confer health benefits to the host when consumed in adequate quantity (Holzapfel *et al*., 2001). *Lactobacillus casei* is a Gram positive anaerobic rod which belongs to the lactic acid bacteria (LAB) group. It is a facultative homo-fermenter found indigenous in dairy products such as *Nono* and cheese. *Nono* is a locally fermented milk drink consumed in Nigeria. It is a functional food which contains high numbers of lactic acid bacteria (LAB) which include *L. casei*. However, unhygienic and unaseptic milking, processing, packaging and vending procedures, could render the food product susceptible to contamination with *Escherichia coli* and other enteric pathogens. *L. casei* is well-known for its wide probiotic values such as immune modulation, cholesterol regulation, non-toxicity, antibacterial activity *inter alia*; and has been given Generally Regarded as Safe (GRAS) status, as there has not been any established risk to humans (Gaynor, 2012). However, a limitation that exists to the probiotic activity of this bacterium is its antibiotic resistance and the likelihood of transfer of this factor to the pathogens it is meant to control- possibly through horizontal gene transfer.

Prebiotics are non-digestible substances that confer beneficial physiological effect on the host when consumed, by selectively stimulating the favourable growth or activity of beneficial bacteria such as *Lactobacilli* and *Bifidobacteria* (Roberfroid, 2007). Commonly known prebiotics are oligofructose, inulin, galactooligosaccharides, lactulose and breast milk oligosaccharides. Prebiotics have also been identified in fruits and vegetables such as Chicory, bananas,*Vernoniaamygdalina*(Onugbu) and *Ocimum gratissimum*(Nchuanwu)(Roberfroid, 2007; Ezeonu*et al*., 2016). The prebiotic activities of *V. amygdalina* and *O. gratissimum* aqueous leaf extracts in the protection of rabbits (*Oryctolagus cuniculus*) have been demonstrated by Ezeonu *et al.,* (2012). *O. gratissimum* is one of the leafy vegetables consumed by Nigerians. It grows usually as a small shrub with many branches and simple oval leaves. The leaves are used as food additives, where it serves medicinal and nutritive values, as well as add aroma or flavor to the food (Okoye and Madumelu, 2013). According to Edeoga and Eriata (2011), it is mainly used as spice. However, it also serves a lot of medicinal purposes which include antibacterial, antifungal, and antihelminthic. Beyond its medicinal value, *O. gratissimum* has prebiotic potential.

Escherichia coli is one of the top seven pathogens of public health concern (CDC, 2014). Avian pathogenic *E. coli* (APEC) is the pathogen responsible for chicken colibacillosis. Colibacillosis is an intestinal infection andit is one of the major infections that threaten biodiversity conservation of poultry (Lutful-Kabir, 2010).

Cases of antibiotic resistance have made it more complex to control the effect of *E. coli* infections. Thus, this work is directed at observing the role of *L. casei* and *O. gratissimum* in the prevention and control of colibacillosis in chicken.

1.1. Statement of the Problem

Colibacillosis in chicken is caused by avian pathogenic *E. coli*. It is a threat to the biodiversity conservation of chicken and other poultry birds, especially at their neonate stage and adversely affects expected economic returns on the poultry by the farmers; thus, the disease condition has been controlled over the years with conventional antibiotics. However, this control pattern adopted by most Nigerian farmers has major draw-backs which are high antibiotic float in the human ecosystem and emergence of multiple drug resistant forms of *E. coli*. Therefore there is need to explore natural treatment alternatives such as probiotics and prebiotics, which are the focus of this research.

Lactobacillus casei is a probiotic microorganism whose usefulness has been documented by many researchers and its benefits alongside other probiotics are usually highest in young animals because they have not yet developed a stable gut microflora. *Nono* is a 24 hour fermented milk product consumed locally in Nigeria and serves as a natural habitat for *L. casei* and other Lactic acid bacteria. It is a functional food with a lot of probiotic advantage. However, poor hygienic standards during milking, processing and retailing of the milk product, tampers with the integrity of the food

byexposing it to contamination by *E. coli* and other enteric pathogens; thereby, posing a great food safety issue in consumption of the milk product.

Ocimum gratissimum has been used widely in cooking and as a medicinal plant in sub-saharan Africa. However, it does contain certain classes of carbohydrates that may serve as prebiotics. For the purpose of this research its potentials are to be investigated as a natural alternative for the treatment and control of chicken colibacillosis.

1.2. Aim of the Study

The aim of this study was to evaluate the probiotic activities of *Lactobacillus sp* andprebiotic activities of *Ocimum gratissimum*against Avian Pathogenic *E. coli*.

1.3. Objectives of the Study

Objectives of the study are to;

- Isolate and characterize *Lactobacillus* species and avian pathogenic *E. coli* from *Nono*.
- Evaluate the probiotic potentials of selected *Lactobacillus* isolate.
- Evaluate the antibiogram of the *Lactobacillus* and avian pathogenic *E. coli* isolates.
- Determine the prebiotic and phytochemical components of *Ocimum gratissimum*.

- Determine the in-vitro antibacterial activity of *O. gratissimum* against avian pathogenic *E. coli*.
- Evaluate the effect of probiotic *Lactobacillus* and *O. gratissimum* extract on growth performance of the chicken population.
- Evaluate the effect of probiotic *Lactobacillus* and *O. gratissimum* extract on haematology and blood chemistry profile of the chicken population.
- Evaluate the effect of *Lactobacillus* and *O. gratissimum* extract on the intestinal and caecal microflora of the chicken population.
- Evaluate the effect of *Lactobacillus* and *O. gratissimum* extract on the intestinal and caecal tissues of the chicken population.

1.4 Significance of Study

This study will show *Lactobacillus* sp. and *O. gratissimum* as useful alternatives in the control of colibacillosis in chicken. The research will help to promote the use of probiotics and prebiotics as antibiotic alternatives in colibacillosis prevention and treatment.

CHAPTER TWO

LITERATUTRE REVIEW

2.1 Biodiversity Conservation- Poultry Perspective

Biodiversity refers to the variety of life on earth at all levels such as genetic, microbial, animal and plant levels with all the ecological and evolutionary processes that sustain it (Rautava *et al*., 2002). It does not only include species that are considered rare, threatened or endangered, but every living thing including microorganisms. Biodiversity conservation is about saving life on earth in all its forms and keeping natural ecosystems functional and healthy. The world governments recognize the importance of biodiversity conservation and thus, have set certain frame works aimed at achieving these goals; frame works such as: enactment of biodiversity act in different nations such as India, Australia *inter alia*; setting up of conventions on biological diversity conservation, fixing of international biodiversity agreements *et cetera*. The main threats to biodiversity conservation include: spread of diseases and invasive species, loss and degradation of habitat, climate change, unsustainable use of natural resources and change to the aquatic and water flows (Mutia, 2009).

Poultry is a group of domestic birds reared to serve as food to man and as a means of income to farmers. Some of the birds in this group are chicken, turkey, duck, guinea fowl *et cetera*. A major threat to the conservation of this group of birds is the spread of disease, of whose effect extends to economic loss to the farmers. Over the years, somany diseases have threatened the existence of poultry birds, diseases ranging from

bacterial to fungal and also viral- such as colibacillosis, coccidiosis, and avian influenza respectively. Some of these diseases end up as epidemics and also pandemics (as seen in the case of Avian Influenza). Just as diseases pose threat to poultry biodiversity conservation, likewise, the conventional disease treatment model adopted by poultry farmers and veterinarians has turned out to equally be a threat to poultry biodiversity conservation *viz*: the use of antibiotics.

Antibiotic use in disease treatment has spanned through several years. Not only are they used in poultry disease treatment, they are also used for treatment in humans. A major unpleasant issue that has emerged through this treatment model is antibiotic resistance. Antibiotic resistance exhibited by pathogens has made disease treatment and control more difficult. In the case of poultry it has turned out to be a threat to their biodiversity existence, thereby, suggesting a need for other alternatives to tackle antibiotic resistance- probiotics and prebiotics.

2.2 Antibiotic Resistance- A Threat to Disease Treatment

The issue of antibiotic resistance has grown over the years, cutting across a range of bacterial infections from tuberculosis to *Staphylococcus* (MRSA), to other Gram negative sepsis. Antibiotic resistance could be seen as an equal opportunity threat, which spans across all the continents of the world (Ostroff, 2015). It threatens to reverse the decades of progress made in infectious disease control, and as such it has climbed the ladder of public health priorities. According to CDC (2013), the world

economic forum has included antibiotic resistance as a global risk- not just a global health risk.The use of antibiotics in food producing animals is a practice *en vogue* for decades in combating farm animal pathogens, as well as food-borne pathogens.

2.2.1 Antibiotic Resistance in Farm Animals and Food-borne Pathogens

Antibiotic influx in food animals' system is the main stem for resistance seen in zoonotic and food-borne pathogens. The resistance occurs via shifts in antibiotic use in farm animals. Zoonotic pathogens are disease causing microorganisms that affect the well-being and possibly threaten the existence of animals (food animals), while food-borne pathogens are disease causing microorganisms that affect man but are gotten from infected food consumption.

The use of antibiotics in disease treatment of food animals is a practice that engages certain stake holders such as farmers, veterinarians, wild life experts and food safety professionals. Virtually most feeds for growing animals are supplemented with antimicrobials in various doses, ranging from sub-therapeutic concentrations to full therapeutic doses. According to Wenger (2012), it is estimated that the volume of antibiotics used in food animals exceeds the use in humans worldwide. Virtually all the classes of antimicrobials used for humans are also being used in food animals, including cephalosporins, fluoroquinolones, glycopeptides and streptogramins (Aarestrup *et al*., 2008). The massive influx of antibiotics as an animal enhancement option has aided the escalated rate of antibiotic resistance in zoonotic and food-borne

pathogens. This resistance is usually passed on from one microbe to the next via horizontal gene transfer, which goes through various vehicles to eventually get to humans.

2.2.2 Antibiotic Resistance- Linking Human and Animal Health

The epidemiology of antimicrobial resistance at the human-animal interface is invariably complex. It involves a lot of potential transmission routes and vehicles, antimicrobial selection pressures and other ecological drivers such as direct contact via the food chain, water, air, manured and sludge-fertilized soils; which transfer antibiotic resistant bacteria from animal husbandry, aquaculture and related agricultural practices to humans (Marshall and Levy, 2011). Low dose and prolonged courses of antibiotics among food animals create ideal selective pressures for the propagation of resistant strains. Spread of resistant strains may not just be directly through food, water, and animal waste application to agricultural soils; it could be greatly aided by horizontal transfer of genetic elements such as plasmids via bacterial conjugation. Certain evidences exist in the works of researchers on the proof of animal-to-human transfer of resistant bacteria on farms using antibiotics for treatment and/or non-therapeutic purposes. Marshall and Levy (2011), reported in their research work, the transfer of Tetracycline-resistant *E.coli* from Chickens to the animal caretakers and their families through plasmids, in the United States of America. The transferable plasmids of the Tetracycline- resistant *E. coli* strains were found in the

gut flora of the caretakers and their families. They went on to posit that the animal caretakers served as conduits of transfer from the chicken at the farm, to their families. A similar work was equally done by same researchers on Gentamicin-resistant *E.coli* from U.S. chickens to the poultry workers through direct contact.They also reported the isolation of Apramycin-resistant *E. coli* and *Salmonella typhimurium* from hospital patients that consume Belgian cattle. Apramycin is an antibiotic used only in animals, but in this case, Apramycin-resistant bacteria were isolated from humans, which suggest an animal-human antibiotic resistance transfer.

Conclusively, 'farm-to-fork' phenomenon is the major route that links antibiotics resistance from animals to humans. This really implies that uncontrolled antibiotics use in food animals would eventually move up to get to man.

2.3. *E. coli* as a Pathogen

Escherichia coli is one of the five bacteria of potential world health concern (CDC 2014). It is a Gram negative, rod-shaped, facultatively anaerobic non- spore former (Madigan& Martinko, 2006). It usually lives as a commensal in the gut at an optimum temperature of 37^0C (Fotador *et al*., 2005). However, when growth conditions vary, it could become an opportunistic pathogen (Tarr *et al*., 2005). Pathogenicity of *E. coli* is ascribed to a number of virulence factors and phenotypic traits, which form the basis for classification of *E. coli* strains (Nyenje and Ndip, 2013). These include: entero-haemorrhagic *E. coli* (EHEC) or Shiga toxin producing *E. coli* (STEC) that produce

verocytotoxin or Shiga-like toxin that causes haemorrhagic colitis and haemolytic ureamic syndrome; entero-toxigenic *E. coli* (ETEC) which produces enterotoxin that causes diarrhea; entero-invasive *E. coli* (EIEC) strains which are causative agents of dysentery-like illnesses; entero-aggregative *E. coli* which do not secret heat-labile enterotoxins but adhere to the mucosal cells in aggregative pattern; and diffusely adherent *E. coli* (DAEC) strains that adhere to the surface of epithelial cells (Tarr *et al*., 2005; Gyles, 2006; Wu *et al*., 2011). However, cases of food-borne outbreaks have been mostly associated with EHEC and EAEC strains, with *E. coli* 0157:H7 being widely recognized as the major cause of food-borne illness (Saghaian *et al*., 2006).

2.3.1:*E. coli* as a Food-borne Pathogen

Food-borne outbreaks and sporadic cases have been reported in foods such as unpasteurized milk, under-pasteurized milk, contaminated fresh whole milk, contaminated meat and meat products, leafy green vegetables and foods fertilized with contaminated animal manure (Sartz *et al*., 2008; CDC, 2011); with *E. coli* being one of the recurring implicated pathogens.

E. coli as a food-borne pathogen is a threat to food safety, especially raw foods, foods that do not require thorough cooking before consumption and food products that do not receive adequate antimicrobial treatments before consumption, e.g., salad dressings, fermented sausages, apple cider *et cetera*. This microorganism presents

food-borne illness characterized mainly by haemorrhagic colitis. Its low infectious dose combined with the disease severity, present unique challenges to its prevention. According to U.S.F.D.A. (2015), Hazard Analysis Critical Control Point (HACCP) system, which includes steps lethal to the pathogen, provides a systematic and effective food safety protocol against the pathogen. This implies that the HACCP system should focus on risk elimination rather than on risk reduction, as a result of low infective dose of the pathogen.

In Nigeria, *Nono* is 24 hour fermented milk produced and retailed mainly by the *Fulani* tribe. The milk product is accessible in any part of the country where the *Fulanis* have their settlements. This milk product is also consumed by other tribes that live around these settlements with the aim of deriving certain nutritional benefits which center mainly on male reproductive health. However, certain factors such as unhygienic milking, processing, packaging and vending, hygiene of the food handlers and vendors pose great avenues for *E. coli* and other faecal pathogens contamination. This affects the wholesomeness of the milk product. Contamination occurs either directly from the environment or indirectly from disease vectors such as house flies (*Musca domestica*). Thus, the food product (*Nono*) is a potential source of *E. coli* disease outbreak in Nigeria. In developed countries, automated systems are set in place for milking, pasteurizing, packaging and vending of whole milk in aseptic conditions (CDC, 2014).

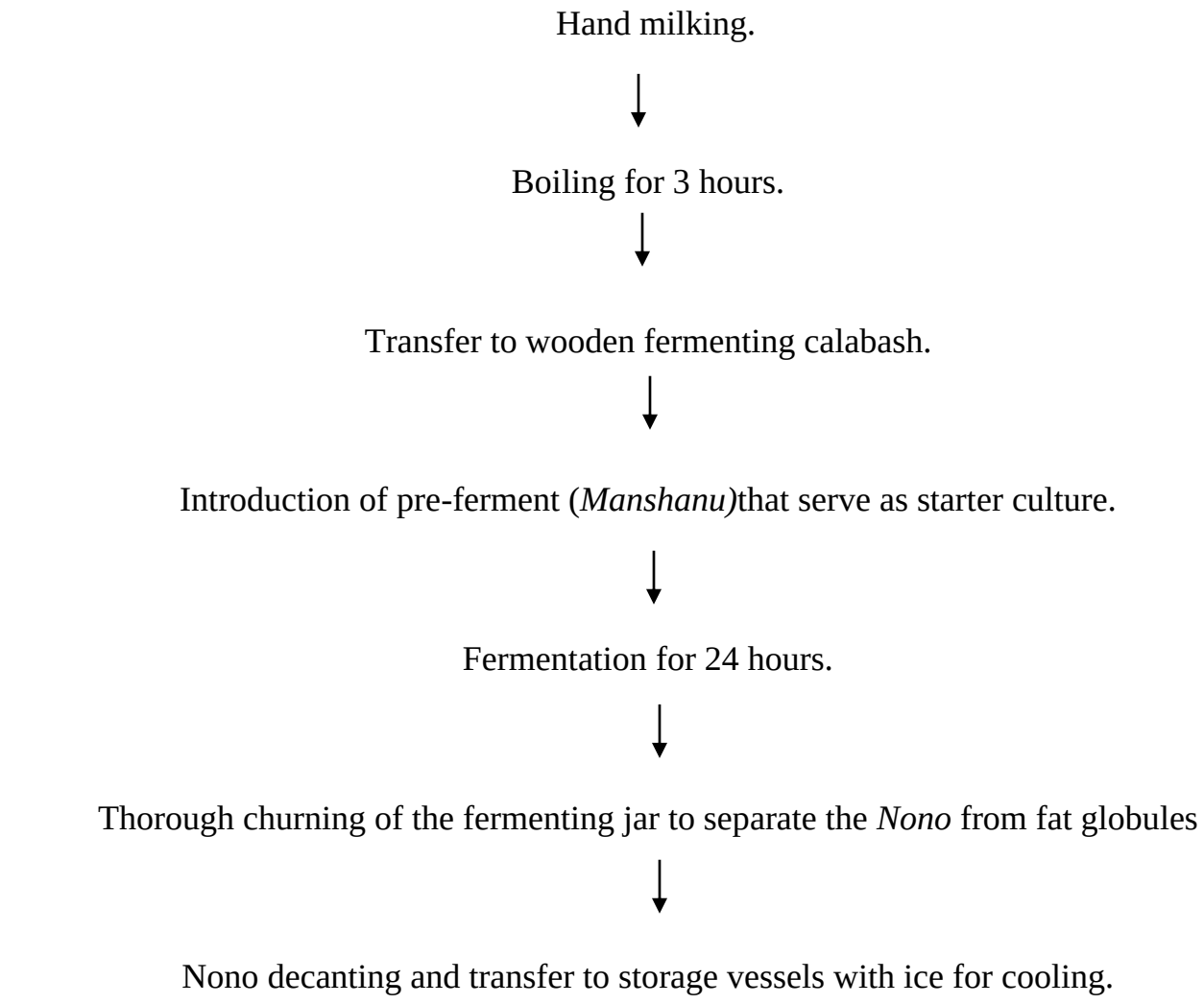

Figure 1: Nono Processing Steps.

2.3.2: *E.coli* as a Farm Animal Pathogen

Disease syndromes usually associated with *E. coli* are known as colibacillosis. In farm animals they include enteric colibacillosis which involves colibacillary dairrhoea and systemic colibacillosis which is mainly caused by invasive strains of *E.coli* (Kariaki *et al*., 2002).

Colibacillary diarrhea occurs most frequently in animals as from 1-3 days after birth and lasts for 2-3 weeks. The disease is often acute, with Entero-toxigenic *E.coli* (ETEC) being the main implicated strain. ETEC are usually able to adhere to the mucoid surface of the villous epithelium of the small intestine by overcoming the mechanical clearance caused by peristalsis. This adherence is usually aided by the presence of fimbriae. Though adherence is essential, it is not responsible for eliciting diarrhea. It is elicited by enterotoxins. These enterotoxins are of two types *viz:* heat Labile and heat stable toxins.

Systemic colibacillosis sets in during a drop in immunoglobulins of the farm animals. It presents with generalized acute infections followed usually by mortality. The external appearance of the dead animals looks normal but internally splenomegaly and organ congestion are noticeable (Witold & Carolyn 2011). In generalized infections, pure cultures of *E. coli* can usually be isolated from most organs and tissues, whereas in localized infections it may only occasionally be isolated from infection sites. The most common *E. coli* strain that causes colibacillosis is 078:K80, which is frequently found in calves, lambs and poultry.

2.4. Colibacillosis in Chicken

E. coli is considered as a member of the normal flora of the poultry intestine. However, certain strains designated Avian pathogenic *E. coli* (APEC), cause colibacillosis characterized by systemic fatal disease(Barnes and Gross, 1997). Colibacillosis in poultry is frequently associated with *E. coli* strains of serotypes 078, K80, 01: K1 and 02:K1 (Kabir *et al*., 2005). Colibacillosis is predominantly found occurring in all age group of chickens, with high prevalence rate in adult layer birds and 1-5 weeks chicks. In a single bird, a large number of different *E. coli* types are present.These are acquired via horizontal contamination from the environment (specifically from other birds, faeces, water and feed) (Ewres *et al*., 2004). Immediately after hatching, the birds start building up their *E. coli* flora (La Ragione *et al*., 2001).

The risk for colibacillosis increases with increasing infection pressure in the environment. Thus, proper house hygiene and avoidance of overcrowding in poultry pens are paramount in reducing the risk factors of the disease (Lutful-Kabir, 2010). Other factors that aid propagation of the infection are duration of exposure, virulence of the strain, breed of the birds and immune status of the birds. An unfavourable housing climate, like an excess of ammonia or dust, renders the respiratory system of the birds more susceptible to APEC infections through colonization of the upper respiratory tract (Manges *et al*., 2007). Avian pathogenic *E. coli* (APEC) strains are

considered to be entero-toxigenic. They produce one or more enterotoxins which start their actions from the small intestine and thereafter, progress to the manifestation of systemic symptoms like, respiratory tract infection, yolk sac infection, swollen head syndrome, salpingitis *et cetera* (Knobl *et al.*, 2001). Colibacillosis in poultry is characterized in its acute form by septicemia, resulting in death.

2.4.1 Pathology of Colibacillosis

APEC is responsible for a considerable number of colibacillosis infections in chicken of different ages. Colibacillosis primarily affects broiler chickens between the ages of 4 to 6 weeks and is responsible for a significant proportion of mortality found in poultry flocks (Hughes *et al*., 2009). Infection with *E. coli* could contaminate the egg before shell formation inside a diseased laying fowl or more so, during the passage of the egg through the cloaca and after laying (Dziva and Mark, 2008). Before hatching, APEC causes yolk sac infections and embryo mortality (Lutful- Kabir, 2010). The chick can also be infected during or shortly after hatching. It is usually believed that APEC causes diseases like salpingitis, respiratory distress, yolk sac infection but not gastroenteritis. However, Zanella *et al*., (2000) were able to experimentally induce diarrhea in chicken using APEC, which suggests that APEC has the ability to elicit intestinal disease and not just diarrheal symptom.

It has been discovered that APEC share identical serotypes and specific virulence genes with human pathogenic *E. coli*, thus establishing their zoonotic potentials –

APEC strains can easily be transmitted to man (Ewres *et al*., 2004). APEC has the ability to spread to humans, where it acts as unpathogenic *E. coli* and a reservoir of virulence genes that will re-transmit to chickens (Rodriguez *et al*., 2005). APEC transmits mainly to humans via consumption of infected poultry meat. Epidemiological reports still suggest that one of the primary causes of human food poisoning is poultry meat, which gets contaminated through unaseptic processing and packaging (Yashoda *et al*., 2001).

2.4.2 Symptoms, Prevention and Treatment

Colibacillosis is usually announced first by the onset of diarrhea. It then progresses to respiratory distress, anorexia and poor growth. The progressive diarrhea equally leads to dehydration, sub-normal body temperatures, mild reddening and congestion of the stomach. Lesions seen at post morterm are pericarditis, peritonitis and air sacculitis (Mellata, 2003). High numbers of *E. coli* are maintained in the poultry house environment through fecal contamination, with the common route of infection being through inhalation of the fecally contaminated dust that contains large numbers of the pathogen.Preventive measures should target towards maintenance of proper poultry house hygiene, use of clean water and uninfected feeds in feeding the birds. Treatment of colibacillosis relies on antimicrobial therapy. However, antibiotic resistance is proving a strong hindrance to this method.

2.5 Emerging Alternatives to the Use of Antibiotics

2.5.1: Probiotics

Probiotics are microorganisms which when administered in adequate amounts confer health benefits to the host. According to Mach (2006), probiotics in the English interpretation means ‘for life’, which opposes the routinely used treatment therapy-antibiotics, which literally means ‘against life’ in English. Lactic acid bacteria and *Bifidobacteria* are the most commonly known probiotics but recent studies have shown that certain *Bacilli*, *Streptococci*, and yeasts are also probiotics (Reid, 2008).

Probiotics are commonly consumed as part of fermented foods as special active live cultures. Such foods are Yoghurt, Soy yoghurts, Pickles, Sauerkraut, Kenkey, Cheese, Salami, Tempeh, Palm wine, *Nono*, Palm sap, Locust beans, Akamu and so on. Probiotics can also be administered as dietary supplements. The WHO standard for any food sold with health claims derived from the addition of probiotics is that it must contain at least 10^6–10^7 cfu of viable probiotic bacteria per gram (FAO/WHO 2001). At the start of 20^{th} century, probiotics were thought to be beneficial to the host by improving host’s intestinal microbial balance, thus, inhibiting pathogens and toxin producing bacteria.

According to Tannock (2003), specific health effects of probiotics that have been investigated and documented include;

- Alleviation of bowel inflammatory syndrome.

- Prevention and treatment of pathogen induced diarrhea.
- Treatment of urinogenital infections.
- Treatment of oral thrush and other dental caries.
- Treatment of Crohn's disease.
- Prevention and treatment of antibiotics associated diarrhea.
- Prevention and treatment of Lactose intolerance.
- Lowering of serum cholesterol.
- Improvement of immune functions.
- Prevention of colon cancer.

Probiotics can be administered in form of powders, tablets, capsules, pastes or sprays. They are not known to have any side effect yet. It is also believed that in-take of about 10^9-10^{10} microorganisms per day will confer better health benefits (Vanderhoof, 2008). Some microbes that have been used as probiotics are *Lactobacillus acidophilus, Bifidobacteria bifidum, Saccharomyces boulardii, Streptococcus thermophilus, Bacillus cereus,Lactobacillus reuterii, Bacillus infantis, Saccharomyces cerevisiae, Lactobacillus plantarum, Lactobacillus casei, Lactobacillus salivarius, Lactobacillus bulgaricus , Bacillus adolescentis, Lactobacillus fermentum, Lactobacillus gasseri, Lactobacillus johnsonii, Lactococcus lactis, Lactobacillus paracasei inter alia* (Sgouras *et al*., 2004).

2.5.1.1:Characteristics of Organisms that Serve as Probiotics

According to Fuller (2007), the following are the desired characteristics expected from microorganisms that will serve as probiotics;

- They should be non-pathogenic.
- They should be non-toxic.
- They should have high viability.
- They should be stable on storage and in the field.
- They should be able to survive in and colonize the gut.
- They should be amenable to cultivation in an industrial scale.
- They should be able to secret antimicrobial agents.

These properties stated by Fuller (2007), are very essential for the well-being of the individuals that take these probiotics and also for the benefit of industrialists that will manufacture them. Toxicity is a very crucial issue to be thought of before approving any ingestible matter for use by man. Microorganisms to be used as probiotics are expected to confer health benefits to man and not to cause more health problems, thus, for an organism to be accepted as a probiotic, it must be non-toxic.Pathogenicity is another undesired property in any microorganism to be used as a probiotic. This is due to the fact that pathogens are disease causing organisms. Viability is an interesting property that is advantageous to the industrialist. These microorganisms to be used as probiotics are needed by a very large population. It is expected that such

organisms should produce high yields with little amounts of substrates during industrial production, in order to serve the population and also yield large profits to the industrialist. There will be no industrial production of these microorganisms if they are not amenable to cultivation in an industrial scale. Likewise, if there is no industrial production of these probiotics, there is no way the teeming population of the world can have access to them. Therefore, microorganisms that serve as probiotics to man should be amenable to industrial cultivation. Also high viability ensures a successful competitive inhibition against pathogens in the gut (Saggioro *et al.*, 2005).

Probiotic organisms are to remain stable during storage. After production of these organisms, they are usually packaged as powders, tablets, capsules, pastes or sprays and stored for later dispachment and sales. Therefore, it is expected that those microorganisms should not mutate during storage. It is also important that the storage conditions of these organisms be written by the manufacturer in order to properly direct those who intend to buy in large quantities.

Since the main route of administration of these probiotics is oral, it is therefore expected that they should be able to withstand the gut conditions such as acidic pH, bile activity, and colonize the gut effectively in order to confer expected health benefits. Production of antimicrobial substances by probiotic organisms is essential for their effectiveness against pathogens. Antimicrobial activity targets the enteric pathogens (Welman, 2009). It involves the production of some substances such as

organic acids (lactic, acetic, propionic acids), carbon dioxide, hydrogen peroxide, diacetyl, low molecular weight antimicrobial substances and bacteriocins (Dunne *et al.*, 2001). Some examples are seen in *Lactobacillus reuterii*, (a member of normal microflora of human and many other animals) which produces a low molecular weight antimicrobial substance called reuterin. Subspecies of *Lactococcus lactis* produce a class I bacteriocin known as nisin A, which serves as a food grade antibiotics (Rautava *et al.*, 2002); *Enterococcus feacalis* DS16 produces a class I bacteriocin known as cytolysin; *Lactobacillus plantarum* produces a class II bacteriocin known as plantaricin S; *Lactobacillus acidophilus* produces a class III bacteriocin known as acidophilucin A (Pelletier *et al.*, 2001). Bacteriocin production is dependent on certain factors such as the species of microorganisms, nutrients available in the medium, pH of medium, incubation temperature and time.

2.5.1.2:Health Benefits of Using Probiotics

The benefits of probiotics intake are that they help in;

- Prevention and treatment of antibiotic associated diarrhea.
- Treatment of lactose intolerance.
- Prevention of colon cancer.
- Lowering of serum cholesterol.
- Improvement of eye sight.
- Prevention of oral thrush and dental caries.

- Improvement of immune functions and prevention against infections.

Antibiotic associated diarrhea results from an imbalance in the colonic microbiota caused by antibiotic therapy. This microbiota alteration changes carbohydrate metabolism with decreased short chain fatty acid absorption, resulting in diarrhea due to osmotic imbalance. The introduction of probiotics into the gut will help keep the gut osmotic concentration in order and prevent the occurrence of diarrhea.

Lactose intolerance is a situation that occurs in individuals who do not have the ability to tolerate milk and any other lactose containing product in their gastro intestinal tract. This is because the intestinal walls lack the ability to produce the enzyme lactase, which is responsible for degrading lactose to glucose and galactose. Thus, the introduction of probiotics, especially the lactic acid bacteria will bring about the degradation of lactose by these organisms, in the gut of those lactose intolerant individuals (Yavuzdurmaz, 2007).

Investigations show that *Lactobacillus bulgaricus* has demonstrated anti-mutagenic effects because of their ability to bind with heterocyclic amines (which are carcinogens formed in cooked meat) thus preventing colon cancer.

Lactic acid bacteria lower serum cholesterol by breaking down bile in the gut, thus, inhibiting its re-absorption which enters the blood as cholesterol.

Saccharomyces cerevisiae have shown the ability to improve eye-sight. They are good sources of beta carotene when ingested through foods like palm wine, bread etc. The liver then converts the beta carotene to vitamin A which is readily absorbed by the retinal cells of the eye (Bernstein, 2006).

Lactic acid bacteria affect pathogens by means of competitive inhibition and they improve immune functions by increasing the number of immunoglobulin A-producing plasma cells, improving phagocytosis, as well as increasing the proportion of T-lymphocytes and natural killer cells. This work aims at discovering the role of *Lactobacillis casei* in the prevention and treatment of colibacillosis in chicken.

2.5.2: Prebiotics

A prebiotic is a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring health benefit(s) upon the host. Prebiotics are generally oligomers made up of 4 to 10 monomeric hexose units (Gibson and Roberfroid, 1995; Usha and Natarajan, 2012). Many dietary fibers, especially soluble fibers, exhibit some prebiotic activity; however, Roberfroid (2000) only identified two groupings of nutritional compounds that meet his definition. These two groupings or sub-categories can be described as inulin-type prebiotics and galactooligosaccharides (GOS). Prebiotics are “indigestible fermented food substrates that selectively stimulate the growth, composition, and activity of microflora in gastrointestinal tract thereby improving the hosts’ health and

well-being" (Roberfroid, 2000). Lactulose was used 50 years ago as a prebiotic formula supplement to increase the number of *Lactobacillus* strains in infants' intestines (Marimuthu *et al*., 2014). Fructooligosaccharides, inulin, oligofructose, lactulose, and galactooligosaccharides have been identified as prebiotics due to characteristics such as resistance to gastric acidity and hydrolysis by mammalian enzymes and they are fermented by gastrointestinal microflora to further selectively stimulate the growth and activity of beneficial microorganisms. The number of new compounds which have gut resistant properties and selective fermentability by intestinal microorganisms are identified and developed as prebiotics (Gibson and Fuller, 2000.) These include oligosaccharides (isomaltooligosaccharides, lactosucrose, xylooligosaccharides, and glucooligosaccharides), sugar alcohols, and polysaccharides (starch, resistant starch, and modified starch), (Cummings *et al*., 2001).

Prebiotics must provide selective stimulation of thegrowth or activity of beneficial native bacteria. Since prebioticsare non-viable, stability is not a concern, but safe consumption levels must be established.These prebiotic substances survive digestion in the stomach and reach the colon where they are metabolized by the bacteria, thereby directly providing the host with energy and metabolic substrates. Efficient prebiotics usually have a specific fermentation in the colon and have the ability to alter the faecal microflora composition towards a more beneficial community structure (Kolida *et al*., 2002; Chakraborti, 2011). Thus, prebiotics exert their

beneficial effects on the host indirectly; by stimulating functions of the intestinal microflora.

Prebiotics which are complex carbohydrates are non- digestible by the gastric juice, pancreatic and brush border enzymes and selectively stimulate the growth of intestinal microflora. These carbohydrates are commonly found in fruits,vegetables and plant products (Menne *et al*., 2000; Rastall *et al*., 2005). In the large intestine, prebiotics, in addition to their selective effects on intestinal bacteria, influence many aspects ofbowel function through fermentation (Cummings *et al*., 2001). These substances are fermentedby bacteria such as *Bifidobacteria* and *Lactobacilli* in the colon, to produce short chain fatty acids (SCFA) such as propionic, lactic, acetic, folic, and butyric acids. Hydrogen gas (H_2) and carbondioxide (CO_2) are also major products of prebiotic metabolism (Wang and Gibson, 1993; Cummings *et al*., 2001; De Vuyst *et al.*, 2005; Saulnier *et al*., 2007). According to Cummings *et al* (2001), besides the stimulation ofintestinal bacteria, fermentability is an important property for evaluating or screening potential prebiotics. However, recent studies show that there may be other candidate-prebiotics such as xylitol, sorbitol, mannitol and lactulose (Chakraborti, 2011).

2.5.2.1: Sources of Prebiotics

Traditional dietary sources of prebioticsinclude soybeans, inulin sources (such as Jerusalem artichoke, jicama, and chicory root), raw oats, unrefinedwheat, unrefined

barley, and yacon (Jurgonski *et al*., 2010). Ezeonu *et al*., (2016) also demonstrated the role of *Vernonia amygdalina*, which is a popular Nigerian vegetable, as a prebiotic. Newburg (2005), included breast milk as a prebiotic. Some of the oligosaccharides that naturally occur in breast milk are believed to play important role in the development of a healthy immune system in infants. The breast feeding infants have flora dominated by *Lactobacilli* and *Bifidobacteria*. These are part of the baby's defence against pathogens, whichis an important primer for the immune system. These flora are nurtured by the oligosaccharides of breastmilk, which is considered to be the original prebiotic. While some peptides, proteins, and certain lipids are potential prebiotics, non-digestible carbohydrates, in particular non-digestible oligosaccharides, have received the most attention (Morais and Jacob, 2006).

2.5.2.2: Health Effects of Prebiotics

Fermentation of oligofructose in the colon is due to thepresence of intestinal microflora, which confers beneficial effects to humans. These include increasing the numbersof probiotics in the colon, increasing calcium absorption, increasing fecal weight, shortening gastrointestinal transittime, and possibly, lowering blood lipid levels (Marimuthu *et al*., 2014). Prebiotics also play immune-modulatory roles (Ezeonu *et al*., 2016), regulate fatty liver disease, blood sugar, constipation and prevents diarrhea.

Improved Gut Mucosal Barrier & Immune Function

The gastrointestinal tract is one of the most important components of the body's defensivesystem. In addition to providing non-specific protection in the form of a physical barrier againsttoxins and pathogenic organisms, the intestinal tract also provides specific protection in the formof gut-associated lymphoid tissue (GALT). GALT represents the largest immune organ in the body and consists of a highly complex network of aggregated and non-aggregated immune cells(Watzl *et al*., 2005). Research indicates prebiotics modulate both intestinal and systemicimmunity largely through their association with gut microflora.

Prebiotic support of health-promoting intestinal microorganisms leads to increased competitionwith pathogens for colonization sites, up regulated GALT expression of secretory IgA andimmune-stimulating cytokines, and enhanced production of short chain fatty acids and otherantimicrobial substances that create an inhospitable environment for pathogen growth (Hosono *etal*., 2003). Prebiotics have been shown to further enhance the integrity of theintestinal mucosa by increasing villous height, augmenting mucin release, and enhancing healthymucosal biofilm composition (Kleessen and Blaut, 2005). The morphological and functionalenhancements prebiotics bring to the gut all improve colonization resistance and reduce the riskof pathogen translocation.

Prebiotics such as inulin, inulin-type fructans, galactooligosaccharides, and lactulose have beenshown to enhance colonization resistance against a variety of

enteropathogenic organisms, including *Clostridium difficile, Clostridium perfringens* and *E. coli* (Shoaf*et al*., 2006). Research indicates some prebiotic-likesubstances may also be able to directly stimulate immune cells. Yeast beta-glucans have beenshown to activate receptors on phagocytes, NK cells, and certain classes of T- and B lymphocytes and a novel class of oligosaccharides known as nigeroligosaccharides has been found to augment splenocyte proliferation and production of immune-potentiating cytokines such as interleukin-12 and interferon-γ (Murosaki *et al*., 1999).

Carcinogenesis and Reduction in Colon Cancer Risk

Carcinogenic substances introduced into the intestinal tract from exogenous dietary sources, or produced endogenously by the gut microflora, represent an environmental insult thought to play a role in the initial stages of cancer. In vitro and animal studies have revealed the potential of prebiotics to enhance detoxification processes in colon cells, reduce toxic metabolite production in the gut, and protect against colonic tumor development. In animal models, inulin- typefructans, galactooligosaccharides, and xylooligosaccharides have been shown to suppress chemically induced colon cancer and precancerous colon lesions (Wijnands *et al*., 2001; Hsu *et al*., 2004; Pool-Zobel, 2005).

This effect is potentiated by the presence of lactic acid bacteria and associated with microflora fermentation and production of butyrate. Health-promoting bacteria inhibit

the growth of pathogenic bacteria and thus decrease the production of carcinogenic substances such as ammonia, and tumor-promoting bacterial enzymes such as beta-glucuronidase. At the same time, bacterial growth increases biomass and thus stool bulk and accelerates colonic transit time decreasing exposure of the colon to potential carcinogens. Prebiotics reduce both the incidence and multiplicity of aberrant crypt foci and colontumors in animal models and research indicates synbiotics have an even more pronounced preventive effect (Rowland *et al*., 1998).

Enhancement of Mineral Absorption

Several animal studies have demonstrated that inulin-type fructans (Beyen *et al*., 2002), galactooligosaccharides (Chonan *et al*., 1995), isomaltooligosaccharides (Kashimura *et al*.,1996), lactitol, (Mineo *et al*., 2002) and lactulose (Brommage *et al*., 1993) substantially enhancemineral absorption, especially calcium and magnesium. The combination of inulin and oligofructose has been shown to increase calcium and magnesium absorption more effectively than either oligosaccharide alone. In addition to augmenting calcium and magnesium absorption, inulin type fructans have been shown to protect animals from developing symptoms associated with magnesium deficiency and to correct osteopenia. A number of clinical trials involving adolescents, post-menopausal women, and adult men have confirmed an enhancement of mineral absorption mediated by inulin-type fructans (Scholz-Ahrens *et al*., 2007). Increased colonic mineral absorption results from fermentation of inulin- type fructans

which leads to higher concentrations of short-chain fatty acids, a lower colon pH, and enhanced mineral solubility andbioavailability.

Promotion of Normal Colon Transit Time

Constipation is an exceedingly common clinical problem affecting large segments of the population including the elderly, pregnant and nursing women, people on weight loss diets, andpeople with disrupted daily schedules such as variable shift workers and business travelers (Brandt, 2001). Prebiotics increase fecal bulk and optimize stool consistency primarily by increasing fecal microbial mass. This increase in fecal bulk stimulates passage through the colon, shortening transit time. Colonic water re-absorption is reduced, stoolbecomes softer and heavier, and stool frequency increases. Together these factors alleviate constipation and improve colon evacuation. In a study of constipated elderly adults, 20 grams per day of inulin- type fructans had a significantly better laxative effect than lactose (Kleessen *et al*., 1997).

A mixture of inulin-type fructans and galactooligosaccharides has been repeatedly shown to improve the stool frequency and consistency of bottle- fed infants similar to that of breast-fedinfants (Moro *et al*., 2002). Administration of isomaltooligosaccharides has been shown toincrease stool frequency and wet stool output in constipated elderly men (Chen *et al*., 2001).Xylooligosaccharides have been shown to reduce severe constipation in pregnant woman (Tateyama *et al*., 2005) and

lactulose administration has a long clinical history of alleviatingconstipation (Schumann, 2002).

2.5.3. *Ocimum gratissimum*

African basil, *O. gratissimum* L., (*Nchuanwu*)is a perennial herbaceous, drought tolerant plant with lime-green pubescent leaves, a characteristically strong fragrance and a slight pungency. In Nigeria and several other countries, the plant plays important roles in traditional medicine preparations for stomach up-set and for treatment of sunstroke, headache and influenza. In the coastal areas of Nigeria, the plant is used in the treatment of epilepsy, high fever, and diarrhea (Effraim *et al*., 2003), while in the savannah areas leaf decoctions are used to treat mental illness (Akinmoladun *et al*., 2007). Other uses include the treatment of fungal infections, fevers, colds, and catarrh (Ijeh *et al*., 2005). The plant is known to contain phenolic compounds with therapeutic potential (Vierra and Simon, 2000). *O. gratissimum* is a shrub up to 1.9 m in height with stems that are branched. The leaves measure up to 10 × 5 cm, and are ovate to ovate-lanceolate, sub-acuminate to acuminate at apex, cuneate and decurrent at base with a coarselycrenate, serrate margin, pubescent and dotted on both the sides (Ijeh *et al*., 2005). The leaves show the presence of covering and glandular trichomes. Stomata are rare or absent on the upper surface while they are present on the lower surface. The peduncles are densely pubescent. Calyx is up to 5 mm long campanulate and 5-7 m long, greenish-white to greenish-yellow in colour.

Plate 1: *Ocimum gratissimum* Plant.

2.5.3.1. Food Values of *O. gratissimum*

Proximate Composition

The proximate composition of the leaves and stems of *O.grastissimum* has been studied by various researchers (Idris *et al*., 2010). The leaves and stems contain some amount of moisture, ash, crude fibers, crude lipid, crude protein, carbohydrates and energy. These proximate compositions are essential in formation of bones, teeth, hair, and the outer layer of the skin and help to maintain the blood vessels and other tissue in the body of humans and animals. Fajohunbo and Egbeyale (2010) reported a carbohydrate composition of 13.40 g and went further to state that carbohydrate is the dominant nutrient in *O. gratissimum* when compared to its crude fibre, ash and crude protein values. Ezeonu *et al*., (2012) reported the likelihood of prebiotics being contained in *O. gratissimum* as a green leafy vegetable and since prebiotics are carbohydrate complexes, it suggests that the plant could contain a good quantity of some classes of prebiotics.

Mineral composition

O. gratisimum is composed of several minerals. These include potassium, calcium, phosphorous, magnesium, iron and copper; which are necessary for the proper functioning of all living cells and thus present in plant and animal tissues. Calcium is a major component of all healthy diet and a mineral necessary for life. Phosphorous is necessary in the formation of bones and teeth. Magnesium is essential to all living

cells and plays a major role in the functions of important biological polyphosphate compounds like ATP, DNA and RNA. Copper helps to produce red and white blood cells and triggers the release of iron to form haemoglobin, the substance that carries oxygen around the body. Also iron is another component of *O. gratissimum* which is important in a number of physiological processes as a constituent of some enzymes and in activation of other enzymes.

Chemical Composition

Numerous publications have presented data on the composition of the essential oils of *O. gratissimum*. In early investigations of the Nigeria variety of *O. gratissimum*, the essential oil was found to possess appreciable antibacterial activity against a wide range of organisms. Thymol was identified as the major principal essential oil which was responsible for its antibacterial activity. *O. gratissimum* from Europe contain eugenol as the dominant component as well as traces of *Ocimene* and *Myrcene* (Agbogidi *et al*., 2014). Analysis of volatile oils from the leaves and flowers from different locations in Nigeria confirmed the occurrence of thymol as the main constituent but eugenol was not detected. However, a recent study of the central Nigeria grown *O. gratissimum*, essential oil yielded eugenol (61.9%) as the most abundant compound (Agbogidi*et al*., 2014).

2.5.3.2. Health Benefits of *Ocimum gratissimum*

Traditional Uses

O. gratissimum has been used extensively in the traditional system of medicine in many countries. In the Northeast of Brazil, it is used for medicinal, condiment and culinary purpose. The flowers and the leaves of this plant are rich in essential oils so it is used in preparation of teas and infusion (Rabelo *et al*., 2003). In the coastal areas of Nigeria, the plant is used in the treatment of epilepsy, high fever, and diarrhea (Effraim *et al*., 2003). In the savannah areas decoctions of the leaves are used to treats mental illness (Akinmoladum *et al*., 2007). *O. gratissimum* is used by the Ibos of southeastern Nigeria in the management of the baby's cord to keep the wound surface sterile. It is also used in the treatment of fungal infections, fever, cold and catarrh (Ijeh *et al*., 2005). Brazilian tropical forest inhabitants use a decoction of *O. gratissimum* roots as a sedative for children (Cristiana *et al*., 2006). People in Kenyan and sub Saharan African communities use this plant for various purpose and sniffed as a treatment for blocked nostrils. They are also used for abdominal pains, sore eyes, ear infections, coughs, barrenness, fever, convulsions and tooth gargle, regulation of menstruation and as a cure for prolapsed of the rectum (Matasyoh *et al*., 2007). In India, the whole plant has been used for the treatment of sunstroke, headache, and influenza, as a diaphoretic, antipyretic and for its anti-inflammatory activity (Tania *et al*., 2006). Different tribes in Nigeria use the leaf extract in treatment of diarrhea, while the cold leaf infusions are used for the relief of stomach upset and haemorrhoids

(Kabir *et al*., 2005). The plant is commonly used in folk medicine to treat different diseases such as upper respiratory tract infections, diarrhea, headache, diseases of the eye, skin diseases, pneumonia, cough, fever and conjuctivitis (Adebolu and Salau, 2005). The infusion of *O. gratissimum* leaves is used as pulmonary antisepticum, antitussivum and antispasmodicum (Ngassoum *et al*., 2003).

Antimicrobial Activities

An investigation of antifungal activity of the essential oil obtained by steam-distillation (1.1%w/w) of the aerial parts of *O. gratissimum* and of an ethanolic extract from the steam-distillation residue was carried out using the agar diffusion method. The result revealed that the essential oil inhibited the growth of all fungi tested, including the phytopathogens, *Botryosphaeria rhodina and Rhizoctonia* spp. and two strains of *Alternaria* sp., while the extract from the residue was inactive. The antifungal activity of eugenol was evaluated against a species of *Alternaria* isolated from tomato and *Penicillium chrysogenum.* The minimal inhibitory concentrations of eugenol were 0.16 and 0.31 mg/disc for *Alternaria* sp. and *P. chrysogenum*, respectively (Agbogidi*et al*., 2014).

The antibacterial activity of different extracts from the leaves of *O. gratissimum* was tested against *Staphylococcus aureus*, *Escherichia coli* and *Salmonella typhi* and *Salmonella typhimurium*, pathogenic bacteria that cause diarrrhoea. Extracts evaluated included cold water extract, hot water extract and steam distillation extract. Only the

steam distillation extract had inhibitory effects on the selected bacterial and the minimum inhibitory concentration ranged from 0.1% for *S. aureus* to 0.01% for *E. coli* and *S. typhimurium,* and 0.001% fo*r S.typhi* (Adebolu and Salau, 2005).

The essential oil of *O. gratissimum* inhibited *S. aureus* at a concentration of 0.75mg/ml. The essential oil was also active against members of the family enterobacteriaceae. The minimum inhibitory concentrations for *Shigella flexineri*, *Salmonella enteritidis, Escherichia coli, Klebsiella* sp., and *Proteus mirabilis* were at concentrations ranging from 3 to 12 mg/ml. The minimum bactericidal concentration of the essential oil was within a two-fold dilution of the MIC for these organisms. The compound that showed antibacterial activity in the essential oil of *O. gratissimum* was identified as eugenol (Orafidiya *et al*., 2006). Ngassoum*et al*., (2003) tested *in vitro* antifungal activity of thirteen essential oils obtained from plants against dermatophytes of the tested oils. *O. gratissimum* was found to be more active, inhibiting 80% of the dermatophyte strains tested and producing zones greater than 10 mm in diameter. Hydro-distilled volatile oils from the leaves of *O. gratissimum* from Meru District in eastern Kenya were evaluated for antimicrobial activity. The antimicrobial activity of the essential oils were evaluated against both Gram positive (*S.aureus,* Bacillus spp.) and Gram negative *(E. coli, P. aeruinosae, S. typhi, K. pneumoniae, P. mirabilis)* bacterial and a pathogenic fungus *Candida albicans*. The minimum inhibitory concentration of oil for Gram negative bacterial ranged from 107 to 750 mg/ml and 93.7 to 150 mg/ml for gram positive bacterial. The minimum

inhibitory concentration for the fungus *C. albicans* was 50 mg/ml. the minimum inhibitory concentration values for chloramphenicol ranged from 22.5 to 31.3 mg/ml. The oil had pronounced antibacterial and antifungal activities on all the microbes (Matasyoh *et al*., 2007).

Antidiarrheal Effect

The antidiarrheal activities of leaf extracts of *O. gratissimum* were investigated by disc diffusion and tube dilution methods. The extracts were active against *Aeromonas sobria, E. coli, Plesiomonas shigelloides, S. typhi* and *Shigella dysenteriae.* The leaf extracts were most active against *S.dysenteriae* and least active against *S. typhi.* The sensitivity of the organisms measured in terms of zone of inhibitory ranged from 8.00 to 19.50 mm. The minimum inhibitory concentration was from 4 to 50 mg/ml, while the minimum bactericidal concentration ranged from 8.00 to 62 mg/ml (Agbogidi *et al*., 2014). *O. gratissimum* leaf extracts have been extensively demonstrated to be effective against the various aetiologic agents of diarrhea, including *Shigella*. The study investigated the effects of *O.gratissimum* essential oil at sub-inhibitory concentration of 0.74 and 1.0 mg/ml on virulent and multidrug-resistant strains of 22 *Shigella* isolates from Nigeria compared with untreated *Shigella* strains.

CHAPTER THREE

MATERIALS AND METHODS

3.1: Collection of Samples

Nono samples were obtained from *Fulani* vendors in Awka metropolis at Dike park, Nise road, Amansea road, and at the milking farm in Umunya, Anambra State, Nigeria.Samples obtained were aseptically transferred to sterile specimen bottles and preserved at 4^0C for further analyses. Sample collection was supported with physical observation of the production process of the milk product.

Fresh *Ocimum gratissimum* leaves were purchased from Eke-Awka market, in Awka South Local Government Area, Anambra State. The leaves were washed with clean water, dried at room temperature for 14 days and were ground into fine powder.

Broiler chicks (*Gallus domesticus*) were obtained from Aroma Farms, Awka, Anambra State Nigeria, as day-old chicks and were raised till they got to three weeks old. They were provided with feeds starting with top starter for the first seven days, and top finishers for the remaining weeks.

Note: Nono producers, vendors and consumers were also interviewed orally and with a structured questionnaire (Appendix 1) to assess their knowledge of hygienic processing and retailing of the milk product and methods of preservation of the finished product and their responses were noted.

3.2: Isolation of Organisms

Lactobacillus and *Escherichia coli* isolates were obtained from *Nono*using one in ten fold serial dilutions in sterile peptone water, and culturing on De Mann Rogosa and Sharpe (MRS) agar and Eosin methylene blue agar respectively. Cultures were incubated at 35^0C for 24 h in an anaerobe jarfor *Lactobacillus* and aerobically for *E. coli* according to the methods of Makut *et al*., (2014).

3.3: Biochemical Characterization of Isolates

3.3.1: *E. coli* Characterization

3.3.1.1: Gram Staining

A thin smear of the organism was made on a clean microscopic slide. It was air dried and then heat-fixed by passing briefly over flame. Two drops of crystal violet were added to the smear for 1 minute, and then rinsed with clean water. Lugol's Iodine was added for 1 minute and washed. It was then decolorized by flooding with acetone for 30 seconds. The film was rinsed with water and counter stained with Safranin for 10 seconds; it was rinsed with water again and allowed to dry. Microscopic observation was made using oil immersion objective lens (Cheesbrough, 2006).

3.3.1.2: Motility Test

The microorganism was stabbed into a sterilized motility test media (Sigma-Aldrich M1053) contained in a sterile test tube, using a sterile inoculating needle. It was incubated for 18-48 h at 35-37^0C and observed for diffused lines of turbidity emerging from the original line of inoculation.

3.3.1.3: Indole Test

Kovac's reagent (0.5 ml) was added to 5ml of a 48 h peptone water culture of the test organisms. The mixture was shaken thoroughly and allowed to stand for 10 minutes. (Cheesbrough, 2006).

3.3.1.4: Methyl Red and Voges Proskaeur Reaction

Methyl Red Test

About 3 drops of methyl red indicator were added to 5 ml of 24 h peptone water culture of the test organisms in sterile test tubes. The tubes were incubated for 24 h at 37^0C and observed for colour change (Cheesbrough, 2006).

Voges Proskauer Test

Three drops of alpha –napthol and potassium hydroxide (also called Barrit's reagent B) were added to 5 ml of 24 h peptone water culture of the test organisms in sterile test tubes. The cultures were allowed to settle for about 15 minutes for colour development to occur. A red colour appearance was indicated a positive result, while a yellowish colour indicated a negative result (Cheesbrough, 2006).

3.3.1.5: Citrate Utilization Test

A 0.1 ml aliquot ofeach test organism was inoculated into Simmon's citrate medium and incubated 48h at 35^0C. Colour change from green to blue indicated a positive result, otherwise negative (Cheesbrough, 2006).

3.3.1.6: Sugar Fermentation Tests

Sugars such as glucose, sucrose, lactose and maltose were added in peptone water in 1% (w/v) and with two drops of Bromothymol blue indicator, and then 1.5 ml aliquot each was distributed in standard assay tubes,each containing an inverted Durham tube. The sugar solutionswere sterilized by autoclaving at 115^0C for 15 minutes and 200 µl of the bacterial samples were inoculated in each tube, and then incubated at 37 ºC; colour change and gas production was observed after 48 h(Oliveira *et al*., 2006).

3.3.2: *Lactobacillus* Characterization

3.3.2.1: Gram Staining

A thin smear of the organism was made on a clean microscopic slide. It was air dried and then heat-fixed by passing briefly over flame. Two drops of crystal violet were added to the smear for 1 minute, and then rinsed with clean water. Lugol's Iodine was added for 1 minute and washed. It was then decolorized by flooding with acetone for 30 seconds. The film was rinsed with water and counter stained with Safranin for 10 seconds; it was rinsed with water again and allowed to dry. Microscopic observation was made using oil immersion objective lens (Cheesbrough, 2006).

3.3.2.2:Catalase Test

A small part of the test colony was collected using a sterile wire loop and immersed into a sterile test tube containing 2-3ml 30% Hydrogen peroxide solution and observed for the appearance of effervescence (Cheesbrough, 2006).

3.3.2.3: Oxidase Test

This was done by placing 2-3 drops of Tetramethyl-P-phenylene diamine dihydrochloride on a sterile filter paper placed in a sterile Petri dish. Thereafter, a 24 hour culture was smeared on the reagent-soaked area of the filter paper, using a sterile glass rod and observed for blue colouration within 10-30 seconds (Cheesbrough, 2006).

3.3.2.4: Sugar Fermentation Tests

Sugars such as glucose and lactose were added in peptone water in 1% (w/v) and with two drops of Bromothymol blue indicator, and then 1.5 ml aliquot each was distributed in standard assay tubes,each containing an inverted Durham tube. The sugar solutionswere sterilized by autoclaving at 115^0C for 15 minutes and 200 µl of the bacterial samples were inoculated in each tube, and then incubated at 37 ºC; colour change and gas production was observed after 48 h (Oliveira *et al*., 2006).

3.4: Probiotic Assesment Tests

3.4.1: Acid Tolerance Test

MRS broth (10ml) was dispensed into 20 test tubes and adjusted to pH values of 2.5, 3.0, 4.0 and 4.2, with HCl. The test tubes were inoculated with 0.1 ml of overnight MRS broth culture of the *Lactobacillus* species and incubated microaerophilically at 37^0C for 3 h. The absorbance values of the cultures were checked, before and after 3 h

of incubation, spectrophotometrically at wavelength of 600 nm; uninnoculated sterile MRS broth served as the blank(Awan and Rahman, 2005).

3.4.2: Cell Surface Hydrophobicity Test

This assay was carried out to measure the ability of the cells of *Lactobacillus* species to adhere to intestinal mucosa. Overnight cultures of the *Lactobacillus* species were centrifuged at 8,000 rpm for 10 minutes. The cells were washed three times with phosphate buffer saline (PBS) and suspended in 1.2 ml of PBS. The absorbances of the bacterial cells were adjusted to 1.0 at 560 nm in the spectrophotometer. 0.6 ml of xylene was added to 3 ml of the cell suspension. The mixture was thoroughly vortexed for 2 minutes and allowed for the xylene to separate completely (approximately 1 h at 37^0C). The aqueous phase was carefully removed, and the remnant transferred to a cuvette. The absorbance values were measured spectrophotometrically at 560 nm. Percentage hydrophobicity was calculated:

$$\% \text{ hydrophobicity} = \frac{A_0 - A}{A_0} \times \frac{100}{1}$$

A_0 = Absorbance values of the mixture before addition of xylene

A = Absorbance values of the mixture after addition and removal of xylene. (Duary *et al*., 2011).

3.4.3: Bile tolerance

The agar well-diffusion assay was used for bile tolerance test. A pour plate of 0.2 ml overnight culture of each isolate was made with molten and cooled MRS agar, and allowed to solidify. Wells of 10mm in diameter were made in each agar plate, and0.2ml of fresh bovine bile was placed in each well. The plates were incubated microaerophically at 37^{o}C for 36 h. Diameters of zone of inhibition around the wells were observed and recorded (Vinderola *et al.*, 2008; Hyronimus *et al*., 2000; Dunne *et al.*, 2001).

3.4.4: NaCl tolerance

Salt tolerance was tested with 3%, 6.5% and 10% (w/v) NaCl in MRS broth. Isolated bacterial cultures were inoculated and incubated at 37^{o}C for 24 h. Turbidity indicates a positive result. (Hyronimus *et al*.,2000).

3.4.5: Crude Bacteriocin Production Assay

The*Lactobacillus* sp. was grown in MRS broth for 24 h at 35^{0}C in an anaerobic chamber. Cell free supernatant was obtained by centrifuging at 4000 rpm for 10 minutes. Ammonium sulphate (0.425g to 5ml concentration) was used to directly precipitate the crude bacteriocin, after which the mixture was refrigerated overnight, and vortexed again at same speed and time. The supernatant was discarded while the trapped precipitate was dissolved using phosphate buffer pH 6.0, and was assayed for antibacterial activity, using the agar well diffusion method (Joshi *et al*., 2006).

3.4.6: *In-vitro* Antibacterial Assay of *Lactobacillus* sp.

The agar well-diffusion assay was used. A 10 ml aliquot of MRS broth was inoculated with the*Lactobacillus* culture and incubated at 37^0C for 48 h in an anaerobic jar. After incubation, the culture was subjected to centrifugation (8000 rpm for 10 minutes), followed by decanting of the supernatant to obtain the cell-free supernatant (CFS). A 100µl aliquot of the CFS was placed in wells cut in Nutrient agar plates (20ml) seeded with *Escherichia coli* and incubated at 35^0C for 24 h and the diameters of zones of inhibition were measured (Ronnqvist *et al*., 2007).

3.5: Antibiotic Sensitivity Testing

The *Lactobacillus* and *E. coli*isolates were inoculated into MRS broth and nutrient broth respectively and incubated at 30^0Cfor 24 h. The isolates (0.1 ml) were spread-plated on MRS agar and Mueller Hinton agar plates respectively,using sterile swab glass spreader.OPTU-discs (10 antibiotics in a single ring namely Amoxicillin(30µg), Septrin(30µg), Ciprofloxacin(10µg), Gentamycin(10 µg) Streptomycin(30µg), Pefloxacin(10 µg), Ampiclox(30µg), Erythromycin (10µg), Zinnacef(20µg) and Rocephin(25µg)) were placed on the top of the agar plates and were incubated at 37ºC over night. Resistance was defined as the absence of a growth inhibition zone around the discs (Vlková *et al*., 2006).

3.6: Analyses of *O. gratissimum* Extracts

3.6.1: Quantitative Analysis of *O.gratissimum* Extracts

3.6.1.1: Determination of Alkaloids

Quantitative determination of alkaloid content of the *O. gratissimum* extract was carried out according to the procedure described by Adewole (2014). Five grams of plant sample was weighed into a 250 ml beaker and 200 ml of 20% acetic acid in ethanol was added and covered and allowed to stand for 4 h at 25^0C. This was filtered with filter paper and the filtrate was concentrated using a water bath to one quarter of the original volume. Concentrated ammonium hydroxide was added drop-wise to the extract until a precipitate was complete. The solution was allowed to settle and the precipitate was collected and washed with dilute NH_4OH, and filtered with a filter paper. The residue on the filter paper is the alkaloid, which was dried in the oven at 80^0C. The alkaloid content was calculated and expressed as a percentage of the weight of the sample analyzed thus:

$$\% \text{ alkaloid} = \frac{\text{weight of filter paper with residue} - \text{weight of filter paper}}{\text{Weight of sample analyzed}} \times 100.$$

3.6.1.2: Flavonoid Determination

A 0.5 ml aliquot of the plant ethanolic extract was mixed with 2 ml of distilled water and subsequently with 0.15 ml of 5% NaNO2 solution. After 6 min, 0.15 ml of 10% AlCl3 solution was added and allowed to stand for 6 min, then 2 ml of 4% NaOH

solution was added to the mixture. Water was added to the mixture to bring the final volume to 5 ml, the mixture was thoroughly mixed and allowed to stand for another 15 min. Absorbance of the mixture was determined at 510 nm with water serving as the blank. The reference standard was prepared with catechin concentrations. Result was expressed as mg catechin equivalents per 100 g of sample, and converted to percentage (Barros *et al*., 2007).

3.6.1.3: Saponin Determination

This was carried out according to the procedure described by Adewole (2014). Five grams of the plant sample was put into 20% acetic acid in ethanol and allowed to stand in a water bath at 50^0C for 24 h. This was filtered and the extract was concentrated using a water bath to one-quarter of the original volume. Concentrated NH_4OH was added drop-wise to the extract until the precipitate was complete. The whole solution was allowed to settle and the precipitate was collected by filtration and weighed. The saponin content was weighed and calculated thus;

$$\% \text{ saponin} = \frac{(\text{weight of filter paper + residue}) - (\text{weight of filter paper})}{\text{Weight of sample analyzed}} \times 100$$

3.6.1.4: Determination of Total Phenol Content

The total phenolcontent of the sample was determined using the method of Barros *et al*., (2007). The ethanolic extract solution (1 ml) was mixed with Folin and Ciocalteu's phenol reagent (1 ml). After 3 min, saturated sodium carbonate solution (1

ml) was added to the mixture and adjusted to 10 ml with distilled water. The reaction was kept in the dark for 90 min, after which the absorbance was read at 725 nm. Gallic acid was used to make standard curve (10-50 µg; Y = 0.013x-0.022; R2 = 0.987) and the result was expressed as mg of gallic acid equivalent per gram of extract and then converted to percentage.

3.6.1.5: Terpenoid Determination

Two grams of grounded plant sample was weighed and soaked in 50 ml 95% ethanol for 24 h. The extract was filtered and the filterate was further extracted with petroleum ether using a separating funnel. The weight of the ether extract was noted and taken as total terpenoid (Gayathri *et al*., 2014).

3.6.1.6: Ascorbic Acid Determination

This was determined according to the method of Klein and Perry (1982). 20 mg of dried leaf powder were extracted with 10 ml of 1% metaphosphoric acid. It was allowed to stand for 45 min at room temperature and was filtered with Whatmann No. 4 filter paper. A 1 ml aliquot of the filterate was mixed with 9 ml of 50 µM 2,6-dichlorophenolindophenol sodium salt hydrate and the absorbance was measured at 515 nm using the spectrophotometer after 30 min. Ascorbic acid was calculated from the calibration curve of authentic L-ascorbic acid and result was expressed as mg ascorbic acid equivalent per gram of dried sample.

3.6.2: Prebiotic Assessment of *O. gratissimum*

Prebiotic assessment was performed according to the methods described by St. John *et al.*, (1996) and Ezeonu *et al*., (2016). Ten grams each of room-dried and grounded leaf samples were immersed in 50 ml distilled water and 85% ethanol in a beaker for 24 h. The extracts were obtained by sieving the soaked leaves with Whatmann No. 1 filter paper and allowed for 72 h for evaporation of the solvents and concentration of the extracts.A distance of 0.5 cm was measured from the bottom ofthin layer chromatography silica gel 60 F (Merck) plate. Using a pencil, a line was drawn across the plate at the 0.5 cm mark. Aqueousand ethanolic extracts of *O. gratissimum*were spotted on the plate on separate lanes, alongside with Inulin and Fructose standards, equally on separate lanes. The TLC plate was developed in Butanol-Actone-Water (5:4:1 v/v/v). The fructopolysaccharides present in the plant extracts were visualized by spraying the chromatogram with Urea–Phosphoric acid reagent. The sprayed and air dried chromatogram was placed in an oven set at 110^{o}C for 5-10 min.

Chromatogram was scanned with HP 4890 digital scanner and densitometry of scanned images were quantitatively analyzed using UN-SCANT-ITTM gel software, version 6.1 (Silk scientific, Orem, Utah).

3.6.3: *In-vitro* Antibacterial Assessment of *O. gratissimum*Ethanolic Extract

The tube dilution assay was employed to first determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the ethanolic plant extract. Two-fold dilution of 500 mg of the plant ethanolic extract was made serially in 10% Dimethylsulfoxide, to get 250 mg/ml, 125 mg/ml, 62.5 mg/ml, 31.25 mg/ml, 15.625 mg/ml,7.813 mg/ml, 3.906 mg/ml and 1.953 mg/ml. Thereafter, 1 ml aliquot of each diluted extract was transferred into test tubes containing 1 ml peptone water with 0.1 ml 24 h *Escherichia coli* culture. The set-up was incubated for 24 h at room temperature and turbidity was checked for in each tube. The agar well diffusion method was employed to determine the zones of inhibition of the MIC with Ciprofloxacin as the standard. A 100 µl aliquot of each concentration of plant extract was placed separately in a well cut in sterile Eosin methylene Blue Agar platethat was already seeded with *E. coli*. The plates were incubated in an anaerobic chamber at 35^0C for 24 h and the diameters of zone of inhibition were measured (Kabir *et al*., 2005).

3.7*In-vivo* Analyses

3.7.1: Avian Pathogenicity Screening of *E. coli* Isolates

Twenty 3-week old broiler chicks were orally infected with 10^7 cfu/ml of *E. coli* in phosphate buffered saline, using sterile pipette. The chicks were then monitored for

thirty days for pathological signs such as malaise and occurrence of watery and bloody stools (Kabir *et al*., 2005a; Ezema, 2013).

3.7.2: Molecular Identification of Isolates

E. coli isolates that elicited bloody diarrhoea in the birds, as well as the *Lactobacillus* sp were selected and subjected to molecular identification. All original samples were subjected to a purity check. Molecular assays were then carried out on each sample using nucleic acid as a template. A proprietary formulation [microLYSIS®-PLUS (MLP), Microzone, UK] was subjected to the rapid heating and cooling of a thermal cycler, to lyse the cells and release deoxyribonucleic acid (DNA). Following DNA extraction, Polymerase Chain Reaction (PCR) was employed to amplify copies of the rDNA *in vitro*. The quality of the PCR product was assessed by undertaking gel electrophoresis.

PCR purification step was carried out to remove unutilized dNTPs, primers, polymerase and other PCR mixture compounds and obtain a highly purified DNA template for sequencing. This procedure also allowed concentration of low yield amplicons. Sequencing reactions were undertaken using BigDye® Terminator v3.1 kit from Applied Biosystems (Life Technologies, UK) which utilises fluorescent labelling of the chain terminator ddNTPs, to permit sequencing.

Removal of excess unincorporated dye terminators was carried out to ensure a problem-free electrophoresis of fluorescently labelled sequencing reaction products on

the capillary array AB 3130 Genetic Analyzer (DS1) DyeEx™ 2.0 (Qiagen, UK). Modules containing prehydrated gel-filtration resin were optimized for clean-up of sequencing reactions containing Big Dye® terminators. Dye removal was followed by suspension of the purified products in highly deionized formamide Hi-Di™ (Life Technologies, UK) to prevent rapid sample evaporation and secondary structure formation.

Samples were loaded onto the AB 3130 Genetic Analyzer and sequencing undertaken to determine the order of the nucleotide bases (adenine, guanine, cytosine, and thymine) in the DNA oligonucleotide. Following sequencing, identifications were undertaken by comparing the sequence obtained with those available from the European Molecular Biology Laboratory (EMBL) database via the European Bioinformatics Institute (EBI). The strains were identified using 16s rDNA sequencing analyses (Macrogen, 2014).

3.7.3: Chicken Groups

Six groups of ten 3-week old broiler chickswere used as stated below:

- Group A: Healthy control.
- Group B: Infected with APEC without treatment.
- Group C: Infected with APEC and treated with 15g/l Norfloxacin (Antibiotic control).
- Group D: Infected with APEC and controlled with *Lactobacillus.*
- Group E: Given oral dose of*Lactobacillus* 48 h before infection with APEC.
- Group F: Infected with APEC and treated with 40g/l *O. gratissimum.*

Test chicken samples were known to be diseased by the discharge of watery and bloody stool.

3.7.4: Innoculation Pattern of Test Microorganisms for the Broiler Groups

Groups B, C, D and F were orally dosedinitiallywith 1.5ml of 1.3 x 10^7 cfu/ml of *Escherichia coli* mixed with 0.5 ml phosphate buffer saline (pH 6) with the aid of a sterile pipette and left for a period of two days to give room for proper pathogen incubation and disease establishment. Thereafter, group D was dosed orally with 1.5ml of 1.1 x 10^9 cfu/ml of *L. casei*in order to initiate competitive inhibition. Group C was treated with 15g/L Norfloxacin, group F was treated with 40g/L of *O. gratissimum* ethanolic extract, while group B was left without treatment as stated earlier.

Group E was given an oral initial dose 1.5ml of 1.1 x 10^9 cfu/ml of *L. casei*isolated from *Nono* and left for two days before infusing 1.5ml of 1.3 x 10^7 cfu/ml of *E. coli*. This group was used to ascertain the peventive ability of the *Nono* isolate on colibacillosis(Pascual *et al*., 2009; Emmanuel& Obiezue, 2014).

3.7.5: Microbial Enumeration

Intestinaland caecal lavage was performed on dead chickens from each group with 1 ml of phosphate buffer saline. Lavage fluid was serially diluted and plated on Eosine Methylene Blue (EMB) agar, Mc Conkey agar and De Mann Rogosa and Sharpe (MRS) agar. Colony forming units from lavage cultures after 24 h were used to determine the intestinal and caecal*E. coli* burden. This helped to reveal the position of *L. casei*and *O. gratissimum*in prophylaxis and control of the disease and also helped

to reveal the level of effective control between the natural antibacterial agents and Norfloxacin (Pascual *et al*., 2009).

3.7.6: Growth Performance Determination

3.7.6.1: Weight Determination

The body weights of the chicken were appropriately taken weekly using Metler weighing balance of 0.01 g sensitivity.

3.7.6.2: Determination of Percentage Weight Gain

This was determined with the formular;

Weight gain (%) = w_f-w_i/w_i x 100; where

W_f =final weight

W_i= initial weight.

3.7.6.3: Determination of Percentage Specific Growth Rate

The method of Radhakrishman *et al*., (2015) was adopted. This was determined using the formular;

SGR (%) = ln w_f- ln w_i/ number of experimental days x 100; where

W_f =final weight; W_i= initial weight.

3.7.7: Haematological Analyses

3.7.7.1: Total White Blood Cell Count

This was done in order to monitor leucocyte roles in the research owing to the fact that they play role in body defence. Blood samples were collected from the chickens via the under-side of their wings and transferred to an EDTA bottle. 0.02ml of the blood samples were mixed with 0.038 of Tursk diluent in a test tube. A little aliquot was used to fill the counting chamber of the already charged Neubauer chamber. This set-up was charged again for 5-10minutes by placing the counting chamber on a damp towel. Thereafter, the under-side of the chamber was cleaned and placed under the microscope where it was viewed and counted using x10 objective lens (Cheesbrough, 2006).

3.7.7.2: Differential White Blood Cell Count

This test was performed in order to have a detailed view on the roles of each leucocyte component during the research. A thin blood film was made on a slide. Four drops of Leishmann stain and eight drops of dilution buffer were added and mixed and then allowed to stand for 8-10 minutes. The stain was washed off and slide was allowed to dry before it was viewed and counted under x40 magnification with the microscope (Cheesbrough, 2006).

3.7.7.3: Haemoglobin Determination (g/dl)

Haemoglobin was determined using an automated haemoglobin reader which displays haemoglobin results in a digital pattern.

3.7.7.4: Occult Blood Determination

This was used to determine the presence of blood in the stool samples. A soft stool smear was made on the occult blood paper and was observed for colour change. Presence of bluish colouration indicated a positive result.

3.7.8: Blood Electrolyte Determination

3.7.8.1. Determination of sodium (Na^+)

Freshly collected blood was centrifuged and the serum was collected. To each of the labeled test tubes A, B, C and D, 1.0 ml of filtrate reagent was added. Then 50µl of serum sample was added to the test tube B,C and D while distilled water was added to the blank test tube (A). The tubes were shaked vigorously and mixed continuously for 3 minutes. The tubes were centrifuged at 1,500 RPM for 10 minutes and the supernatant was tested ensuring that the protein precipitate was not disturbed. A 1.0ml aliquot of acid reagent followed by 50µL of supernatant and 50µL of color reagent were added and mixed. The absorbance of the labelled tubes were spectrophotometrically read at 550 nm and calculation was made thus;

$$\frac{\text{Abs. of sample-Abs. of Standard (STD)}}{\text{Abs. of blank-Abs. of STD}} \times \text{Conc of STD (mEq/L)} = \text{Conc of Sodium}$$

(Ochei and Kolhatkar, 2008).

3.7.7.2. Determination of chlorides (Cl^-)

Test tubes A, B, C and D were labelled and 1.5ml chloride reagent was added into each test tube. 0.01ml (10µl) of calibrator was added in tube A while serum samples were added in the other tubes and were all mixed. The tubes were incubated at room temperature for 5 minutes. The absorbances of all the tubes were spectrophotometrically read at a wave length of 520 nm**.** Calculation was made thus;

$$\frac{\text{Abs. of unknown}}{\text{Abs. of calibrator}} \times \text{concentration of calibrator} = \text{concentration of chloride}$$

(Ochei and Kolhatkar, 2008).

3.7.7.3. Determination of potassium (K^+)

Into each labeled test tubes (A-D), 1.0 ml of potassium reagent was added. Thereafter 0.01 ml (10µl) of serum sample was added to each of the labeled tubes and mixed. The tubes were allowed to stand for 3 minutes at room temperature. After 3 minutes the absorbance was read at 500 nm. Calculation was made thus;

$$\frac{\text{Abs. of sample-Abs. of Standard (STD)}}{\text{Abs. of blank-Abs. of STD}} \times \text{Conc of STD (mEq/L)} = \text{Conc of Potassium}$$

(Ochei and Kolhatkar, 2008).

3.7.7.4. Determination of bi-carbonate (HCO_3)

A 1.0 ml aliquot of carbon dioxide reagent was placed into each of the test tubes labeled A-D and incubated for 3 minutes at the temperature of 37^0C. Thereafter, 5µl (0.005 ml) of water, standard and sample were transferred to cuvettes labelled A, B, C and D, mixed gently and incubated for 5 minutes. The absorbance read at 340nm. Calculation was made thus;

CO_2 content (mmol/L) = Abs. blank-Abs. sample x concentration of standard.

Abs. blank-Abs.standard

(Ochei and Kolhatkar, 2008).

3.7.8: Blood Chemistry Determination

3.7.8.1. Determination of serum aspartate aminotransferase

The blood of the chicks were collected through the wing vein and transferred into sterile specimen tube without EDTA. The serum was transferred into another test tube and immediately refrigerated to maintain the high level of enzyme activity until further analysis. A 0.1 ml aliquot of the sample was pipetted into a test tube, mixed with 0.5 ml of buffer and incubated for exactly 1h at 37°C. A 0.5 ml aliquot of the chromogen solution was mixed with the solution and allowed to stand for 20 min at 20°C to 25°C. After the time elapsed, 5.0 ml of 0.4N NaOH was added. The solution was allowed to stand for 5 minutes at room temperature. The absorbance was read against a blank at 546 nm (Reitman and Frankel, 1957).

3.7.8.2. Determination of serum alanine aminotransferase

This was done according to the method of Reitman and Frankel (1957).The blood of the chicks were collected through the wing vein and transferred into specimen tube without EDTA. The serum was transferred into another test tube and immediately refrigerated to maintain the high level of enzyme activity until further analysis.

A 0.1 ml aliquot of the sample was pipette into a test tube, mixed with 0.5 ml of buffer and incubated for exactly 30 min at 37°C. Also, 0.5ml of the chromogen

solution was mixed with the solution and allowed to stand for 20 min at 20°C to 25°C. After the time elapsed, 5.0 ml of 0.4N NaOH was added. The solution was allowed to stand for 5 minutes at room temperature. The absorbances were read against a blank at 546 nm (Ochei and Kolhatkar, 2008).

3.7.8.3. Determination of Acid Phosphatase Enzyme activity

This was done according to the method of Babson and Read (1959). Into each of the test tubes labeled B (blank) and T (test), 0.5 ml of the working reagent was pipetted and incubated at 37°C using a water bath for 3 min. After the incubation, 0.1 ml of the blood sample was added to the test tube T, mixed and incubated again at 37°C for exactly 30 min. A 5.0 ml aliquot of 0.02 N NaOH was pipetted in each labeled tube. The absorbance of the content of labeled test tube T was read against blank at 450 nm and the calculation made (Ochei and Kolhatkar, 2008).

3.7.8.4. Determination of serum urea

Three specimen tubes were labelled A, B and C. 10µl serum sample was added into test tube A (sample), 10µl of standard (CAL) was added into test tube B (standard), 10µl of distilled water was added into test tube labeled C; while 100 µl of reagent R1 (EDTA) was added into tubes A, B and C, mixed and incubated for 10 minutes at the temperature of 37^0C. The absorbance (Abs) was read at 546nm and calculation was made thus;

$$\text{Urea concentration (mg/dl)} = \frac{\text{Abs sample}}{\text{Abs standard}} \times \text{Concentration of standard}$$

(Reddy *et al*., 2011).

3.7.8.5. Determination of serum creatinine

This was determined with alkaline picrate method using the creatinine kit according to the method of Reddy *et al*., (2011). A 2 ml aliquot of picric acid reagent in a test tube was added to 0.2 ml of serum (for deproteinization of serum), mixed well and centrifuged at 3000 rpm to obtain a clear supernatant. A 100 µl aliquot of buffer reagent was added to 1.1 ml of supernatant, 0.1 ml of standard creatinine and 0.1 ml of distilled water to prepare the test solution, standard and blank respectively. A 0.1 ml aliquot of picric reagent was added to blank and standard. The test tubes were mixed well and kept at room temperature for 20 minutes. The alkaline picrate reacts with the creatinine to form an orange coloured complex. The absorbance was read at 520 nm. The serum creatinine concentration was calculated thus;

$$\text{Serum creatinine concentration (mg/dl)} = \frac{\text{Abs sample}}{\text{Abs standard}} \times 2$$

3.7.8.6. Determination of C-reactive Protein

Serum samples were obtained from whole blood samples obtained from the test chicken samples. Double dilutions of the serum were done using isotonic saline (1:2, 1:4, 1:8, 1:16, 1:32). One drop of each serum dilution was placed on a white tile, following one drop of C-RP reagent to the test circle. The drops were mixed using a disposable stirrer ensuring coverage of the test circle with the mixture. The slides were gently and evenly rocked for two minutes while examining the test slides for agglutination. The calculation of the serum C-RP concentrations was done by multiplying the dilution factor (2,4,8,16) by the detection limit. Which implies that if

the agglutination titre appears at 1:8, the approximate serum C-RP concentration is 8 x 6 (mg/L), where 6 is a constant (Ochei and Kolhatkar, 2008).

3.7.9: Gross Morphological Examination

This was performed on the birds by exsanguination, followed by opening up of the chicks from the lateral view, with the help of dissecting tools. Proper and hygienic evisceration aided in revealing of the intestine and caecum.

3.7.10: Histopathological Examination of Tissues

Intestinal and caecum samples from each chicken group were excised and immediately fixed in 10% neutral buffered formalin. The tissues were dehydrated into graded concentrations of alcohol (70%, 80%, 90% and 100%) for one hour each. The tissues were cleared overnight using of xylene. After blocking using soft paraffin, serial sections of 4µm thickness were made and stained with haematoxylin and eosin stain and photomicrograph was immediately taken (Ikele *et al*., 2014).

3.7.10.1: Degree of Tissue Change (DTC).

This was determined according to the method of Poleksic and Mitrovic-Tutundzic (1994). The alteration in the tissues were classified in progressive stages of tissue damage and allocated scores

Stage 1. First stage lesions; slight damage and can be possibly reversed when environmental conditions are improved.

Stage 2. More severe damage leading to malfunctions on tissues.

Stage 3. Very severe and irreparable damage.

The degree of tissue change was evaluated as the degree of the number of lesion types within each of the three stages multiplied by the stage coefficient which represents the numerical value of DTC.

DTC= $(10^0\sum_i) + (10^1\sum_{ii}) + (10^2\sum_{iii})$

where i, ii and iii correspond to the sum of number of laterations found in stages i, ii and iii.

3.8: Determination of Mortality:

Mortality of the chicken population was determined by noting the number of dead chicks in each group and the day(s) of the week the deaths occurred. Percentage mortality is calculated as;

$$\frac{\text{no. of deaths per group}}{\text{total no. of chicks per group}} \times 100.$$

3.9: Data Collation and Analyses

All the data were analyzed using SPSS version 17, significant difference ($p<0.05$) was obtained using Analysis of variance (ANOVA), and difference between means were partitioned using Duncan Multiple Range Test.

CHAPTER FOUR

RESULTS

4.1: *In-vitro* Analyses

4.1.1: Responses of Nono Producers and Consumers Regarding Product Safety Issues

The interviewed respondents were 69% male and 31% female. Approximately 98% of the respondents opted for “No” towards the knowledge of the presence of germs (faecal contaminants) in Nono while 100% of the respondents admitted to the fact that flies perch on the wooden Nono stirrer with which they serve and drink the product as well as the scooping plates being exposed. A frequency of 88% of the respondents admitted that Nono vendors do not wash their hands before sales but they said they are fine with it, as it is a part of the normal serving procedures they have lived with from childhood (Table 1). All the respondents answered that they do not have abdominal up-sets after taking the product and that they like the product a lot. The pictures of investigation on the production and consumption safety are shown on Appendix 2.

Table 1: Respondents' Answers to Knowledge of Aseptic Nono Processing and Vending

Questionnaire Items	Frequency (%)	
	Y	**N**
Presence of Enteric Pathogens in Nono	2	98
House fly infestation of Vending Utensils	100	0
Unhygienic Food Handlers	88	2
Feeling of Diarrhea after Product Consumption	0	100

Key:

Y= yes

N= No

4.1.2: Prevalence of *E. coli* in Nono Samples

All the five hundred Nono samples examined showedpresumptive presence of *E. coli*as indicated by production of colonies with metallic sheen on Eosine Methylene Blue agar and biochemical characteristics(Table 2).

4.1.3: Probiotic Attributes of*Lactobacillus* Isolate

The biochemical identification of the*Lactobacillus* isolate is also shown on Table 2 while its probiotic screening is shown on Table 3. One isolate whose cell free supernatant and crude bacteriocin gave the highest zone of inhibition against *E. coli* was selected.

4.1.4: Antimicrobial Susceptibility of *Lactobacillus*and*E. coli* Isolates

Tables 4and 5, show the antimicrobial susceptibility of*E. coli* and *Lactobacillus*which were selected for the study. The *E. coli* isolate was susceptible to two out of seven tested antibiotics while the *Lactobacillus* isolate was susceptible to seven out of ten antibiotics.

4.1.5: Phytochemical Composition of*Ocimum gratissimum*

The phytochemical analyses of *O. gratissimum* ethanolic extractshow several phytochemical constituents in different quantities including Saponin, Flavonoid, Ascorbic acid *inter alia* (Table 6).

4.1.6: Identification of Prebiotics Present in*Ocimum gratissimum*

Thin layer chromatography of *O. gratissimum*ethanolic and aqueous extracts revealed the presence of Fructooligosaccharides(Plate 2, Table 7).

Table 2: Biochemical Characteristics of *Lactobacillus* and *E. coli* Isolates

Isolate Codes	Characteristics													
	Colony Morphology	Gram Reaction	Motility	Catalase Test	Oxidase Test	Indole Test	Methyl Red	Voges Proskauer	Citrate Test	Glucose Fermentation	Lactose Fermentation	Sucrose Fermentation	Maltose Fermentation	Presumptive Identification
P	Circular colonies with metallic sheen on EMB agar.	- (rods)	+	ND	ND	+	+	-	-	+	+	ND	ND	*E. coli*
LB	Punctiformmilkish colonies on MRS agar.	+ (short rods)	ND	-	+	ND	ND	ND	ND	+	+	+	+	***Lactobacillus***

ND = not done, + = positive, - = negative.

Table 3: Probiotic Attributes of *Lactobacillus* Isolate

Screening Parameters	**Results**
Antibacterial activity against *E. coli*	+(10 mm diameter zone of inhibition).
Tolerance to Acidity	+
Tolerance to Bile	+
Cell Hydrophobicity Test	+
Crude Bacteriocin activity against *E. coli*	+(10.4 mm diameter zone of inhibition).
Microaerophilic Growth	+
Tolerance to 10% NaCl	+

+ = Positive

- = Negative

Table 4: Antimicrobial Susceptibility of *E.coli*

Antibiotics	**Results**	**Diameter Zone of Inhibition (mm)**
Gentamycin	-	10
Erythromycin	+	0
Tetracycline	+	0
Ciprofloxacin	-	13
Ampicillin	+	0
Amoxicillin	+	0
Florfenicol	+	0

+ = Resistant to Antibiotic

- = Not resistant to Antibiotic

Table 5: Antimicrobial Susceptibility of *Lactobacillus* Isolate

Antibiotics	**Results**	**Diameter zone of Inhibition (mm)**
Amoxicillin	+	0
Septrin	-	19
Ciprofloxacin	-	16
Gentamycin	+	0
Streptomycin	-	14
Pefloxacin	-	10
Ampiclox	+	0
Erythromycin	-	20
Zinnacef	-	15
Rocephin	-	16

+ = Resistant to antibiotic

- = Not resistant to antibiotic

Table 6: Phytochemical Composition of *Ocimum gratissimum*

Parameters	**Relative Abundance**	**Actual Quantity(%)**
Alkaloid	++	0.79
Saponin	+++	1.90
Flavonoid	+	0.399
Tannin	+	0.197
Terpenoid	+	0.22
Phenol	+	0.327
Ascorbic acid	++	0.934

+ = slightly present

++ = very present

+++ = strongly present

Table 7: Thin Layer Chromatography Showing the Prebiotic Constituents of *Ocimum gratissimum*

Solvents	Prebiotics present (mg/g sample)		
	Inulin	Fructose	Other Fructooligosaccharides
Water extract	0	9.27	24.904
Ethanol extract	0	12.311	14.21

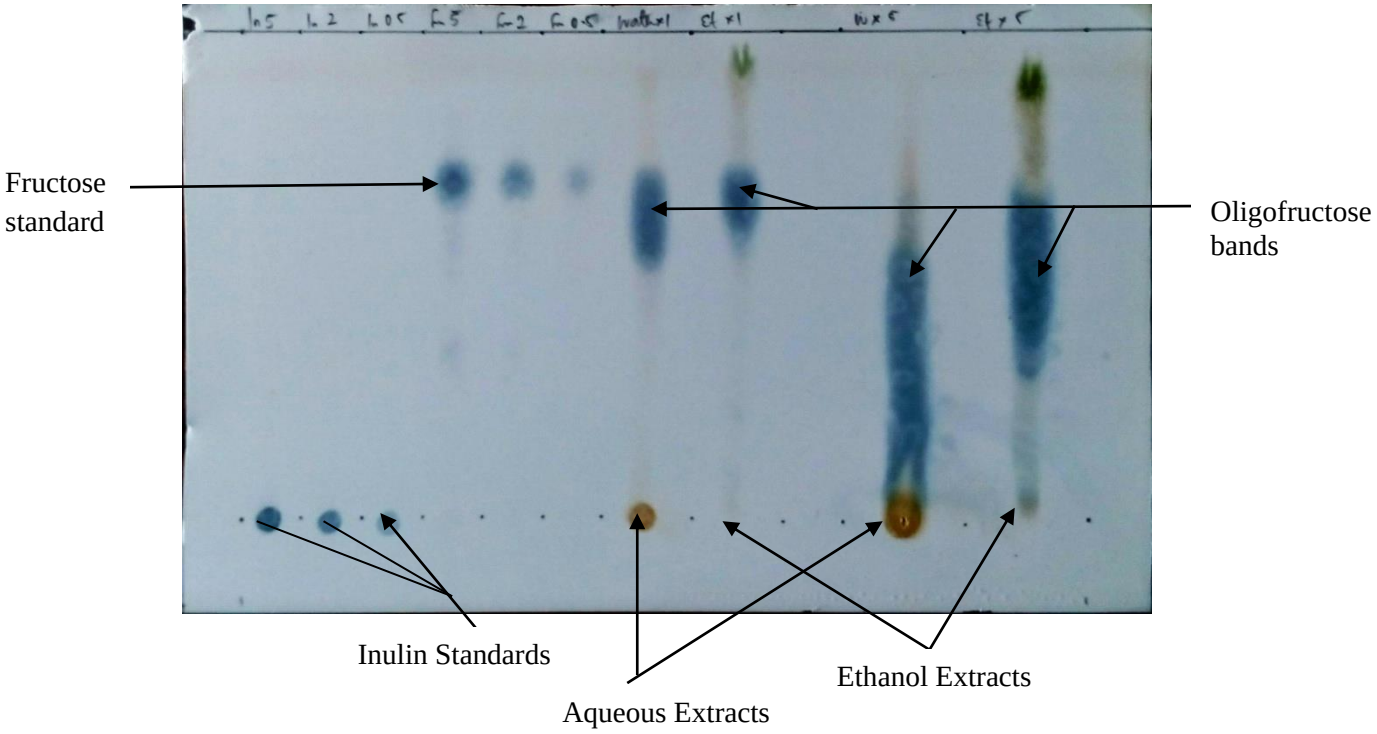

Plate 2: Thin Layer Chromatogram of *O. gratissimum* showing the Separation Bands

4.1.7: Antibacterial Activity of Ethanolic Extract of *O. gratissimum* against *E. coli*

Table 8 shows the antibacterial activity of ethanolic extract of *O. gratissimum* against *E. coli*. The extract had its Minimum Inhibitory Concentration (MIC) as 31.25 mg/ml with 13 mm zone of inhibition.

4.2 *In-vivo* Analyses

4.2.1: Selection ofAvian Pathogenic *E. coli* Isolate

A total of four *E. coli* isolates produced bloody diarrhea in the chicks and were thus classified as Avian Pathogenic *E. coli* (APEC). One isolate which gave the most severity of infection in the chicks was selected and stocked for further use.

4.2.2: Molecular Identification of Isolates

Molecular identification of the Avian pathogenic *E. coli* and *Lactobacillus* isolates revealed them as *E. coli* O157:H7 strain sakaii and *Lactobacillus casei* as shown on Appendix 3 and 4 respectively.

Table 8: Antibacterial Activity of *O. gratissimum* Ethanolic Extract against Avian pathogenic *E. coli*

Concentrations (mg/ml)	Results	Diameter Zone of Inhibition(mm)
250	+	15
125	+	14
62.5	+	13.6
31.25	+	13
16.625	-	0
7.812	-	0
3.906	-	0
1.953	-	0
Ciprofloxacin	+	12

+ = Zones of Inhibition Present.

- = Zones of Inhibition Absent.

4.2.3: Effects of Oral Administration of *L. casei* and *O. gratissimum* Extract on the Growth Performance of Broiler Chicks.

There was significant difference ($P< 0.05$) amongst the chicken groups throughout the experiment. The undiseased chicks (control) had the best overall performance, increasing in weight from 892.95±149.45g to 2975.00±84.95g over a period of four weeks.Among the infected chicks, the best performance was recorded in the group treated with *O. gratissimum* with weight increase from 685.00±64.27g to 2030.00±300.42g, with percentage weight gain of 224.2% and specific growth rate of 4.2%; while the worst performance was observed in untreated group, which showed a progressive decrease in weight from 658.80±69.55g to 500.00±60.00g, percentage weight loss of 17.9% and specific growth rate of 0.7% (Tables 9-11).

Table 9: Mean Live Weight (g) of the Chicken Groups

Weeks	Treatment Groups					
	A	**B**	**C**	**D**	**E**	**F**
1	892.95±149.45^{b}	658.80 ± 69.55^{a}	632.80±67.80^{a}	853.21± 101.03^{b}	898.40± 100.93^{b}	685.00± 64.27^{a}
2	2246.50± 136.95^{b}	1006.10± 132.95^{a}	1030.00 ± 37.42^{a}	1120.20± 130.52^{a}	1230.00± 218.68^{ab}	1305.00± 140.50^{ab}
3	2360.40± 90.65^{d}	565.60 ± 108.21^{a}	870.67 ± 52.70^{b}	965.00 ± 170^{b}	1115.00± 220.60^{c}	1160.50± 168.14^{c}
4	2975.00± 84.95^{c}	500.00 ± 60.00^{a}	1605.00± 200.84^{b}	1520.00± 210.5^{b}	1730.00± 160.93^{b}	2030.00± 300.42^{bc}
B/L	844.04±132.2^{a}	609.26±48.33^{b}	585.46±46.45^{c}	802.17±126.66^{a}	842.20±113.33^{a}	626.14±48.08^{b}

Mean along the same row with different suffixes are significantly different ($P<0.05$).

Key:

A: undiseased.
B: diseased without treatment.
C: diseased & treated with Norfloxacin.
D: diseased & treated with *L. casei*.
E: prophylactic group; F: diseased & treated with *O. gratissimum*.
B/L:Baseline.

Table 10: Percentage Weight Gain of Chicken Groups throughout Experimental Weeks

Experimental Groups	Weight Gain (g)	Percentage (%)
A	2130.96	252.4
B	-109.26	-17.9
C	1019.54	174.1
D	717.83	89.5
E	887.80	105.4
F	403.86	224.2

Table 11: Specific Growth Rate of Chicken Groups throughout the Experimental Weeks

Experimental Groups	Specific Growth Rate (%)
A	4.5
B	-0.7
C	3.6
D	2.28
E	2.57
F	4.2

4.2.4. Effects of Oral Administration of *L. casei* and *O. gratissimum* Extract on Blood Electrolyte Levels of Broiler Chicks.

There were significant differences($p < 0.05$) amongst the chick groups in the Sodium, Chlorine and Bicarbonate levels tested.However, there was no significant difference ($p>0.05$) in their Potassium levels. The undiseased chicks (control) had the highest sodium, chlorine and bicarbonate levels; increasing from 128.00±1.15 to 145.00±3.06, 106.00±1.5 to 155.00±1.15 and17.00±1.54 to 27.00±1.15 respectively, over a period of four weeks. Among the infected chicks, the highest sodium, chlorine and bicarbonate levels were recorded in the group treated with *O. gratissimum* with electrolyte level increase of 128.30±1.45 to 140.00±1.15, 115.00±1.15 to 140.00±1.15 and 16.35±1.20 to 25.30±1.45 respectively over a period of four weeks. Infected and untreated group showed a decrease in chlorine levels from 115.65±1.75 to 105.00±1.15 over same monitoring period. Other groups were within range but the undiseased group and *O. gratissimum* treated group showed remarkable electrolyte levels (Tables 12 to 15).

Table 12: Mean Sodium levels (mEq/L) of the Chicken Groups

Weeks	Treatment Groups					
	A	**B**	**C**	**D**	**E**	**F**
1	128.00±1.15^{b}	136.66 ± 1.75^{c}	116.62±88^{a}	140.32 ± 1.45^{d}	115.00± 1.15^{a}	128.30± 1.45^{b}
2	134.00 ± 1.15^{b}	135.32± 1.45^{b}	136.65 ± 1.75^{b}	128.00 ± 1.15^{a}	135.00 ± 1.10^{b}	130.05 ± 1.15^{a}
3	145.00 ± 1.15^{c}	132.32 ± 1.20^{a}	140.32 ± 1.45^{b}	134.00 ± 1.15^{a}	135.00 ± 1.75^{a}	135.00 ± 1.10^{a}
4	145.00 ± 3.06^{c}	130.00 ± 1.15^{a}	135.32 ± 1.40^{ab}	136.00 ± 1.15^{abc}	139.30 ± 1.40^{b}	140.00 ± 1.15b^{b}
B/L	124.00±1.15^{b}	138.34±1.45^{c}	110.22±1.5^{a}	136.00±1.15^{c}	110.00±1.15^{a}	125.00±1.10^{b}

Mean along the same row with different suffixes are significantly different (P<0.05).

Key:
A: undiseased.
B: diseased without treatment.
C: diseased & treated with Norfloxacin.
D: diseased & treated with *L. casei*.
E: prophylactic group.
F: diseased & treated with *O. gratissimum*.
B/L: Baseline.

Table 13: Mean Potassium Levels (mEq/L) of the Chicken Groups

Weeks	Treatment Groups					
	A	**B**	**C**	**D**	**E**	**F**
1	2.20±1.00[a]	3.05 ± .85[a]	2.45±1.90[a]	3.12 ±.95[a]	2.55±1.15[a]	2.21± 1.15[a]
2	3.15 ± 1.20[a]	2.12± 1.05[a]	2.10 ± 1.35[a]	2.85 ±.60[a]	2.90 ±.55[a]	2.83 ±. 62[a]
3	3.37± 1.0[a]	2.35 ± 1.15[a]	2.00 ± 1.05[a]	2.15 ± 1.00[a]	2.85 ±.83[a]	2.75 ± 1.00[a]
4	2.15 ± 1.80[a]	2.25 ± .80[a]	2.20± 1.10[a]	2.20 ± 1.10[a]	2.20 ± 1.15[a]	2.55 ±.60[a]
B/L	2.15±1.00[a]	3.00±1.05[a]	2.40±1.10[a]	3.02±1.10[a]	2.50±1.15[a]	2.16±1.00[a]

Mean along the same row with same suffixes are not significantly different (P>0.05).

Key:

A: undiseased.
B: diseased without treatment.
C: diseased & treated with Norfloxacin.
D: diseased & treated with *L. casei*.
E: prophylactic group.
F: diseased & treated with *O. gratissimum*.
B/L: Baseline.

Table 14: Mean Chlorine Levels (mEq/L) of the Chicken Groups

Weeks	Treatment Groups					
	A	**B**	**C**	**D**	**E**	**F**
1	106.00±1.5^{b}	115.65 ± .1.75^{a}	105.00±1.15^{a}	120.30 ± 1.20^{d}	122.00± 1.15^{cd}	115.00± 1.15^{c}
2	130.00 ± 1.15^{c}	120.30± 1.45^{a}	125.30 ± 1.45^{b}	125.00 ± 1.15^{bc}	126.00 ± 1.15^{bc}	120.00 ± 1.15^{a}
3	138.00± 1.15^{d}	115.60 ± 1.40^{a}	120.65 ± 1.75^{b}	120.00 ± 1.15^{b}	122.00 ± 1.15^{bc}	125.00 ± 1.15^{c}
4	155.00 ± 1.15^{d}	105.00 ± .1.15^{a}	135.60 ± 1.45^{c}	130.30 ± 1.5 ^{b}	135.30 ± 1.20^{c}	140.00 ± 1.15^{c}
B/L	94.06±1.15^{a}	105.55±1.40^{a}	96.20±1.75^{a}	116.60±0.06^{a}	116.28±1.08^{a}	106.00±1.15^{a}

Mean along the same row with different suffixes are significantly different (P<0.05).

Key:

A: undiseased.
B: diseased without treatment.
C: diseased & treated with Norfloxacin.
D: diseased & treated with *L. casei*.
E: prophylactic group.
F: diseased & treated with *O. gratissimum*.
B/L: Baseline.

Table 15: Mean Bicarbonate Levels (mEq/L) of the Chicken Groups

Weeks	Treatment Groups					
	A	**B**	**C**	**D**	**E**	**F**
1	17.00±1.54^{ab}	22.30±.85^{cd}	15.30±1.45^{a}	25.00±1.15^{d}	20.10±1.15^{bc}	16.35±1.20^{ab}
2	20.65± 1.75^{ab}	28.00±.58^{cd}	18.65±.85^{cd}	28.21±1.15^{d}	24.67±1.15^{bc}	18.10±1.75^{a}
3	25.30±1.45^{a}	20.00±15$^{\cdot a}$	18.00± 1.15^{a}	25.67±34.5^{a}	22.02±1.15^{a}	17.60±1.80^{a}
4	27.00±1.15^{d}	15.00±1.15^{a}	24.00±1.10^{bc}	21.00±1.10^{b}	22.02±1.15^{bc}	25.30±1.45^{cd}
B/L	14.00±1.15^{a}	25.20±1.15^{b}	20.33±1.10^{b}	22.21±1.10^{b}	17.01±1.15^{a}	14.35±1.45^{a}

Mean along the same row with different suffixes are significantly different (P<0.05).

Key:
A: undiseased.
B: diseased without treatment.
C: diseased & treated with Norfloxacin.
D: diseased & treated with *L. casei*.
E: prophylactic group.
F: diseased & treated with *O. gratissimum*.
B/L: Baseline.

4.2.5: Effects of Oral Administration of *L. casei* and *O. gratissimum* Extract on the Haematological Parameters of Broiler Chicks.

There were significant differences($p<0.05$) in haemoglobin values of mice groups, with the untreated group showing severe blood loss at the fourth week of monitoring. Total white blood cell count showed significant differences ($p<0.05$) amongst the groups, with infected not treated group giving the highest count of 14100.30±1.20 at the end of four weeks monitoring. *O. gratissimum* treated group and *L. casei* prophylactic group gave relatively higher counts of 13900.00±0.00 and 12885.00±12.50 respectively at the end of same monitoring period. The differential count showed no significant differences ($p>0.05$) in the eosinophil, basophil and monocyte readings, while there were significant differences ($p<0.05$) in the neutrophil and lymphocyte values amongst the chicken groups. Undiseased group showed progressive increase in neutrophil levels, from 28.30±1.75 to 30.30±1.20 in the four-week monitoring period. Other infected chicken groups showed declining levels of neutrophil, however, the untreated group and diseased/treated with *L.casei* group, had increased neutrophil levels by the fourth week, with the *L. casei* treated group having the highest level of 31.60±1.45 among all the infected chicken groups. *O. gratissimum* treated group and *L. casei* prophylactic group gave the first two highest lymphocyte readings of 79.00±1.15 and 72.30±1.45 respectively, at the end of the four-week monitoring period (Tabels 16 to 22).

Table 16: Mean Hemoglobin Levels (g/dl) of the Chicken Groups

Weeks	Treatment Groups					
	A	**B**	**C**	**D**	**E**	**F**
1	5.20±2.20[a]	4.20±1.25[a]	4.35± 1.35[a]	4.65±1.21[a]	5.65±1.25[a]	6.41±.95[a]
2	6.40±1.35[a]	4.35±1.20[a]	6.22±1.25[a]	6.25±1.25[a]	6.35±1.35[a]	5.35±1.25[a]
3	6.50±1.15[a]	3.60±.55[b]	5.05±1.15[a]	4.10±1.15[a]	5.35±1.20[a]	6.45±1.25[a]
4	6.05±1.15[a]	2.45±.85[b]	8.35±1.25[a]	9.50±1.15[a]	7.35±1.20[a]	11.25±1.35[a]
B/L	5.05±1.05[a]	4.86±.55[a]	4.66±.33[a]	4.82±1.05[a]	5.25±1.26[a]	5.15±1.05[a]

Mean along the same row with different suffixes are significantly different ($P<0.05$).

Key:

A: undiseased.
B: diseased without treatment.
C: diseased & treated with Norfloxacin.
D: diseased & treated with *L. casei*.
E: prophylactic group.
F: diseased & treated with *O. gratissimum*.
B/L: Baseline.

Table 17: Mean Total White Blood Cell Levels (cells/L) of the Chicken Groups

Weeks	Treatment Groups					
	A	**B**	**C**	**D**	**E**	**F**
1	9190.00±1.10^{a}	11495.65±1.45^{cd}	10599.00±1.15^{bc}	9900.00±1.15^{ab}	10630.65±0.65^{bc}	11800.00±1.15^{c}
2	11890.00±1.15^{c}	13100.65±1.45^{f}	10800.30±1.20^{a}	12400.65±.88^{d}	1200000±1.15^{b}	12600.30±2.00^{e}
3	10800.65±1.45^{a}	12900.00±1.15^{d}	13200.30±.85^{e}	11800.30±1.20^{b}	13400.30±1.20^{f}	12100.00±1.15^{c}
4	12797.65±1.45^{a}	14100.30±1.20^{b}	12600.20±1.20^{a}	12700.00±1.70^{a}	12885.00±12.50^{a}	13900.00±0.03^{ab}
B/L	9050.25±1.2^{a}	9215.00± 1.2^{a}	9118.00±1.15^{a}	9224.65 ± 1.2^{a}	9218.36±1.15^{a}	9110.33±1.00^{a}

Mean along the same row with different suffixes are significantly different (P<0.05).

Key:

A: undiseased.
B: diseased without treatment.
C: diseased & treated with Norfloxacin.
D: diseased & treated with *L. casei*.
E: prophylactic group.
F: diseased & treated with *O. gratissimum*.
B/L: Baseline.

Table 18: Mean Neutrophil Levels of the Chicken Groups

Weeks	Treatment Groups					
	A	**B**	**C**	**D**	**E**	**F**
1	28.30 ± 1.75^{b}	26.20 ± 1.15^{ab}	35.30 ± 0.82^{c}	37.00 ± 1.15^{c}	28.15 ± 1.15^{b}	24.20 ± 1.15^{a}
2	$28.00\pm1.10b^{c}$	22.20 ± 1.45^{a}	33.00 ± 1.15^{c}	25.30 ± 1.45^{ab}	29.35 ± 1.40^{bc}	24.00 ± 1.15^{a}
3	$28.20\pm1.15b^{c}$	22.60 ± 1.45^{ab}	29.65 ± 1.75^{c}	25.50 ± 1.20^{ab}	30.25 ± 1.20^{c}	22.30 ± 1.45^{a}
4	30.30 ± 1.20^{d}	26.00 ± 1.15^{c}	$28.65\pm.82^{c}$	31.60 ± 1.45^{d}	23.00 ± 1.15^{b}	18.30 ± 1.45^{a}
B/L	26.20 ± 1.10^{a}	26.05 ± 1.15^{a}	32.65 ± 1.15^{b}	32.60 ± 1.12^{b}	26.15 ± 1.15^{a}	24.70 ± 1.10^{a}

Mean along the same row with different suffixes are significantly different ($P<0.05$).

Key:

A: undiseased.
B: diseased without treatment.
C: diseased & treated with Norfloxacin.
D: diseased & treated with *L. casei*.
E: prophylactic group.
F: diseased & treated with *O. gratissimum*.
B/L: Baseline.

Table 19: Mean Lymphocyte Levels of the Chicken Groups

	A	B	C	D	E	F
Weeks			**Treatment Groups**			
2	65.60±1.75^{c}	64.30±1.45^{c}	61.60±1.45^{bc}	60.05±1.10^{ab}	61.00±.82^{bc}	59.00±1.15^{a}
3	65.60±1.20^{ab}	68.60±1.20^{bc}	62.65±1.70^{a}	70.00±1.10^{bc}	61.60±1.70^{a}	74.00±1.15^{c}
4	65.60±6.60^{ab}	68.40±1.40^{bc}	68.00±1.10^{b}	64.00±1.70^{a}	72.30±1.45^{c}	79.00±1.15^{d}
B/L	60.06±1.75^{a}	60.25±1.15^{a}	51.33±1.50^{b}	50.15±1.20^{b}	60.33±.80^{a}	60.25±1.10^{a}

Mean along the same row with different suffixes are significantly different (P<0.05).

Key:

A: undiseased.
B: diseased without treatment.
C: diseased & treated with Norfloxacin.
D: diseased & treated with *L. casei*.
E: prophylactic group.
F: diseased & treated with *O. gratissimum*.
B/L: Baseline.

Table 20: Mean Eosinophil Levels of the Chicken Groups

Weeks	Treatment Groups					
	A	**B**	**C**	**D**	**E**	**F**
1	0.65±.65[a]	1.00±1.00[a]	1.33±1.33[a]	0.00±0.00[a]	0.67±.67[a]	0.70±.28[a]
2	0.65±.65[a]	0.65±.65[a]	1.33±1.33[a]	0.65±.65[a]	1.00±1.00[a]	1.00±1.00[a]
3	0.65±.65[a]	1.00±1.00[a]	1.00±1.00[a]	1.00±1.00[a]	1.00±100[a]	0.67±.67[a]
4	0.00±.00[a]	1.00±1.00[a]	0.67±.67[a]	1.00±1.00[a]	1.33±1.33[a]	0.70±.27[a]
B/L	0.65±.65[a]	1.00±1.00[a]	0.67±.67[a]	0.00±0.00[a]	0.67±.67[a]	0.70±.25[a]

Mean along the same row with same suffixes are not significantly different (P>0.05).

Key:

A: undiseased.
B: diseased without treatment.
C: diseased & treated with Norfloxacin.
D: diseased & treated with *L. casei*.
E: prophylactic group.
F: diseased & treated with *O. gratissimum*.
B/L: Baseline.

Table 21: Mean Basophil Levels of the Chicken Groups

Weeks	**Treatment Groups**					
	A	**B**	**C**	**D**	**E**	**F**
1	0 .00±.00[a]	0.67±.33[a]	0.00±0.00[a]	0.33±33[a]	0.00±.00[a]	0.67±.03[a]
2	0.00±.001[a]	0.33±33[a]	0.33±.33[a]	0.00±00[a]	0.00±.00[a]	0.00±.00[a]
3	0.00±.00[a]	0.00±.00[a]	0.33±.33[a]	0.67±.33[a]	0.00±.00[a]	0.67±.03[a]
4	0.33±.33[a]	0.33±.33[a]	0.67±.33[a]	0.00±.00[a]	0.00±.00[a]	0.00±.00[a]
B/L	0.00±.00[a]	0.00±.00[a]	0.33±.33[a]	0.33±33[a]	0.67±.03[a]	0.00±.00[a]

Mean along the same row with same suffixes are not significantly different (P>0.05).

Key:

A: undiseased.
B: diseased without treatment.
C: diseased & treated with Norfloxacin.
D: diseased & treated with *L. casei*.
E: prophylactic group.
F: diseased & treated with *O. gratissimum*.
B/L: Baseline.

Table 22: Mean Monocyte Levels of the Chicken Groups

Weeks	Treatment Groups					
	A	**B**	**C**	**D**	**E**	**F**
1	1.00±.52[a]	1.37±.58[a]	1.67±.65[a]	1.00±.68[a]	1.00±67[a]	1.00±.65[a]
2	1.00±.67[a]	1.00±67[a]	2.00±.116[a]	1.48±.06[a]	1.33±.58[a]	1.48±.58[a]
3	1.33±.05[a]	.66±.66[a]	2.00±.48[a]	2.260±.58[a]	1.33±.58[a]	1.88±.62[a]
4	2.00±1.05[a]	.58±16[a]	2.48±.67[a]	2.67±.67[a]	1.37±.33[a]	2.00±.58[a]
B/L	1.00±.37[a]	1.00±67[a]	1.00±.68[a]	1.33±.58[a]	1.00±.33[a]	1.00±37[a]

Mean along the same row with same suffixes are not significantly different (P>0.05).

Key:

A: undiseased.
B: diseased without treatment.
C: diseased & treated with Norfloxacin.
D: diseased & treated with *L. casei*.
E: prophylactic group.
F: diseased & treated with *O. gratissimum*.
B/L: Baseline.

4.2.6: Effects of Oral Administration of *L. casei* and *O. gratissimum* Extract on the Blood Chemistry Parameters of Broiler Chicks.

There were significant differences ($p<0.05$) amongst the chicken groups for all the parameters observed within the four-week monitoring period. The untreated group gave the highest acid phosphatase, serum aspartate amino transferase,serum alanine amino transferase, urea, creatinine and C-reactive protein levels of 47.19±6.26, 88.00±16.64, 48.67±6.66, 17.41±3.22, 1.16±0.16 and 18.00±6.00, respectively at the end of the fourth week of monitoring (Tables 23-28).

4.2.7: Effects of Oral Administration of *L. casei* and *O. gratissimum* on the Microbial Counts of the Broiler Chicks.

Microbial counts of the intestine and caecum of the chicken groups obtained during the research period are shown on Figures 2-8.

4.2.8: Effects of Oral Administration of *L. casei* and *O. gratissimum* on the Intestinal and Caecum Tissues of the Broiler Chicks.

4.2.8.1: Gross Morphological Examination.

The gross morphological examination of the intestinal tissues of the chicks showed thatinfected birds had enlarged Bursa of Fabricus and ulceration of the small intestine compared to control (Plates 3-7).

Table 23: Mean Acid Phosphatase Levels of the Test Chickens

Weeks	Treatment Groups					
	A	**B**	**C**	**D**	**E**	**F**
1	17.83±5.12^{a}	24.65 ± 12.71^{b}	15.82±2.47^{a}	20.12 ± 4.75^{a}	19.73± 14.75^{a}	19.77± 6.34^{a}
2	5.36 ± 4.41^{a}	27.78± 6.73^{c}	16.42 ± 11.53^{b}	18.85 ± 5.70^{b}	20.18 ± 6.60^{b}	27.15 ± 6.91^{c}
3	18.10 ± 5.87^{a}	44.94 ± 7.27^{c}	23.70 ± 4.77^{b}	28.83 ± 7.10^{c}	20.64 ± 17.73^{a}	32.31 ± 13.83^{d}
4	28.87 ± 10.03^{a}	47.19 ± 6.26^{c}	24.56± 5.40^{a}	26.75 ± 6.95^{a}	30.43 ± 11.30^{b}	32.07 ± 8.48^{b}
B/L	15.15± 3.33^{a}	17.33± 6.66^{a}	15.06± 2.15^{a}	16.28± 4.05^{a}	15.25± 6.33^{a}	15.36± 6.30^{a}

Mean along the same row with different suffixes are significantly different (P<0.05).

Key:

A: undiseased.
B: diseased without treatment.
C: diseased & treated with Norfloxacin.
D: diseased & treated with *L. casei*.
E: prophylactic group.
F: diseased & treated with *O. gratissimum*.
B/L: Baseline.

Table 24: Mean Serum Aspartate Amino Transferase Levels of the Test Chickens

Weeks	Treatment Groups					
	A	**B**	**C**	**D**	**E**	**F**
1	26.33±2.52^{a}	51.33 ± 23.97^{ab}	68.00±8.19^{ab}	73.33 ± 10.41^{b}	61.33±7.62^{ab}	67.33±1.50^{ab}
2	21.00 ± 7.94^{a}	50.00± 11.53^{b}	55.33 ± 10.02^{b}	64.67 ± 13.05^{c}	75.33 ± 7.57^{c}	58.33±4.95^{b}
3	33.33 ± 7.64^{a}	74.33 ± 7.02^{c}	52.67 ± 16.86^{ab}	62.33 ± 4.16^{b}	63.33 ± 7.64^{b}	66.67 ±7.62^{b}
4	35.00 ± 7.55^{a}	88.00 ± 16.64^{c}	74.00 ± 6.56^{b}	74.00 ± 9.64^{b}	75.00 ± 6.68^{b}	75.00 ± 8.36^{b}
B/L	24.12± 3.33^{a}	33.86± 10.24^{a}	34.24± 3.35^{a}	32.68± 4.05^{a}	35.15± 6.33^{a}	35.66± 7.62^{a}

Mean along the same row with different suffixes are significantly different (P<0.05).

Key:

A: undiseased.
B: diseased without treatment.
C: diseased & treated with Norfloxacin.
D: diseased & treated with *L. casei*.
E: prophylactic group.
F: diseased & treated with *O. gratissimum*.
B/L: Baseline.

Table 25: Mean Serum Alanine Amino Transferase Levels of the Test Chickens

Weeks	Treatment Groups					
	A	**B**	**C**	**D**	**E**	**F**
1	31.67±7.51^{a}	36.33 ± 16.50^{a}	33.67±8.74^{a}	54.33 ± 22.90^{b}	43.67± 27.10^{ab}	33.67± 8.10^{a}
2	27.00 ± 8.19^{a}	62.00± 9.54^{c}	39.00 ± 3.61^{b}	50.33± 22.74^{c}	42.33 ± 7.51^{b}	52.67 ± 18.50^{c}
3	34.00 ± 8.19^{a}	63.67 ± 6.66^{c}	47.67 ± 4.93^{ab}	59.33 ± 13.58^{b}	55.67 ± 4.73^{b}	48.00 ± 2.65^{ab}
4	36.00 ± 5.29^{a}	48.67 ± 6.66^{b}	27.00 ± 7.55^{a}	33.33 ± 12.58^{a}	36.67± 21.08^{a}	29.33 ± 7.37^{a}
B/L	28.22± 5.35^{a}	31.00± 6.16^{a}	29.66± 3.13^{a}	33.00± 8.19^{a}	33.33± 7.26^{a}	28.67± 7.33^{a}

Mean along the same row with different suffixes are significantly different (P<0.05).

Key:

A: undiseased.
B: diseased without treatment.
C: diseased & treated with Norfloxacin.
D: diseased & treated with *L. casei*.
E: prophylactic group.
F: diseased & treated with *O. gratissimum*.
B/L: Baseline.

Table 26: Mean Urea Levels (mg/dl) of the Test Chickens

Weeks	Treatment Groups					
	A	**B**	**C**	**D**	**E**	**F**
1	5.92±1.44[a]	8.19 ± 2.26[a]	6.22±2.81[a]	7.33 ± .59[a]	7.86± 1.38[a]	7.31± 1.87[a]
2	5.28 ± 1.65[a]	12.55± 2.81[b]	6.62 ± 1.46[a]	8.15 ± 1.08[ab]	9.00 ± 2.73[ab]	8.56 ± 2.33[ab]
3	6.06± 1.52[a]	11.96 ± 1.34[b]	8.87 ± 3.02[ab]	9.73 ± 4.10[ab]	9.41 ± .89[ab]	9.33 ± 1.28[ab]
4	7.02± .78[a]	17.41 ± 3.22[b]	9.40 ± 2.33[a]	9.83 ± 3.15[a]	10.83 ± 1.54[a]	10.34 ± 2.97[a]
B/L	4.19± 2.81[a]	4.92± .78[a]	4.33± .59[a]	4.22± 1.45[a]	4.31± 1.12[a]	4.86± 2.55[a]

Mean along the same row with different suffixes are significantly different (P<0.05).

Key:

A: undiseased.
B: diseased without treatment.
C: diseased & treated with Norfloxacin.
D: diseased & treated with *L. casei*.
E: prophylactic group.
F: diseased & treated with *O. gratissimum*.
B/L: Baseline.

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Table 27: Mean Creatinine Levels (mg/dl) of the Test Chickens

Weeks	Treatment Groups					
	A	**B**	**C**	**D**	**E**	**F**
1	0.17± .08[a]	0.32 ± .30[a]	0.42±.17[a]	0.17 ± .03[a]	0.13± .08[a]	0.17± .02[a]
2	0.13 ± .04[a]	1.10± .24[b]	0.32 ± .05[a]	0.38 ± .07[a]	0.37 ± .15[a]	0.38 ± .05[a]
3	0.83 ± .60[a]	2.55 ± 1.96[b]	0.52 ± .19[a]	0.65 ± .08[a]	0.81 ± .33[a]	0.45 ± .32[a]
4	0.51 ± .31[a]	1.16 ± .16[b]	0.57 ± .25[a]	0.39 ± .25[a]	0.85 ± .48[a]	0.22 ± .11[a]
B/L	0.13± .03[a]	0.24± .33[a]	0.22± .25[a]	0.13± .17[a]	0.09± .15[a]	0.15± .33[a]

Mean along the same row with different suffixes are significantly different ($P<0.05$).

Key:

A: undiseased.
B: diseased without treatment.
C: diseased & treated with Norfloxacin.
D: diseased & treated with *L. casei*.
E: prophylactic group.
F: diseased & treated with *O. gratissimum*.
B/L: Baseline.

Table 28: Mean C-reactive Protein Levels of the Test Chickens

Weeks	Treatment Groups					
	A	**B**	**C**	**D**	**E**	**F**
1	8.00±3.46[a]	12.00 ± 6.00[b]	8.00±3.46[a]	6.00 ± 0.00[a]	8.00± 3.46[a]	8.00± 3.46[a]
2	8.00 ± 3.46[a]	16.00± 9.17[b]	12.00 ± 6.00[a]	10.00 ± 6.93[a]	10.00 ± 6.93[a]	12.00 ± 10.39[a]
3	8.00 ± 3.46[a]	20.00 ± 3.46[c]	4.67 ± 2.31[a]	8.00 ± 3.46[b]	10.00 ± 3.46[b]	12.00 ± 6.00[b]
4	10.00 ± 3.46[a]	18.00 ± 6.00[b]	6.00 ± 0.00[a]	6.00 ± 0.00[a]	8.00 ± 3.46[a]	10.00 ± 3.46[a]
B/L	4.00± 1.15[a]	4.17± 1.33[a]	4.31± .23[a]	4.45± .15[a]	4.93± .05[a]	4.15± 2.45[a]

Mean along the same row with different suffixes are significantly different ($P<0.05$).

Key:

A: undiseased.
B: diseased without treatment.
C: diseased & treated with Norfloxacin.
D: diseased & treated with *L. casei*.
E: prophylactic group.
F: diseased & treated with *O. gratissimum*.
B/L: Baseline.

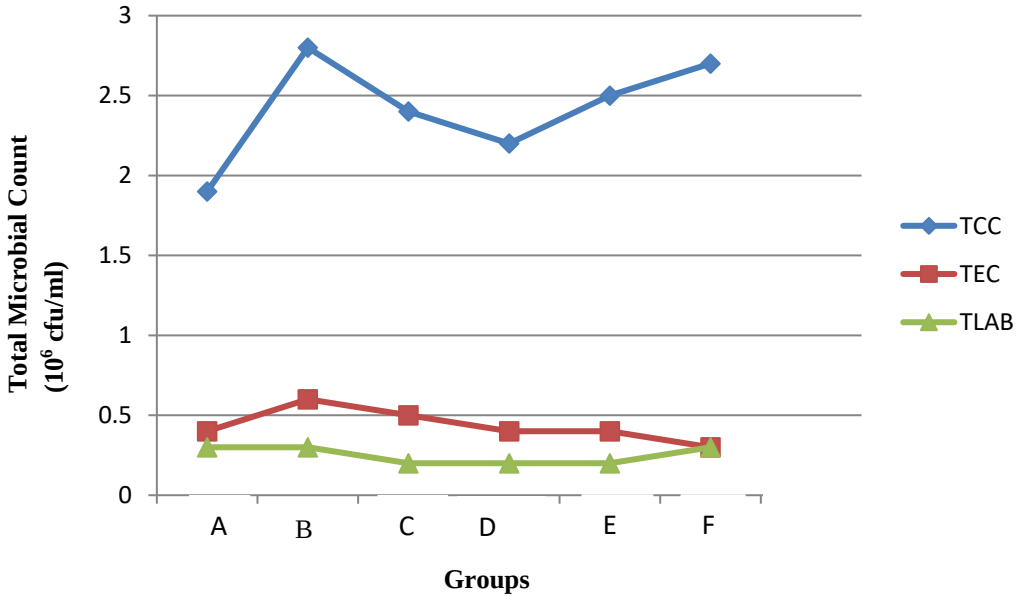

Figure 2: Mean Microbial Counts of the Chicken Stool before Infection.

A: Healthy control.

B: Diseased without Treatment.

C: Antibiotic control using Norfloxacin (15g/L).

D: Probiotic control using *Lactobacillus*

E: Probiotic prophylaxis using *Lactobacillus*

F: Prebiotic control using *O. gratissimum*.

TCC: Total Coliform Count.

TEC: Total *E.coli* Count.

TLAB: Total Lactic Acid Bacteria Count.

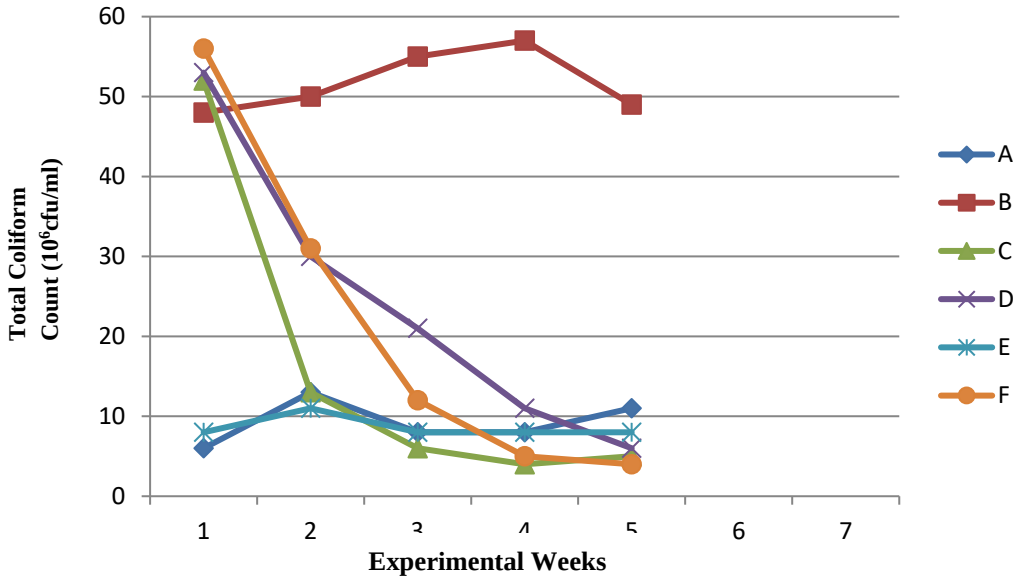

Figure 3: Mean Intestinal Total Coliform Count of the Test Chicken.

A: Healthy control.

B: Diseased without Treatment.

C: Antibiotic control using Norfloxacin (15g/L).

D: Probiotic control using *Lactobacillus*

E: Probiotic prophylaxis using *Lactobacillus*

F: Prebiotic control using *O. gratissimum*.

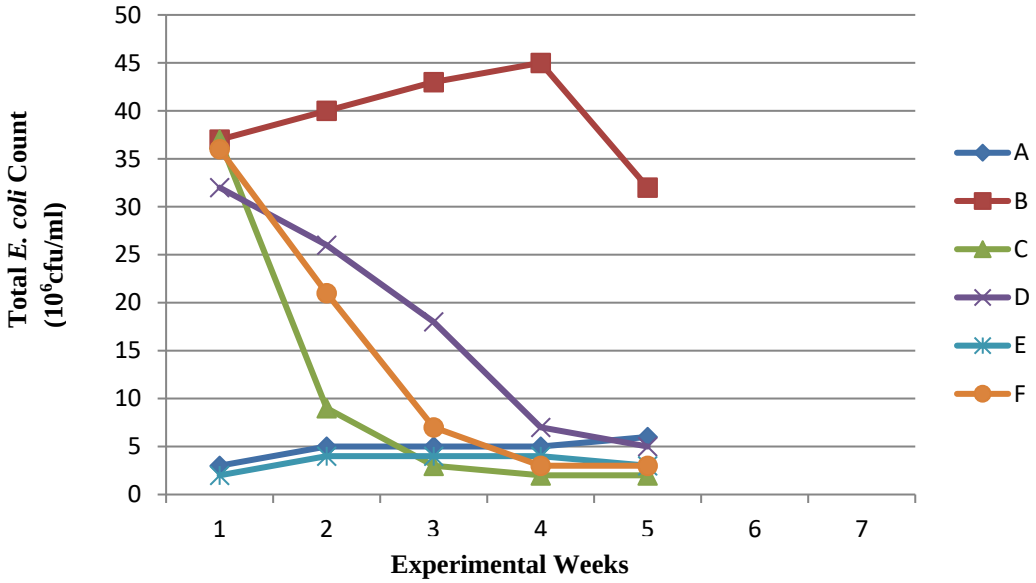

Figure 4: Mean Intestinal Total *E. coli* Count of the Test Chicken.

A: Healthy control.

B: Diseased without Treatment.

C: Antibiotic control using Norfloxacin (15g/L).

D: Probiotic control using *Lactobacillus*

E: Probiotic prophylaxis using *Lactobacillus*

F: Prebiotic control using *O. gratissimum*.

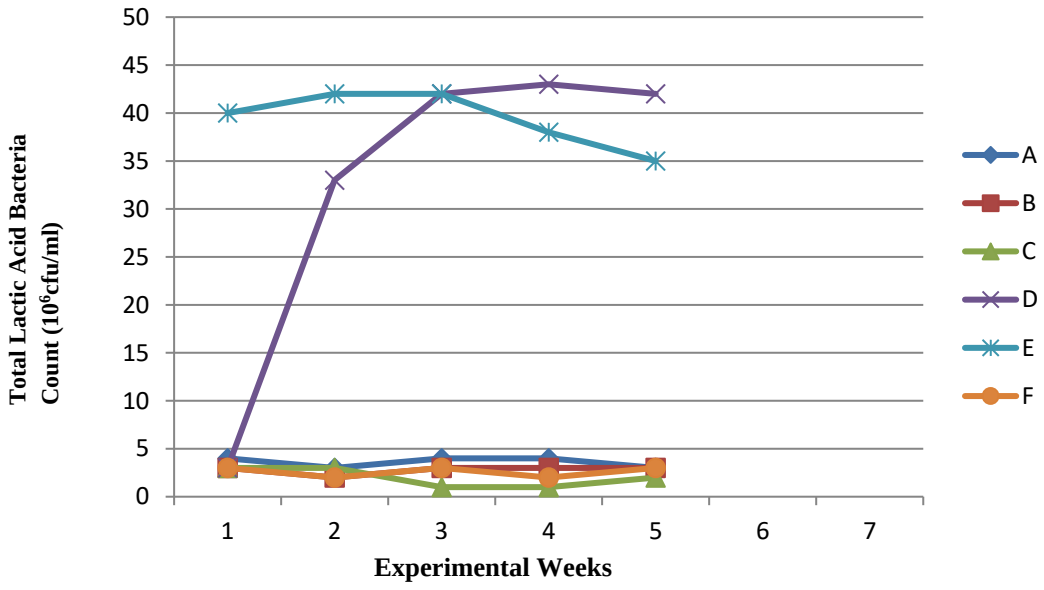

Figure 5: Mean Intestinal Total Lactic Acid Bacteria Count of the Test Chicken.

A: Healthy control.

B: Diseased without Treatment.

C: Antibiotic control using Norfloxacin (15g/L).

D: Probiotic control using *Lactobacillus*

E: Probiotic prophylaxis using *Lactobacillus*

F: Prebiotic control using *O. gratissimum*.

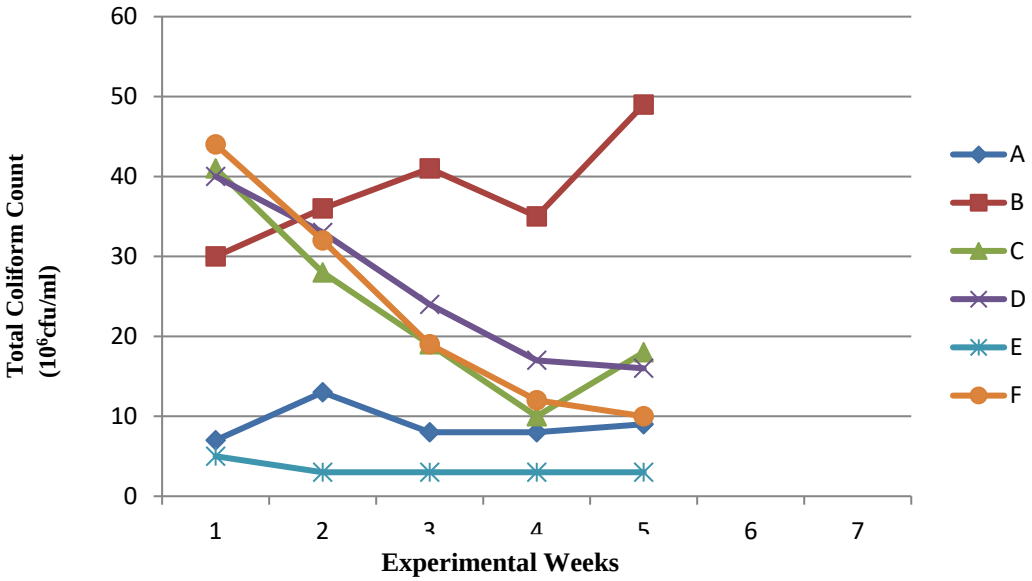

Figure 6: Mean Caecal Total Coliform Count of the Test Chickens

A: Healthy control.

B: Diseased without Treatment.

C: Antibiotic control using Norfloxacin (15g/L).

D: Probiotic control using *Lactobacillus*

E: Probiotic prophylaxis using *Lactobacillus*

F: Prebiotic control using *O. gratissimum*.

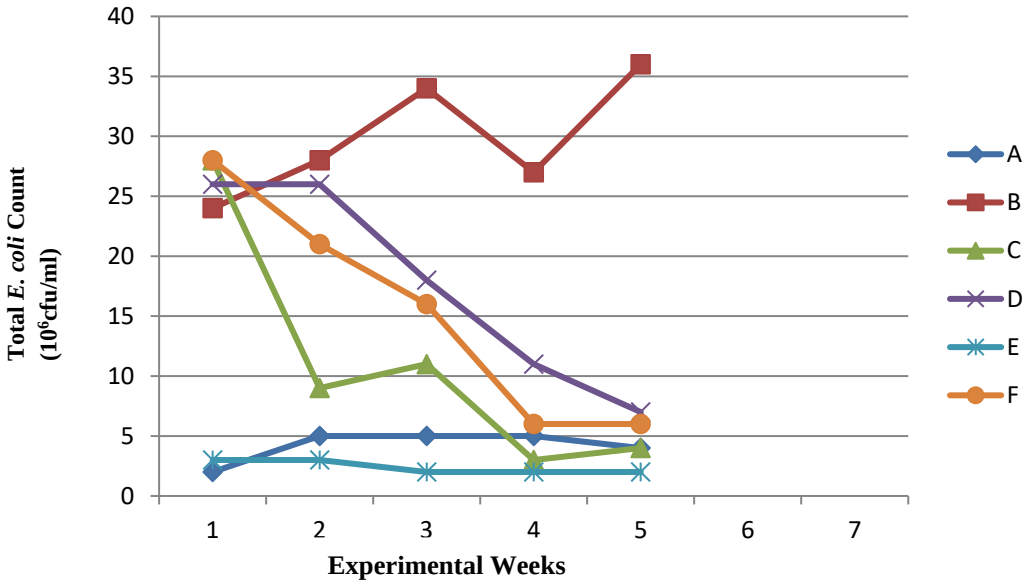

Figure 7: Mean Caecal Total *E. coli* count of the Test Chicken.

A: Healthy control.

B: Diseased without Treatment.

C: Antibiotic control using Norfloxacin (15g/L).

D: Probiotic control using *Lactobacillus*

E: Probiotic prophylaxis using *Lactobacillus*

F: Prebiotic control using *O. gratissimum*.

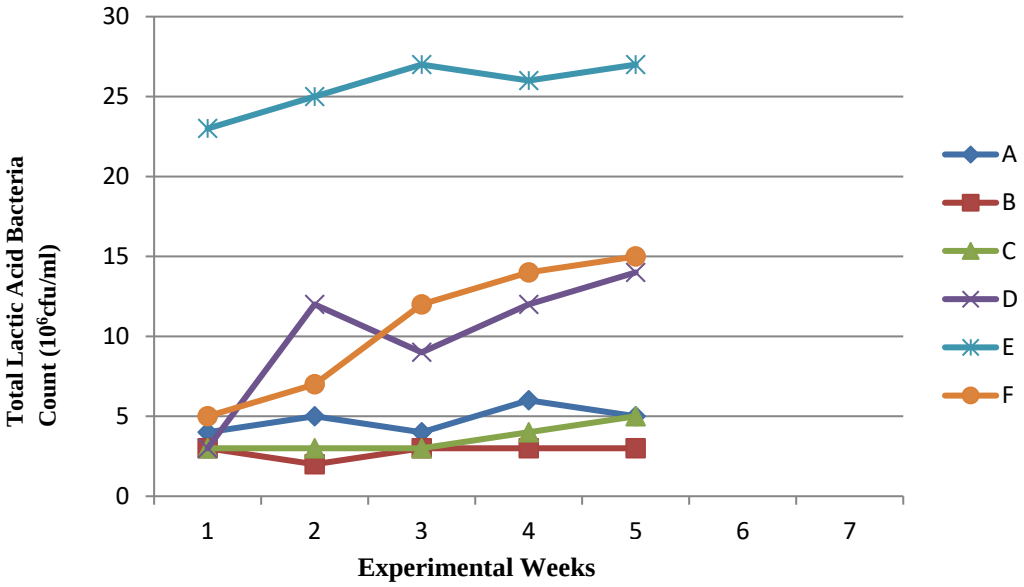

Figure 8: Mean Caecal Total Lactic Acid Bacteria Count

A: Healthy control.

B: Diseased without Treatment.

C: Antibiotic control using Norfloxacin (15g/L).

D: Probiotic control using *Lactobacillus*

E: Probiotic prophylaxis using *Lactobacillus*

F: Prebiotic control using *O. gratissimum*.

Plate 3: Photograph of Dead Broiler Chick during the Experiment.

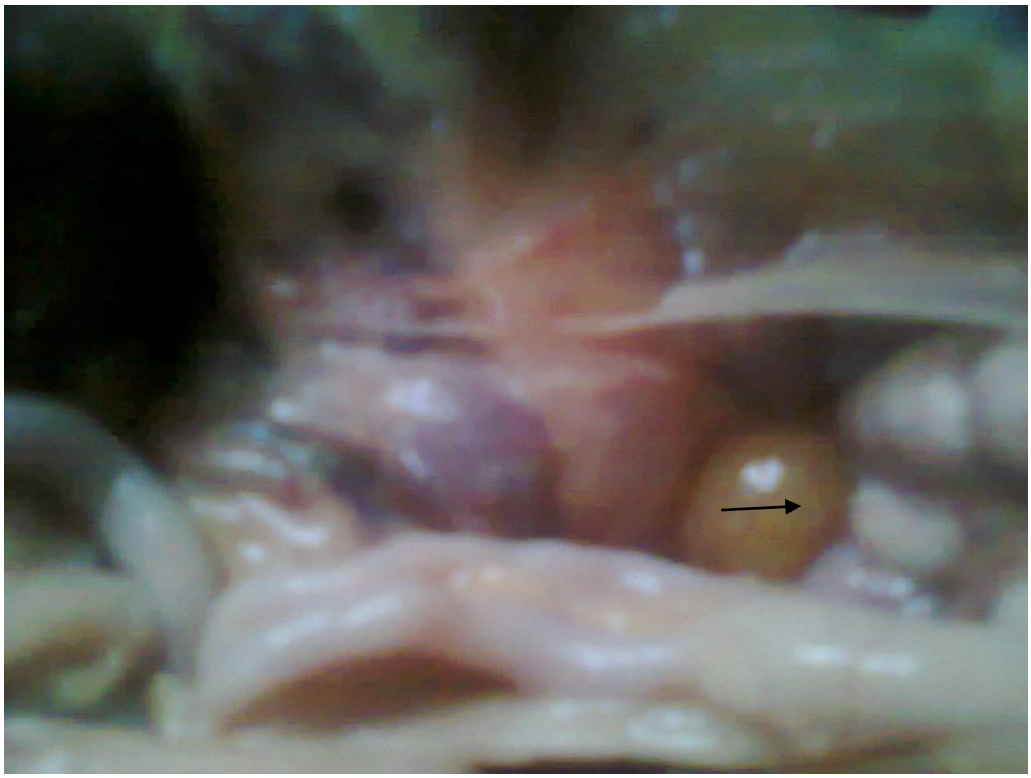

Plate 4: Gross Morphology Photograph of Group A (Healthy Control) Showing Intact Bursa (Black arrow).

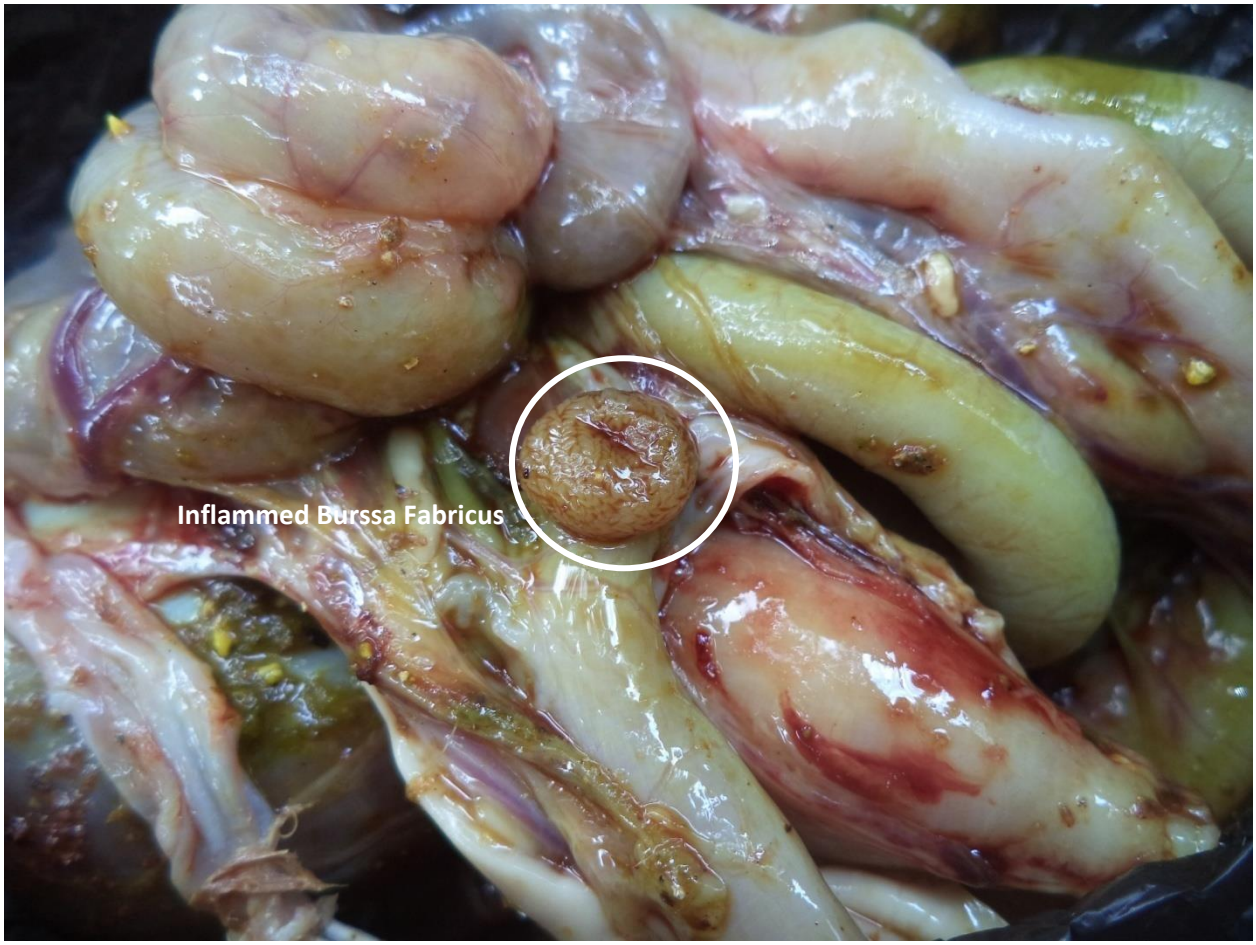

Plate 5: Gross Morphology Photograph of Infected Chick showing Inflammed Burssa Fabricus

Plate 6: Dissection of Broiler Chick in Group B (infected not treated) showing Severe Intestinal Ulceration.

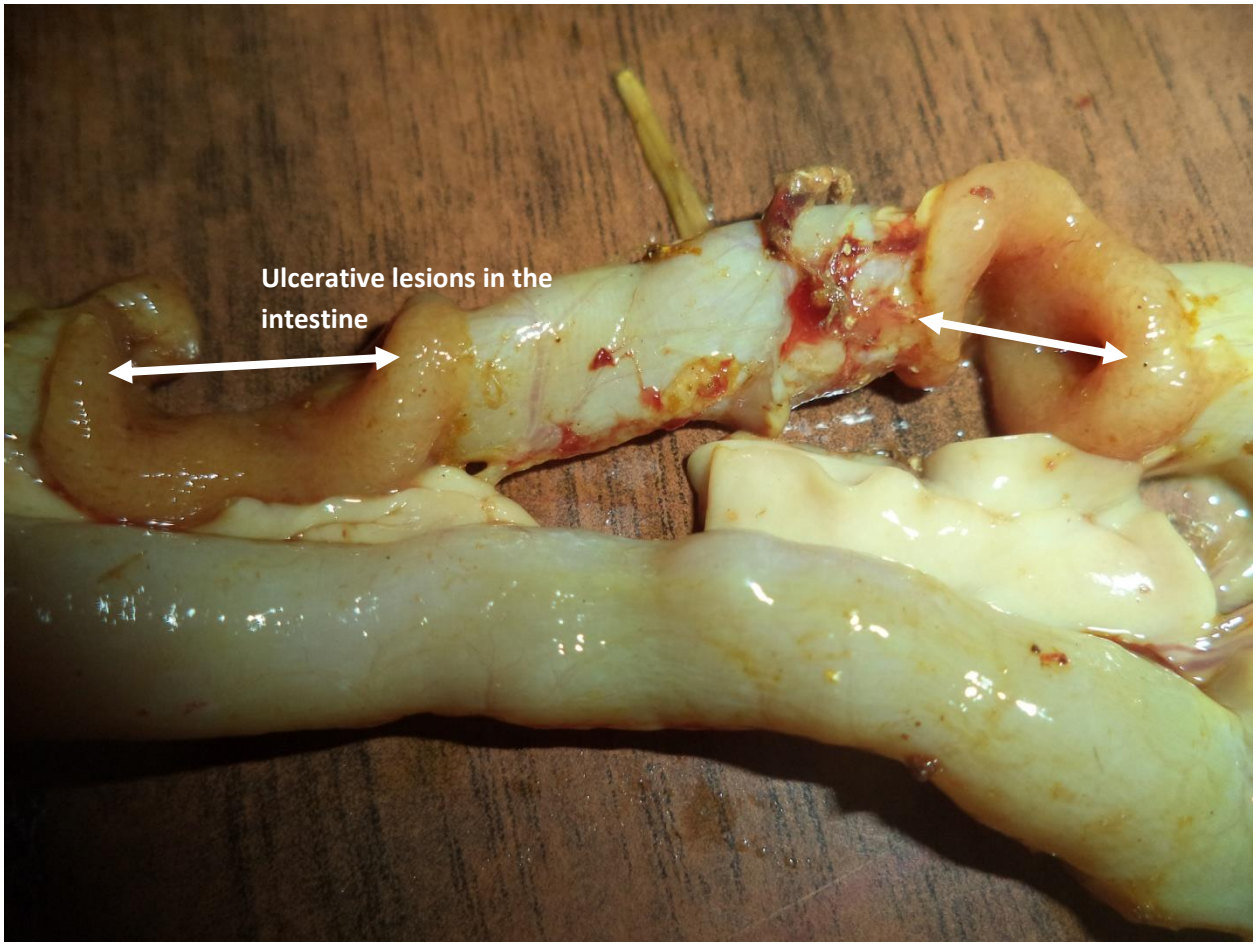

Plate 7: Gross Morphology Photograph of the Small Intestine of an Infected Chick at the End of the Experiment showing Severe Ulcerative Lesion in the Intestine.

4.2.8.2: Histopathology of Small Intestine Tissues.

Histopathological examination of tissues of the small intestine showed that infection of the chicks caused gross distortion of the intestinal architecture compared to the uninfected chicks (control) as shown in Plates 8 and 9. However, the different treatments used in different groups of chicks achieved different levels of restoration of the intestinal tissues (Plates 10-13). The best results were seen with standard antibiotic (Plate 10) and group given *L.casei* prophylaxis (Plate12).

4.2.8.3:Histopathology of Caecal Tissues.

Histopathological examination of tissues of the caecum showed that infection of the chicks caused distortion of the caecal architecture compared to the uninfected chicks (control) as shown in Plates 14 and 15. However, the different treatments used in different groups of chicks achieved different levels of restoration of the caecal tissues (Plates 16-19). The best results were seen with standard antibiotic (Plate 16) and group given *L. casei* prophylaxis (Plate 18).

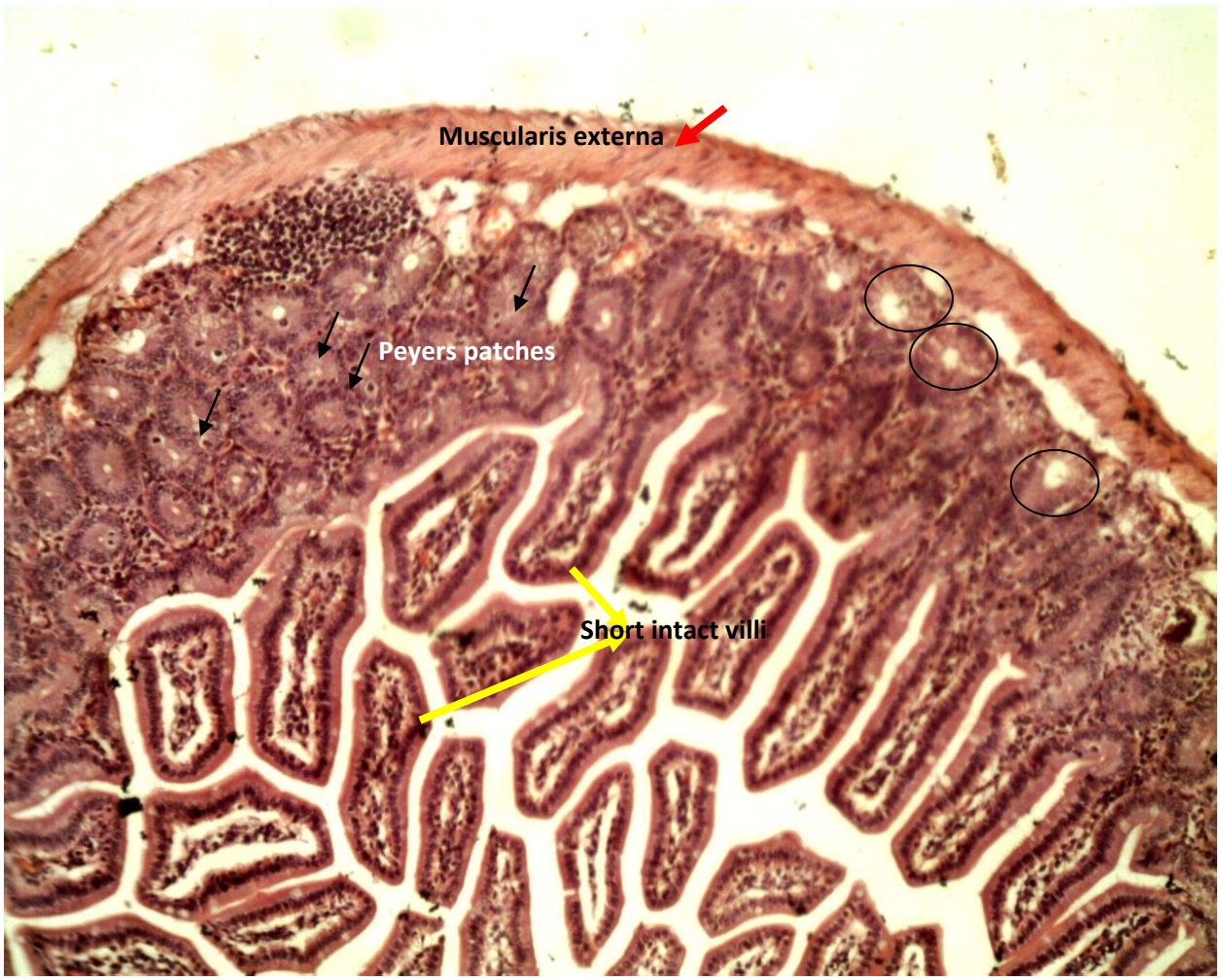

Plate 8:Photomicrograph of small intestine of normal chicken (Group A), showing a normal typical ileum with: (1) Clear diffused lymphoid tissues usually called peyers patches (black arrow) which is a unique feature in ileum and is usually seen in the submucosal region and the muscularis externa is equally intact (red arrow) and also with presence of paneth cells (circles) (2). More prominent and short villi (yellow arrow). H&E.mag. 100X.

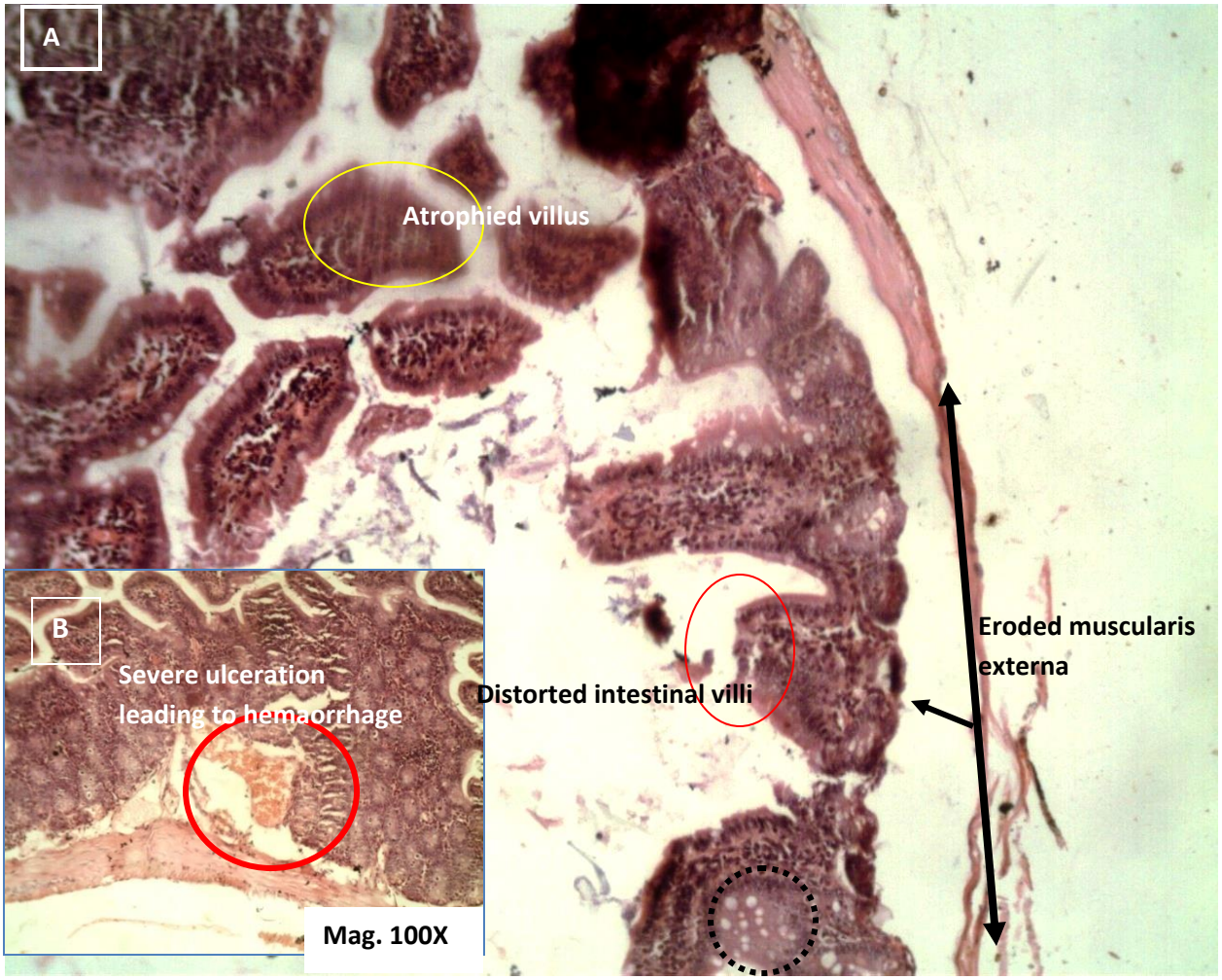

Plate 9: Photomicrograph of duodenum of Group B chicken (infected not treated) showing: (1). Severe erosion of the muscularis externa down to the submucosa (black arrow) with loss of mucosal epithelial cells (2). Distortion/or complete breaking of the intestinal villi and almost complete loss of the villi (red circles) and also villous atrophy was equally observed (yellow circle). (3). There is severe reduction/ or degeneration of goblet cells (dotted circle). (4). Severe observable intestinal hemorrhage due to ulceration is seen (red circle). H&E. mag. 100X.

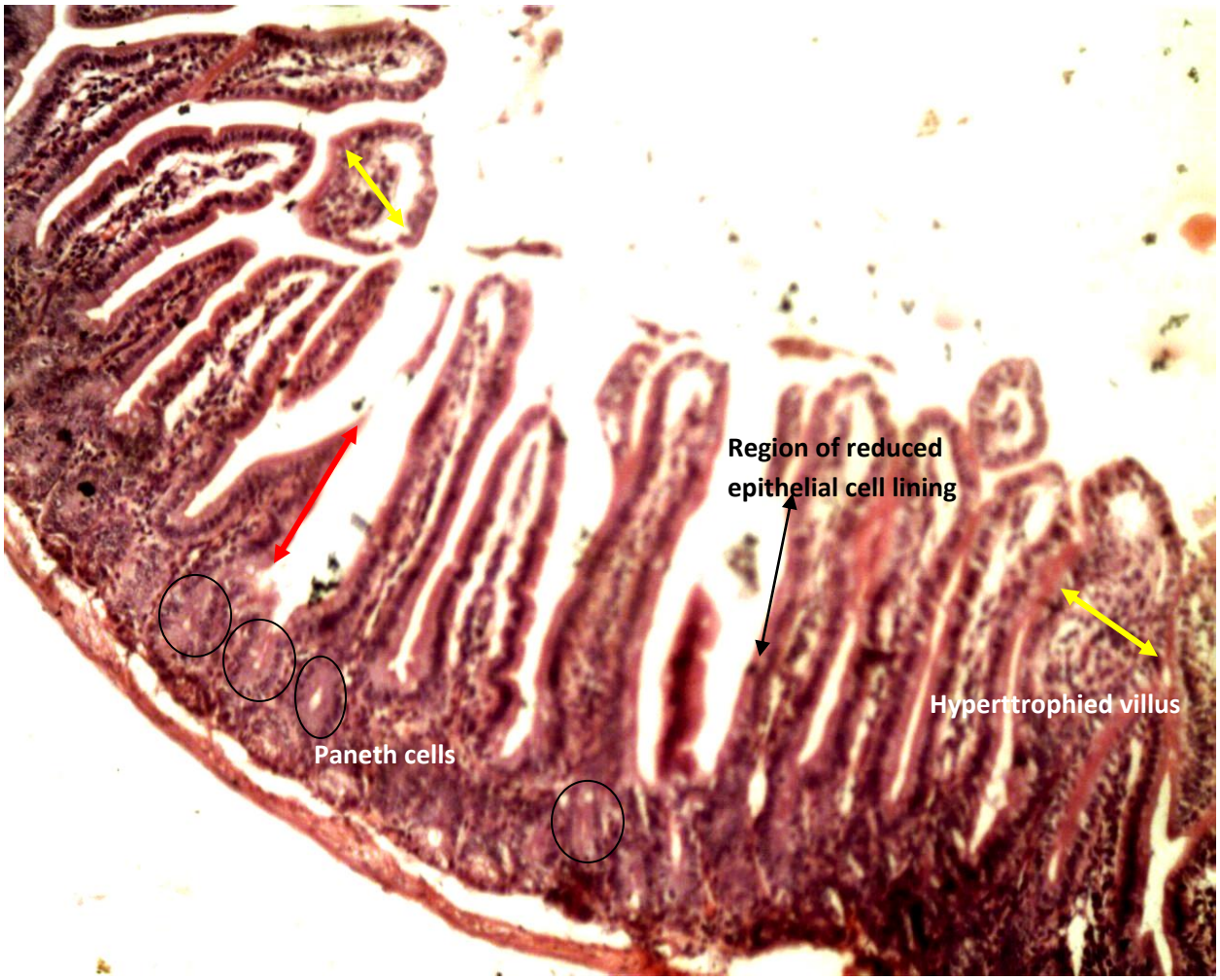

Plate 10: Photomicrograph of duodenum of Group C chicken (antibiotic control) showing:(1) Minor reduction of epithelial cells lining(black arrow head) in the villi although nuclei of the columnar epithelium are evident and clear. (2) Minor reduction of villous length (red arrow head). (3) Increased size of the villi which is usually one of the characteristics pathological changes observed in cases of mucosal hypertrophy (yellow arrow head). (4) That the paneth cells (circles) are observed and increased in number and they are usually the key effectors of innate mucosal defense. H&E. mag. 100X.

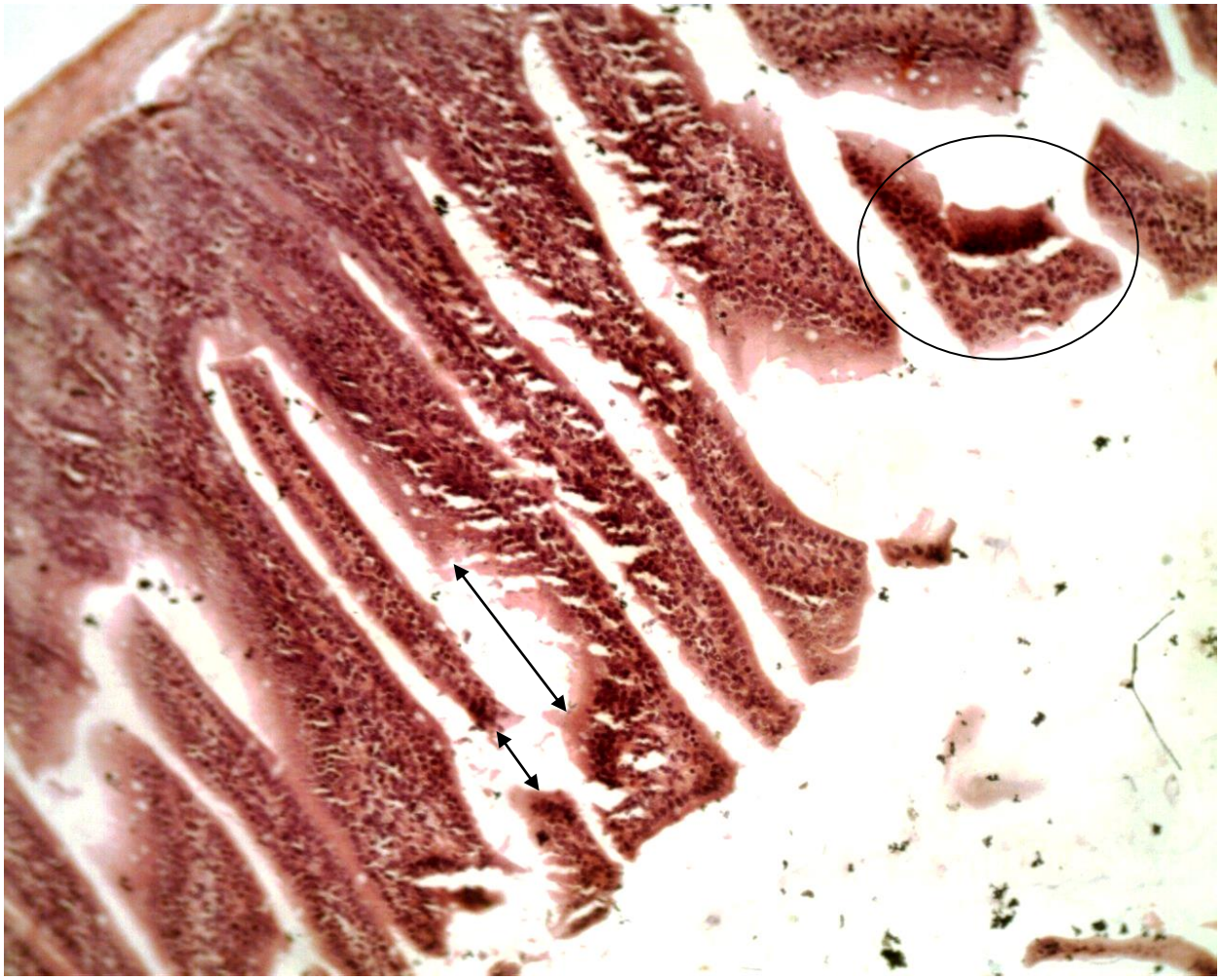

Plate 11: Photomicrograph of duodenum of Group D chicken (treatment Group using *Lactobacillus*) showing: (1) Regeneration of villi although some appear eroded and deformed (circles). (2) Increased number of goblet cells that are visible in each villus (black arrows). H&E. mag. 100X.

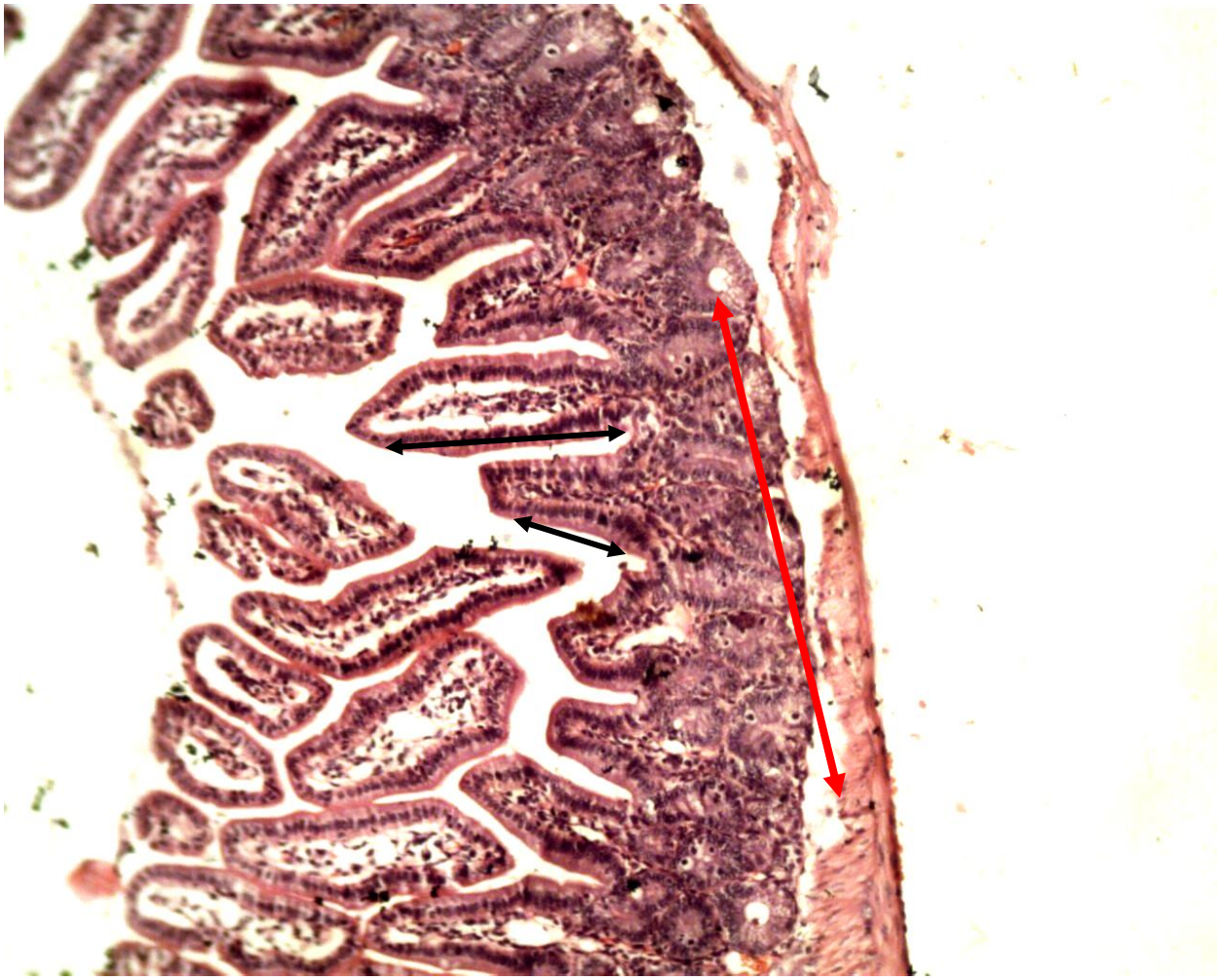

Plate 12: Photomicrograph of small intestine of Group E chicken (prophylactic Group, using *Lactobacillus*) showing: (1) Minor eroded muscularis externa (red arrow head) with reduced thickness with presence of small inflammatory infilterates around the area of tissue loss. (2) Villi differing in sizes and depth (balck arrow heads). (3) Increased epithelial cell and paneth cell numbers. H&E. mag. 100X.

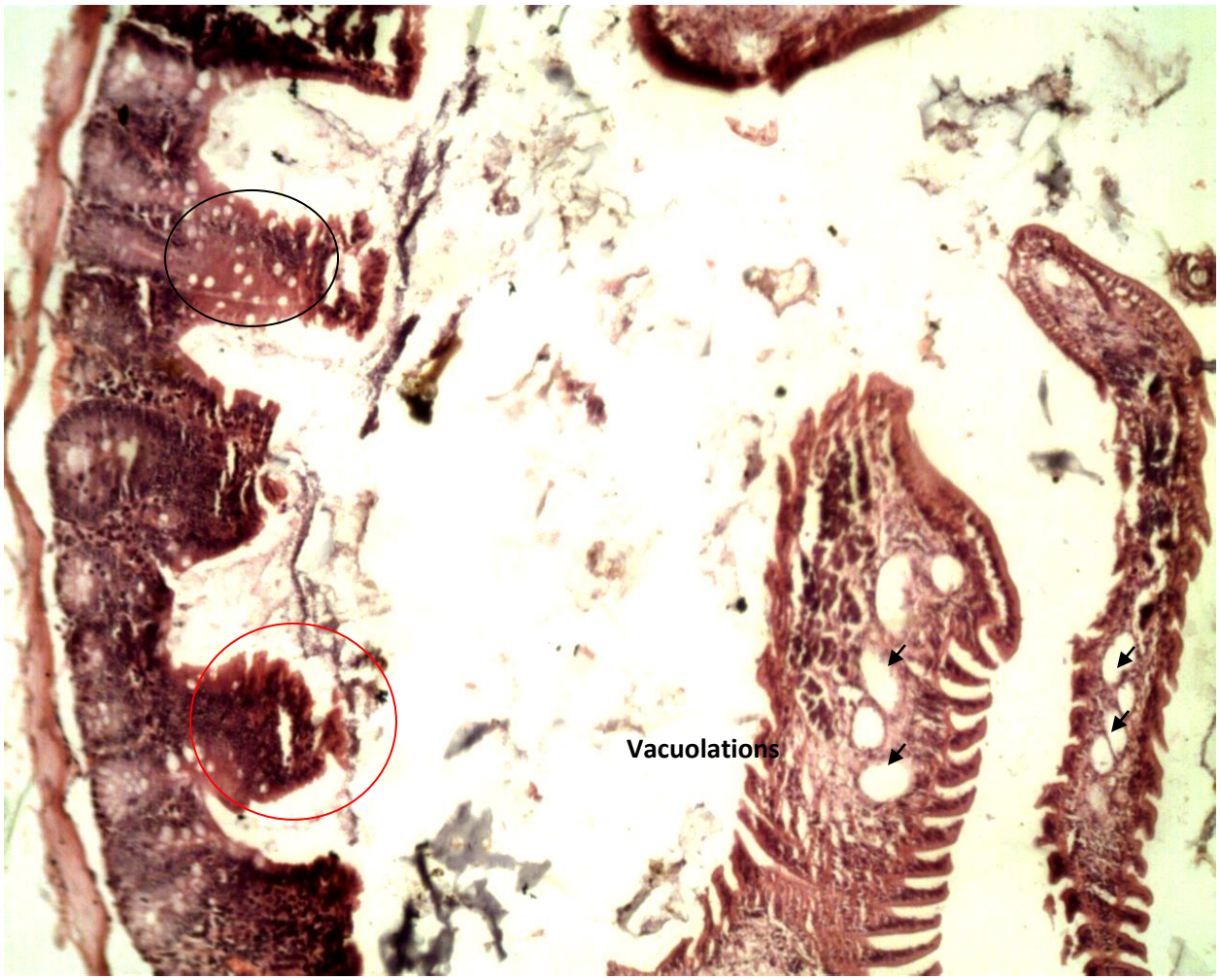

Plate 13: Photomicrograph of small intestine of Group F (diseased and treated with *O. gratissimum*) showing: (1) Gradual villous regeneration (red circle).(2) Regeneration of goblet cells which are usually the frontline of innate host defense (black circle). (3) Vacuolation of epithelial cells which is seen at the apical portion of epithelial cells of the upper third of the villus keeping for mucosal lipidosis(black arrow). H&E. mag. 100X.

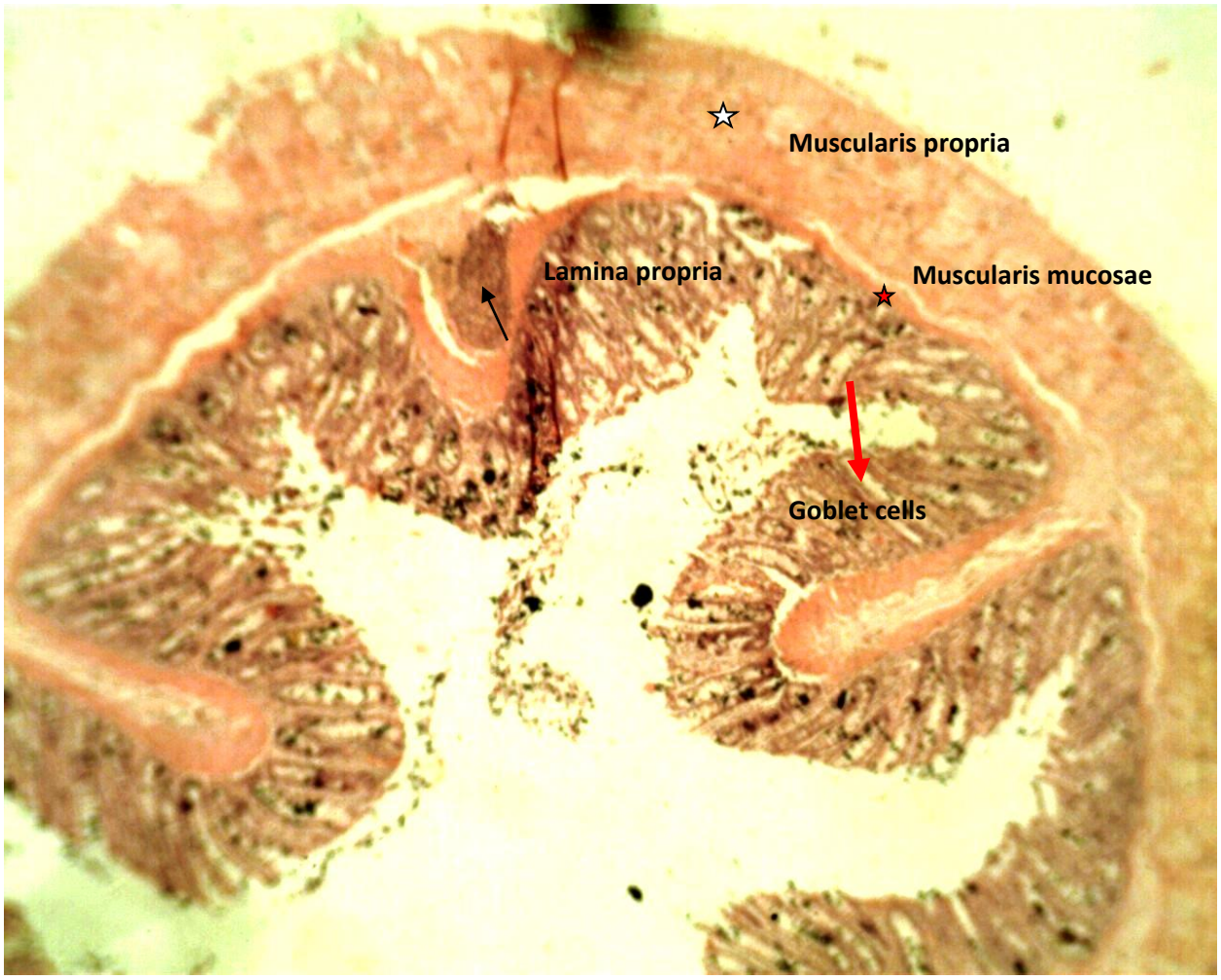

Plate 14:Photomicrograph of caecumof Group A chicken (healthy control) showing normal rectal architecture with: (1) Lamina propria (black arrow). (2) Muscularis propria(star). (3) Goblet cells (red arrow). (4) Muscularis mucosae (red star). All the structures of the caecal regions are intact. H&E. mag. 100X.

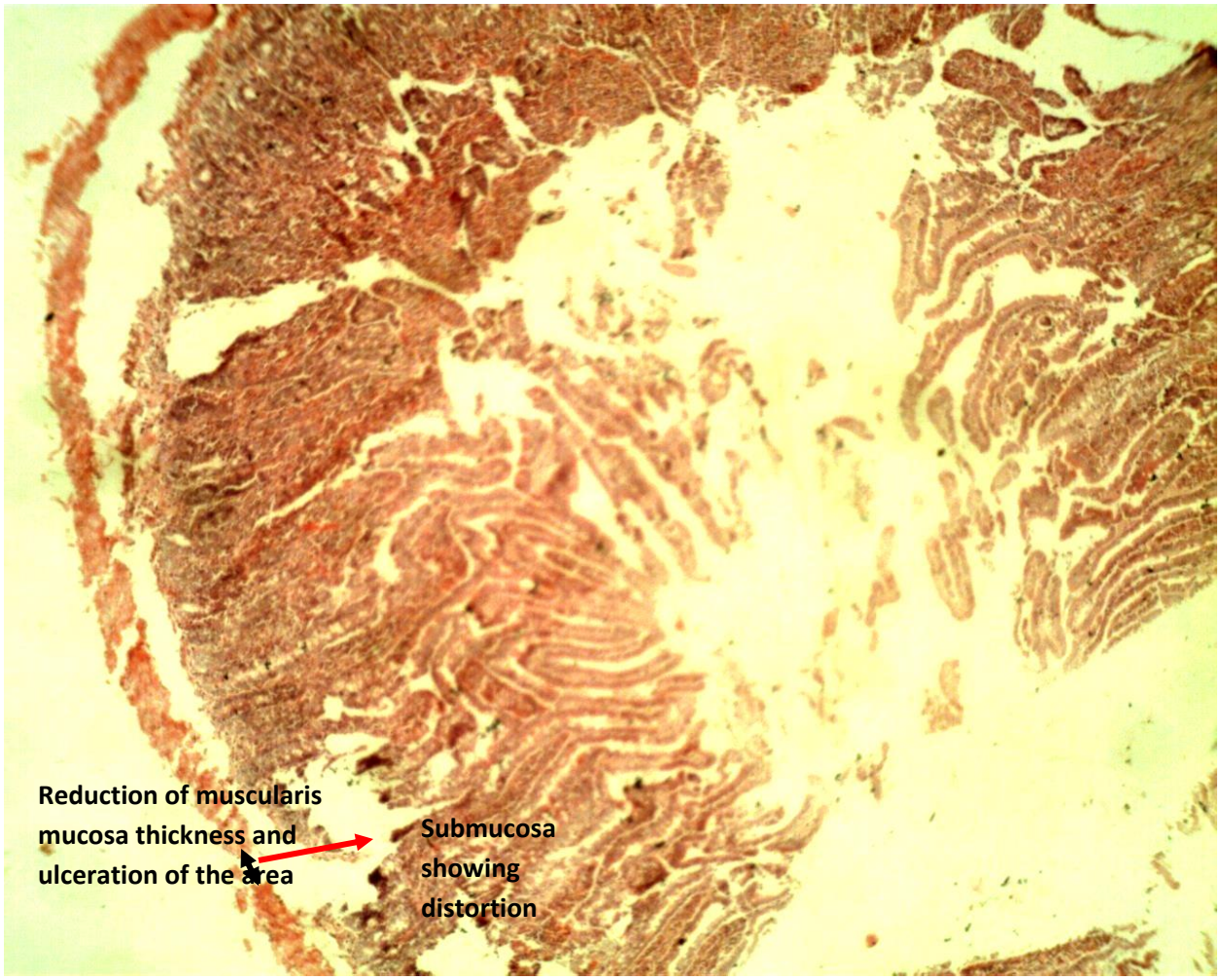

Plate 15:Photomicrograph of caecumof Group B (negative control) showing reduction of thickness of the muscularis mucosae (black arrow head) due to ulceration of the area which went deep into the submucosa (red arrow) leading to ulcerative lesion. H&E. mag. 100X.

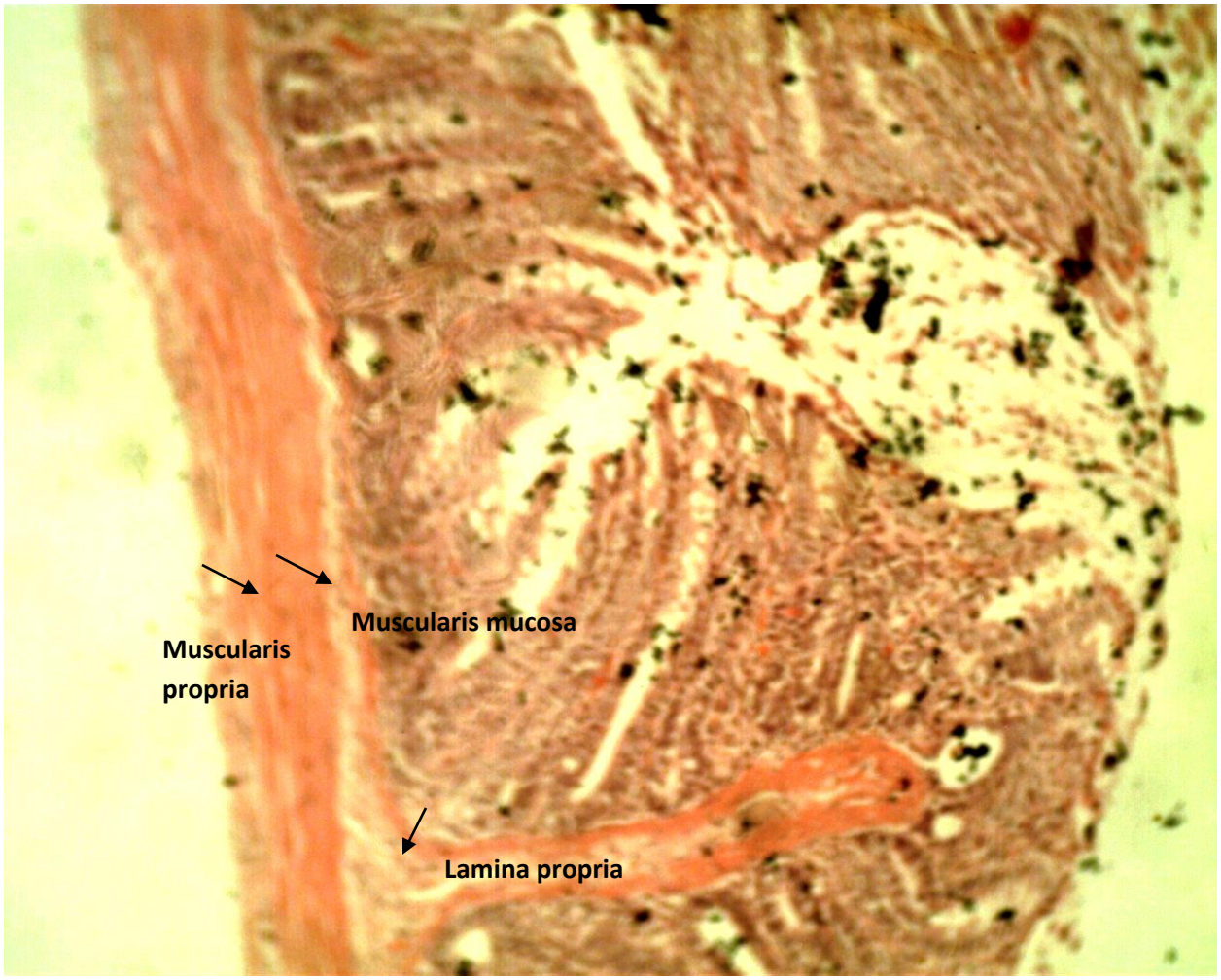

Plate 16: Photomicrograph of caecumof Group C chicken (antibiotic control) showing no observable architectural distortion either in the muscularis mucosa, muscularis propria and lamina propria. All structures are intact. H&E. mag. 100X.

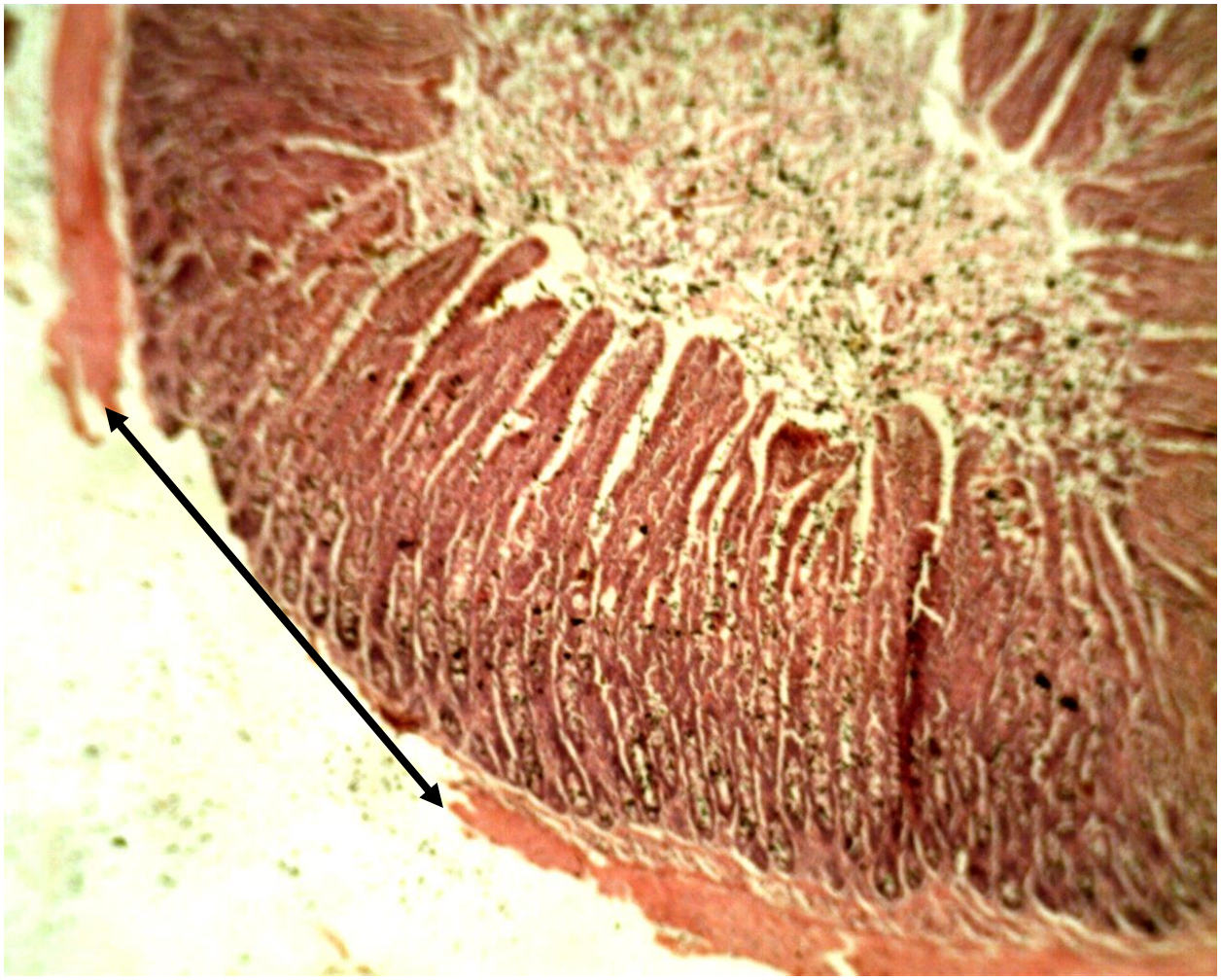

Plate 17:Photomicrograph of caecumof Group D chicken (diseased and treated with *Lactobacillus*) showing erosion of the muscularis propria (arrow head). H&E. mag. 100X.

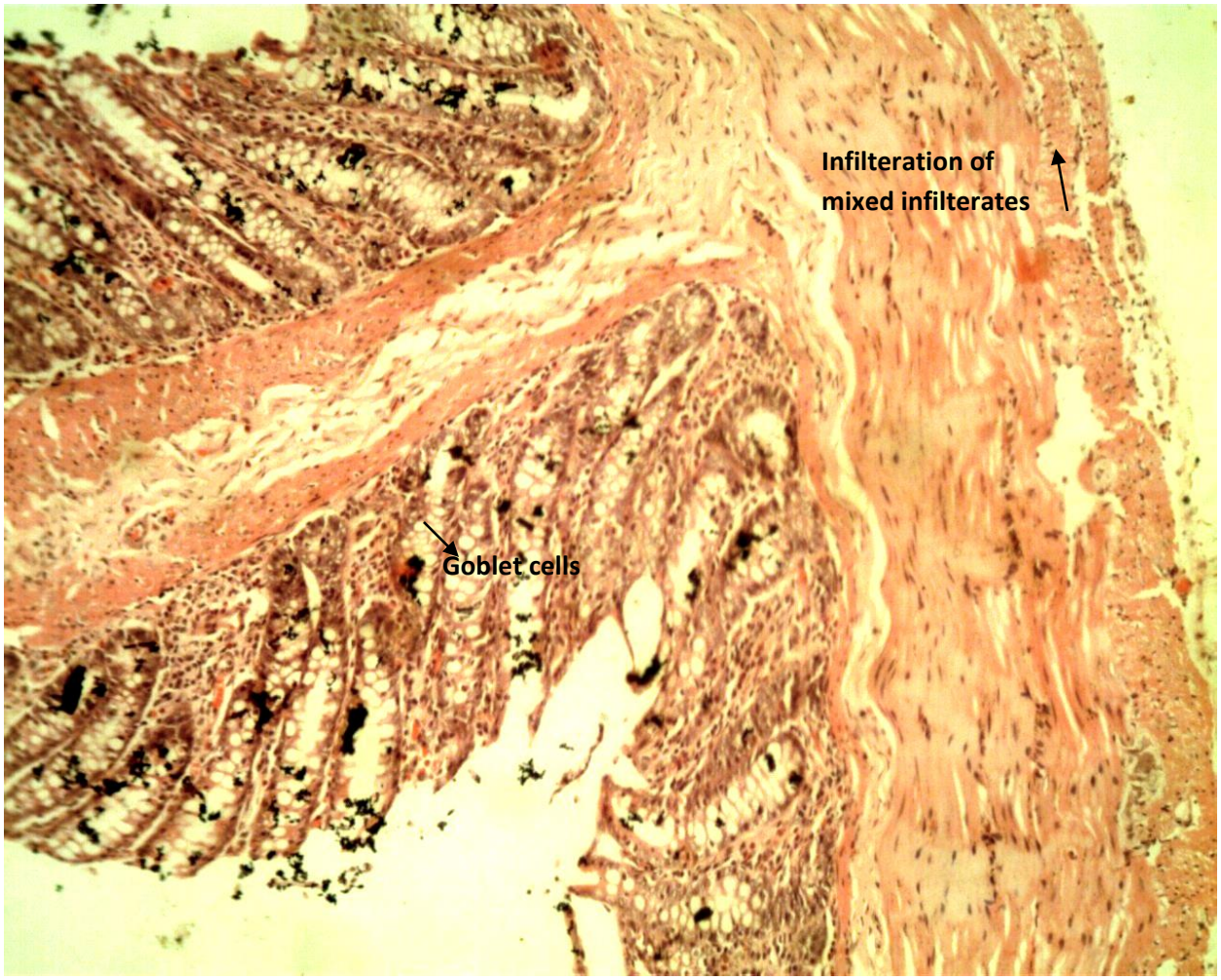

Plate 18:Photomicrograph of caecumof Group E chicken (prophylactic group using *Lactobacillus*) showing:(1) Infilteration of lymphocytic infilterates (black arrow) around the mucosal (2) Goblet cellswhich are present and clearly defined. H&E. mag. 100X.

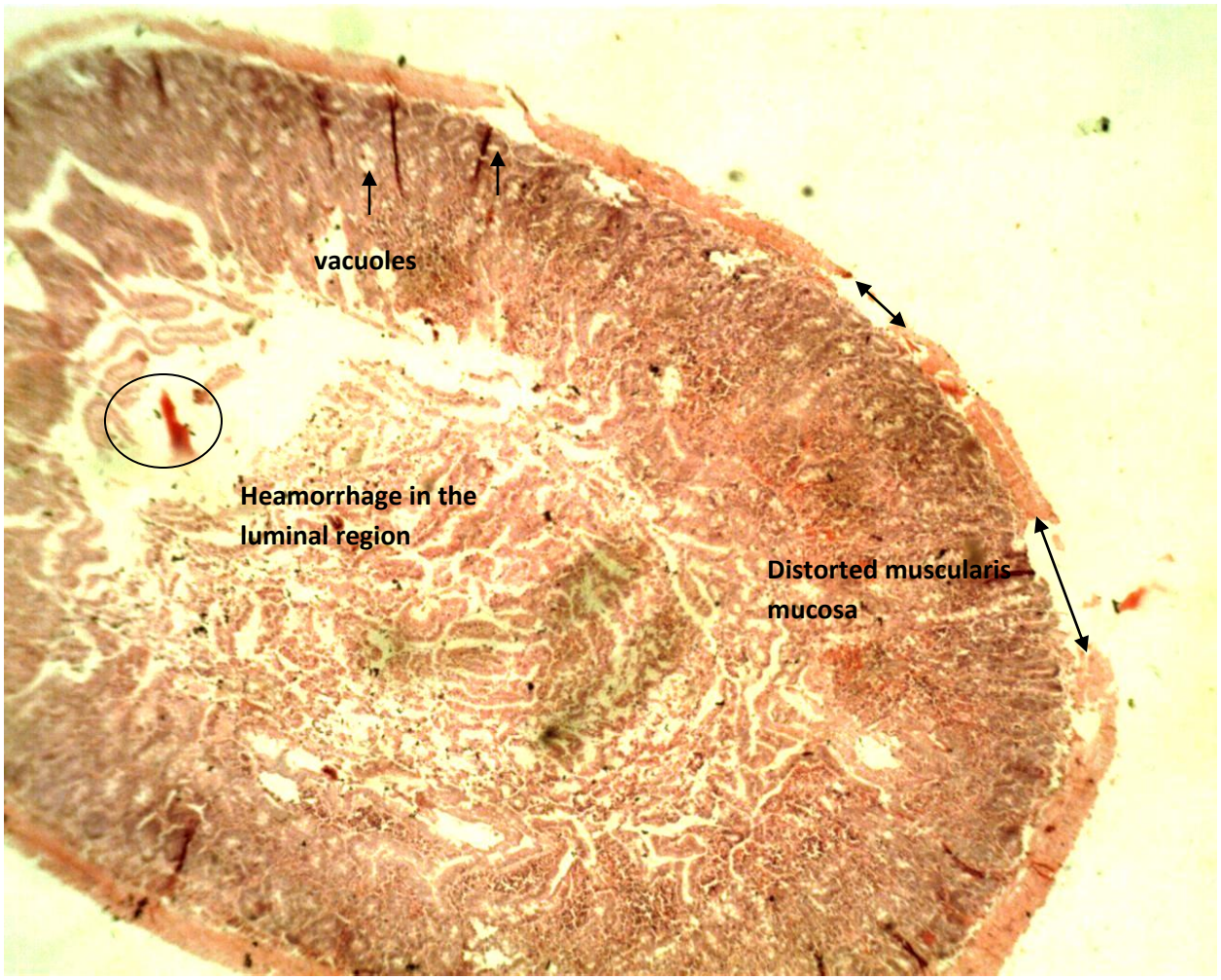

Plate 19:Photomicrograph of caecum of Group F chicken (diseased and treated with *O. gratissimum* extract) showing: (1)Minor hemorrhage in the lumen (2) Distorted muscularis mucosa (arrow head). (3) Vacuolation present keeping for minor onset of accumulation of lipids which is called mucosal lipidosis. (H&E. mag. 100X).

4.2.8.4: Degree of Intestinal Tissue Change.

Degree of tissue change model revealed that infection of the chicks caused a high degeneration in the intestinal architecture compared to the uninfected chicks (control) which had no intestinal tissue damage. However, the least tissue damage from different treatment groups was seen in antibiotic treated, *L. casei* treated and *L. casei* prophylactic groups (Figure 9).

4.2.8.5: Degree of Caecal Tissue Change.

Degree of tissue change model revealed that infection of the chicks resulted in a high degeneration in the caecal architecture compared to the uninfected chicks (control) which had no caecal tissue damage. However, the least tissue degeneration from the different treatment groups was seen in *L. casei* treatment group and *L. casei* prophylactic group; while antibiotic treated group also had no tissue damage (Figure 10).

4.2.9: Effects of Oral Administration of *L.casei* and *O. gratissimum* Extract on Mortality Rate of the Broiler Chicks.

The mortality of the chick groupswas observed over the four-week monitoring period. Only the untreated group recorded 60 % death of its population (Table 29).

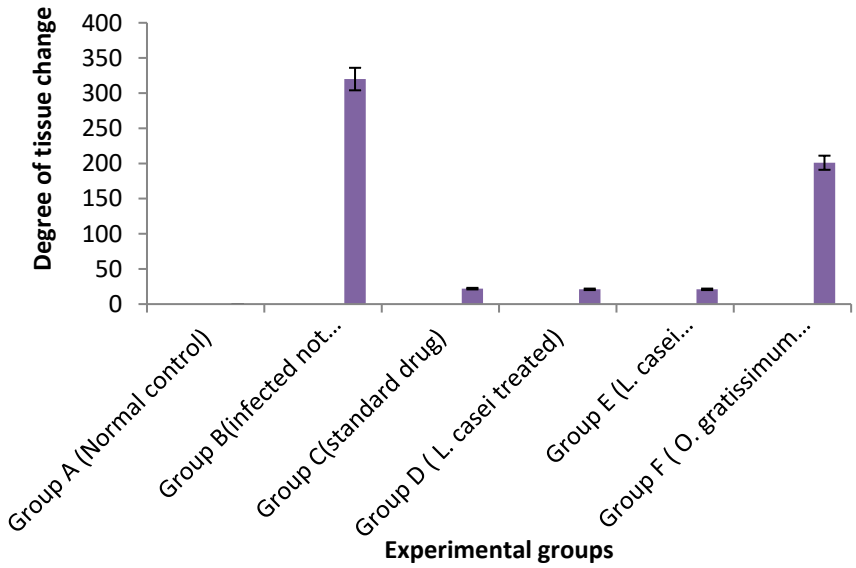

Figure 9: Degree of Tissue Change of Chicken Intestine

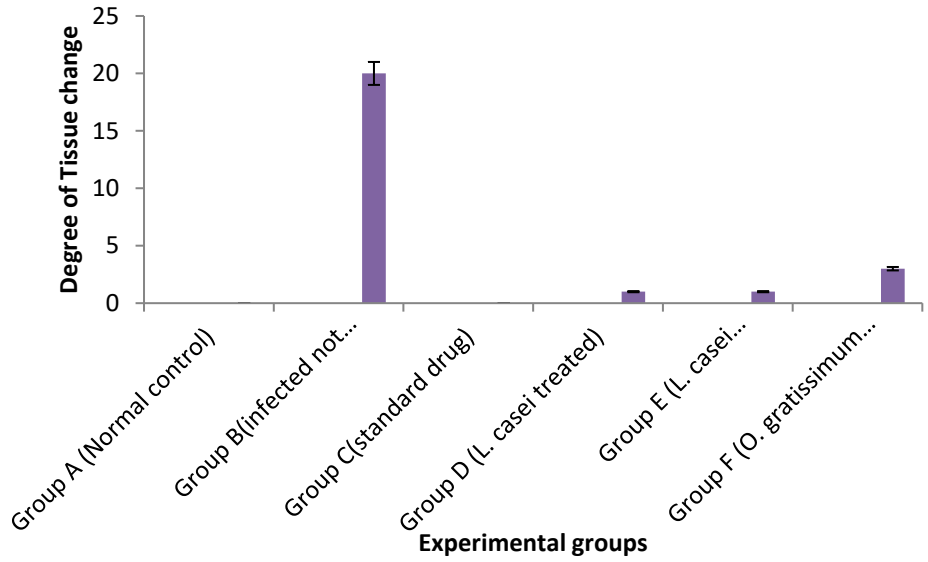

Figure 10: Degree of Tissue Change of Chicken Caecum

Table 29: Mortality Rate of Chicken Groups

Weeks	**Chicken Groups (n = 60)**					
	A	**B**	**C**	**D**	**E**	**F**
1	0	0	0	0	0	0
2	0	4	0	0	0	0
3	0	2	0	0	0	0
4	0	0	0	0	0	0
Total	0	6	0	0	0	0
Percentage (%)	0	60	0	0	0	0

A: Healthy control.

B: Diseased without Treatment.

C: Antibiotic control using Norfloxacin (15g/L).

D: Probiotic control using *Lactobacillus*

E: Probiotic prophylaxis using *Lactobacillus*

F: Prebiotic control using *O. gratissimum*.

CHAPTER FIVE

DISCUSSION

Chicken colibacillosis is caused by strains of *Escherichiacoli* known as Avian Pathogenic *E. coli* (APEC). *E. coli* is a member of the Enterobacteriaceae and thus its infectious route is usually the faecal-oral route, with its transmission vehicles being food, feed, water and disease vectors such as *Musca domestica* (Makut *et al*., 2014).

Table 1 which shows the questionnaire responses of persons involved in processing and vending of *Nono* as well as the end consumers on their knowledge of maintenance of strict hygiene in the production and sales of *Nono*, reveals that they are aware of the unhygienic practices in *Nono* production and vending. However, they were unaware of faecal contamination of the product and do not feel any form of abdominal disturbance after product consumption. They argued that if germs were present in the product most of them would be long dead since it is a product they have been taking since childhood. Scientifically, the possible explanations for this could be that most of the consumers of the product are tolerant to the faecal organisms present in the milk product. Likewise, it could also be that non-virulent strains of *E. coli* are what they are ingesting. But these serve as pointers to the fact that*Nono* could be a potential source of enteric food-borne disease outbreak in case of emergence of a new virulent strain of *E. coli* or any other enteric pathogen present in the product. Some respondents claimed that nothing is wrong with *Nono*, rather the milk product has been beneficial

to them health-wise such as in controlling arthritis, freshening of their skin and boosting their sperm quality.

Physical investigation of the processing, packaging and vending systems of this milk product at the *Fulani* farm in Umunya and at the several vending points in Awka and its environs, revealed unaseptic processing, packaging and vending procedures as shown on Appendix 2. It was also observed that the relationship existing between house flies (*Musca domestica*) and *Nono* is synonymous to 'the relationship between man and the air he breathes'. Thus, faecal contamination is not far-fetched and this jeopardizes the little hygiene levels maintained at the control points of *Nono* production.

The presence of *E. coli* in all presumptively examined *Nono* samples further suggests faecal contamination of the milk product. This finding corresponds with the works of some other authors such as Abdulkadir and Mugadi (2012), and Obande and Azua (2013)) - who found *E. coli* as one of the bacterial contaminants of *Nono*. Makut *et al*., (2014) reported an occurence of 80% for *E. coli* when assessing contaminants present in *Nono*. They also reported 30% occurrence of *E. coli* in *Manshanu*, which is the starter culture used in *Nono* fermentation. These findings suggest that *Nono* is a possible source of public health epidemic due to failure to meet up with NAFDAC (2009) standards for approval of food safety of dairy products, which requires a zero *E. coli* count as one of the approval criteria. The fact that the population size of the

Fulanis and their surrounding neighbours who could get *E. coli* infection by consuming the food product are quite high makes it a greater health risk. According to Kudva *et al*., (1995) HACCP methods for the control of *E. coli* contamination of foods should focus mainly on the prevention and elimination of the pathogen as a risk factor, rather than reduction of the pathogen load in the food products, in order to provide a systematic and effective food safety protocol against the pathogen.This implies that stringent hygiene is paramount in every production step of *Nono*, from the milking point to the consumption point. Biochemical characteristics of the *E. coli* and *Lactobacillus* isolates are shown on Table 2.

Table 3 which shows the probiotic screening of the *Lactobacillus*, shows it had tolerance to acidic pH, 10% NaCl and bile, as well as good adherence to xylene. The isolate had *in vitro* antibacterial activity against APEC with a 10 mm diameter zone of inhibition and equally exhibited crude bacteriocin activity against APEC with a 10.4 mm diameter zone of inhibition.

Table 4 shows the antimicrobial susceptibility of *E. coli*, with the isolate exhibiting multi-drug resistance to antibiotics such as erythromycin, tetracycline, ampicillin, amoxicillin and florphenicol. These are some of the conventional antibiotics used by man for therapeutic purposes and this result corresponds with that of Abd El Tawab *et al*., (2015). This buttresses the issue of antibiotics resistance by microorganisms, which is an existing draw-back to disease prevention and control.

Table 5 shows the antimicrobial susceptibility of *Lactobacillus* with the isolate exhibiting resistance to amoxicillin and gentamycin. According to Gogineni *et al*., (2013), antibiotic resistance in probiotic organisms hampers the efficacy of their antimicrobial activity due to the possibility of horizontal gene transfer of the drug resistant genes to other pathogens, even after evicting the main pathogen of interest. However, the *L. casei* isolate used in this work could be said to be non-multidrug resistant, which is an added advantage in portraying its probiotic roles in animal health. Thus, the isolate was adopted as one of the natural therapies in controlling colibacillosis in broiler chicks.

Ocimum gratissimum was adopted as the second natural therapy for the control of colibacillosis elicited in broilers by the multi-drug resistant APEC. Table 6 shows the phytochemical components of the plant's ethanolic extract to include flavonoid, tannin, saponin, alkaloid and phenol. These are antibacterial and immunnomodulatory components. Phenol and tannin inhibit colon cancer, which implies that they have positive activities in maintaining colon health (Akinmoladun *et al*., 2007). It will thus play a synergistic role with the prebiotics contained in the plant in protecting the caecum of broilers during oral administration of the plant extract to infected birds. There is also the presence of antioxidants such as terpenoids and ascorbic acid in the ethanolic plant extract. Antioxidants mop up free radicals generated by host adaptive immune response towards bacteriosis thereby ensuring that the rate of free radicals

produced do not exceed the antioxidant capacity of the host, thereby preventing the occurrence of oxidative stress (Akinmoladun *et al*., 2007).

Table 7 and Plate 2 show the presence and quantities of prebiotics contained in *O. gratissimum* ethanolic and water extracts. The preence of fructooligosaccharides in the plant extracts agrees with the findings of Ezeonu *et al*., (2012). There was no inulin as contained in bananas and chicory reported by Jurgonski *et al*., (2010). Fructooligosaccharides and inulin are fructopolysaccharides but they differ in their chain lengths, with inulin having a longer chain length Kolida *et al*., (2002). Fructooligosaccharides are carbohydrate components and according to Fajohunbo and Egbeyale (2010) the carbohydrate component of *O. gratissimum* is 13.40 g/100g and has a higher value in the plant when compared to the values of crude protein, ash and crude fibre. This explains the high content of fructooligosaccharides in the plant. The findings of Ezeonu *et al*., (2012) suggested that green leafy vegetables such as *Vernonia amygdalina* and *O. gratissimum* could contain different classes of prebiotics. Ezeonu *et al*., (2016) went further to demonstrate the prebiotic content of *Vernonia amygdalina* using liquid-liquid fractionation and thin layer chromatography. This research work confirms that *O. gratissimum* contains fructooligosaccharides as prebiotics. Aqueous extract of *O. gratissimum*leaves gave a higher yield of the prebiotic as against the ethanolic extract as already shown in Table 7. This shows that prebiotics are more soluble in water (Roberfroid, 2007). However, ethanolic extract (85% v/v ethanol) was used in this research as a novel approach at assessing plant

prebiotics *in vivo* since most previous researchers used aqueous extract. Ethanolic extraction gives better yield of antibacterial and antioxidant phytochemicals while aqueous method yields more prebiotics from the leaves as suggested by Ladipo *et al*., (2010) and Ezeonu *et al*., (2016). Since the antibacterial and prebiotic activities of the vegetable were needed for a successful *in vivo* study, 85% (v/v) ethanol was used in extraction to fairly harness both advantages which are essential to the research. Table 8 shows that *O. gratissimum* had a Minimum Inhibitory Concentration (MIC) of 31.25 mg/ml against *E. coli*. However, the work of Ladipo *et al*.,(2010), recorded 50mg/ml against *E. coli*.

In vivo assay focused on enteritis symptoms in 3-week old chicks (*Gallus domesticus*), according to the method of Daud *et al*., (2014) was used to monitor presentation of colibacillosis in neonate chicks. Four (E, J, O and P) out of the twenty *E. coli* isolates randomly selected from the *Nono* screening possessed the ability to elicit pathogenic signs of bloody diarrhea in neonate broiler chicks. This indicates avian pathogenicity potential. The avian pathogenicity screening of the isolates took a period of one month in order to capture variations in the individual immunities of the test chicks. *E. coli* isolate P (*E. coli* P) gave the most severe diarrheal signs and was selected for further studies.

The isolate was characterized molecularly with16s rDNA sequencing and was identified as *E. coli* O157:H7 strain sakaii as shown on Appendix 3. *E. coli* O157:H7

has been implicated in human infections (Abd El Tawab *et al*., 2015). However, this research shows it has the ability to elicit enteritis infection in neonate broilers.

The Lactobacillus isolate was also confirmed to be *Lactobacillus casei* through molecular typing using 16s rDNA sequencing as shown on Appendix 4. The activities of *L. casei* as a probiotic and *O. gratissimum* as a prebiotic were compared against that of Norfloxacin which is a conventional antibiotic used in the treatment of colibacillosis in chicken. There is no published work yet on *L. casei* probiotic role in chicken colibacillosis. However, there is a published work on probiotic *E. coli* Nissle 1917 against chicken colibacillosis by Huff *et al*., (2006). There is also no published work yet on the prebiotic role of *O. gratissimum* in chicken colibacillosis. However, there are mice model works on the probiotic isolate and on the medicinal plant (in assessing its phytochemical roles but not prebiotic roles).

All the chicken groups with the exception of Group B showed weight gain and increased specific growth rate throughout the weeks of monitoring. Group B (diseased without treatment) showed weight loss (Tables 9-11). Blood electrolyte readings showed significant differences($p<0.05$) for sodium, chlorine and bicarbonate levels but not for potassium levels ($p>0.05$) for all the groups (Tables 12-15). Blood electrolyte levels were used to ascertain the severity of the diarrhea. Group B showed great loss in chlorine and bicarbonate levels, while probiotic and prebiotic groups had good levels of blood electrolyte. This suggests that *L. casei* and *O. gratissimum* have

the ability to control electrolyte loss in the event of the diarrhea. Aizenabor and Anyaehie (2012) stated that *O. gratissimum* maintains blood electrolyte balance in an indirect manner by inhibiting intestinal motility during its anti-diarrheal activity, thereby preventing electrolyte loss.

Changes in the mean haematological parameters showed a significant difference ($p<0.05$) in the haemoglobin readings amongst the groups. Haemoglobin levels were monitored to check for anaemic conditions. The Group B had declined haemoglobin levels at the end of the fourth week due to blood loss in diarrheal course as shown in Table 16. Tables 17-22 show the total and differential white blood cell counts. There were significant differences ($P<0.05$) in their total white blood cell, neutrophil and lymphocyte counts. Among the infected chicks, untreated group had the highest total white blood cell count, which indicates activity of adaptive immune system against the pathogen. *O. gratissimum* treated group had the second highest total white blood cell count, which suggests the ability of the plant extract to boost immune system (Table 17). *L. casei* elicited more neutrophil levels in Group D chickens as shown in Table 18. This agrees with the works of Ani and Anyamene (2014) which suggested that *L. casei* elicits good neutrophil response, which plays major role in inflammations. In other words, the probiotic plays a role in the control of intestinal inflammations. Table 19 shows that Group F had the highest lymphocyte count at the fourth week and this suggests immune boosting ability of *O. gratissimum*, since lymphocytes are involved in humoral and cellular immune responses. This agrees with

the work of Nweze and Ekwe (2012). Eosinophils, basophils and monocytes showed no significant differences ($p>0.05$) amongst the test groups for all the weeks of experiment, for the probiotic, prebiotic and antibiotic activities. This agrees with the findings of Ani and Anyamene (2014) for the probiotics, and Nweze and Ekwe(2012) for the prebiotics.

Tables 23-25 show the levels of the liver enzymes- serum Acid phosphatase (ACP), serum Aspartate amino-transferase (AST) and serum Alanine Transferase (ALT) respectively. There was significant difference ($p<0.05$) in the ACP levels among the groups. The final ACP readings (week 4) show that Group B had very high ACP level when compared to other groups. Likewise, the final AST levels (week 4) also show that Group B had very high AST readings when compared to other treatment groups, except for Group A (healthy control). The AST readings were high in all groups, save for Group A, with Group B having the highest readings. Final ALT readings were normal for all groups, with no significant difference ($p>0.05$) in the treatment groups, except for Group B which showed a significant difference ($p<0.05$). These liver enzyme values suggest impairment of the liver in Group B, which is indicative of a level of systemic colibacillosis. For the other groups (excluding Group A), there was slight liver malfunctioning as clearly indicated by the AST readings. However, a level of control was established in the treatment groups by the probiotics, prebiotics and antibiotics, as seen in their final ACP and ALT values.

The kidney function of chicken groups was monitored by determination of urea and creatinine levels (Tables 26 and 27 respectively). The final urea and creatinine levels showed no significant differences ($p>0.05$) among the groups, save for Group B. This indicates that only Group B birds suffered a level of kidney impairment, which is suggestive of systemic colibacillosis for the group members. Table 28 shows the C-reactive protein readings. There was no significant difference ($p>0.05$) in the chicken groups, except for Group B. C-reactive protein indicates inflammation levels. This implies that there was gastro-intestinal wall inflammation, suggestive of systemic colibacillosis. This suggests that the probiotic, prebiotic and antibiotics could control intestinal inflammations, thus, showing that these natural alternatives could perform same function as the standard drug.

Figure 2 shows the mean microbial counts of the faecal samples obtained from the experimental chickens before commencement of the *in vivo* research. Figures 3-8 show the trend taken by the microbial population in the intestine and caecum. It was discovered that there was a decrease in all the microbial population categories- Total coliform count (TCC), Total *E. coli* count (TEC) and Total Lactic acid bacteria count (TLC) in the intestine of Group C chicken (antibiotic treated group). This explains that the in-take of Norfloxacin affected both the pathogen of interest and also the beneficial microorganisms. This shows another draw-back in antibiotics use in disease treatment aside from emergence of resistant microbial strains. Norfloxacin treatment lasted for a week for the diarrhea to halt. However, its effect on the microbial

population lingered for extra two weeks post-treatment before there could be a rejuvenation of the intestinal microbial ecosystem. *L. casei* treatment and prophylactic groups (D and E respectively) showed a significant control of the pathogen both by pathogen growth inhibition and prevention of effective pathogen adherence to the intestinal walls. The diarrhea course was halted within the fifth day of the treatment with the probiotic and this was before the antibiotic could achieve a total diarrhea halt. This situation agrees with the suggestion of Gogineni (2013) that probiotic treatment of intestinal diseases cuts the infection cycle shorter when compared to that of antibiotics treatment. This equally agrees with the works of Ani and Anyamene (2014) who demonstrated the activity of *L. casei* against *Shigella* in mice model.

O. gratissimum gave a significant decrease in the intestinal microbial ecosystem affecting TCC and TEC more than TLC population in Group F. This is because the main activity of the extract in the small intestine is antibacterial. This is brought about as a function of its phytochemicals and not necessarily the prebiotic function. Treatment with the plant extract lasted for two weeks before diarrhea could be completely stopped and a normal formed stool seen. However, Group B (diseased without treatment) member chickens shed high loads of *E. coli* and blood in their stool to the point of mortality as shown on Table 29.

Similar microbial trend in the intestine (for Groups A-E) took place at the caecum, which serves the function of colon in birds. Group F showed a different pattern in

their caecal microbial population response. This is seen in the notable increase in TLC at the third and fourth week of administering *O. gratissimum*. This suggests that there was a selective growth support for the lactic acid bacteria population in the caecum, which indicates that the accumulation of fructooligosaccharides from the plant extract over the weeks played a notable prebiotic role. This is based on the fact that prebiotics do not get digested in the alimentary canal, rather, they escape digestion and move to the colon where they selectively stimulate the growth of beneficial bacteria (Roberfroid, 2007). One can therefore suggest that *O. gratissimum* can serve as prebiotic for lactic acid bacteria.

Gross morphological examination of the intestinal tract showed swollen Burssa fabricus and ulcerative lesions in the intestine of infected chicks (Plates 4-7). The histological examination of the intestinal and caecal tissues are shown and described in Plates 8-19. Small intestine of control group (Group A) maintained its normal histological architecture. The presence of Peyer's patch (small masses of lymphatic tissues found in the ileum) formed an important part of the immune system monitoring with regards to activities of the pathogen in the treatment groups. The presence of normal muscularis externa and villi conformations indicate adequate contraction of circular muscle layers for proper chyme mixing and normal food absorption respectively. The presence of intact Paneth cell conformation is indicative of normal alpha defensin secretion, which is responsible for epithelial cells' renewal in the intestinal tissues. The severity of intestinal histopathological changes observed

in the treatment groups varied at diverse degrees. The untreated group had severe erosion of muscularis externa down to the submucosa which affected muscular layers responsible for chyme mixing, leading to improper food mixing and digestion as a result of *E. coli* intestinal wall attack. The complete degeneration of the villi and villous pertubation (atrophy) is indicative of impaired absorption of food and presence of intestinal inflammations. The presence of intestinal haemorrhage and ulceration is indicative of *E. coli*induced diarrhea. Intestinal histopathology of antibiotic treatment group indicated a progressive recovery of the intestinal wall architecture by Norfloxacin. The Paneth cell hyperplasia observed is indicative of over-secretion of alpha defensins as an innate immune response for the protection of the mucosal linings of epithelial cells. Villi regeneration and increased goblet cells seen in the *L. casei* treatment group is indicative of the attempts of the probiotic to restore the intestinal architecture formally distorted by *E. coli* infection. Observed hyperplasia of the Paneth cells, minor eroded muscularis and intact villi proved the preventive potency of *L. casei* against APEC. Paneth cell hyperplasia elicited by *L. casei* prophylaxis was to ensure more production of alpha defensins for the protection of the epithelial cell linings of the small intestine, thus preventing APEC from having a successful attachment and distortion of intestinal walls.*O. gratissimum* treatment group indicated epithelilal cells' vacoulisation which gave room for influx of lipids (mucosal lipidosis) as an immune response attempt to heal intestinal wall injury created by APEC (Hosono *et al*., 2003; Shoaf *et al*., 2006). There was also increased

number of goblet cells in the treatment group, which indicates the efficacy of *O. gratissimum* in the control of APEC.

Caecal histology of the control group indicated intact muscularis mucosa, muscularis propria, goblet cells and laminar propria. Histo-architecture of untreated group indicated tissue erosion of muscularis mucosa and submucosa, necrotic and flattened epithelial cells. Antibiotic treatment group showed no caecum architectural alterations, *L. casei* treatment group had superficial erosion of the muscularis propria which did not extend deeply into the muscularis mucosa, thus showing that the probiotic restored the caecum walls. *L. casei* prophylactic group indicated infilteration of lymphocytes around the mucosal region in a bid to protect the caecal walls from APEC attack, implying the ability of the probiotic to elicit adaptive immune responses as stated by Ani and Anyamene (2014). *O. gratissimum* treatment group indicated minor haemorrhage in the lumen, as well as distorted muscularis mucosa resulting from APEC infection. However, the presence of vacoulations is an innate immune responseelicited by fructooligosaccharides as a means to stimulate accumulation of lipids (mucosal lipidosis) which is used to repair degenerated areas of the caecum tissues (Hosono *et al*., 2003).

Figures 9 and 10 show the degree of tissue change in the intestine and caecum, according to the model of Poleksic and Mitrovic-Tutundzic (1994). Figure 9 shows that Group A had no tissue degeneration of intestinal architecture, Group B had the

highest distortion of intestinal walls which is irreparable, followed by Group F which shows intestinal wall distortion but reparable, while Groups C, D and E, had minor and reparable intestinal wall distortions. Figure 10 shows the summary of tissue change in the caecum. Groups Aand C had no caecal wall degeneration, Group B had irreparable caecal wall degeneration, while the rest of the groups had reparable minor caecal wall distortions. This implies that the prebiotic role of *O. gratissimum* could stimulate the repair of the caecal walls by selectively supporting the growth of lactic acid bacteria which in turn trigger host innate and adaptive immune responses. Likewise, *L. casei* could stimulate the repair of the caecum in Group D and equally reverse the effect of caecum damage by *E. coli's* attempt to colonize the caecal walls in Group E (*L. casei* prophylactic group).

CONCLUSION

This research work has shown that *L. casei* has good probiotic attributes that could be harnessed in the prevention and treatment of chicken colibacillosis. A good number of the organism in the gastro-intestinal tract of chickens can prevent the establishment of Avian pathogenic *E. coli* (APEC) as well as control the disease condition. *O. gratissimum* which is a common Nigerian vegetable rich in prebiotics, especially fructooligosaccharides can selectively encourage the growth of lactic acid bacteria (LAB) in the caecum and slightly in the intestine of chicken. This plant has good therapeutic use against APEC and equally acts as a boost to chicken immune system.

These suggest that the use of probiotics and prebiotics as functional food ingredients could be a potent biological means to aid the biodiversity conservation of poultry birds from threats like colibacillosis especially at their neo-natal stage (1 to 5 weeks old). Nono testing positive for the presence of fecal contaminants suggests the need for adoption of aseptic processing and vending procedures.

RECOMMENDATIONS

Further researches could be carried out on;

- Monitoring the antioxidant roles of*O. gratissimum*in the control of chicken colibacillosis.
- Monitoring the roles of *O. gratissimum* aqueous extract in the control of colibacillosis in neonate broilers.
- Identifying the prebiotics present in*O. gratissimum* using other solvents and extraction methods apart from ethanol, cold maceration and thin layer chromatography used in this work.

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APPENDICES

Appendix 1

NNAMDI AZIKIWE UNIVERSITY, P.M.B. 5025, AWKA.

FACULTY OF BIO-SCIENCES

DEPARTMENT OF APPLIED MICROBIOLOGY AND BREWING

QUESTIONNAIRE BY IKELE ONYEKA MICHAEL, 2013487009F

PLEASE TICK AGAINST YOUR PREFFERD ANSWER CHOICE, 'Y' FOR YES AND 'N' FOR NO.

NAME OF RESPONDENT:

AGE:

SEX:

LEVEL OF EDUCATION:

Do you know that germs are in Nono? Y ☐ N ☐

Do you know that the wooden stirrer is perched on by flies? Y ☐ N ☐

Do you know that the sellers do not wash their hands before sales? Y ☐ N ☐

DO you know that the scooping plate is left exposed? Y ☐ N ☐

Do you feel diarrhea days after drinking Nono? Y ☐ N ☐

Do you really like Nono? Y ☐ N ☐

Appendix 2

Unhygienic *Nono* Milking, Processing, Packaging and Vending Investigation.

Plate 20: *Nono* Milking Farm Umunya, Anambra State; Showing the Cattle Ranch/Milking Environment (star) and Milking Vessel (Black arrow).

Plate 21: Umunya Milking Farm, Showing One of the *Nono* Storage Vessels Containing the Scooper inside the Milk Product (Black arrow) and the Improperly Covered Food Handler (star).

Plate 22: Polythene Bag of Ice (Double Headed Arrow) Immersed into the Milk Product during Storage at the Farm.

Plate 23: Amansea *Nono* Vending Point showing Exposed Vending Utensils(Black Arrow).

Plate 24:*Nono*Vendor using the Exposed

Plate 25: AConsumer Taking the Milk

Table 30: Distribution of Respondents by Gender

Gender	Frequency	Percentage
Male	61	61
Female	39	39
Total	100	100

Table 31: Distribution of Respondents by Highest Educational Level Attained

Highest Educational Level	Frequency	Percentage
Primary	6	6
Secondary	26	26
Tertairy	5	5
Quranic	63	63
Total	100	100

Table 32: Respondents' Answers to Knowledge of Aseptic Processing and Vending of Nono.

Responses	Frequency		Percentage		Total
	Y	N	Y	N	
a) Nono has Germs	2	98	2	98	100
b) Flies perch on Stirrer	100	0	100	0	100
c) Vendors sell with unwashed Hands	88	12	88	12	100
d) Scooping Plate is left Exposed	100	0	100	0	100

Table 33: Respondents' Answers to Abdominal Up-set feeling after Nono Consumption.

Responses	Frequency		Percentage		Total
	Y	N	Y	N	
a) Diarrhea after Nono consumption	0	100	0	100	100

Table 34: Respondents' Answers to Choice of Nono as a Milk Drink.

Responses	Frequency		Percentage		Total
	Y	N	Y	N	
a) I really like Nono	100	0	100	0	100

APPENDICES

Appendix 1

Gene Sequencing of Avian Pathogenic *E. coli*

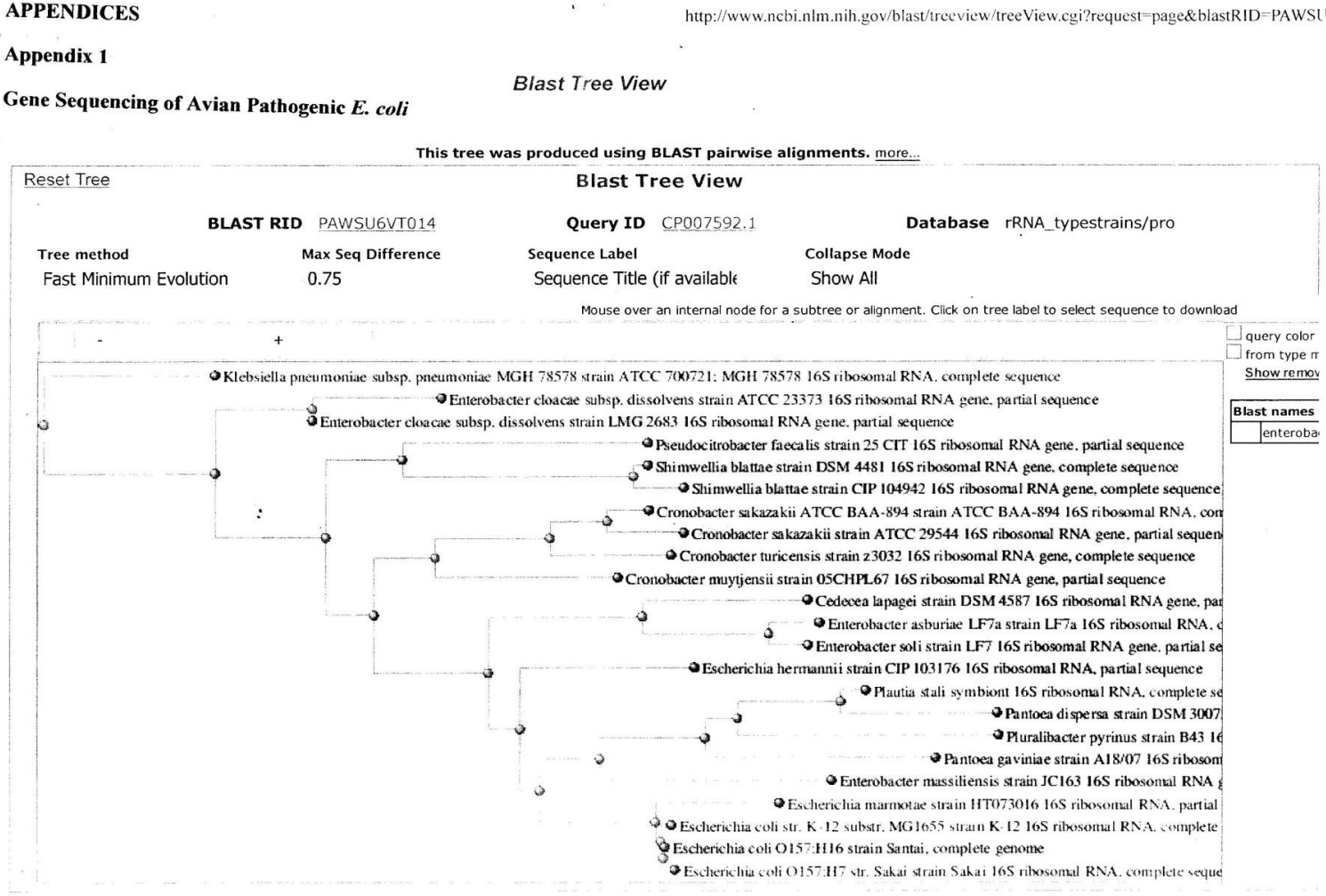

Plate 26: Phylogenetic Treeof Avian Pathogenic *E. coli.*

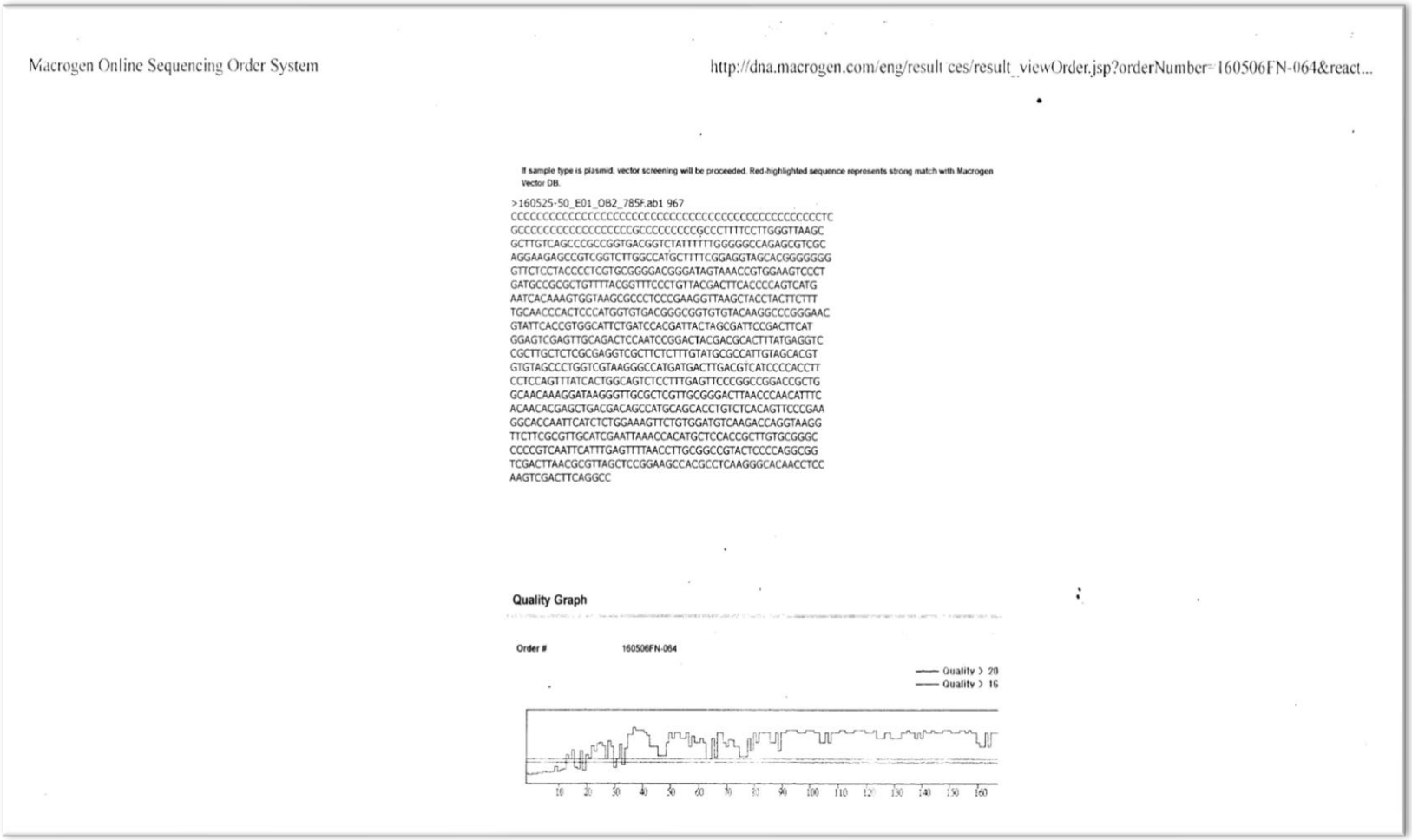

Plate 27: Gene Sequence of Avian Pathogenic *E. coli.*

Appendix 4

Genetic Identification of *Lactobacillus casei.*

CABI IDENTIFICATION REPORT

Our ref: YN3/15/H133
Your ref:
Reporting to:
Mr. Ikele, Onyeka M.
Nnamdi Azikiwe University
Awka
Anambra State
Nigeria
Date: 09 August, 2014.

CONFIDENTIAL
Enquiry YN3/15/H115 Final Identification Report
Date received: 07/07/2014 Date started: 07/07/2014 Date completed: 09/07/2014
Description of material received:

The customer submitted 2 samples for microbial identification

Result:

Customer sample	IMI Number	Identification and comments
LB1	**504030:**	**Identified as:** *Lactobacillus casei*

Process: This isolate was identified using partial 16S rDNA sequencing analysis, using the FASTA algorithm with the Prokaryote database from EBI.

Result: Top matches of up >99% were made to sequences assigned to this species, *L.delbrueckii subsp. bulgaricus, L. lactis* including the Validated Type Strain sequence (AB130738).

Comment: Members of this species are common fermented food organisms which can be found in milk, kefir grains, lactating animals as probiotics etc. These species are not known to be pathogenic to man, and ACDP have categorised them as hazard group 1 organisms

Destination: This material will be discarded.

Lactobacillus casei

ATGCCCAATTTAGAGGAGCTTTGGGCTTACCTGAATGATAAATTCCGTGAAGAGTTGACCCCAGTCGGCT
ACAGCACATGGATTCAAACAGCCAAACCCGTTAAATTGACCAAAGATAAACTCGAAATCGAAGTCCCGGC
ATCGTTGCATAAGGCTTACTGGGAGAAAAATCTGGTCACCAAAGTCGTGGAAGGGGTCTATGAATTTGCC
CAGCTGGAAGTCGATCCGGTGATCATGACCAAAGACGAGTTACAGCCGGTCACGACGCACCAGCAACCAG
CGACTGCCGATGATGATGATCAACAACTAACTTTTAAGGCGAAAACGCATCTCAATCCGAAATACACGTT
TGACCGGTTCGTGATCGGCAAAGGCAACCAAATGGCGCATGCCGCGACGTTAGCGGTTGCCGAAGCTCCC
GGCACGACGTATAATCCGCTGTTTATTTATGGTGGCGTCGGTTTGGGCAAGACGCACTTGATGCAGGCTA
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CGACTTCATTAACTCGATTCAAACCAAGCAGCAGGAGCAATTTCGTCAGGAGTATCGCAATGTTGACCTG
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GCAGGAGCGGCTGGTGTCGCGGTTTAACAAAGGCTTGTCCGTTGACGTGACGCCGCCTGATCTCGAAACC
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CACGAAAAAATTATGTCGGCAATGACGACGAATGAAGATCTTAAAGCCCAAGTCGTCGAACTGCGAAATA
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TCCACAGGTGCATAACTTTTCCGGCTGTTGTTTCTCAAAGTTTTCCACAGTTTGAACACGGCCTATTACT
ATTACTTAAAAAGCTTTATATTATATATATAAATAAACGTACGGGAGGCTCTTATGAAATTTACGATTAC
CCGATCCACATTCTTGAAAACCTTGAATGACGTTGCCCGGGCTATTTCAACCAAAACCACGATTCCGATC
CTGACTGGTTTAAAAATCGTCCTCACTGATACGGGACTGGTACTCACCGGTAGTGATGCCGATATTTCGA

Appendix 5

Statistical Tables

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
Acid phosphatase	0[a,b]	.	.	.
Healthy	3	14.5700	3.72125	2.14846
Diseased	3	17.2167	5.52777	3.19146
antibiotics	3	16.9833	1.69134	.97650
probiotics	3	15.6800	3.77747	2.18092
prophylaxis	3	16.6933	2.45732	1.41874
prebiotics	3	14.9033	1.67804	.96882
C-reactive protein	0[a,b]	.	.	.
Healthy	3	10.0000	3.46410	2.00000
Diseased	3	8.0000	3.46410	2.00000
antibiotics	3	10.0000	3.46410	2.00000
probiotics	3	6.0000	.00000[c]	.00000
prophylaxis	3	6.0000	.00000[c]	.00000
prebiotics	3	8.0000	3.46410	2.00000
Urea	0[a,b]	.	.	.
Healthy	3	4.3300	.31048	.17926
Diseased	3	6.4533	.89489	.51667
antibiotics	3	7.6200	.79542	.45924
probiotics	3	5.4733	.21197	.12238
prophylaxis	3	6.4400	.25239	.14572
prebiotics	3	7.4100	2.47443	1.42861
creatinine	0[a,b]	.	.	.
Healthy	3	.0967	.02517	.01453
Diseased	3	.1767	.08963	.05175
antibiotics	3	.2300	.18735	.10817
probiotics	3	.1200	.06245	.03606
prophylaxis	3	.1100	.03606	.02082
prebiotics	3	.6467	.97388	.56227

One-Sample Test

	Test Value = 0					
					95% Confidence Interval of the Difference	
	t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
healthy	6.782	2	.021	14.57000	5.3259	23.8141
diseased	5.395	2	.033	17.21667	3.4849	30.9484
antibiotics	17.392	2	.003	16.98333	12.7818	21.1849
probiotics	7.190	2	.019	15.68000	6.2962	25.0638
prophylaxis	11.766	2	.007	16.69333	10.5890	22.7977
prebiotics	15.383	2	.004	14.90333	10.7348	19.0718
healthy	5.000	2	.038	10.00000	1.3947	18.6053
diseased	4.000	2	.057	8.00000	-.6053	16.6053
antibiotics	5.000	2	.038	10.00000	1.3947	18.6053
prebiotics	4.000	2	.057	8.00000	-.6053	16.6053
healthy	24.155	2	.002	4.33000	3.5587	5.1013
diseased	12.490	2	.006	6.45333	4.2303	8.6764
antibiotics	16.593	2	.004	7.62000	5.6441	9.5959
probiotics	44.723	2	.000	5.47333	4.9468	5.9999
prophylaxis	44.195	2	.001	6.44000	5.8130	7.0670
prebiotics	5.187	2	.035	7.41000	1.2632	13.5568
healthy	6.653	2	.022	.09667	.0342	.1592
diseased	3.414	2	.076	.17667	-.0460	.3993
antibiotics	2.126	2	.167	.23000	-.2354	.6954
probiotics	3.328	2	.080	.12000	-.0351	.2751
prophylaxis	5.284	2	.034	.11000	.0204	.1996
prebiotics	1.150	2	.369	.64667	-1.7726	3.0659

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
healthy	3	17.8333	5.12254	2.95750
diseased	3	24.6533	12.71112	7.33877
antibiotics	3	15.8167	2.47351	1.42808
probiotics	3	20.1233	4.75456	2.74505
prophylaxis	3	19.7300	14.75411	8.51829
prebiotics	3	19.7733	6.33825	3.65939
healthy	3	8.0000	3.46410	2.00000
diseased	3	12.0000	6.00000	3.46410
antibiotics	3	8.0000	3.46410	2.00000
probiotics	3	6.0000	.00000[a]	.00000
prophylaxis	3	8.0000	3.46410	2.00000
prebiotics	3	10.0000	6.92820	4.00000
healthy	3	5.9267	1.44091	.83191
diseased	3	8.1933	2.26014	1.30489
antibiotics	3	6.2200	2.80854	1.62151
prebiotics	3	7.3333	.59011	.34070
prophylaxis	3	7.8600	1.38481	.79952
prebiotics	3	7.3100	1.86540	1.07699
creatinine	0[b,c]	.	.	.
healthy	3	.1700	.08185	.04726
diseased	3	.3267	.29569	.17072
antibiotics	3	.4200	.16643	.09609
probiotics	3	.1733	.03215	.01856
prohpylaxis	3	.1333	.08145	.04702
prebiotics	3	.1667	.02082	.01202

One-Sample Test

	Test Value = 0					
					95% Confidence Interval of the Difference	
	t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
healthy	6.030	2	.026	17.83333	5.1082	30.5584
diseased	3.359	2	.078	24.65333	-6.9228	56.2295
antibiotics	11.075	2	.008	15.81667	9.6721	21.9612
probiotics	7.331	2	.018	20.12333	8.3124	31.9343
prophylaxis	2.316	2	.147	19.73000	-16.9212	56.3812
prebiotics	5.403	2	.033	19.77333	4.0282	35.5184
healthy	4.000	2	.057	8.00000	-.6053	16.6053
diseased	3.464	2	.074	12.00000	-2.9048	26.9048
antibiotics	4.000	2	.057	8.00000	-.6053	16.6053
prophylaxis	4.000	2	.057	8.00000	-.6053	16.6053
prebiotics	2.500	2	.130	10.00000	-7.2106	27.2106
healthy	7.124	2	.019	5.92667	2.3472	9.5061
diseased	6.279	2	.024	8.19333	2.5788	13.8078
antibiotics	3.836	2	.062	6.22000	-.7568	13.1968
prebiotics	21.524	2	.002	7.33333	5.8674	8.7993
prophylaxis	9.831	2	.010	7.86000	4.4199	11.3001
prebiotics	6.787	2	.021	7.31000	2.6761	11.9439
healthy	3.597	2	.069	.17000	-.0333	.3733
diseased	1.913	2	.196	.32667	-.4079	1.0612
antibiotics	4.371	2	.049	.42000	.0066	.8334
probiotics	9.339	2	.011	.17333	.0935	.2532
prohpylaxis	2.836	2	.105	.13333	-.0690	.3357
prebiotics	13.868	2	.005	.16667	.1150	.2184

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
Acid phosphataseB	0[a,b]	.	.	.
healthy	3	5.3633	4.40873	2.54538
diseased	3	27.7800	6.73460	3.88823
antibiotics	3	16.4167	11.53434	6.65936
probiotics	3	18.8467	5.69021	3.28525
prophylaxis	3	20.1767	6.59723	3.80891
prebiotics	3	27.1467	6.91123	3.99020
c-reactive proteinB	0[a,b]	.	.	.
healthy	3	8.0000	3.46410	2.00000
diseased	3	16.0000	9.16515	5.29150
antibiotics	3	12.0000	6.00000	3.46410
probiotics	3	10.0000	6.92820	4.00000
prophylaxis	3	10.0000	6.92820	4.00000
prebiotics	3	12.0000	10.39230	6.00000
ureaB	0[a,b]	.	.	.
healthy	3	5.2800	1.64557	.95007
diseased	3	12.5500	2.81206	1.62355
antibiotics	3	6.6267	1.45583	.84052
probiotics	3	8.1500	1.07893	.62292
prophylaxis	3	9.0033	2.73184	1.57723
prebiotics	3	8.5533	2.33011	1.34529
creatinineB	0[a,b]	.	.	.
healthy	3	.1267	.03786	.02186
diseased	3	1.0967	.24090	.13908
antibiotics	3	.3167	.04726	.02728
probiotics	3	.3800	.06557	.03786
prophylaxis	3	.3733	.14503	.08373
prebiotics	2	.3750	.04950	.03500

One-Sample Test

	Test Value = 0					
					95% Confidence Interval of the Difference	
	t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
healthy	2.107	2	.170	5.36333	-5.5886	16.3152
diseased	7.145	2	.019	27.78000	11.0503	44.5097
antibiotics	2.465	2	.133	16.41667	-12.2362	45.0696
probiotics	5.737	2	.029	18.84667	4.7114	32.9819
prophylaxis	5.297	2	.034	20.17667	3.7882	36.5651
prebiotics	6.803	2	.021	27.14667	9.9782	44.3151
healthy	4.000	2	.057	8.00000	-.6053	16.6053
diseased	3.024	2	.094	16.00000	-6.7675	38.7675
antibiotics	3.464	2	.074	12.00000	-2.9048	26.9048
probiotics	2.500	2	.130	10.00000	-7.2106	27.2106
prophylaxis	2.500	2	.130	10.00000	-7.2106	27.2106
prebiotics	2.000	2	.184	12.00000	-13.8159	37.8159
healthy	5.557	2	.031	5.28000	1.1922	9.3678
diseased	7.730	2	.016	12.55000	5.5644	19.5356
antibiotics	7.884	2	.016	6.62667	3.0102	10.2431
probiotics	13.083	2	.006	8.15000	5.4698	10.8302
prophylaxis	5.708	2	.029	9.00333	2.2171	15.7896
prebiotics	6.358	2	.024	8.55333	2.7650	14.3417
healthy	5.795	2	.029	.12667	.0326	.2207
diseased	7.885	2	.016	1.09667	.4982	1.6951
antibiotics	11.606	2	.007	.31667	.1993	.4341
probiotics	10.037	2	.010	.38000	.2171	.5429
prophylaxis	4.459	2	.047	.37333	.0131	.7336
prebiotics	10.714	1	.059	.37500	-.0697	.8197