ANTIBIOTIC RESISTANCE PROFILE OF SOME FOODBORNE BACTERIA FROM *Allium fistulosum* (SPRING ONIONS)

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BENIN CITY.

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CERTIFICATION

This is to certify that this work was carried out by **Mr ANEMA MICHAEL IKHAGUEBOR** with **Matriculation Number PG/LSC0201809,** in the Department of Microbiology, Faculty of Life Sciences, University of Benin, Benin City.

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CERTIFICATION OF THESIS

We the undersign attest and declare that the thesis of Mr. ANEMA MICHAEL IKHAGUEBOR, Titled: ANTIBIOTIC RESISTANCE PROFILE OF SOME FOOD BORNE BACTERIA FROM Allium fistulosum (SPRING ONIONS) has successfully passed the anti-plaglarism test and does not violate any copyright regulations.

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Prof. B. A. Omogbai **Date**

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DEDICATION

I dedicate this work whole heartedly to my family and to the Almighty God who gave me His infinite mercy.

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ABSTRACT

Allium fistulosum (Spring onions) also known as scallions or green onions are vegetables derived from various species of the genus *Allium*. The leaves and bulbs which are used as vegetables can either be eaten raw or cooked, chopped into other dishes and used as garnishes. These onions which require cultivation temperature ranging from 10^{o} C to 22^{o} C and irrigation during dry periods can be contaminated by various types of bacteria. This study therefore investigated the antibiotic resistance profile of some food-borne bacteria isolates from spring onions.

Twenty-four (24) samples of spring onions were obtained from vendors across eight markets in Benin metropolis. Ten grams (10g) of spring onions was blended with a high speed homogenizer and the homogenate was serially diluted using peptone water. Standard microbiological methods were used to evaluate the bacteria load present in the samples. The bacteria were identified using both phenotypic and molecular techniques with the aid of polymerase chain reaction (PCR). Antibiotic sensitivity test was carried out using Kirby-Bauer disk diffusion method on Mueller Hinton agar. The results were interpreted by measuring the zones of inhibition around each antibiotic disc in millimeters. Isolates that resisted one or more antibiotics from three or more distinct antibiotics classes were classified as multidrug resistant (MDR) isolates. The multidrug resistant (MDR) food-borne bacterial isolates were then subjected to plasmid profiling.

The heterotrophic bacterial count showed that spring onions from Oba market had the highest count ($8.71 \pm 1.80 \times 10^{6}$cfu/g) while Oluku had the lowest ($5.71 \pm 1.50 \times 10^{6}$cfu/g). The bacteria isolates were *Enterobacter aerogenes, Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus, Bacillus cereus, Proteus vulgaris* and *Serratia marcescens*. The percentage occurrence of isolates ranged from 25% to 100% with *Bacillus cereus* having the highest and *Enterobacter aerogenes* the lowest. *Serratia marcescens* was resistant to all antibiotics used while *Enterobacter aerogenes* was the least resistant. The multiple antibiotic resistance index (MAR I) ranged from 0.25 to 1.0 for all bacteria isolated. Two of the isolates (*Klebsiella pneumoniae* and *Proteus vulgaris*) had plasmids of size 1000 base pair (1.0 Kb). The presence of these food-borne bacterial isolates could be due to use of contaminated irrigation water, improper handling during harvesting and point of sales. The bacteria isolates

which were multi-drug resistant and plasmid mediated pose a grave threat to public health. Spring onions should therefore be properly cooked prior to consumption.

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF STUDY

Spring onions (also referred to as scallions or green onions) are vegetables derived from various species in the genus *Allium*. Spring onions generally have a milder taste as most onions and its close relative including garlic, shallot, leek, chive and chinese onions. The bulbs and leaves of the onions can be used as food. The leaves are used as vegetables and can be eaten either raw or cooked (Frithch and Friesen, 2002). Spring onions can be contaminated by enteric bacteria during cultivation and/or handling.

Bacteria are ubiquitous in nature. They are found in/on humans, animals, plant, soil, water and other extreme environment. Some of them are normal flora, while others are direct pathogenic or opportunistic pathogens of plants and animals. Among these pathogens, some strains have the potentials to move from animals to humans or from plants to humans, leading to infections with disease severities. Some of such pathogens of public health relevance are *Escherichia coli, Salmonella, Klebsiella, Staphylococcus aureus, Proteus vulgaris* and *Bacillus cereus* (Flemming and Wuertz, 2019).

Escherichia coli are bacteria found in the environment, food and intestines of people and animals. *E. coli* is a large and diverse group of bacteria belonging to the family Enterobacteriaceae. It is a Gram negative, rod shaped, non-sporulating, non-fastidious, motile and facultative anaerobic bacterium. Although most strains of *E. coli* are harmless, others can cause sicknesses. Some kinds of *E. coli* can cause diarrhea while others cause urinary tract infections, respiratory illness, pneumonia and other illnesses. Many strains of *E. coli* cause diarrhea, one of which are *E. coli* (STEC) and *E. coli* 0157.47 (Singleton, 1999).

Salmonella is a genus of rod-shaped, gram-negative bacteria of the family Enterobacteriaceae. The two known species of *Salmonella* are *Salmonella* enteric and *Salmonella bongori-Salmonella. Salmonella species* are non-spore forming predominantly non-motile. Enterobacteria *Salmonella* species are intra-cellular pathogens and while certain serotypes cause illness. Most infections are due to ingestion of food contaminated by animal feces or by human feces, such as by a food service worker at a commercial eatery. *Salmonella* serotypes can be divided into two main groups; typhoidal and non-typhoidal (Su and Chiu, 2007).

Non-typhoidal serotypes are zoonotic and can be transferred from animal-to-animal and they usually invade only the gastro intestinal tract and cause *Salmonello*sis. Symptoms of which can be solved without antibiotics. However, non-typhoidal *Salmonella* can be invasive and cause paratyphoid fever which regains immediate treatment with antibiotics.

*Klebsiella*is a genus of Gram negative, rod-shaped bacteria that belongs to the family Enterobacteriaceae are commonly found in the environment, particularly in water and soil, and can also be found in the human gut and respiratory tract (Ryan and Ray, 2004). *Klebsiella* spp. are pathogenic meaning they can cause disease in humans including; *Klebsiella pneumoniae* and *Klebsiella oxytoca* which causes urinary tract infections, pneumonia, and bloodstream infections (Ristuccra and Cunha, 1984). However, *Staphylococcus* is a genus of Gram positive, spherical bacteria that are commonly found on the skin and mucus membranes of humans and animals. Some species of *Staphylococcus* can cause a range of infections from mild to severe. *Staphylococcus aureus* can cause skin infections, pneumonia, bloodstream infections and endocarditis. *Staphylococcus epidermidis* is commonly found on the skin and can cause infections in people with compromised immune systems and *Staphylococcus saprophytis* can cause urinary tract infections.

Antibiotics include range of powerful indications that are used to treat diseases caused by bacteria as they destroy or slow down the growth of bacteria. For decades, the marked increased in antibiotic usage has accelerated. The natural phenomena antibiotic resistance happens when bacteria develop the ability to defeat the drug that is designed to destroy them (Gould, 2016).

However, the impedance of antibiotic resistance is greater in developing countries, because of the comprehensive misuse of antibiotics lacking inspection and poor quality of drugs. Antibiotic resistance emerges not only in pathogenic and disease-causing organism but also in commensal strains like *Escherichia coli,* which are widely used as an indicator organism for the microbiological quality of water and food (Laxminarayan *et al.*,2013).

1.2 Aim and Objectives

The aim of this study was to determine the antibiotic resistance profile of some foodborne bacteria isolates from spring onions.

The objectives were to:

1. isolate, enumerate and identify bacteria from spring onions;
2. determine the antibiotic resistance profile of the isolates;
3. extract plasmid DNA, determine the plasmid profiling and
4. molecularly confirm the identities of phenotypically identified bacteria strains.

CHAPTER TWO

LITERATURE REVIEW

2.1 Plant Description

Spring onions (also referred to as Scallions or green onions) are vegetables derived from various species in the genus *Allium*. Spring onions generally have a milder taste than most onions and its close relative include garlic, shallot, leek, chive and Chinese onions. Although, the bulbs of many *Allium* species are used as food, the defining characteristics of spring onions species is that they lack a fully develop bulb. Instead, the *Allium* species referred to as spring onions make use of the hollow, tabular green leaves growing directly from bulb. These leaves are used as a vegetable and can be eaten either raw or cooked. Often the leaves are used as a vegetable and can be eaten raw or cooked. Often the leaves are chopped into other dishes and used as garnishes (Fritsch and Friesen, 2002).

2.1.1 Etymology and names

The word spring onions or scallion can be traced back to the Ancient Greek as askolonion as described by Greek writer Theophrastus. This name, in turn, is believed to originate from the name of the ancient Canaanite city of Ashkehon (Fritsch and Friesen, 2002).

Various other names are used throughout the world to describe spring onions or scallion including green onion, stick, table onion, salad onion, onion stick, long onion, baby onion, precious onion, wild onion, yard onion, gibbon, syboe, scallion and shallot.

2.1.2 Varieties

Species and cultivars that may be used as spring onions are

1) *Allium cepa*;

a) White Lisbon

b) White Lisbon Winter hardy- an extra-hardy variety for over wintering

c) Callot

d) *Allium cepa* var. *cepa*- most of the cultivars grown in the west as spring onions belong to this variety. Spring onions from *Allium cepa* var.*cepa* (common onion) are usually from a young plant, harvested before a bulb or sometimes soon after slight bulbing.

e) *Allium cepa* var. aggregation (formerly *A. ascalonium* - commonly called shallots or sometimes eschalot

2) A. chinense

3) *A. fistulosum*, the Welsh onion – does not form bulbs even when mature, and is grown in the West almost exclusively as a Spring onions or Salad onions.

4) *A. xproliferum*- sometimes used as spring onions (Brewster, 1994).

2.1.3 Germination

Spring onions generally take 7 – 14 days to germinate depending on the variety.

2.1.4 Culinary Uses

Spring onions may be cooked or used raw as a part of salads, salsas or Asian recipes. Diced spring onions are used in soup, noodle and sea food dishes, sandwiches, curries and as part of a stir fry. In Nepal, it can be used in different meat item fillings like momo (dumpling), choyla (meat intertwined with spring onion and spices). In China, Spring onion is commonly used together with ginger and garlic to cook a wide variety of vegetables and meat.

This combination is often called the "holy trinity" at Chinese cooking much like the mire pox (celery onions and carriots) in French cuisine or the holy trinity in Cajun cuisine. The white part of the onion is usually fried with the other ingredients while the green part is usually chopped to decorate finished food (Rombauer *et al.*, 2006).

2.1.5 Bioactive composition of *A. fistulosum*

Plants serve as a reservoir for bioactive compounds; they have a diverse range of chemical structures and are associated with the medicinal potential of plants (Balkrishna *et al.*, 2021).The bioactivity, functionality, quantity, and applications of various plant-derived chemical components are affected by a variety of internal and external factors, such as geographical location, altitude, climate change, temperature, seasonal variations, etc. *Allium fistulosum* also have rich bioactive composition. Several researchers have reported a variety of bioactive compounds from *A. fistulosum*. Vlase*et al*. (2013) reported the flavonoids as isoquercitrin, kaempferol, quercetol, quercitrin; phenolic compounds as ferulic acids, p-coumaric acid; sterols as β-sitosterol, campesterol, stigmasterol and allicin from whole plant. Along with these compounds steroidalsapogenins, such as yuccagenin, cinnamic acid amide, typheramide, alfrutamide, fistuloimidates andOnionins A1, A2, and A3, were also reported in *A. fistulosum*. Subsequently, documented several flavonoids, such as kaempferol, quercetin, p-coumaric, and ferulic acid, from the aerial parts, bulbs were found to be rich in welsonins A1, coumaran derivatives, cinnamic acid amides, and hydroxy phenol. The leaves contain flavonoids, saponins, steroids and β-sitosterol. Tigu*et al*. (2021) reported that the leaves also contain alliin, allicin, 4-hydroxybenzoic, and p-coumaric acid. Terada *et al*. (2006) reported fistulosin from the roots of *A. fistulosum*. Lastly, seeds contain tianshic acid, p-hydroxybenzoic acid, vanillic acid, daucosterol.

2.2 Antimicrobial Resistance

Antimicrobial resistance (AMR) occurs when microbes evolve mechanisms that protect them from the effects of antimicrobials (drugs used to treat infections). All classes of microbes can evolve resistance where the drugs are no longer effective. Fungi evolve antifungal resistance. Viruses evolve antiviral resistance. Protozoa resistance and bacterial evolve antibiotic resistance. Together all of these come under the umbrella of antimicrobial resistance.

Microbes resistant to multiple antimicrobials are called multi drugs resistance (MDR) and are sometimes referred to as a superbug (Magiorakos *et al.*,2012). Although antimicrobial resistance is a naturally occurring process it is often the return of improper usage of the drugs and management of the infections (Saha and Sarkar, 2021).

Antibiotic resistance is a major subject of AMR that applies specially to bacteria that becomes resistant to antibiotics. Antibiotic resistance in bacteria can arise naturally by genetic mutation or by one species acquiring resistance from another. Resistance can appear spontaneously because of random mutations but also arises through spreading of resistant genes through horizontal gene transfer. However, extended use of antibiotic is appeal to encourage selection for mutations which can render antibiotic ineffective (Dabour *et al.*, 2016).

Rising drug resistance is caused mainly by use of antimicrobials in humans and other animals and spread of resistant strains between the two. Growing resistance has also been linked to releasing inadequately treated effluent from the pharmaceutical industry, especially in countries where bulk drugs are manufactured (Murray *et al.*, 2022).

Antibiotics increase selective pressure in bacterial populations, killing vulnerable bacteria. This increases the percentage of resistant bacteria with routine growing. Even at very low levels of antibiotic, resistant bacteria can have a growth advantage and grow faster than vulnerable bacteria.

In 2019, there were around 1.27million deaths globally caused by bacteria AMR. Infections caused by resistant microbes are more difficult to treat, requiring highly doses of antimicrobial drugs, more expensive antibiotics, or alternative medication which may prove more toxic. These approaches may also cost more.

The prevention of antibiotic misuse, which can lead to antibiotic resistance, includes taking antibiotics only repler prescribed. Narrow spectrum antibiotics are preferred over broad spectrum antibiotics, when possible, as effectively and accurately targeting specific organisms is use likely to cause resistance, as well as side effects. For people who take these medications at home, education about proper use is essential. Health care providers can minimize spread of resistant infections by use of proper sanitation and hygiene; include hand washing and disinfection between patients and show encouragement to the same patient, visitors and family members (Gerber, 2017).

Antimicrobial resistance increases globally due to increase prescription and dispensing of antibiotic drugs in developing countries. Estimates are that 700,000 to several million deaths result per year and continue to pose a major public health threat worldwide. Each year in the United States, 2.8 million people become infected with bacteria that are resistant to antibiotics, 35,000 people die and 55 billion is spent on increased health care costs and productivity. According to World Health Organization (WHO) estimates of 350 million deaths could be caused by AMR by 2050. By then the yearly death toll will be 10 million, according to a United Nations report (WHO, 2022).

There are public calls for global collective action to address the threat that include proposals for international treaties on antimicrobial resistance. The burden of worldwide antibiotic resistance if not completely identified, both low and middle income countries with weaker health care systems is most affected, in the mortality being the highest in Sub-Saharan Africa. During the Covid-19 pandemic, priorities changed with action against anti-microbial resistance slowing due to scientist and governments focusing more on SARS-COV-2 research, making the threat of AMR increased during the pandemic (Murray, *et al.*, 2022).

2.2.1 Causes of Antimicrobial Resistance

Antimicrobial resistance is mainly caused by the overuse/misuse of antimicrobials. This leads to microbes either evolving a defense against drugs used to treat them, or certain strains of microbes that have a natural resistance to antimicrobials becoming much more prevalent than the ones that are easily defeated with medication. While antimicrobial resistance does not occur naturally over time, the use of antimicrobial agents in a variety of settings both within the health care industry and outside has led to antimicrobial resistance becoming increasingly more prevalent (Holmes, *et al.*, 2016).

Although many microbes develop resistance to antibiotics over time through natural mutation, overprescribing and in appropriate prescription of antibiotics have accelerated the problem. It is possible that as many as in prescription written for antibiotics are unnecessary. Every year, approximately 154 million prescriptions for antibiotic are written. Of these, up to 40 million are unnecessary or in appropriate for the condition that the patient has. Microbes may naturally develop resistance through genetic mutations that occur during cell division and although random mutations are rare, many microbes reproduce frequently and rapidly, increasing the changes of members of the population acquiring a mutation that increases resistance. Many individual stop taking antibiotics when they begin to feel better. When this occurs, it is possible that the microbes are able to continue to reproduce; this can lead to an infection by bacteria that are less susceptible or even resistant to an antibiotic (Michael, *et al.*, 2014).

2.2.2 Natural occurrence

Antimicrobial resistance can evolve naturally due to continued exposure to antimicrobials. Natural selection means that organisms that are able to adapt to their environment, survive and continue to produce offspring. As a result, the types of microorganisms that are able to survive over time with continued attack by certain antimicrobial agents will naturally become

more prevalent in the environment, and those without this resistance will become obsolete (Holmes, *et al.,* 2016).

2.2.3 Self-medication

In 89% of countries, antibiotics can only be prescribed by a doctor and supplied by a pharmacy. Self-medication by consumers is defined as "the taking of medicines on one's own initiative or on another person's suggestion, who is not a certified medical professional", and it has been identified as one of the primary reasons for the evolution of antimicrobial resistance. Self-medication with antibiotics is an unstable way of using them by a common practice in resource constrained countries. The practice exposes individuals to the risk of bacteria that have developed antimicrobial resistance. Many people resort to this out of necessity, when access to a physician is unavailable due to lockdown and GP surgery closures or when the patients have a limited amount of time or money to see a prescribing doctor. This increased access makes it extremely easy to obtain antimicrobials and an example in India, where in the state of Punjab 73% of the population resorted to treating their minor health issues and chronic illnesses through self-medication (Rather, *et al.,* 2017).

Self-medication is higher outside the hospital environment and this is linked to higher use of antibiotics, with the majority of antibiotics being used in the community rather than hospitals. The prevalence of self-medication in low- and middle-income countries (LMICs) ranges from 8.1% to very high at 93%. Accessibility, affordability and conditions of health facilities, as well as the health-seeking behavior, are factors that influence self-medication in low- and middle-income countries. Two significant issues with self-medication are the lack of knowledge of the public on firstly, the dangerous effects of certain antimicrobials (for example, ciprofloxacin which can cause tendonitis, tendon rupture and aortic dissection) and secondly, broad microbial resistance when seeking medical care if the infection is not clearing. In order to determine the public's knowledge and preconceived notions on

antibiotics resistance, a screening of 3,537 articles published in Europe, Asia and North America was done. Of the 55,225 total people surveyed in the articles, 70% had heard of antibiotic resistance previously, but 88% of those people thought it referred to some type of physical change in the human body. With so many people around the world with the ability to self-medication using antibiotics and a vast majority unaware of what antimicrobial resistance is, it makes the increase of antimicrobial resistance and its global negative impact much more likely (Rather, *et al.,* 2017).

2.2.4Clinical misuse

Clinical misuse by health care professionals is another contributor to increased anti-microbial resistance. Studies done in the US showed that the indication for treatment of antibiotics, choice of the agent used and the duration of therapy was in lowest in up to 50% of the cases studied. In another study in an intensive care unit in a major hospital in France, it was shown that 30% to 60% of prescribed antibiotics were unnecessary. These inappropriate uses of anti-microbial agents promoted the evolution of anti-microbial resistance by evolution of antimicrobial resistance by supporting the bacteria in developing genetic alterations that lead to resistance (Fleming-Dutra, *et al.,* 2016).

2.3 History of some food borne bacteria

Infectious food borne diseases are of great concern throughout the world, as they are responsible for considerable morbidity and mortality, especially in developing countries (Guerrant, *et al.,* 1990). It has been reported that diarrheal diseases cause approximately three million deaths worldwide per year countries (Guerrant, *et al.,* 2002).

The major causes of food borne diseases differ significantly in developing countries. In the developed world, the most common cause of infectious food borne diseases are viruses,

whereas elsewhere bacterial agents such as enterotoxigenic *Escherichia coli* (ETEC) and *Campylobacter*, *Shigella*and *Salmonella Spp.* have been reported to account for most diarrhea infections (Philip, *et al.,* 2009).

Diarrheagenic *Escherichia coli* are an important cause of endemic and epidemic diarrhea worldwide (Nataro, *et al.,* 1998). These organisms are currently classified in six categories as follows: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), diffusely adhering *E. coli* (DAEC) enteroaggregative *E. coli* (EAEC), and enterohemorrhagic *E. coli* (EHEC) (Nataro *et al.,* 1998).

2.3.1 *Escherichia coli*

Escherichia coli is a Gram-negative, facultative anaerobic, rod-shaped, coliform bacterium of the genus *Escherichia* that is commonly found in the lower intestine of warm-blooded organisms (Tenaillon *et al.,* 2010). Most *E. coli* strains are harmless, but some serotypes such as EPEC, and ETEC are pathogenic and can cause serious food poisoning in their hosts, and are occasionally responsible for food contamination incidents that prompt product recalls (Vogt and Dippold, 2005). Most strains are part of the normal microbiota of the gut and are harmless or even beneficial to humans (although these strains tend to be less studied than the pathogenic ones). For example, some strains of *E. coli* benefit their hosts by producing vitamin K_2or by preventing the colonization of the intestine by pathogenic bacteria. These mutually beneficial relationships between *E. coli* and humans are a type of mutualistic biological relationship where both the humans and the *E. coli* are benefitting each other. *E. coli* is expelled into the environment within fecal matter. The bacterium grows massively in fresh fecal matter under aerobic conditions for three days, but its numbers decline slowly afterwards. *Escherichia coli* and other facultative anaerobes constitute about 0.1% of gut microbiotaand fecal–oral transmission is the major route through which pathogenic strains of the bacterium cause disease. Cells are able to

survive outside the body for a limited amount of time, which makes them potential indicator organisms to test environmental samples for fecal contamination. A growing body of research, though, has examined environmentally persistent *E. coli* which can survive for many days and grow outside a host (Montealegre *et al.*,2018).

The bacterium can be grown and cultured easily and inexpensively in a laboratory setting, and has been intensively investigated for over 60 years. *E. coli* is a chemoheterotroph whose chemically defined medium must include a source of carbon and energy (Tortora, *et al.*, 2010). *E. coli* is the most widely studied prokaryotic model organism, and an important species in the fields of biotechnology and microbiology, where it has served as the host organism for the majority of work with recombinant DNA. Under favorable conditions, it takes as little as 20 minutes to reproduce.

2.3.1.2 Culture growth

Optimum growth of *Escherichia coli* occurs at 37 °C (99 °F), but some laboratory strains can multiply at temperatures up to 49 °C (120 °F) (Fotadar, *et al.*,2005).*E. coli* grows in a variety of defined laboratory media, such as lysogeny broth, or any medium that contains glucose, ammonium phosphate monobasic, sodium chloride, magnesium sulfate, potassium phosphate dibasic, and water. Growth can be driven by aerobic or anaerobic respiration, using a large variety of redox pairs, including the oxidation of pyruvic acid, formic acid, hydrogen, and amino acids, and the reduction of substrates such as oxygen, nitrate, fumarate, dimethyl sulfoxide, and trimethylamine N-oxide. *E.coli* is classified as a facultative anaerobe. It uses oxygen when it is present and available. It can, however, continue to grow in the absence of oxygen using fermentation or anaerobic respiration. Respiration type is managed in part by the arc system. The ability to continue growing in the absence of oxygen is an advantage to

bacteria because their survival is increased in environments where water predominates (Tortora *et al.*,2010).

2.3.1.3 Genetic adaptation

Escherichia coli and related bacteria possess the ability to transfer DNA via bacterial conjugation or transduction, which allows genetic material to spread horizontally through an existing population. The process of transduction, which uses the bacterial virus called a bacteriophage (Brüssow *et al.*,2004), is where the spread of the gene encoding for the Shiga toxin from the *Shigella* bacteria to *E. coli* helped produce *E. coli* O157:H7, the Shiga toxin-producing strain of *E. coli*.

2.3.2 *Salmonella*spp.

*Salmonella*spp. isa genus of rod-shaped (Bacillus) Gram-negative bacteria of the family Enterobacteriaceae. The two known species of *Salmonella* are *Salmonella enterica* and *Salmonella bongori*. *S. enterica* is the type species and is further divided into six subspecies that include over 2,650 serotypes. *Salmonella* was named after Daniel Elmer Salmon (1850–1914), an American veterinary surgeon (Traaschel, *et al.*, 2022).

Salmonella species are non-spore-forming, predominantly motile enterobacteria with cell diameters between about 0.7 and 1.5 μm, lengths from 2 to 5 μm, and peritrichous flagella (all around the cell body, allowing them to move). They are chemotrophs, obtaining their energy from oxidation and reduction reactions, using organic sources. They are also facultative anaerobes, capable of generating adenosine triphosphate with oxygen (aerobically) when it is available, or using other electron acceptors or fermentation (anaerobically) when oxygen is not available (Fàbrega and Vila, 2013).

Salmonella species are intracellular pathogens of which certain serotypes cause illness such as salmonellosis. Most infections are due to the ingestion of food contaminated by feces.

Typhoidal *Salmonella* serotypes can only be transferred between humans and can cause foodborne illness as well as typhoid and paratyphoid fever. Typhoid fever is caused by typhoidal *Salmonella* invading the bloodstream, as well as spreading throughout the body, invading organs, and secreting endotoxins (the septic form). This can lead to life-threatening hypovolemic shock and septic shock, and requires intensive care including antibiotics.

Non-typhoidal*Salmonella* serotypes are zoonotic and can be transferred from animals and between humans. They usually invade only the gastrointestinal tract and cause salmonellosis, the symptoms of which can be resolved without antibiotics. However, in sub-Saharan Africa, non-typhoidal *Salmonella* can be invasive and cause paratyphoid fever, which requires immediate antibiotic treatment. Clinical manifestations of Typhoidal *Salmonella* can lead to diarrhea. It can include anorexia, abdominal pain, tenderness, and bloating, nausea and vomiting, diarrhea and bloody diarrhea (Philip *et at.*, 2009).

2.3.2.2 Pathogenicity

Most infections are due to ingestion of food contaminated by animal feces, or by human feces, such as by a food-service worker at a commercial eatery. *Salmonella* serotypes can be divided into two main groups typhoidal and non-typhoidal. Typhoidal serotypes include *Salmonella typhi* and *Salmonella paratyphi*A, which are adapted to humans and do not occur in other animals. Non-typhoidal serotypes are more common, and usually cause self-limiting gastrointestinal disease. They can infect a range of animals, and are zoonotic, meaning they can be transferred between humans and other animals. *Salmonella* pathogenicity and host interaction has been studied extensively since the 2010s. Most of the important virulent genes of *Salmonella* are encoded in five pathogenicity islands; the so-called *Salmonella* pathogenicity islands (SPIs). These are chromosomal encoded and

have significant contribution to bacterial-host interaction. More traits like plasmids, flagella or biofilm-related proteins can contribute in the infection. SPIs are characterized to be regulated by complex and fine-tuned regulatory networks that allow the gene expression only in the presence of the right environmental stresses (Fàbrega and Vila, 2013).

2.3.3 *Klebsiella* spp.

Klebsiella spp**.** is a genus of Gram-negative, oxidase-negative, rod-shaped bacteria with a prominent polysaccharide-based capsule (Ryan and Ray, 2004).*Klebsiella* is named after German-Swiss microbiologist Edwin Klebs (1834–1913). Carl Friedlander described *Klebsiella bacillus* which is why it was termed Friedlander bacillus for many years. The species of *Klebsiella* are all gram-negative and usually non-motile. They tend to be shorter and thicker when compared to others in the family Enterobacteriaceae.

Klebsiella species are found everywhere in nature. This is thought to be due to distinct sub-lineages developing specific niche adaptations, with associated biochemical adaptations which make them better suited to a particular environment. They can be found in water, soil, plants, insects and other animals including humans, including as part of the human and animal's normal flora in the nose, mouth and intestines (Dworkin *et al.*,2006).

2.3.3.1 Features

Klebsiella bacteria tend to be rounder and thicker than other members of the family Enterobacteriaceae. They typically occur as straight rods with rounded or slightly pointed ends. They can be found singly, in pairs, or in short chains. Diplobacillaryforms are commonly found *in vivo*.

They have no specific growth requirements and grow well on standard laboratory media but grow best between 35 and 37 °C and at pH 7.2.The species are facultative anaerobes, and most strains can survive with citrate and glucose as their sole carbon sources and ammonia as

their sole nitrogen source. Members of the genus produce a prominent capsule, or slime layer, which can beused for serologic identification, but molecular serotyping may replace this method (Brisse *et al.*,2004).

Members of the genus *Klebsiella* typically express two types of antigens on their cell surfaces. The first, O antigen is a component of the lipopolysaccharide (LPS), of which 9 varieties exist. The second is K antigen, a capsular polysaccharide with more than 80 varieties. Both contribute to pathogenicity and form the basis for Sero grouping. Based on those two major antigenic determinants several vaccines have been designed (Brisse, *et al.*, 2004).

2.3.3.2 *Klebsiella* spp.in humans

Klebsiella species are routinely found in the human nose, mouth, and gastrointestinal tract as normal flora; however, they can also behave as opportunistic human pathogens.*Klebsiella* species are known to also infect a variety of other animals, both as normal flora and opportunistic pathogens.*Klebsiella* organisms can lead to a wide range of disease states, notably pneumonia, urinary tract infections, sepsis, meningitis, diarrhea, peritonitis and soft tissue infections. *Klebsiella* species have also been implicated in the pathogenesis ofankylosing spondylitis and other spondyloarthropathies. The majority of human *Klebsiella* infections are caused by *K. pneumoniae*, followed by *K. oxytoca*. Infections are more common in the very young, very old, and those with other underlying diseases, such as cancer and most infections involve contamination of an invasive medical device.

During the last 40 years, many trials for constructing effective *K. pneumoniae* vaccines have been tried and new techniques were followed to construct vaccines against *Klebsiella*. However, currently, no *Klebsiella* vaccine has been licensed for use in the US. *K. pneumoniae* is the most common cause of nosocomial respiratory tract and premature intensive care infections, and the second-most frequent cause of Gram-negative bacteraemia

and urinary tract infections. Drug-resistant isolates remain an important hospital-acquired bacterial pathogen, add significantly to hospital stays, and are especially problematic in high-impact medical areas such as intensive care units. This antimicrobial resistance is thought to be attributable mainly to multidrug efflux pumps (Ogawa, *et al.,* 2005). The ability of *K. pneumoniae* to colonize the hospital environment, including carpeting, sinks, flowers, and various surfaces, as well as the skin of patients and hospital staff, has been identified as a major factor in the spread of hospital-acquired infections (Bagley, 1985).

2.3.3.3 *Klebsiella* spp. in animals

In addition to certain *Klebsiella* spp. being discovered as human pathogens, others such as *K. variicola* have been identified as emerging pathogens in humans and animals alike. For instance, *K. variicola* has been identified as one of the causes of bovine mastitis (Davidson, *et al.,* 2015).

2.3.3.4 *Klebsiella* spp. in plants

In plant systems, *Klebsiella* can be found in a variety of plant hosts. *K. pneumoniae* and *K. oxytoca* are able to fix atmospheric nitrogen into a form that can be used by plants, thus are called associative nitrogen fixers or diazotrophs (Dworkin *et al.,* 2006). The bacteria attach strongly to root hairs and less strongly to the surface of the zone of elongation and the root cap mucilage. They are bacteria of interest in an agricultural context, due to their ability to increase crop yields under agricultural conditions. Their high numbers in plants are thought to be at least partly attributable to their lack of a flagellum, as flagella are known to induce plant defences (Fouts *et al.,*2008). Additionally, *K. variicola* is known to associate with a number of different plants including banana trees, sugarcane and has been isolated from the fungal gardens of leaf-cutter ants (Wei Chun Yan *et al.,* 2014).

2.3.4 *Proteus* sp.

*Proteus*spp. is a genus of Gram-negative bacteria. It is a rod shaped, aerobic and motile bacteria, which is able to migrate across surfaces due its "swarming" characteristic in temperatures between 20 and 37 °C. Their size generally ranges from 0.4 to 0.8 μm in diameter and 1.0–3.0 μm in length. They tend to have an ammonia smell (Olsen and Motarjemi, 2014). *Proteus* bacilli are widely distributed in nature as saprophytes, being found in decomposing animal matter, sewage, manure soil, the mammalian intestine, and human and animal faeces. They are opportunistic pathogens, commonly responsible for urinary and septic infections, often nosocomial.

The term *Proteus* signifies changeability of form, as personified in the Homeric poems in *Proteus*, "the old man of the sea", who tends the seal flocks of Poseidon and has the gift of endless transformation. The first use of the term "*Proteus*" in bacteriological nomenclature was made by Hauser (1885), who described under this term three types of organisms which he isolated from putrefied meat.

2.3.4.1 Clinical significance

Three species *P. vulgaris, P. mirabilis,* and *P. Penneri* are opportunistic human pathogens. *Proteus* includes pathogens responsible for many human urinary tract infections (Guentzel, 1996).*P. mirabilis* causes wound and urinary tract infections. Most strains of *P. mirabilis* are sensitive to ampicillin and cephalosporins. *P. vulgaris* is not sensitive to these antibiotics but ticarcillin. However, this organism is isolated less often in the naturally in the intestines of humans and a wide variety of animals, and in manure, soil, and polluted waters. *P. mirabilis*, once attached to the urinary tract, infects the kidney more commonly than *E. coli. P. mirabilis* is often found as a free-living organism in soil and water.

About 10–15% of kidney stones are struvite stones, caused by alkalinization of the urine by the action of the urease enzyme (which splits urea into ammonia and carbon dioxide) of *Proteus* (and other) bacterial species (Olsen and Motarjerni, 2014).

A surveillance study conducted between 2000-2005 found that women (69%) are at a higher risk of developing *Proteus vulgaris* infections. *Proteus vulgaris* and *Proteus penneri* are easily isolated from individuals in long term care facilities and hospitals and from patients with underlying diseases or compromised immune systems *P. mirabilis* causes 90% of *Proteus* infections

Patients with recurrent infections, those with structured abnormalities of the urinary tract, those who have had urethral instrumentation, and those whose infections where acquired in the hospital have an increased frequency of infections caused by *Proteus* and other organisms (e.g. *Klebsiella*, *Enterobacter, Pseudomonas, Enterococci* and *Staphylococci*) (Laupland *et at.*, 2007).

P. vulgaris is highly resistant to antibiotics because of the plasmids present in the bacterium, making infections extremely difficult to cure. This is because the plasmids have carried drug resistant makers on them (Laupland *et at.*, 2007).

2.3.5 *Bacillus cereus*

Bacillus cereus is a Gram-positive rod-shaped bacterium commonly found in soil, food, and marine sponges (Paul *et al.*, 2021). The specific name, *cereus*, meaning "waxy" in Latin, refers to the appearance of colonies grown on blood agar. Some strains are harmful to humans and cause foodborne illness due to their spore-forming nature, while other strains can be beneficial as probiotics for animals, and even exhibit mutualism with certain plants. *B. cereus* bacteria may be aerobes or facultative anaerobes, and like other members of the genus *Bacillus*, can produce protective endospores. They have a wide range of virulence factors, including phospholipase C, cereulide, sphingomyelinase, metalloproteases,

and cytotoxin K, many of which are regulated via quorum sensing *B. cereus* strains exhibit flagellar motility.

The *Bacillus cereus* group comprises seven closely related species: *B. cereus sensustricto* (referred to herein as *B. cereus*), *B. anthracis*, *B. thuringiensis*, *B. mycoides*, *B. pseudomycoides*, and *B. cytotoxicus* (Guinebretière *et al.*, 2010); or as six species in a *Bacillus cereus* sensulato: *B. weihenstephanensis*, *B. mycoides*, *B. pseudomycoides*, *B. cereus*, *B. thuringiensis*, and *B. anthracis (Kolstø et al., 2009)*. A phylogenomic analysis combined with average nucleotide identity (ANI) analysis revealed that the *B. anthracis* species also includes strains annotated as *B. cereus* and *B. thuringiensis.*

Bacillus cereus a spore forming anaerobe that causes diseases by producing toxins causes two distinct syndromes in humans. The first syndrome is an emetic disorder without diarrhea, which is attributed to *cereulide,* a small cyclic dodecadepsipeptide that resists heat and acid (Philip *et al.,*2009). Diarrhea caused by *Bacillus cereus* has been attributed to heat-labile enterotoxin, which can be divided into at least three different types, including hemolysin, nonhemolytic enterotoxin and cytotoxin (Philip *et al.,* 2009).

Most causes resolve within 1-2 days but *B. cereus* emetic toxin can occasionally cause fulminant hepatic failure.Treatment of an illness caused by *B. cereus* is supportive. Diagnosis is made by isolating the organism from food or emesis (Philip *et al.*, 2009).

2.3.5.1 Microbiology of *Bacillus cereus*

B. cereus is a rod-shaped bacterium with a Gram-positive cell envelope. Depending on the strain, it may be aerobic or facultatively anaerobic. Most strains are mesophilic, having an optimal temperature between 25 °C and 37 °C, and neutralophilic, preferring neutral pH, but some have been found to grow in environments with much more extreme conditions (Drobniewski *et al.,* 1993).

These bacteria are both spore-forming and biofilm-forming, presenting a large challenge to the food industry due to their contamination capability. Biofilms of *B. cereus* most commonly form on air-liquid interfaces or on hard surfaces such as glass. *B. cereus* displaysflagellar motility, which has been shown to aid in biofilm formation via an increased ability to reach surfaces suitable for biofilm formation, to spread the biofilm over a larger surface area, and to recruit planktonic, or single, free-living bacteria. Biofilm formation may also occur while in spore form due to varying adhesion ability of spores.

Their flagella are peritrichous, meaning there are many flagella located all around the cell body that can bundle together at a single location on the cell to propel it. This flagellar property also allows the cell to change directions of movement depending on where on the cell the flagellum filaments come together to generate movement (Rilley *et al.*, 2018). Some studies and observations have shown that silica particles the size of a few nanometers have been deposited in a spore coat layer in the extracytoplasmic region of the *Bacillus cereus* spore. The layer was first discovered by the use of scanning transmission electron microscopy (STEM), however the images taken did not have resolution high enough to determine the precise location of the silica. Some investigators hypothesize that the layer helps different spores from sticking together. It has also been shown to provide some resistance to acidic environments. The silica coat is related to the permeability of the cell's inner membrane. Strong mineral acids are able to break down spore permeability barriers and kill the spore. However, when the spore has a silica coating, it may reduce the permeability of the membrane and provide resistance to many acids (Ryuichi *et al.*, 2010).

2.3.6 *Staphylococcus aureus*

*Staphylococcus aureus*is a Gram-positive spherically shaped bacterium, a member of the Bacillota, and is a usual member of the microbiota of the body, frequently found in the upper respiratory tract and on the skin. It is often positive for catalase and nitrate reduction and is a facultative anaerobe, meaning that it can grow without oxygen. Although *S. aureus* usually acts as a commensal of the human microbiota, it can also become an opportunistic pathogen, being a cause of skin infections including abscesses, respiratory infections such as sinusitis and food poisoning. Pathogenic strains often promote infections by producing virulent factors such as potent protein toxins and the expression of a cell-surface protein that binds and inactivates antibodies. *S. aureus* is one of the leading pathogens for deaths associated with antimicrobial resistance and the emergence of antibiotic resistance strains, such as methicillin- resistant *S. aureus*(MRSA) (Masalha *et al.*, 2001).

2.3.6.1 Microbiology of *Staphylococcus aureus*

Staphylococcus aureus is a facultataive anaerobic Gram-positvecoccal (round) bacterium also known as "golden staph" and "orostaphira". *S. aureus* is nonmotile and does not form spores. In medical literature, the bacterium is often referred to as *S. aureus*, *Staph aureus* or *Staph a..S. areus* appears as *staphylococcus* (grape-like clusters) when viewed through a microscope and has large, round, golden-yellow colonies, often with hemolysis, when grown on blood agar plates. *Staphylococcus aureus* reproduces asexually by binary fusion. Complete separation of the daughter cell is mediated by *S. aureus* autolysin and in its absence or targeted inhibition, the daughter cells remain attached to one another and appear as clusters (Varrone *et al.*, 2014). *Staphylococcus aureus* is catalase-positve (meaning it can produce the enzyme catalase). Catalase converts hydrogen peroxide (H_2O_2) to water and oxygen. Catalase-activity tests are sometimes used to distinguish *staphylococcus* from enterococci and streptococci. Previously, *S. aureus* was differentiated from other staphylococci by the

coagulase test. However, not all *S. aureus* strains are coagulase-positive and incorrect species identification can impact effective treatment and control measures (Matthews *et al.*, 1997).

2.3.6.2 Role in health

In humans, *S. aureus* can be present in the upper respiratory tract, gut mucosa and skin as a member of the normal microbiota (Wollina, 2017). However, because *S. aureus* can cause disease under certain host and environmental conditions, it is characterized as a parthobiont.

In the United States, MRSA infections alone are estimated to cost the healthcare system over $3.2 billion annually (Roberts, *et al.,* 2009). These infections account for nearly 20,000 deaths each year in the U.S, exceeding those caused by HIV/AIDS, Parkison's disease and homicide. Annually, over 119,000 bloodstream infections in the U.S are attributed to *S. aureus. S.aureus* infections are ranked as one of the costliest healthcare-associated infections (HAIs), with each case averaging $23,000 to $46,000 in treatment and hospital resource utilization. While *S. aureus* usually acts as a commensal bacterium, asymptomatically colonizing about 30% sometimes cause disease(Tong, *et al.*, 2015). In particular, *S. aureus* is one of the most common causes of bacteremia and infective endocarditis. Additionally, it can cause various skin and soft infections, particularly when skin or mucosal barriers have been breached.

Staphylococcus aureus infections can spread through contact with pus from an infected wound, skin-to-skin contact with an infected person and contact with objects used by an infected person such as towels, sheets, clothing or athletic equipment. Joint replacements put a person at a particular risk of septic arthritis, staphylococcal endocarditis (infection of the heart valves) and pneumonia (Kuehnert *et al.,* 2005). Food handlers or other surfaces such as processing equipment are usually where *Staphylococcus aureus* gets access to the foods which accounts to up to one third of food borne gastrointestinal illnesses.

2.3.6.2.1. Clinical Significance and Disease Burden

Staphylococcus aureus is one of the leading causes of both community-acquired and healthcare-associated infections worldwide. It is a major etiological agent of skin and soft tissue infections, bacteremia, pneumonia, osteomyelitis, septic arthritis, and infective endocarditis (Lowy, 1998; Tong *et al.,* 2015). In particular, *S. aureus* is among the most common causes of bloodstream infections and infective endocarditis, conditions associated with high morbidity and mortality.

In the United States, infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) alone are estimated to cost the healthcare system over $3.2 billion annually (Roberts *et al.,* 2009). These infections account for nearly 20,000 deaths per year, exceeding mortality figures attributed to HIV/AIDS, Parkinson's disease, and homicide. Annually, over 119,000 bloodstream infections in the U.S. are linked to *S. aureus*, underscoring its public health significance.

From an economic perspective, *S. aureus* infections rank among the costliest healthcare-associated infections (HAIs), with treatment and hospital resource utilization averaging between $23,000 and $46,000 per case (Roberts *et al.,* 2009). The burden is even greater in low- and middle-income countries, where limited diagnostic capacity and antimicrobial resistance complicate disease management.

2.3.6.2.2. Transmission Pathways and Risk Factors

Transmission of *S. aureus* occurs primarily through direct contact. This includes contact with pus or exudates from infected wounds, skin-to-skin contact with colonized or infected individuals, and indirect contact through contaminated fomites such as towels, clothing, bed

linens, and athletic equipment (Kuehnert *et al.*, 2005). In healthcare settings, invasive devices such as catheters, prosthetic joints, and surgical implants significantly increase susceptibility to severe infections, including septic arthritis, staphylococcal endocarditis, and pneumonia.

Joint replacements, in particular, place individuals at elevated risk due to the organism's ability to form biofilms on artificial surfaces, thereby enhancing persistence and resistance to host immune responses and antimicrobial agents (Otto, 2008).

2.3.6.3 *Staphylococcus aureus* as a Foodborne Pathogen

2.3.6.3.1. Sources of Food Contamination

Staphylococcus aureus is a major cause of foodborne gastrointestinal illness globally. Contamination of foods typically occurs through human handling, as the bacterium is frequently present on the skin, in the nasal passages, and on the hands of food handlers. Food handlers with poor personal hygiene, cuts, burns, or skin infections are particularly significant sources of contamination (Kadariya *et al.*, 2014).

In addition to humans, contaminated food contact surfaces such as processing equipment, utensils, cutting boards, and packaging materials also serve as important reservoirs. It is estimated that *S. aureus* accounts for up to one-third of foodborne gastrointestinal illnesses associated with bacterial toxins, especially in improperly handled or stored foods.

2.3.6.3.2. *Staphylococcal* Food Poisoning

Staphylococcal food poisoning results from the ingestion of pre-formed enterotoxins rather than live bacterial cells. Commonly implicated foods include cooked meats, poultry, dairy

products, pastries, salads, and ready-to-eat foods that are handled extensively and stored at ambient temperatures (Le Loir *et al.,* 2003; Bennett and Monday, 2003).

The heat stability of staphylococcal enterotoxins presents a major food safety challenge, as normal cooking or reheating temperatures may destroy the bacteria but not the toxins. Consequently, prevention strategies must focus on hygienic food handling, temperature control, and exclusion of infected food handlers rather than reliance on cooking alone.

2.3.6.3.3. Public Health Implications

The dual role of *Staphylococcus aureus* as both a commensal organism and a potent pathogen presents significant public health challenges. In food safety contexts, asymptomatic carriers among food handlers contribute substantially to contamination events, often without detection. In clinical settings, increasing antimicrobial resistance, particularly MRSA, further complicates treatment outcomes.

The intersection between foodborne exposure, human colonization, and clinical infection highlights the importance of integrated surveillance approaches consistent with the One Health framework**,** which recognizes the interconnectedness of human health, food safety, and environmental hygiene.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Sample collection

Samples of spring onions were bought from eight markets in Benin City namely: Oba Market, Lagos Street, Ramat Park, New Benin, University of Benin Teaching Hospital (UBTH) vendor, Oluku, Ikpoba Hill and Uselu Markets.

3.2 Preparation of samples

Ten (10) grams of spring onions was blended and homogenized with the help of high-speed homogenizer at 10,000rpm for 1min (Ultra Turrax T25 basic, IKA, USA).

3.3 Enrichment and isolation of Bacteria.

Each homogeneous sample was inoculated on several media, MacConkey agar (Thermoscientific United States) for isolation of *Escherichia coli and Proteus vulgaris,* Eosin methylene Blue (EMB) for isolation of *Klebsiella, Enterobacter* and *Serratia marcescens,* Selenite F Broth (Himedia, India) an enrichment medium for isolation of *Salmonella*, Salmonella-Shigella agar for isolation of *Shigella,*Mannitol salt agar (MSA) for the isolation of *Staphylococcus* and *Bacillus cereus* agar base for the isolation of *Bacillus cereus.*

3.4 Isolation of Bacteria

One gram (1g) of homogenized sample was collected and dissolved in 9ml of sterile distilled water in a test tube and shaken vigorously for 1min. Thereafter, a 10-fold serial dilution of up to 10^{-3} was carried out. The serial dilution was carried out by transferring 1ml from stock solution into 9.0ml of sterile water in a test tube to obtain 10^{-1} dilution. Other dilutions (10^{-2} – 10^{-3}) were subsequently prepared by 10- fold dilution method.

An aliquot of 1ml each was transferred to a Petri plate containing various media and was incubated at 37^{o}C for 24h for bacteria growth. Following successful growths of

microorganisms, the colonies were counted and the results per dilution count were recorded as CFU/g.

3.5 Sub-culturing

Different colonies of diverse cultural characteristics were observed on the agar plates. Sterile inoculating loop was used to pick a portion of each dissimilar colony and streaked on freshly prepared sterilized nutrient agar to obtain pure culture of the isolates. The plates were then incubated at 37℃ for 24h after which the morphological characteristics of the isolates were observed.

3.6 Identification of bacteria isolates.

The isolates were identified using colony morphology on agar plates, Gram stain and biochemical tests.

3.6.1 Cultural characterization

Each colony morphology e.g, shape, color, margin, surface, elevation, cell type, cell arrangement was determined.

3.6.2 Gram staining

A thin smear of the isolates was made on different slides using a wire loop and left to dry and then heat-fixed and allowed to cool. The different smears were covered with crystal violet stain for 30sec and washed off immediately with sterile water. The smear were then covered with Lugol's iodine for 30 sec and immediately washed with sterile water. The smears were further decolourized with alcohol and washed off rapidly and then covered with safranin for 30 sec and washed off. The stained smears were then air-dried then a few drops of oil immersion were added and viewed under the microscope using x100 objective lens. The Gram-positive isolates appeared as purple while the Gram-negative isolates showed as pink colouration (Holt,1994).

3.6.3 Potassium Hydroxide (KOH) Test

The potassium hydroxide (KOH) test was used to determine or confirm Gram-negative bacteria. To quickly differentiate between Gram-negative and Gram-positive bacteria as a complement to Gram staining, KOH breaks down the thin peptidoglycan bacterial cell walls of Gram-negative bacteria but does not affect the thick layer of Gram-positive cell walls. Disruption of bacterial cell walls lyses the cell and releases its contents, including the genetic material. A drop of 3% KOH solution was applied on a labelled clean microscope slide and smeared with pure isolated culture using a loop. It was stirred carefully and observed to see if the solution turned to be viscous or dense suspension, which formed a slimy or mucoid string within 60 seconds. The appearance of dense suspension which formed slimy substance indicated positive result showing the presence of Gram-negative bacteria while appearance of non-slimy viscous suspension was negative result (Cheesbrough, 2000).

3.7 Biochemical Test

3.7.1 Catalase Test

This test was used to distinguish between bacteria that produce the catalase enzyme, such as Staphylococci, and bacteria that do not, such as Streptococci. Catalase catalyzes the breakdown of hydrogen peroxide (H_2O_2) to oxygen (O_2) and water (H_2O). In this test, 2mL of hydrogen peroxide solution was poured into a test tube, and some colonies of the test organism were picked and immersed into the H_2O_2 solution using a sterile glass rod. The bacteria that generated catalase (positive result) produced gas bubbles (oxygen), but those that did not possess catalase enzyme had none (negative result) (Cheesbrough, 2000).

3.7.2 Cytochrome oxidase test:

In this test; a piece of fitter paper was placed in Petri dish and 3 drops of freshly prepared oxidase reagent was added. Using a piece of glass rod, a colony of the test organism was

smeared on the paper. The appearance of blue/purple colouration signaled a positive result.(Cheesbrough, 2000).

3.7.3 Urease test

This test was used to demonstrate the ability of the isolates to produce the enzyme urease which splits urea forming ammonia. The test is usually used to differentiate organisms like *Proteus* from other non-urease positive organisms (Baker and Breach 1979). A loopful of the isolates were used to inoculate Bijou bottles of urea agar base. The Bijou bottles were incubated at 37^{o}C. A change in colour from yellow to red confirmed the presence of urease (Cheesbrough, 2000).

3.7.4 Citrate utilization test

Slopes of the Simon's citrate medium in bijou bottles were prepared as recommended by the manufacturer. Using a sterile straight wire, the slope was first streaked with a saline suspension of the test organism and the butt was stabbed. It was then incubated at 35^{o}C for 48 hrs. A change in color from green to bright blue indicated positive for citrate test while no change in color of medium indicated negative citrate test (Cheesbrough, 2000).

3.7.5 Indole test

The test organism was inoculated into Bijou bottles containing 3ml of sterile tryptone water and incubated at 37℃for 48h. Thereafter 0.5ml of Kovac's reagent was then added and shaken gently. It was then examined for a red color in the surface layer after 10 min. A red surface layer indicated positive indole test while no red surface layer indicated negative indole test (Cheesbrough, 2000)

3.7.6 Sugar Fermentation Test

Many bacteria species can be differentiated on the basis of the sugars they utilize and ferment. The fermentation medium was prepared by the addition of 0.1 g of peptone, 0.1 g of

sodium chloride and 0.1 g of fermentable sugar (glucose, lactose, mannitol, arabinose, sucrose and mannose) in 10 ml of distilled water. 4 ml of the medium was pipetted into Bijou bottles containing Durham tubes. 1 ml of phenol red indicator was also added to the tubes. The Bijou bottles containing the sugar solution were inoculated with the test bacterial isolates and incubated at 37^{o}C for 24-48 h. After incubation, a change of colour from red to yellow indicates acid production and the presence of gas in the inverted Durham tubes was indicative of gas production (kirk *et al.*, 1975).

3.8 Antibiotics susceptibility assays

Antibiotic susceptibility testing (AST) was performed using the Kirby-Bauer disk diffusion method on Mueller Hinton Agar (Oxoid, Basing stroke, UK). Excess moisture was removed from the plates prior to use by drying. Eight antibiotics were tested for the Gram-negative bacteria; Amoxicillin 30µg (AUG), Cefuroxime 30µg (CRX), Gentamicin 10µg (GEN), Ceftazimidine 5µg (CAZ), Ampicillin 10µg (AMP), Ofloxacin 5µg (OFL), Ciprofloxacin 5µg (CPR) and Nitrofurantoin 300µg (NIT) and while seven antibiotics were tested for the Gram-positive bacteria, Amoxicillin 30µg (AUG), Ceftazimidine 5µg (CAZ), Cefuroxime 30µg (CRX), Doxycyclin 30µg (DOXY), Ampicilin 10µg (AMP), Ofloxacin 5µg (OFL) and Ciprofloxacin 5µg (CPR). Results were then interpreted by measuring the zones of inhibition around each antibiotic disc in millimetres. Zones were recorded and interpreted using the guidelines of clinical and laboratory standard institute (CLSI) (CLSI, 2020). Isolates that resisted one or more antibiotics from three or more distinct antibiotics classes were classified as multidrug resistant (MDR) isolates. The multidrug resistant (MDR) food borne bacteria isolates were then subjected to plasmid profiling.

3.9 Extraction of plasmid DNA and plasmid profiling.

The first step was to harvest the cell culture using distilled water (nuclease free water). A total of 250ml of the nuclease-free water was pipetted into four 1.5ml centrifuge tubes. A loopful of the test bacteria culture was collected and inoculated into a clean 1.5ml tube. The tubes were centrifuged in a micro-centrifuge at full speed for 20 seconds. The supernatants were then discarded. 250µl of ZymoPure^{TM} Pi (Red) was then added to the bacterial cell pellet and resuspended completely by vortexing. After which 250µl at ZymoPure P2 (Green) was then added and immediately mixed by gently inverting the tube 6-8 times. It was then allowed to sit at room temperature for 3 min. The cell was completely used when the solution appeared clear purple and viscous.

250µl of ice cold ZymoPure P3 (Yellow) was added and mixed thoroughly by inversion. The tubes were further inverted 4 times after which the sample turned completely yellow and a yellowish precipitate formed. It was incubated for 5 minutes and centrifuged for 5 minutes at 16000xg.

600µl of supernatant was then transferred into clean 1.5ml micro-centrifuge tube. Thereafter, 275ml of ZymoPure binding buffer was added to the clear lysate and mixed thoroughly by inverting the capped tube 8 times. The mixture was then transferred into a collection tube placed in a ZymoSpin^{TM} II-P column and incubated at room temperature for 2 minutes and then centrifuge at 5000xg for 1 min.800µl of ZymoPure wash 1 was added to the Zymo-Spin column and centrifuge at 5000g for 1 min and the flow through was discarded.

800µl of ZymoPure wash 2 was added to theZymo-Spin II-P column and centrifuged at 5000 x g for 1 min. 200µl of ZymoPure wash 2 was added to the ZymoSpin II-P columns and centrifuge at 5000 g for 1 min and the flow through was discarded. The Zymo-Spin II-P columns were centrifuge at 10,000 x g for 1 min in order to remove any residue wash buffer.

The Zymo-Spin II-P column was the transferred into a clean 1.5ml tube and the 25µl of ZymoPure Elution Buffer was then added differently to the column matrix. It was then incubated at room temperature for 2 min and then centrifuged at 10,000 x g for 1 min in a micro-centrifuge. The eluted plasmid DNA was then stored at 20^{o}C.

Agarose gel-electrophoresis was then used to resolve the eluted plasmid DNA on the bases of molecular weight. 10µl of 100bp DNA ladder was loaded mixed with loading dyes and loaded in the first well. After this, 10µl of samples containing the DNA loading dyes were loaded into the other wells. The gel electrophoresis was then run at 120V for 45min. Thereafter, the gel was transferred to the UV trans-illuminator in order to get the copy of the gel image.

3.10 Molecular identification of isolates.

3.10.1 Bacteria DNA extraction

The DNA was extracted using the standard protocol stated by Trindade *et al.* (2007). Briefly, Single colonies grown on medium were transferred to 1.5 ml of liquid medium and cultures were grown on a shaker for 48 h at 28 ºC. After this period, cultures were centrifuged at 4600g for 5 min. The resulting pellets were resuspended in 520 µl of TE buffer (10 mMTris-HCl, 1mM EDTA, pH 8.0). Fifteen microliters of 20% SDS and 3 µl of Proteinase K (20 mg/ml) were then added. The mixture was incubated for 1 hour at 37 ºC, then 100 µl of 5 M NaCl and 80 µL of a 10% CTAB solution in 0.7 M NaCl were added and votexed. The suspension was incubated for 10 min at 65 ºC and kept on ice for 15 min. An equal volume of chloroform: isoamyl alcohol (24:1) was added, followed by incubation on ice for 5 min and centrifugation at 7200g for 20 min. The aqueous phase was then transferred to a new tube and isopropanol (1: 0.6) was added and DNA precipitated at –20 ºC for 16 h. DNA was collected by centrifugation at 13000g for 10 min, washed with 500 µl of 70% ethanol, air-

dried at room temperature for approximately three hours and finally dissolved in 50 µl of TE buffer.

3.10.2 PCR Analysis

3.10.2.1 Bacteria PCR

The PCR sequencing preparation cocktail consisted of 10 µl of 5x GoTaq colourless reaction, 3 µl of 25mM $MgCl_2$, 1 µl of 10 mM of dNTPs mix, 1 µl of 10 pmol each 27F 5'- AGA GTT TGA TCM TGG CTC AG-3' and - 1525R, 5′-AAGGAGGTGATCCAGCC-3′ primers and 0.3units of Taq DNA polymerase (Promega, USA) made up to 42 µl with sterile distilled water 8µl DNA template. PCR was carried out in aGeneAmp 9700 PCR System Thermocycler (Applied Biosystem Inc., USA) with a PCR profile consisting of an initial denaturation at 94°C for 5 min; followed by 30 cycles consisting of 94°C for 30 s, 50°C for 60s and 72°C for 1 min 30 seconds; and a final termination at 72°C for 10 min. It was chilled at 4^0C GEL (Wawrik *et al.,* 2005).

3.10.2.2 Integrity

The integrity of the amplified gene fragment was checked on a 1% Agarose gel ran to confirm amplification. The buffer (1xTAE buffer) was prepared and subsequently used to prepare 1.5% agarose gel. The suspension was boiled in a microwave for 5 min. The molten agarose was allowed to cool to 60°C and stained with 3µl of 0.5 g/ml ethidium bromide (which absorbs invisible UV light and transmits the energy as visible orange light). A comb was inserted into the slots of the casting tray and the molten agarose was poured into the tray. The gel was allowed to solidify for 20 min to form the wells. The 1xTAE buffer was poured into the gel tank to barely submerge the gel. Two microliter (2µl) of 10x blue gel loading dye (which gives colour and density to the samples to make it easy to load into the wells and monitor the progress of the gel) was added to 4µl of each PCR product and loaded into the wells after the 100bp DNA ladder was loaded into well 1. The gel was electrophoresed at

120V for 45 min visualized by ultraviolet trans-illumination and photographed. The sizes of the PCR products were estimated by comparison with the mobility of a 100bp molecular weight ladder that was ran alongside experimental samples in the gel.

3.10.3 Purification of amplified product

After gel integrity, the amplified fragments were ethanol purified in order to remove the PCR reagents. Briefly, 7.6 µl of Na acetate 3M and 240 µl of 95% ethanol were added to each about 40µl PCR amplified product in a new sterile 1.5 µl tube Eppendorf, mix thoroughly by vortexing and kept at -20°C for at least 30 min. Centrifugation for 10 min at 13000 g was carried out and 4°C followed by removal of supernatant (inverted tube on trash once) after which the pellet were washed by adding 150 µl of 70% ethanol and mixed, then centrifuged for 15 min at 7500 g and 4°C. Again, all supernatant were removed (inverting tube on trash) and inverting tube on paper tissue and dried in the fume hood at room temperature for 10-15 min. Then re-suspended with 20 µl of sterile distilled water and kept in -20^{o}C prior to sequencing. The purified fragment was checked on a 1.5% Agarose gel ran on a voltage of 110V for about 1hr to confirm the presence of the purified product and quantified using a nanodrop of model 2000 from Thermos-Scientific.

3.10.4 Sequencing

The amplified fragments were sequenced using a Genetic Analyzer 3130xl sequencer from Applied Biosystems using manufacturers' manual while the sequencing kit used was that of BigDye terminator v3.1 cycle sequencing kit. Bio- Edit software and MEGA 6 were used for all genetic analysis.

3.11 Phylogenetic Analysis

Sequenced organisms were uploaded in a bio-edit software and cluster W ran to align sequences. The cluster W aligned sequence were then uploaded to the Mega12 software and

the phylogenetic tree was constructed using the unweighted pair method with arithmetic mean (UPGMA) (Santamaria and Theron, 2009).

CHAPTER FOUR

RESULTS

Table 4.1 shows the heterotrophic bacterial count (CFU/g) of spring onions bought from markets in Benin metropolis. From the Table 4.1, it showed that Oba market had the highest heterotrophic bacterial count ($8.71 \pm 1.80 \times 10^6 \mathrm{CFU/g}$) while Oluku market had the lowest heterotrophic bacterial count ($5.71 \pm 1.50 \times 10^6 \mathrm{CFU/g}$) in the spring onions.

Table 4.1: Total Heterotrophic bacterial count in spring onions

Markets	**Bacterial Counts ($x10^6$ cfu/g)**
Lagos street	6.71 ± 1.50^b
Uselu	7.43 ± 1.27^b
Oba	8.71 ± 1.80^c
New Benin	7.86 ± 1.35^b
Oluku	5.71 ± 1.50^a
Ramat Park	6.43 ± 1.27^b
UBTH	6.86 ± 1.35^b
Ikpoba Hill P- value = 0.016	7.71 ± 1.80^b

NOTE: Values are presented as mean ± std deviation. Similar superscripts within a column are not significantly different. P < 0.05.

Table 4.2 shows the cultural, morphological and biochemical characteristics of the bacteria isolated from spring onions. It showed that seven bacterial organisms were isolated. They are *Enterobacter aerogenes, Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus, Bacillus cereus, Proteus vulgaris* and *Serratia marcescens.* From the cultural characteristics of the isolates, *Klebsiella pneumonia* and *Serratia marcescens* had raised elevation while the other five isolates had flat elevation. Their colour was creamy. Some had irregular shape while others had circular shape. However, based on their morphological characteristics, five of the isolates are Gram-negative while the other two are Gram-positive. The Gram-negative bacteria are *Enterobacter aerogenes, Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris* and *Serratia marcescens* while the Gram positive bacteria are *Staphylococcus aureus* and *Bacillus cereus.* From the Biochemical test *Enterobacter aerogenes, Escherichia coli*and *Klebsiella pneumoniae* had gas formations while the other four had no gas formation. Only *Serratia marcescens* was able to ferment mannitol sugar. All the organisms produced catalase enzymes.

Table 4.2: Morphological and biochemical characteristic of bacteria isolates from spring onions

Cultural	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Elevation	Flat	Flat	Flat	Raised	Flat	Flat	Raised
Margin	Undulate	Undulate	Entire	smooth	Undulate	Entire	Entire
Color	Cream	Cream	Cream	Cream	Cream	Cream	Cream
Shape	Irregular	Irregular	Circular	Irregular	Irregular	Circular	Circular
Size	Large	Large	Small	Small	Large	Medium	Medium
Morphological **Gr. diff. agar**	EMB	MCC	EMB	MSA	BCA	MCC	EMB
Color	Pink	Pink	Pink	Yellow	Blue	Black	Opaque
Staining **Gram stain**	-	-	-	+	+	-	-
Biochemical **KOH test**	+	+	+	-	-	+	+
Catalase	+	+	+	+	+	+	+
MR test	-	+	-	-	-	-	-
VP test	+	-	-	-	-	-	+
Indole	-	+	-	-	-	+	-
Citrate	+	-	+	+	+	+	+
Oxidase	-	-	-	-	-	-	-

Motility	+	+	-	-	+	+	+
Urease	-	-	+	+	-	+	-
Glucose	+	+	+	+	+	+	+
Sucrose	+	+	-	+	+	-	+
Lactose	+	+	-	+	+	-	-
Mannitol	-	-	-	-	-	-	+
Gas formation	+	+	+	-	-	-	-
H_2S formation	-	--	+	-	-	+	-
Identity	*Enterobacteraerogenes*	*Escherichia coli*	*Klebsiellapneumoniae*	*Staphylococcus aureus*	*Bacillus cereus*	*Proteus vulgaris*	*Serratiamarcescens*

KEY: EMB: Eosin Methylene Blue Agar (EMB), MCC: MacConkey agar, MSA: Mannitol salt agar, BCA: *Bacillus cereus* agar

Figure 4.1 shows the percentage occurrence of the bacteria isolated from the different markets. *Bacillus cereus* had the highest occurrence (100%), followed by *Staphylococcus aureus* and *Escherichia coli* (75%) which are higher than *Proteus vulgaris* (37.5%), while *Klebsiella pneumoniae, Serratia marcescens* and *Enterobacter aerogenes* had the lowest occurrence(25%)

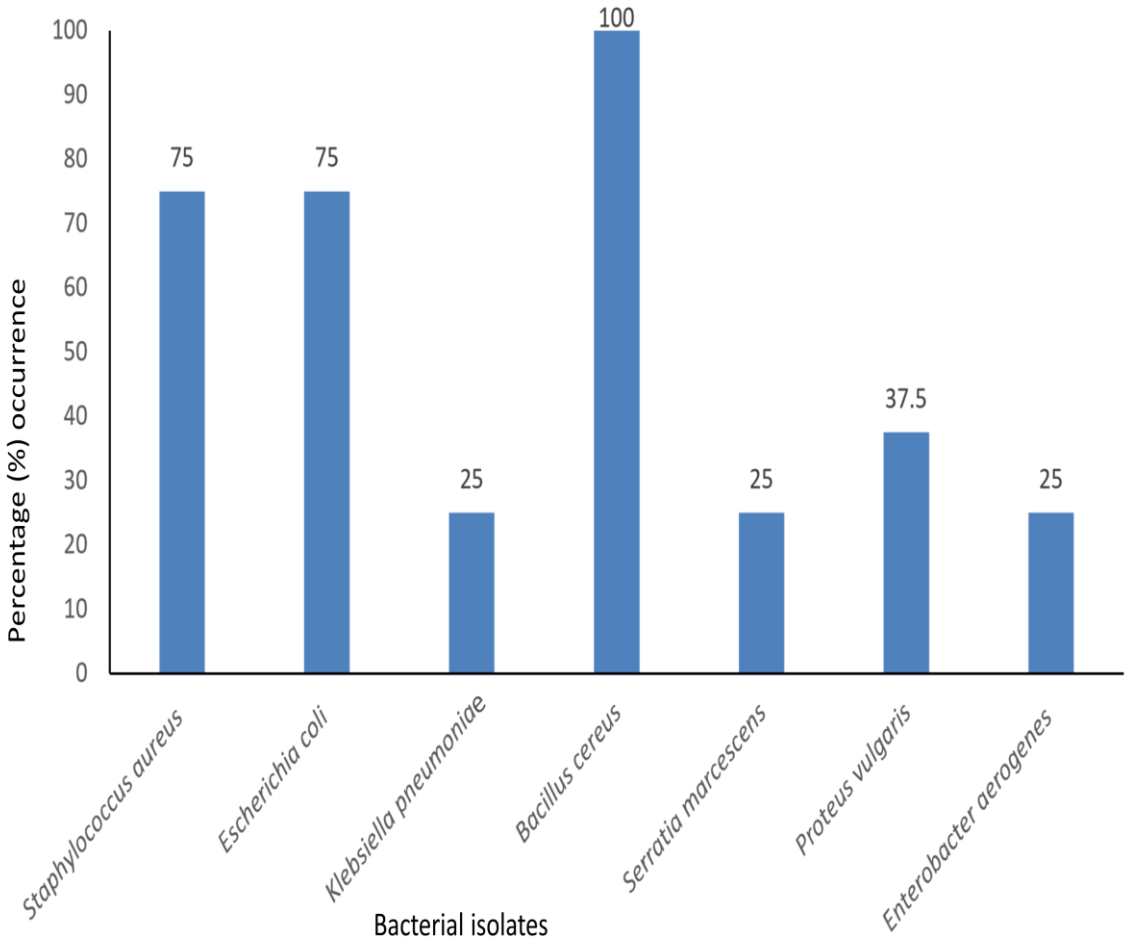

Figure 1: Percentage distribution of bacteria isolated from spring onions

Plate 4.1 shows the agarose gel showing positive amplification of the 16S regions amplified from selected bacteria samples. The bacteria species identified includes *Bacillus cereus* which is represented in Lane OA1, *Escherichia coli* in Lane OL1, *Proteus vulgaris* in Lane OA2 and *Klebsiella pneumoniae* in Lane OB1 respectively at 1500bp.

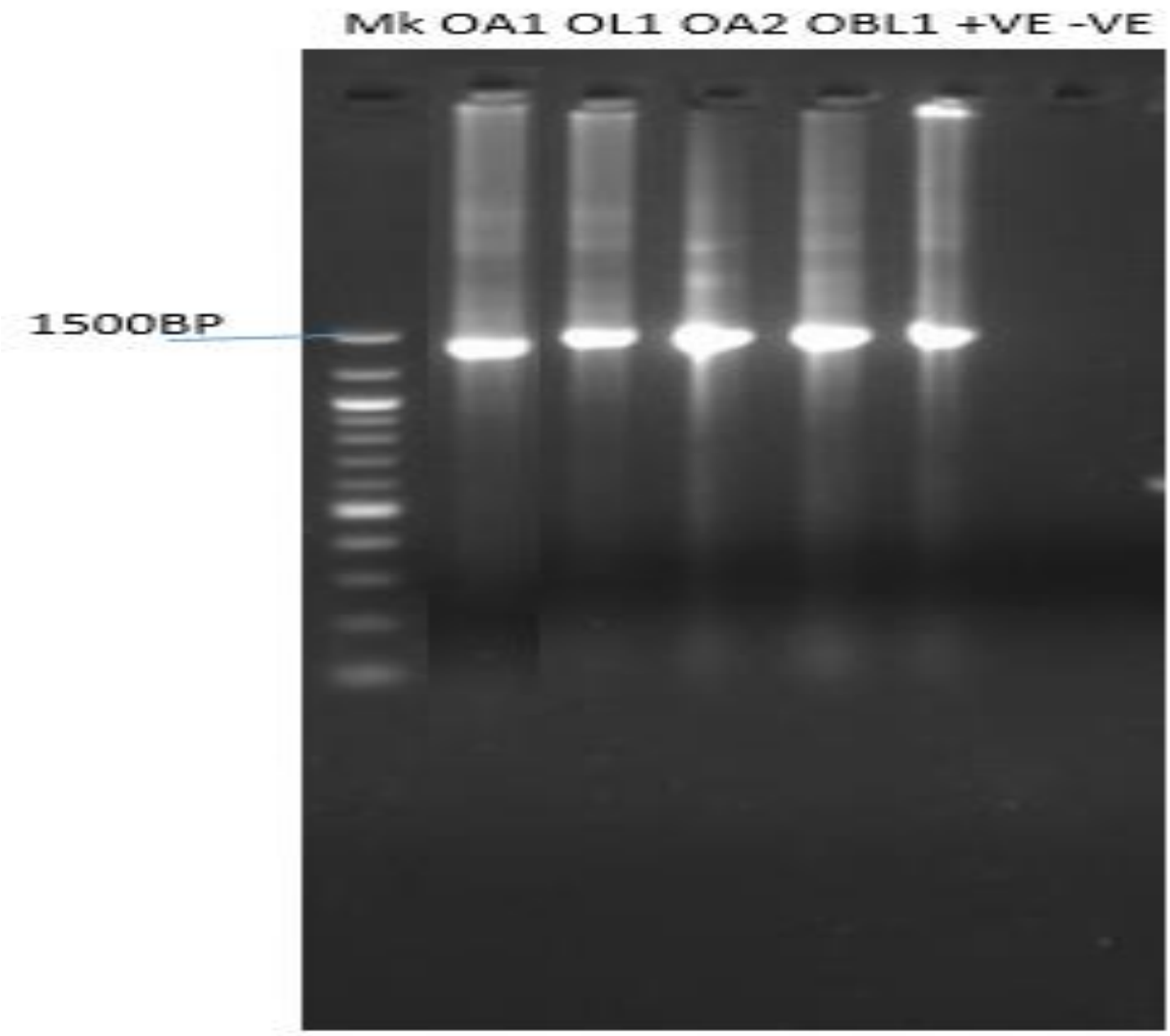

Plate 4.1: Agarose Gel showing the positive amplification of the 16S regions amplified from the selected bacteria samples. Lane OA1: *Bacillus cereus,* Lane OL1: *Escherichia coli,* Lane OA2: *Proteus vulgaris,* Lane OBL1: *Klebsiella pneumoniae.* MK: Size marker DNA ladder (100bp-1.5kb), positive (+ve) and negative (-ve) markers

Table 4.3 shows the NCBI blast sequence identity of the isolates edited sequences. The percentage sequence homology for *Bacillus cereus* (PV453807) was 99.80%, *Escherichia coli*(PV453808) was 99.80%, *Proteus vulgaris* (PV453809) was 99.80% and *Klebsiella pneumoniae* (PV453810) 100%.

Table 4.3: NCBI Blast of the sequence identity of bacteria isolates

Sample ID	Organism	Max Score	Total Score	Query Cover (%)	E value	Percentage Identities (%)	Accession Number
OA1	***Bacillus cereus***	**1934**	**1934**	**98**	**0**	**99.80**	**PV453807**
OL1	***Escherichia coli***	**1836**	**1836**	**100**	**0**	**99.80**	**PV453808**
OA2	***Proteus vulgaris***	**1836**	**1836**	**100**	**0**	**99.80**	**PV453809**
OBL1	***Klebsiellapneumoniae***	**1821**	**1821**	**100**	**0**	**100.00**	**PV453810**

Figure 4.2 shows the Phylogenetic tree of the bacteria isolates. It showed that the closest strain to *Klebsiella pneumoniae* was *Klebsiella pneumoniae*CP021696.1, *Escherichia coli* was *Escherichia coli* MW368769.1, *Proteus vulgaris* was *Proteus vulgaris* LN681563.1 and *Bacillus cereus* was *Bacillus cereus* LN890127.1.

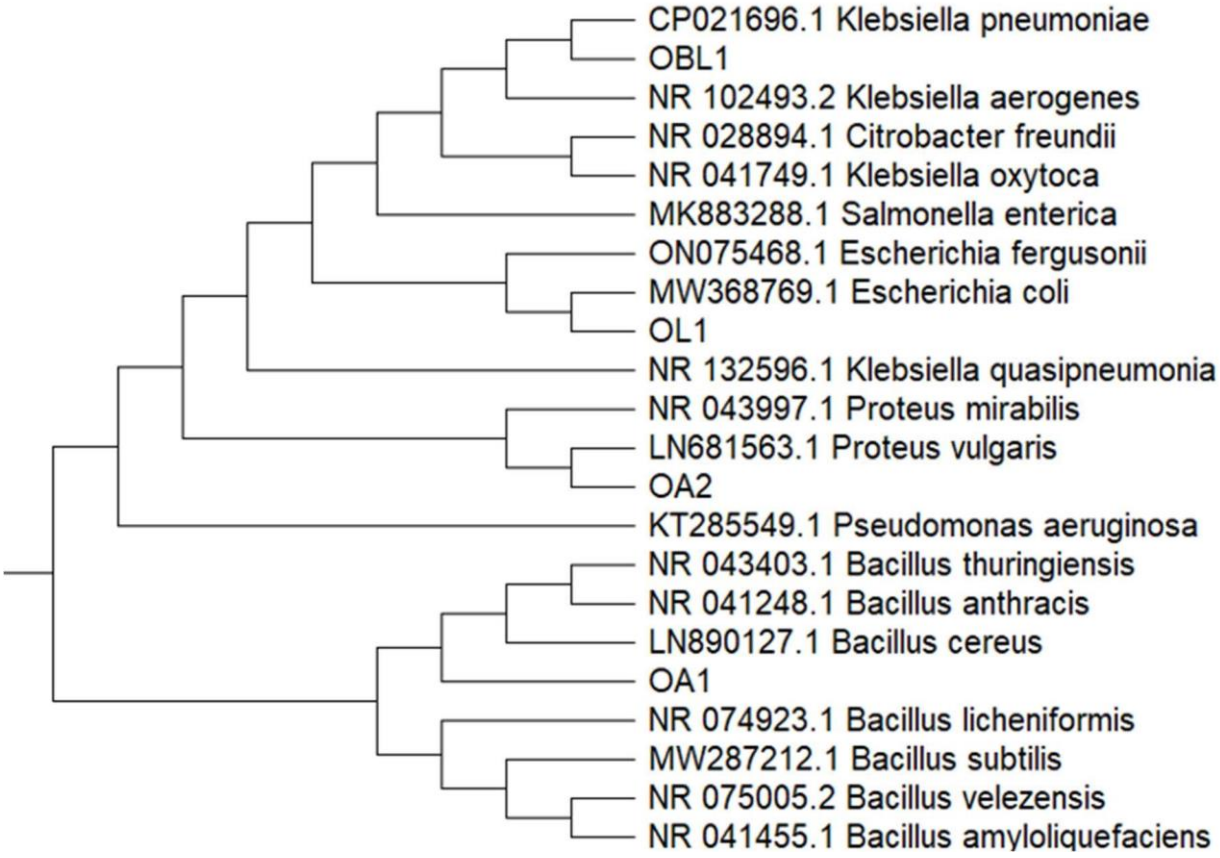

Figure 4.2: Phylogenetic tree of the bacteria isolates

Table 4.4 shows the antibiotics sensitivity pattern of the Gram negative bacteria isolated from the spring onions using the disc diffusion method. Eight antibiotics were used to test for the sensitivity patterns of the isolates. *Escherichia coli, Klebsiella pneumoniae* and *Serratia marcescens* were resistant to cefuroxime while *Proteus vulgaris* and *Enterobacter aerogenes* were susceptible to the drug. The isolates used were susceptible to gentamicin, ciprofloxacin and ofloxacin except *Serratia marcescens* which was resistant to gentamicin and ciprofloxacin and *Proteus vulgaris* which was of intermediate sensitivity to ofloxacin. Furthermore, *Escherichia coli* and *Serratia marcescens* were resistant to Nitrofurantoin, while *Klebsiella pneumonia* and *Enterobacter aerogenes* were susceptible and *Proteus vulgaris* was of intermediate sensitivity to the drug.

Table 4.4: Antibiotics sensitivity pattern of gram-negative bacteria isolated from spring onions

	Zone of inhibition (mm)								
Bacterial isolates	**CAZ**	**CRX**	**GEN**	**CPR**	**OFL**	**AUG**	**NIT**	**AMP**	**MAR-Index**
Escherichia coli (6)	12.0 (R)	10.0 (R)	19.0 (S)	21.0 (S)	18.0 (S)	12.0 (R)	14.0 (R)	10.0 (R)	0.63
Klebsiellapneumonia(2)	0.0 (R)	8.0 (R)	19.0 (S)	22.0 (S)	16.0 (S)	0.0 (R)	18.0 (S)	0.0 (R)	0.50
Serratiamarcescens(2)	0.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)	0.0 (S)	0.0 (R)	0.0 (R)	0.0 (R)	1.0
Proteus vulgaris(4)	0.0 (R)	18.0 (S)	20.0 (S)	26.0 (S)	14.0 (I)	0.0 (R)	16.0 (I)	10.0 (R)	0.38
Enterobacteraerogenes(2)	16.0 (I)	18.0 (S)	20.0 (S)	23.0 (S)	22.0 (S)	0.0 (R)	18.0 (S)	14.0 (R)	0.25

KEY: - AUG-Amoxicillin, CAZ-Ceftazimidine, CRX-Cefuroxime, GEN-Gentamicin, AMP-Ampicillin, OFL-Ofloxacin, CPR-Ciprofloxacin, NIT-Nitrofurantoin, S-Sensitive, R-Resistances, I-Intermediate, MAR-I – Multiple antibiotic resistance index

On Table 4.5, is shown the antibiotic sensitivity pattern of Gram-positive bacteria isolated from spring onions. *Bacillus cereus* was resistant to all the antibiotics except ofloxacinto which it was susceptible. *Staphylococcus aureus* was susceptible to ofloxacin and doxycycline but was resistant to the other five antibiotics.

Table 4.5: Antibiotics sensitivity pattern of Gram-positive bacteria isolated from spring onions

	Zone of inhibition (mm)							
Bacterial isolates	**CAZ**	**CRX**	**CPR**	**OFL**	**AUG**	**DOXY**	**AMP**	**MAR-Index**
Staphylococcus aureus(6)	12.0 (R)	0.0 (R)	0.0 (R)	16.0 (S)	0.0 (R)	18.0 (S)	14.0 (R)	0.71
Bacillus cereus(8)	12.0 (R)	0.0 (R)	0.0 (R)	12.0 (S)	0.0 (R)	12.0 (R)	0.0 (R)	0.86

KEY: - AUG-Amoxicillin, CAZ-Ceftazimidine, CRX-Cefuroxime, DOXY-Doxycycline, AMP-Ampicilin, OFL-Ofloxacin, CPR-Ciprofloxacin, NIT-Nitrofurantoin, S-Sensitive, R-Resistances, ITM-Intermediate, MAR-I – Multiple antibiotic resistance in

Plate 4.2 shows the plasmid profile of the antibiotic resistant bacteria isolates analyzed with 1% agarose gel electrophoresis. In this plate, four isolates which were more resistant to the antibiotic applied were analyzed. They include *Klebsiella pneumoniae* CP021696.1, *Proteus vulgaris* LN681563.1, *Bacillus cereus* LN890127.1 and *Escherichia coli* MW368769.1. It showed that *Klebsiella pneumoniae* CP021696.1 and *Proteus vulgaris* LN681563.1 had plasmids while *Bacillus cereus* LN890127.1 and *Escherichia coli* MW368769.1 had no plasmids. When compared to the ladder of molecular weight 100bp-1kbp. It showed that the plasmid in *Klebsiella pneumoniae* CP021696.1 and *Proteus vulgaris* LN681563.1 had a molecular weight of 1000bp (Table 4.6).

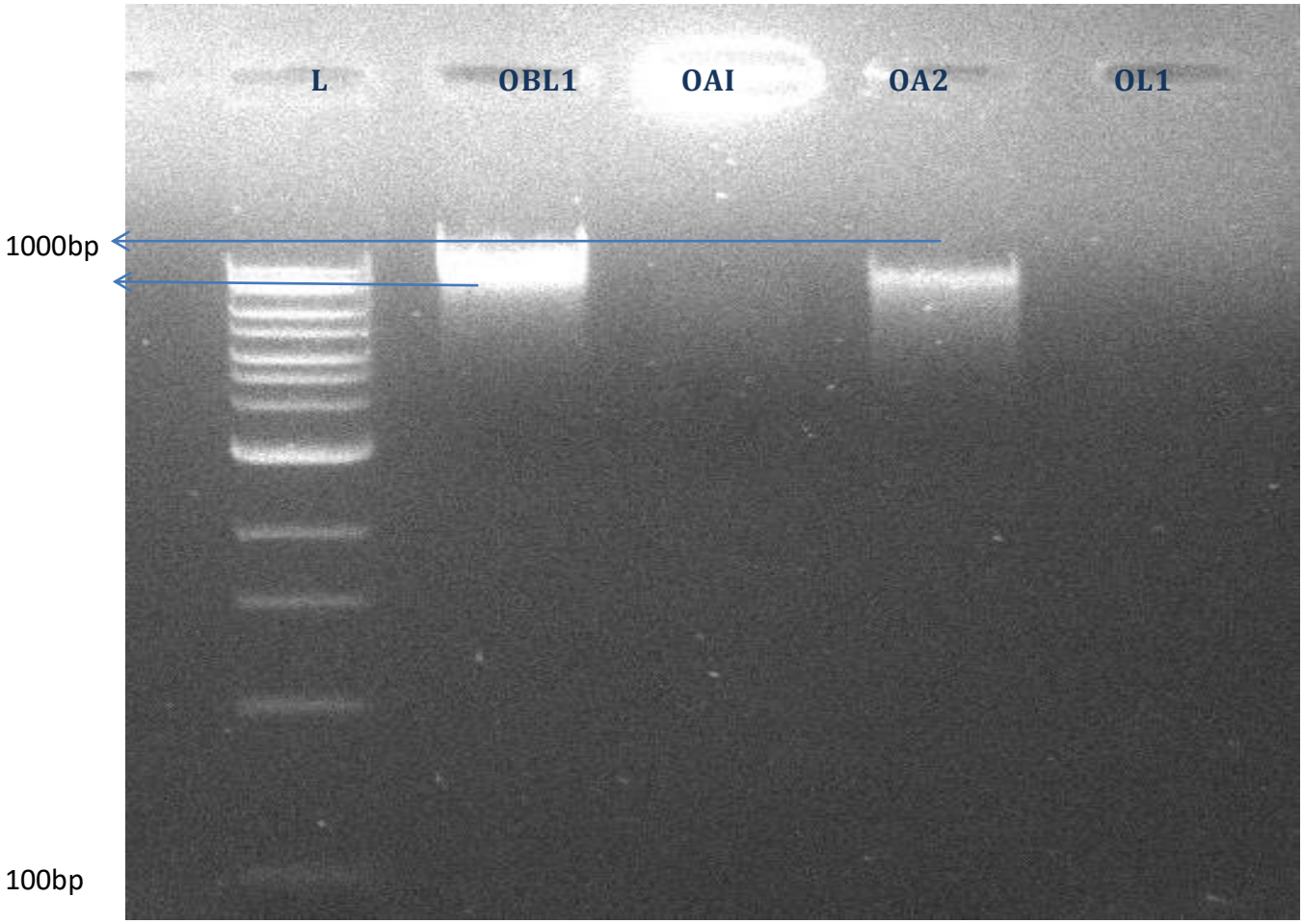

Plate 4.2: Plasmid profile of the Antibiotic resistance Bacterial isolates analyzed with 1% agarose gel electrophoresis. L is 100bp-1kb ladder (molecular marker). Lanes OBL1 and OA2 were positive for plasmid geneswhile OA1 and OL1 were negative for plasmid genes.

KEY:

OBL1 –*Klebsiellapneumoniae*

OA2 – *Proteus vulgaris*

OAI – *Bacillus cereus*

OL1 – *Escherichia coli*

Table 4.6: Occurrence and estimated sizes of plasmids in antibiotic-resistant bacteria isolates

Bacterial Isolate	**Lane ID**	**Plasmid Detected**	**Approximate Band Size (bp)**	**Result**
Klebsiella pneumoniae	OBL1	Yes	~1000 bp (1.0 kb)	Positive
Proteus vulgaris	OA2	Yes	~1000 bp (1.0 kb)	Positive
Bacillus cereus	OA1	No	–	Negative
Escherichia coli	OL1	No	–	Negative

CHAPTER FIVE

5.1 DISCUSSION

Foodborne disease, also known as foodborne infection, foodborne illness, or food poisoning is any illness caused by eating or drinking the contaminated food or water that contains pathogenic microbes (viruses, bacteria, protozoa, helminths), as well as chemical or natural toxins (Mead *et al.,* 1999). Foodborne infections affect both men and women, all age groups, all seasons and both rural and urban areas and they can occur in sporadic or epidemic (Painter *et al.,* 2013).

Contamination that occurs during production, collection, transporting and preparation as well as during processing makes food a possible source of human infection. Potential sources of food contamination include feaces- contaminated water or soil unsanitary handling and use of unclean utensils and equipment (Slifko*et al.,* 2000). The presence of ready-to-eat foods, milk and milk products, meat and meat products put the consumer at risk and also causes the producers to suffer significant financial losses (Syne*et al.,* 2013).

The findings of this study demonstrate that spring onions (*Allium fistulosum*) sold in major markets across Benin City are contaminated with heterotrophic bacteria and serve as reservoirs of potentially pathogenic and antibiotic-resistant microorganisms. The heterotrophic bacterial counts which ranged from 5.71×10^6 to 8.71×10^6cfu/g**,** far exceed the microbiological safety limits recommended for fresh vegetables intended for raw consumption. According to the International Commission on Microbiological Specifications for Foods (ICMSF, 2011), aerobic plate counts above 10^5cfu/g are indicative of poor hygienic quality and increased public health risk.

The variations in bacterial load among the markets, with Oba Market having the highest counts and Oluku Market the lowest, was statistically significant ($p < 0.05$). This disparity may be due to differences in environmental sanitation, market congestion, waste management practices, and the degree of post-harvest handling. Oba Market is characterized by intense human traffic, prolonged exposure of produce to dust and flies, and frequent contact with contaminated surfaces. Similar patterns were reported by Ogbonna, *et al.,* (2019)**,** who documented heterotrophic bacterial counts in the range of 10^6–10^7cfu/g in vegetables stored under unhygienic market conditions in Nigeria. Likewise, Igba *et al*. (2021)**,** in a systematic review of fresh vegetables and ready-to-eat salads in Nigeria, reported that virtually all studies reviewed recorded bacterial loads above acceptable limits, emphasizing that contamination of vegetables in Nigerian markets is widespread.

However, the isolation of several bacterial species, namely *Enterobacter aerogenes, Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus, Bacillus cereus, Proteus vulgaris,* and *Serratia marcescens,* further confirms extensive microbial contamination of spring onions in Benin City. These organisms which are associated with soil, water, fecal matter, and human handling can cause contamination at multiple stages of production such as cultivation, harvesting, transportation and point of sales. The predominance of Gram-negative bacteria, particularly members of the Enterobacteriaceae, aligns with the ecology of leafy vegetable contamination (Omoya *et al.*,(2024).

The isolation of *Staphylococcus aureus* in this study strongly suggests human-associated contamination**,** as the organism is a normal flora of the skin, nose and hands. This observation corroborates the findings of Ugwu (2024)**,** who reported frequent isolation of *Staphylococcus aureus* and *Escherichia coli* from leafy vegetables sold in Enugu markets, attributing contamination largely to poor personal hygiene among vendors. Similarly, Igba *et al.* (2021)

identified *Staphylococcus aureus* as one of the most frequently isolated bacteria from Nigerian vegetables, reinforcing the role of handling practices in vegetable contamination.

The consistent isolation and 100% occurrence of *Bacillus cereus* in this study shows its dominance in spring onions suggesting contamination from soil and dust during cultivation and marketing, being that Bacillus cereus is usually a microbe associated with food. This finding agrees with Omoya *et al*. (2024), who reported *Bacillus cereus* as the most prevalent organism (22.07%) on vegetables sold in Akure, and with earlier Nigerian studies by Adesemoye *et al*. (2006) and Ogbonna *et al*. (2019)**.** The public health relevance of *Bacillus cereus* is heightened by its ability to produce enterotoxins capable of causing food poisoning, especially when contaminated foods are eaten raw.

Moreover, the high occurrence of *Escherichia coli* (75%) observed in this study which is on the high side, as *Escherichia coli* is a well-established indicator of fecal contamination and water quality, suggests the use of contaminated irrigation water or untreated organic manure during cultivation. Bahira *et al*. (2020) further demonstrated that the public health significance of *Escerichia coli* contamination by isolating the pathogenic *Escherichia coli* O157:H7 from raw salad vegetables in Sokoto, Nigeria can harbor not only indicator organisms but also highly virulent strains capable of causing severe foodborne disease.

Molecular identification using 16S rRNA gene sequencing confirmed the identities of selected isolates with sequence homology values ranging from 99.80% to 100%**,** validating the phenotypic identification methods used in this study. The phylogenetic analysis showed close clustering of the isolates with reference strains previously associated with food contamination and human infections worldwide. This aligns with the observations of Antunes *et al.* (2021), who used phenotypic and genomic tools to characterize antibiotic-resistant

Escherichia coli from fresh vegetables in the United Arab Emirates, highlighting the global relevance of molecular confirmation in food microbiology studies.

Moreso, the antibiotic susceptibility pattern revealed widespread resistances, particularly among the Gram-negative bacteria to commonly used beta-lactam antibiotics such as ampicillin, amoxicillin, cefuroxime and ceftazidime. These resistances trend mirrors findings from several Nigerian studies. For instance, Ogbonna *et al*. (2019**)** who reported resistances of vegetable-associated bacteria to ampicillin and ceftazidime, while Katyayani *et al*. (2022) documented multiple antibiotic resistances among *Escherichia coli* isolates from leafy vegetables sold around Joseph Ayo Babalola University. Aslo, Alhumaid *et al*. (2011) who also reported high resistance rates to ampicillin among bacteria isolated from fresh vegetables in Saudi Arabia indicates that resistance to beta-lactam antibiotics is not restricted to Nigeria alone, it represents a broader global trend.

The relatively high susceptibility of most isolates to fluoroquinolones (ciprofloxacin and ofloxacin) and gentamicin observed in this study is consistent with the findings of Omoya*et al*. (2024)**,** who reported high sensitivity of vegetable-associated bacteria to ciprofloxacin and gentamicin in Akure. However, the presence of high Multiple Antibiotic Resistance (MAR) indices, particularly in *Serratia marcescens* (MAR = 1.0), indicates that these organisms originated from environments with frequent antibiotic exposure. According to Krumperman (1983), MAR values greater than 0.2 signify high-risk sources of contamination, suggesting that spring onions sold in Benin City may contribute to the dissemination of multidrug-resistant bacteria.

The resistance patterns observed among Gram-positive isolates further compound public health concerns. *Bacillus cereus* exhibited resistance to most antibiotics tested except ofloxacin, while *Staphylococcus aureus* was resistant to multiple antibiotics including

ampicillin and ceftazidime. These findings are consistent with reports by Taiwo *et al*. (2011) and Olowe *et al*. (2013), who documented high resistance rates among Gram-positive foodborne bacteria in Nigeria.

Plasmid profiling revealed the presence of plasmids (~1000 bp) in *Klebsiella pneumoniae* and *Proteus vulgaris*, suggesting that antibiotic resistance in these isolates may be plasmid-mediated. This observation aligns with global findings *by* Antunes *et al*. (2021), who reported plasmid-associated resistance genes such as *blaCTX-M-15* in *Escherichia coli* from salad vegetables. The absence of detectable plasmids in *Escherichia coli* and *Bacillus cereus* in this study does not preclude resistance, as chromosomal resistance mechanisms may also play significant roles. As emphasized by Davies and Davies (2010)**,** plasmid-mediated resistance poses a major public health threat due to its ability to spread horizontally among bacteria within the food chain and human gut microbiota.

Overall, the detection of multidrug-resistant and potentially pathogenic bacteria in spring onions sold in Benin Metropolis presents serious public health implications. Comparable findings from Nigeria **(**Ugwu, 2024; Omoya *et al.*, 2024; Igba *et al.,* 2021**)** and other parts of the world **(**Alhumaid *et al.*, 2011; Adzitey, 2018; Antunes *et al.,* 2021; Shapiro *et al.,* 2021**)** demonstrate that contamination of fresh vegetables with antibiotic-resistant bacteria is a global challenge. Given that spring onions are often consumed raw or lightly processed, the risk of foodborne infection and transmission of antimicrobial resistance is obtainable, particularly among vulnerable populations such as children, the elderly and immunocompromised individuals.

5.2. CONTRIBUTION TO KNOWLEDGE

This study has made several significant contributions to existing knowledge in the field of food and environmental microbiology, particularly within the context of fresh vegetable safety in Nigeria.

1. The study provides updated and location-specific data on the bacteriological quality of spring onions (*Allium fistulosum*) sold in selected markets in Benin City, Edo State. While previous studies in Nigeria have focused largely on commonly consumed leafy vegetables such as lettuce, spinach and cabbage, this research expands the scope of knowledge by highlighting spring onions as an importance but often overlooked vehicle for microbial contamination.
2. The study contributes to knowledge by quantifying heterotrophic bacterial loads of spring onions obtained from different markets and statistically demonstrating significant variations among sampling locations. This underscores the influence of market sanitation, handling practices and environmental exposure on microbial contamination levels, thereby providing empirical evidence to support public health interventions at the market level.
3. The identification of both Gram-negative and Gram-positive bacteria, including *Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus, Bacillus cereus, Proteus vulgaris, Enterobater aerogenes* and *Serratia marcescens*, contributes to the growing body of evidence that fresh vegetables in open markets can serve as reservoirs of opportunistic and foodborne pathogens. The confirmation of these isolates using molecular (16S rRNA) techniques further strengthens the reliability of the findings and adds molecular data to existing Nigerian studies that relied solely on phenotypic methods.

4. The study provides valuable insight into the antibiotic resistance profiles and Multiple Antibiotic Resistance (MAR) indices of bacteria associated with fresh vegetables. The detection of high MAR indices, particularly among *Serratiamarcescens* and *Bacillus cereus*, contributes to knowledge on the role of vegetables in the dissemination of multidrug-resistant bacteria along the food chain.
5. The detection of plasmid-mediated resistance in selected isolates contributes to understanding the genetic basis of antimicrobial resistance in foodborne bacteria in Nigeria. This finding reinforces the public health relevance of fresh produce as potential conduits for horizontal transfer of resistance genes between environmental and human-associated bacteria.

5.3. RECOMMENDATIONS

Based on the findings of this study, the following recommendations are made to improve food safety and reduce public health risks associated with the consumption of fresh vegetables such as spring onions (*Alliumfistulosum*):

1. **Improved Agricultural Practices:** Farmers should be encouraged to adopt good agricultural practices (GAP), particularly the use of clean and treated water for irrigation and properly composted organic manure to minimize fecal contamination of vegetables at the farm level.
2. **Market Hygiene and Sanitation:** Local government authorities should enforce improved sanitation in open markets, including regular waste disposal, provision of clean water, and routine environmental sanitation exercises to reduce microbial contamination of fresh produce.
3. **Vendor Education and Training:** Vegetable vendors should be sensitized on the importance of personal hygiene, proper handling, and safe storage of vegetables.

Regular training programs on food safety practices should be organized by public health and environmental health officers.

4. **Consumer Awareness:** Consumers should be educated on the importance of thoroughly washing vegetables with clean water and, where possible, using appropriate food-grade disinfectants before consumption, especially for vegetables intended to be eaten raw.
5. **Antibiotic Stewardship:** Relevant regulatory agencies should strengthen policies on the prudent use of antibiotics in agriculture and human medicine to curb the spread of antimicrobial resistance through the food chain.
6. **Routine Monitoring and Surveillance:** Periodic microbiological surveillance of fresh vegetables sold in markets should be carried out by food safety authorities to ensure compliance with acceptable microbiological standards.
7. **Further Research:** Future studies should include a wider range of vegetables, seasonal variations, and larger sample sizes, as well as advanced molecular techniques such as whole-genome sequencing to better understand resistance mechanisms and transmission dynamics.

5.4. CONCLUSION

This study has demonstrated that spring onions sold in selected markets in Benin City, Edo State, are heavily contaminated with heterotrophic bacteria, including potentially pathogenic and multidrug-resistant species. The bacterial loads recorded exceeded internationally recommended microbiological limits for fresh vegetables, indicating poor hygienic quality and a potential risk to public health. The isolation of fecal indicator organisms such as *Escherichia coli*, human-associated bacteria such as *Staphylococcus aureus*, and spore-forming bacteria such as *Bacillus cereus* suggests contamination from multiple sources

including soil, irrigation water, market environment, and human handling. The antibiotic susceptibility patterns revealed widespread resistance to commonly used antibiotics, with high Multiple Antibiotic Resistance indices pointing to exposure to environments with frequent antibiotic use. The presence of plasmid-mediated resistance in selected isolates further emphasizes the role of fresh vegetables as potential vehicles for the transmission of antimicrobial resistance genes. Given that spring onions are often consumed raw or minimally processed, their contamination poses a significant public health concern.

The findings of this study highlight the urgent need for improved agricultural practices, enhanced market sanitation, effective antibiotic stewardship, and increased consumer awareness to ensure the microbiological safety of fresh vegetables such as spring onions. Addressing these issues will contribute significantly to reducing foodborne infections and limiting the spread of antimicrobial resistance in Nigeria.

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APENDIX I

KEY:- AUG-Amoxicillin, CAZ-Ceftazimidine, CRX-Cefuroxime, GEN-Gentamicine, AMP-Ampicilin, OFL-Ofloxacin, CPR-Ciprofloxacin, NIT-Nitrofurantoin, S-Sensitive, R-Resistances, ITM-Intermediate

Antibiotic	**Disc code**	**Resistant > or = (mm)**	**Intermediate (mm)**	**Susceptible < or = (mm)**
Ceftazimidime (5)	CAZ	14	15-17	18
Cefuroxime	CRX	14	15-17	18
Gentamycin	GEN	12	13-14	15
Ciprofloxacin	CPR	15	16-20	21
Ofloxacin	OFL	12	13-17	18
Amoxicillin clavulanate	AUG	13	14-17	18
Nitrofurantoin	NIT	14	15-17	18
Ampicillin	AMP	11	12-19	20

Distribution of the bacteria isolated from spring onions sold in different markets

Bacteria isolates	**Oluku**	**Uselu**	**New Benin**	**Oba**	**Lagos Street**	**Ramat Park**	**UBTH**	**Ikpoba Hill**	**Percentage Distribution (%)**
Staphylococcus aureus	+	+	-	+	+	+	-	+	75
Escherichia coli	+	+	-	+	+	+	-	+	75
Klebsiella pneumonia	-	-	+	-	-	-	+	-	25
Bacillus cereus	+	+	+	+	+	+	+	+	100
Serratiamarcescens	-	-	+	-	-	-	+	-	25
Proteus vulgaris	+	-	-	+	+	-	-	+	37.5
Enterobacteraerogenes	-	-	-	+	-	-	-	+	25

Key: + = Present, - = Ab

APENDIX II

>OA1 *Bacillus cereus*

TTGGGCCCAACGTCGCATGCTGATCCTGGCTCAGGATGAACGCTGGGGGGGTGC

CTAATACATGCAAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCG

GCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCG

GGAAACCGGGGCTAATACCGGATAACATTTTGAACTGCATGGTTCGAAATTGAA

AGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTG

AGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGC

CACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAAT

CTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCT

TTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTG

GCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCG

CGGTAATACGTAGGTGGCAGGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCG

CAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCAT

TGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCG

GTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTC

TGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCT

GGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTA

GTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTG

AAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAAT

TCGAAGCAACGCGAAGAACCTTACCAGGTCTTGGCATCCTCTGAAAACCCTAGA

GG

>OL1 *Escherichia coli*

AATGATCATGGCGCAGATTGAACGCTGGCGGCCGGCCTAACACATGCAAGTCGA

ACGGTAACAGGAAGCAGCTTGCTGCTTTGCTGACGAGTGGCGGACGGGTGAGTA

ATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAAT

ACCGCATAACGTCGCAAGCACAAAGAGGGGGACCTTAGGGCCTCTTGCCATCGG

ATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACG

ATCCCTAGCTGGTCTGAGAGGATGACCAGCAACACTGGAACTGAGACACGGTCC

AGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGA

TGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCG

GGGAGGAAGGGAGTAAAGTTAATACCTTTGCTCATTGACGTTACCCGCAGAAGA

AGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTT

AATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTG

AAATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCG

TAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGA

ATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAG

CGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTC

GACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGA

CCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCG

CACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTG

GTCTTGACATCCACGGAAGTTTTCAGAAA

>OA2 *Proteus vulgaris*

TGATCCTGGCTCAGATTGAACGCTGGCGGAAGGCCTAACACATGCAAGTCGAGC

GGTAACAGGAGAAAGCTTGCTTTCTTGCTGACGAGCGGCGGACGGGTGAGTAAT

GTATGGGGATCTGCCCGATAGAGGGGGATAACTACTGGAAACGGTGGCTAATAC

CGCATGACGTCTACGGACCAAAGCAGGGGCTCTTCGGACCTTGCGCTATCGGAT

GAACCCATATGGGATTAGCTAGTAGGTGAGGTAATGGCTCACCTAGGCAACGAT

CTCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAG

ACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATG

CAGCCATGCCGCGTGTATGAAGAAGGCCTTAGGGTTGTAAAGTACTTTCAGCGG

GGAGGAAGGTGATAAAGTTAATACCTTTGTCAATTGACGTTACCCGCAGAAGAA

GCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTA

ATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCAATTAAGTCAGATGTGA

AAGCCCCGAGCTTAACTTGGGAATTGCATCTGAAACTGGTTGGCTAGAGTCTTGT

AGAGGGGGGTAGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAA

TACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGC

GTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTCG

ATTTAGAGGTTGTGGTCTTGAACTGTGGCTTCTGGAGCTAACGCGTTAAATCGAC

CGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCG

CACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTA

CTCTTGACATCCAGCGAATCCTTTAAAAA

>OBL1 *Klebsiellapneumoniae*

TGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGC

GGTAGCACAGAGAGCTTGCTCTCGGGTGACGAGCGGCGGACGGGTGAGTAATGT

CTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCG

CATAACGTCGCAAGACCAAAGTGGGGGACCTTCGGGCCTCATGCCATCAGATGT

GCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCC

CTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGAC

TCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCA

GCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGGGG

AGGAAGGCGATGAGGTTAATAACCTCATCGATTGACGTTACCCTGCAGAAGAAG

CACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAA

TCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAA

ATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTA

GAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAAT

ACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCG

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ACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGG

TCTTGACATCCACAGAACTTTCCAGA