1.0 INTRODUCTION

Helicobacter pylori (*H. pylori*) which was previously called *Campylobacter pylori*, is a helix shaped, Gram-negative bacterium found in the stomach (Kuster *et al*., 2006). The organism measures about 3 micrometers long with a diameter of about 0.5 micrometer (Blaser and Atherton, 2004). It is microaerophilic and has an enzyme hydrogenase which it uses to obtain energy by oxidizing molecular hydrogen (H_2) produced by other intestinal bacteria (Jonathan and Robert, 2002). *H. pylori* is oxidase, catalase and urease positive (Brooks *et al*., 2007). It was identified in 1982 by Australian scientists Barry Marshall and Robin Warren, in patients with chronic gastritis and gastric ulcers (Konturek, 2003). About 50% of the world's population are said to harbour *H. pylori* in their upper gastrointestinal tract (Malaty, 2007). More than 80% of individuals infected with the bacterium are asymptomatic (Bytzer *et al*., 2011) and infection is more prevalent in developing countries than in Western countries (Yamaoka, 2008). *H. pylori* infection leads to antral gastritis, duodenal (peptic) ulcer disease, gastric ulcers, and gastric carcinoma (Malfertheiner *et al*., 2012). The infection is diagnosed by checking for symptoms especially those relating to stomach discomfort and by doing laboratory tests which could be invasive or non invasive (Myllyluoma, 2007). Invasive test is the most reliable method for diagnosing *H. pylori* infection because of its high specificity and sensitivity, but it requires the use of endoscopy to collect gastric biopsy for biopsy urease test, histology, fluorescent *in situ* hybridization, culture and polymerase chain reaction (Lee *et al*., 2013). The non-invasive tests include $_{13}$C-urea breath test, serology, stool antigen test and stool culture (Tanih *et al*., 2008). The stool antigen test has been found to give a more accurate result than other non-invasive tests in diagnosing *H. pylori* infection (Smith *et al*., 2008). Serological tests are not useful in area where *H. pylori* infection is endemic because it cannot differentiate between previous and recent infection (Jemilohun *et al*., 2010). However, in research settings, two or more non invasive tests could be combined to increase their sensitivities (Smith *et al*., 2008). Comparison of the culture tests has shown that using biopsy specimen is better than stool in the isolation of *H. pylori* because of the toxic effect of the bile salt in stool on the bacterium (Al-Sulami *et al*., 2012). Another test that is frequently included as a screening test for *H. pylori* in dyspeptic patients is the fecal occult blood test which detects gastrointestinal bleeding (Yi-Chia *et al*., 2013).

H. pylori infection is treated with antibiotics that kill the organism, H_2 blockers and proton pump inhibitor (PPI)) that reduce stomach acid and bismuth compounds which protect the stomach lining (Manyi-Loh *et al*., 2010). However, there is often treatment failure due to the high cost of drugs and its non-availability, side effects, non compliance by patients,

inactivation of antibiotic by pH, lack of correlation between in vitro susceptibility test and in vivo efficacy, resistance to the antimicrobial agents used, duration of the treatment and the dosage of the antibiotic (Ndip *et al*., 2008; Ierardi *et al*., 2013). For this, an alternative method was searched for, which would be from natural sources, cheap, readily available, effective, having no side effects and non-antibiotic (Bytzer and O'Morian, 2008). These alternative methods included Probiotics and Prebiotics such as Honey, *Allium sativum L* (garlic), Capsaicin (hot pepper) and *Vaccinium macrocarpon* (Cranberry fruit) (Manyi Loh *et al*., 2010).

Studies have proved that probiotics treat and prevent *H. pylori* infection when used as complementary or alternative medicine (Hamilton-Miller, 2003). Probiotics are live microorganisms, which when administered in adequate amounts confer a health benefit on the host (FAO/WHO 2002). Probiotics organisms include *Lactobacillus* and *Bifidobacteria* which are resident bacterial flora, *Bacillus laterosporus*, *Bacillus subtilis* and *Streptococcus thermophilus* which are transient flora and the yeast *Saccharomyces boulardii* (Sanders, 2007; Adagbada *et al*., 2011). Probiotics are available in foods like yogurt, fermented and unfermented milk, miso, tempeh, some juices, soy beverages, Ogi, Kunun zaki, burukutu, ogiri, ugba and in dietary supplements such as capsules, tablets, and powders (Shah, 2000; Tersoo-Abiem et al., 2010). Probiotics may be contained originally in foods and supplement or be added to them during preparation (NIH, 2007). In this study, *Lactobacillus acidophilus* probiotic was evaluated for its ability to reduce gastric inflammation caused by *H. pylori* infection and prevent bacterial colonization in infected mice.

Aims of the Study

The aims of this study are to compare the sensitivities and specificities of different diagnostic tests used in the detection of *H. pylori* infection and to investigate the effects of using probiotics in the treatment of mice infected with *H. pylori,* as well as the effects of the infection on the stomach and intestine of untreated mice.

Statement of the Problem

The eradication of *H. pylori* has been tried with the combination of antimicrobials, but growing resistance of the bacterium to conventional antimicrobial agents is a source of concern to clinical microbiologists worldwide (Gatta *et al*., 2013). The standard triple treatment and the recent sequential therapy have several adverse effects on humans (Laving, 2013). This has led to the development of alternative anti-*H. pylori* treatments such as probiotics (Hamilton-Miller, 2003; Ierardi *et al*., 2013).

Several *in vitro* studies have shown that the probiotic lactobacilli or their cell-free cultures inhibit or kill *H. pylori* and prevent its adhesion to mammalian epithelial cells, but adequate investigation on probiotics and the potential benefits of their uses have not been done in the developing world (Meeke, 2013). This calls for a more scientific knowledge about probiotics, including their safety and appropriate use (Adagbada *et al*., 2011). Considering the need for up to date research on the health benefit of probiotics in Nigeria, it is anticipated that this present study will contribute to enriching not only the Nigerian community, but the global probiotic activities through the discovery of the process of healing and the duration of treatment of *H. pylori* infection with a probiotic.

2.0 LITERATURE REVIEW

2.1 Gastrointestinal Defences against Pathogens

The gastrointestinal defence is made up of the host gut microbiota, intestinal epithelium and the immune system (Bourlioux *et al.,* 2003). The host microbiota provides the first level of defence seconded by the intestinal epithelium and finally, the immune system. (McCracken and Lorenz, 2001; Myllyluoma, 2007).

2.1.1 Gut Microbiota

Gut microbiota also known as gut flora is made up of diverse microbial species which live in the intestinal tracts of animals and humans (Zoetendal et al., 2006). It has the largest reservoir of host-specific commensal that is stable over time in healthy adults (Mueller *et al*., 2012).

Gut bacteria is beneficial to man because it aids in the digestion of unutilized energy substrates, stimulates cell growth, represses the growth of harmful microorganisms, train the immune system to respond only to pathogens and give defences against some diseases (Guinane and Cotter, 2013). However, in certain conditions, some species become infectious and can predispose the host to cancer (Guarner and Malagelada, 2003).

Humans host about one hundred trillion microorganisms in their guts (Mueller *et al*., 2012). Bacteria make up most of the flora in the colon and about 60% of the dry mass of feaces (Bik *et al*, 2006). The most common bacteria phyla were *Proteobacteria, firmicutes, Bacteroids, Acinetobacters* and *Fusobacteria*, while the most abundant genera were *Helicobacter, Streptococcus* and *Prevotella* (Blaser, 2006). Fungi, protozoa and archaea also make up a part of the gut flora (Guarner and Malagelada, 2003). Studies have found that mucosal microbiota was 50-90% different from fecal microbiota and is stable along the distal gastrointestinal tract from ileum to rectum (Lepage *et al*., 2005).

When the numbers of gut bacteria is altered by taking broad spectrum antibiotics, the host suffers ill health like antibiotic-associated diarrhea (Carman *et al*., 2004). Also antibiotics increase the number of antibiotic-resistant bacteria which invade the host tissue to cause diseases that are difficult to treat ((Beaugerie and Petite, 2004). Other factors that can change the composition of the gut flora are illnesses such as ischemia of the gut, failure to eat, and immune compromise (Knight and Girling, 2003).

A change in the numbers and species of the gut flora results in the reduction of carbohydrates fermentation and bile salt metabolism (Gibson, 2004). Also, the reduction in levels of native bacterial species disrupts their ability to inhibit the growth of harmful species

such as *Clostridium difficile* and *Salmonella kedougou* which lead to diarrhea (Carman *et al*., 2004).

When this change becomes harmful, treatment known as selective digestive tract decontamination (SDD) which kill only the pathogenic bacteria and allow the re-establishment of healthy ones is given (Knight and Girling, 2003). It involves the therapeutic exploitation of the commensal flora using pharmabiotics such as live probiotic bacteria, probiotic derived biologically active metabolites, prebiotics, synbiotics or genetically modified commensal bacteria (O'hara and Shanahan, 2006). Since the lack of gut flora can have such harmful health effects, then probiotics which has anti-inflammatory effects in the gut should be used to improve health (Guinane and Cotter, 2013).

2.1.2 Intestinal Epithelium

The tight epithelial cell barrier forms the second line of defence between the gut luminal contents and the host (McCracken and Lorenz, 2001). The epithelial cells lining the gastrointestinal tract respond to infection by initiating either a non-specific or specific host-defence response (Myllyluoma, 2007).

Adhesion of bacterial to the host cell is important as a first line of action in the disease process (Servin and Coconnier, 2003). Bacteria adheres to the glycoconjugate receptors on the intestinal cell surface (Pretzer *et al*., 2005). This is recognized by the toll-like receptors (TLR) on the epithelial cells (Algood, 2006). Other known recognition receptors are nucleotide-binding oligomerization domain proteins, which recognize both gram-positive and gram-negative bacteria (Amieva and El-omar, 2008. They are located in cell cytoplasm and are implicated in the induction of defensins (Lu and Walker, 2001).

Intestinal epithelial cells also secrete many other mediators including antimicrobial peptides, such as defensins and mucins that are involved in immune responses to potentially pathogenic microorganisms (Servin 2004). The immune-inflammatory reaction is highly important in eliminating pathogens, but this reaction must be controlled to avoid the risk of a more widespread inflammation (Bik et al., 2006). Microbes differ in terms of their ability to induce inflammatory response (Blaser, 2006). The commensal microbiota produces a very mild inflammation response and is therefore tolerated by the mucosa, while modified microbiota induces a more marked response (O'Hara and Shanahan, 2006).

The tight cell-cell junction between epithelial cells permits the passage of small molecules such as ions, and restricts the movement of large molecules like antigens and microorganism (Bik *et al*., 2006). Increased epithelial barrier permeability is frequently

associated with gastrointestinal disorders contributing to both disease onset and persistence (Berkes *et al*., 2003).

2.1.3 The Intestinal Immune System

The third level of defence is the immune system which protects humans and animals against invading pathogens (Guarner and Malagelada, 2003). The gut microbiota is the most important stimulant of the body's immunological defence (Bourlioux et al., 2003). The immune system can be classified into two types of response: innate (nonspecific) and adaptive (specific) immune response (Steinhoff, 2005).

Innate (nonspecific) or "natural" immunity is a rapidly activated host defence that recognizes conserved microbial structures which are not expressed by the host and mounts a nonspecific immune response against these structures (often specific carbohydrates or lipoproteins) (Muellar *et al*., 2012). The activated effectors of innate immunity, such as phagocytic cells, natural killer cells, and the complement system, are able to destroy the invader (Janeway and Medzhitov, 2002). Innate immunity also includes acid in the stomach, lysozyme, lactoferrin and antimicrobial molecules (Shanahan, 2002).

Adaptive (Specific) immune response is usually induced by direct contact between the lymphoid tissue and the potentially pathogenic macromolecules or microorganisms in the intestinal lumen (Janeway and Medzhitov, 2002). It is activated by the infection if the innate immune system is insufficient (Guarner and Malagelada, 2003). Adaptive immunity develops a memory, which enables a rapid and effective response in a re-infection (Bourlioux *et al*., 2003). The mechanism by which this type of immunity operates is based on the recognition as non-self any antigenic structures (often peptides), not expressed in the host (Muellar *et al*., 2012). Antigens are presented to the effector cells of the adaptive immunity by antigen presenting cells (Steinhoff, 2005). Furthermore, intestinal dendritic cells can directly sample the contents of the gut lumen by extending dendrites between epithelial cells (Guinane and Cotter, 2013). The adaptive immune system is a more specific and powerful tool against pathogens, but the primary response mounts slower than in innate immunity (Steinhoff, 2005).

Although, innate and adaptive immunity represent two separate arms of immunity, a close relationship exists between them (Bourlioux *et al.* 2003). The initiation and direction of adaptive immunity is influenced by innate immunity, which regulates its direction via cytokines, T and B cell co-stimulatory mechanisms and antigen presentation (Steinhoff, 2005). Furthermore, pattern recognition receptors, such as toll like receptors (TLRs), in epithelial cells, dendritic cells and macrophages are important in bridging the innate and

adaptive immune responses (Guinane and Cotter, 2013). Continuous formation of immunoglobulin (Ig) A in plasma cells in the lamina propria also plays an important role in the protective function of the mucosa (Steinhoff, 2005). This IgA is transported to the luminal side of the mucosa and released into the bowel as secretory IgA, where it is able to neutralize potentially pathogenic bacteria and viruses (Guarner and malagelada, 2003).

2.2 The Gut Flora *Helicobacter*

The bacterium was initially named *Campylobacter pyloridis*, then *Campylobacter pylori* (after a correction to the latin grammar) (Kuster, 2006). In 1989, DNA sequencing and other data showed that the bacterium did not belong in the *Campylobacter* genus, so it was placed in its own genus, *Helicobacter* (Karlik *et al*., 2009).

The genus *Helicobacter* belongs to a subdivision of Proteobacteria, class Epsilonproteobacteria, order Campylobacterales, family Helicobacteraceae, and consists of over 20 recognized species including *Helicobacter pylori* (Karlik, *et al*., 2009; Correa and Piazuelo, 2012).

Helicobacter pylori (*H. pylori*), produces the enzymes oxidase, catalase, and urease (Brooks *et al*., 2007). It forms biofilms and can convert from spiral to a viable coccoid form which cannot be isolated in culture (Stenström, *et al*., 2008; Dube, *et al*., 2009). These characteristics aid in the survival and epidemiology of the bacterium (Al-sulami *et al*., 2012).

H. pylori possess five major outer membrane protein families which are the adhesins, porins, iron transporters, flagellum-associated proteins, and proteins of unknown function (Mahdavi et al., 2002; Kuster *et al*., 2006). Similar to other typical Gram-negative bacteria, the outer membrane of *H. pylori* consists of phospholipids and lipopolysaccharide (LPS) (Yamako, 2008). The O antigen of LPS may be fucosylated and mimic Lewis blood group antigens found on the gastric epithelium (Kuster *et al*., 2006). The outer membrane also contains cholesterol glucosides, which are found in few other bacteria (Yamaoka and Alm, 2008).

All gastric and enterohepatic *Helicobacter* species are highly motile owing to flagella (Josenhans *et al*., 2000). The characteristic sheathed flagellar filaments of *Helicobacter* are composed of two copolymerized flagellins, FlaA and FlaB (Rust *et al*., 2008). Flagella motility is useful in the colonization of the gastric mucus (Viala *et al*., 2004). *H. pylori* has four to six lophotrichous flagella (Brooks *et al*., 2007).

H. pylori can be demonstrated in tissue by Gram stain, Giemsa stain, haematoxylin-eosin stain, Warthin-Starry silver stain, acridine-orange stain, and phase-contrast microscopy (Viara *et al*., 2002 ; Lee *et al*., 2013).

2.2.1 Genome of *Helicobacter pylori*

Study of the *H. pylori* genome is centered on the attempts to understand the ability of this organism to cause disease (Blaser and Atherton., 2004). About 29% of the loci are in the "pathogenesis" category of the genome database (Baldwin *et al*., 2007). Two of sequenced strains have about 40-kb-long Cag pathogenicity island (a common gene sequence believed to be responsible for pathogenesis) that contains over 40 genes (Oh *et al*., 2006). This pathogenicity island is usually absent from *H. pylori* strains isolated from humans who are carriers of *H. pylori* but remain asymptomatic (Broutet *et al*., 2001).

The cagA gene codes for one of the major *H. pylori* virulence proteins (Hatakeyama, 2004). Bacterial strains with the cagA gene are associated with an ability to cause ulcers (Dixon, 2000). The cagA gene codes for a relatively long protein (Oh *et al*., 2006). The *cag* pathogenicity island (PAI) has about 30 genes, part of which code for a complex type IV secretion system (Hatakeyama, 2004). The low GC-content of the *cag* PAI relative to the rest of the *Helicobacter* genome suggests the island was acquired by horizontal transfer from another bacterial species (Correa and Piazuelo, 2012).

2.2.2 History of *Helicobacter pylori* Infection

Dr. Barry Marshal and Dr. Robin Warren of Perth Western Australia were the first to discover *H. pylori* in the stomach of patients with gastritis and stomach ulcers in 1982, when it was thought that it will be difficult for bacteria to survive in the human acidic stomach (Konturek, 2003). In recognition of their discovery, they were awarded the 2005 Nobel Prize in Medicine (Malfertheiner *et al*., 2012).

Before the research of Marshall and Warren, German scientists had already found spiral-shaped bacteria in the lining of the human stomach in 1875, but they were not able to isolate it (Blaser, 2005). Later, an Italian researcher Giulio Bizzozero found a similary shaped bacteria in the stomach of dogs in 1893 (Egan and O'morain, 2007). In 1899, Professor Walery Jaworski of the Jagiellonian University Kraków found a spiral shaped bacteria which he called *Vibro rugula* among some rod-like bacteria in the sediment of human gastric washings. He was the first to suggest its role in the pathogenesis of gastric diseases (Konturek, 2003).

The presence of curved rods in the stomach of many patients with peptic ulcers and stomach cancer was demonstrated in studies carried out in the early 20th century (Starzyñska and Malfertheiner, 2006). Unfortunately, interest in the bacteria waned in 1954 when an American study published that the bacteria could not be identified in 1180 stomach biopsies (Blaser, 2005). However, in 1970 the bacteria were visualized in the stomach of gastric ulcer

patients. This finding rekindled the interest in understanding the role of bacteria in the stomach of gastric ulcer patients (Starzyñska and Malfertheiner, 2006).

In 1979, Australian pathologist Robin Warren observed the bacterium. He did a further research on it with Australian physician Barry Marshall beginning in 1981. After numerous unsuccessful attempts at culturing the bacteria from the stomach, they finally succeeded in visualizing colonies in 1982, when they unintentionally left their petri dishes incubating for five days over the Easter weekend. In their original paper, Warren and Marshall contended that most stomach ulcers and gastritis were caused by infection from this bacterium and not by stress or spicy food, as had been assumed before (Malfertheiner *et al*., 2012).

Although some doubt was expressed initially, numerous research groups over several years verified the association of *H. pylori* with gastritis and ulcers (Shiotani and Graham, 2002). To demonstrate that *H. pylori* caused gastritis, Marshall drank a beaker of *H. pylori* culture. After some days, he became ill with nausea and vomiting. An endoscopy was carried out on the 10th day of inoculation and it revealed signs of gastritis and the presence of *H. pylori*. From these results it was suggested that *H. pylori* caused the gastritis. Marshall and Warren went on to demonstrate that antibiotics were effective in the treatment of many cases of gastritis (Blaser, 2005).

Thomas Borody, a Sydney gastroenterologist invented the first triple therapy for the treatment of duodenal ulcers in 1987 (Schubert and Peuru, 2008). In 1994, the National Institutes of Health (USA) published an opinion stating that most recurrent duodenal and gastric ulcers were caused by *H. pylori*, and recommended the inclusion of antibiotics in the treatment regimen (NIH, 2004).

2.2.3 Pathogenesis and pathology of *Helicobacter pylori*

H. pylori avoids the acidic stomach lumen by chemotaxis (Kuster *et al*., 2006). With the aid of its flagella, the organism burrows into the mucus lining of the stomach to the underneath of the epithelial cells where there is a neutral pH (Amieva and El-Omar, 2008).

In people producing large amounts of acid, *H. pylori* avoids the acid-secreting parietal cells at the fundus (near the entrance to the stomach) by colonizing near the pyloric antrum (exit to the duodenum) (Kuster *et al*., 2006). The inflammatory response caused by the colonization induces G cells in the antrum to secrete the hormone gastrin, which travels through the bloodstream to parietal cells in the fundus (Blaser and Atherton, 2004). Gastrin stimulates the parietal cells to increase in number and to secrete more acid into the stomach

lumen (Schubert and Peura, 2008). The increased acid load damages the duodenum, which may eventually result in ulcers forming in the duodenum (Schubert and Peura, 2008).

The rest of the stomach is colonized in those who produce normal or reduced amounts of acid (Meeke, 2013). The inflammatory response can result in atrophy of the stomach lining and eventually ulcers in the stomach. This also may increase the risk of stomach cancer (Suerbaum and Michetti, 2002).

Two related mechanisms by which *H. pylori* could promote cancer are the enhanced production of free radicals and an increased rate of host cell mutation. The other proposed mechanism known as "perigenetic pathway" involves enhancement of the transformed host cell phenotype by means of alterations in cell proteins, such as adhesion proteins (Tsuji *et al*., 2003). In the perigenetic mechanism, inflammation-associated signaling molecules, such as tumor necrosis factor alpha (TNF-α), can alter gastric epithelial cell adhesion and lead to the dispersion and migration of mutated epithelial cells without the need for additional mutations in tumor suppressor genes, such as genes that code for cell adhesion proteins (Suganuma *et al*., 2008).

In addition to using chemotaxis to avoid areas of low pH, *H. pylori* also neutralizes the acid in its environment by producing large amounts of urease, which breaks down the urea present in the stomach to carbon dioxide and ammonia. The ammonia, which is basic, then neutralizes stomach acid (Konturek, 2003).

H. pylori harm the stomach and duodenal linings by several mechanisms. The ammonia produced to regulate pH is toxic to epithelial cells while some of the biochemicals produced by *H. pylori* such as proteases, catalase, phospholipases, vacuolating cytotoxin A (VacA) damages epithelial cells, disrupts tight junctions and causes apoptosis (Algood and Cover, 2006).

The mucus environment of the stomach also keeps the bacteria from being swept away into the lumen (Screiber *et al*., 2004). Therefore, *H. pylori* is found in the mucus, on the inner surface of the epithelium, and occasionally inside the epithelial cells (Karlik *et al*., 2009).

The organism adheres to the epithelial cells by producing adhesins which bind to the membrane lipids and carbohydrates (Peterson and Krogfelt, 2003). BabA adhesins binds to the Lewis b antigen displayed on the surface of stomach epithelial cells while SabA binds to increased levels of sialyl-Lewis x antigen expressed on gastric mucosa (Mahdavi *et al*., 2002). Following attachment of *H. pylori* to stomach epithelial cells, the type IV secretion system expressed by the *cag* pathogenicity island (*cag* PAI) injects the inflammation - inducing agent, peptidoglycan, from their own cell wall into the epithelial cells (Shiotani and

Graham, 2002). The injected peptidoglycan is recognized by the cytoplasmic pattern recognition receptor (immune sensor) Nod1, which then stimulates expression of cytokines that promote inflammation (Viala *et al*., 2004).

The type-IV secretion apparatus also injects the *cag* PAI-encoded protein CagA into the stomach's epithelial cells, where it disrupts the cytoskeleton, adherence to adjacent cells, intracellular signaling, cell polarity, and other cellular activities (Backert and Selbach, 2008). Once inside the host cell, membrane-associated tyrosine kinase (TK) phosphorylates the CagA protein on tyrosine residues, which then allosterically activates protein tyrosine phosphatase and protooncogene (Hatakeyama, 2004).

About 50–70% of *H. pylori* strains in Western countries carry the *cag* pathogenicity island (*cag* PAI) (Peek and Crabtree, 2006). Western patients infected with strains carrying the *cag* PAI have a stronger inflammatory response in the stomach and are at a greater risk of developing peptic ulcers or stomach cancer than those infected with strains lacking the island (Kuster *et al*., 2006).

Pathogenic strains of *H. pylori* have been shown to activate the epidermal growth factor receptor (EGFR), a membrane protein with a tyrosine kinase domain. Activation of the epidermal growth factor receptor by *H. pylori* is associated with altered signal transduction and gene expression in host epithelial cells that may contribute to pathogenesis (Broutet *et al*., 2001). It has also been suggested that a C-terminal region of the CagA protein (amino acids 873–1002) are able to regulate host cell gene transcription, independent of protein tyrosine phosphorylation (Baldwin *et al*., 2007).

Helicobacter cysteine-rich proteins (Hcp), particularly HcpA (hp0211), are known to trigger an immune response which causes inflammation (Dumrese *et al*., 2009). Ulcers in the stomach and duodenum result when the consequences of inflammation allow stomach acid and the digestive enzyme pepsin to overwhelm the mechanisms that protects the stomach and duodenal mucous membranes (Shiotani and Graham, 2002).

The majority of *H. pylori* in colonized hosts are free-living, but approximately 20% bind to gastric epithelial cells (Viala *et al*., 2004). *H. pylori* is genetically heterogeneous and lacks clonality, resulting in every *H. pylori* subject carrying distinct strain (Logan and Walker, 2001). The outcome of infection depends on the strain with which one is infected (Módena *et al*., 2007).

A major public health concern in developing countries is the gastric inflammation in children infected with *H. pylori*, which could cause low gastric secretion resulting in impaired “gastric barrier” that is associated with increased susceptibility to enteric infections, diarrhea, malnutrition and growth failure (Thomas *et al*., 2004). In the elderly, infection can

disappear as the stomach becomes increasingly atrophic and inhospitable to colonization (Goodman *et al*., 2005).

2.2.4 Survival of *Helicobacter pylori*

The pathogenesis of *H. pylori* depends on its ability to survive in the harsh gastric acidic environment, withstand the peristalsis and overcome the attack by phagocytes (Olczak *et al*., 2002). During colonization, *H. pylori* elicit an oxidative stress response which induces potentially lethal and mutagenic oxidative DNA adducts in the *H. pylori* genome (O'Rourke *et al*., 2003).

Vulnerability to oxidative stress and oxidative DNA damage is common in many studied bacterial pathogens, including *Neisseria gonorrhoeae, Hemophilus influenzae, Streptococcus pneumoniae, Streptococcus mutans* and *Helicobacter pylori* (Michod *et al*., 2008). For each of these pathogens, surviving the DNA damage induced by oxidative stress appears to be supported by transformation-mediated recombinational repair. This means that transformation and recombinational repair contribute to successful infection (Nitharwal *et al*., 2011).

Transformation (the transfer of DNA from one bacterial cell to another through an intervening medium) appears to be part of an adaptation for DNA repair (Wang and Maier, 2007). *H. pylori* is capable of undergoing transformation naturally and this is done throughout its logarithmic growth (Dorer *et al*., 2010). Many organisms can only do this under certain environmental conditions, such as starvation (Michod *et al*., 2008). In *H. pylori*, homologous recombination is required for repairing DNA double-strand breaks (DSBs) (Nitharwal *et al*., 2011). Natural transformation is increased by DNA damage in *H. pylori*, and a connection exists between the DNA damage response and DNA uptake in *H. pylori*, suggesting that persistence of *H. pylori* in its human host has to do with its natural competence (Dorer *et al*., 2010).

H. pylori mutants that are defective in RuvC protein, have increased sensitivity to DNA-damaging agents and to oxidative stress leading to a reduce survival of the organism within macrophages and subsequent inability to establish successful infection in a mouse model (Loughlin *et al*., 2003). Similarly, RecN protein plays an important role in DNA double-strand breaks repair in *H. pylori*. An *H. pylori recN* mutant displays an attenuated ability to colonize mouse stomachs, highlighting the importance of recombinational DNA repair in the survival of *H. pylori* within its host (Wang and Maier, 2008).

Furthermore, *H. pylori* survive by using its unique acid gated membrane channel which effectively controls the amount of alkali to be produced by the bacterium to combat the

gastric acid production (Weeks *et al*., 2000). When acid is present, the membrane channel increases the amount of urea entering the bacterial cytoplasm by 300-fold (Berger, 2000). This produces sufficient ammonia to neutralize the periplasm (Croxen *et al*., 2006). This channel is regulated by a protein Urel which is an amino doporin family of protein (Loughlin *et al*., 2003). The gene that code for this channel has also been identified (Berger, 2000). It is believed that the genes will be useful as a new therapeutic target in the eradication of H. pylori (Wang and Maier, 2008).

A study by Celli *et al* in 2009, indicated that the helicoidal-shaped *H. pylori* does not bore its way through the mucus gel as was previously suggested, but instead achieves motility by altering the rheological properties of its environment. The organism hydrolyses urea to elevate the pH of its environment which becomes neutral. This modification causes the reduction in the visco-elasticity of the mucin gel in which it was initially elastically confined and unable to translate. The organism then swims freely to penetrate the mucus layer and attach to epithelial cells.

Indeed the breakdown of gastric mucin by *H. pylori* has been previously examined by others, but with apparently contradictory results. Early in vitro studies suggest that *H. pylori* directly compromise the mucus layer by proteolytic degradation of mucin glycoproteins (Worku *et al*., 1999; Lu and Walker, 2001). In later studies however, other researchers conclude that an observed loss of high particle weight glycoprotein in mucus from ulcer patients is not the result of proteolytic enzymes, but perhaps from a carbonate-bicarbonate buffer at the mucosal surface because of the hydrolysis of urea (Allen and Flemstrom, 2005).

2.2.5 Signs and symptoms of *Helicobacter pylori* Infection

Up to 85% of people infected with *H. pylori* never experience symptoms or complications (Bytzer *et al*., 2011). Acute infection is most commonly asymptomatic and maybe associated with epigastric pain, abdominal distention or bloating, belching, nausea, flatulence and halitosis (Ndip *et al*., 2008; Karlik *et al*., 2009). Where this develops into chronic gastritis, the symptoms, if present, are often those of non-ulcer dyspepsia: stomach pains, nausea, bloating, belching, and sometimes vomiting or black stool (Ryan, 2010).

Individuals infected with *H. pylori* have a 10 to 20% lifetime risk of developing peptic ulcers and a 1 to 2% risk of acquiring stomach cancer (Kuster, 2006). Inflammation of the pyloric antrum is more likely to lead to duodenal ulcers, while inflammation of the corpus (body of the stomach) is more likely to lead to gastric ulcers and gastric carcinoma (Suerbaum and Michetti, 2002). Common symptoms of ulcer include pain, discomfort in the

upper abdomen, bloating, feeling full after eating a small amount of food, lack of appetite, nausea, vomiting, dark colored stools, ulcers that bleed (leading to a low blood count) and fatigue (Gisbert and Abraira, 2006). Disease may progress to stomach cancer in those who were infected at an early age (Hong and Yang, 2012). *H. pylori* may play a role in the first stage that leads to common chronic inflammation, but not in further stages leading to carcinogenesis (Brown, 2000).

A meta-analysis conducted in 2009 concluded that the eradication of *H. pylori* reduces gastric cancer risk in previously infected individuals, suggesting that the continued presence of *H. pylori* constituted a relative risk factor of 65% for gastric cancers with the absolute risk increasing from 1.1% to 1.7% (Fuccio *et al*., 2009). *H. pylori* has also been associated with colorectal polyps and colorectal cancer (Wu *et al*., 2013).

Although all the machinery needed for immune defense seems to be present, spontaneous recovery is rare because the immune system is unable to clear the infection (Algood and Cover, 2006). In the absence of treatment, chronic *H. pylori* infection persists for years (Kargel and Basel, 2008).

2.2.6 Diagnosis of *H. pylori*

It is very important to test for *H. pylori* infection in the diagnosis of gastric and duodenal inflammatory disease (Vaira *et al*., 2002). Testing is also a useful means of monitoring the effectiveness of antimicrobial treatment (Ierardi, 2013). A number of different diagnostic test methods which are both invasive and non-invasive, are available for the detection of *H. pylori* (Smith *et al*., 2004).

a. Invasive Methods

This makes use of biopsy specimens collected from inflamed or ulcerated regions of the stomach and duodenum using endoscopy (Gatta *et al*., 2003). The biopsy material can be examined using one, or more, of the following three different test methods:

Histological examination –this is the staining and examination of the tissue samples. It allows both evaluation of cell damage and the detection of *H. pylori* cells in situ (Kuster et al., 2006). Histological identification is regarded as the ‘gold standard’ among the diagnostic tests (Stenström *et al*., 2008).

Urease test – this is a colorimetric test which detects urease enzyme activity in the biopsy sample (Sood, 2006). This method can be used to give a rapid indication of infection at the time of the biopsy (Krogfelt *et al*., 2005).

Culture and isolation of *H. pylori* – the tissue sample may be homogenised, or inoculated directly onto selective agar media (Boyanova, 2003). Typically, cultures are incubated for at least 3-5 days at 35^{o}C under microaerophilic conditions (Krogfelt *et al*., 2005). Isolates can be confirmed as *H. pylori* by Gram staining and biochemical tests which include the production of catalase, oxidase, urease and hydrogen sulphide (H_2S), nitrate reduction, growing in 35% sodium chloride (Nacl), and growing with 1% glycine (Al-Sulami *et al*, 2012). *Helicobacters* are fastidious and requires special culture conditions like a rich growth medium, microaerobic atmosphere (5-7% oxygen level), high humidity and an incubation temperature of 37^{o}C to succeed. Culture has been found to be 91.4% sensitive, 100% specific and 97.15% accurate with biopsy samples taken from both the antrum and corpus sections of the stomach (Lee *et al*, 2013). Isolates which gave positive results by biochemical tests as H. pylori can be confirmed by using polymerase chain reaction (PCR) amplification of the 16SrRNA and genomic sequencing (Windsor et al., 2005, Al-Sulami *et al*., 2012). Meanwhile they are not widely assessable (Karlik, *et al*., 2009). Culturing enables antibiotic susceptibility testing of the strain involved (Krogfelt *et al*., 2005). Culture was necessary for this present study because of the second stage which required the infection of mice with a pure *H. pylori* isolate.

Invasive testing is considered to be the reference method for diagnosing *H. pylori* infection (Gatta *et al*., 2003). The method is highly specific, particularly the histological examination although its sensitivity is partly dependent on the accuracy of the biopsy procedure (Lee *et a*l., 2013). The disadvantages are that histological examination and culture are time consuming and require specialized laboratory facilities with highly trained staff (Myllyluoma, 2007). Endoscopy is also an expensive and demanding procedure requiring highly trained, skilled staff and is quite uncomfortable for the patient (Gatta et al., 2003, Jemilohun *et al*., 2010). Urease testing is much more rapid and less costly, but the specimen to be tested is obtained through invasive procedure (Krogfelt *et al*., 2005).

Histological detection of *H. pylori* provides histological data on inflammation and atrophy, and it also allows the classification of possible gastroduodenal lesions and reveals premalignant alterations in the mucosa (Vaira *et al*., 2002). The sensitivities of the histological test depend mainly on the experience of the pathologist. The sensitivities and specificities usually achieved by histology are both above 95% (Kuster *et al*., 2006).

b. **Non-invasive methods**

Common non-invasive diagnostic tests for *H. pylori* include the following:

Serological assays – these uses whole blood or serum to measure specific *H. pylori* IgG and IgA antibodies which determine if an individual has been infected. The sensitivity and specificity of these assay usually range between 80-90% depending on the method used (Kuster *et al*., 2006). Laboratory based serologic testing using enzyme linked immunosorbent assay (ELISA) technology to detect IgG antibodies is not expensive, however, concerns over its accuracy have limited its use (Smith *et al*., 2008). Large studies have found uniformly high sensitivity (90-100%), but variable specificity (76-96%); the accuracy has ranged from 83-98% (Ricci *et al*., 2007). Serologic tests require validation at the local level, which is impractical in routine practice. Local prevalence of *H. pylori* affects the positive predictive value (PPV) of antibody testing. In areas where the prevalence of *H. pylori* is less than 20 percent, as in the United States, a positive result on serologic testing represents active infection approximately 50% of the time (Jemilohun *et al*., 2010). As the low accuracy of serology would result in inappropriate treatment in significant numbers of patients, guidelines recommend that serologic testing should not be used in low prevalence populations; testing for active infection with stool antigen assay or UBT is recommended in these populations (Smith *et al*., 2008). In patients with newly diagnosed *H. pylori* infection without prior treatment, the differentiation between past or present infection is not relevant. Therefore serologic test is appropriate in the initial workup of the patient (Al-Sulami *et al*, 2012). However, it cannot be used to see if the infection has been eradicated because the test remains positive for years even if the infection is cured. As a result, in a patient with prior history of treated *H. pylori* with recurrent symptoms, a serologic test will not be informative (Ricci *et al*., 2007; Jemilohun *et al*., 2010).

Urea Breath Test (UBT) - in urea breath test (UBT), the patient is given an oral preparation of either non radioisotope carbon -13- ($_{13}$C-) labeled urea, or radioactive isotope carbon-14- ($_{14}$C) labeled urea (Gatta *et al*., 2003). In the presence of a *H. pylori* infection, bacterial urease metabolizes the urea to produce labeled carbon dioxide and ammonia. The labeled carbon diffuses into the bloodstream and is excreted by the lungs (Sood, 2006). To determine the presence of *H. pylori*, this labeled carbon dioxide is measured in the patient breath using a mass spectrophotometer for $_{13}$C-labeled urea and a liquid scintillation for $_{14}$C-labeled urea (Ricci *et al*., 2007). UBT is indicated for the initial diagnosis of *H. pylori* and for follow up of eradication therapy (Ierardi *et al*., 2013). The sensitivity and specificity of UBT are above 95% (Kusters *et al*., 2006). False negatives can result from acid suppression with proton pump inhibitors; therefore test should be done two weeks post acid suppression treatment and

retesting for confirmation of eradication should be done four weeks after the completion of therapy (Myllyluoma, 2007). The breath test and stool test detect *H. pylori* more accurately than the blood test (Smith *et al*., 2008).

Stool Antigen Test- Stool antigen testing identifies active infection and has a sensitivity and specificity above 90% (Kusters *et al*., 2006). This means that it can be used to monitor the eradication of infection by antimicrobial treatment and can also detect repeat infections (Ricci *et al*., 2007). ELISA technique is used in the stool antigen test (Smith *et al*., 2008). The principle of the stool antigen test is that a polyclonal or monoclonal antibody to *H. pylori* is adsorbed to microwells. Diluted patient samples are added to the wells and any *H. pylori* in the fecal sample is bound to the adsorbed antibody. A second *H. pylori* antibody conjugated to peroxidase is added and binds to *H. pylori*. After unbound material is washed off, a substrate is added that reacts with bound peroxidase enzyme to produce a yellow colour, the intensity is measured to estimate H. pylori levels (Gatta *et al*., 2003). Stool antigen testing has been found to be reliable, inexpensive and easy to use (Jemilohun *et al*., 2010).

Stool culture –The recovery of *H.pylori* from feces of infected individuals is important in molecular epidemiological investigations, especially in children who may not successfully have endoscopy (Thomas *et al*., 2004). Dore *et al.*, 2000 and Falsafi *et al*., 2007 confirmed in their study that it is possible to isolate *H. pylori.* The reason why it was difficult to isolate *H. pylori* from stool before now was due to the toxic effect of the bile salt in stool on the bacterium (Chang *et al*., 1999). Also, the optimal conditions for the recovery of H. pylori from stool were not known (Graham and Osato, 2000; Lee *et al*., 2013). Dore et al., 2000 in their study recovered *H. pylori* from stool by treating the stool suspension with cholestyramine which is a basic anion exchange resin that inactivates the bile acids. Stool culture is done in brain heart infusion broth using a *H. pylori* positive stool sample before subculturing on Columbia agar. (Zimmermann and Trampe, 2010)

Faecal Occult Blood Test - A fecal occult blood test is done to find out if there is hidden (occult) blood in the stool (faeces) (Cleveland *et al*., 2010). The hidden blood may be from either upper gastrointestinal bleeding or lower gastrointestinal bleeding (Rocky, 2015). The detection of occult blood requires further investigation that will reveal if it was from peptic ulcer or colorectal cancer or gastric cancer (Shaukat *et al*., 2013). The test can also be used to check for active occult blood loss in anemia or when there are gastrointestinal symptoms (Harewood, and Ahlquist 2000; Rocky, 2015). An estimated 1- 5% of large tested populations have a positive fecal occult blood test (Cleveland *et al*., 2010). There are four clinical methods used in testing for occult blood in feces which detect DNA from cellular material such as from lesions of the intestinal mucosa or antibodies, heme, globin or

porphyrins in blood (Young and Cole, 2009). The methods are stool guaic test, fecal immunochemical testing (FIT), and immunochemical fecal occult blood test (iFOBT), Fecal Porphyrin quantification (HemoQuant) (Shaukat *et al*., 2013). Stool guaiac test for fecal occult blood (gFOBT) which involves smearing some feces onto some absorbent paper that has been treated with a chemical. The addition of a drop of hydrogen peroxide causes a colour change if there is a trace of blood. The principle and faecal DNA test () of this method is that the heme component in hemoglobin which has an effect similar to peroxidase, rapidly breaks down hydrogen peroxide (Cleveland *et al*., 2010). In some settings such as gastric or proximal upper intestinal bleeding the guaiac method may be more sensitive than tests detecting globin because globin is broken down in the upper intestine to a greater extent than is heme (Harewood *et al*., 2002). Hence its utilization as one of the screening tests for *H. pylori* in dyspeptic patients (Yi-Chia *et al*., 2013). Optimal clinical performance of the stool guaiac test depends on preparatory dietary adjustment (Harewood *et al*., 2002). Faecal immunochemical testing (FIT), and immunochemical fecal occult blood test (iFOBT) utilizes specific antibodies to detect globin. FIT screening is more effective in terms of health outcomes and cost compared with guaiac FOBT (Yi-Chia *et al*., 2013). FIT testing which is preffered in recent guidelines has replaced most gFOBT tests as the colon cancer screening test of choice (Rex *et al.*, 2009). Fecal porphyrin quantification (HemoQuant) - Unlike gFOBT and FIT, permits precise quantification of hemoglobin, and can analyze gastric juice, urine, and stool samples (Young and cole, 2009). The heme moiety of intact hemoglobin is chemically converted by oxalic acid and ferrous oxalate or ferrous sulfate to protoporphyrin, and the porphyrin content of both the original sample and of the sample after hemoglobin conversion to porphyrin is quantified by comparative fluorescence against a reference standard. The specificity for hemoglobin is increased by subtracting the fluorescence of a sample blank prepared with citric acid to correct for the probable confusing effect of existing non-specific substances (Cleveland *et al*., 2010). Precise quantification measurement has been very useful in many clinical research applications (Yi-Chia *et al*., 2013). Fecal DNA test – This test is used to find out alterations in human DNA extracted from stool sample which is associated with cancer (Imperial *et al*., 2014). The test looks at twenty three individual DNA alterations, including twenty one specific point alteration in the adenomatous polyposis coli (APC), KRAS, BAT26 and p53 genes (Jin et al., 2006).

In Nigeria, the non-invasive tests are not generally available except immunoglobulin G (IgG) serology. The value of serological tests in a hyper-endemic area like Nigeria is limited because of their low discriminatory power between previous and current infection (Jemilohun *et al*., 2010). However, a combination of stool antigen tests and serology had the

highest diagnostic accuracy in detecting *H.pylori* infection in Nigeria (Smith *et al*., 2008). This is done in research settings, where a combination of at least two methods is often applied in the detection of *H. pylori*, as compared to clinical practice, where it is common to use a single test (Smith *et al*., 2008).

2.2.7 Immunity

Patients infected with H. pylori respond with the development of an immunoglobulin M (IgM) antibody. Subsequently, immunoglobulin G (IgG) and immunoglobulin A (IgA) are produced which persists in high titer in chronically infected persons both systemically and mucosally (Blaser and Atherton, 2004; Brooks *et al*., 2007).

2.2.8 Treatment of *Helicobacter pylori* infection

Once the diagnosis of *H. pylori* is confirmed in a person with peptic ulcer, the normal procedure is to eradicate it and allow the ulcer to heal (Manyi-Loh *et al*., 2010). Previously, the only option was the control of symptom using antacids, H_2-antagonists or proton pump inhibitors alone (Senström *et al*., 2008). Now the treatment of *H. pylori* infection requires at least two antibiotics and an acid inhibitor (Malfertheiner *et al*., 2011). This is also known as the triple therapy and is a standard first-line therapy which consists of proton pump inhibitors such as omeprazole and the antibiotics clarithromycin and amoxicillin (Malfertheiner *et al*., 2007, Meeke, 2013). The triple therapy can be varied by the use of a different proton pump inhibitor, such as pantoprazole or rabeprazole, and replacing amoxicillin with metronidazole for people who are allergic to penicillin (Malfertheiner *et al*., 2012). The regimens are accepted internationally because it had a reported cure of 85-90% (Gatta *et al*., 2013).

The duration of treatment using triple therapies is from 10-14 days (Ierardi *et al*., 2013). The treatment regimens omeprazole, amoxicillin, and clarithromycin (OAC) are administered for 10 days while bismuth subsalicylate, metronidazole, and tetracycline (BMT) is for 14 days and lansoprazole, amoxicillin, and clarithromycin (LAC), has been approved for either 10 days or 14 days of treatment (Lahbabi *et al*., 2013).

Levofloxacin has recently appeared in treatment of *H. pylori* as a second line regimen when classical first-line therapy containing clarithromycin failed (Malfertheiner *et al*., 2012). The regimens are levofloxacin, amoxicillin, and lansoprazole (LAL) (Laving, 2013). However, resistant strains are increasing over the years because of plasmid-mediated horizontally transferable genes encoding quinolone resistance (Ierardi *et al*., 2013). This treatment failure led to the introduction of additional rounds of antibiotic therapy, such as a

quadruple therapy, which adds a bismuth colloid, such as bismuth subsalicylate (Gatta *et al*., 2013). But still, the eradication rate was not achieved (Malfertheiner *et al*., 2012)

A study using amoxicillin and clarithromycin plus omeprazole recorded the eradication rate of 92% in Europe and 50% in a German study in 2011 (Schmid, 1999; Malfertheiner *et al*., 2011). Failure was high in a turkish study where only 32.7% of eradication occurred (Songür *et al*., 2009). In Asia Pai et al., 2003 recorded 82.9% in India and 67.7% in Korea (Hong and Yang, 2012). In American continent, eradication rates range from 78% to 97% (Veldhuyzen *et al*., 2003). These data is characterized by enormous variability, however data from Africa are more homogeneous with eradication rate of 71.0% - 78.2% as was recorded in a multicentric and in Moroccan study respectively (Lahbabi *et al*., 2013).

Treatment regimens that include sequential administration of antibiotics with acid inhibitors have been developed to try and increase the rate of eradication (Laving, 2013). The sequential therapy is a new regimen which administers antimicrobials in a given sequence rather than simultaneously (Greenberg, 2011). This kind of treatment is not actually new because it uses the same established drugs approved for eradication of *H pylori*, but the administration strategy is innovative (Laving, 2013). The sequential regimen is a simple dual therapy including a proton pump inhibitor plus amoxicillin 1 g (both twice daily) given for the first five days, followed by a triple therapy including a proton pump inhibitor, clarithromycin 500 mg, and a nitroimidazole antimicrobial (all twice daily) for the remaining five days. Initial studies of sequential therapy suggested that its superiority over standard triple therapy might be due to improved eradication of clarithromycin resistant strains (Gatta *et al*., 2013).

Nevetheless, treatment of infection is challenged by the rapid rate with which the bacteria acquire resistance to the drugs, poor compliance, an excessively high bacteria load, impaired mucosal immunity, early re-infection and the presence of intracellular bacteria (Laving, 2013). Therefore, the selection of proper antimicrobial therapy is based on susceptibility test results (Lahbabi *et al*., 2013).

Meanwhile, the increasing report of antimicrobial resistance and its negative impact on the eradication of *H. pylori* brought about the search for a new therapeutic approach which will not be an antibiotic, but will be readily available, inexpensive, effective and free from side effect (Mégraud, 2004; Ierardi *et al*., 2013).

Some natural food met these criteria and was proposed as an alternative in the treatment of *H. pylori* (Manyi-Loh et al., 2010). Studies have shown that probiotics treats *H.*

pylori infection and hinders its gastric colonization (Hamilton-Miller, 2003). Utilizing it can decrease the use of antibiotics (Adagbada *et al*., 2011).

2.2.9 Epidemiology and Transmission

H. pylori infection is the most widespread in the world (World Gastroenterology Organization Global Guideline, 2011).Ten to 20% of the 50% of the normal populations infected have symptoms (Malaty, 2007). The actual rate of infection varies from one nation to another (Brown, 2000). The developing nations have higher rate of infection than the developed nations (Kusters et al, 2006). An annual incidence rate of *H. pylori* infection is 4.5% in developing nations and 0.5% in developed and industrialized nations (Adiekha *et al*., 2013). The prevalence rates in the general population ranges from 30-40% in the United States, 80-90% in South America and 70-90% in Africa (Jemilohun *et al*., 2010). Nigeria has a prevalent rate of 91% for adult while children between 5 and 9 years have 82% (Barzilay and Fagan, 2013).

The prevalence of *H. pylori* has been found to increase with age from 20% among teenagers to 50-60% in 60 to 70 years adults (World Gastroenterology Organization Global Guideline, 2011). The higher prevalence among the elderly reflects higher infection rates when they were children rather than infection at later ages (Kusters et al, 2006). In the United States, prevalence appears to be higher in African-American and Hispanic populations, most likely due to socioeconomic factors (Correa and Piazuelo, 2012). The lower rate of infection in the West is largely attributed to higher hygiene standards and widespread use of antibiotics ((World Gastroenterology Organization Global Guideline, 2011).

The possible pathologic outcome of the infection is influenced by the age at which this bacterium is acquired (Adiekha *et al*., 2013). Infection at an early age predisposes an individual to the development of a more intense inflammation that may be followed by atrophic gastritis with a higher subsequent risk of gastric ulcer, gastric cancer or both (Brown, 2000; Hong and Yang, 2012). When the infection is acquired at an older age, the gastric changes are more likely to lead to duodenal ulcer ((Jemilohun *et al*., 2010)).

Infections are usually acquired in early childhood in all countries (Kusters et al, 2006). However, the infection rate of children in developing nations is higher than in industrialized nations, probably due to poor sanitary conditions (Correa and Piazuelo, 2012).

Despite high rates of infection in certain areas of the world, the overall frequency of *H. pylori* infection is declining (Adiekha *et al*., 2013).

H. pylori is contagious, although the exact route of transmission is not known (Correa and Piazuelo, 2012). Person-to-person transmission by either the oral-oral or fecal-oral route

is most likely (Delport and van der Merwe, 2007). These transmission routes has been proved by the isolation of the organism from feces, saliva and dental plaque of some infected people (Adiekha *et al*., 2013). *H. pylori* is transmitted more easily through the gastric mucus than saliva (Brown, 2000). Transmission occurs mainly within families in developed nations and can be acquired from the community in developing countries (Delport and van der Merwe, 2007). *H. pylori* may also be transmitted orally by means of fecal matter through the ingestion of waste-tainted water, so a hygienic environment could help decrease the risk of *H. pylori* infection (Brown, 2000; Al-Sulami *et al*.,2012). Wegermann and McColl in 2006 suggested that infected mothers were the main source of *H. pylori* infection in children (Wagermann and McColl, 2006).

The organism forms biofilms and can convert from helical to coccoid form. These characteristics favour its survival and also play a role in the epidemiology of the bacterium. The coccoid form which is found in water adheres to gastric epithelial in vitro, but it is difficult to be isolated in culture (Karlik *et al*., 2009).

While *H.pylori* remains the most medically important bacterial inhabitant of the human stomach, there are other species of the *Helicobacter* genus for example *H. canis*, *H. rappini*, *H. hepaticus* and *H. pullorum*, which have been identified in other mammals and some birds, and some of these can infect humans (Mobley *et al*., 2001). *Helicobacter* species have also been found to infect the livers of certain mammals and to cause liver disease (Starzynska and Malfertheiner, 2006).

2.2.10 Prevention

Once it was recognized in 1990 that infection with *H. pylori* is the main cause of peptic ulcer disease and is a strong risk factor for gastric cancer, efforts to develop a *Helicobacter pylori*-specific vaccine began (Salama *et al*., 2013). Prophylactic immunization, especially during infancy or early childhood, was expected to be gainful in the United States in 2009, despite the documented gradual loss of *H. pylori* from Western populations (Rupnow *et al*., 2009; Blaser and Falkow, 2009). However, the results of the efforts geared at *H. pylori* vaccine development, both preclinical and early clinical have been disappointing with recent move from animal to human trial (Blanchard and Nedrub, 2010). This is as a result of the difficulty of achieving sterilizing immunity, even in animal models, and there is no consensus on the delivery route, adjuvants and choice of antigen (Kabir, 2007; Salama *et al*., 2013). The most promising preclinical results have been obtained with vaccination strategies that aim to induce protective T cell-mediated immunity rather than humoral

immunity, with local gastric T helper 1 (T_H1) and T helper 17 (T_H17) responses being a prerequisites for protection (Muller and Solnick, 2011).

The recombinant vaccines of *H. pylori* antigens that are ectopically expressed in *Salmonella enterica* vaccine strains, whole-cell *H. pylori* extracts and multi-component, have been delivered parenterally or mucosally with success in mice (Czinn and Blanchard, 2011) *H. pylori* antigens with documented immunogenicity in rodents include the urease enzyme, cytotoxin-associated gene A (CagA), vacuolating cytotoxin (VacA), catalase, neutrophil-activating protein (NAP) and heat shock proteins; these can be delivered by various mucosal routes such as orogastric, intranasal, sublingual and rectal (De Vries *et al*., 2009; Muller and Solnick, 2011).

Despite the strict limitation of *H. pylori* to its gastric niche, systemic immunization via the intraperitoneal or subcutaneous routes can be as effective as mucosal vaccination (Czinn and Blanchard, 2011). In contrast to most other vaccines, *H. pylori*-specific immunization generates prophylactic, as well as therapeutic, immunity in rodent models (Selgrad and Malfertheiner, 2008). Persistence mechanisms that are used by *H. pylori* to overcome and subvert adaptive immunity have been identified as crucial obstacles that preclude sterilizing immunity; therefore, vaccination strategies may need to bypass or override the host immunoregulatory response (Hitzler *et al*., 2011).

Two recently conducted Phase I clinical trials in human volunteers showed antigen-specific humoral and cellular responses, but did not confer satisfactory protection against challenge infection (Aebischer *et al*., 2008; Salama *et al*., 2013). In one trial, intramuscular immunization with three recombinant antigens (CagA, VacA and NAP) adjuvanted with alum induced responses to some or all antigens in the majority of volunteers, irrespective of the exact dose and immunization schedule; T cell responses were observed only against CagA and VacA, but were detectable as late as 24 months post-primary vaccination and are therefore indicative of T cell memory (Malfertheiner *et al*., 2008). Oral immunization with live *Salmonella enterica* subsp. *enterica* serovar Typhi Ty21a expressing *H. pylori* urease or HP0231 provided evidence that the clearance or the reduction of a challenge infection requires T cell-mediated immunity, but the study failed to demonstrate improved infection control in the vaccinated group relative to the non-immunized (but challenged) volunteers Aebischer *et al*., 2008; Salama *et al*., 2013).

Given that the rodent models of *H. pylori*-specific vaccination have revealed useful antigens, adjuvants and delivery routes, the ultimate proof of immunogenicity and protective immunity in humans remains elusive (Muller and Solnick, 2011). The advancement of *H. pylori* vaccine is dependent on a renewed or expanded commitment from the biotechnology

or pharmaceutical industry that could make use of recent advances in our understanding of the host immune response to *H. pylori*. Their continued support is viewed as being essential for promoting *H. pylori* vaccine development in the future (Czinn and Blanchard, 2011).

2.3 Probiotics

Microorganisms that provide health benefit when consumed are referred to as probiotics (Rijkers *et al*., 2011).The introduction of the concept is attributed to Nobelist Elie Metchnikoff who ascribed the long life of Bulgarian peasants to their consumption of fermented milk products in 1907 (Parvez *et al*., 2006). Metchnikoff reasoned that substances like phenols, indoles and ammonia which are by-products formed when *Clostridia* digests proteins were responsible for "intestinal auto-intoxication" which caused the physical changes associated with old age (Bourlioux *et al*., 2002). This gave the knowledge that the fermentation of milk by lactic-acid bacteria caused the lowering of pH due to the fermentation of lactose, leading to the inhibition of the growth of proteolytic bacteria. From this information, Metchnikoff proposed that consuming fermented milk which has the harmless lactic acid bacteria benefits health, so he introduced in his diet sour milk fermented with the bacteria he called "Bulgarian Bacillus" (Parvez *et al*., 2006). His friends in Paris quickly followed his example when they found his health improved and Physicians began prescribing the sour milk diet for their patients (Wang *et al*., 2004).

However, in 1920, Rettger and Cheplin reported that Metchnikoff's "Bulgarian Bacillus", which was later called *Lactobacillus delbrueckii subsp. Bulgaricus*, could not live in the human intestine. They reveled from their experiment that there was a change in the composition of the fecal microbiota of rats and animals fed with Lactobacillus acidophilus. This was described as "transformation of the intestinal flora". Rettger further explored the possibilities of *Lactobacilus acidophilus* and reasoned that bacteria originating from the gut were more likely to produce the desired effect in this environment (Azizpour *et al*., 2009).

Henry Tissier, who was also from the Pasteur Institute and was the first to isolate a *Bifidobacterium*, recommended the administration of *Bifidobacteria* to infants suffering from diarrhea, claiming that this organism would displace the proteolytic bacteria that cause disease (Ishibashi and Yamazaki 2001). It was reasoned that bacteria originating from the gut were more likely to produce the desired effect in the gut, and in 1935 certain strains of *Lactobacillus acidophilus* were found to be very active when implanted in the human digestive tract (Hamilton-Miller, 2003). Trials were carried out using this organism, and encouraging results were obtained especially in the relief of chronic constipation (Rijkers *et al*., 2011).

The benefits of probiotics include the decrease of potentially pathogenic gastro-intestinal microorganisms; the reduction of gastro-intestinal discomfort; the strengthening of the immune system; the improvement of the skin's function; the improvement of bowel regularity; the strengthening of the resistance to cedar pollen allergens; the decrease in body pathogens; the reduction of flatulence and bloating; the protection of DNA; the protection of proteins and lipids from oxidative damage; and the maintaining of individual intestinal microbiota in subjects receiving antibiotic treatment (Rijkers *et al*., 2010).

The increase in the demand for probiotics has led to higher requirements for scientific validation of the assumed beneficial effects conferred by the microorganisms, however, studies on the medical benefits of probiotics are still inconclusive (Rijkers et al., 2011).

Japan introduced yakult, a probiotic fermented food drink in 1935, and in the Northern hemisphere, research and use of probiotics has gained an unprecedented momentum in the last decade (Hamilton-Miller, 2003). Use of probiotics is not uncommon in Europe, but in many developing countries use of probiotics in its present definition is a foreign concept. Some African traditional foods are fermented with lactic acid bacteria, and some may have probiotic properties, but clinical evidence is yet to be deciphered (Jemilohun *et al*., 2010).

2.3.1 Definition of Probiotics

According to Hamilton-Miller *et al*., the term "probiotics" was first introduced in 1953 by Werner Kollath (see Hamilton-Miller *et al*. 2003) to describe organic and inorganic food supplements that are useful in the restoration of health to patients suffering from malnutrition. In contrast to antibiotics, probiotics were defined as microbial derived factors that stimulate the growth of other microorganisms (Hamilton-Miller *et al*., 2003). Lilly and Stilwell in 1965 described probiotics as microorganisms that have effects on other microorganisms (Hamiliton-Miller, 2003). Their idea of probiotics was that substances secreted by one microorganism stimulated the growth of another microorganism (Ishibashi and Yamazaki, 2001). In 1971 Sperti described probiotics as tissue extracts which stimulated microbial growth (Hamilton-Miller *et al*., 2003). In 1974 Parker defined the concept as organisms and substances that have a beneficial effect on the host animal by contributing to its intestinal microbial balance (Ishibashi and Yamazaki, 2001). Later, the definition was greatly improved by Fuller in 1989, who described probiotics as a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance (Schrezenmeir and de Vrese, 2001). He stressed two important facts of probiotics: the viable nature of probiotics and the capacity to help with intestinal balance (Reid *et al*., 2010).

However, a consensus definition of probiotics which was based on the available information and scientific evidence was adopted in 2001, after a joint Food and Agricultural Organization of the United Nations and World Health Organization expert consultation defined probiotics as live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host (FAO/WHO, 2002). The FAO/WHO consultation was also the first to assess of probiotics efficacy which was documented in May 2002, in a document named "Guidelines for the Evaluation of Probiotics in Food" (Hamilton-Miller, 2003).

In the following decades, intestinal lactic acid bacterial species with alleged health beneficial properties have been introduced as probiotics, including *Lactobacillus rhamnosus*, *Lactobacillus casei* and *Lactobacillus johnsonii* (Tannok, 2003)

2.3.2 Properties of Probiotics

1. Probiotics must be alive when administered (Knut, 2001). Scientists are concerned of the viability and reproducibility of probiotics on a large scale, as well as the viability and stability during use and storage and finally the ability to survive in the intestinal ecosystem (Reid *et al*., 2010). This aspect represents a major challenge for scientific and industrial investigations because several difficulties arise, such as variability in the site for probiotic use (oral, vaginal, intestinal) and mode of application (Floch *et al*., 2011).
2. The probiotic candidate must be a microbe or combination of microbes (Axelsson *et al.*, 2010). It is commonly admitted that the action of most probiotics on the host are strain-specific and cannot be extended to other probiotics of the same genus or species (Hamilton-Miller *et al*., 2003). Therefore, precise identification of the strain, i.e. genotypic and phenotypic characterization of the tested microorganism is important (Gueimonde and Collado, 2012).
3. An effective probiotic should be non-pathogenic, non-toxic and be beneficial to the health of the host (Hamilton-Miller, 2003). The 2002 FAO/WHO guidelines recommend that, though bacteria may be Generally Recognized as Safe (GRAS), the safety of the potential probiotic should be assessed (Syndman, 2008). The assessment includes the determination of antibiotic resistance patterns, production of certain metabolites (e.g., D-lactate production, bile salt deconjugation), side-effects to consumers, ability of the strain under evaluation to produce toxin or has a hemolytic potentials (Colin *et al*., 2014). Three levels of study are necessary to ascertain the safety and efficiency of probiotics: *in vitro* studies, animal studies, and ultimately, clinical trials (Reid *et al*., 2010).

4. The probiotic should be easy to process and cost effective, have good sensory properties and be isolated from the same species as its intended host. Dairy products are mainly used as carriers for probiotics after their extraction (Knut, 2001).

2.3.3 Application of Probiotics

The clinical application of probiotics has been in the prevention and treatment of gastrointestinal infections and diseases (Floch et al., 2011). Probiotics is used to control chronic gastrointestinal inflammatory diseases, such as ulcerative colitis and pochitis (Rowland *et al*., 2010). The consumption of probiotics has been associated with the improvement of a diversity of health conditions, including lactose intolerance, high cholesterol and rheumatoid arthritis (Reid *et al*., 2010). Also there is evidence of beneficial effects of probiotics with respect to the developme nt of dental caries, allergy and cancer (Rijkers *et al*., 2011).

2.3.4 Dosage of Probiotics

Probiotics are generally sold as capsules, powder, tablets, liquid, or incorporated into food. The specific number of CFUs contained in a given dose or serving of food can vary between brands. (Kliger and Cohrseen, 2008). The concentration of probiotics in research trials and in food or other products varies greatly, and there are no international standards regarding the levels of bacteria required (Parvez *et al*., 2006).

A wide range of dosages for Lactobacillus sp. and other probiotics range from 100 million to 1.8 trillion CFUs per day (Kligler and Cohrssen, 2008). Most studies examined dosages in the range of 1 to 20 billion CFUs per day, although exact dosages for specific indications varied within this range (Rijkers *et al*., 2011). Generally, higher dosages of probiotics (i.e., more than 5 billion CFUs per day in children and more than 10 billion CFUs per day in adults) were associated with a more significant study outcome (Floch et al., 2011). There is no evidence that higher dosages are unsafe; however, they may be more expensive and unnecessary (Rijkers *et al*., 2011). The dosages of S. boulardii in most studies range between 250 mg and 500 mg per day (Kligler and Cohrssen, 2008).

2.3.5 Mechanism of Probiotic Action

Based on a report by Oelschlaeger (Oelschlaeger, 2010), the effects of probiotics may be classified in three modes of action:

1. Probiotics do modulate the host's defences including the innate and acquired immune system. This mode of action is important for the prevention and treatment of infectious diseases.
2. Probiotics can also have a direct effect on other microorganisms, commensal or pathogenic ones. This helps in the prevention and treatment of infections and restoration of the microbial equilibrium in the gut.
3. Furthermore, probiotics inactivate microbial products like toxins and host products, e.g. bile salts and food ingredients resulting in the inactivation of toxins and detoxification of host and food.

2.3.6 Side Effect of Probiotics

Some side effects of probiotics are linked to digestive problems. Gas, bloating and stomach cramps are common effects of probiotics, especially in people who are eating probiotics foods alongside with taking probiotics supplements (Alvarez-Olmos and Oberhelman, 2001; Kligler and Cohrssen, 2008).

Some studies had shown that probiotic products like yogurts could be a cause of obesity (Ehrlich, 2009). In patients on immunosuppressants, impaired immune systems, and those who have a compromised intestinal barrier or underlying health problems, probiotics could over-stimulate the immune system, causing unhealthy metabolic activities, or gene transfer which leads to probiotic infections like *Lactobacillus* septicaemia and severe fungal infections (Sanders *et al*., 2010).Perhaps, the biggest potential danger of probiotics is the risk of transferring antibiotic resistance from probiotics to more deadly microorganisms (Labia *et al*., 2008). This is why scientists have strict rules on which bacteria can qualify as probiotic (Kligler and Cohrssen, 2008).

Other researchers concluded that probiotic supplements are generally considered safe for use by healthy people: in 143 studies that included a total of over 7,500 participants, no serious adverse effects of probiotics was noted (Madsen, 2001; Snydman, 2008).

2.3.7 *Lactobacillus*

The genus *Lactobacillus* are rod shaped, Gram-positive, non-spore forming, non pigmented, catalase negative and microaerophilic lactic acid bacteria with variable metabolic characteristics, such as the production of diacetyl, hydrogen peroxide and lactic acid (Axelsson *et al*., 2010). Most species have multiple requirements for amino acids and vitamins, resulting in the abundance of lactic acid bacteria in communities with these necessities (Ljunah and Wadström, 2006). *Lactobacilli* are often associated with animal oral

cavities and intestines, plant leaves, as well as decaying plant or animal matter such as rotting vegetables, feacal matter and compost (Ayad *et al*., 2004). Most are free-living or act as commensals to man and animals in the oral cavity, intestinal tract and vagina, where they play a beneficial role, though some are opportunistic pathogens (Deegan *et al*., 2006).

Lactobacilli are used in the food industry because their growth lowers the carbohydrate content and the pH of the foods they ferment due to the production of lactic acid which inhibits the growth of most other microorganisms including the most common human pathogens, thereby allowing these foods to have a prolonged shelf life (Axelsson *et al*., 2010). The acidity also changes the texture and flavour of the foods due to precipitation of some proteins and the biochemical conversions involved in the bacterial growth (Ayad *et al*., 2004).

Lactobacilli are important group of probiotic bacteria that inhibit undesirable microflora in the human gut and create a healthy equilibrium between beneficial and potential intestinal pathogens (Yuan-Kun, 2009).

2.3.8 Role of Probiotics in the Treatment of *H. pylori* Infection

In vitro studies of the immune cell response of host intestinal epithelium to probiotic strains has demonstrated the mechanism of probiotic action on *H. pylori* (Myllyluoma, 2007). Lactic acid bacteria and *Bifidobacteria* probiotics are able to produce organic acids, hydrogen peroxide and carbon dioxide which inhibit potential pathogens (Yuan-Kun, 2009). Also, defined antimicrobial substances have been produced by many probiotics (Servin, 2004).

Coconnier *et al*., found that *L. acidophilus* LB strain produced an anti-*Helicobacter* substances that were different from lactic acid (Myllyluoma, 2007). It has been shown that *L. johnsonii* La 1 release a non bacteriocin antimicrobial substances while, *Bifidobacterium* strains release heat stable protienaceous antimicrobial (Collado *et al*., 2005). Several probiotics species, such as *L.salivarius, L. gasseri* and *L. acidophilus*, have shown growth inhibition or anti-adhesion capacity against *H. pylori* in gastric epithelial cell model (Tsai *et al*., 2004).

Probiotics act on pathogens by co-aggregating with the pathogens and exposing them to high doses of potential growth inhibiting factors (Casena *et al*., 2001). Probiotics also inhibit the adhesion of pathogens by steric hindrances, where the receptor sites are covered in a non-specific manner or by competing for specific carbohydrates receptors that would otherwise be available to pathogens (Oelschlaeger, 2010).

Ingesting lactic acid bacteria has been found to exert a suppressive effect on *H. pylori* infection in both animals and humans, and supplementing with Lactobacillus- and

Bifidobacterium -containing yogurt improved the rates of eradication of *H. pylori* in humans (Wang *et al.*, 2004).

Probiotics can be used as a complement or alternative to antibiotics in the treatment of *H. pylori* infection (Myllyluoma, 2007). As a complement to antibiotics, probiotics may have the potential to reduce the adverse effects of triple anti-*Helicobacter* treatment and to improve the eradication rate (Ierardi *et al*., 2013). In the study by Myllyluoma 2007, supplementation improved the eradication rate but did not alleviate the adverse effects of the anti-Helicobacter treatment. In contrast, Armuzzi *et al*., (2001a, 2001b) reported in two separate studies that *Lactobacillus rhamnosus* GG was able to reduce the occurrence of adverse effects, such as diarrhea, taste disturbances, nausea and bloating. Moreover, Sheu *et al*., 2002 reported that *L. acidophilus* La5 and *B. lactis* Bb 12 containing yogurt was able to increase the eradication rate and also decrease several side effects of the triple therapy.

Probiotics as an alternative to antimicrobials have also been the focus of several studies. Administration of culture supernatant or fermented milk containing the strain of *L. acidophilus* La 1 decreased *H. pylori* urease activity, measured by 13C-UBT in adults and in children (Michetti *et al*., 1999; Cruchet *et al*., 2003). However, Felly *et al*., 2001 and Pantoflickova *et al*., 2003 found by histological analysis a decrease in inflammation but there was no eradication of H. pylori.

2.3.9 Experimental studies using mice

Animal models are necessary tools in biomedical research (Kuramoto *et al*., 2012). They have been used from the early days of scientific discovery and are still useful in the understanding of the functions of individual genes, the mechanisms of different diseases, and the effectiveness and the toxicities of various medicines and chemicals (Oakley *et al*, 2008). Their physiology, genetics and function of specific genes showed that they can easily be compared to human (Kuramoto *et al*., 2012). Mouse models of many human diseases have also been developed to advance the studies of disease pathogenesis, and to evaluate the effectiveness and toxicities of various candidate drugs (Oakley *et al*., 2008). A survey of animal models found that the mouse is the overwhelmingly preferred laboratory animal; the most widely used mouse and rat strains are C57BL/6 mice, BALB/c mice, Wistar mice and Sprague-Dawley rats (Beerman *et al*., 2004).

According to the animal resource centre, the average weight of a male wistar mice are 77g for three weeks, 122g for four weeks, 178g for 5 weeks, 225g for 6 weeks, 273g for 7 weeks, 305g for 8 weeks and 327g for 9 weeks (Kuramoto *et al*., 2012).

Various probiotics have shown favourable effects in animal models of *H. pylori* infection (Oelschlaeger, 2010). Studies have shown highly protective and therapeutic effect of oral administration of *L. salivarius* on *H. pylori* infected gnotobiotics BALB/c mice model (Hamilton-Miller, 2003). Similarly, Coconnier *et al*., 1998 reported that *L. acidophilus* strain LB was able to protect against *H. pylori* infection in conventional mice (Myllyluoma, 2007). Probiotics combination containing *L. acidophilus* R0052 and *L. rhamnosus* R0011 reduced the effects of *H. pylori* infection in a C57BL/6 mice model by reducing *H. pylori* colonization and alleviating *H. pylori* induced inflammation of the stomach (Johnson-Henry *et al*., 2004).

3.0 MATERIALS AND METHODS

3.1 MATERIALS

1. Mice
2. Animal cages
3. Water and feeding dish
4. Urethral pediatric probe
5. Dissecting board

6. Equipment

a. Distiller (Manesty)
b. Anaerobic jar (Oxoid)
c. Chemical balance (Metler)
d. Weighing container (A plastic box)
e. Light microscope(Olympus CX21)
f. Photomicrograph microscope (Olympus CH)
g. Rotary Microtome (Reichert-Jung)
h. Automatic tissue processor (Shandon)
i. Endoscopy unit

7. Laboratory materials

a. Sterile Universal container (Stericon)
b. Microscope slide (Micropoint)
c. Bijou bottles (Pyrex)
d. Test tubes (Pyrex)
e. Pasteur pipette
f. Disposable specimen dropper
g. Petri dishes (Stericon)
h. Cornical flasks-250ml, 500ml, 1000ml (Pyrex)
i. Measuring cylinder
j. Tourniquet
k. Syringes and needle (Axoject)
l. Needle disposal unit
m. Adhesive bandages tape
n. Autoclave tape
o. Gauze
p. Cotton wool
q. Alcohol wipes

r. Stainless Steel pot
s. Glass bowl
t. Glass rod
u. Procelin mortar and pestle
v. Sterile Swab sticks (Evapon)
w. Stainless scissors
x. Filter paper (Whatman 1)
y. Grease pencil

8. Reagents

a. Kovac's Oxidase reagent (BDH)
b. Nitrate broth (BDH)
c. Sulphanilic acetic acid reagent (BDH)
d. Alpha-Naphthylamine reagent (BDH)
e. Motility indole urea (MIU) medium (BDH)
f. Oxidation fermentation medium (Oxoid)
g. Hydrogen peroxide (Labtech)
h. Gram staining reagent (Labtech)
i. Giemsa staining reagent (Labtech)
j. Haematoxylin Eosin Staining reagents (BDH)
k. Sulphanilic acetic acid reagent (BDH)
l. alpha-Naphthylamine reagent (BDH)
m. Motility indole urea (MIU) medium (BDH)
n. Oxidation fermentation medium (Oxoid)

9. Test Kits

a. One step fecal occult blood test kit (Abon)
b. *H. pylori* one step test device for feces (Diastop)
c. *H.pylori* one step test device for serum/plasma (Diastop)

10. Fresh Cow Milk

3.2 CULTURE MEDIA

a. Columbia Blood Agar Base (Oxoid)
b. Brain Heart Infusion Broth (Lab M)
c. Lactobacilli MRS Agar (Acumedia)

3.3 METHODS

3.3.1 Study designs and Subjects

This study was in two phases.

Phase 1:

a. Both serum and stool samples were collected from 200 patients between the ages of 18 and 75 years who enrolled in the Gastroenterology unit of the Medical Out Patient Clinic of the Federal Teaching Hospital Abakaliki. Serum was used to detect *Helicobacter pylori* antibody, while stool was used to detect *H. pylori* antigen, fecal occult blood and also for the isolation of bacteria.
b. Gastric biopsy collected from a dyspeptic patient visiting the Gastroenterology unit of the Niger Foundation Hospital Enugu was cultured to isolate *Helicobacter pylori*.
c. Exclusion criteria were: antibiotic treatment during the previous two months, the use of H_2-receptor antagonists, bismuth or proton pump inhibitors (PPI) during the previous five years, the use of probiotic products during the previous month, gastric surgery and pregnancy.

Phase 2:

One hundred and twenty six (126) two weeks old male albino wistar mice from Faculty of Veterinary Medicine, University of Nigeria Nsukka were divided into three groups A, B and C, which were the negative control, positive control and test respectively. Mice in groups B and C were infected with *H. pylori* isolate from a gastric biopsy. Mice in group C were given probiotics one week after post challenge with *H. pylori*. The effect was noted through histopathological studies of the duodenum and the stomach using the positive and negative controls.

3.3.2 Ethics

All participants gave their written informed consent approved by the Ethics Committee of Federal Teaching Hospital Abakaliki (Appendix 1).

3.3.3 Mice Maintenance (Hamilton-Miller, 2003)

1. Screening for infection.

   The mice used in this study were first screened by a veterinary doctor. Only those without intestinal helminthiasis were used for this study to ensure that the pathological change that will be observed in the mice was caused by *H. pylori* infection only

2. Housing.

Rubber cages having smaller space between the bars were used in this study to house the mice. Mice were kept in a well ventilated out building at a temperature of 22^{o}C to 30^{o}C and were maintained in a natural environment all through the study. The mice were kept in sevens in eighteen cages marked as cage A1-A6, B7-B12 and C13-C18.

3. Feeding.

Due to the scarcity of rodent pellets the Veterinary advised that growers mash be used to feed mice. Clean drinking water was available at all times (Appendix 2).

4. Weighing.

Each mouse was weighed weekly. An empty plastic box which also served as a transfer container was weighed. Each mouse was transferred to the plastic container by holding the tail base. This was placed in a weighing balance, weighed and carefully returned to the cage. The weight of mouse was calculated by subtracting the weight of the container alone from the weight of mouse and container.

3.3.4 Sample Collection

1. Blood:

The patient was made to sit comfortably in a chair with the arm hyper-extended. A tourniquet was tied about 4 inches above the selected venipuncture site as the patient made a fist. The portion was cleaned with an alcohol swab in a circular fashion from the site working outward. This was allowed to air dry. Grasping the arm firmly and drawing the skin taut with the thumb to anchor the vein, the needle was swiftly inserted through the skin and into the lumen of the vein at 15 to 30 degree angle with the surface of the arm. Blood was withdrawn to the 4 ml mark on the syringe. The tourniquet was removed before the needle. A guaze was pressed on the spot to avoid hematoma. The sample was delivered into clean dry labeled 10ml pyrex test tube (Sood et al., 2006).

2. Stool:

Patients who were positive for *H. pylori* serology test were instructed to collect stool directly into a sterile wide mouth universal container. Stools from 1-12 months old healthy infants were collected in a sterile wide mouth container.

3. Gastric Tissue Biopsy:

This was performed by a consultant Gastroenterologist. Tissue sample from the stomach of a patient was got through esophagogastroduodenoscopy commonly known as an endoscopy or EGD. The endoscope was inserted down the patient's throat, through the esophagus, and into the stomach and upper small intestine. Air was put into the endoscope to

help visibility. After visual inspection, tissue sample for biopsy and culture was taken from the antrum and corpus of the stomach. The procedure lasted for about 10 minutes (Pietrangelo, 2012).

4. Gastric and duodenal tissue from mice:

Mice were killed by spinal dislocation, the structures of the head, neck and limbs were pinned. A cut was made through the abdominal wall of the dead mice (See Appendix 3). The diaphragm, heart, thymus gland, lungs, coelom, liver, esophagus, stomach, spleen, pancrease, small intestine and its sections (for example duodenum, jejunum and ileum), and colon were located. The lobes of the liver was pushed aside to show the caudate lobe which wrapped around the stomach. The stomach was cut off and a portion was put into brain heart infusion for culture while the remaining portion was put immediately in 10% formalin solution for histology. Observing the small intestine, the duodenum which was the first stretch of the intestine leading from the stomach was cut and put immediately into 10% formalin solution.

3.3.5 Serological Tests

For each volunteer (study 1), a blood sample and a stool sample was obtained.

1. *H. pylori* one step test for Serum:

Serum was separated from blood soon after collection. Test was carried out following the manufacturer's instruction. Result was read at 10 minutes. Positive had two distinct red lines at the control and test region. Negative results had one line appearing at the control region and non in the test region. Invalid results had no line at the control region. Despite an inbuilt procedural control, known negative and positive control standard were tested along with the samples.

2. *H. pylori* one step test for feces:

A little fecal sample was diluted with buffer. This was emulsified. Test was carried out following the manufacturer's instruction. The result was read after 10 minutes. Positive had two distinct red lines, one at control region and the other at the test region. Negative had one line appearing in the control region while invalid had no line appearing.

3. *H. pylori* one step fecal occult blood test:

The specimen collection stick from the tube was used to stab the feces in 3 different sites. The collection stick was transferred into the specimen collection tube which contained the extraction buffer. The tube was shaken vigorously to mix the specimen and the extraction buffer. Holding the tube upright, the tip was broken off and inverted to transfer 10 drops of the extracted specimen (approximately 500µl) to the reaction tube. Test strip was removed from the sealed pouch and immersed into the extracted specimen. At 5 minutes, the result

was read. Positive had two distinct red lines at the control line region and the test line region. Negative had only one red line in the control line region. Invalid did not have line in the control region.

3.3.6 Isolation and Identification:

1. Stool culture for *Helicobacter pylori*

Using the method by Zimmermann and Trampe, 2010; 1 gram of *H. pylori* antigen positive fecal sample was suspended in 1.5ml of saline (0.45% Nacl) and an aliquot of this preparation was added to brain heart infusion broth, incubate microaerobically using 10% carbon dioxide for 18 hours at 37^{o}C. A loopful was streaked on Columbia agar, incubated microaerobically for 12 days at 37^{o}C.

2. Stool culture for *Lactobacillus acidophilus*

A loopful of stool samples from 10 infants aged between one to twelve months who were feeding on milk was diluted in sterile 1% peptone water. Plates of MRS agar were prepared according to the manufacturer's instruction. Duplicate plates were prepared for each dilution. A loopful was inoculated onto MRS agar (De-Mann, Rogosa and Sharpe agar) and incubated anaerobically at 32^{o}C for 48 hours. Distinct and well isolated colonies were sub-cultured and examined by Gram staining and tested for catalase, oxidase, indole, motility, nitrate reduction and sugar fermentation (Babatunde et al., 2014).

3. Gastric biopsy specimen

One from the antrum and one from the corpus were divided into three parts. A smear was prepared from one part by scraping the biopsy on the slide. The smear was used for a modified Gram stain with carbol fuchsin as the counterstain (Boyanova, 2003; Lee *et al*., 2013). Another part of the biopsy specimen was placed in urea (10%) agar medium, incubated at 37^{o}C and observed for colour change after 30 minutes and 3 hours (Boyanova, 2003). The remaining part of the specimen was grinded in a glass grinder using 0.1ml sterile saline (Lee *et al*.,, 2013). The homogenate was inoculated onto Columbia blood agar base (oxoid) with 10% defribrinated sheep blood and vancomycin (10mg/l), trimethoprim (5mg/l), polymixin B (2500u/l) and amphotericin B (2mg/l) to make it selective for Helicobacter pylori (Boyanova, 2003). The plates were incubated for 5-12 days in a microaerobic atmosphere (Oxoid pak) at 37^{o}C. *H. pylori* was identified by Gram staining of suspect colonies, lack of aerobic growth on blood agar and testing for the presence of urease, oxidase and catalase (Boyanova, 2003; Krogfelt et al., 2005).

4. Gastric and duodenal tissue from mice:

Mice were killed by spinal dislocation, the structures of the head, neck and limbs were pinned. A cut was made through the abdominal wall of the dead mice as shown in figure 3. The diaphragm, heart, thymus gland, lungs, coelom, liver, esophagus, stomach, spleen, pancrease, small intestine and its sections (for example duodenum, jejunum and ileum), and colon were located. The lobes of the liver was pushed aside to show the caudate lobe which wrapped around the stomach. The stomach was cut off and a portion was put into brain heart infusion for culture while the remaining portion was put immediately in 10% formalin solution for histology. Observing the small intestine, the duodenum which was the first stretch of the intestine leading from the stomach was cut and put immediately into 10% formalin solution for histology.

3.3.7 Bacteria Identification

1. Biochemical tests

(a) Oxidase test: Filter paper spot method (Shields and Cathcart, 2013).

A piece of filter paper (Whatman 1) was placed in a clean dry petri dish. A sterile wood stick was used to pick a well-isolated colony from a fresh (18- to 24-hour culture) nutrient agar plate and rubbed onto a small piece of filter paper. 1 or 2 drops of 1% Kovács oxidase reagent (1% aqueous solution of Tetramethyl para phenylene diamine hydrochloride – T_4M_3-P-Ph-$2NH_2$) was placed on the organism smear. The development of dark purple colour within 5 to10 seconds showed a positive test, while a dark purple within 60 to 90 showed delayed oxidase positive and oxidase negative if the color does not change or it takes longer than 2 minutes.

(b) Catalase test: **Tube method** (Reiner, 2013).

2 ml of 15% H_2O_2 was poured into a 12 x 75-mm test tube (10). Using a wooden applicator stick and being careful not to pick agar, a small amount of organism from a well-isolated 18- to 24-hour colony was collected and place into the test tube. This was placed against a dark background. The production of active bubble (O_2 + water) in the tube showed a positive reaction. No bubble formation (no catalase enzyme to hydrolyze the hydrogen peroxide) represents a catalase-negative reaction.

(c) Urease breath test (UBT) for biopsy specimen:

The patient were given an oral preparation of either non radioisotope carbon -13- ($_{13}$C-) labeled urea. After 30 minutes, the patient breathed into a mass spectrophotometer for $_{13}$C-labeled urea. The measurement was taken.

(d) Urease test: Using a sterile straight wire a colony of the test organism was collected and inoculated into a tube of sterile Motility Indole Urea medium (MIU). An Indole paper strip was placed placed at the neck of the MIU tube above the medium. The tube was stoppered and incubated at 37^{o}C overnight. Red-pink medium showed positive test. Turbidity throughout the medium spreading from the stab-line showed motility. Reddening of the indole paper strip showed indole production.

(e) Nitrate reduction test: 0.5ml of sterile nitrate broth was inoculated with a heavy growth of the test organism. This was incubated at 37^{o}C for 4 hours. One drop of sulphanilic acid reagent and 1 drop of alpha-naphthylamine reagent was added to the inoculated broth. This was mixed by shaking. A red colouration shows positive test.

(f) Sugar fermentation test: The medium was prepared using 1% sugar (fructose, glucose, lactose and galactose) in peptone water. Two drops of 0.01% neutral red indicator was added. After mixing thoroughly, 5ml was dispensed into bijou bottles. The bottles were sterilized by autoclaving at 121^{o}C and 15lb pressure for 15 minutes. A heavy growth of the test organism was inoculated to the bottom of the two bottles using a sterile straight wire. The inoculum in one bottle was covered with 10mm deep layer of sterile paraffin oil. This was incubated at 37^{o}C for 24 to 48 hours. Both tubes showing yellow colour indicated fermentation, but open tube showing yellow and sealed tube showing green indicated oxidation. Gas production was determined from the inverted Durham tube in the broth.

1. **Gram's staining**: Smear was allowed to air dry then fixed by rapidly passing the slide with smear uppermost, three times through the flame of a Bunsen burner. Smear was allowed to cool before covering with crystal violet stain for 60 seconds. Stain was washed with clean water and covered with lugols iodine for 60 seconds. Iodine was washed off with clean water and decolourized with acetone-alcohol for few seconds. Smear was covered with 10% carbol fuchsin for 2 minutes. This was washed off in clean water and placed in a draining rack for the smear to air dry. Smear was examined microscopically using 100x objective (oil immersion). Gram positive bacteria appeared dark purple, Gram negative bacteria was pale to dark red, nuclei of pus cells were pale red. The morphology of bacteria was also noted.

2. **Giemsa staining**: The air dried smear was fixed by covering with methanol for 2-3 minutes. The smear was allowed to air dry. The slide was placed in a petri-dish (supported on each side by a thin piece of stick) with the smear downwards. Diluted Giemsa stain (1 in 20) was poured into the dish and covered with a lid. The inversion avoids stain deposits on smear. The smear was left to stain for 30 minutes. Stain was washed from the dish and smear rinsed with

buffered water. The slide was placed in a draining rack for the smear to air dry. Smear was examined microscopically using x100 objective (oil immersion). Bacteria cell appear blue mauve to purple.

3. Histological technique as decribed by Sood, 2006:

a. Slide preparation

The stages in slide preparation included fixation, dehydration, clearing and dealcoholization, wax impregnation and infilteration, blocking or tissue embedding, sectioning/microtomy, mounting of section on the slide, and staining.

Fixation: The tissue was fixed in 10% formol saline in a container with tight fitting lid for a week.

Dehydration: Water was removed from the tissue by using different grade of alcohol ranging from 50% to absolute alcohol for 30 minutes.

The procedure were-

50% Alcohol	30 minutes
70% Alcohol	30 minutes
90% Alcohol	30 minutes
Absolute Alcohol 1	30 minutes
Absolute Alcohol 11	30 minutes
Absolute Alcohol 111	30 minutes

b. Clearing/Dealcoholization: The dehydrated section of tissue was cleared by removing the alcohol from tissue by immersing it through 3 changes of xylene for 30 minutes each.

c. Wax Impregnation/Infilteration: The cleared tissue was impregnated with wax in the hot oven at a temperature of 60^{o}C by passing it through 3 changes of molten paraffin wax for 30 minutes each.

d. Embedding: The impregnated tissue was embedded with molten paraffin wax in the embedding mould and allowed to solidify.

e. Mounting on wooden block: The paraffin block of tissue was attached to a wooden block with the aid of a hot spatula held in-between wooden blocks and paraffin wax. The spatula melted the wax to enable block attach.

f. Microtomy: The block of tissue was cooled in ice block for easy sectioning which was done using the rotary microtome. Tissue was sectioned at 4 micron. Using a forcep the cut section was placed on a flat surface with 50% alcohol before transferring to the warm water bath set at 10^{o}C below the melting point of wax. The warm water expanded the section and removed folds. The tissue section was picked with a clean slide and labeled.

g. Staining

This was done using the Haematoxylin and Eosin method. The section was dried in the hot plate and allowed to cool before staining. Tissue section was dewaxed by immersion in xylene for 5 minutes. The xylene was removed by rinsing in absolute alcohol, then 90% alcohol, 70% alcohol and 50% alcohol. The tissue section was hydrated by washing in water. The tissue section was stained in haematoxylin for 5 minutes, washed in 2 changes of water, differentiated with 1% acid alcohol, washed in water and blued in running tap water for 5 minutes. The tissue section was conterstaine in Eosin for 2 minutes, washed with running tap water till the excess eosin was removed. The tissue section was dehydrated in alcohol, cleared in xylene and mounted in DPX. This was viewed in the photomicrograph microscope and result showed the nuclei as blue black, while cytoplasm appeared in shade of red or pink.

3.3.8 Distribution of Mice According to the Day of Sacrifice

Using the method by Hamilton-Miller 2003, one hundred and twenty six albino wistar mice were divided into three groups A, B and C (Appendix 7). Mice in groups A were the negative control, group B were the positive control while group C were the test. Group A had forty two mice, group B had forty two mice and group C had forty two mice. Six out of the forty two mice from each group were sacrificed on day ten post inoculation with *H. pylori*. This was repeated on days fourteen, twenty one, twenty eight, thirty five and sixty. One inoculated mouse from each group was reserved on each day of sacrifice.

3.3.9 Inoculation of Mice with *H.pylori*

The mice in group B and C were inoculated intragastrically with 1 ml of suspension (bacterial inoculum) prepared with fresh *Helicobacter pylori* bacteria on two consecutive days after fasting overnight, with urethral pediatric probe N 04. Group A was inoculated using the same technique but with 1 ml of Brain Heart Infusion broth. Treatment with a probiotic strain of *Lactobacillus acidophilus* was initiated after 1 week post challenge with *Helicobacter pylori* strain in mice of group C. 6 animals from each group were killed by spinal dislocation at 10, 14, 21, 28, 35 and 60 days post inoculation. The stomach and duodenum were collected, washed with sterile saline, and divided for histopathological studies. The histopathological changes noted and evaluated were the site of inflammation, its composition,intensity, level of the infiltrate in the gastric and duodenal wall, the presence of erosions or ulceration and the presence of gastritis and/or duodenitis and the eradication rate of *Helicobacter pylori.* 6 smears from group B were also stained by Giemsa and were viewed

using oil immersion (x1000) to assess the presence of *H. pylori* in the stomach and duodenum.

3.3.9 Preparation of Probiotic (see Appendix 8)

3.4.0 Treatment of Mice Using Probiotic Drink

Treatment of mice in Group C with probiotic drink was started after a week of infection with *H. pylori*. The mice were fed only on probiotic drinks in a drinking dish. The concentration of probiotic fed was 5×10^9cfu/ml at night.

3.4.1 Preparation of culture media (See Appendix 9)

3.4.2 Statistical Analysis

Data was analyzed using SPSS, version 20.0 (SPSS Inc. Chicago Illinois). Results were presented as number (percentages) for qualitative variables. Categorical variables were compared with Pearson's Chi square. Significant P-value was taken as <0.05.

4.0 RESULTS

Result of *H. pylori* Blood Antibody Test Result in Male and Female Patients in Age Groups 18-40 years and 41-75 years.

From Table 1, the blood antibody test showed that seven (14.0%) males and seventeen (34.0%) females were positive for *H. pylori* infection in age group 18-40 years, while Table 2, shows that thirteen (26.0%) males and twenty (40.0%) females were positive in age group 41-75 years. In the two age groups, a total of fifty seven (28.5%) Patients tested positive to *H. pylori* blood antibody test, while one hundred and forty three (71.5%) were negative (Tables 1 and 2).

Result of *H. pylori* Stool Antigen Test Result in Male and Female Patients in Age Group 18-40years and 41-75 years.

In the stool antigen test, ten (20.0%) males and twenty two (44.0%) females were positive for *H. pylori* infection in the age group 18-40 years (Table 3), while thirteen (26.0%) males and twenty six (52%) females were positive in age group 41-75 years (Table 4). In the two age groups, a total of seventy one (35.5.0%) Patients were positive for *H. pylori* stool antigen test, while one hundred and twenty nine (64.5%) were negative (Tables 3 and 4).

Result of Fecal Occult Blood Test Result in Male and Female Patients in Age Group 18-40years and 41-75 years.

The fecal occult blood test showed that ten (20.0%) males and twelve (24.0%) females were positive for *H. pylori* infection in age group 18-40 years (Table 5), while eight (16.0%) males and ten (20.0%) females were positive in age group 41-75 years (Table 6). In the two age groups, a total of forty (20.0%) Patients were positive for fecal occult blood test while one hundred and sixty Patients (80.0%) were negative (Table 5 and 6).

Comparison of the *H. pylori* Blood Antibody Test Result, *H. pylori* Stool Antigen Test Result, and Fecal Occult Blood Test Result.

The *H. pylori* blood antibody test result, *H. pylori* stool antigen test result, and fecal occult blood test result was compared in Table 7. The result shows that out of the two hundred persons tested, fifty seven (28.5%) were positive for *H. pylori* blood antibody, seventy one (35.5%) were positive for *H. pylori* stool antigen, while forty (20.0%) were positive for fecal occult blood. Although there was a numerical difference, the result was not statistically significant ($P>0.05$).

Table 1: Result of *H. pylori* Blood Antibody Test Result in Male and Female Patients in Age Group 18-40years

Sex	18 – 40 years		Total	$\chi 2$	p-value
	Positive	Negative			
Male	7 (14.0%)	43 (86.0%)	50 (100. 0%)	5.482	0.019
Female	17 (34.0%)	33 (66.0%)	50 (100.0%)		
Total	24 (24.0%)	76 (76.0%)	100 (100.0%)		

Table 2: Result of *H. pylori* Blood Antibody Test Result in Male and Female Patients in Age Group 41-75years

Sex	41 – 75 years		Total	$\chi 2$	p-value
	Positive	Negative			
Male	13 (26.0%)	37 (74.0%)	50 (100.0%)	2.216	0.137
Female	20 (40.0%)	30 (60.0%)	50 (100.0%)		
Total	33 (33.0%)	67 (67.0%)	100 (100.0%)		

Table 3: Result of *H. pylori* Stool Antigen Test Result in Male and Female Patients in Age Group 18-40years

Sex	18 – 40 years		Total	$\chi 2$	p-value
	Positive	Negative			
Male	10 (20.0%)	40 (80.0%)	50 (100.0%)	6.618	0.010
Female	22 (44.0%)	28 (56.0%)	50 (100.0%)		
Total	32 (32.0%)	68 (68.0%)	100 (100.0%)		

Table 4: Result of *H. pylori* Stool Antigen Test Result in Male and Female Patients in Age Group 41-75years

Sex	41-75years		Total	$\chi 2$	p-value
	Positive	Negative			
Male	13 (26.0%)	37 (74.0%)	50 (100.0%)	7.104	0.008
Female	26 (52.0%)	24 (48.0%)	50 (100.0%)		
Total	39 (39.0%)	61 (61.0%)	100 (100.0%)		

Table 5: Result of Fecal Occult Blood Test Result in Male and Female Patients in Age Group 18-40years

Sex	18 – 40 years		Total	$\chi 2$	p-value
	Positive	Negative			
Male	10 (20.0%)	40 (80.0%)	50 (100.0%)	0.233	0.629
Female	12 (24.0%)	38 (76.0%)	50 (100.0%)		
Total	22 (22.0%)	78 (78.0%)	100 (100.0%)		

Table 6: Result of Fecal Occult Blood Test Result in Male and Female Patients in Age Group 41 - 75years

Sex	41 – 75 years		Total	$\chi 2$	p-value
	Positive	Negative			
Male	8 (16.0%)	42 (84.0%)	50 (100.0%)	0.271	0.603
Female	10 (20.0%)	40 (80.0%)	50 (100.0%)		
Total	18 (18.0%)	82 (82.0%)	100 (100.0%)		

Table 7: The positivity of *H. pylori* Blood Antibody Test Result, *H. pylori* Stool Antigen Test Result, and Fecal Occult Blood (FOB) Test Result

Parameters	Antibody Test	Antigen Test	FOB Test	$\chi 2$	P-value	Decision
Age Group						
18 – 40years	24 (24.0%)	32 (32.0%)	22 (22.0%)	1.663	0.435	Not
41 – 75years	33 (33.0%)	39 (39.0%)	18 (18.0%)			significant
Total	57 (28.5%)	71 (35.5%)	40 (20.0%)			
Sex						
Male	20 (20.0%)	23 (23.0%)	18 (18.0%)	1.814	0.404	Not
Female	37 (37.0%)	48 (48.0%)	22 (22.0%)			significant
Total	57 (28.5%)	71 (35.5%)	40 (20.0%)			

Measure of Agreement of *H. pylori* Blood Antibody Test Result and *H. pylori* Stool Antigen Test Result.

From Table 8, the measure of agreement between *H. pylori* blood antibody test and *H. pylori* stool antigen test showed that a total of fifty seven (28.5%) patients were positive for *H. pylori* blood antibody test while seventy one (35.5%) patients were positive for *H. pylori* stool antigen test. This implies that fourteen (7.0%) patients who tested positive for *H. pylori* stool antigen test were negative to *H. pylori* blood antibody test. The measure of agreement between the blood antibody test and the stool antigen test were the 57 patients who tested positive to both tests. This was significant ($P<0.05$), indicating that *H. pylori* stool antigen test was more sensitive than the *H. pylori* blood antibody test.

Measure of Agreement of *H. pylori* Blood Antibody Test Result and Fecal Occult Blood Test Result.

The measure of agreement of *H. pylori* antibody test result and fecal occult blood test in Table 9, shows that fifty seven (28.5%) patients were positive for *H. pylori* blood antibody test while forty (20.0%) patients were positive for fecal occult blood test. Seventeen (8.5%) patients who were positive for *H. pylori* blood antibody test were negative to fecal occult blood test. The results showed forty (20.0%) patients having a positive significant agreement in the two tests ($P<0.05$). Therefore, *H. pylori* blood antibody test was more sensitive than fecal occult blood test (Table 9).

Measure of Agreement of *H. pylori* Stool Antigen Test Result and Fecal Occult Blood Test Result.

From Table 10, the measure of agreement between *H. pylori* stool antigen test and Fecal occult blood test showed that a total of seventy one (35.5%) patients were positive for *H. pylori* stool antigen test while forty (20.0%) patients were positive for fecal occult blood test. This implies that thirty one (15.5%) patients who tested positive for *H. pylori* stool antigen test were negative to fecal occult blood test. The measure of agreement between the *H. pylori* stool antigen test and the fecal occult blood test were the 40 (20.0%)) patients who tested positive to both tests. This was significant ($P<0.05$), indicating that *H. pylori* stool antigen test was more sensitive than the fecal occult blood test.

Table 8: Measure of Agreement of *H. pylori* Blood Antibody Test Result and *H. pylori* Stool Antigen Test Result

		Positive	**Negative**	**Total**	**Kappa**	**P-value**	**Decision**
H. pylori Antibody Test	*H. pylori* Antigen Test						
	Positive	57(28.5%)	0 (0.0%)	57(28.5%)			
	Negative	14(7.0%)	129(64.5%)	143(71.5%)	0.625	0.000	Significant
	Total	71 (35.5%)	129(64.5%)	200(100.0%)			

Table 9: Measure of Agreement of *H. pylori* Blood Antibody Test Result and Fecal Occult Blood Test Result

Age Group		Positive	Negative	Total	Kappa	P-value	Decision
Fecal Occult Blood Test	*H. pylori* Blood Antibody Test						
	Positive	40 (20.0%)	0 (0.0%)	40 (20.0%)			
	Negative	17(8.5%)	143 (71.5%)	160(71.0%)	0.625	0.000	Significant
	Total	57 (28.5%)	143(71.5%)	200 (100.0%)			

Table 10: Measure of Agreement of *H. pylori* Stool Antigen Test Result and Fecal Occult Blood Test Result

Age Group		Positive	Negative	Total	Kappa	P-value	Decision
		H. pylori Stool Antigen Test					
Fecal Occult Blood	Positive	40 (20.0%)	0(0.0%)	40 (20.0%)			
	Negative	31 (15.5%)	129 (64.5%)	160 (80.0%)	0.633	0.008	Significant
	Total	71 (35.5%)	129(64.5%)	200 (100%)			

Isolation of *Helicobacter pylori*

Fresh stool specimens collected from the seventy (71) persons who tested positive to *H. pylori* stool antigen test as shown in Table 7 were cultured for the isolation of *H. pylori*. Also, one (1) gastric biopsy from a dyspepetic patient who tested positive to both *H. pylori* blood antibody test and *H. pylori* stool antigen test was also cultured for the isolation of *H. pylori*. None of the stool specimens yielded any growth, but the biopsy specimen yielded the growth of an organism which was identified as shown in Table 11 using biochemical, morphological and physiological attributes. The isolate was gray in colour, circular, translucent, 2 mm in size and Gram negative spiral organism. It was motile and reacted positively to catalase, oxidase, urease. It did not reduce nitrate and was resistant to Nalidixic acid. Further identification was done using PCR and DNA sequencing by Macrogen Laboratory USA (see Table 13 and Appendix 5). The isolate was identified as *H. pylori*.

Furthermore, a portion of the stomach and duodenum of the infected mice in group B (positive control) was cultured and there was growth. The isolate was identified using the morphological and biochemical characteristics as recorded in Table 11.

Isolation of *Lactobacillus acidophilus* from Stool

The isolate from infant feaces was identified from the colonial, morphology and biochemical tests as shown in Table 12. The isolate which was white, Gram positive rods, 2mm in size, reduced nitrate, grew at 4.5% Nacl, reacted negatively to urease, indole, catalase, coagulase, and oxidase test. Fructose, lactose, galactose was fermented with the production of acid but glucose was not fermented. Further identification was done using PCR and DNA sequencing by Macrogen Laboratory USA (see Table 13 and Appendix 6). The isolate was identified as *Lactobacillus acidophilus.*

DNA Sequence of Isolated Organism

This was done by Macrogen Laboratory Maryland, USA. Result is shown in Table 13 and Appendix 5 and 6. Isolate A1 was identified as *Helicobacter pylori,* while B1 was *Lactobacillus acidophilus*.

Table 11: Biochemical, Morphological and Physiological Attributes of *H. pylori* Isolate

S.No	Test	Result
1	Colour	Gray
2	Size	2mm
3	Gram Stain	Gram Negative
4	Shape	Helical and Spiral Rod Shaped
5	Motility	Motile
6	Catalase	Positive
7	Oxidase	Positive
8	Urease	Strongly Positive
9	Nitrate Reduction	Negative
10	Nalidixic Acid Sensitivity	Resistance
	Isolate Identification :	*Helicobacter pylori*

Table 12: Biochemical, Morphological and Physiological Attributes of *L.acidophilus* Isolate

S.No	Test	Result
1	Colour	White
2	Size	2mm
3	Gram Stain	Gram Positive
4	Shape	Rod Shaped
5	Motility	Non Motile
6	Indole	Negative
7	Urease	Negative
8	Catalase	Negative
9	Coagulase	Negative
10	Nitrate Reduction	Positive
11	Growth at 4.5% Sodium Chloride	Positive
12	Glucose Fermentation	Negative
13	Fructose Fermentation	Positive , Acidic, No Gas
14	Galactose Fermentation	Positive, Acidic, No Gas
15	Lactose Fermentation	Positive, Acidic, No Gas
	Isolate Identification:	*Lactobacillus acidophilus*

Table 13: The DNA Sequence of *Helicobacter pylori* and *Lactobacillus acidophilus*

	Label	Length			
		Normal	QV≥16	QV≥20	GC%
A1	*Helicobacter pylori* F	942	941	941	55.0
	Helicobacter pylori R	749	739	732	55.0
B1	*Lactobacillus acidophilus F*	947	107	92	50.0
	Lactobacillus acidophilus R	747	176	165	52.0

Weekly Weight of Mice

Mice in group A (negative control), group B (positive control) and group C (test) were weighed weekly and the average weight recorded in Table 14. The difference in the average weight of the mice in the three groups was not significant in the second and third week ($P>0.05$). Subsequently, there was a significant difference in the weight of mice in groups A and B, groups A and C and in groups B and C from the fourth week to the ninth week ($P<0.05$). Mice in group A had a sequential addition in weight of an average of 23.7g per week, while those in groups B had 12.5g and C had 15.0g (Appendix 10).

Table 14: Weekly Weight of Mice

Weeks	**Average Weight (g)**					**P-value**	**Decision**
	Group A	Group B	Group C	Difference A &B	Difference A &C		
2weeks	54.4±6.2	52.5±8.4	54.4±7.6	1.9	0	0.421	Not significant
3weeks	79.0±7.9	77.6±8.2	79.4±8.2	1.4	-0.4	0.574	Not significant
4weeks	104.2±9.2	95.5±8.3	95.2±9.9	8.5	8.8	0.000	Significant
5weeks	128.7±10.0	107.7±8.5	104.0±9.9	21.0	24.0	0.000	Significant
6weeks	156.0±12.4	116.7±10.7	125.7±10.2	39.3	30.3	0.000	Significant
7weeks	180.8±12.6	123.2±13.3	147.7±13.0	57.6	33.1	0.000	Significant
8weeks	205.0±12.9	127.1±15.2	154.2±14.0	77.9	50.8	0.000	Significant
9weeks	230.0±11.5	129.9±18.8	159.5±15.0	100.1	70.5	0.000	Significant

Comparison of the Histopathological Changes in the Stomach and Duodenum of Mice Infected with *H. pylori*

From figure 1, the photomicrograph of the stomach of group A mice at ten days post inoculation with *H. pylori* shows normal gastric epithelium and creptes, group B mice shows that the normal architecture of the stomach was changing to dysplastic epithelium with increase in the number of cells (hyperplasia), while group C mice shows the regneration of the stomach epithelium in some areas with partial removal of the hyalinized dysplastic epithelium.

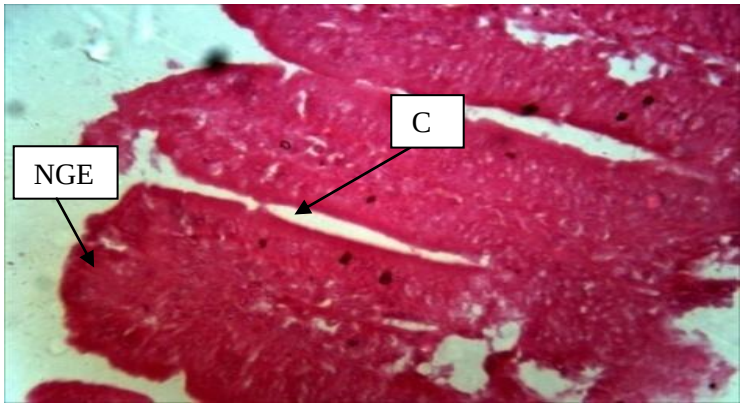
Figure 2 shows the result of the changes in the duodenum of mice of group A, B and C at day ten. The duodenum of group A shows normal duodenal epithelium, group B shows mild proliferation of the duodenal epithelium with the cells still distinct and relatively healthy, while group C shows that the duodenal epithelium were being regenerated.

From figure 3, the photomicrograph of the stomach of group A mice at fourteen days post inoculation with *H. pylori* shows normal gastric epithelium architecture which was well perfused, group B mice shows that the tissues were being destroyed by the organisms injected making the cells indstinct, matted and necrotic, while group C mice shows some focal area of necrosis evidenced by loss of tissue but the epithelium was undergoing regeneration.

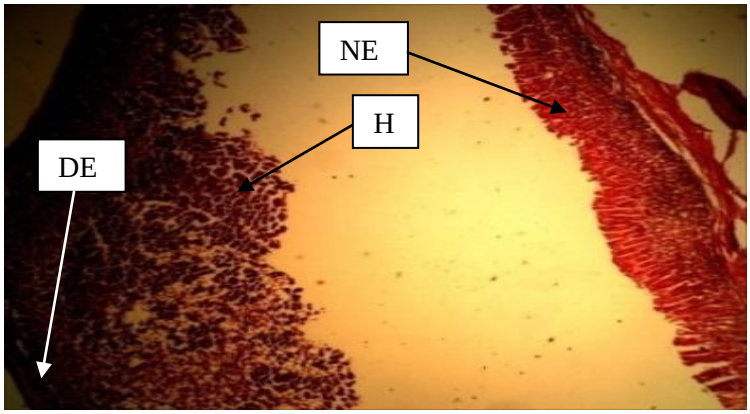
From figure 5, the photomicrograph of the stomach of group A mice at twenty one days post inoculation with *H. pylori* shows normal gastric epithelium and creptes, group B mice shows that one side of the epithelium was grossly matted while the other side had cellular debris which came out of a flushed epithelium, while group C mice shows that the necrotic tissue was being replaced by regenerating gastric epithelium.

Figure 6 shows the photomicrograph of the changes in the duodenum of mice of group A, B and C at day twenty one. The duodenum of group A shows normal duodenal epithelium, group B shows the duodenum having matted cells in some areas and partially distinct cells in others, however, there was necrosis, while group C shows that the duodenum was regenerating at certain parts with the replacement of the normal folding.

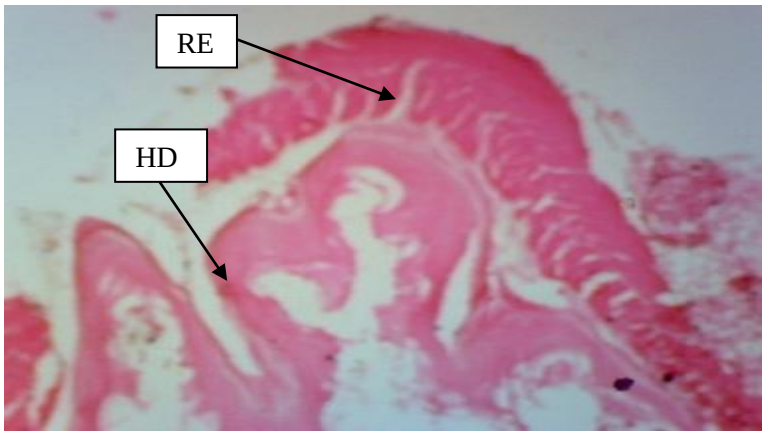
From figure 7, the photomicrograph of the stomach of group A mice at twenty eight days post inoculation with *H. pylori* shows normal gastric epithelium that was well perfused, group B mice shows the thinning out of the gastric epithelium, while group C mice shows that the gastric epithelium was well regenerated.



$A_{10}S$ (Negative control) x400



$B_{10}S$ (Positive control) x40



$C_{10}S$ (Test) x60

Figure 1: Day 10 A, B, and C stomach stained with HxE

Key:

NGE = Normal Gastric Epithelium

NE = Normal epithelium

DE = Dysplastic epithelium

NE = Normal epithelium

C = Crepte

H = Hyperplasia

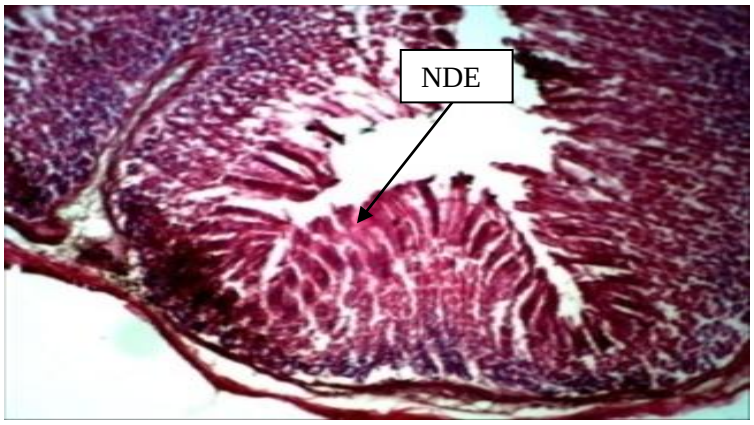
RE = Regenerated epithelium

HD = Hyalinized dysplastic epithelium

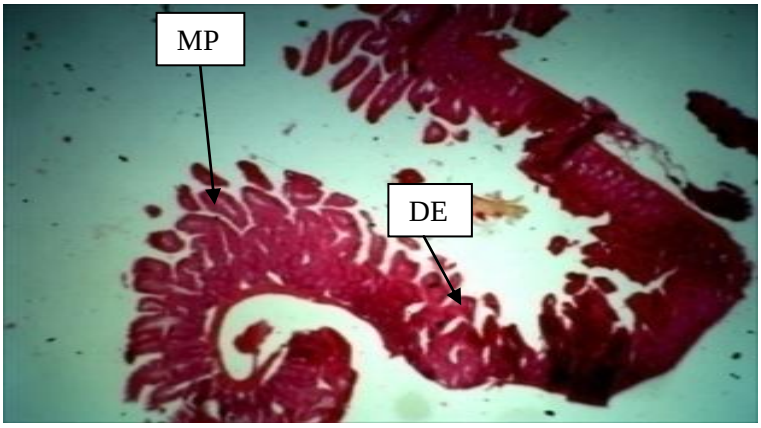
$A_{10}S$= Group A stomach at 10 days

$B_{10}S$= Group B stomach at 10 days

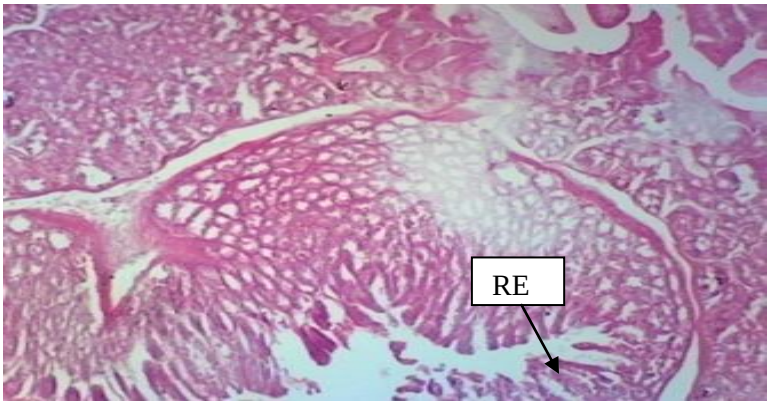
$C_{10}S$ = Group C stomach at 10 days



$A_{10}D$ (negative control) x100



$B_{10}D$ (positive control) x100



$C_{10}D$ (test) x150

Figure 2: Day 10 A, B, and C duodenum stained with HxE

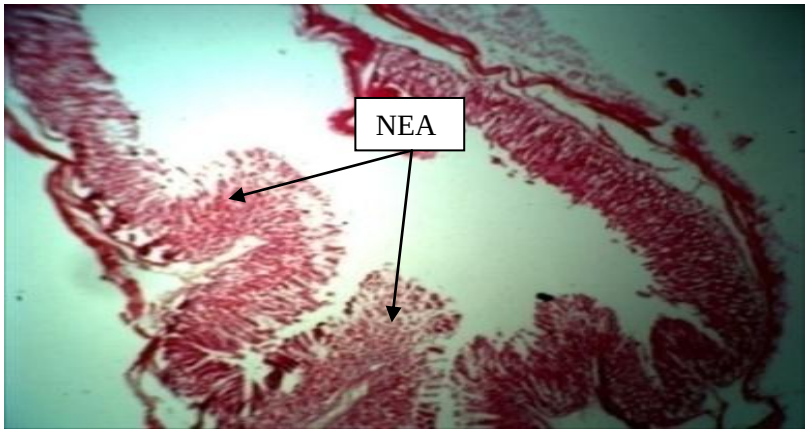
Key:

NDE = Normal duodenal epithelium MP = Mild proliferation

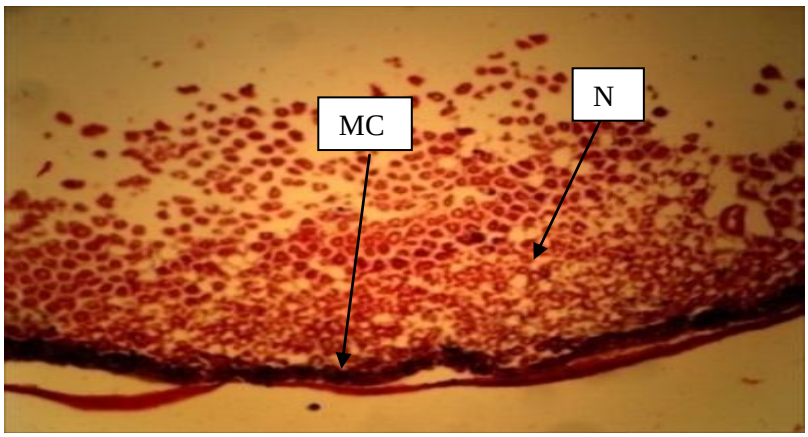
DE = Duodenal epithelium RE = Regenerated epithelium

$A_{10}D$ = Group A duodenum at 10 days $B_{10}D$ = Group B duodenum at 10 days

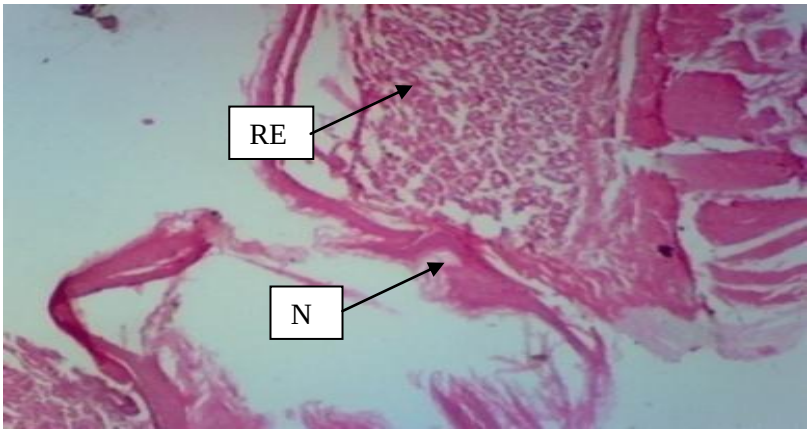
$C_{10}D$ = Group C duodenum at 10 days



$A_{14}S$ (Negative control) x400



$B_{14}S$ (Positive control) x100



$C_{14}S$ (Test) x150

Figure 3: Day 14 A, B and C stomach stained with HxE

Key:

NEA = Normal Epithelial Architecture

MC = Matting of cells

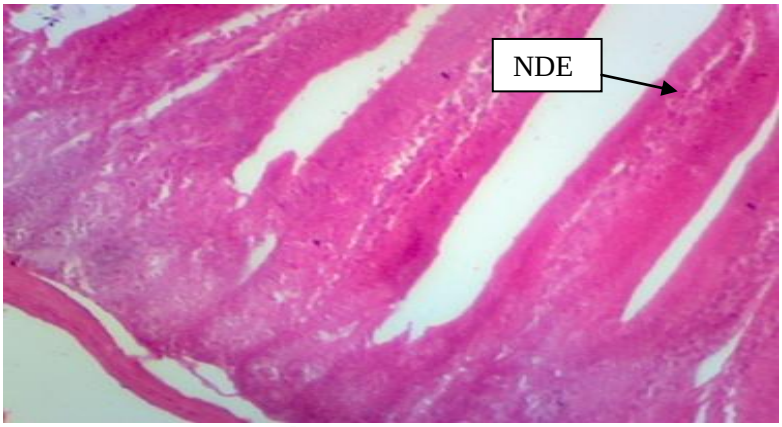
N = Necrosis

RE = Regenerating epithelium

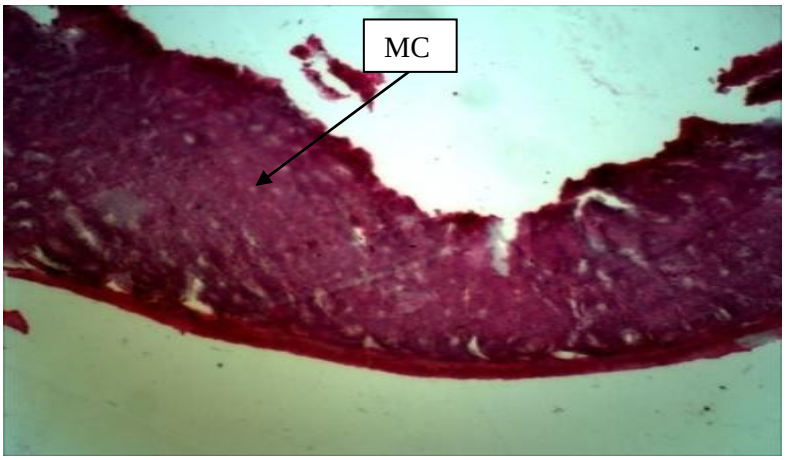
$A_{14}S$= Group A stomach at 14 days

$B_{14}S$= Group B stomach at 14 days

$C_{14}S$ = Group C stomach at 14 days



$A_{14}D$ (Negative control) x400



$B_{14}D$ (Positive control) x100

$C_{14}D$ (Test) x60

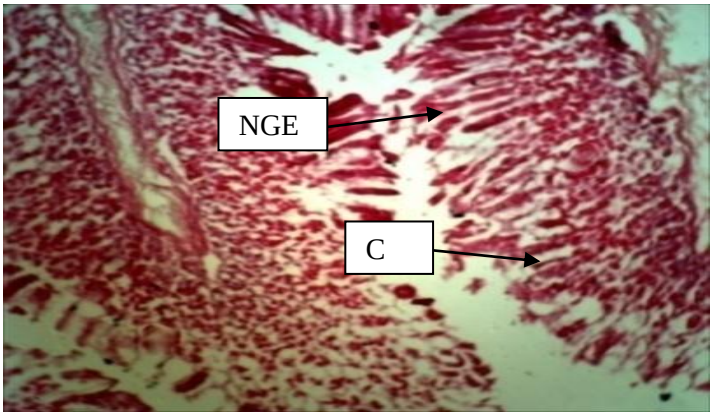
Figure 4: Day 14 A, B, and C duodenum stained with HxE

Key:

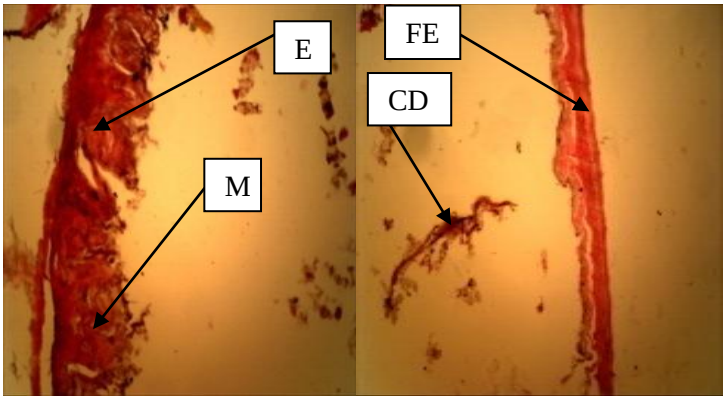
NDE = Normal Duodenal Epithelium MC = Matted cells

RDE = Regeneration of the Duodenal Epithelium $A_{14}D$ = Group A duodenum at 14 days

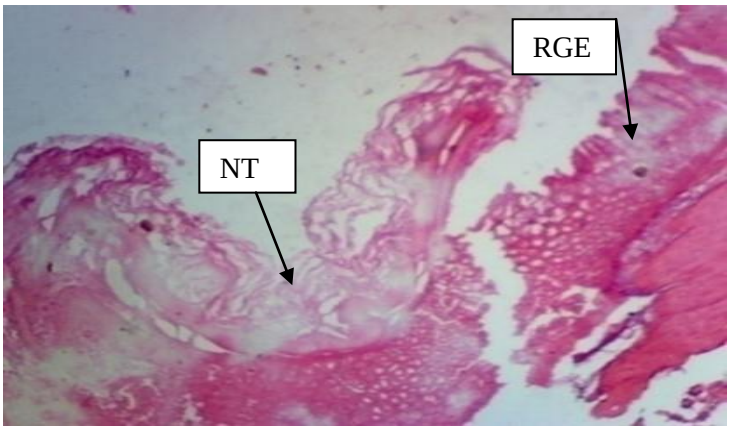
$B_{14}D$ = Group B duodenum at 14 days $C_{14}D$ = Group C duodenum at 14 days



$A_{21}S$ (Negative control) x400



$B_{21}S$ (Positive control) x100



$C_{21}S$ (Test) x150

Figure 5: Day 21 A, B and C stomach stained with HxE

Key:

NGE = Normal Gastric Epithelium

C = Creptes

E = Epithelium

M = Matting

CD = Cellular Debris

FE = Flushed Epithelium

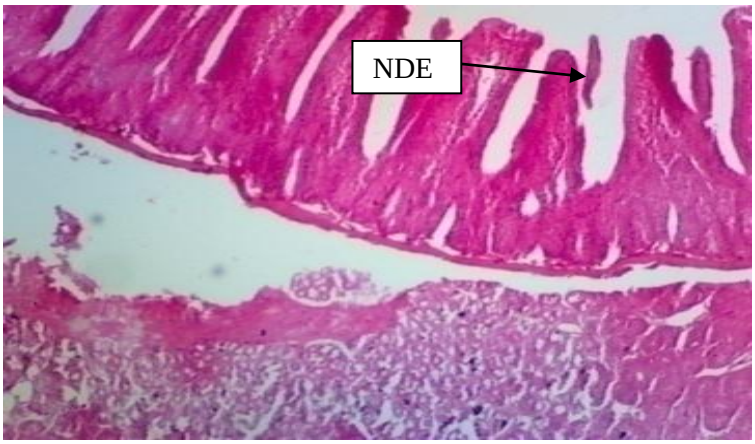
NT = Necrotic tissue

RGE = Regenerating gastric epithelium

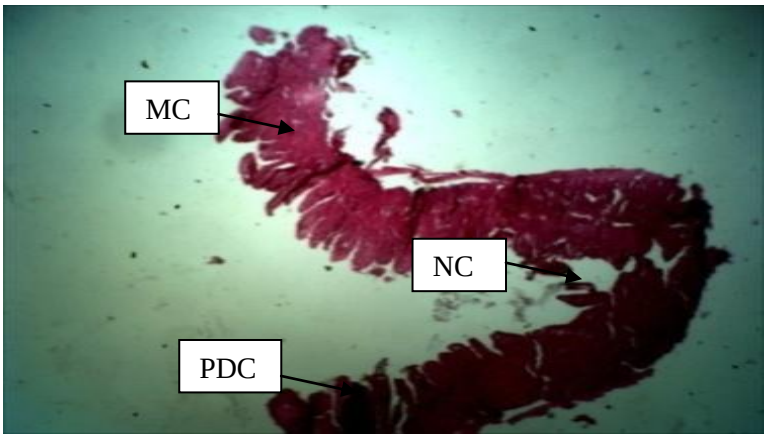
$A_{21}S$= Group A stomach at 21 days

$B_{21}S$= Group B stomach at 21 days

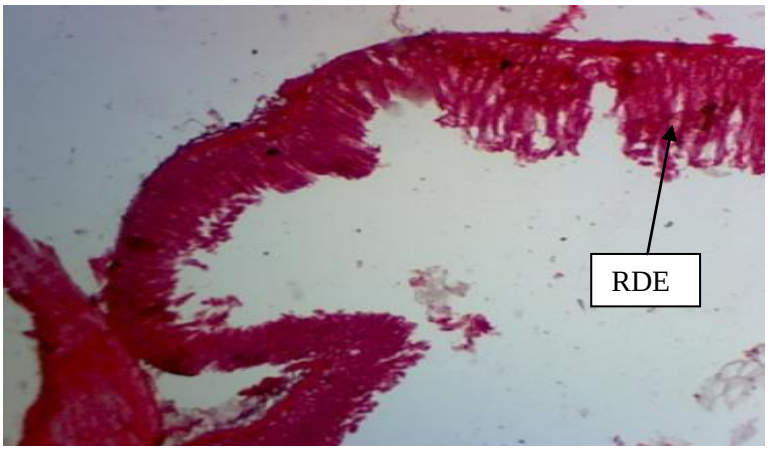
$C_{21}S$ = Group C stomach at 21 days



$A_{21}D$ (Negative control) x400



$B_{21}D$ (Positive control) x40



$C_{21}D$ (Test) x60

Figure 6: Day 21 A, B and C duodenum stained with HxE

Key:

NDE = Normal duodenal epithelium

MC = Matted cells

NC = Necrotic change

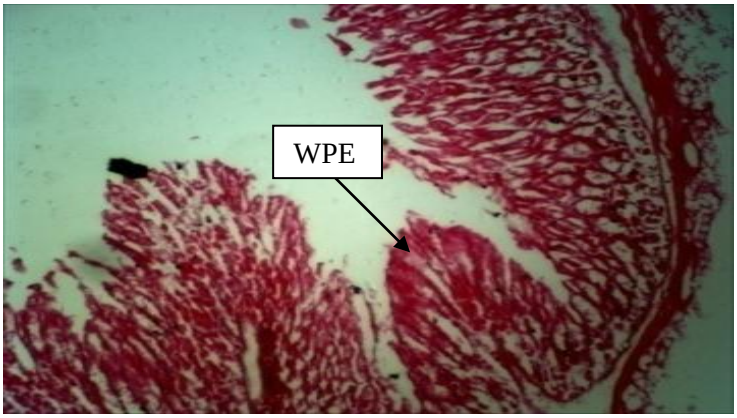
PDC = Partially distinct cells

RDE= Regenerated duodenal epithelium

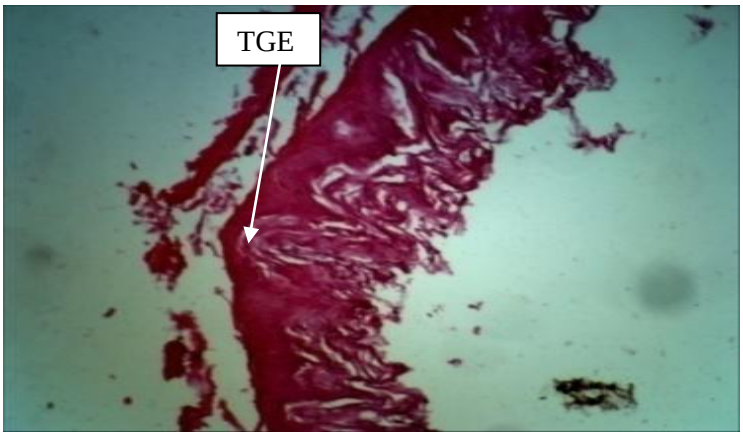
$A_{21}D$ = Group A duodenum at 21 days

$B_{21}D$ = Group B duodenum at 21 days

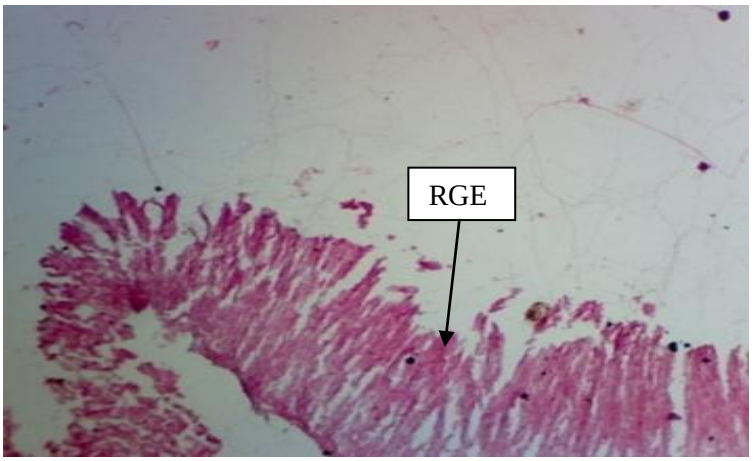
$C_{21}D$ = Group C duodenum at 21 days



$A_{28}S$ (Negative control) x400



$B_{28}S$ (Positive control) x100



$C_{28}S$ (Test) x60

Figure 7: Day 28 A, B and C stomach stained with HxE

Key:

WPE = Well perfused epithelium

TGE = Thin Gastric epithelium

RGE= Regenerated Gastric Epithelium

$A_{28}S$= Group A stomach at 28 days

$B_{28}S$= Group B stomach at 28 days

$C_{28}S$ = Group C stomach at 28 days

Figure 8 shows the photomicrograph of the changes in the duodenum of mice of group A, B and C at day twenty eight. The duodenum of group A shows normal duodenal epithelium, group B shows coagulative necrosis of the duodenal epithelium with the cell having no nucleus and appearing as a ghost of itself, while group C shows that duodenal epithelium has completely returned to normal.

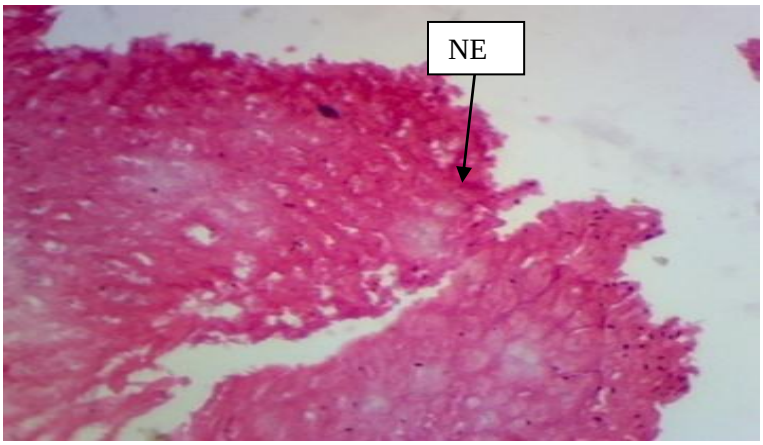
From figure 9, the photomicrograph of the stomach of group A mice at thirty five days post inoculation with *H. pylori* shows normal gastric epithelium, group B mice shows that the thinned gastric epithelium have some areas with extensive fibrocollagenous hyalinization of the submucousal area, while group C mice shows shows thinning of the gastric epithelium with some areas of necrosis amid cell regeneration.

Figure 10 shows the photomicrograph of the changes in the duodenum of mice of group A, B and C at day thirty five. The duodenum of group A shows normal duodenal epithelium, group B shows coagulative necrosis of the duodenal epithelium, while group C shows that duodenal epithelium was well regenerated, even though there was thinning of the duodenal wall.

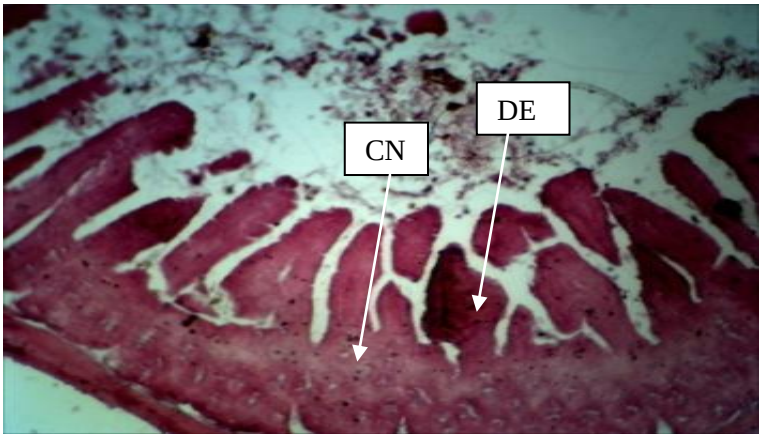
From figure 11, the photomicrograph of the stomach of group A mice at sixty days post inoculation with *H. pylori* shows normal gastric epithelium, group B mice shows that the the gastric epithelium was completely thinned out with areas of necrosis and converted to fibrotic tissue, while group C mice shows shows thinning of the gastric epithelium amid cell regeneration.

Figure 12 shows the photomicrograph of the changes in the duodenum of mice of group A, B and C at day sixty. The duodenum of group A shows normal duodenal epithelium, group B shows coagulative necrosis of the duodenal epithelium with the nucleus appearing ghost-like, while group C shows that duodenal epithelium was well regenerated with focal area of necrosis.

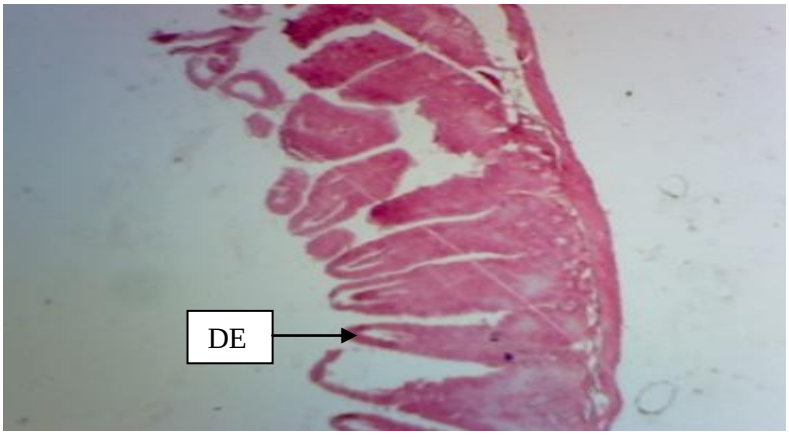
The gastric tissue from representatives of Mice A (Negative control) and Mice B (Positive control) were stained with Giemsa as shown in figure 13. The result revealed a comma shaped organism in the crepte of mice in Group B, while there was none in group A.



$A_{28}D$ (Negative control) x100



$B_{28}D$ (Positive control) x100



$C_{28}D$ (Test) x60

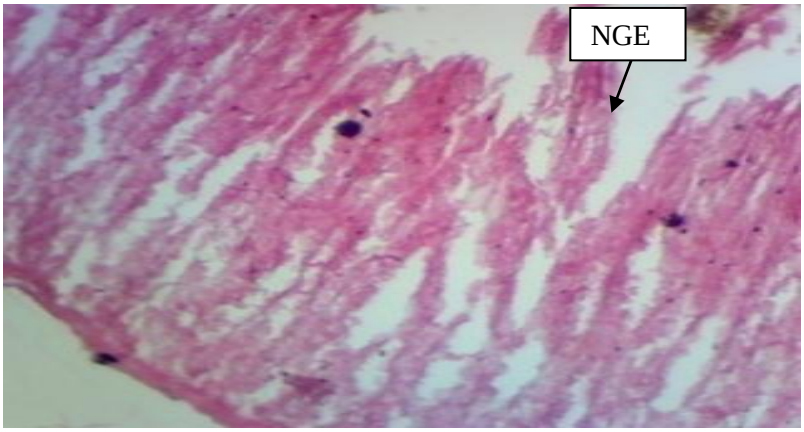
Figure 8: Day 28 A, B and C duodenum stained with HxE

Key:

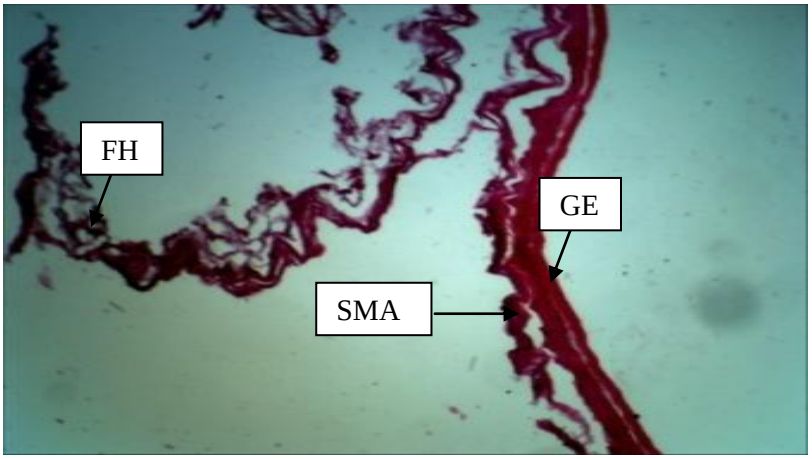
NE = Normal epithelium DE = Duodenal epithelium

CN = Coagulative necrosis $A_{28}D$ = Group A duodenum at 28 days

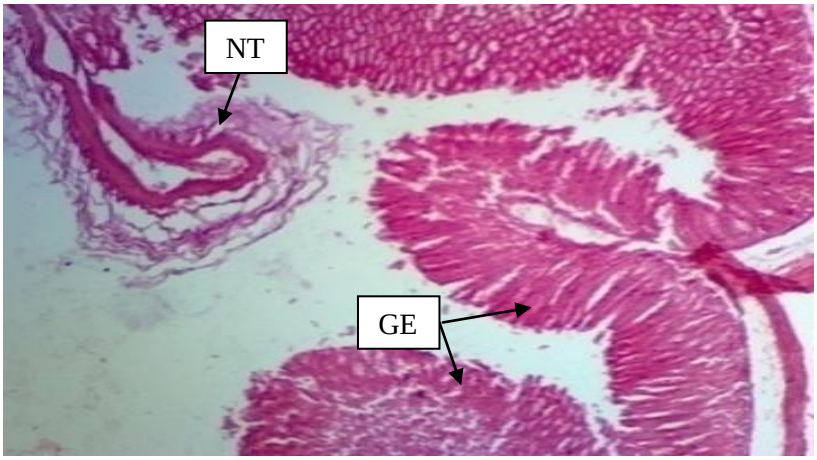
$B_{28}D$ = Group B duodenum at 28 days $C_{28}D$ = Group C duodenum at 28 days



$A_{35}S$ (Negative control) x150



$B_{35}S$ (Positive control) x100



$C_{35}S$ (Test) x150

Figure 9: Day 35 A5 stomach (Negative control) x150 stained with H&E

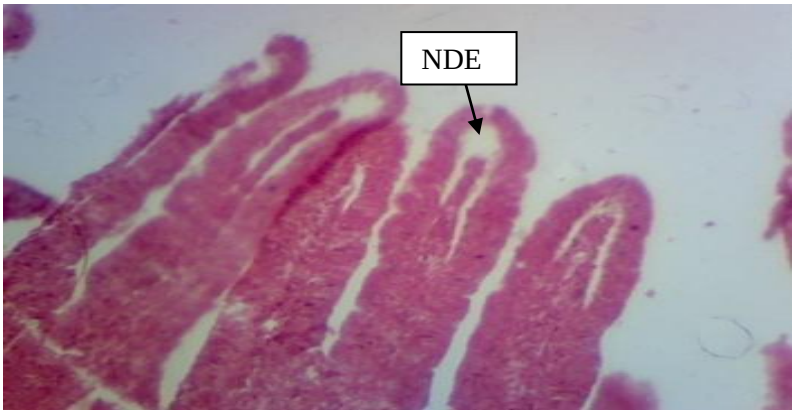
Key:

NGE = Normal gastric epithelium FH= Fibrocollagenous hyalinization

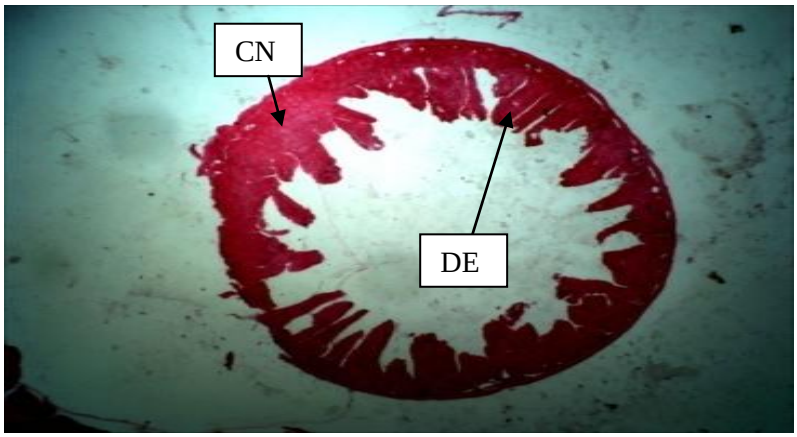
GE= Gastric epithelium SMA= Submucousal area

NT= Necrotic tissue $A_{35}S$= Group A stomach at 35 days

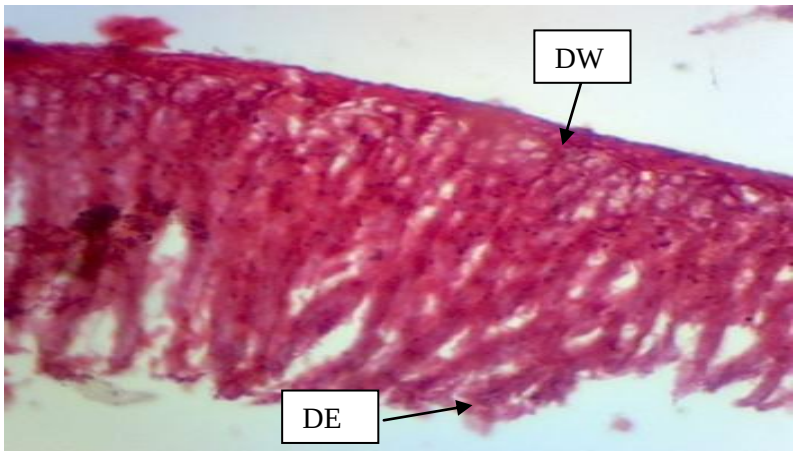
$B_{35}S$= Group B stomach at 35 days $C_{35}S$ = Group C stomach at 35 days



$A_{35}D$ (Negative control) x150



$B_{35}D$ (Positive control) x40



$C_{35}D$ (Test) x150

Figure 10: Day 35 A5, B5 and C5 duodenum stained with H&E

Key:

NDE = Normal duodenal epithelium

CN = Coagulating necrosis

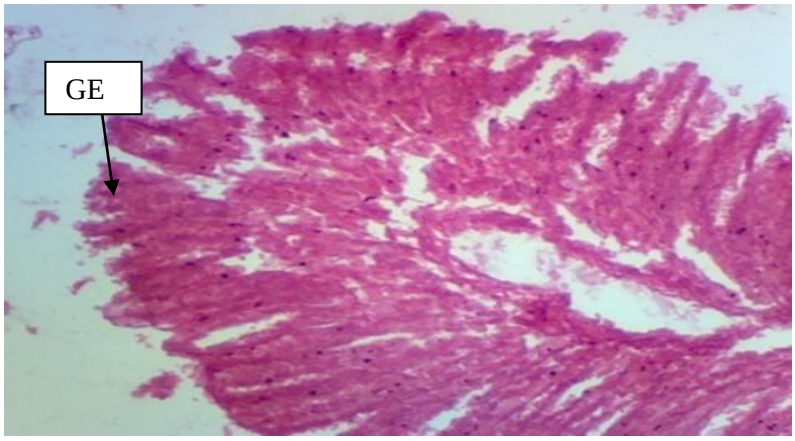
DE = Duodenal epithelium

DW= Duodenal wall

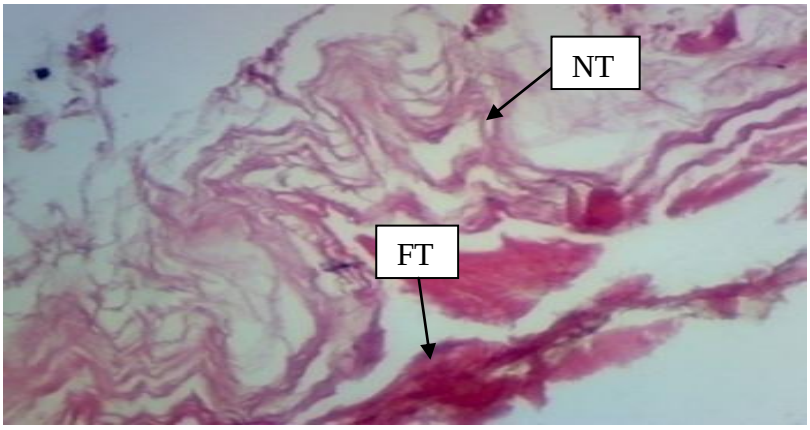
$A_{35}D$ = Group A duodenum at 35 days

$B_{35}D$ = Group B duodenum at 35 days

$C_{35}D$ = Group C duodenum at 35 days



$A_{60}S$ (Negative control) x150



$B_{60}S$ (Positive ontrol) x150

$C_{60}S$ (Test) x150

Figure 11: Day 60 A6, B6 and C6 stomach stained with H&E

Key:

GE = Gastric epithelium

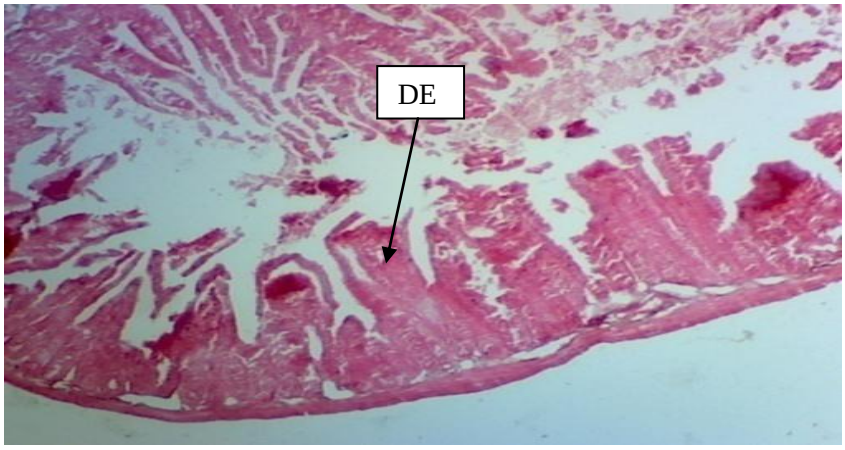
NT = Necrotic tissue

FT = Fibrotic tissue

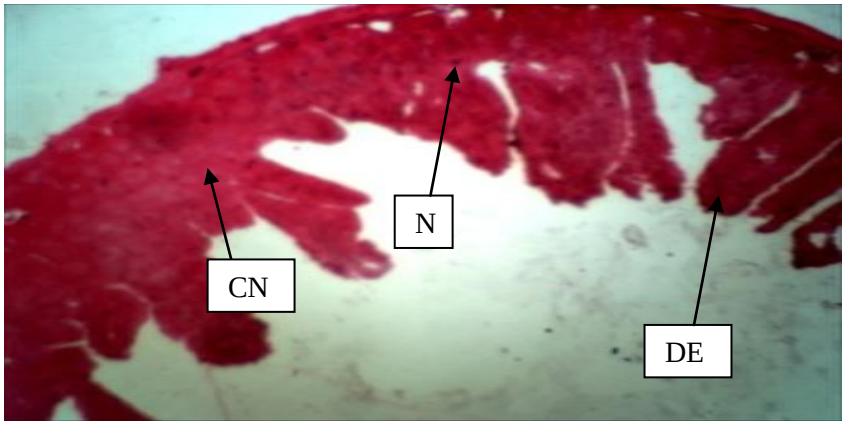
$A_{60}S$= Group A stomach at 60 days

$B_{60}S$= Group B stomach at 60 days

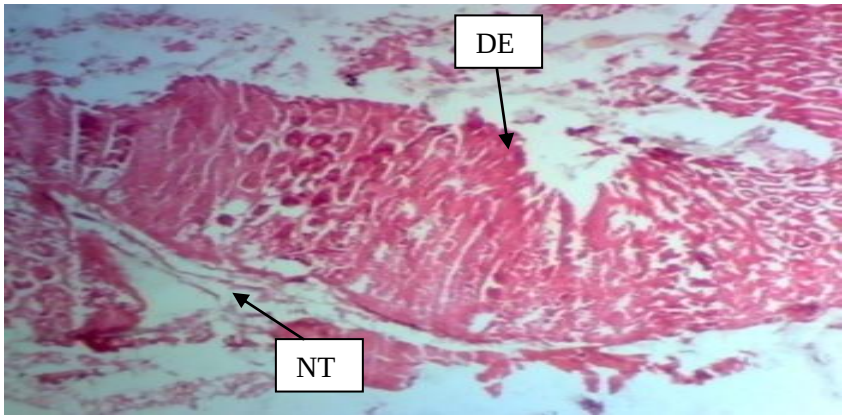
$C_{60}S$ = Group C stomach at 60 days



$A_{60}D$ (Negative control) x60



$B_{60}D$ (Positive control) x100



$C_{60}D$ (Test) x60

Figure 12: Day 60 A6, B6, C6 duodenum stained with H&E

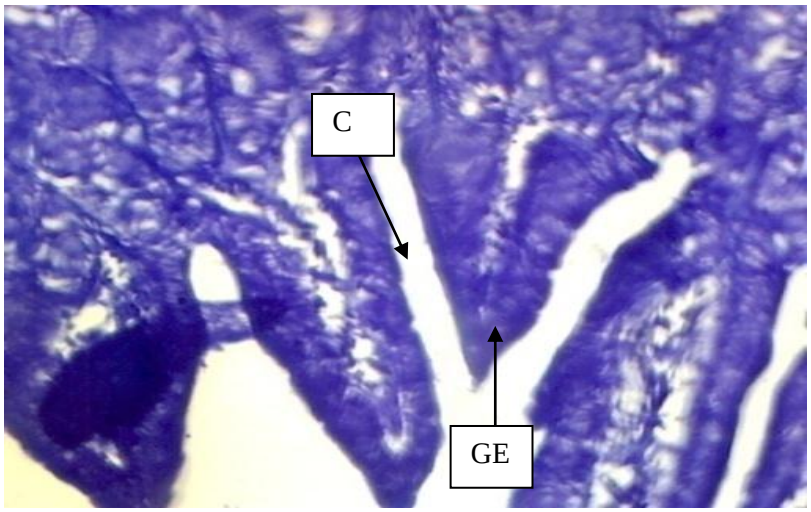
Key:

DE = Duodenal epithelial cells (DE) CN = Coagulating Necrosis

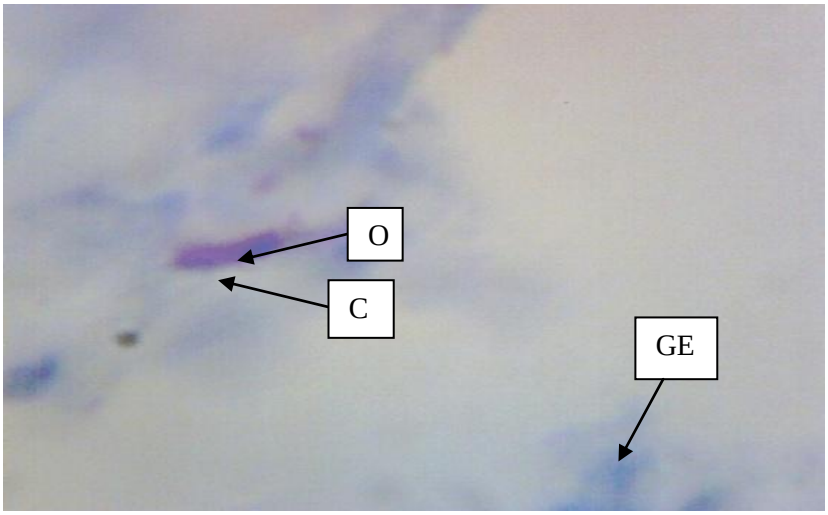
DE = Duodenal epithelium N = Nucleus

NT = Necrotic tissue $A_{60}D$= Group A duodenum at 60 days

$B_{60}D$= Group B stomach at 60 days $C_{60}D$ = Group C duodenum at 60 days



$A_{10}S$ (Negative control) x100



$B_{10}S$ (Positive control) x100

Figure 13: A1 and B1 stomach stained with Giemsa

Key:

C = Crept

GE = Gastric epithelium

O = Organism

$A_{10}S$= Group A stomach at 10 days

$B_{10}S$= Group B stomach at 10 days

5.0 DISCUSSION

The results of this research have shown that *H. pylori* stool antigen test was more sensitive than *H. pylori* antibody test and fecal occult blood test in the detection of *H. pylori* infection (Table 7). Of the two hundred patients tested, 57 (28.5%) were positive for blood antibody test (Tables 1 and 2), 71 (35.5%) were positive for stool antigen test (Tables 3 and 4), while 40 (20.0%) were positive for the fecal occult blood test (Tables 5 and 6). The measure of agreement between the *H. pylori* antibody test, *H. pylori* stool antigen test and fecal occult blood test showed that there was a positive significant agreement between the three tests ($P<0.05$) implying that the three methods of testing for *H. pylori* infection were independent (Tables 8, 9 and 10). *H. pylori* stool antigen test was more sensitive than *H. pylori* antibody test and fecal occult blood test with a difference of 14.0% (Table 8) and 31.0% respectively (Table 10). *H. pylori* antibody test was more sensitive than fecal occult blood test with a difference of 17.0% (Table 9). This is in agreement with other researchers who had reported that *H. pylori* stool antigen test was more sensitive than other screening test for the detection of recent infection by *H. pylori* (Oluwasola *et al*., 2002; Otegbayo *et al*., 2004; Smith *et al*., 2008). They recommended that *H. pylori* stool antigen test be used in a low prevalence population. Jemilohun *et al*., (2010) and Smith *et al*., (2008) in their studies proposed a combination of stool antigen tests and H. pylori blood antibody test in Nigeria because of the high endemicity of *H. pylori*. From our result, *H. pylori* stool antigen test and the *H. pylori* blood antibody test were better diagnostic tools than the fecal occult blood test (Table 7).

In this study, *H. pylori* antibody test, *H. pylori* stool antigen test and fecal occult blood test had more female testing positive than men. This result did not differ from the research of Zhu *et al*, which found a higher infection rate in females than men (Zhu et al., 2014). Also, a research carried out on Kuwait indigenes found more females infected with *H. pylori* than men (Waleed *et al*., 2010). The same study also found that the expatriate in Kuwait had more men having *H. pylori* infection than female (Waleed *et al*., 2010). Naja *et al*., 2007 and Yasir *et al*., 2010 found that men had a higher infection rate than women. The discrepancy in these results may be due to geographical location, habit and acquisition of resistant strain (Ierardi *et al*., 2013). However, it has been reported that the higher rate of infection in females than males may be as a result of the hormonal differences between the two genders. Sex steroid hormones like estradiol and testosterone as well as the difference in the gene of the two sexes may affect the immune responses to diseases (Klein *et al*., 2012). A Study has shown that any form of infection in female appears to affect the cycling sex hormones negatively by elongating the oestrus cycle from four days to eight days. This

stretch reduces the oestrogen level. Oestrogen and progesterone are the hormones which protect women from disease (Klein et al., 2010). Also female acquire *H .pylori* at a tender age than male (Waleed *et al*., 2010). But, this comparison is limited in this present study because the study was carried out on patients visiting the medical out-patient clinic which did not represent an epidemiological picture of the rate. Females are likely to report to hospital than their male counterparts when infected or when pregnant (Yingying *et al*., 2013).

The study also revealed the infection rate of *H. pylori* in age group 18-40 years and group 41-75 years (Table 7). The division of age into two groups in this study was done for convenience without consideration to the rate of infection in a particular age. The result showed that those in age group 41-75 years tested more positive for *H. pylori* blood antibody test and *H. pylori* stool antigen test than those in age group 18- 40 years, but the fecal occult blood test had those in age group 18-40 years testing more positive than age group 41-75 years (Table 7). Other studies had found that *H. pylori* seroprevalence increased significantly with age. Studies by Naja *et al*, found that the seropositivity rate increased from those who were under 60 years to those between the age of sixty and seventy and peaked at those greater than seventy years (Naja *et al*., 2007). Waleed *et al.*, (2010) also revealed that *H. pylori* infection increased with age which was in agreement with this study. The higher rate of infection in age group 41-75 years could be due to the occurrence of this infection in the developing countries at an early age, with chronic infection continuing into adulthood. Another explanation may be as a result of a constant infection rate over time or by a birth cohort effect, with decreasing rates in subsequent generations. In the developed countries, the prevalence among children is low but rises in proportion throughout adult life at a rate of approximately 1% per year (Naja et al., 2007). However, it is not possible to compare the two age groups because the screening in this study was not random but was done on selected population. Tania and Karen (2014), reported that older persons seek medical help than the younger ones.

Fecal occult blood test was included in this study because the gastroenterologists in Federal Teaching Hospital Abakaliki requested it among *H. pylori* serum antibody and stool antigen tests. Testing for occult blood in the stool was used as the only screening test for dyspeptic patients because of its availability and affordability in the rural areas (Yi-Chia *et al*., 2013).The high positivity rate recorded by those in age group 18-40 years in the fecal occult blood test as shown in Table 7, may be due to the non specificity of fecal occult blood test for *H. pylori* infection. It does not diagnose *H. pylori* infection but detects gastrointestinal bleeding which may be caused by ulcer and cancer (Yi-Chia *et al*., 2013). Therefore, it is possible that the high positivity rate in the younger group may be from other causes.

The stool samples of the seventy one patients who were positive for *H. pylori* stool antigen test were cultured and none yielded the growth of *H. pylori*. Also, Falsafi *et al*., 2007 did not isolate *H. pylori* from stool, but Dore *et al*., 2000 did after treating stool samples with cholestyramine (basic anion exchange) which inactivated the bile acids that prevented the growth of *H. pylori*. At the time of this study, cholestyramine was scarce. However, *H. pylori* was isolated from biopsy culture which other researchers reported as a more reliable method of diagnosis (Boyanova, 2003; Lee *et al*., 2013). *H. pylori* isolates were identified as shown in Table 11 and 13 and also in Appendix 5. The isolate was stored on agar slant in the refrigerator till when required.

The isolate was used to infect eighty four (84) two weeks old, male wistar mice in groups B (positive control) and C (test) as shown in Table 7. Forty two (42) mice in group A (Negative control) were not infected. All through the study, a total of thirty six mice from each group were sacrificed and six (6) mice in each group were reserved (Appendix 7). The sacrifice of six (6) mice on each day of sacrifice was due to the delicate and fragile nature of mice's gastrointestinal structure which denatures easily during histopathological processing, and the reason for the reserve was to forestall occurrences that may demand the use of extra mice. The distribution of mice according to the day of sacrifice was done in accordance with the research done by Hamilton-Miller in 2003 (Appendix 7). The first day of sacrifice was on the tenth day post infection in consideration of Marshall's endoscopy result which revealed signs of gastritis and the presence of *H. pylori* ten days after he drank a beaker of *H. pylori* culture (Blaser, 2005).

Subsequently, the infected mice in group C were fed with a probiotic *Lactobacillus acidophilus* (*L. acidophilus*) which was isolated from the feaces of breastfeeding infants and identified as shown in Tables 12 and 13 and in Appendix 6. This study used *L. acidophilus* isolate from human as probiotic because the *H. pylori* used in infecting mice was from a human's biopsy. This was in accordance with the study by Knut, 2001 which reported that the use of probiotics originating from the host gave a better treatment result. The probiotic *L. acidophilus* in yogurt was administered to mice in group C after one week post challenge with *H. pylori* suspension. Trials in which fermented milk products or whole cultures of lactobacilli were used tended to show better results than when the probiotic was taken in the form of bacteria alone (Hamilton-Miller, 2003). Treatment of the mice in group C with probiotic lasted for eight weeks and five days.

The weekly weight in gram of normal (group A), infected (group B) and infected but treated mice (group C) was taken from the second week of birth to the ninth week which was the duration of the study as shown on Table 14. Mice in group A maintained a normal

increase in weight with an average of 23.7g per week, while groups B and C had an average of 12.4g per week and 15.0g per week respectively (Appendix 10). The gain in weight per week by group A mice was in agreement with the report of Animal Resource Centre, 2015 which stated an average of 25g per week. The slight increase in weight per week of mice in group B was drastically reduced from week 5 (which was the twenty one days post inoculation) to week 9 (which was the sixty days post inoculation). Group C showed a decrease in weight on week 4 and 5 (which was the fourteenth and twenty one days post inoculation) but it gradually increased through week 6 (twenty eight days post inoculation) to 9 (Sixty days post inoculation) as shown in Tables 14 and Appendix 10. The reduction in the weight of mice in group B was more than group C, because mice in group B had *H. pylori* infection which made them ill. It was observed that the sick mice were not feeding well. The treatment of group C mice with probiotics may have led to increase in weight at week 6 through week 9 (Table 14).

This study showed the photomicrograph of the changes in the gastric and duodenal epithelium of the mice in the three groups (Figure 1-13). The photomicrograph of mice in group A showed normal gastric and duodenal epithelium from day ten to the sixtieth day. On the tenth day post inoculation with *H. pylori*, the mice in group B had dysplastic and hyperplastic gastric epithelium, while the duodenal epithelium had mild proliferation of the cells. But the dysplastic and hyperplastic cells in group C mice had regenerated with some normal cells, while the duodenum was well regenerated (Figures 3 and 6). This may be because of the introduction of probiotics on the seventh day post inoculation for group C mice (Figure 1 and 2).

On day 14, both the gastric and the duodenal epithelium of group B mice were matted. However, the action of the probiotic in group C mice caused the regeneration of the gastric and duodenal epithelial cells amidst necrosis (Figure 3 and 4).

By day twenty one, the gastric pathology of group B mice had degenerated to the formation of debris by the matted cells with some part of the epithelium appearing flushed while the matted cells in the duodenum appeared partially distinct. Meanwhile, the necrotic tissues in the gastric and duodenal epithelium of mice in group C were being replaced with normal tissue (Figure 5 and 6). This inflammatory process in group B mice was definitely resulting to atrophy and ulcers (Suerbaum and Michetti, 2002). There was an on-going healing process in group C mice.

The photomicrograph of the twenty eight day showed a thinning out of the gastric epithelium of the mice in group B, while the duodenal cells had coagulation and squamous metaplasia which depicts malignancy. The inflammatory process was resulting to atrophy and

ulcers, atrophy and malignancy (Suerbaum and Michetti, 2002). Tsuji *et al*, reported that gastric and duodenal cells become cancerous when the infection with *H. pylori* causes the production of free radicals and increased host mutation (Tsuji *et al*., 2003). The complete regeneration of both the gastric and duodenal epithelium in group C mice in Figures 7 and 8 shows that the probiotic *L. acidophilus* prevented and treated the H. pylori infection in group C mice.

There was a progression of the pathology in group B mice whose thinned gastric epithelium had extensive fibrocollagenous hyalinization of the submucousal area, with the duodenum still showing coagulating necrosis on the thirty fifth day. The mice appeared physically weak, emaciated and had difficulty feeding. However, the mice in group C which had complete regeneration of its gastric and duodenale epithelium on the twenty-eight day, showed the gastric epithelium becoming necrotic amid cell regeneration while the duodenal epithelium remained healthy, revealing that the stomach may be more predisposed to *H. pylori* infection than the duodenum. The reversal implies that the effect of drug was no longer beneficial (Figure 9 and 10).

By the sixtieth day, mice in group B had their gastric epithelium completely thinned with some areas converting to fibrotic tissues, while the duodenum showed coagulating necrosis with the nucleus appearing ghost like. The Mice in group C had their gastric epithelium thinning out amidst cell regeneration while the duodenum had started reversing with the duodenal epithelium changing into necrotic tissues (Figure 11 and 12). Previous studies have reported that treatment with probiotic partially relieved damage to gastric tissue caused by *H. pylori* infection (Myllyluoma, 2007; Chenoll *et al*., 2010). But, Patel *et al*., 2013 proposed that the long-term intake of products containing probiotic strains may have a favorable effect on *H. pylori* infection in humans, particularly by reducing the risk of developing disorders associated with high degrees of gastric inflammation. This is not in support of this research finding which showed that the duration of treatment was twenty eight days.

Futhermore, the cure seen in Group C mice at the twenty eight day was in agreement with research done by Chenoll *et al*., 2010 who found that after 21 days, mice treated with probiotic developed significantly fewer ulcers than the control group. However, the gradual reversal to necrosis in this study may be from the formation of resistant mutants against the one probiotic used.

Also, the difference in the pathology of the gastric and the duodenal epithelium as was seen in the photomicrographs of group B and C shows that the *H. pylori* infection

affected both organs differently because the colonization pattern is not the same (Kuster *et al.*, 2006).

The presence of *H. pylori* infection in mice was demonstrated by histological analysis using Giemsa stain and culture. A curved-like organism was seen in the Giemsa stain of the stomach tissue of mice in group B, while none was in group A mice (Figure 13). Culture of the stomach tissue from group B yielded growth of *H. pylori*, while that of group A had no growth.

Taken together, the results of our work concurred with the findings of Hamilton-Miller in 2003, Myllyluoma in 2007 and Rosania in 2012, which showed that probiotics and their product prevented and treated *H. pylori* infection.

6.0 CONCLUSION

This study showed that the culture of gastric biopsy was more reliable than stool culture in the isolation of *Helicobacter pylori*. The antigen test was more sensitive than the antibody test. Also, the probiotic *Lactobacillus acidophilus* effectively treated and prevented *Helicobacter pylori* infection but the beneficial effect terminated after the twenty-eight day. This implies that duration is very important in the treatment of *H. pylori* infection using probiotic. Because of the reversal noted in group C mice at thirty five days post inoculation, there is need to add another anti-*Helicobacter pylori* agent as a synergy or give booster doses of probiotic. Some studies have tested the association of a multi-strain probiotic mixture with antibiotic therapy. The results of two of these studies showed a reduction in the side effects of antibiotic therapy and a higher eradication rate than that obtained with a single strain (Cremonini *et al*., 2002; Myllyluoma *et al*., 2005), but the third study by Yoon *et al*., 2011 did not obtain any significant result. Probiotic should be used as a complement in medical practice rather than as an alternative.

Additional studies should be done on the production of probiotics that harbor high numbers of viable organism at the time of consumption, their safety and appropriate use to get the maximum effect. The choice of probiotic strain(s), the use of a wide range of probiotic strains (*Lactobacillus acidophilus, L. johnsonii, L. gasseri, lactobacillus GG, Bifidobacterium longum,* and *bioyoghurts),* and the quantification of doses need attention in future trials.

Given the prevalence of *H. pylori* infection in the developing countries and the role it plays in several serious medical concerns including cancer, the ability to rapidly identify its potential vaccine candidates using genomic sequencing and the understanding of the pathogenesis of *H. pylori* should be a priority in the research community. More data bases need to be built up in Nigeria on the role of probiotics in the treatment and prevention of *H. pylori* infection. The education of the public through lectures and symposium on *H. pylori* infection and the benefits of probiotics in the treatment of *H. pylori* infection should be done regularly especially among medical practitioners as called for by Anukam in 2006.

CONTRIBUTION TO SCIENCE

This present study revealed that *H. pylori* stool antigen test was more sensitive in the detection of *H. pylori*, gastric biopsy sample was better in the isolation of *H. pylori* than stool sample and that after 28 days (approximately 1 month) of administration of a probiotic *Lactobacill*, the effect was no longer beneficial. This implies that duration is very important in the treatment of *H. pylori* infection using probiotic.

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APPENDIX 1

Ethical Committee Approval

APPENDIX 2

Consent Form

CONSENT TO BE A RESEARCH PARTICIPANT

NNAMDI AZIKIWE UNIVERSITY AWKA

A. Purpose and background

Mrs. Egwu-Oko, Uchenna Tony (PhD Student) in the department of Applied Microbiology at the Nnamdi Azikiwe University Awka is conducting a research study entitled "The role of probiotics in the treatment of *Helicobacter pylori* infection". The purpose of this study is to help understand whether or not an administered probiotic dietary supplement can suppress the colonization of *H. pylori* in a mouse model. The study will also consider the effect of dosage in the treatment therapy. You are being asked to participate in this study because you are a patient having gastroduodenal disease and require the analysis of your stool or blood sample or endoscopy for the diagnosis of the infection.

B. Procedures

If you agree to be in the study, the following will occur-

1. You will produce a stool sample for culture and 4ml of your blood withdrawn for serology. If you are for endoscopy a small biopsy sample will be collected from your stomach into a culture media.
2. The cultured sample will be transferred to the laboratory of the Federal Teaching Hospital Abakaliki for analyses. The duration will take about 72hours to 168 hours.
3. Risks and Discomfort-

The research will not pose any risks. Discomfort may be as a result of the treatment of your diagnosis e.g surgery.

C. Confidentiality

Participants in research may involve a loss of privacy; however, your records will be handled as confidential as possible. Only Professor Umeh, Chibuzor .N. will have access to this study. No individual identities will be used in any reports or publications that may result from the study.

D. Benefits

Participants in this research will have a direct benefit in the free analysis of their samples. Reports of tests will be given to the Patient's Clinician. Also the research outcome may help health professionals to better understand how to treat infections caused by *Helcobacter pylori*.

E. Costs

The test will be done free of charge.

F. Payment

You will be given your transport fare to and from the hospital (on one visit).

G. Questions

If you have any question or concerns about participation in this study, you should first talk with the investigator. Mrs. Egwu-Oko, Uchenna Tony. 08035015166.Or email ladyuctony@yahoo.com. You may also contact the Ethics and Research Committee, Ebonyi State University Teaching Hospital (EBSUTH) Abakaliki. The Research and Ethics Committee of EBSUTH has reviewed this project for the protection of human and animal participants in research.

H. Consent

You will be given a copy of this consent form to keep. Your participation is voluntary and there is no penalty if you do not participate.

I give my consent to participate in this study:

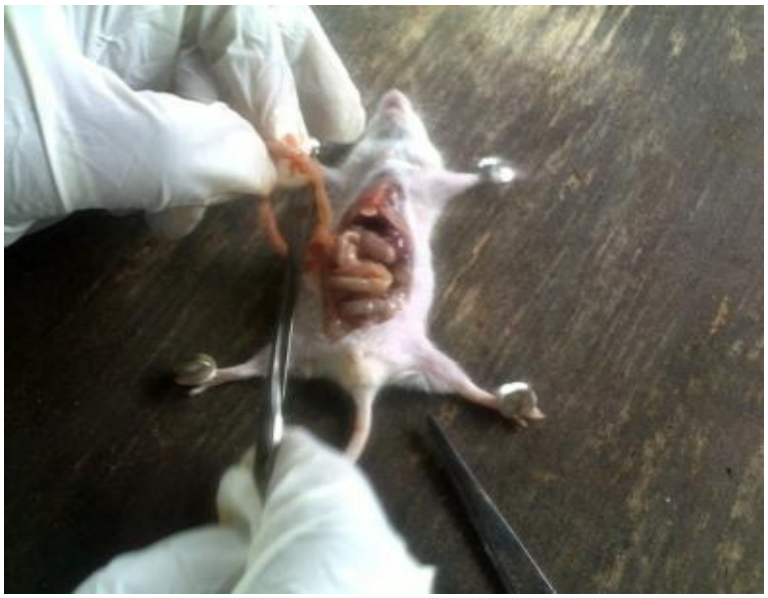
Signature of study participant: Date:

Signature of person obtaining consent: Date:

APPENDIX 3

Mice feeding dish

APPENDIX 4



Dissection of Mice

APPENDIX 5

Close

MACROGEN
Advancing through Genomics

Basic Information

- Order #1015UMDN-093
- Req #

Download

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Chromatogram

Analysis Report

Req#	1	Read Length(Normal)	942
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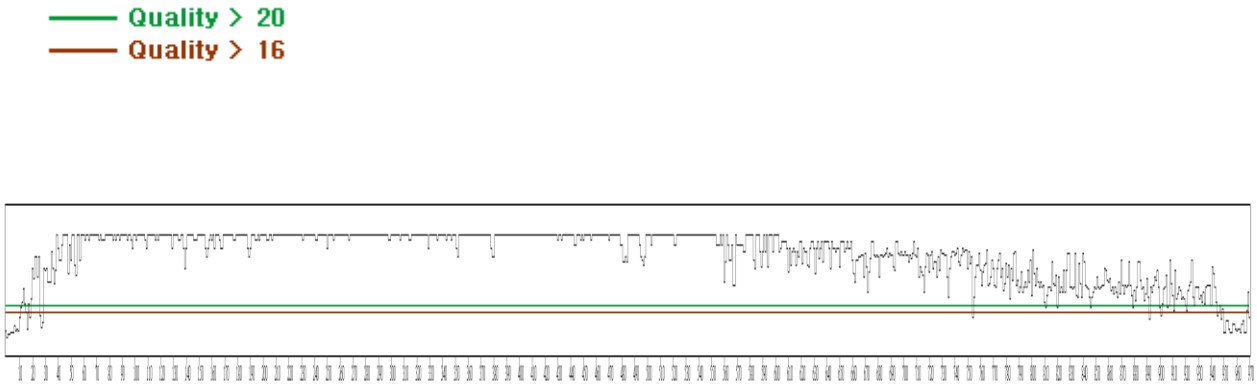
Quality Graph



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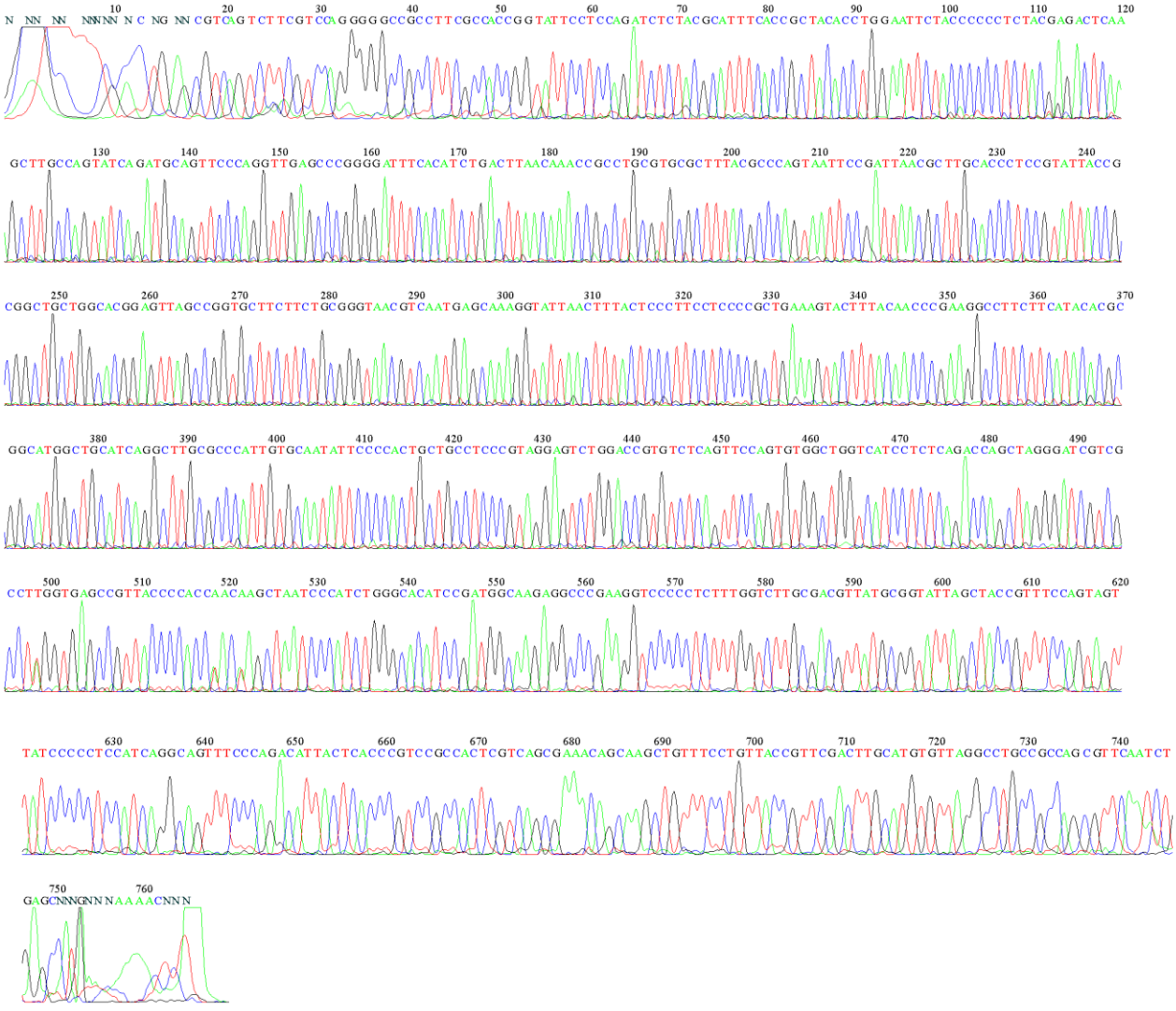

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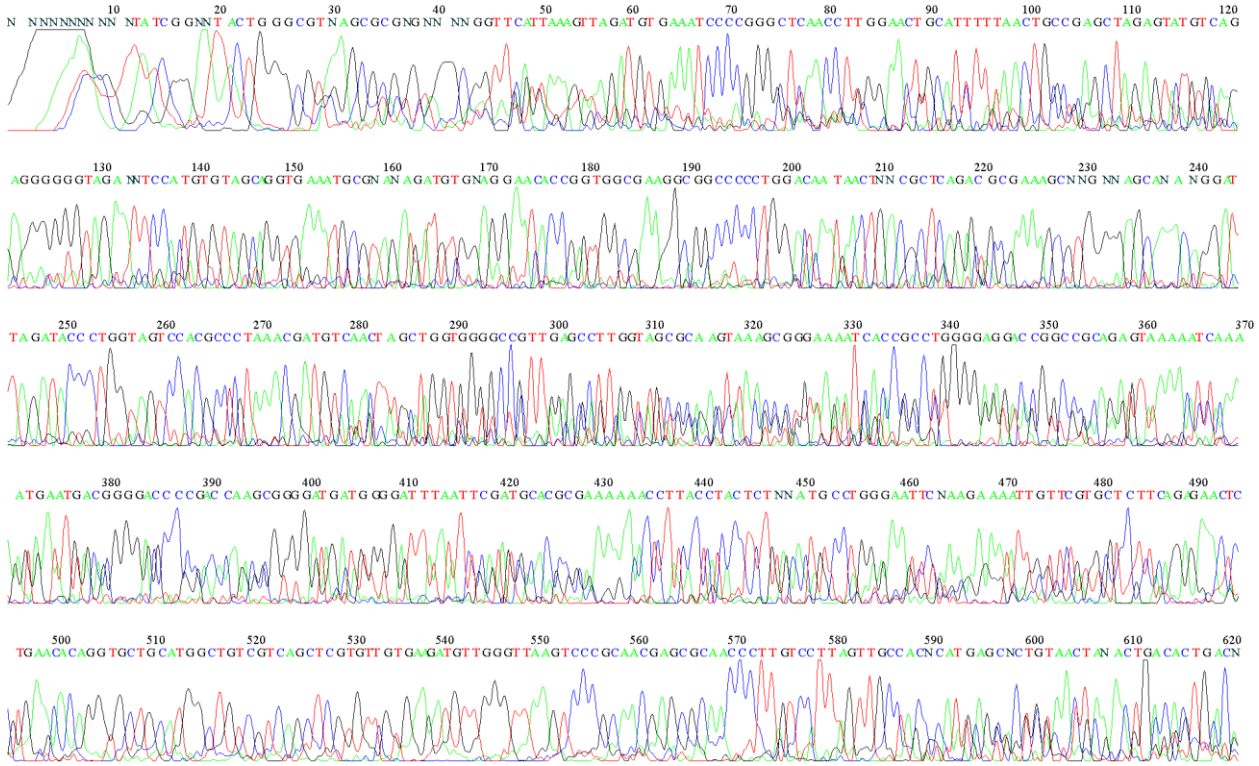
TNNACNNNGGNNNNNCCNNAAA

Gene sequencing of *Helicobacter pylori*

APPENDIX 6

File: B1_518F.ab1 Run Ended: 2014/10/16 5:11:14 Signal G:416 A:452 C:330 T:338

Sample: B1_518F Lane: 64 Base spacing: 13.946369 985 bases in 11745 scans Page 1 of 2



>141015-18_A16_B1_518F.ab1 985

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Gene Sequencing of *Lactobacillus acidophilus*

APPENDIX 7

Distribution of Mice According to the Day of Sacrifice

Day	Sacrificed Mice			Reserved	Total
	Group A	Group B	Group C	Mice	
10	6	6	6	3	21
14	6	6	6	3	21
21	6	6	6	3	21
28	6	6	6	3	21
35	6	6	6	3	21
60	6	6	6	3	21
Total	36	36	36	18	126

A = Negative control

B = Positive control

C = Test

APPENDIX 8

Preparation of Probiotic

Three cups of fresh cow milk from Hausa quarters Nkwagu Abakaliki, was poured into a stainless steel pot. This was heated on the Bunsen burner to about 80^{o}C to kill any undesirable bacteria and to denature the milk proteins so that milk sets rather than form curds. The milk was cooled to about 45^{o}C before *Lactobacillus acidophilus* (10^{7}) colonies was added to a litre of milk. The temperature was maintained by covering the pan with aluminum. This was allowed to sit overnight to allow fermentation to give the Probiotic drink used in this study. (Shah *et al*., 2000; Babatunde *et al*., 2014).

APPENDIX 9

Media Preparation

Brain Heart Infusion Broth

Dehydrated commercial infusion media (Lab M, code no Lab 49) weighing 37g Brain heart was dispensed in 1litre of distilled water. This was soaked for 10 minutes, swirled to mix and warmed gently to dissolve. This was dispensed into bijou bottles and sterilized by autoclaving for 15 minutes at 121^{o}C. Preparation was stored in the refrigerator until when needed.

Columbian Blood Agar Base

Columbian blood agar base powder ((Oxoid, code no m0331)) weighing 39g was suspended in 1litre of distilled water. This was boiled to completely dissolve the media before sterilizing by autoclaving at 121^{o}C for 15minutes. The media was cooled to 50^{o}C before adding 5% sterile defribinated blood. When gelled, the preparation was stored in the refrigerator until when needed.

Lactobacilli MRS agar

Lactobacilli MRS agar ((Acumedia, code no 7543A)) weighing 70g was suspended in 1litre of distilled water. This was heated with frequent agitation and allowed to boil for one minute to completely dissolve the medium. It was sterilized by autoclaving at 121^{o}C for 15 minutes until when needed.

APPENDIX 10

Weekly Weight gain by Mice

Weeks	Weight Gained Per Week (g)		
	Group A	Group B	Group C
2-3 weeks	24.6	35.1	25.0
3-4 weeks	25.2	17.9	15.8
4-5 weeks	24.5	12.2	8.8
5-6 weeks	27.3	9.0	21.7
6-7 weeks	24.8	6.5	22.0
7-8 weeks	14.2	3.9	6.5
8-9 weeks	25.0	2.8	5.3
Total	165.6	87.4	105.1
Average	23.7	12.5	15.0