# MOLECULAR CHARACTERIZATION AND ANTIBIOTIC RESISTANCE PATTERNS OF NON-TYPHOIDAL *SALMONELLA* FROM FRESH PRODUCE

**BY**

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**A RESEARCH PROJECT SUBMITTED TO THE DEPARTMENT OF BIOLOGICAL SCIENCES, COLLEGE OF BASIC AND APPLIED SCIENCES, MOUNTAIN TOP UNIVERSITY, MAKOGI, IBAFO, OGUN STATE, NIGERIA**

**IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF BACHEOR OF SCIENCE (B.Sc.) IN BIOTECHNOLOGY**

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## DECLARATION

I hereby declare that this project report written under the supervision of Dr. G. B. Akanni is a product of my research work information derived from various sources has been duly acknowledged in the text and a list of references provided. This research project report has not been previously presented anywhere for the award of any degree or certificate.

__________________________ _______________________

ADESANYA DANIEL O. Date

## CERTIFICATION

This is to certify that this research project titled "**MOLECULAR CHARACTERIZATION AND ANTIBIOTIC RESISTANCE PATTERNS OF NON-TYPHOIDAL *SALMONELLA* FROM FRESH PRODUCE"** was carried out by ADESANYA, Daniel Oluwapelumi, with matriculation number 17010104003. This project meets the requirement governing the award of Bachelor of Science (B.Sc.) Degree in Biotechnology from the Department of Biological sciences of Mountain Top University, Ogun State, Nigeria and is approved for its contribution to knowledge and literacy presentation.

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DR O.T. KAYODE DATE

Ag. HEAD OF DEPARTMENT

## DEDICATION

This project is dedicated to God Almighty for providing me with good health, and the grace to complete this project; to my beloved parents, Mr. and Mrs. Adesanya, for their prayers, counsel, and sacrifice. I also dedicate this work to my spiritual father Dr. D.K. Olukoya, for the prayers all through this journey and the words of advice that helped me understand the essence of studying Biotechnology just before entering this great Institution. I love you all and God bless in Jesus name.

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**ABSTRACT**

Microbial contamination of fruits and vegetables is a major public health concern with several outbreaks of foodborne diseases linked to its consumption. There has been increasing reports on the occurrence of non-typhoidal *Salmonella* (NTS) contamination of fresh produce worldwide. A total of 63 fresh produce samples were obtained from road side vendors in Lagos and Ogun State. The samples ($n$ = 12) were positive for Salmonella species on XLD and HEA agar. The 12 isolates of *Salmonella* species were confirmed with Gram staining, catalase test and oxidase test. Molecular typing of the isolates was performed using *Salmonella* spp specific PCR. Antimicrobial susceptibility testing using Kirk-Bauer disk diffusion method was carried out. Antimicrobial resistance (AMR) was observed in 100% (12 isolates) of all the isolates with each exhibiting resistance to more than three antimicrobial agents, hence; all were multidrug resistant (MDR). The occurrence of MDR *Salmonella* spp. in this study indicates fresh produce from roadside vendors poses a high risk to human health. Therefore, proper precautions must be taken to prevent contamination of fresh fruits and vegetables from Farm to Fork. Inappropriate use of antibiotics in farm animals should be regulated and antibiotic stewardship would help greatly in slowing down the spread of resistant NT *Salmonella*.

Keywords: Fresh produce, Non-typhoidal *Salmonella*, antimicrobial resistance, multi drug resistance, antibiotic stewardship.

# CHAPTER 1

# 1 INTRODUCTION

## 1.1 Background to the Study

Fresh produce consumption has risen globally, owing to greater knowledge of the advantages of a healthy diet in human well-being with the UK and US government's recommended "Five a Day" and "Nine a Day" servings of fresh produce initiatives respectively (Anon, 2007). As a result, consumers are demanding more options, such as minimally processed, pre-packaged, ready-to-eat fruit and vegetables and availability of out-of-season fresh produce (Everis 2004). Fresh produce consumption has risen over the last two decades for a variety of reasons. For example, people are more concerned about remaining healthy and eating properly, and as a result, a wide selection of local and imported fruit has been accessible in all seasons to meet this demand (Warriner *et al*., 2009). From 1990 to 2004, the global consumption of fresh produce increased by an average of 4.5 percent every year (EU, 2007).

Fruits and vegetables have grown in popularity across the world as a proven component of healthy diets, as well as a source of minerals, nutrients, dietary fibre, and vitamins for humans (O'Shea *et al*., 2012). Government health agencies in several countries recommended intake of fruits and vegetables to protect against a variety of maladies, including eye disorders, malignancies, and cardiovascular disorders. Reduced intake of fruits and vegetables leads to poor health and an increased risk of non-communicable diseases (NCDs), according to the World Health Organization (WHO, 2017). Inadequate consumption of fruits and vegetables has been linked to an estimated 5 million deaths worldwide (Afshin *et al*., 2019).

Furthermore, new data reveal those fast-food specialties such as pizza, burgers, and chow mein are substantial contributors to obesity and multivitamin deficiency in children and young individuals (Rickman *et al*., 2007). In order to promote healthy living and well-being; regular consumption of fruits and vegetables is encouraged.

Simultaneously, occurrences of foodborne diseases linked to fresh produce intake have risen (Warriner *et al*., 2009). Despite the numerous health advantages of these fresh crop, its manufacturing process is complicated, involving multiple essential processes that may compromise microbiological safety (Kawamoto *et al*., 2015; Abatcha *et al*., 2018). As a

result, there are an increasing number of cases and outbreaks of foodborne pathogens linked to the ingestion of contaminated fresh produce (Kawamoto *et al*., 2015; Abatcha *et al*., 2018).

Microbial contamination can occur at any point in the farm-to-consumer supply chain (production, harvest, processing, wholesale storage, transit, or retailing and handling in the house), and it can come from a variety of sources (WHO/FAO, 2008). Fresh fruits and vegetables can occasionally become infected with pathogens, which are hazardous bacteria, viruses, fungi, protozoa etc. Members of the Enterobacteriaceae family are the most prevalent bacterial pathogens connected with foodborne diseases. They are usually included in microbiological criteria for safe and quality fresh produce, and their presence is fundamentally linked to food hygiene and safety (Rajwar *et al*., 2015). Foodborne pathogens are a diverse group of microorganisms that contaminate food and water at various stages of processing (Hanson *et al*., 2012). According to the WHO, food poisoning claims the lives of over 200,000 Nigerians each year (Onyeneho *et al*., 2013). While the whole scope of the burden and expense of contaminated food is unclear, the impact on global health, trade, and development is thought to be enormous. Foodborne pathogens have been studied in Nigeria, with *Staphylococcus aureus*, *Salmonella* spp., *Campylobacter* spp., *Listeria monocytogenes*, *Escherichia coli*, accounting for over 90% of annual food poisoning cases (Eni *et al*., 2010; Onyeneho *et al*., 2013).

Foodborne disease outbreaks and cases linked to fresh produce have grown dramatically during the previous two decades. Because a substantial part of this produce is eaten raw, the frequency of foodborne outbreaks linked to it has risen concurrently (Lynch *et al*., 2009; Olaimat *et al*., 2012). Globalization and expanding international trade can further raise the danger, particularly if the product comes from nations with lower safety regulations (Newell *et al*., 2010). Increased produce consumption and better foodborne illness surveillance may be contributing to the rise in outbreaks related with produce. The number of outbreaks reported in the United States and the European Union (defined as the occurrence of two or more instances of identical disease caused by the consumption of a common meal) reflects just a small percentage of the total number of outbreaks that occur (Arendt *et al*., 2013). Salmonella and *E. coli* O157:H7 are renowned for causing major outbreaks of foodborne disease linked to fresh produce on a regular basis (Warriner *et al*., 2009). In the United States, about 1 million cases of Non-typhoidal *S. enterica* are reported each year, with an estimated 27.2 percent of these cases resulting in hospitalisation (Hoffmann *et al*., 2012). *Salmonella* spp. were responsible for 18% of all single-etiology outbreaks between 1998 and 2008, as

well as the bulk of hospitalizations (44%) and fatalities (30%). In the same time span, *Salmonella* spp. were responsible for 53% of all multistate outbreaks (Gould *et al*., 2013). *S. enterica* Enteritidis was responsible for the most multistate outbreaks (by case count) between 2009 and 2015. In addition, Salmonella spp. also caused nearly twice as many single-etiology outbreaks (30%) as in the preceding decade (Dewey-Mattia *et al*., 2018). Non-typhoidal *S. enterica* infections are projected to cost over 3.3 billion dollars per year, more than any of the 14 main foodborne pathogens combined (Hoffmann *et al*., 2012).

Food can be contaminated at any point in the food chain therefore treatments must be performed when appropriate at every stage, the prevalence of food-related illnesses owing to fresh produce necessitates stronger control interventions and enhanced preventative methods globally (Kozak *et al*., 2013).

## 1.2 Statement of the Problem

Fresh cut fruits are contaminated during the process of handling, slicing and exposure to microorganisms in the surrounding. Due to the nature of the production of fresh cut fruits and vegetables, they can become easy mediums for the transmission of pathogenic microorganisms. If fruits from different sources or locations are kept together, the process of cross contamination and recontamination could occur easily. Humans become infected after ingesting contaminated fresh cut fruits. Pathogenic microorganisms have become resistant towards antibiotics which are the first line of action humans take when infected.

This project will focus on the presence of Non-typhoidal *Salmonella* in fresh produce, such as fruits and vegetables sliced and sold at the market, the resistance towards antibiotics and the mechanisms that aid the antibiotic resistance.

## 1.3 Aims and Objectives of the Study

To isolate and identify of *Salmonella* species in fresh cut fruits from locations in Lagos state and Ogun state.

To determine the incidence of bacterial infection caused by non-typhoidal *Salmonella species* which is hazardous to the health of people.

To determine the molecular mechanisms responsible for antibiotic resistance in *Salmonella* species with regards to non-typhoidal *Salmonella* serovars.

### 1.4 Significance of the Study

Microorganisms are more likely to contaminate fresh produce (fresh cut fruits) due to the availability of nutrients, the study aims to discover these organisms and the impacts they have on humans who consume them, precisely in the areas of salmonellosis and antibiotic resistance.

# CHAPTER 2

## 2 LITERATURE REVIEW

### 2.1 Introduction

Fresh fruits and vegetables contribute to a healthy and balanced diet and can help prevent chronic illnesses such as heart disease, cancer, diabetes, and obesity, as well as various micronutrient shortages, particularly in impoverished nations (Septembre-Malaterre *et al*., 2018). Fresh vegetables and fruits are significant sources of vitamins such as vitamins B, C, and K, minerals such as calcium, sodium, iron, magnesium, and dietary fibre (Yahia *et al*., 2019). It is highly suggested that you consume 3-5 servings of fruits and vegetables per day to decrease your risk of illness (Denis *et al*., 2016). In recent years, distinct consumption patterns of fresh produce have emerged. The largest rate of consumption was recorded in Asia followed by countries in Europe, Northern America, Oceania, and then Africa (FAO, 2020). Europe's consumption rate was found to be somewhat greater than that of Northern America, which saw a significant drop in consumption per capita all across the years. In comparison to Asia, Europe, and North America, Oceania saw a consistent increase in consumption, but it was significantly lower (FAO 2020). Over the previous 23 years, Africa's consumption rate has risen steadily, although at a slower rate; but in comparison to Europe, North and South America, and Oceania, the consumption patterns of fruits and vegetables in Africa and Asia is still very low (FAO 2020).

The presence of a positive correlation whereby global consumption of fresh fruits and vegetables is rising, as well as an increase in microbial contamination is posing a serious concern (Snyder *et al*., 2018). Fresh produce has the capacity to host a variety of microbial pollutants such as Gram-negative and Gram-positive bacteria, protozoa, viruses, and fungus such as yeast and moulds despite the fact that their consumption has been shown to promote overall health (Eni *et al*., 2010). Organic fertilizers, such as manure, municipal sludge, and faecal polluted water, are the most common sources of contamination, whereas pathogens

from humans, animals, and the environment are minor sources of fruit contamination (Hanning *et al*., 2009).

Fresh produce especially vegetables are becoming more recognized as major carriers of human diseases (Ramees *et al*., 2017). Fresh vegetables are often consumed raw or barely cooked to maintain their flavour and nutrient content, therefore, they are suitable vehicle for pathogen transmission to human, thus, can become a source of food-borne illnesses and disease outbreaks (Mir *et al*., 2018).

The epidemiology of foodborne diseases has altered dramatically in the last two decades (Henao *et al*., 2015). The development and re-emergence of foodborne pathogens has aggravated this trend (Kawamoto *et al*., 2015). Disease-causing etiological agents may be found in a wide range of microorganisms, including parasites. Bacterial microbes are recorded to have the highest number of different species followed by parasites then viruses and lastly fungi (Balali *et al.,* 2020).

Pathogenic viruses (Hepatitis A, Norovirus, and Rotavirus), bacteria (Campylobacter jejuni, Escherichia coli, Listeria, and Salmonella), and intoxication caused by toxins produced by pathogens such as Clostridium perfringens, Staphylococcus aureus, and Bacillus cereus) are the most common causes of foodborne disease or illness (Sharif *et al*., 2018). The growing number of etiological agents (Balali *et al*., 2020) leads to disease outbreaks, posing a serious health risk to people and the rest of the globe (Adegoke *et al*., 2018). In many countries both developed and developing, significant knowledge of food-borne illnesses linked to freshly consumed fruits and vegetables, as well as the water used in the preparation of foods, is critical in combating the situation. Foodborne disease is strongly linked to microbial contamination, according to several studies (Bintsis, 2018; Manjunath *et al*., 2018; Semanda *et al*., 2018). The ingestion of pathogenic microorganisms or their toxins, among other things, has been linked to epidemics of illnesses such as typhoid fever, dysentery, diarrhoea, and even cholera (Bhunia, 2018b).

## 2.2 Foodborne Disease Outbreaks Associated with Fresh Produce

The shift in recent years from more animal-based foodborne outbreaks to more fruit and vegetable-related illnesses might be attributed to rising produce intake (Fatica et al., 2011).

The categories used for classifying food vehicles and the typical pathogens found in food types are described in Table 2.1. Majority of the pathogens found are of bacterial nature but included are also viral pathogens and parasites.

**Table 2.1**: Description of the Categories Used for Classifying Food Vehicles and the typical pathogens found

| Category | Food vehicle | Pathogens | References |
|---|---|---|---|
| Salad | Every salad-related produce item: Bar salad, vegetable salad, tossed salad, coleslaw, French salad, Greek salad | *Clostridium botulinum*, *Shigella* spp., Noroviruses | Doona, 2015; Ölmez, 2016; Callejon, 2015 |
| Leafy vegetables | Every leaf-related produce item: Iceberg lettuce, romaine lettuce, fresh spinach, baby spinach, shredded lettuce | *Shigella* spp., *Listeria monocytogenes, Escherichia coli* O157:H7, Hepatitis A, *Salmonella* spp. (*S*. Enteritidis, *S*. Newport, *S*. Napoli, *S*. Java) | Doona, 2015; Ölmez, 2016; Callejon, 2015 |
| Tomato | Tomato | *Salmonella* Strathcona, *Salmonella* Typhimurium | Ölmez, 2016; Callejon, 2015 |
| Other vegetables | The rest of the vegetables that didn't fit into the preceding categories: carrots, pepper, beetroot, onion, peas, cucumber | Hepatitis A, Noroviruses; *Shigella* sonnei; *Yersinia*; *Salmonella* spp. *(S*. Newport*, S*. Saintpaul*)* | Doona, 2015; Ölmez, 2016; Callejon, 2015 |
| Sprouts | Every sprouts-related produce item: Alfalfa sprouts, bean sprouts, Radish sprouts | *Escherichia coli* O157:H7; *Salmonella* spp. (*S*. Weltevreden, *S*. Bareilly, *S*. Stanley, *S*. Newport) | Doona, 2015; Ölmez, 2016; Callejon, 2015 |
| Berries Every berry-related produce | Every berry-related produce: Strawberries, raspberries, blueberries, blackberries | Hepatitis A, Noroviruses; *Salmonella* spp.; *Cyclospora* spp. | Doona, 2015; Callejon, 2015 |
| Melon-like | Melon, watermelon, cantaloupe | *S*. Newport, *S*. Typhimurium; *L*. monocytogenes | Ölmez, 2016; Doona, 2015; Callejon, 2015 |
| Fruit juices | Every unpasteurized juice-related produce: Apple juice and cider unpasteurized, orange juice unpasteurized, fresh fruit | *Escherichia coli* O157:H7; *Shigella spp.*; *Salmonella enterica* | Doona, 2015; Vantarakis, 2011; Callejon, 2015; Kaczmarek *et al*., 2019 |

| | juice | | |
|---|---|---|---|
| Other fruits | Fruits not listed in the preceding categories: Banana, mango, grapes, pineapple, papaya | *Salmonella* spp. (*S* Agona, *S.* Braenderup) | Ölmez, 2016; Callejon, 2015 |

The incidence of multistate epidemics linked to food has risen, as has the number of people killed as a result of these outbreaks. These results imply that multistate produce-related epidemics are on the rise in the United States (Table 2.2); posing a serious public health risk (Carstens *et al*., 2019).

**Table 2.2**: Multiple state foodborne outbreaks involving *Salmonella* spp., *Listeria monocytogenes*, *E. coli* O157:H7, Hepatitis A virus and *Cyclospora cayetanensis* infections in the U.S. from 2010 to 2017 with associated fresh produce categories.

(Source: Carstens *et al*., 2019)

| Fresh produce category | Multiple state outbreaks, (2010 to 2017) n = 83 | Illnesses n = 4501 | Hospitalizations n = 1117 | Deaths n = 55 |
|---|---|---|---|---|
| Fruits (total) | 25 | 1443 | 530 | 42 |
| Melons | 10 | 578 | 276 | 38 |
| Papaya | 7 | 418 | 113 | 3 |
| Mango | 3 | 181 | 49 | 0 |
| Avocado | 1 | 59 | 7 | 0 |
| Grapes | 1 | 27 | 10 | 0 |
| Coconut | 1 | 14 | 2 | 0 |
| Stone fruits | 1 | 2 | 2 | 1 |
| Unspecified fruit | 1 | 7 | 1 | 0 |
| Vegetables (total) | 58 | 3215 | 657 | 13 |
| Sprouts | 16 | 603 | 99 | 3 |
| Lettuce | 8 | 144 | 43 | 1 |
| Cucumber | 7 | 1375 | 297 | 7 |
| Leafy greens* | 7 | 181 | 58 | 2 |
| Romaine | 7 | 358 | 100 | 0 |
| Tomatoes | 6 | 434 | 35 | 0 |

| | | | | |
|---|---|---|---|---|
| Spinach | 3 | 22 | 4 | 0 |
| Peppers | 2 | 53 | 13 | 0 |
| Onions | 1 | 29 | 6 | 0 |
| Cabbage | 1 | 16 | 2 | 0 |

*Includes leafy greens not categorized as lettuce, Romaine, or spinach.

From the year 2010 to 2017, 85 multiple state outbreaks linked to fresh fruit with proven etiologies occurred in the United States (Table 2.2). These outbreaks were caused by a total of five diseases: 83 were caused by three bacterial infections, while the remaining two were caused by Hepatitis A and *Cyclospora cayetanensis*. During the study period, bacterial infections were the primary source of multiple state outbreaks linked to fresh produce (Carstens *et al*., 2019). With 32 distinct verified serotypes, *S. enterica* was connected to almost half of the multistate outbreaks attributable to bacterial infections (67.5%). The serotypes Newport (10), Enteritis (6), and Javiana were the most common single-etiology outbreaks of *S. enterica* infection (5). 27.4% of the outbreaks were caused by pathogenic *E. coli*, whereas 4.8 percent were caused by *L. monocytogenes*.

Although *S. enterica* was the cause of the bulk of documented illnesses (81.1%) and hospitalizations (66.2%). The bulk of reported deaths (67.3%) were caused by *L. monocytogenes*, which were primarily caused by a single epidemic (33 known deaths). Approximately, 69.9% of outbreaks were connected to vegetables, whereas the rest were linked to fruits (Table 2.2); nevertheless, outbreaks linked to fruits resulted in more deaths than outbreaks linked to vegetables (Table 2.2). Sprouts and lettuce were the most commonly recognized food vehicles within the vegetable group, followed by cucumbers, Romaine, and leafy greens, while outbreaks related with cucumbers accounted for over half of all illnesses attributed to vegetables. In the fruit group, melons were the most common dietary vehicle for bacterial outbreaks.

## 2.3 Contamination Routes of Fresh Produce

Fruits and vegetables can be contaminated at any step from farm to fork and this ranges from preharvest to harvest and finally postharvest processes (Gil *et al*., 2015).

### 2.3.1 Contamination of fresh Produce Pre-harvest

Preharvest contamination can come from a variety of sources, including the soil in which fruits and vegetables are grown, as well as water used for irrigation. Other sources include water used to apply insecticides and fungicides, faeces, dust, improperly composted manure, and finally human interaction with these vegetables at various times (Balali *et al*., 2020).

Local and migratory birds frequent fields for food and shelter and may act as vectors for foodborne diseases (Cernicchiaro *et al*., 2012). Furthermore, bacterial pathogens such as *E. coli* O157:H7, Campylobacter, Shigella, *Salmonella enterica*, and *Listeria monocytogenes* have been found in nematodes, and some insects (Khamesipour *et al*., 2018).

In Africa, the use of irrigation as a technique of farming during the dry season is a common practice. In Sub-Saharan Africa, however, many vegetable crops are grown in fresh form utilizing irrigation (Uyttendaele *et al*., 2015). Irrigation water is a known source of foodborne pathogen contamination, and its quality is used to determine the safety of produce. Rivers, collected rainfall, aquifers, and groundwater are examples of natural sources of irrigation water. As a result, certain microbes have the potential to contaminate the plants and, as a result, the customers (Uyttendaele *et al*., 2015).

### 2.3.2 Contamination of Fresh Produce during Harvest

Contamination at this stage occurs through direct contact with infected equipment, transport containers, knives and tools, as well as human hands and gloves. Pathogens can be carried by mechanical harvesting equipment, such as vegetable cutters and corers, and spread to fresh food during harvesting (McEvoy *et al*., 2009; Yang *et al*., 2012). Food worker transmissions are thought to be responsible for 20% of all foodborne bacterial infections (Greig *et al*., 2007). Food worker transmissions were blamed for 647 foodborne outbreaks in the United States during the 2000s, resulting in 54,888 illnesses (Greig *et al*., 2007). Out of these outbreaks, 23 % (151) were linked to the intake of *S. enterica*-contaminated food, with 5 % (7) linked to produce and resulting in 1263 cases.

Pathogen transmission from food workers to food products can occur as a result of inadequate sanitary circumstances (personal or environmental), working while unwell, or a lack of sufficient food safety training and farming practices (Carstens *et al*., 2019).

### 2.3.3 Contamination of Fresh Produce during Post-harvest Processes

Transportation techniques, processing equipment, dust, and washing water are all causes of contamination post-harvest (Gil *et al*., 2015). Washing/flushing, shredding and cutting, drying, and packing are some of the processes in which fresh produced is slightly processed. When fresh produce is washed in pond or river water, it is more susceptible to contamination since these waters are more likely to carry harmful microorganisms (Uyttendaele *et al*., 2015).

Contamination of produce can occur as a result of contaminated equipment or cross-contamination with other produce. Many researches have looked at how product items get contaminated or cross-contaminated during processing (Buchholz *et al*., 2014; Smolinski *et al*., 2018). *S. enterica* and *E. coli* O157:H7, for example, have been found to cross-contaminate spinach, cilantro, and Romaine lettuce during pilot plant-scale processing, such as washing and flushing (Smolinski *et al*., 2018).

In Africa, vegetables are cleaned using readily available water sources such as rivers and ponds near the production or selling location (Acheampong, 2015). Containers used by farmers and fruit and vegetable merchants to wash vegetables are rarely cleansed after use, and even when they are, the water is reused multiple times, allowing for cross-contamination (Acheampong, 2015). To guarantee the safety and avoidance of microbiological contamination, washing containers should be cleaned both before and after use.

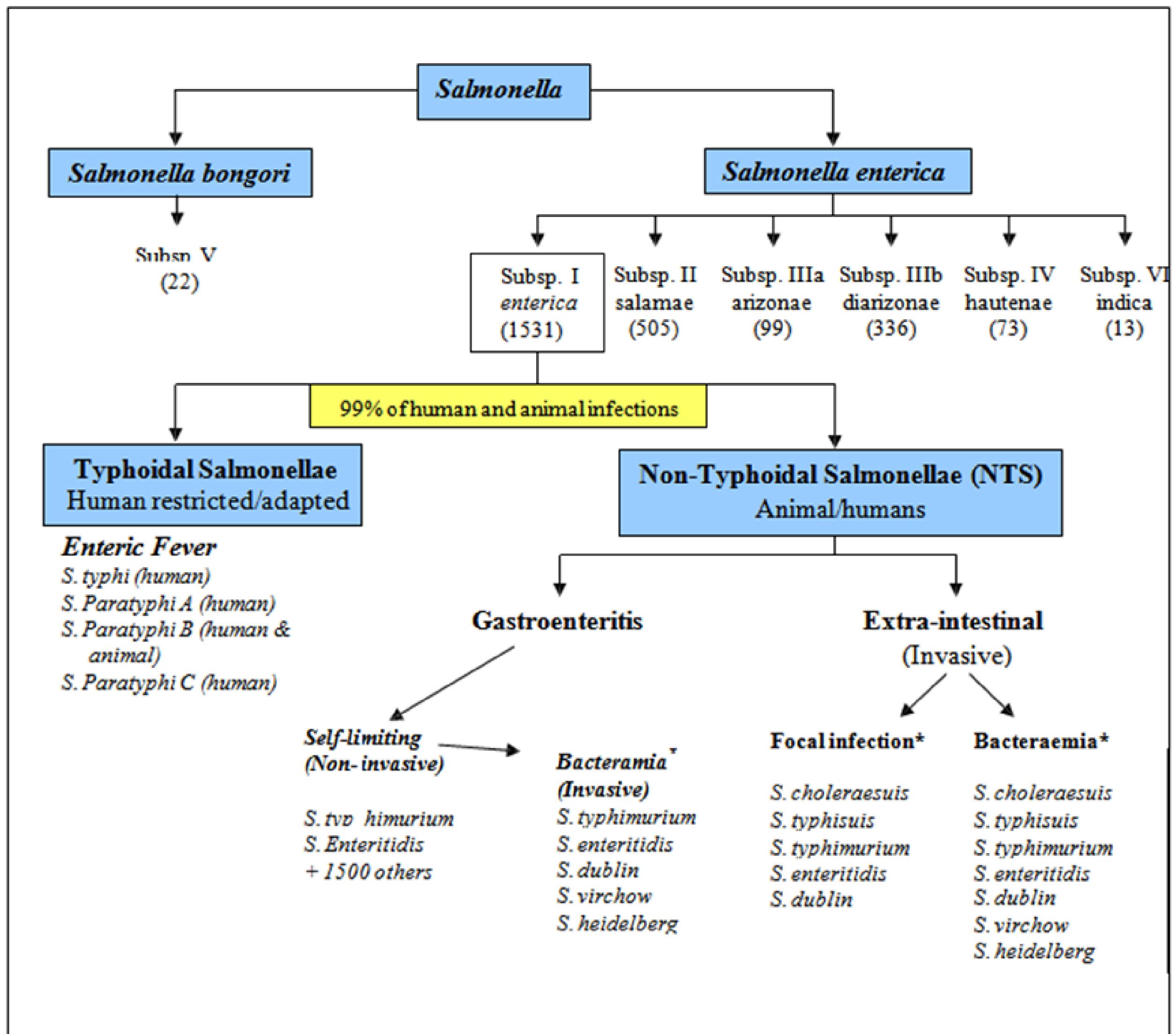
### 2.4 *Salmonella* Species

*Salmonella* is a Gram-negative, facultatively anaerobic, non-spore producing rod-shaped bacillus belonging to the Enterobacteriaceae family with cell diameters ranging from 0.7 to 1.5 m and lengths ranging from 2 to 5 m. (Tindall *et al*., 2005). They are chemotrophs and motile, with most possessing peritrichous flagella, with the exception of *S*. Gallinarum and *S*. Pullorum, which are non-motile and highly harmful to poultry (Bhunia, 2008). Salmonella are non-fastidious bacteria that may live and proliferate in a variety of environments outside of a living host cell (Pui *et al*., 2011). Temperatures vary from 7 to 48°C and pH levels ranging from 6.5 to 7.5(Pui *et al*., 2011). *Salmonella* is a heat-sensitive bacterium that may be destroyed in 15 to 20 minutes at 60 degrees Celsius, which is the temperature at which milk is pasteurized (Adams *et al*., 2008).

#### 2.4.1 Classification of *Salmonella* Species

According to the current World Health Organization and American Society of Microbiology nomenclature, the genus *Salmonella* is divided into two species, *Salmonella* enterica and *Salmonella bongori*, based on sequence analysis variations. Based on biochemical profiles and genetic relatedness, *S. enterica* is divided into six subspecies (*enterica, salamae, arizonae, diarizonae, houtenae, and indica*) (Brenner *et al*., 2000). More than 2500 serovars of *Salmonella enterica* exist, with around 80 of them being linked to Salmonellosis in both animals and humans (de Freitas Neto *et al*., 2010). The most often reported serotypes associated with human foodborne infections in the United States include *S. enterica, S.*

*Typhimurium, S. Enteritidis, S. Hadar, S. Newport, S. Heidelberg, and S. Javiana* (Suresh *et al.*, 2006). *S. bongori*, on the other hand, has 20 serotypes and is most often linked with cold-blooded animals, although it can also infect people (Bhunia, 2008).




Numbers in brackets indicate the total number of serotypes included in each subspecies.
* Common serotypes are listed but other serotypes may cause bacteraemia or focal infection; subsp., subspecies.

**Figure 2.1**: Classification of the *Salmonella* genus.

(Adapted from Akyala and Alsam, 2015)

Based on the reaction to particular antibodies, members of these seven subspecies can be classified into one of the more than 2,500 known serotypes or serovars. *Salmonella* is categorized into various O classes and serovars based on the expression of somatic lipopolysaccharide O antigen and flagellar H antigen, according to the Kauffman-White classification system. (Kauffmann *et al.,* 1952).

*Salmonella* is classified into Typhoidal and Non-typhoidal *Salmonella* (NTS) depending on the infections they cause in humans.

This is shown in table 2.3 which indicates the differences between the serovars under *Salmonella subsp. I enterica*

**Table 2.3**: Summary of the differences between NTS and typhoidal serovars associated with disease in humans.

(Source: Gal-Mor O *et al*., 2014)

| Criteria | NTS Serovars | Typhoidal Serovars |
|---|---|---|
| Serovars | Mainly the serovars *Typhimurium* and *Enteriditis*. Although 1500 other serovars of *S. enterica* ssp. 1 are known | *Typhi, Paratyphi* and *Sendai* |
| Host Range | Broad | Human-restricted |
| Epidemiology | Worldwide | Endemic in developing countries especially those in south east Asia, Africa and South America |
| Reservoirs | Farm animals, Fresh produce, pets | None, mode of transmission is human to human |
| Clinical Manifestations | Self-limiting gastroenteritis in immunocompetent individuals (diarrhoea, vomiting, cramps)<br>In immunocompromised patients, disease is associated with invasive extraintestinal infections | Invasive, systemic disease in immunocompetent individuals (fever, chills, abdominal pain, rash, nausea, diarrhoea) |
| Course/ Duration of Disease | - Short incubation period (6–24 h)<br>- Brief duration of symptoms (less than 10 days)<br>- Long-term carriage has not been observed | - Long incubation period (7–21 days)<br>- Extended duration of symptoms (up to 3 weeks) |
| Human Immune Response | Robust intestinal inflammation, neutrophil recruitment, Th1 response | Minimal intestinal inflammation, leukopenia Th1 response |
| Genetic Basis of Disease Differences and Host Specificity | - Low degree of genome degradation<br>- Able to use terminal electron acceptors for anaerobic respiration in the inflamed gut<br>- Unique virulence factors (e.g., fimbriae, SPI-14) | - 5% of the genome is degraded (e.g., inactivated metabolic and virulence factor genes)<br>- Unique virulence factors and pathogenicity islands (e.g., Vi antigen, SPIs 7, 15, 17, and 18) |
| Vaccination | No vaccine presently available for humans | - Killed whole cell parenteral vaccine,<br>- live attenuated oral vaccine (Ty21a),<br>- Vi polysaccharide capsule-based vaccine |

| Animal Models of Human Disease | - Streptomycin-pretreated mice<br>- Calves<br>- Non-human primates | - Mouse infection with S. Typhimurium<br>- Tlr11$^{-/-}$ mice<br>- Humanized mice |
|---|---|---|

## 2.5 Non-Typhoidal *Salmonella*

Nontyphoidal salmonella is a major pathogen that has been linked to food poisoning across the world. Non-typhoidal salmonella is primarily a zoonotic pathogen that colonizes important livestock species such as cattle, pigs, and poultry in an asymptomatic state. They comprise of all serotypes of *Salmonella* subspecies I enterica (within the species *Salmonella enterica*) excluding *S.* Typhi, Paratyphi A, Paratyphi B, Paratyphi C, and *S.* Sendai, as shown in Figure 1. Examples include: *S.* Typhimurium, *S.* Enteritidis, *S.* Newport, *S.* Infantis, *S.* Saintpaul, *S.* Dublin, *S.* Virchow etc. Non-typhoidal Salmonellosis is the name given to Salmonellosis caused by these serotypes (Gal-Mor O *et al*., 2014).

### 2.5.1 Non-Typhoidal *Salmonella*: Pathogenesis

The majority of human salmonellosis infections are linked to infected water and food supplies such as poultry, eggs, beef, and meat products. Direct interaction with contaminated pet animals and person to person transmission, especially in hospitals, are other modes of transmission (Chen H-M *et al*., 2013). Nontyphoidal Salmonellosis is a leading cause of food poisoning around the world and the most prevalent *Salmonella* infection (Abatcha *et al*., 2020). They are significant foodborne pathogens that can cause self-limiting gastroenteritis, bacteraemia, and extraintestinal focal infections in rare cases. (Harish *et al*., 2017).

The infection's symptoms usually develop one week or more after consuming contaminated food and continue for one to seven days (Crump *et al*., 2008). Depending on the strain, the infectious dosage of *Salmonella* varies between 1 and $10^{10}$ CFU/g. Salmonellosis can be caused by $10^{10}$ cells from a single dietary item, according to one epidemic (Bhunia, 2008). *Salmonella* infection susceptibility is influenced by host factors such as age, immunological state, underlying disease, and digestive tract health (Pui *et al*., 2011). ***Salmonella typhimurium* and *Salmonella enteritidis*** are the major pathogens that induce non-typhoidal Salmonellosis, which is also a significant concern in developed countries (Crump *et al*., 2004).

## 2.6 Survival Mechanisms of Non-Typhoidal *Salmonella* in Fresh Produce

Nutrient availability, UV light, poisonous substances generated by the plant, competition from other microbes, and desiccation all have an impact on microbes' capacity to survive on fresh produce (Whipps *et al*., 2008).

### 2.6.1 Enteric Fitness on the Plant Surface

The plant's above-ground surface, known as the Phyllosphere, provides a potentially unfriendly habitat for an intestinal pathogen. Bacteria are exposed to strong doses of UV radiation, a lack of nutrition, an aerobic environment, and a wide range of temperature conditions in the Phyllosphere (Heaton *et al*., 2008; Whipps *et al*., 2008). The intestine environment, on the other hand, is UV-protected, nutrient-rich, anaerobic, and has little temperature variation. Six enteric bacteria and viruses were put into cantaloupe, lettuce, and bell pepper plants in a single research that was conducted under regulated temperature and humidity conditions. After 14 days, the pathogens were still detected in plant settings, indicating that enterics may survive in the phyllosphere (Stine *et al*., 2005).

### 2.6.2 Interactions between *Salmonella* Spp and Plants

The interior plant environment is naturally accessed through stomatal openings on the leaf surface. During photosynthesis, when sugars are generated at these locations, *Salmonella* spp may be attracted to the open stomata. Although Salmonella isn't attracted to open stomata that aren't generating sugars, this association is thought to be dependent on nutrition acquisition rather than penetration into interior plant tissues (Kroupitski *et al*., 2009). Salmonella spp. can survive in the plant environment, however the Phyllosphere's water and nutrient conditions appear to be insufficient for Salmonella spp. to use plants as an optimum home. The bacteria may be able to survive in the plant environment by forming biofilms on or within the plants (Fatica *et al*., 2011).

Fresh produce that has been cut has more water activity and more easily available nutrients at cut surfaces than fresh fruit that has not been cut, allowing a range of foodborne bacteria to thrive (WHO/FAO, 2008).

Enteric pathogens such as *E*. coli O157:H7 and *Salmonella* can form biofilms or internalise inside plant tissue when they come into contact with plant surfaces (Aruscavage *et al*., 2006), and bacterial fimbriae or flagella can aid plant infection. Biofilms form on fresh produce when bacterial cells clump together in exopolysaccharide compounds that shield the organisms from environmental challenges such as desiccation and bactericidal chemicals (Morris *et al*., 2003). As a result, bacteria in biofilms will have a higher chance of surviving.

These aggregates will comprise anywhere from 30% to 80% of the entire bacterial population on a leaf surface (Morris *et al*., 2003).

*S. Thompson* was observed on lettuce leaves using episcopic differential interference contrast microscopy and epifluorescence, and the aggregated cells seemed slimy, indicating the development of a biofilm on the lettuce leaves (Warner *et al*., 2008).

## 2.7 Outbreaks of Non-Typhoidal *Salmonella* in Fresh Produce

Molecular Subtyping is an essential epidemiological technique for tracking the source of infection and determining the epidemiological relationship between *Salmonella* Isolates from food and environmental sources (Ait Melloud *et al*., 2001).

**Table 2.4**: Non-typhoidal *Salmonella* (NTS) serotypes associated with multiple state fresh produce outbreaks in the U.S. from 2010 to 2017.

| Category of fresh produce | Associated *S. enterica* serotype(s) |
|---|---|
| Tomato | *Newport, Javiana, Saint Paul, Hartford* |
| Sprouts | *Newport, Cubana, Enteritidis, München, Kentucky, Ready, Abony, Braenderup, Montevideo* |
| Papaya | *Agona, Thompson, Gaminara, Kiambu, Seftenberg, Braenderup, Urbana, Infantis, Newport* |
| Cantaloupe | *Uganda, Panama, Typhimurium, Newport, Baildon, Minnesota* |
| Mango | *Braenderup, Worthington, Minnesota, Infantis* |
| Cucumber | *Javiana, Saint Paul, Newport, Poona, Oslo* |
| Romaine | *Newport, Enteritidis* |
| Grapes | *Saint Paul* |
| Avocado | *Enteritidis* |
| Peppers | *Anatum* |
| Onions | *Javiana* |
| Leafy greens | *Enteritidis, Javiana* |
| Melon | *Newport* |
| Coconut | *Chailey* |

(Source: Carstens *et al*., 2019)

From 2010 to 2017, *S. enterica* was identified as the causative agent in 56 multistate outbreaks linked to fresh produce (Carstens *et al*., 2019). These outbreaks in fresh produce

(table 2.4) were connected to a total of 3778 cases, resulting in a 28.3% hospitalization rate and 16 fatalities.

About 94% of *Salmonella* illnesses are foodborne, and persistent contamination with irrigation water has been proven to be a prevalent route of crop contamination in *Salmonella* outbreaks involving vegetables. Epidemiological studies back up the idea that contaminated irrigation water and animal manure serve as enteric pathogen transmission vehicles for fresh produce (Scallan *et al*. 2011).

Between 1999 and 2008, 880 deaths were reported in Lagos Nigeria out of the 85,187 confirmed cases of *Salmonella*-related diseases, giving a case-fatality rate of 1.03 percent (Akinyemi *et al*., 2012). The lack of epidemiological surveillance systems brings about difficulty in determining the true incidence of Salmonella-associated diseases, especially in developing countries like Nigeria (Akinyemi *et al*., 2012). Many instances go undocumented, and many milder cases go undiagnosed or unreported (Olowe *et al*., 2007). Ingestion of polluted irrigated vegetables has been related to an increasing number of human Salmonellosis cases (Lee *et al*., 2012). The current rainfall pattern in Nigeria results in a prolonged dry season during the cropping season, which has an impact on crop development and necessitates irrigation (Nwauwa *et al*., 2010). Due to the fact that *Salmonella* infection is widespread in Nigeria (Adabara *et al*., 2012), genetic diversity study of Salmonella strains is critical for infection epidemiology.

Antibiotic drug resistance is a major problem since certain instances of Salmonellosis are severe and necessitate antimicrobial therapy (Marrero-Ortiz *et al*., 2012). *Salmonella* strains are becoming increasingly resistant, making treating patients with serious illnesses more challenging (Adzitey *et al*., 2012). As a result, multidrug-resistant *Salmonella* has become a major scientific topic as well as a serious food-safety problem (Adzitey *et al*., 2012).

## 2.8 Antimicrobial Resistance of Pathogens in Fresh Produce

*Salmonella* has become much more common across the world over the years. Table 3 summarizes the frequency of *Salmonella* spp. in vegetables based on different research. The incidence varies from 0.4 percent to 97.9 % (Sant'Ana *et al*., 2011; Najwa *et al*., 2015). Leafy green vegetables in Malaysia (Najwa *et al*., 2015) had the greatest prevalence rate of 97.9%, followed by 28%, 27%, and 21.5 percent from a similar nation (Salleh *et al*., 2003; Nillian *et al*., 2011; Abatcha *et al*., 2018). According to these data, Malaysian vegetables had the

greatest level of *Salmonella* spp. contamination, followed by Iran (29 %) (Mehrabian *et al*., 2009). Sant'Ana *et al*. 2011, found that Brazilian vegetables had a low level of contamination.

**Table 2.5**: Prevalence (%) of Salmonella species in vegetables from various countries

| Country | Sample sources | Common serovars | Prevalence (%) | Reference |
|---|---|---|---|---|
| Iran | Cabbage-lettuce | *S. Typhimurium, S. Dublin, S. Enteritidis, S. Infantis, S. Montevideo, S. Derby* | 29 | Mehrabian *et al*. (2009) |
| Malaysia | Coriander, water spinach, bean sprouts, amaranth green, amaranth red, water spinach, | *S. Weltevreden, S. Corvallis, S. Brancaster, S. Typhimurium*<br>*S. Albany, S. Richmond, S. Braenderup, S. Enteritidis* | 21.5 | Abatcha *et al*. (2018) |
| | Asiatic pennywort, Long bean, winged bean and water dropwort | *S. Typhimurium and S. Enteritidis* | 97.9 | Najwa *et al*. (2015) |
| | Tomato, capsicum, cucumber, Carrot, Cabbage and Lettuce | *S. Typhimurium* and *S. Enteritidis* | 28 | Nillian *et al*. (2011 |
| | Selom, pegaga, kankong and Kesum | *S. Weltevreden, S. Agona, S. Seftenberg* and *S. Albany* | 27 | Salleh *et al*. (2003) |
| Brazil | Salads, collard greens, arugula, watercress, chicory, cabbage, spinach, Swiss hard, and colewort | *S. Typhimurium* and *S. enterica subsp. enterica O:47: z4, z23:* | 0.4 | Sant'Ana *et al*. (2011) |
| | Celery, watercress, beet, broccoli, zucchini, white round onion, cilantro, cabbage, cauliflower, spinach, Romaine lettuce, potato, parsley | *S. Typhimurium, S. Arizonae, S. Choleraesuis, S. Gallinarum,*<br>*S. Anatum, S. Houtenae, S. Agona, S. Enteritidis, S. Salamae,* | 5.7 | Quiroz-Santiago *et al*. (2009) |
| Nigeria | Spinach, Corchorus olitorus spp., sorrel, bitter leaf, and waterleaf | *S. Hadar, S. Vinohrady* | 6.3 | Raufu *et al*. (2014) |
| | Cabbage, lettuce, cucumber, tomatoes, green pepper | *S. Typhimurium* | 13.9 | Abakpa *et al*. (2015) |
| | Onion flakes, tomatoes, lettuce | *S. Typhimurium, S. Enteriditis, S. Derby, S. Newport* | 22.0 | Bagudo *et al*. (2014) |
| Pakistan | Carrot, coriander, cucumber, radish, cabbage, and tomato | *Salmonella spp.* | 8 | Razzaq *et al*. (2014) |
| India | Coriander, mint, carrots, radish | *S. Anatum, S. Bsilla, S. Newport,*<br>*S. Saintpaul, S. Teko, S. Virchow, and S. Weltevreden* | 3.6 | Singh *et al*. (2007) |

In Malaysia, most vegetables are sold at room temperature in the wet market, allowing harmful microbes to thrive and multiply (Puspanadan *et al*., 2012). At the same time, at the retail level, inappropriate handling and sanitary standards play a key role as a source of cross-contamination on vegetables and other fresh produce (Nillian *et al*., 2011). In this survey, several serovars were reported by different nations. In Malaysia (Najwa et al., 2015; Nillian *et al*., 2011), Iran (Mehrabian *et al*., 2009), and Nigeria, for example, *S. Typhimurium* and *S. enteritidis* were stated to be the major *Salmonella* serovars (Bagudo *et al*., 2014).

Antimicrobial drug resistance indicates that the antibiotic is ineffective in treating clinical illness caused by a certain bacterial infection (Alcaine *et al*., 2007). Table 2.6 shows the antibiotic resistance of *Salmonella* serovars from vegetables, as determined by many investigations.

**Table 2.6**: Prevalence (%) of Antimicrobial Resistance *Salmonella* among raw vegetables from various studies.

| Antimicrobial | Prevalence (%) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Najwa *et al*. (2015) | Kqueen *et al*. (2008) | Raufu *et al*. (2014) | Tasnim *et al*. (2016 | Singh *et al*. (2007) | Abatcha *et al*. (2018) | Overall Prevalence (%) |
| Amikacin | - | - | - | - | 28.6 | - | 28.6 |
| Amoxycillin-clavunic acid | 81.3 | - | 0.0 | 100 | 5.7 | 2.1 | 37.8 |
| Ampicillin | 100 | 29 | 0.0 | - | 11.4 | 26.7 | 33.4 |
| Apramycin | - | - | 0.0 | - | - | - | 0.0 |
| Cephalothin | 75 | - | - | - | 54.3 | 4.8 | 44.7 |
| Ciprofloxacin | 50 | - | 0.0 | 0.0 | 2.9 | 0.0 | 10.6 |
| Chloramphenicol | 6.3 | 11 | 0.0 | 0.0 | 5.7 | 21.9 | 7.4 |
| Cefotaxime | - | 0.0 | 0.0 | - | - | - | 0.0 |
| Ceftriaxone | - | 0.0 | - | - | 0.0 | - | 0.0 |
| Colistin | - | - | 0.0 | - | 22.9 | - | 11.4 |
| Ceftiofur | - | - | 0.0 | - | - | - | 0.0 |
| Ceftazidime | - | - | - | - | 25.7 | - | 25.7 |
| Cefoperazone | - | - | - | - | 48.6 | - | 48.6 |
| Cephotaxime | - | - | - | - | 40 | - | 40 |
| Erythromycin | 100 | - | - | 64.70 | - | - | 82.3 |
| Florfenicol | - | - | 8.0 | - | - | - | 8.0 |
| Furazolidone | - | - | - | - | 62.9 | - | 62.9 |
| Gentamycin | 0.0 | - | 0.0 | 76.47 | 28.6 | 3.2 | 21.6 |
| Kanamycin | - | - | - | - | 85.7 | 11.2 | 48.5 |
| Nalidixic acid | 0.0 | 36 | 14 | 23.53 | 85.7 | 12.8 | 28.7 |
| Streptomycin | 50 | 47 | 38 | 100 | 0.0 | 62.6 | 49.6 |
| Trimethoprim-sulphamethoxazole | 6.3 | 25 | - | - | - | 16.6 | 15.9 |
| Tetracycline | 12.5 | 85 | 8 | 0.0 | 51.4 | 44.3 | 33.5 |
| Spectinomycin | - | 0.0 | - | - | - | - | 0.0 |
| Sulphamethoxazole | - | - | 23 | - | 0.0 | 44.3 | 22.4 |
| Trimethoprim | - | - | 31 | - | 22.9 | - | 26.9 |

(Source: Abatcha *et al*., 2020)

The level of resistance varies depending on the country, the sample size, and the type of study. Several findings, however, were in agreement. In all of the surveys, there was no evidence of resistance to apramycin, cefotaxime, ceftriaxone, ceftiofur, or spectinomycin (Abatcha *et al*., 2020). According to Abatcha *et* al., 2020, it was reported that Salmonella isolates were frequently resistant to Erythromycin, furazolidone, streptomycin, Cefoperazone, kanamycin, cephalothin, and amoxicillin-clavulanic acid. Trimethoprim-sulphamethoxazole, florfenicol, colistin, ciprofloxacin, and chloramphenicol all had a lower resistance level. Salmonella species from vegetables are becoming increasingly resistant, according to all of the research included in Table 2.6, making treatment of clinical infections more challenging.

All *Salmonella* spp. identified from vegetables in Nigeria, according to Abakpa *et al*. (2015), were multidrug resistant (MDR). The development of MDR *Salmonella* isolates implies that these isolates may have come from locations where antibiotics are widely abused or utilized in animal production as therapeutic, prophylactic, and growth boosters (Singh *et al*., 2013; Abatcha *et al*., 2015).

## 2.9 Mechanisms Of Antimicrobial Resistance by Non Typhoidal Salmonella

The following are mechanisms of resistance displayed by NTS:

1. Release of microbial enzymes that either inhibit or destroy the antibiotic
2. Alteration of antibiotic binding targets
3. Enhanced export of antibiotic by efflux pumps
4. Alteration/ loss of drug entry ports (porins)

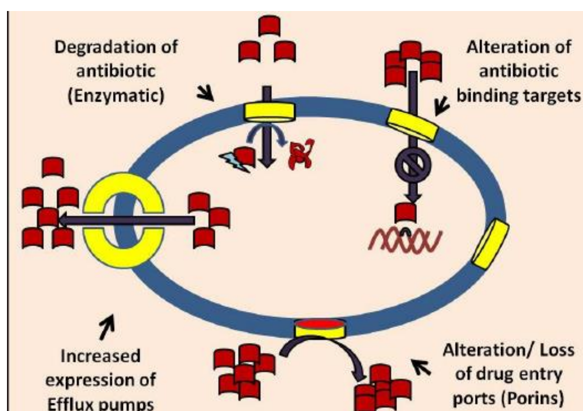



**Figure 2.2**: Mechanisms of resistance displayed by NTS

(Source: Andersen *et al*., 2015)

### 2.9.1 Chloramphenicol

It interacts with the conserved sequences of the 23S rRNA of the 50S subunit's peptidyl transferase cavity. As a consequence, it prevents t-RNA from binding to the A site of the ribosome, blocking protein synthesis. (Yoneyama *et al*., 2006).

For a long time, this broad-spectrum antibiotic, active against both Gram-positive and Gram-negative species, has been used to treat Salmonellosis in both humans and animals. Due to the emergence of resistance, this antibiotic's use has been restricted (Gunell *et al*., 2009). *Salmonella* has acquired resistance to chloramphenicol through two mechanisms:

1. The development of the non-enzymatic chloramphenicol resistance gene *cmlA* and the *flo* gene against the synthetic fluorinated analogue of chloramphenicol, florfenicol, both of which code for efflux pumps that prevent the antibiotic from reaching its target site (Adesiji *et al*., 2014)
2. Chloramphenicol acetyltransferases (*CAT*), which are found on plasmids. *Cat* enzymes are classified into two categories: *Cat A* and *Cat B*, with *Cat* B being detected on integrons in Nontyphoidal *Salmonella* serovar *Typhimurium, Derby, Enteritidis,* and *Haardy*. The efflux pump encoding genes –*flo* and *cmlA* – have been discovered in a number of *Salmonella* serotypes, including Typhimurium, Albany, Newport, and Agona. (Adesiji *et al*., 2014; Gunell *et al*., 2009).

### 2.9.2 Aminoglycosides

The bacterial ribosome is the primary focus of action; via hydrogen bonds, AGs interact with the 30S subunit's 16S r RNA near the A site. They trigger mRNA translation to be misread and terminated prematurely (Kapoor *et al*., 2017). To get there, it must travel across the cytoplasmic membrane, which necessitates an energy-dependent active bacterial transport system that involves oxygen and an active proton motive force. As a result, AG only function in aerobic environments and have no action against anaerobic bacteria. These AG have a synergistic effect with antibiotics that inhibit cell wall synthesis (such as β-lactam and glycopeptides) since they allow AG to enter the cell more deeply and at lower doses (Kapoor *et al*., 2017).

Reduced permeability and antibiotic absorption, alteration of the target site, and finally enzymatic modification are the three main mechanisms by which *Salmonella* may become immune to aminoglycoside antibiotics (Davies *et al*., 1978).

2.9.3 Fluoroquinolones

Quinolones and their derivatives are synthetic broad-spectrum antibiotics that function by preventing the unwinding and replication of bacterial DNA (Angulo et al., 2000). Because of their low toxicity and broad spectrum of action, they have been used in human and veterinary medicine to treat serious infections, and the development of resistance to these novel antibiotics poses a real threat. Various fluoroquinolones, such as enrofloxacin, difloxacin, marbofloxacin, and sarafloxacin, have been used in food animals to treat and avoid infections (Angulo *et al*., 2000). The main mechanisms of resistance to quinolones that have been recognized are:

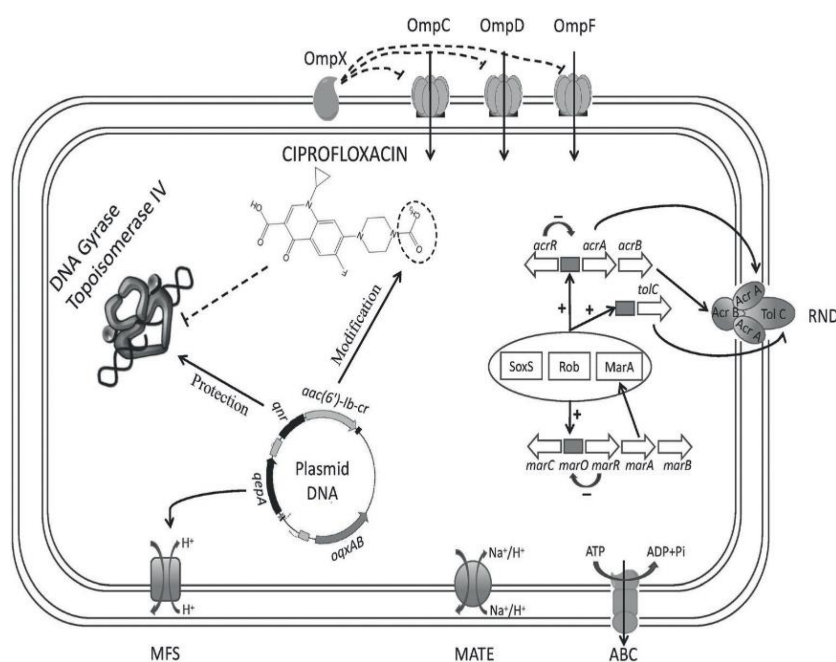



**Figure 2.3**: Mechanisms of resistance to Quinolones

(Source: Li *et al.*, 2018)

The target protein is structurally changed by chromosomal mutations in the QRDRs of the genes encoding DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV, decreasing its drug-binding affinity (Li *et al*., 2018). The AcrAB-TolC efflux network continues to be the major mechanism generating quinolone resistance in *S. Typhimurium* DT104 strains, with minor contribution from *gyrA* mutations, while for increased fluoroquinolone resistance in *S. Typhimurium* DT204 strains, both active efflux and accumulation of target gene mutations are required. (Baucheron *et al*., 2004).

Reduced outer membrane permeability and increased expression of efflux pumps are both caused by chromosomal mutations. Quinolone-resistant genes encoded on plasmids can

generate *Qnr* target defence proteins and *AAC (60)-Ib-cr* acetyltransferase variants capable of modifying quinolones, or (*QepA* and *OqxAB*) efflux pumps that actively eject quinolones (*Li et al*., 2018).

### 2.9.4 Tetracycline

Tetracyclines, inhibit t-RNA binding to the A site by acting on the conserved sequences of the 30S ribosomal subunit's 16S r RNA (Yoneyama *et al.,* 2006). Examples include tetracycline, chlortetracycline, doxycycline, and minocycline etc. This broad-spectrum antibiotic has been widely used in the treatment and prevention of infections in humans and animals, as well as subtherapeutic growth promoters in animal feeds (Chopra *et al*., 2001).

Tetracycline resistance in *Salmonella* is caused by the presence of newly acquired genes that code for energy-dependent tetracycline efflux or proteins that shield tetracycline's target site, the ribosome, from its action. Tetracycline resistant genes (*tet*) have been identified, and these genes code for membrane bound efflux proteins. The tetracycline cation complex is exchanged for a proton by these efflux proteins. The most common *tet* genes in *Salmonella* are found in the *Salmonella* genomic island and belong to groups A, B, C, D, G, and H. These genes are frequently found on mobile genetic elements such as plasmids, transposons, and integrons, and are also found together with genes that code for antibiotic resistance (Adesiji *et al*., 2014; Frye *et al*., 2013).

### 2.9.5 Sulphonamides and Trimethoprim

Sulphonamides and trimethoprim also function on the bacteria's folic acid pathway, preventing the formation of dihydrofolic acid. Since these antibiotics are synthetic, naturally occurring enzymes are unable to degrade or alter them (Adesiji *et al*., 2014; Cosby *et al*., 2015). These antibiotics target bacteria selectively and may therefore be used to treat systemic infections. The *sul1, sul2, and sul3* genes, which encode the drug insensitive dihydropterase synthetase (DHS) enzyme, are primarily responsible for sulphonamides resistance in Salmonella. (Adesiji *et al*., 2014; Anjum *et al*., 2011). In *Salmonella* with class 1 integrons and *aadA* and *dfrA* gene cassettes, the *sul3* gene can sometimes be detected, allowing isolates to survive co-trimoxazole, a common therapeutic combination. DHFR (Dihydrofolate reductase) encoding genes *dhfr* or *dfr*, or both, are responsible for trimethoprim resistance (Harish *et al*., 2017).

### 2.9.6 Beta lactam drugs

The most common mechanism of resistance to beta lactams in *Salmonella* is the secretion of beta-lactamases into the cytoplasmic environment. These enzymes hydrolyse the beta-lactam

ring structure, resulting in beta-amino acids with little antimicrobial action. (Harish *et al.*, 2017).

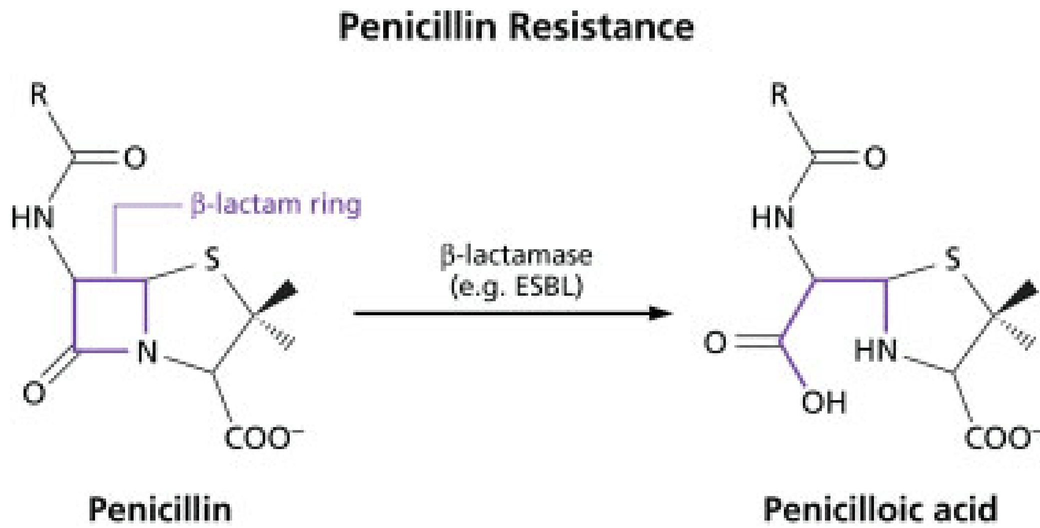



**Figure 2.4**: Hydrolysis of the beta-lactam ring structure

(Source: Harris, Patrick 2015)

Primary mechanisms of Beta-lactam resistance in *Salmonella* include the following:

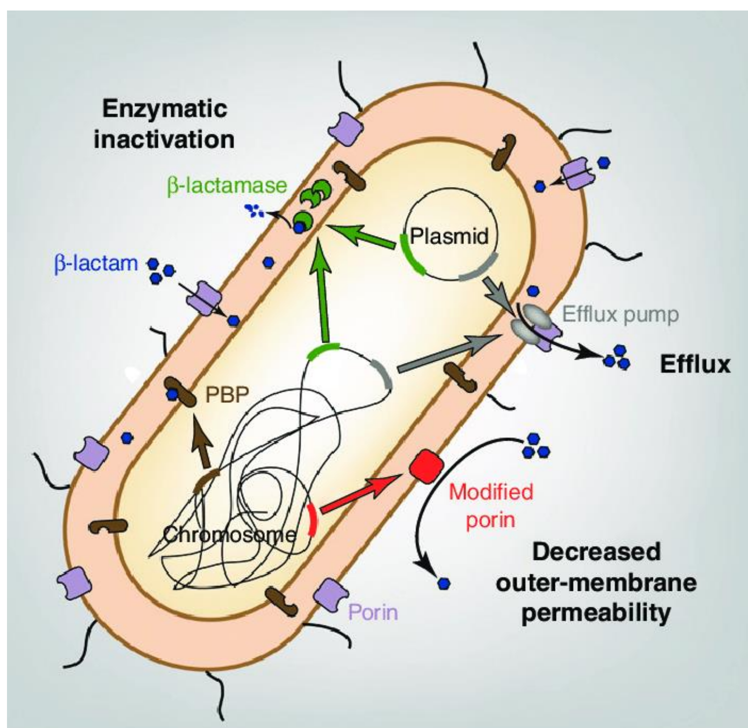



**Figure 2.5**: Primary mechanisms of b-lactam resistance in *Salmonella*

(Source: Nordmann *et al.*, 2012)

Enzymatic inactivation of the antibiotic by enzymes encoded on the chromosome and/or plasmid that have hydrolytic action against Beta-lactam molecules. Reduced permeability of the outer membrane due to the development of modified porins, a lack of porin expression, or a change in the types of porins present in the outer membrane. Ejection of the antibiotic to the outside of the bacterium through the development of an efflux pump (Nordmann *et al.*, 2012).

## 2.10 Multidrug Resistance

NTS multidrug resistance phenotype suggests resistance to three or more antimicrobial agents. For example, ampicillin, chloramphenicol, streptomycin, sulphonamides, and tetracycline (ACSSuT), according to the National Antimicrobial Resistance Monitoring System (NARMS) 2014 surveillance study on enteric pathogens (Harish *et al.*, 2017). ASSuT phenotype without chloramphenicol resistance has appeared as a significant phenotype in recent years. ACSSuT phenotype plus resistance to amoxicillin-clavulanic acid and ceftriaxone (ACSSuTAuCx) is another essential phenotype. *Typhimurium, Dublin, Heidelberg*, and *Newport* serovars are the most common Nontyphoidal *Salmonella* serovars with a multidrug tolerant phenotype (Harish *et al.*, 2017).

## 2.11 Antimicrobial Resistance Genes in Non-Typhoidal *Salmonella*

Most *Salmonella* strains have a unique collection of virulence characteristics, such as the capacity to invade and adhere to surfaces, as well as the ability to produce toxins, which are activated in the infected host and define the pathogenic potential (Tenor et al., 2004). *Salmonella* infection is mostly determined by the host's and the bacterium's condition. While host variables such as genetics, environment, and age impact an individual's ability to get illness, virulence genes or virulence factors determine the bacterium's pathogenicity (Ahmer et al., 1999).

*Salmonella* spp. requires a large number of genes to achieve maximum virulence, because it represents a complex combination of interactions inside its host (Lhocine et al., 2015). For the majority of the genes, discrete chromosomal groupings called "Salmonella pathogenicity islands" (SPIs) were discovered (Karunasagar et al. 2012; Que et al., 2013). Essential virulence factors are encoded by genes found on Salmonella pathogenic islands (SPIs), whilst others are encoded by genes found on chromosomes or virulence plasmids (pSLT) (Fàbrega *et al.*, 2013).

The characteristics of resistance to different antimicrobial drugs in salmonella with the associated AMR genes is summarised in Table 2.7

**Table 2.7**: Characteristics of resistance to different antimicrobial drugs in *Salmonella*. (Adapted from Alcaine *et al.*, 2007)

| Antimicrobial drug class | Common Resistance Genes | Salmonella serotypes |
|---|---|---|
| Aminoglycosides | ***aac*(3)-IV, *aac*(3)-IVa, *aac*C2, *str*A, *str*B, *aph*(3)-IIA, *aad*A1, *aad*A2, *aad*B** | *Agona, Anatum, Blockley, Bredeney, Derby, Give, Hadar, Heidelberg, Kentucky, London, Infantis, Saintpaul, Newport, Typhimurium* |
| Beta-lactams | $\textbf{\textit{bla}}_{\textbf{CMY-2}}$, $\textbf{\textit{bla}}_{\textbf{CTX-M9}}$, $\textbf{\textit{bla}}_{\textbf{TEM-1}}$, $\textbf{\textit{bla}}_{\textbf{TEM-53}}$, $\textbf{\textit{bla}}_{\textbf{CARB2}}$, $\textbf{\textit{bla}}_{\textbf{OXA-30}}$ | *Anatum, Agona, Blockley, Dublin, Enteritidis, Haardt, Muenchen, Newport, Stanley, Typhimurium, Virchow* |
| Chloramphenicol | ***cat*1, *cat*2, *cml*A, *flo*R** | *Albany, Agona, Derby, Enteritidis, Haardy, Kiambo, Newport, Typhimurium* |
| Quinolones | ***gyr*A, *gyr*B, *par*C**[a] | *Enteritidis, Typhimurium* |
| Tetracyclines | ***tet*(A), *tet*(B)** | *Agona, Anatum, Blockley, Bredeney, Colorado, Derby, Dublin, Enteritidis, Haardt, Hadar, Heidelberg, Infantis, Orion, Senftenberg, Typhimurium* |
| Sulfonamides | ***sul*1, *sul*2, *sul*3** | *Agona, Albany, Anatum, Brandenburg, Derby, Enteritidis, Hadar, Heidelberg, Orion, Rissen, Typhimurium* |

The incidence/occurrence of Salmonella in fresh produce is a public health implication. More so, the recent reports of AMR and MDR Salmonella strains is of concern. Therefore, the need to investigate the process of Salmonella with AMR patterns in fresh produce in Lagos and Ogun State is crucial.

# CHAPTER 3

## 3 METHODOLOGY

### 3.1 Sample Collection

Samples of seven different fresh produce namely; lettuce, cucumber, pineapple, watermelon, carrot, cabbage and pawpaw were obtained from road side fruit vendors at different locations around Lagos State(6.5244° N, 3.3792° E) and Ogun State(6.9980° N, 3.4737° E). The fresh produce samples were stored in sterile polyethylene bags and then immediately taken to the laboratory for microbial analysis.

**Table 3.1**: Fresh produce samples and their corresponding location

| **Fresh produce sample** | **Location** | | |
|---|---|---|---|
| Lettuce | Jakande (L) | Ibafo (O) | Magboro (O) |
| (*n* = 9) | 3 | 3 | 3 |
| Cabbage | Yaba (L) | Ibafo (O) | Magboro (O) |
| (*n* = 9) | 3 | 3 | 3 |
| Pine apple | Magodo (L) | Ibafo (O) | Magboro (O) |
| (*n* = 9) | 3 | 3 | 3 |
| Water melon | Magodo(L) | Ibafo (O) | Magboro (O) |
| (*n* = 9) | 3 | 3 | 3 |
| Cucumber | Jakande(L) | Ibafo (O) | Magboro (O) |
| (*n* = 9) | 3 | 3 | 3 |
| Carrot | Yaba (L) | Ibafo (O) | Magboro (O) |
| (*n* = 9) | 3 | 3 | 3 |
| Pawpaw | Yaba (L) | Ibafo (O) | Magboro (O) |
| (*n* = 9) | 3 | 3 | 3 |

Key notes: (L) - Lagos state. (O) - Ogun state.

### 3.2 Apparatus and Equipment Used

Apparatus used include: stomaching 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 slide, 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, Inoculating loop, Centrifuge, Heating block, Gel electrophoresis tanks, Gel documentation system, Microscope.

## 3.3 Media And Reagents Used

For isolation of *Salmonella* species:

Buffered peptone water, Rappaport-Vassiliadis-Soya (RVS)**,** Xylose lysine Deoxycholate (XLD), Brain Heart Infusion Broth (BHI), Hektoen Enteric Agar (HEA), 20 % Glycerol, Distilled water, 70% ethanol.

For molecular identification:

Agarose, 1x TAE buffer, *Taq*Man Master mix, Nuclease free water, Ethidium Bromide.

For biochemical test:

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

Antibiotic Susceptibility Test

Mueller-Hinton agar

## 3.4 Preparation of Culture Media

### 3.4.1 Buffer Peptone Water

Buffered Peptone water (BPW) is a microbial growth medium composed of peptic digest of animal tissue and sodium chloride. The pH of the medium is 7.2±0.2 at 25 °C and is rich in tryptophan. Buffered Peptone Water is also a nonselective broth medium which can be used as a primary enrichment medium for the growth of bacteria.

<u>Preparation</u>

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 cotton wool that is wrapped in aluminium foil.

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 flask.

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

### 3.4.2 Rappaport-Vassiliadis-Soya Peptone

The RVS broth (Oxoid, England) is used as a selective enrichment medium for the isolation of Salmonellae from food and environmental specimens. It has the ability to selectively enable the growth of Salmonella species and suppress the growth of other species. The characteristics of Salmonella include:

I. The ability to survive at high osmotic pressure is one of them.
II. To multiply at pH values that are relatively low.
III. To be more resistant to malachite green in comparison to other plants.
IV. Have nutritional needs that are not required by other Enterobacteriaceae.

<u>Preparation</u>

1. 26.75g was Suspended into 1 litre of distilled water (based on the manufacturer's instructions) and heat gently to dissolve using a hot plate stirrer.
2. 10ml volumes were dispensed into tubes and sterilized by autoclaving at 120°C for 15 minutes.

### 3.4.3 Xylose Lysine Deoxycholate Agar

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

<u>Preparation</u>

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.

### 3.4.4 Hektoen Enteric Agar

Hektoen Enteric Agar (HEA) is both a selective and differential medium developed for isolating and distinguishing members of the Salmonella species.

<u>Preparation</u>

1. The medium 72.66 grams was suspended in 1000 ml distilled water and mixed thoroughly. The mixture was heated with frequent agitation (using a 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. The inoculum was spread round evenly to obtain well-separated colonies. Incubate for 18-24 hours at 37°C.

### 3.4.5 Brain Heart Infusion

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

Preparation

1. The dehydrated medium was dissolved in the appropriate volume 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 $121^0$C for 15minutes.

### 3.4.6 Mueller-Hinton Agar

Mueller-Hinton Agar is mostly used for antimicrobial susceptibility testing (AST). The Clinical & Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing have both selected it as the standard medium for the Bauer-Kirby method (EUCAST).

Preparation

1. 38g of dehydrated medium was dissolved in the appropriate volume of distilled water based on manufacturer's instructions(1 litre) 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 hot plate magnetic stirrer to completely dissolve the powder.
3. The mixture was then Sterilized by autoclaving at 121°C for 15 minutes.

## 3.5 Isolation of *Salmonella* Species

### 3.5.1 Primary Enrichment

Twenty-five (25g) of the sample was put in a sterile stomacher bag containing 225ml of 1% peptone water (enrichment broth) and then homogenized using the stomacher at 180 rpm for 2 minutes. The homogenized sample was then transferred into conical flasks and incubated for 24 hours at $37^0$C (Najwa *et al.*, 2015).

### 3.5.2 Secondary Enrichment

This was performed for the detection of *Salmonella*, the overnight incubated primary enrichment media containing BPW and the homogenized sample was used to inoculate the secondary enrichment media. 1ml of sample pipetted from the primary enrichment was dispensed into 9mls RVS in test tubes and incubated for 24hrs at 42°C (Najwa *et al.*, 2015).

### 3.5.3 Plating Of the Agar

Using an inoculating loop, the secondary enrichment was streaked onto plates containing XLD agar which is the agar for culturing and presumptive confirmation of *Salmonella* spp. They were then incubated inversely at 37°C in an incubator for 24hrs (Najwa *et al.*, 2015).

After 24 hours, the formation of black colonies on the plate indicated the presence of presumptive *Salmonella* spp. whilst pink colonies indicated presumptive *Shigella* and white colonies indicated presumptive *E.coli*.

The black colonies which indicate presumptive *Salmonella* spp. were sub cultured on HEA in order to confirm the colonies. If the colonies after 24 hours of incubation appear clustered, a loopful of the colonies is sub-cultured again on another plate containing HEA.

## 3.6 Cryopreservation of Isolates

However, if they appear distinct, a loopful was inoculated into a sterile Eppendorf tube containing 1ml of brain heart infusion and 750ul of 20 % sterile glycerol as cryoprotectant and it was stored in a $-4^0$C freezer.

## 3.7 Biochemical Test

### 3.7.1 Gram Staining

The inoculating loop was sterilized on a flame of a Bunsen burner, then a smear of suspension was created with a loopful of the isolate on a sterile slide, this was air dried and heat fixed. Drops of crystal Violet were added to the slide and kept for about 30 seconds and rinsed with water. It was then flooded with gram's iodine for 1 minute and rinsed with water. 70% alcohol (decolorizing agent) was added for about 10-20 seconds and rinsed with water. Counterstain Safranin was added for about 1 minute and rinsed with water. It was then air dried and Observed under Microscope.

### 3.7.2 Catalase Test

Using an inoculating loop, a small amount of the isolate was transferred to the surface of a clean, dry glass slide, a drop of 3% $H_2O_2$ was added and observed for the evolution of oxygen bubbles.

### 3.7.3 Oxidase Test

An oxidase disc was used. An isolated colony to be tested was picked and rubbed on the disc. It was observed for colour change within 10 seconds.

## 3.8 Molecular Identification

### 3.8.1 Activation of Isolates

Cryopreserved *Salmonella* isolates were taken out of the freezer and allowed to thaw at room temperature. A 100µl of *Salmonella* isolates were added to the Eppendorf tubes containing 200µl of sterile BHI broth and incubated at 37°C overnight to activate the isolates.

### 3.8.2 DNA Extraction

The isolates were centrifuged at 10,000RPM for 5minutes and the supernatant was decanted. 1ml of sterile distilled water was added to the Eppendorf tube, vortexed and centrifuged again at 10,000 RPM for 5 minutes, the supernatant was discarded and the process was repeated. Afterwards, 200$\mu$l of sterile nuclease free water was then pipetted into the Eppendorf tube containing the pellets, vortexed and then it was placed in the heating block to boil at 100$^{o}$C for 15minutes, the solution was then placed in ice to cool, the content of the Eppendorf tube was then centrifuged finally at 14,000RPM for 5 minutes.

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

### 3.8.3 Polymerase Chain Reaction (PCR)

The components of the PCR and primers used for *Salmonella spp.* identification are shown in Table 3.2 and Table 3.3 below. The PCR cocktail was prepared and transferred into a PCR tube and was placed in the thermocycler. The PCR was carried with initial denaturation at 95°C for 5 min; 35 cycles of 95°C for 2 min; 42°C for 30 s and 72°C for 4 min; and a final elongation step at 72°C for 10 min. Negative control was included which involved replacing the template DNA with sterile water. The PCR products were confirmed by electrophoresis and visualized under UV light with a Gel Documentation system.

**Table 3.2**: PCR reaction components used for *Salmonella* spp., 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 |

**Table 3.3**: Primers

| Primer | Target gene | Target | PCR product size (bp) | Sequences | Reference |
|---|---|---|---|---|---|
| STM4057-f | STM4057 | Salmonella subspecies I | 137 | 5’ -GGTGG CCTCG ATGAT TCCCG-3’ | Kim *et al.* (2006a) |
| STM4057-r | | | | 5’ -CCCAC TTGTA GCGAG CGCCG-3’ | |

**Table 3.4**: Protocol for Thermocycler

| Analysis | Step | Temperature | Time |
|---|---|---|---|
| 1x | Initial denaturation | $95^0$C | 5 min |
| 35x | Denaturation | $95^0$C | 2 min |
| | Annealing | $42^0$C | 30 sec |
| | Polymerization | $72^0$C | 4 min |
| 1x | Final polymerization | $72^0$C | 10 min |

| 1x | Hold | $4^0$C | ∞ |
|---|---|---|---|

3.9 Agarose Gel Electrophoresis

The agarose was prepared using dry agarose powder, 1.8g of the agarose powder was dissolved in 100ml of 1x TAE buffer. The mixture was then boiled until a clear solution was gotten. 3µl of ethidium bromide was added to the mixture using a micropipette. It is then swirled and left to cool but not solidify, the content of the flask is then transferred into the gel container with the combs in place, after, it is left to solidify and the comb is gently removed. 1x TAE buffer is poured into the gel container. 3$\mu$l of DNA ladder was added to the fist well and 4$\mu$l of the amplicon (one sample per well) were then pipetted into each well that was formed after removing the comb. The tank was connected to the power pack and left to run at 100 volts for 45 mins and the gel is viewed using the gel documentation system for results.

3.10 Antibiotic Susceptibility Test

The antibiotic susceptibility test was performed according to Kirby-Bauer standard disk diffusion technique and Clinical Laboratory Standards Institute (CLSI) standards. The disc diffusion test was performed on Mueller-Hinton agar (Oxoid, England) for each isolate (Najwa *et al*., 2015; Kebede *et al.,* 2016).

Brain Heart Infusion broth (OXOID, England) was prepared into test tubes and autoclaved. *Salmonella* isolates were injected into 5ml of BHI and incubated at 37°C for 24 hours. MH agar was prepared and autoclaved after which was poured into sterile Petri plates and allowed to solidify.

Each isolate culture was compared to 0.5 McFarland turbidity standards (if necessary, adjusted by adding sterile saline into tubes until culture was more turbid). Swab sticks were used to inoculate isolates on Mueller-Hinton agar, and inoculated plates were kept at room temperature for 30 minutes to enable drying (Kebede *et al.,* 2016).

Antibiotic-impregnated discs (Cell tech Diagnostic, Belgium Inc.) were distributed over the surface of Muller-Hinton agar cultures and incubated for 20 hours at $37^0$C. Salmonella isolates were tested using the disk diffusion method for susceptibility to the following 12 antibiotics: amoxicillin/clavulanate (30 $\mu$g), cefotaxime (25 $\mu$g), imipenem/ cilastatin (10/10$\mu$g), ofloxacin(5$\mu$g), gentamicin (10 $\mu$g), nalidixic acid (30 $\mu$g), Nitrofurantoin (300

$\mu$g), cefuroxime (300$\mu$g), ceftriaxone sulbactam (45 $\mu$g), ampliclox (10 $\mu$g), cefexime (5 $\mu$g), Levofloxacin (5 $\mu$g), utilizing the disk diffusion technique in accordance with the Clinical Laboratory Standards Institute's guidelines (CLSI, 2020). According to an established interpretative chart (CLSI, 2020), the diameters of the zones of inhibition were measured to the closest millimeter and categorized as resistant, intermediate, or susceptible.

3.11 Precautions

- Personal protective technique was observed, such as wearing of covered shoe, nose cover, gloves, lab coat, etc.
- Aseptic techniques were observed at every stage of work.
- Cross contamination of the samples was avoided.
- Ensured that the samples were always properly labelled.
- Ensured that the inoculating loop cooled before picking the organism when subculturing in order not to kill the organism of interest.

CHAPTER 4

# 4 RESULTS AND DISCUSSION

## 4.1 Results

The existence of suspected *Salmonella* colonies was checked on the XLD plates. On XLD plates, the development of with black colonies was observed.

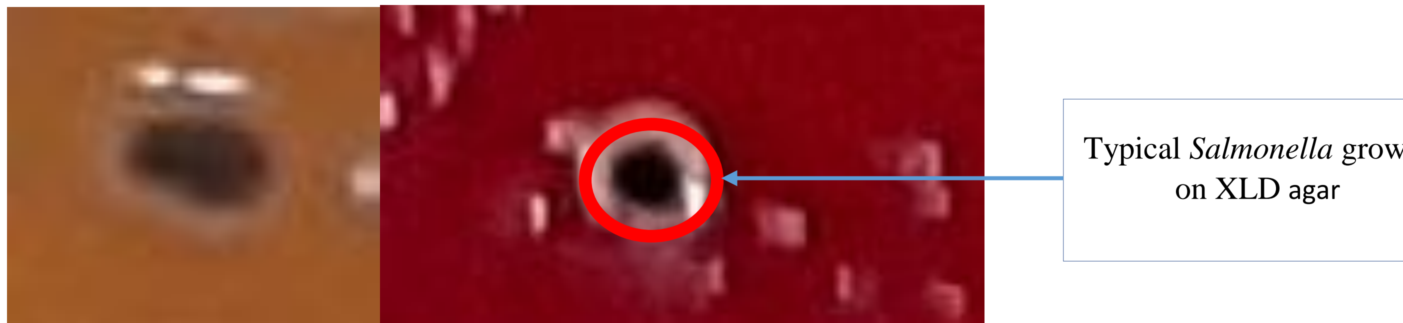



**Plate 4.1**: Examples of Presumptive *Salmonella* spp. Colonies on XLD agar

A total of 63 samples were tested, and 12 of them tested positive for salmonella as presumptive *Salmonella* colonies.

| Isolates Coding | Description |
|---|---|
| 2SILS1 | Ibafo lettuce |
| 2SILS2 | Ibafo lettuce |
| SIRS1 | Ibafo Carrot |
| SIRS2 | Ibafo Carrot |
| 2SIRS1 | Ibafo Carrot |
| 2SGWS1 | Magboro Watermelon |
| 2SGWS2 | Magboro Watermelon |
| SJCS1 | Jakande Cucumber |
| SJCS2 | Jakande Cucumber |
| 3SMWS2 | Magodo Watermelon |
| 3SMWS1 | Magodo Watermelon |
| 2SGRS1 | Magboro Carrot |

**Table 4.1**: Description of *Salmonella* positive isolates

These presumptive *Salmonella* colonies were further sub-cultured on HEA plates (for further nourishing and confirmation) which produced black colonies as presumptive *Salmonella*.

For confirmation, a loopful of presumptive *Salmonella* colonies was taken from the agar plates and inoculated for biochemical testing.

Gram staining was used to start the process of biochemically identifying the isolates. The isolates from the fresh produce samples were all Gram-negative and rod shaped. More biochemical assays and PCR amplification were used to identify all Gram-negative isolates.

The samples were positive for catalase test and negative for oxidase test.

**Table 4.2**: Biochemical test result and observation

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

For the molecular identification of the samples, PCR amplicons were run on a 1.8% agarose gel electrophoresis and each isolate produced a 137-bp product using *Salmonella* specific (STM4057) primers (as shown in Kim *et al*., 2006a; Park *et al*., 2009). This was used to confirm *Salmonella* positive isolates.

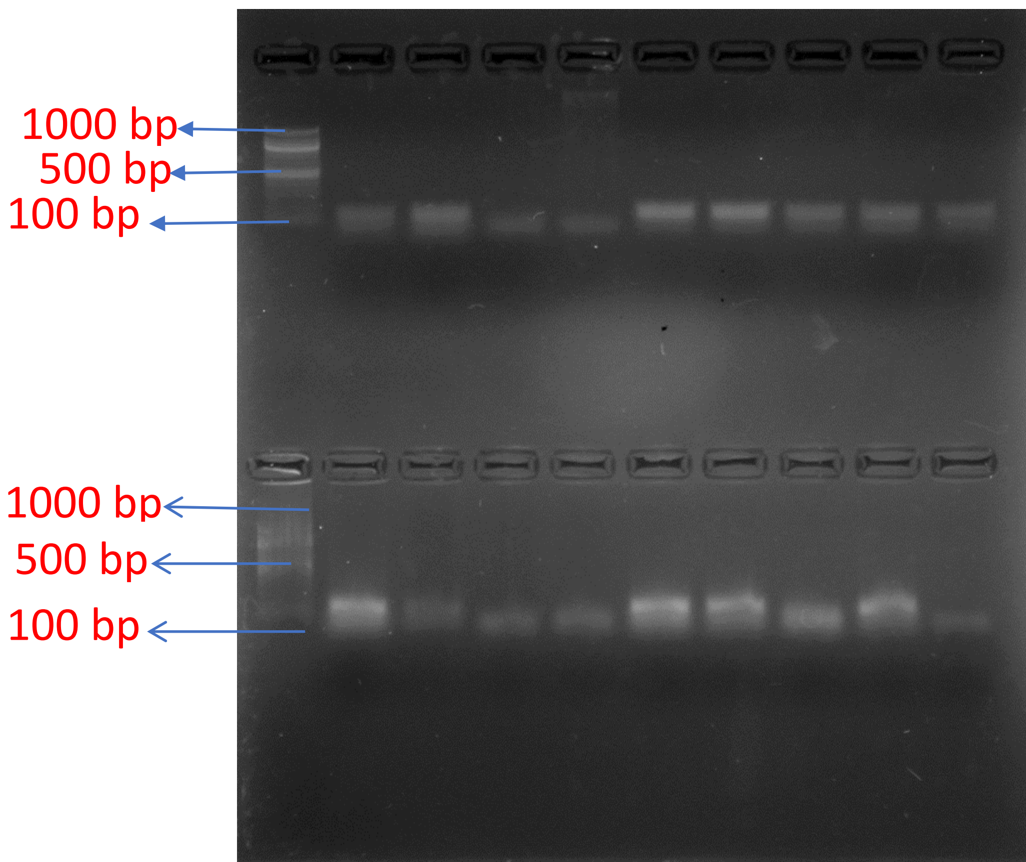



**Plate 4.2**: Agarose Gel electrophoresis of PCR amplicon for *Salmonella* spp.

**Plate 4.3**: Examples of disc diffusion test performed on Mueller-Hinton agar

The diameters of the zones of inhibition formed on Mueller-Hinton Agar from the disk diffusion technique performed according to the Clinical Laboratory Standards Institute (CLSI) standard was measured and categorized as resistant, intermediate, or susceptible according to the established interpretative chart from CLSI 2020.

**Table 4.3**: Diameter of zone of inhibition around antimicrobial agents to nearest millimeter

| Salmonella Isolates | AUG | CTX | IMP | OFX | GM | NA | NF | CXM | CRO | ACX | ZEM | LBC |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2SILS1 | - | - | - | 12 | - | - | 5 | - | 8 | - | - | 7 |
| 2SILS2 | 11 | - | - | 8 | - | - | - | - | 7 | - | - | 10 |
| SIRS1 | - | - | - | - | - | - | - | - | 12 | - | - | 8 |
| SIRS2 | - | 12 | - | - | - | - | - | - | 11 | - | 9 | 16 |
| 2SIRS1 | - | - | - | 10 | 9 | 10 | - | - | 14 | - | 9 | 17 |
| 2SGWS1 | - | - | - | - | - | - | - | - | 8 | - | - | 11 |
| 2SGWS2 | - | 14 | - | 25 | 11 | 11 | - | - | 13 | 11 | - | 16 |
| SJCS1 | - | - | - | - | 19 | - | 9 | - | 15 | - | - | 21 |
| SJCS2 | - | 19 | - | 18 | 12 | 11 | - | - | 23 | - | 15 | 25 |
| 3SMWS2 | - | 11 | - | - | 8 | - | - | - | 12 | - | 8 | 13 |
| 3SMWS1 | - | - | - | - | 11 | - | - | - | 17 | - | 10 | 11 |
| 2SGRS1 | - | - | - | - | 10 | - | - | - | 19 | 11 | - | 12 |

Key: AUG: Amoxicillin/clavulanate (30 $\mu$g), CTX: Cefotaxime (25 $\mu$g), IMP: Imipenem/ cilastatin (10/10$\mu$g), OFX: Ofloxacin(5$\mu$g), GM: Gentamycin (10 $\mu$g), NA: Nalidixic acid (30 $\mu$g), NF: Nitrofurantoin (300 $\mu$g), CXM: Cefuroxime (300$\mu$g), CRO: Ceftriaxone sulbactam (45 $\mu$g), ACX: Ampiclox (10 $\mu$g), ZEM: Cefexime (5 $\mu$g), LBC: Levofloxacin (5 $\mu$g)

Multidrug resistance by the 12 isolates to all the antibiotics used in this study was observed. The importance of this resistance is highly significant because these antibiotics are commonly used nowadays

The most ineffective antibiotics were: AUG, CTX, IMP, NA, NF, CXM, ACX, ZEM as they all had 100% resistance in all of the 12 isolates. This was followed by GM and CRO with both having 91.67% resistance in 11 out of the 12 isolates. OFX had 83.33% resistance (10 out of 12 isolates) and LBC had 75% resistance (from 9 out of 12 isolates, although one from the three that were not resistant was categorized as intermediate resistant).

**Table 4.4**: Classification of the diameters of the zones of inhibition according to CLSI interpretative chart

| Salmonella Isolates | Multidrug resistance patterns | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2SILS1 | AUG | CTX | IMP | OFX | GM | NA | NF | CXM | CRO | ACX | ZEM | LBC |
| 2SILS2 | AUG | CTX | IMP | OFX | GM | NA | NF | CXM | CRO | ACX | ZEM | LBC |
| SIRS1 | AUG | CTX | IMP | OFX | GM | NA | NF | CXM | CRO | ACX | ZEM | LBC |
| SIRS2 | AUG | CTX | IMP | OFX | GM | NA | NF | CXM | CRO | ACX | ZEM | LBC |
| 2SIRS1 | AUG | CTX | IMP | OFX | GM | NA | NF | CXM | CRO | ACX | ZEM | **LBC**[*] |
| 2SGWS1 | AUG | CTX | IMP | OFX | GM | NA | NF | CXM | CRO | ACX | ZEM | LBC |
| 2SGWS2 | AUG | CTX | IMP | + | GM | NA | NF | CXM | CRO | ACX | ZEM | LBC |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SJCS1 | AUG | CTX | IMP | OFX | + | NA | NF | CXM | CRO | ACX | ZEM | + |
| SJCS2 | AUG | CTX | IMP | + | GM | NA | NF | CXM | + | ACX | ZEM | + |
| 3SMWS2 | AUG | CTX | IMP | OFX | GM | NA | NF | CXM | CRO | ACX | ZEM | LBC |
| 3SMWS1 | AUG | CTX | IMP | OFX | GM | NA | NF | CXM | CRO | ACX | ZEM | LBC |
| 2SGRS1 | AUG | CTX | IMP | OFX | GM | NA | NF | CXM | CRO | ACX | ZEM | LBC |

Key- Resistant: AUG, CTX, IMP, OFX, GM, NA, NF, CXM, CRO, ACX, ZEM, LBC

Intermediate resistance: **LBC***

Susceptible: +

Although there was multiple resistance, OFX and LBC were the most effective antibiotics particularly LBC. These two antibiotics are under the Quinolones (Fluoroquinolones) antimicrobial drug classification. NA is also under this classification but not as potent as shown in Tables 4.2 and 4.3. They function by preventing the unwinding and replication of bacterial DNA (Angulo *et al.,* 2000)

The disk contents used were the same as those described in the CLSI 2020 document except for that of : Amoxicillin-clavulanate (AUG), Cefotaxime(CTX), Cefuroxime(CXM), Ceftriaxone sulbactam(CRO). The disk content in the document for Amoxicillin-clavulanate is 20/10 $\mu$g whereas 30 $\mu$g was used for this experiment, the disk content in the document for Cefotaxime is 30 $\mu$g whereas 25 $\mu$g was used, the disk content in the document for Cefuroxime is 30 $\mu$g whereas 300 $\mu$g was used, the disk content in the document for Ceftriaxone sulbactam is 30 $\mu$g whereas 45 $\mu$g was used in this study.

According to CLSI 2020, the antimicrobial agents are classified under the following Test/ Report Groups

**Table 4.5**: Classification of Antimicrobial Agent

| Test/ Report Groups | Antimicrobial Agents |
|---|---|
| **PENICILLINS** | ACX: Ampiclox, (Ampicillin and cloxacillin) |
| **B-LACTAM COMBINATION AGENTS** | AUG: Amoxicillin/clavulanate |
| **CEPHEMS** | |
| (PARENTERAL including cephalosporins I, II, III and IV) | CTX: Cefotaxime (cephalosporins III)<br>CRO: Ceftriaxone sulbactam (cephalosporins III) |
| (ORAL) | CXM: Cefuroxime (also parenteral cephalosporins II) |

| | ZEM: Cefixime |
|---|---|
| **CARBAPENEMS** | IMP: Imipenem/ cilastatin |
| **AMINOGLYCOSIDES** | GM: Gentamicin |
| **QUINOLONES (FLUOROQUINOLONES)** | OFX: Ofloxacin<br>LBC: Levofloxacin<br>NA: Nalidixic acid |
| **NITROFURANS** | NF: Nitrofurantoin |

## 4.2 Discussion

All *Salmonella* Isolates identified from the fresh produce were multidrug resistant (MDR) in agreement with Abakpa *et al*. (2015). The emergence of MDR *Salmonella* isolates suggests that they may have originated in areas where antibiotics are commonly misused or used as medicinal and growth promoters in animal husbandry and the faeces used as organic manure for fresh produce production as reported by Singh *et al*., 2013 and Abatcha *et al*., 2015. Multiple resistance by the 12 isolates to all the antibiotics means the isolates can be termed as Multi-Drug Resistant (MDR) because resistance to two or more antibiotics tested was exhibited (Yang *et al.,* 2002 and de Freitas Neto *et al.,* 2010). This is alarming to public health as it reduces the effectiveness of first line antibiotics in combating various Non-typhoidal Salmonellosis like self-limiting gastroenteritis, bacteraemia, and extraintestinal focal infections in rare cases and makes the choice of antibiotics more difficult in the therapy of these diseases.

Tetracycline, Ampiclox, and Amoxicillin are popular antibiotics used to treat infections in farm animals (Economou *et al*., 2015). Antibiotics are given to the entire farm's livestock herd during treatment, which can contribute to antimicrobial resistance and impact the intestinal microbiota of healthy animals if not performed appropriately. Non-typhoidal S*almonella* (NTS) a foodborne pathogen, has been discovered in the faeces of animals, including chickens and cattle, and may be transmitted by wildlife that roams and forages in fields or inadequately composted manure (as confirmed with Cernicchiaro *et al*., 2012). NT *Salmonella* may be able to survive in plants via infiltrating the Phyllosphere which is the plant's above surface (Stine *et al*., 2005), or by internalizing and producing biofilms on or within the plants (Fatica *et al*., 2011).

Antibiotics such as Tetracyclines, Sulfonamides, Macrolides, Fluoroquinolones, and Beta-lactams are generally utilized as growth promoters in farms, in addition to disease treatment and prevention (Eagar *et al*., 2012; Van Boeckel *et al*., 2015). Food animals in metropolitan settings are often fed preserved foods that include residues of antibiotics for rapid growth as a result of increased demand for meat, overpopulation, and limited space (Chattopadhyay *et al*., 2014). In contrast, animals in rural regions usually eat natural grass and grains. Certain factors like these might explain the high rates of resistance to these antibiotics. The high degree of resistance to Beta-lactam medicines is concerning because extended Beta-lactams, such as Ceftriaxone, are the medication of choice for treating Salmonellosis in children and pregnant women (Parry *et al*., 2008).

*Salmonella* resistance to Cephalosporins (ceftriaxone) is also a massive problem, and as Mthembu *et al*., 2019 pointed out, this antibiotic is one of the more recently approved antibiotics for medical usage. Furthermore, the identification of fluoroquinolone-resistant *Salmonella* spp. isolates is extremely alarming because it is on the WHO's high priority list as stated by Tacconelli *et al*., 2017. If not controlled effectively the rising incidence of MDR *Salmonella*, which has numerous antibiotic resistances, might lead to *Salmonella* evolving into a super bacterium (Campioni *et al*., 2014). With this in mind, there is a need to increase epidemiological investigations of Salmonella infections, and more research is necessary to strengthen our understanding of the development of MDR and the food safety problems it poses to consumers' health.

Further research is needed to type *Salmonella* isolates to the serovar level in order to identify the prevalent *Salmonella* serovars in fresh produce at farms and market places. The isolates' resistome and virulome will be characterized in detail by whole-genome sequencing of chosen samples as described by Mthembu *et al*., 2019. Frequent monitoring and expanded surveillance systems will act as an early warning system for antibiotic-resistant *Salmonella*, allowing easier detection of any possible infection much faster, limit antibiotic resistance at the farm level, and most importantly reduce public health risk.

# CHAPTER 5

# 5 CONCLUSION AND RECOMMENDATIONS

## 5.1 Conclusion

The occurrence of multidrug-resistant *Salmonella* spp., from this study indicates that fresh produce is a major vehicle for antimicrobial resistant Non Typhoidal *Salmonella*. The contamination could have occurred on the farm lands and the environment in which it was marketed. Humans who ingest this fresh produce are at risk of severe infections with difficult-to-treat Salmonella spp., as indicated by the multidrug resistance phenotype presented by these isolates.

## 5.2 Recommendations

To assure the safety and quality of these fresh produce, extensive supervision and surveillance is required. As a result, proper precautions must be taken to prevent contamination of fresh fruits and vegetables from Farm to Fork. Professionals and anyone working in the food production business, including farmers and market women, should be made aware of the potential danger connected with particular techniques and the possibility of contamination. They should be taught to understand the origins of the etiological agents that cause pollution and the illnesses that follow from them.

Antibiotic resistance has emerged, implying that antibiotics are being used excessively in human and agricultural settings, posing a growing threat to human health. Antibiotic use must be controlled and used judiciously in order to prevent the spread of antibiotic resistance among *Salmonella* serovars, this is because antibiotic resistance can be delayed but not halted as it is a normal phase in which bacteria adapt. As a result, we still need new antibiotics to combat resistant bacteria, as well as new diagnostic tests to map the resistance's progression. Therefore, changing how antibiotics are used is perhaps the singular intervention required to significantly delay the growth and spread of antibiotic-resistant infections. This process is termed antibiotic stewardship and it is the obligation to only select and use the best antibiotics properly and safely only when they are required to treat illness. Consumers may help by following basic and fundamental hygienic principles while preparing and storing food which could also help prevent infections and reduce the spread of resistance as antibiotics are used less often if infections are avoided in the first place.

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