OCCURENCE OF NON-TYPHOIDAL *SALMONELLA* IN FRESH PRODUCE SOLD IN LAGOS AND OGUN STATE.

BY

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A PROJECTSUBMITTED TO THE DEPARTMENT OF BIOLOGICAL SCIENCES, COLLEGE OF BASIC AND APPLIED SCIENCES, MOUNTAIN TOP UNVIERSITY, MAKOGI, IBAFO, OGUN STATE, NIGERIA.

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DECLARATION

I, YUSUF, HANNAH OJOCHENEMI hereby declare that the project report written under the supervision of DR. O.E FAYEMI is a product of my own research work. Information derived from various sources have been duly acknowledged in the text and a list of reference provided.

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YUSUF HANNAH O.

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DATE

CERTIFICATION

This is to certify that this research project titled "**OCCURENCE OF NON TYPHOIDAL *Salmonella* IN FRESH PRODUCE SOLD IN LAGOS AND OGUN STATE**" was carried out by Yusuf, Hannah Ojochenemi with matriculation number 17010101003. This project report meets the requirements governing the award of Bachelor of Science (B.Sc.) Degree in Microbiology, Department of Biological Sciences of Mountain Top University, Ogun State, Nigeria and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

I dedicate this research to Almighty God for the strength, wisdom, divine direction and grace given unto me to carry out this project successfully.

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TABLE OF CONTENTS

LISTS OF TABLES

LISTS OF FIGURES

ABSTRACT

Non-typhoidal *Salmonella spp.* is one of the most common pathogens associated with foodborne disease in Africa**.** Fresh produce consumption has increased as a result of the promotion of better health and living. Fruits and vegetables are usually minimally processed and eaten raw, which may expose consumers to an elevated risk of foodborne diseases if contaminated. As a result, reports of ready-to-eat fruits and vegetables-related disease outbreaks have increased significantly in recent years, and information about these events is frequently linked to *Salmonella* contamination. To date, outbreaks of *Salmonella spp*. from fresh vegetables have not been reported in Nigeria. Fresh fruits and vegetables were collected from street vendors in local street marketsin Lagos and Ogun states for the purpose of isolating *Salmonella spp*. from these fruits and vegetables. HEA- Hektoen enteric agar plates was used to confirm the presumptive *Salmonella* isolates that were streaked from XLD- Xylose Lysine Deoxycholate Agar which were used to test a total of 63 Samplesfor the presence of *Salmonella spp.* Only 16 Samples out of 63 samples tested positive for Nontyphoidal *Salmonella spp,* hence molecular identification of selected isolates (n = 16). Ibafo cucumber had the greatest total viable count of 7.8 log10(cfu/g). The morphological and biological properties of the *Salmonella* isolates were evaluated and confirmed presumptive positive using catalase test, oxidase test and Capsule test.The presence of nontyphoidal *Salmonella spp* was confirmed using *Salmonella* Specific PCR.

Molecular confirmation of the *Non-typhoidal Salmonella* identification was performed using *Salmonella* specific primers for S. Enterica. The presence of *Salmonella* in fresh produce is of public health concern in Ogun and Lagos state.

Key words: Fresh Produces, *Nontyphoidal Salmonella*, Biochemical tests, *Salmonella.*

CHAPTER ONE

1.0 BACKGROUND TO THE STUDY

Growing customer desire for fresh and healthier convenience meals has fueled the growth of the fresh produce business which has seen significant growth over the last two decades. While fresh produce consumption has increased, particularly lettuce, watermelons, cucumbers, pine apples, cabbages, pawpaw, and carrots, which are generally classified as ready-to-eat foods, foodborne outbreaks associated with fresh produce have also increased in recent years, with the majority of outbreaks occurring in developing countries. World Health Organization (WHO) (2006). It has climbed from 12 percent in the 1990s to 24 percent in the 2010s, with fresh produce being the most commonly associated with foodborne diseases (Pezzoli *et al.*, 2008; Sivapalasingm *et al*., 2004).

There are three key causes that could contribute to a rise in the number of illnesses that are reported. One thing that is undeniably true is that the globalization of the food industry has resulted in the spread of foodborne outbreaks between countries and even continents, with a consequent increase in the number of people who have been affected by the same tainted produce. Secondly, the changes in agricultural and processing practices that have occurred across the food chain as a result of efforts to increase supply may have increased the danger of disease transmission and cross-contamination between foods. In addition, technological advancements in the areas of microbiological detection and identification, together with advancements in monitoring systems, have all had a role in the rise in the number of illnesses reported. Thousands of outbreaks and 23,748 illnesses were reported in Nigeria, with fresh produce outpacing other food-borne outbreak sources such as poultry, beef, and seafood. The produce category alone accounted for more outbreaks and illnesses than any other category, with the majority of illnesses associated with vegetables (Hedberg, MacDonald & Osterholm, 1999; Beuchat & Ryu, 1997). *Salmonella spp., Shigella spp.,* and pathogenic *Escherichia coli* were the causal agents that were most frequently identified (Berger *et al*., 2010). Foodborne outbreaks involving fresh-cut fruits and vegetables are frequently connected with foodborne pathogens because they are

frequently ingested raw after only limited processing. The primary reason for this is that they are frequently consumed raw after

very minimal processing (Betts., 2014). There is no kill phase in the processing that can guarantee the complete removal of pathogenic germs associated with fresh produce. Following contamination in the field, it is not possible to entirely eradicate harmful germs from fresh-cut produce once it has been harvested. To manage the food safety hazards associated with fresh produce, it is essential to identify the primary sources of contamination as well as the pathways of transmission of pathogenic bacteria to the fresh produce (Betts., 2014). Inadequately packed, washed, and transported fresh produce can become contaminated with bacteria such as *Escherichia coli, Salmonella,* and *Shigella,* amongst other species, leading to food poisoning (Kirezieva *et al*., 2015).

Salmonella spp. is one of the most common pathogens associated with foodborne disease in Africa. They are classified into two species: *Salmonella bongori* and *Salmonella enterica,* and have been isolated from a wide variety of foods, including fresh fruits and raw vegetables (Beuchat 2002). They are typically found in fresh produce because of an infected food handler who practices poor personal hygiene, or in products harvested from sewage-polluted area.

Salmonella enterica are divided into typhoidal and Non-typhoidal. Typhoid fever serovars (Typhi and paratyphi) are divided into two groups, typhoidal serovars Typhi and paratyphi that cause typhoid fever while non-typhoidal *Salmonella* (NTS) serovars that have a broader host range and are frequently Zoonotic (infecting other animals (Johnson *et al*., 2006) Recently published research has revealed that NTS have emerged as a significant source of invasive bloodstream infection in Africa (Johnson *et al.,* 2006), particularly in young children suffering from malaria and malnutrition, and that these infections can result in gastrointestinal and bacteriological disorders in these children.

1.1 PROBLEM STATEMENT

Nontyphoidal *Salmonella* (NTS) is one of the most commonly reported bacterial foodborne zoonoses, accounting for the severity of all reported case hazards caused by the consumption of

Nontyphoidal *Salmonella* contaminated fresh produce (fruits and vegetables), as well as examining the possible routes of Nontyphoidal *Salmonella* contamination in fresh produce..

1.2 JUSTIFICATION OF THE STUDY

Investigation of NTS in fresh produce is required to determine the safety status of these fresh produce and to reduce foodborne illnesses connected with fresh produce. It is also necessary to know the specific microorganism that may be present in the items and cause illness or death in people.

1.3 OBJECTIVE OF STUDY

To isolate and identify Non-typhoidal *Salmonella* associated with street vended fresh produce (fresh fruits and vegetables)

To assess the risk of eating infected or unwholesome fresh produces in (Lagos and Ogun state).

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 FRESH PRODUCE AND BACTERIAL CONTAMINATION

Fresh, minimally processed fruits and vegetables are an important part of a healthy, well-balanced diet. The nutritional and other benefits of a regular intake of fruits and vegetables are widely recognized internationally, according to (Lerici *et al*., 2000). Their consumption has increased as a result of the promotion of healthier lifestyles, there has been a worldwide increase in recent years (Betts, 2014). These products are frequently ready-to-eat (RTE). Consumers frequently do not treat these foods prior to eating to guarantee that pollutants such as chemical residues or harmful bacteria are effectively removed or inactivated (Egan, & Dennis, 2012). Their increased consumption, combined with globalization and large-scale production of RTE foodstuffs (Olaimat and Holley, 2012), has resulted in a further increase in desirability and consumption. Their role in reducing the risk of lifestyle-related illnesses like heart disease, diabetes, and cancer has resulted in a further increase in desirability and consumption (Ijabadeniyi, 2010).

Figure 2.1: Diagram of Fresh Produce (John Murray, 2016)

Government health officials in several places advocate its intake. For example, the Food and Drug Administration (FDA) and the World Health Organization (WHO) recommend eating 5–9 servings of fruits and vegetables per day because proper fresh produce intake alone could save 2.7 million lives per year (Johnston *et al*, 2006) by protecting against a variety of illnesses such as eye diseases, cancers, and cardiovascular diseases.

However, fruits and vegetables, particularly raw leafy greens, are rapidly becoming recognized as major carriers for the spread of human diseases previously associated with animal-based meals (Pezzoli *et al*., 2008). There is currently little understanding of where contamination occurs in the food chain or the process by which human infections colonize and thrive on or in fruits and vegetables (Berger *et al*., 2010).

Consumption of fresh fruits and vegetables has been linked to danger for consumers, despite its health benefits (Beuchat, 2002). Foodborne illness outbreaks linked to the use of ready-to-eat veggies have been on the rise (Pezzoli *et al*., 2008). Garg *et al*., 2010 revealed that bacterial outbreaks have been epidemiologically connected to the eating of a wide range of vegetables, and to a lesser extent, fruits. Bacteria, viruses, and parasites are pathogens that can cause human disease and can be found in irrigation water or in the soil where food is grown. On a global basis, however, bacteria pose the biggest threat in terms of serious illness and the number of people at risk of infection. Furthermore, bacterial pathogens such as *Salmonella spp*., *Shigella spp*., Shiga toxigenic *E. coli* (STEC), *Listeria monocytogenes,* and *Campylobacter spp*. have been found in vegetables under monitoring.

Members of the Enterobacteriaceae family such as Shigella and *Salmonella* are the most common pathogenic bacteria found in fresh produce. These gram-negative, straight rods range in diameter from 0.3 to 1.5 meters, are facultative anaerobes, oxidase-negative, and have a respiratory and fermentative metabolism. They frequently use glucose as their only carbon source and thrive on peptone and meat extracts. They don't produce spores, aren't acid fast or halophilic, yet they can withstand bile salts (Brenner, 1984). They inhabit animals' intestinal systems, and their presence has long been associated with food cleanliness and safety.

Testing methods for members of the Enterobacteriaceae family such as Shigella and *Salmonella* were originally used on milk and then expanded to include all meals (Ayers and Johnson, 1915; Shippen, 1915; Weinzirl and Harris, 1928; Tanner and Windsor, 1929). The majority of *Salmonella* species are considered human pathogens, albeit they differ in their features and severity of sickness. Achard and Bensaud (1897) isolated the paratyphoid bacilli, which Schottmuller confirmed as culturally and serologically separate from the typhoid bacilli (1911).

2.2 *SALMONELLA*

Salmonella infections results in illnesses known as salmonellosis, a frequent foodborne infection in humans. *Salmonella spp*. exposure can cause anything from minor symptoms to severe disease, and it can even be fatal. *Salmonella spp*. have been identified from the environment and are carried by a variety of domestic and wild animals and birds (Jay *et al*. 2003)

2.2.1 Taxonomy of *Salmonella*

Salmonella are Gram-negative, facultatively anaerobic bacteria that may thrive on a variety of relatively simple media. They are distinguishable from other members of the family by their biochemical properties and antigenic structure (Greenwood *et al*., 2016). *Salmonella* enterica and *Salmonella* bongori are the only two main species in the genus *Salmonella*

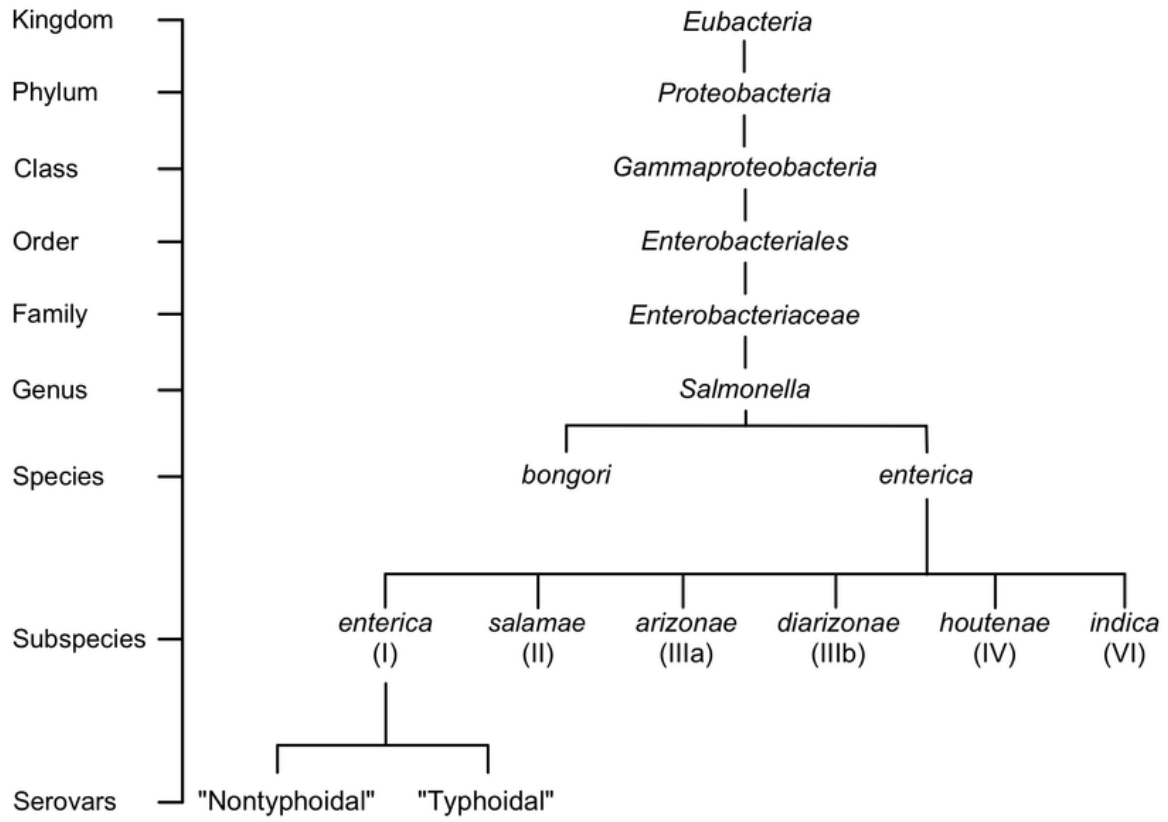

Figure 2.2 Classification of *Salmonella spp* (Keith D. Mackenzie, 2017)

Salmonella enterica is split into six subspecies, each with its own set of biochemical traits and vulnerability to bacteriophage Felix 01 lysis (Grimont and Weill, 2007). Subspecies I, II, IIIa, IIIb, IV, and VII are the seven phylogenetic groups of *Salmonella* enterica. There are 1367 serovars in Subspecies I, some of which are frequently isolated from sick birds and animals, including humans. Rotger and Casadess (1999) found that the other subspecies primarily colonize cold-blooded vertebrates. Su and Chiu (2007) claim that , because of their close kinship as determined by DNA hybridization tests, all *Salmonella* species were officially designated as a single species, S. enterica, in 2005. *Salmonella* strains were previously named after their original isolation sites, such as *Salmonella* london and *Salmonella* indiana. This approach was replaced by phage typing, which is a categorization system based on the sensitivity of isolates to various bacteriophages (Pui*etal*,. 2011). Phage typing has proven to be quite useful in distinguishing between *Salmonella typhimurium*, virchow, enteritidis, and typhi strains (Greenwood *et al*., 2016).*Salmonella* serotypes, on the other hand, can be divided into typhoidal and nontyphoidal *Salmonella* based on the clinical symptoms they cause (Haeusler and Curtis, 2013). Some

Salmonella serovars are host-adapted to specific animal species and have a wide range of disease severity; others, such as *S. Typhimurium*, have a broad host range and can infect a variety of animals, including humans (Jayal. 2003; Wallis 2006). Infections with S. Typhi and S. Paratyphi are specifically linked to infections in humans, which result in a serious condition known as enteric fever. Typhoid and paratyphoid fever are clinical syndromes caused by *Salmonella Typhi* and *Salmonella Paratyphi*, respectively. In affluent countries, enteric fever is uncommon, with the bulk of instances linked to international travel (Darby and Sheorey, 2008).

Salmonella infections are a major public health concern around the world, especially in underdeveloped nations, where they are a major cause of illness and mortality (Bisi-Johnson and Obi, 2012). They are responsible for a wide range of infectious illnesses in people and animals. The most frequent of these diseases is gastroenteritis, which is characterized by bacterial proliferation in the sub mucosa of the intestine and diarrhea brought on by the inflammatory response and, possibly, toxins (Grimont and Weill,2007).

2.2.2 Pathogenicity of *Salmonella*

Salmonella species are intracellular pathogens which are facultative in nature. *Salmonella* can infect a variety of cell types, including epithelial cells, M cells, macrophages, and dendritic cells (Jantsch*et al*., 2011). *Salmonella*, as a facultative anaerobic bacteria, uses oxygen to produce ATP in an aerobic environment (i.e.when oxygen is available). (LaRock *et al*, 2015). Nontyphoidal *Salmonella* serovars (NTS), the causal agents of gastroenteritis, share this path of infection. However, from a clinical standpoint, the diseases caused by these two distinct families of bacteria are vastly different. While NTS gastroenteritis has a rapid start after a short incubation period (12-72 hours) and a short duration (10 days), typhoid fever has a much longer incubation time (median of 5 to 9 days) and a much longer duration of symptoms (fever persists for approximately three weeks). In immunocompetent patients, gastroenteritis is an infection that is limited to the intestine and mesenteric lymph nodes. Typhoid fever, on the other hand, is a systemic infection in which S. Typhi colonizes the liver, spleen, and bone marrow as well as the gut and mesenteric lymph nodes. (Tsolis *et al*,. 2009, Santos *et al*,. 2011). The short clinical course of gastroenteritis suggests that the onset of an adaptive immune response results in clearance of the infection. On the contrary, S.Typhi can persist in human tissue for long periods

of time, indicating that the organism has a greater propensity to evade immune responses than nontyphoidal *Salmonella* serovars. (Jantsch *et al*., 2011).

2.2.3 Typhoidal *Salmonella*

Salmonella serotypes that are strictly suited to humans or higher primates, such as *Salmonella* Typhi, Paratyphi A, Paratyphi B, and Paratyphi C, cause typhoid disease. *Salmonella*e enter the bloodstream through the lymphatic system of the colon (typhoid form) and are transferred to various organs (liver, spleen, kidneys) to establish secondary foci in the systemic form of the disease (septic form). As well as vomiting and diarrhea. In severe cases, enough liquid and electrolytes are lost to disrupt water-salt metabolism, lower circulating blood volume and arterial pressure, and result in hypovolemic shock. Septic shock is also a possibility. In severe salmonellosis, mixed shock (with signs of both hypovolemic and septic shock) is more common. Renal involvement due to hypoxia and toxemia can result in oliguria and azotemia in severe cases.

2.2.3.1 Virulence factors of *typhoidal Salmonella*

Virulence factors in *Salmonella* Typhi are involved in the various stages of infection, namely: the production of toxins (LPS) endotoxin, enterotoxin, cytotoxin), colonization, adhesion and invasion, as well as survival inside the host cells (Madigan and Martinko 2007).

Vi antigen:

The capsular Vi antigen is a linear alpha 1–4 homopolymer connected to galactose aminouronic acid, which is acetylated at the C3 site in a variety of ways. This antigen is thought to prevent non-specific opsonization by limiting phagocytosis and complement C3 activation. (Hart *et al*., 2016) The development of a polysaccharide capsule known as the Vi antigen is one of the primary traits that separates S. Typhi from (non-typhoid *Salmonella*) NTS. (Wilson *et al*., 2001) found that the Vi capsule suppresses phagocytosis and imparts serum resistance, most likely by sheltering the O-antigen from antibodies (Hart *et al*., 2016). The via B locus within *Salmonella* pathogenicity island (SPI)-7 encodes the Vi capsule, as well as the type III secretion system (T3SS) effector SopE and a type IVB pilus. (Pickard *et al*,. 2003)

The SPI-1, SPI-2 and type III secretion systems:

Common to both typhoidal and NTS are two pathogenicity-island encoded type III secretion systems (T3SS): the SPI-1 and SPI-2 T3SS, which are essential for *Salmonella* virulence. In S. Typhi, the SPI-1 T3SS is also required for invasion of nonphagocytic cells (Bishop, 2007), but the importance of the SPI-2 T3SS is less clear. Disruption of the SPI-2 T3SS did not influence the survival of S. Typhi in THP-1 and human monocyte-derived macrophages (Forest *et al*,. 2010) however, S. Typhi strains with transposon insertions in the SPI-2 components ssaQ, ssaP, or ssaN were negatively selected against during competitive growth in human macrophages (Sabbagh, S. C., *et al*,. 2012). The role of SPI-2 during the intracellular lifestyle of typhoidal serovars therefore warrants further investigation.

Endotoxin of *Salmonella* Typhi:

Gram-negative bacteria's outer membrane contains a large amount of endotoxin (OM). Endotoxins have been discovered to have a key role in the pathogenesis of Gram-negative bacterial infections. It is a potent mediator of a variety of pathological effects in humans, mostly in the gastrointestinal tract. As a result, they're also called enterotoxins. Lethal toxicity, pyrogenicity, and tissue necrotizing activity are examples of harmful behaviors, as well as numerous favorable ones connected to immunostimulation. (Mahamuni *et al*,. 2017). Endotoxins are high-molecular weight complexes, of lipopolysaccharides (LPS) which is the major component of bacterial cell wall (Mahamuni *et al*,. 2017). It's a heat stable toxic substance released by gram negative bacteria's after disruption of cell envelopes (Beutler *et al*,. 2003, Bishop *et al*,. 2005). The role of endotoxins in bacterial pathogenesis and their chemical characterization as lipopolysaccharide (LPS) have been studied earlier (Raetz and Whitfield 2002, Reynolds *et al*,. 2007). Chemically, LPS consist of a hydrophilic polysaccharide covalently linked to a hydrophobic lipid portion which is termed as lipid A, which anchors the molecules in the outer membrane (OM) (Brandenburg and Wiese 2004). Endotoxins play a major role in human disease states that created interest to investigate the pathogenicity of the producing bacteria (Mahamuni *et al*,. 2017). Lipopolysaccharide found to be an important activator for the activation of immune system that leads to non- specific inflammatory immune response (Buyse *et al*,. 2007)

2.2.3.2 Non typhoidal *Salmonella*

A) Non-invasive

Food poisoning is the most common symptom of *Salmonella* infection with non-typhoidal serotypes. When a person consumes foods containing a high concentration of bacteria, they become infected. Infants and little children are significantly more vulnerable to infection, which can be easily transmitted by consuming a small number of bacteria through contaminated food or water. In order to cause disease in healthy individuals, *Salmonella* must enter through the digestive tract in high quantities. Only until live *Salmonella* (not only *Salmonella*-produced toxins) has entered the gastrointestinal tract can an infection begin. Some of the *Salmonella* are killed in the stomach, but the ones that survive reach the small intestine and grow in tissues. The majority of eaten bacteria are destroyed by gastric acidity, however *Salmonella* has evolved a level of tolerance to acidic environments that allows a fraction of ingested bacteria to survive(Garcia-del *et al*,.1993)Bacterial colonies can also get stuck in the mucus produced by the esophagus.

There are over 2,000 non-typhoidal *Salmonella* serotypes identified, which are thought to be responsible for up to 1.4 million illnesses in the United States each year. Infants, the elderly, organ transplant recipients, and the immunocompromised are all at risk for serious illness. (Graham *et al., 2009).*

B) Invasive

While nontyphoidal serotypes are usually associated with gastrointestinal sickness in affluent nations, they can cause serious bloodstream infections in Sub-Saharan Africa and are the most commonly isolated bacterium from the blood of people who have a fever. In 2012, a case fatality rate of 20–25 percent was observed in Africa from nontyphoidal *Salmonella* bloodstream infections. *Salmonella enterica Typhimurium* or *Salmonella enterica Enteritidis* are the most common causes of invasive nontyphoidal *Salmonella* infection (iNTS). A new strain of *Salmonella* Typhimurium (ST313) appeared 75 years ago in the southeast of Africa, followed by a second wave from central Africa 18 years later. This second wave of iNTS may have started in the Congo Basin and acquired a gene that made it resistant to the antibiotic chloramphenicol early on. The genetic makeup of iNTS is changing, and it is becoming more typhoid-like, with

the ability to spread quickly throughout the human body. Fever, hepatosplenomegaly, and respiratory symptoms are among the symptoms mentioned, with gastrointestinal symptoms generally absent. (*Feasey et al,. 2012)*

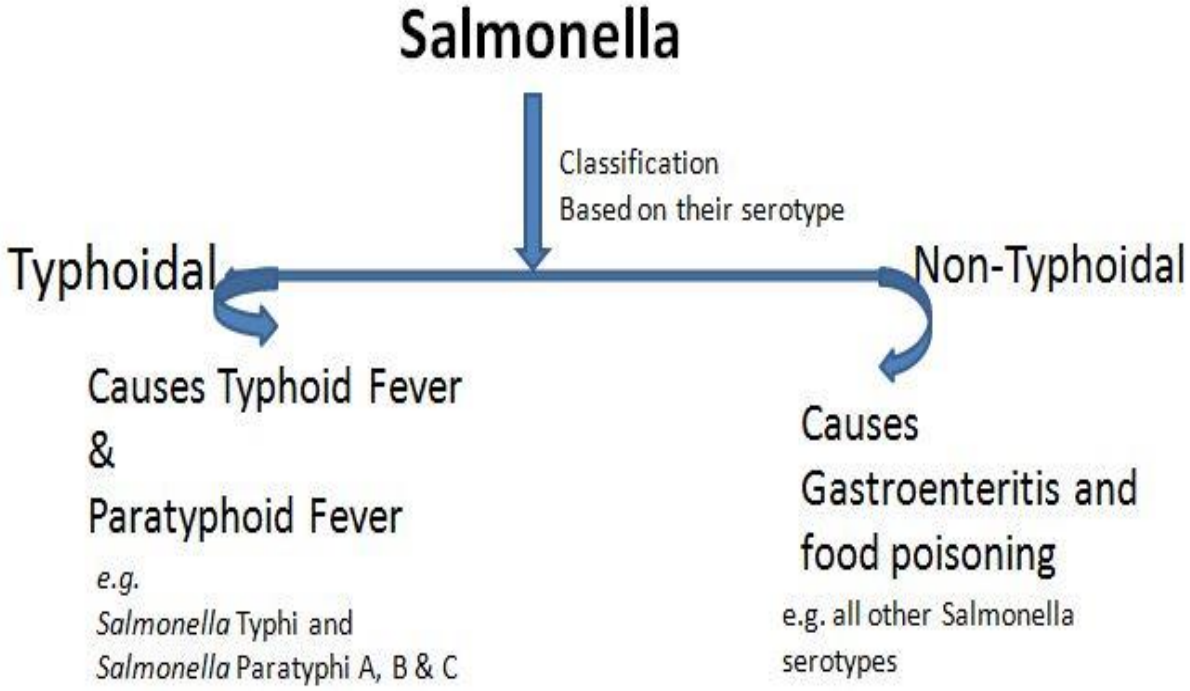

Figure 2.3 Classification based on their serotype. (Acharya Tankeshwar, 2021)

This can range from having no effect to colonization of the gastrointestinal tract without symptoms of illness (asymptomatic infection) to colonization with acute gastroenteritis-like symptoms. Abdominal cramps, nausea, diarrhoea, mild fever, vomiting, dehydration, headache, and/or prostration are common gastroenteritis symptoms. The symptoms last 2–7 days and the incubation period is 8–72 hours (typically 24–48 hours) (WHO/FAO 2002; Darby and Sheorey, 2008). Severe sickness, such as septicemia, can develop, especially in immunocompromised people.

Salmonella spp. enters the bloodstream, causing symptoms like high fever, lethargy, abdominal and chest discomfort, chills, and anorexia, which can be fatal (in less than 1 percent of cases. As a result of infection, a tiny number of people acquire secondary conditions such arthritis, meningitis, or pneumonia (Hohmann 2001; WHO/FAO 2002; FDA 2009). At the commencement of sickness, *Salmonella* spp. are found in huge numbers in the feces of infected people. In the event of non-typhoid disease, bacterial shedding lasts about 4 weeks in adults and 7 weeks in children after the illness. Individuals in 0.5 percent of non-typhoid cases become long-term carriers and continue to shed the bacteria on a regular basis (Jay *et al*. 2003; Crum-Cianflone 2008).

2.3 SOURCES AND ROUTES OF NONTYPHOIDAL *SALMONELLA* CONTAMINATION IN FRESH-CUT PRODUCE

Contaminated produce has been linked to a number of foodborne outbreaks around the world in recent years. These foodborne outbreaks are not only dangerous to people's health, but they also cost the food business a lot of money (Hussain and Dawson, 2013). According to a recent analysis by the Center for Science in the Public Interest (CSPI), fresh produce was the source of the most outbreaks in the United States from 2002 to 2011.

Manure, irrigation water, soil, fecal contamination, infected seeds, geographical location, and climate change are all current contamination sources in the field. If not effectively regulated according to good manufacturing procedures, postharvest steps such as harvesting, pre-cooling,

processing operations, washing and sanitizing, storage packaging, and transfer can lead to Nontyphoidal *Salmonella* contamination. One of the main sources of contamination has been

identified as irrigation water and irrigation methods (Kim *et al*., 2012). High quantities of fecal coliforms come from the use of contaminated surface water or untreated sewage water, which leads to the spread of Non-typhoidal *Salmonella* to fresh produce. Spray irrigation systems, in comparison to other irrigation technologies such as surface irrigation and drip irrigation, represent a higher risk of pathogenic bacteria contamination due to the direct deposition of contaminated water onto the edible areas of the product (Aruscavage*et al*., 2006; Majowicz*et al*., 2006; Graham *et al*., 2011). Organic fertilizers, such as animal manures and slurries (Beuchat, 1996), abattoir wastes and sewage sludge, transfer pathogens to the field directly, and run-off can pollute irrigation water. Growers have access to comprehensive recommendations that educate them on proper waste treatment and application timing, with the goal of limiting crop contamination.

2.4 PREVENTION OF *SALMONELLA* IN FRESH PRODUCE

After coming into contact with raw meat or poultry, wash your hands, cutting boards, utensils, and countertops. Between handling different types of foods, wash your hands (fresh fruits and vegetables). Before eating, properly wash fresh fruits and vegetables. Keep different foods and fresh produce separately to avoid cross contamination i.e Keep raw meat, poultry, and seafood separate from ready-to-eat foods, such as salads . Wash and sterilize refrigerators in which fresh produce are stored. Avoid storing fresh produce in ambient temperature, to prevent spoilage of fresh produce, keep the refrigerator temperature below 40 degrees Fahrenheit. Farmers should use sterile irrigation water.

CHAPTER THREE

3.0 MATERIALS AND METHOD

3.1 SAMPLE COLLECTION

A total of 63 fruit and vegetable samples (9 samples each) were purchased from selected areas in various open markets in Ogun and Lagos states. . The purchased samples were placed in sterile food-grade bags and kept on ice packs in cooling boxes and then transported to the laboratory where they were immediately analyzed.

Table 3.1 Fresh produce samples location and frequency of collection.

Fresh produce sample	Location		
Lettuce	Jakande (L)	Ibafo (O)	Magboro (O)
Number of times sampled	3	3	3
Cabbage	Yaba (L)	Ibafo (O)	Magboro (O)
Number of times sampled	3	3	3
Pine apple	Magodo (L)	Ibafo (O)	Magboro (O)
Number of times sampled	3	3	3
Water melon	Magodo(L)	Yaba (O)	Magboro (O)
Number of times sampled	3	3	3
Cucumber	Jakande(L)	Ibafo (O)	Magboro (O)
No of times	3	3	3
Carrot	Yaba (L)	Ibafo (O)	Magboro (O)
No of times	3	3	3
Pawpaw	Yaba (L)	Ibafo (O)	Magboro (O)
No of times	3	3	3

3.2 APPARATUS AND EQUIPMENT USED

Apparatus used include: stomacher bags, wash bottles, petri-dishes, measuring cylinder, glass pipettes, beakers, conical flasks, glass spreader, inoculating loop, wash bottles, eppendorf tubes, micro pipette (with their tips), test tubes and foil corks (with their racks), PCR tubes, glass slides, oxidase test disc.

Equipment used: Analytical balance, Hot plate stirrer, Autoclave, Vortex mixer, Water distiller, , Water bath (set at 50°C and 100°C), Incubators (37°C and 42°C), Bunsen burner, , Centrifuge, Heating block, Thermal cycler, Gel electrophoresis tanks, Gel documentation system, Microscope.

3.3 MEDIA AND REAGENTS USED

The work area was sterilized by using 70% ethanol to inactivate any microbial contaminants.

For isolation of *Salmonella* species:

Buffered peptone water, Rappaport-Vassiliadis-Soya (RVS) Broth**,** Nutrient Agar, Xylose lysine Deoxycholate Agar(XLD), Brain Hearth Infusion Broth (BHI), Hektoen Enteric Agar (HEA),20 % Glycerol , Distilled water.

For molecular identification:

Agarose, 1x TAE buffer, master mix, specific primers, Nuclease free water, Ethidium Bromide.

For biochemical test:

Crystal Violet, Iodine, alcohol (95%), Safranin, 3% Hydrogen Peroxide.

3.4 PREPARATION OF CULTURE MEDIA

3.4.1 Buffered peptone water (BPW)

Peptone water is a microbiological growth medium made up of sodium chloride and peptic digest of animal tissue. The medium has a pH of 7.2 ± 0.2 at 25°C and is high in tryptophan. Buffered Peptone Water is a nonselective broth medium that can be used to grow bacteria as a primary enrichment media.

Preparation

1. 10g of the dehydrated medium was dissolved in 1litre of distilled water in a conical flask and was mixed thoroughly. The conical flask is then closed with a foil cork.

2. The mixture was then stirred for a while using the magnetic stirrer hot plate to dissolve the powder completely.

3. 225ml of the 1% was then dispensed into conical flasks.

4. The conical flasks containing the media was then autoclaved at 121°C for 15mins.

3.4.2 Rappaport-vassiliadis-soya broth (RVS)

The use of Soya Peptone Broth (RVS Broth) as a selective enrichment medium for the separation of *Salmonellae* from food and environmental specimens has been recommended (OXOID, England). It has the same ability to exploit all of the properties of *Salmonella* species as other Enterobacteriaceae. These include:

I. The ability to survive at high osmotic pressure.
II. The ability to multiply at pH levels that are lower than normal.
III. Compared to other plants, it is more resistant to malachite green.
IV. Have dietary requirements that aren't as high as those of others.

Preparation

1. 26.75g was suspended into 1 litre of distilled water (based on the manufacturer's instructions) and heated gently to dissolve using a hot plate stirrer.
2. 9 ml volumes were dispensed into tubes and sterilized by autoclaving at 121°C for 15 minutes.

3.4.3 Nutrient agar

Nutritional agar is a multipurpose nutrient media for the cultivation of microbes that can sustain the development of a variety of non-fastidious organisms. For the isolation and detection of total count of mesophilic organisms, it was prepared according to the manufacturer's instructions.

Preparation

1. The dehydrated medium was dissolved in the appropriate volume of distilled water i.e. 28g of Nutrient agar in 1 litre of distilled water based on manufacturer's instructions in a conical flask and mixed thoroughly. The conical flask is then closed using a foil cork (made up of cotton wool wrapped in aluminium foil).

2. The mixture was stirred for a while using the magnetic stirrer hot plate to dissolve the powder completely and was then sterilized by autoclaving at 121°C for 15mins.

3. The medium was then allowed to cool and poured aseptically into sterile petri dishes and left to solidify.

Note: The medium appears opalescent and is light amber in colour.

3.4.4 Xylose lysine deoxycholate (xld) agar

Xylose Lysine Deoxycholate Agar is a selective growth medium used for the isolation of *Salmonella spp.* from clinical and food samples.

Preparation

1. The dehydrated medium (57g) was suspended in 1000ml distilled water according to the manufacturer's instructions and mixed thoroughly. The mixture was heated with frequent agitation (using hot plate stirrer) to completely dissolve the powder.

2. This agar is not to be autoclaved as instructed by the manufacturer. The agar was allowed to cool and poured aseptically into sterile Petri-dishes and left to solidify.

3.4.5 Hektoen enteric agar

Hektoen Enteric Agar is a selective and differential medium designed to isolate and differentiate members of the species of *Salmonella.*

Preparation

1. 72.66 grams of the medium was suspended in 1000 ml distilled water and mixed thoroughly. The mixture was heated with frequent agitation (using hot plate stirrer) to completely dissolve the powder.

2. It is not to be autoclaved as instructed by the manufacturer. The agar was allowed to cool and poured aseptically into sterile Petri-dishes and left to solidify.

3.4.6 Brain heart infusion broth

Brain Heart Infusion (BHI) broth is a general-purpose liquid medium for the cultivation and maintenance of a wide range of fastidious and non-fastidious microorganisms, including aerobic and anaerobic bacteria, yeast, and molds from a variety of clinical and non-clinical specimens.

Preparation

1. 37 grams of the dehydrated medium was dissolved in 1000 ml of distilled water based on manufacturer's instructions in a conical flask and was mixed thoroughly. The conical flask is then closed using a foil cork (made up of cotton wool wrapped in aluminium foil).
2. The mixture was stirred for a while using the magnetic stirrer to completely dissolve the powder.
3. 5ml of the media was then dispensed into various test tubes and covered with foil cork and was then sterilized by autoclaving at 121^0C for 15minutes.

3.5 ISOLATION OF *SALMONELLA* SPECIES

3.5.1 Primary enrichment

Twenty-five grams of each fresh produce (fruits and vegetables) were placed in a sterile stomacher bag containing 225 milliliters of 1 percent peptone water (enrichment broth) and homogenized for two minutes at 180 revolutions per minute using the stomacher. After homogenization, the material was transferred to conical flasks and incubated at 37°C for 24 hours (Najwa *et al*., 2015).

3.5.2 Secondary enrichment

The overnight incubated primary enrichment media containing BPW and the homogenized sample were used to inoculate the secondary enrichment media for *Salmonella* detection. 1 mL of primary enrichment sample was pipetted into test tubes containing 9 mL Rappaport-Vassiliadis-Soya (RVS) and incubated for 24 ho at 42°C (Najwa *et al*., 2015).

3.5.3. Plating of the inoculum on agar

The secondary enrichment was streaked onto plates containing Xylose-lysine-Deoxycholate (XLD) agar, which was used for culture and presumptive confirmation of *Salmonella spp*. using a sterile inoculating loop and incubated for 24 h at 37°C. (Najwa *et al*., 2015).

Black colonies on the plate after 24 hours indicated the presence of putative *Salmonella* spp., pink colonies indicated presumptive *Shigella*, and white colonies suggested presumptive *E. coli.*

To confirm the colonies, the black colonies that indicated presumptive *Salmonella spp.* were sub-cultured on Hektoen Enteric Agar (HEA). Black colonies on HEA confirms the presence of presumptive *Salmonella spp.* Colonies that appeared clustered after incubation were sub-cultured of fresh HEA plates in order to obtain a pure culture.

3.6 Cryopreservation of isolates

A loopful of pure cultured presumptive *Salmonella spp.* was inoculated into a sterile Eppendorf tube containing 750µl of brain heart infusion broth and 250µl of 20% sterile glycerol which serves as cryoprotectant and it was stored in a -4^0C freezer.

3.7 BIOCHEMICAL TEST

3.7.1 Grams staining technique

The inoculating loop was sterilized with a bunsen burner flame, and then a pure culture was smeared on a sterile slide and heat fixed by passing it fast across the flame with the smear facing up. For staining, the slides were placed on the staining rack. The smear was covered in crystal violet stain and left for 1 minute before being carefully wiped off under running tap water. The smear was then flooded with Gram's iodine, which was allowed to sit for 1 minute before being drained off under a gentle running tap. The slide was then washed with a decolorizing chemical (70 percent alcohol) and let to stand for 10 seconds. After that, the slide was cleaned under running tap water, drained fully, and counterstained for 30 minutes with safranin. The slide was then blot dried with filter paper after being washed under gently running tap water until no color appeared in the effluent (Olutiola *et al*., 2000).

3.7.2 Catalase tests

Using a sterilized inoculating loop, the pure culture was smeared on a sterile slide. The smear was then treated with a drop of hydrogen peroxide. The outcome was then examined. The existence of oxygen bubbles indicated that catalase was present, while the absence of bubbles indicated that catalase was absent. (Olutiola*et al*., 2000)

3.7.3 Oxidase tests

The pure culture was smeared on the filter paper, along with a few drops of the oxidase reagent, and the results were examined. Oxidase positive cultures produced a purple tint in less than 10 seconds. Oxidase negative cultures did not develop any purple colour. (Olutiola*et al*., 2000)

3.7.4 Coagulase tests

On a sterile slide, a loopful of natural saline was added and emulsified with a loopful of 24 hour cultures until a homogeneous suspension was formed. The suspension was then given a drop of human plasma and swirled for 5 seconds. Clumping that did not re-emulsify revealed a coagulase positive result. The absence of clumping indicated a coagulase negative result.(Olutiola *et al*., 2000).

3.8 MOLECULAR IDENTIFICATION

3.8.1 Activation of isolates

Isolates were taken out of the freezer and allowed to thaw at room temperature. 1ml of BHI was added to Eppendorf tubes and autoclaved. 100µl of *Salmonella* isolates were added to the Eppendorf tubes containing the BHI and incubated at 37◦C for 24 h bringing about the activation of the isolates.

3.8.2 DNA extraction

The isolates were centrifuged for 5 minutes at 10,000RPM, then the supernatant was decanted. The Eppendorf tube was filled with 1ml of sterile distilled water, vortexed, and centrifuged at 10,000 RPM for 5 minutes, the supernatant was discarded, and the process was repeated. After which, 200 µl of sterile nuclease-free water was pipetted into the Eppendorf tube containing the pellets, vortexed, and then placed in the heating block to boil at 100◦C for 15 minutes, the solution was then placed in ice to cool, and the contents of the Eppendorf tube were centrifuged for 5 minutes at 14,000RPM.

A new set of Eppendorf tubes were labelled with the corresponding codes of the isolates and 150μl supernatant containing the extracted DNA was then transferred into the new Eppendorf tubes and stored in the freezer at -20^{o}C for further analysis.

3.8.3 Polymerase chain reaction (PCR)

The components of the PCR used to identify *Salmonella* spp. are listed in table 3.2 below. The PCR cocktail was made and then transferred to a PCR tube before being placed in the thermocycler. Initial denaturation was performed at 95°C for 5 minutes, followed by 35 cycles of 95°C for 2 minutes, 42°C for 30 s, and 72°C for 4 minutes, followed by a final elongation step at 72°C for 10 minutes. There were also negative control reactions provided. The template DNA was replaced with sterile water for the negative controls. Electrophoresis was used to confirm the PCR products, and a Gel Documentation system was used to visualize them under UV light.

Table 3.2: PCR reaction components used for 16s rRNA amplification

No.	Component	Initial concentration	Final concentration	Volume/rxn
1	Master mix	5x	1x	2µl
2	Forward primer	20µm	0.25µm	0.125µl
3	Reverse primer	20µm	0.25µm	0.125µl
4	DNA			2µl
5	dH_2O			5.75µl
6	Total			10µl

Primer	Target gene (synonym)	Target	PCR product size (bp)	Sequences	Reference
STM4057-f	STM4057	*Salmonella* subspecies I	137	5’ -GGTGG CCTCG ATGAT TCCCG-3’	Kim *et al.* (2017)
STM4057-r				5’ -CCCAC TTGTA GCGAG	

CGCCG-3’

Table 3.3: PROTOCOL FOR THERMAL CYCLER

Analysis	Step	Temperature	Time
.1x	Initial denaturation	95^0c	5 min
35x	Denaturation	95^0c	2 min
	Annealing	42^0c	30 sec
	Polymerization	72^0c	4 min
1x	Final polymerization	72^0c	10 min
1x	Hold	4^0c	∞

3.8.4 Gel electrophoresis using agarose

Dry Agarose powder was used to make the agarose, and 1.8g of it was dissolved in 100ml of 1x TAE buffer. The mixture was heated until a clear solution was obtained. A micropipette was used to add 3 µl of ethidium bromide to the mixture. It was then spun, allowed to cool and then transferred to the gel tray which results in a slab with the combs in place, that create wells in the slab. It was then allowed to solidify before carefully removing the combs. The gel tray containing the slab was placed into the gel tank and 1x TAE buffer was added to the gel tank. 3 µl of DNA ladder was added to the first well, and 4 µl of amplicon (one sample per well) was pipetted into each well that was produced. The tank was attached to the power pack and the gel was ran at 100 volts for 45 minutes. The resulting gel was inspected using the gel documentation system.

3.9 PRECAUTIONS

- Personal protective equipment, such as a covered shoe, a nasal cover, gloves, and a lab coat, were observed.
- At every stage of the project, aseptic practices were observed.
- There was no cross-contamination of the samples.
- Ensured that the samples were appropriately labeled at all times.
- To avoid killing the organism of interest, the inoculating loop was allowed to cool before making contact with the organism for sub-culturing.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 RESULTS

Fresh produce samples obtained from Lagos and Ogun state open markets were subjected to microbiological analysis. A total of 63 samples was analyzed but presumptive *Salmonella spp.* was not found in all of the samples. Only 16 samples had colonies that were black, elevated, round, and smooth on HEA. This shows that presumptive *Salmonella spp.* were present in the samples.

All suspected *Salmonella* colonies were collected from the agar plates and inoculated for biochemical identification of the isolates according to their various techniques. Gram-negative and rod-shaped isolates were found in all of the fresh produce samples. The samples passed the catalase test but failed the oxidase test.

Biochemical testing, which involved Gram staining for the identification of Gram-negative isolates, Catalase test, Oxidase test and Coagulase test, however, decreased the presumptive *Salmonella* samples to 12 (2SILS1, 2SILS2, SIRS1, SIRS2,2SIRS1,2SGWS1,2SGWS2, SJCS1, SJCS2,3SMWS2,3SMWS1, 2SGRS1)

Polymerase chain reaction was carried out for the confirmation of the 12 presumptive *Salmonella* isolates. PCR amplicons were conducted on a 1.8 percent agarose gel electrophoresis for molecular identification of the samples, and each isolate yielded a 137-bp positive result using STM4057 primers (as shown in Kim *et al*., 2017). *Salmonella* positive isolates were confirmed using this method.

Figure 4.1: HEA plate with growth of *Salmonella spp*.

Table 4.1: The total viable count (Log_{10}cfu/g) for each sample from different location.

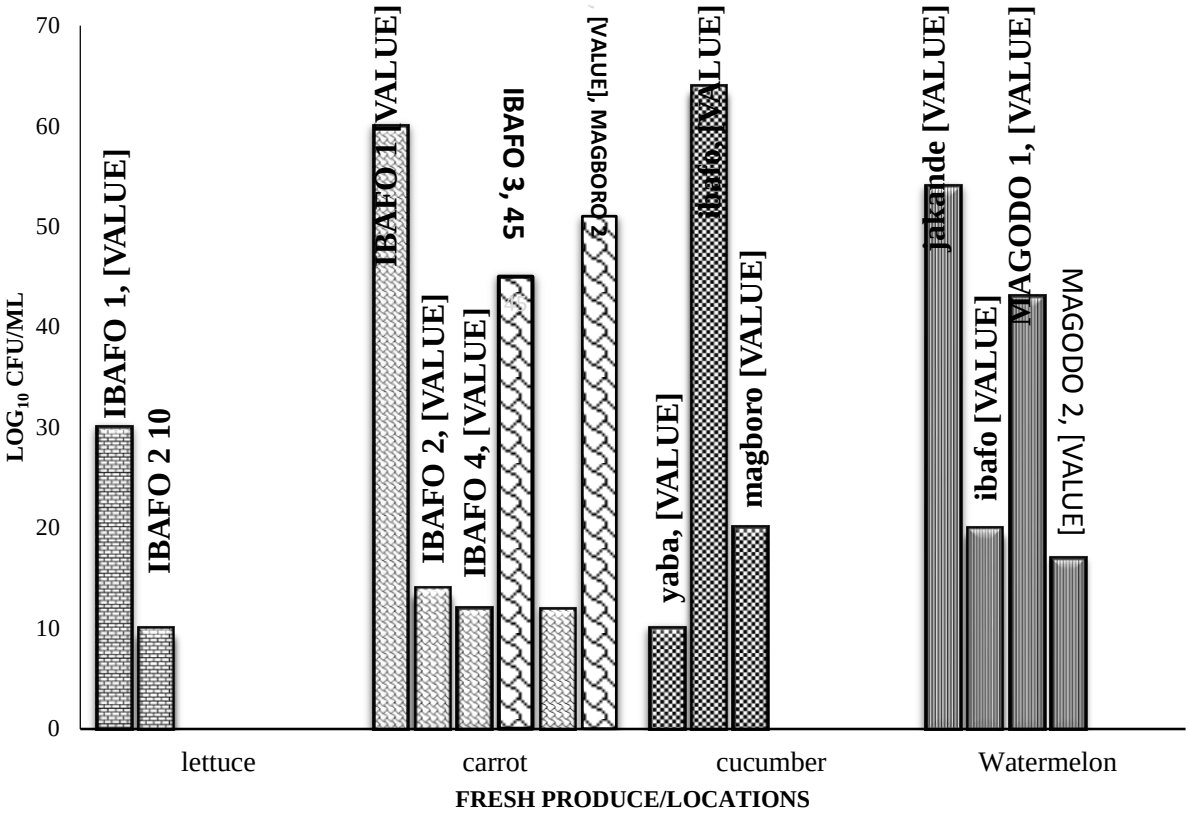

Table 4.2 Result of Biochemical Test for *Salmonella spp*

Biochemical tests	Result	Observation
Catalase test	Positive	Presence of bubbles
Gram staining	Positive	Pink colour
Oxidase test	Negative	No colour change observed

4.1 MOLECULAR IDENTIFICATION

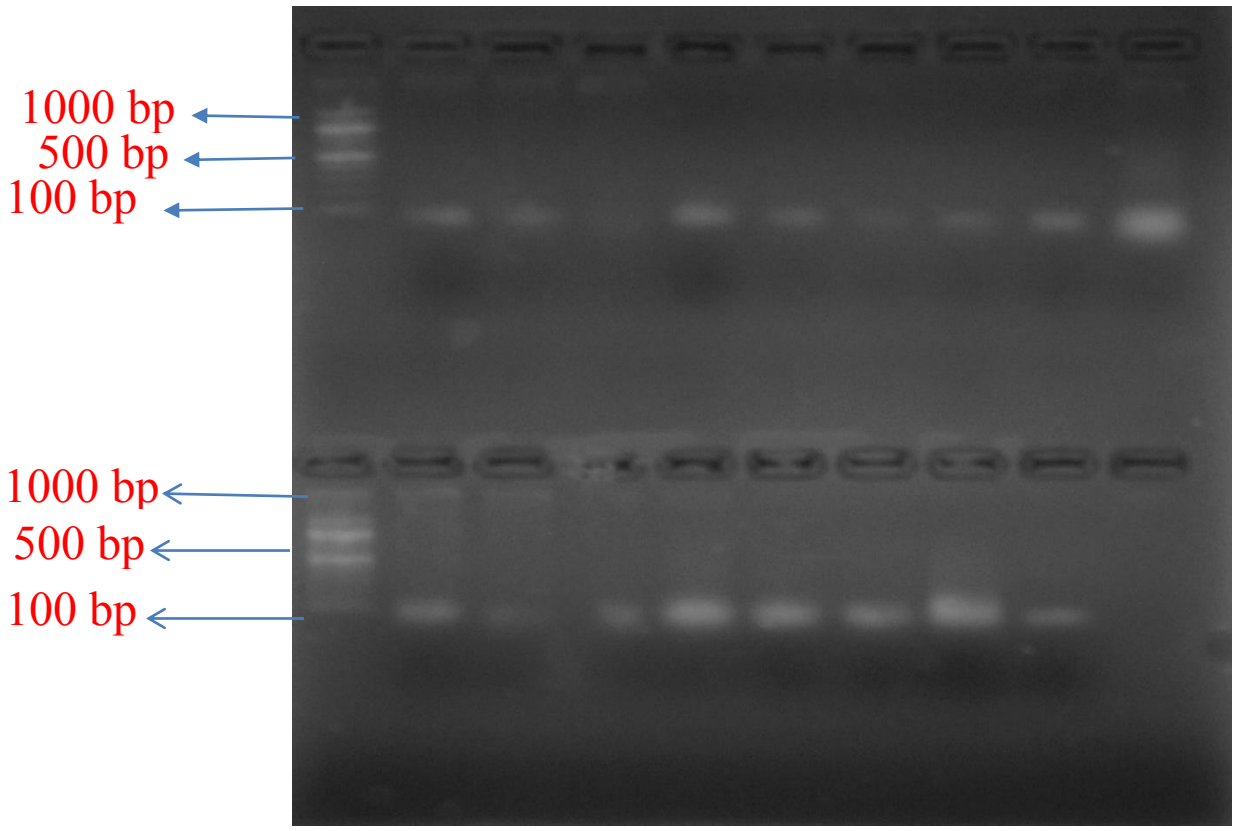

Figure 4.2: Agarose Gel electrophoresis of PCR amplicon for (16 rRNA amplification)

SPECIFIC
SALMONELLA

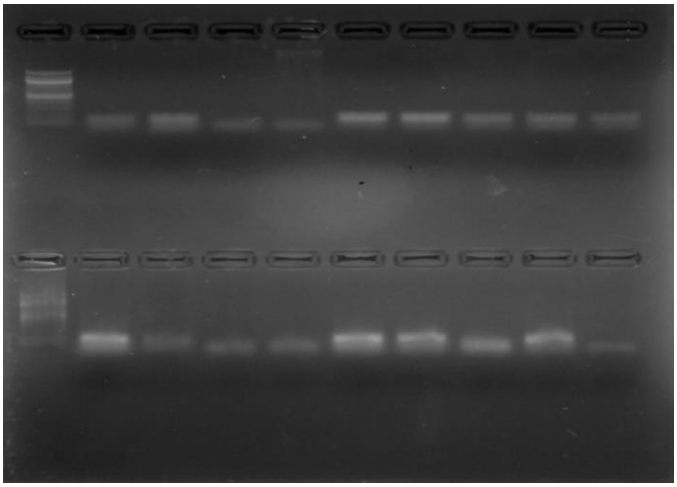

Figure 4.3 Agarose Gel electrophoresis of PCR amplicon for (Specific *Salmonella*)

There is a significant difference between the samples base on their Genetic Variances, Morphological Properties and their Epidemiology.

4.2 DISCUSSION

Non-typhoidal *Salmonella* (NTS), a foodborne pathogen, has been detected in the faeces of animals such as chickens and cattle, and may be spread by wildlife that roams and forages in fields or insufficiently decomposed dung (Cernicchiaro *et al*., 2012).

As a result of the small number of African countries who publish their surveillance data, there is relatively little information on NTS for the continent. Nonetheless, data from a South African veterinary diagnostic laboratory found that *Salmonella Typhimurium* was the most prevalent *Salmonella* serovar from 1996 to 2006 (Kudakwashe *et al*., 2015).

It has previously been reported in Several U.S studies (Johnston *et al*., 2005, Moll *et al*., 2006, Tauxe*et al*., 2010) showed that *Salmonella* frequency in fresh produce is usually lower than in cooked food, according to research. Several outbreaks of salmonellosis in the United States have involved fresh fruits, mainly melons. *S. miami* and *S. bareilly* infections were linked to the

consumption of precut watermelon in 1955 (Gayler*et al*., 1955). In more recent outbreaks, *S. oranienburg* (CDS, 1979) and *S. javiana* (Blostein., 1991) were implicated as causative agents of outbreaks associated with the consumption of watermelon. Laboratory studies have shown that S. derby and S. typhi can grow on cubes, of fresh watermelon (Escartin*et al*., 1989).

In this study, a total of 63 samples were collected from watermelon, carrots, pawpaw, pineapple, cucumber, lettuce and cabbage in open markets of Ogun and Lagos states for a period of 3 months. The samples were analyzed for the presence of *Salmonella spp* and to characterize the non-typhoidal *Salmonella* serotype.

12 samples (19%) was confirmed to be positive using biochemical tests and they were characterized as *Salmonella Typhimurium* using PCR. Ogun state had the highest level of non-typhoidal *Salmonella*, it ranged from Carrot (3), Lettuce (2), from Ibafo and watermelon (2) from Magboro, this was followed by samples from Lagos state which included Cucumber(2) from Jakande, Watermelon(2) and Carrots (1) from Magodo.

The presence of *Salmonella* could be due to the unhygienic practices and use of non-sterile water by farmers and vendors which is commonly observed in Nigeria including exposing these fresh produce to ambient temperature which is conducive for the proliferation of *Salmonella* contaminants, as well as the cross contamination of these fresh produce as observed in the case of the fresh produce obtained from Ibafo, Ogun State.

The presence of non-typhoidal *Salmonella* isolated from these fresh produce(fruits and vegetables) is of serious public health concern and should be taken into consideration as these could cause a series of food poisoning and Salmonellosis.

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATION

In Africa, outbreaks of foodborne diseases linked to fresh fruits and vegetables are still common. There is strong evidence that microbial contamination of fresh produce is widespread and contributes to the burden of foodborne illness. *E. coli* and *Salmonella species* were the most common pathogens connected to fresh vegetable outbreaks in this research. *Salmonella* infections were mostly associated to fresh-cut fruits in areas of Ibafo and Magboro One of the primary causes of the rise in enteric pathogenic bacteria-related outbreaks involving fresh-cut fruit is *Salmonella species* and it is a serious problem for the fresh produce business.

This study observed the presence of Non-typhoidal *Salmonella* in fresh produce (fruits and vegetables) sold in the open markets of Ogun and Lagos States, Nigeria. The presence of *Salmonella* indicates the lack of hygiene by these marketers, which serves as a public health concern. Good agricultural practices should be observed from the pre-harvest, harvest and post-harvest stages of these fresh produce.

Fresh fruits sold on the side of the road are normally Unhygienic and must be carefully washed before consumption. The sources of these fresh fruits must also be tracked to verify they were not exposed to any organic manure or contaminated water.

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