CHAPTER ONE

1.1 Introduction

Ready-to-eat (RTE) vegetables, particularly salads prepared from raw cabbage, carrots, and cucumbers, are a convenient and increasingly popular food choice among urban populations in Nigeria. These vegetables, when consumed raw, retain their nutrients and fibre content, but they also bypass the microbial decontamination step that cooking provides. In developing countries like Nigeria, RTE vegetables are often sold by street vendors operating in unregulated environments, with limited access to potable water, refrigeration, or sanitary facilities (Iwu *et al*., 2020). This creates a high-risk for contamination with pathogenic microorganisms, especially Gram-negative bacteria.

Food-borne diseases caused by bacteria are among the most common health concerns globally, especially in resource-limited settings where food hygiene practices are poorly regulated. The World Health Organisation estimates that unsafe food causes about 600 million cases of food-borne illnesses every year. Thus, resulting in about 420,000 deaths recorded, with a significant proportion of these cases occurring in Africa (WHO, 2022).

Gram-negative bacteria are especially significant in food safety due to their structural resistance to many environmental factors and antibiotics. The outer membrane of Gram-negative bacteria contains lipopolysaccharide (LPS), which can act as an endotoxin and contributes to their pathogenicity and resistance to several antibiotics (Silhavy *et al.*, 2010).

Common Gram-negative pathogens isolated from vegetables include *Escherichia coli, Klebsiella pneumoniae , Salmonella enterica, Shigella* spp.*, Pseudomonas aeruginosa, Proteus* spp.*, Citrobacter* spp.*, and Enterobacter* spp. (Adeoye *et al.*, 2019; Nwachukwu *et al.*, 2021). These organisms are often introduced during post-harvest handling, through contaminated water, utensils, vendor hands, or soil residues (Adeoye *et al.*, 2019)

Escherichia coli, particularly the enterotoxigenic and enterohemorrhagic strains, are among the most dangerous, capable of causing diarrhoea, hemorrhagic colitis, and haemolytic uremic syndrome (HUS) (Scallan *et al*., 2011). *Salmonella enterica* is associated with typhoid fever and gastroenteritis and has been isolated from street-vended vegetables in multiple studies across Nigeria (Nwankwo *et al.*, 2020). *Klebsiella pneumoniae* is another opportunistic pathogen frequently found in contaminated water and raw food; it is associated with pneumonia, urinary tract infections, and septicaemia, particularly in immunocompromised individuals (Podschun and Ullmann, 1998). *Proteus mirabilis* and *Citrobacter freundii* are commonly associated with wound infections, urinary tract infections, and food poisoning (Bauer *et al*., 2019).

Beyond their presence, the emergence of multidrug-resistant (MDR) strains of these bacteria to the cephalosporins, tetracyclines, and fluoroquinolones in isolates from vegetable and water samples in Nigerian urban centres have been reported (Odu and Essien, 2020; Ogunbanwo *et al.*, 2022).

There is limited research on associated Gram-negative resistant bacteria pathogens in the face of widespread consumption of RTE vegetables in Benin City, Nigeria. Additionally, there is insufficient empirical evidence to prove that water sources used by vendors are safe and not a contamination point. The (research) questions that therefore comes to mind are: what is the microbial load of street-vended vegetable salads in Benin City? What Gram-negative bacteria are present in these salads and their water sources? Are the isolated organisms resistant to commonly used antibiotics? Is there evidence to suggest that municipal water is a medium of bacterial transmission onto RTE vegetables in Benin City? Bridging this gap in knowledge is critical to effective policy making and risk communication to safeguard consumer health.

Therefore, this study evaluated the microbiological quality of RTE vegetables from Benin city as well as the antibiogram of some selected Gram-negative bacteria isolates and their possible

transfer by water. Results from our earlier study will generate much needed data on the microbial hazards posed by consumption RTE vegetables in urban Nigeria. They will help regulatory agencies in designing effective food safety policies, inform public health campaigns and guide efforts to improve hygiene practices among food vendors.

1.2 Aim and Objectives of the Study

1.2.1 Aim of the Study

This study aimed to determine the prevalence and antibiogram of Gram-negative bacteria isolates linked to ready-to-eat vegetables procured from unregulated street food vendors in Benin City, Nigeria.

1.2.2 Specific Objectives

The specific objectives of this study include the following;

i. determine the total bacterial, coliform, and *E. coli* counts in RTE vegetable salads and water samples used for the preparation of such salads.
ii. isolate and identify selected Gram-negative bacteria species from the samples using their cultural, morphological and biochemical characteristics.
iii. assess the antibiotic susceptibility profiles of selected bacteria isolates.
iv. confirm the identity of selected bacteria isolates using 16S rRNA sequence analysis.

CHAPTER TWO

LITERATURE REVIEW

Street-vended foods are an important component of urban food systems in several lower/middle-income countries (Madlala *et al*., 2019; Taye *et al.*, 2023). They nourish and sustain millions of Nigerians. But, most of these foods are prepared and sold in conditions that might promote microbial contamination. Ready-to-eat (RTE) vegetables which are not usually cooked before consumption, invoke specific public health risks of causing human disease when associated with pathogenic and multi drug resistant bacteria (Havelaar *et al*., 2010).

It reviews literature pertinent to the microbial risks posed by RTE vegetables, characteristics and resistance profiles of food borne Gram-negative pathogens, and considers the potential contribution of water to contamination in this chapter.

2.1 Ready-to-eat (RTE) Foods and Vegetables -Socio-Economic and Nutritional Importance

Ready-to-eat (RTE) foods are those defined as food which have been prepared for consumption without the need of additional cooking or other processing that would eliminate microbial hazards (FAO, 2004; FDA,2025). RTE items include pre-washed/packaged salads, fruit slices, raw vegetables between mixed salad and "street-salads", sandwiches etc., RTE vegetables are raw or cooled processed veggies for instant consumption (Mengistu *et al.*, 2022). Examples include leafy greens (lettuce, spinach), fruit vegetables (sliced tomatoes and cucumbers), root vegetable (carrots and cabbages) and mixed salad products. They are appreciated for their nutrients: vitamins A, C, K; minerals: Fe or K; dietary fibre; bioactive phytochemicals (e.g. antioxidants) (Slavin., 2012). RTE vegetables provide cheap nutrient specific sources of micronutrients to urban consumers, particularly critical in low-middle-income countries (LMICs), and contribute to food security (FAO and WHO, 2024). They are, however, eaten raw with no thermal kill step making safety down to hygiene at every stage from pre-harvest

(soil, dung and irrigation water), harvest(s) and post-harvest washings-, handling-, transport-, storage and display-conditions (FAO & WHO 2024).

2.2 Food Safety Regulation: Street Vending and Hygiene Practices in Nigeria

Essentially there are multiple agencies involved with most of the regulatory framework as it relates to food in Nigeria: National Agency for Food and Drug Administration and Control (NAFDAC), Standards Organisation of Nigeria (SON) amongst others some Nigerian centres exist such as the Nigeria Centre for Disease Control (NCDC) plus many other Ministries, Health and Agriculture. Such policies exist at the national level (National Policy on Food Safety and Implementation Strategy, 2014; National Policy on Food Hygiene and Safety; National Environmental (Sanitation and Waste Control) Regulations 2009), through Acts such as the National Agency for Food and Drug Administration and Control (NAFDAC) Act (1993, amended 2004), Standards Organisation of Nigeria [SON] Act,2015), Public Health Law Cap.164), Guidelines from relevant agencies like the National Primary Health Care Development Agency(NPHCDA)(Guidelines on Food Hygiene); or even Federal Ministry of Heath guidelinesonFood Vendorsand Handlers(the Terms food vendors and food handlers will be used interchangeably because they refer to persons such as street hawkers or stalls selling foodto passers by using their hands). These frameworks collectively set standards for food production, processing, storage and distribution of all food for human consumption or sale as well as the vending of foods while also outlining institutional roles for regulatory agencies, environmental health officers and local authorities to avert or contain public health risks relating to food. Still, the majority of RTE merchants do business in the informal sector and many work without a license and lack proper monitoring (Akinbule *et al.*, 2022).

Speciffically, Ibrahim and Adeola (2024) examined food safety awarness and practices among street food vendors using a survey of 316 vendors in their study entitled, Assessing Food Safety Awareness and Practices Among Street Food Vendors in Katsina State. In this regard, although

the awareness of general hygiene (wiping utensils or not working when sick) was relatively high, adherence to more essential practices such as use of gloves and cleanliness of vending sites were found to be low.

Vendors were also categorized as low risk (green), moderate risk (amber), and high risk based on hygiene compliance by the study. Though food safety was broadly understood, vendors were mostly categorized as moderate to high risk due to inadequate hygiene infrastructure and resources. The poor adherence to good practices in safe handling, water use and waste disposal showed increased risk of contamination identifying the need for vendor training, improved sanitation facilities, and enforcement of food safety regulations (Ibrahim and Adeola 2024).

The third study conducted at Federal University of Agriculture, Abeokuta evaluated the hygiene practices of street food vendors: reflections in terms of supply of water, waste disposal management, utensil cleaning and vendor Knowledge–Practice Alignment (Akinbule et al. 2022). This reveals a classic divide between regulatory requirements, vendor knowledge and practice (personal hygiene, environmental sanitation or utensil/food handling).

Overall, while there are numerous food safety policies in place, the oversight of street food is lacking. Although NAFDAC and state-level health authorities are overburdened with manpower, coordination and funding (Business Day 2025).

2.3 Global ramifications and Policy Defifi cits in Microbial Surveillance in Food

The global health ramifications of the dissemination of antibiotic-resistant Gram-negative bacteria within food and water reservoirs are far-reaching. Introduction Multidrug resistant Gram negative pathogens have been designated as critical research and intervention priorities based on their rapid dissemination capacity, high mortality association (WHO 2017). However, the lack of linkages among clinical surveillance and environmental health systems is common. In many low-income countries, systematic food and water monitoring programs are not in place or have poor outreach, which results in outbreaks unreported or unsourced from their

environmental origin establishment (Lopes *et al*., 2023). Additionally, informal food sectors and public utilities often function in poorly regulated environments making regulatory enforcement and public health response more difficult (Grace., 2015).

Risk reduction efforts include increasing availability of water treatment infrastructure, promoting training and tracking of street vendors, strengthening monitoring systems, and expanding the promotion of antibiotics fight in agriculture and human medicine (Aina *et al.*, 2024). While the burden of antimicrobial resistance is increasing in Nigeria, surveillance systems are weak (Igbinosa, 2024; Eze *et al*., 2025). The integration of clinical laboratories, environmental monitoring bodies, and food safety regulators is limited, hampering our ability to track and respond to outbreaks associated with environmental sources (NCDC 2024). This is compounded by underreporting of many community-acquired infections, partly due to the absence of systematic microbial testing in food and water sectors (Bain *et al.*,2012).

While Nigeria has made progress in antimicrobial surveillance, including its two surveillance systems—the Antimicrobial Resistance Surveillance System (AMRESS) through the Nigerian Centre for Disease Control (NCDC), and National Surveillance for Antimicrobials Used in Human Medicine system for national drug sale records held by the National Agency for Food and Drug Administration and Control (NAFDAC)—the high burden of AMR is still largely recognised due to capacity gaps in local government areas and informal sectors, especially given prior restricted implementation of concepts such as One Health Ghana NCDC1976202220. The One Health sector, which provides synergy between human health, environmental and animal health is evidently needed. Strengthening water treatment infrastructure, regulating food vending practices and raising public awareness will be key to slowing the spread of Gram-negative resistant bacteria through Nigerian communities (Alabi *et al.*, 2025).

2.4 Gram-Negative Bacteria and Their Public Health Importance

Gram-negative bacteria are a wide category of organisms with a unique double membrane structure that contains an outer membrane made of lipopolysaccharide (LPS). This structure often provides some protection against them environmental factors and some antimicrobials (Palusiak *et al.* 2022). Among these important genera (e.g. *Escherichia*, *Klebseilla*, *Proteus*, *Pseudomonas*, *Salmonella* and *Acinetobacter*) Common causes of community and nosocomial infections including, gastrointestinal disease (e.g., diarrhea and related symptoms), urinary tract infections, sepsis, and pneumonia are these bacteria (Palusiak *et al.* 2022). They pose a global threat of antibiotic resistance due to their capacity to obtain plasmid-mediated resistance genes including those coding extended spectrum β-lactamases, AmpC β-lactamases and lately carbapenemises (Maduakor, Ajiromanus and Onyemelukwe 2022; Igbinosa *et al.*, 2023). In addition, environmental reservoirs and transmission routes from agriculture to consumers (soil, water, manure and food chains) are responsible for the spread of Gram-negative bacteria (Igbinosa *et al.*, 2023).

2.5. Gram-Negative Bacteria in RTE Salads

Ready-to-eat (RTE) salads frequently harbour Gram-negative bacteria, such as *Escherichia coli*, *Klebsiella*, *Pseudomonas*, *Proteus* or *Salmonella* and other Enterobacteriaceae. Evidence has also shown that street food is an important source of food borne pathogens, particularly Gram negative bacteria, aided by the ubiquitous presence of unregulated vendors (especially in developing and transitional economies). In Dhaka, Bangladesh, a higher prevalence of MDR E. coli, Klebsiella spp., and Pseudomonas spp. has been identified. in street foods with different food items. These vendors work in unsanitary conditions: untreated water, no refrigeration or personal hygiene presence. Hence, contamination occurs during food processing, packaging or display (Islam *et al.*, 2019).

From irrigation water, human handling, utensils to food contact surfaces are sources of external contamination which RTE vegetables especially those that are eaten raw are highly susceptible (Barro *et al.*, 2006). In fact, systematic reviews have shown that in many different parts of the world RTE salads are frequently contaminated with Gram-negative bacteria including *E. coli*, *Klebsiella*, *Salmonella* etc all of who may carry antibiotic resistance genes (e.g.,ESBLs).

Abacha (traditional salad) sold by the RTE vendors in Enugu metropolis has been sampled recently, with 184 (≈36.8%) of 500 samples contaminated and indicated the production of 752 isolates from different species of bacteria (Nwosu *et al*., 2025). Preliminary study on microbiological quality of ready-to-eat African salad from supermarkets in Port Harcourt, Nigeria found that counts for RTE African salads exceeded acceptable limits while coliform and aerobic bacterial loads were extremely high showing poor sanitation and increased opportunity for exposure to pathogenic bacteria (Amadi and Nwankwo 2021). Fruits and vegetables surrounded with Gram negative bacterial contamination in a micro-bial data from Uselu market, Benin City isolates were often multidrug-resistant strains of bacteria (Omoruyi and Nosa-Cole., 2024). These observations show widespread contamination, not limited to RTE salads per se but existing across the supply chain.

In urban markets of Lagos, Ibadan and Abuja, total bacterial counts have 10–40 times greater than International Commission on Microbiological Specification for Foods (ICMSF) recommended levels while coliform counts reported so much higher than ICMSF level for fresh produce as well as reuse of wash water, lack of refrigeration and poor hygiene amongst vendors (Iwu *et al.,* 2020) further aggravates the situation.

2.6. Contamination of RTE Salads with Water

Gram-negative bacteria is an important source of exposure through unsafe public water supplies. While in numerous metropolitan territories, breaking water supply, matured pipelines and casualties of sub-extra facility framework were empowered by contamination (Zhang *et*

al., 2020). Bacteria can also survive and replicate in the biofilm developed in pipes or storage tanks even within chlorinated water systems (Levy *et al*., 2020).

Studies of environmental monitoring in South America and Southeast Asia have shown that antibiotic resistant Enterobacteriaceae, Acinetobacter spp., and Pseudomonas spp. are present. in drinking water samples (Zhou *et al*., 2020). The closest relative of these samples had been identified to sometimes also harplace resistance genes (bla_CTX-M, bla_NDM and mcr-1), making environmental reservoirs key nodes for the horizontal evolution of antibiotics resistance determinants(Arif *et al.*, 2020; Zhou *et al.*, 2020).

Background: Gram-negative bacteria are frequently recovered from public water supplies in Nigeria. Published data from Abuja showed the detection of high levels of bacteria and isolation of Enterobacteriaceae resistant to cephalosporins and tetracyclines in municipal tap water samples (Okonko *et al.*, 2008). Detection of MDR Pseudomonas spp. is also conducted in another study, which was carry out at Ogun State. and E. coli in sachet water which is a common replacement for tap water in cities (Igbeneghu and Abdu, 2014).

Klebisiella spp, Proteus spp, Citrobacter spp. are frequently detected in both vegetable and drinking water isolates (Okonko *et al.*, 2009; Odu and Essien, 2020). They may lead to urinary tract infections, gastroenteritis, septicaemia and meningit. The introduction of the pathogens into foods by this means was evident in street-vended foods because of cross-contamination resulting from water, poor handling and storage (Umeh *et al.*, 2022).

Evidence at the field scale in Germany was shown that the use of recycled water during irrigation meant significantly greater numbers and concentrations of resistance genes sul1 and integrons (intI1) were found to persist in underlying groundwater due their infiltration, teaching us that this is an additional reservoir and transmission pathway for antimicrobial resistance (Kampouris *et al.*, 2022). In line, over-arching environmental studies show that irrigation groundwater becomes increasingly polluted via human activities and thus resistant bacteria

(e.g. Escherichia coli) can survive there and later transfer to vegetables, especially those irrigated from borehole or shallow aquifers (Iwu, *et al.*, 2020). Similarly, a study conducted in China investigating the water > soil > plant continuum in agricultural ecosystems has shown that irrigation water including groundwater sources is capable of harbouring shared microbial communities and resistance determinants with vegetables as beta-lactam and carbapenem-resistant bacteria are found frequently in both irrigation water and fresh produce, suggesting direct pathways for microbial transfer (Soler *et al*., 2025)

2.7 Antibiotic Resistence from Food borne Bacteria

For example world over, several research indicate that food borne bacteria often harbour antibiotic-resistance determinants and demonstrate how ready-to-eat (RTE) foods and street foods could serve as vehicles for resistant strains . Recent literature and review articles describe increasing resistance among the more common Gram-negative food borne pathogens (such as Escherichia coli, Klebsiella, Salmonella and Pseudomonas), including opposition to third-generation cephalosporins and fluoroquinolones that compromise clinical treatment options for food borne sicknesses (Grudlewska-Buda *et al*., 2023; Ahmed *et al.*, 2024). Multilocus resistance profiles have been documented, as well as transferable resistance genes (e.g., ESBLs and plasmid-borne quinolone determinants), in retail and street-sold RTE products, indicating that contamination along the farm-to-fork chain (irrigation/wash water, processing, handling) can populate resistant bacteria into the food supply system through cross-contamination following postharvest activities (Grudlewska-Buda *et al.*, 2023; Fusaro *et al.* 2024). These findings also support WHO global warning about the potential role of AMR in food systems in dictating the wider AMR crisis and highlights the need for integrated across food, animal and human sectors (One health), treatment between processing and improved water/sanitation provision, and antibacterial agent interventions to steward appropriate use of antibiotics among agricultural medical (WHO, 2023).

Data show recurrent isolation of Enterobacteriaceae, including E. coli and Klebsiellaa with multi-drug resistance eliminating available therapeutic options (e.g., Onohuean, 2025), in Ready-To-Eat (RTE) and street-vended foods, Studies all scream the presence of multiple antibiotic resistant bacteria from RTEs. Country-level investigations (e.g., market and street food surveys, restaurant sampling) have determined contaminated wash water, lack of vendor hygiene & others' storage are major contributors to contamination and AMR spread (Chau *et al.,* 2017; Vestrheim *et al.,* 2015; Klištincová *et al.,* 2024; Fusaro *et al.,* 2024). The gaps in coordinated AMR surveillance in food revealed by these regional scoping reviews therefore highlight the need to strengthen One-Health surveillance, vendor training and access to safe water in food processing and vending environments. (WHO, 2023)

One major global concern is the wide-scale use of antibiotics in food production (e.g. poultry and meat) that contribute to the selection of resistant strains transmitted to the consumer through undercooked or raw foods (Marshall and Levy, 2011). In Nigeria, high levels of resistance to antibiotics, like ampicillin, streptomycin and tetracycline were reported among enteric bacteria isolated from foods (Ogunbanwo *et al.,* 2022). The presence of beta-lactam resistance through extended-spectrum beta lactamase (ESBL) production is well documented among strains of Klebsiella and Enterobacter isolated from environmental matrices such as vegetables and water sources (Iroha *et al*., 2015).

More than 40% of ready to eat street food in another study performed in Enugu State were positive for multidrug resistant (MDR) E. coli, in some instances also resistant to fluoroquinolones and aminoglycosides (Eze *et al.,* 2020). These findings corroborate earlier reports of the potential for food borne disease outbreaks associated with street-vended foods in Lagos and Kano (Islam *et al.,* 2019). This trend highlights the importance of ongoing antibiogram monitoring to guide treatment guidelines and ultimately, to mitigate difficult-to-treat infections outbreaks.

The difficulty with enforcement efforts is exacerbated by limited oversight from food safety regulatory agencies and the socioeconomic importance of street vending. Nevertheless, such risky transmission route of resistant pathogenic bacteria to the broader community via street food will remain ongoing at high end unless safeguards are exhibited. Genetic similarities have been shown between street food–isolated bacteria and those causing community-acquired infections, suggesting an environmental link for public health (Davis *et al*., 2015).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area

This study was conducted in Benin City, Edo State, Nigeria. The areas from which samples were collected included Agip junction, Adesuwa junction, Ring-road market, Akpakpava modern market, BIU cafeteria, 2nd Ugbor, Ugbor village market, Country home road junction and Benin Sapele Road by MRS Petrol Station, opposite Ecobank building, and Outside ShopRite mall; all within Benin City, Nigeria. The sample collection sites were located in the Benin Metropolis, a densely populated, urban area with high human and animal interactions and an extensive informal food vending sector. Ready to eat (RTE) vegetables, such as salads made with cabbage, carrot, and cucumber are frequently sold by street vendors, but little or no regulatory oversight exists.

3.2 Sample Collection

A total of thirty (30) RTE vegetable salads and five (5) samples of water were collected aseptically for six (6) weeks from June to July, 2024. All vegetable specimens were placed into sterile stomacher bags and all water samples (500 mL each) into sterile screw-cap bottles. All samples were labelled, put in ice-cooled boxes and transported to the Microbiology Laboratory of Benson Idahosa University, Benin City for immediate processing within 2 hours.

3.3 Isolation and Enumeration of Bacteria Species

Vegetable samples were homogenised using a sterile blender in a ratio of 25 g of vegetable to 225 mL sterile Buffered Peptone Water (SBPW). Homogenates were serially diluted (10^{-1} to 10^{-7}) for microbial analysis. The water samples were inoculated directly into the respective agar plates without serial dilution. (0.1 ml) aliquots of 10^{-5} - 10^{-7} dilutions of the RTE vegetable and the undiluted water samples were used to inoculate sterile plates of nutrient, McConkey and EMB agars for the enumeration of total heterotrophic bacterial, coliform and

E. coli counts respectively following the standard spread plate technique. The plates were incubated aerobically at 37°C for 24 - 48h, and the results were expressed as colony-forming units per gram (CFU/g) for vegetables and per ml (CFU/ml) for water samples. All colonies growing on the nutrient agar plates were counted as total heterotrophic bacteria. Only colonies producing pink coloration on McConkey agar plates were recorded as coliform, while colonies showing a green metallic sheen on EMB agar were counted as *E. coli* (Cheesbrough, 2010).

3.4 Isolation and Identification of Bacterial Isolates Using their Cultural, Morphological and Biochemical Characteristics

Pure colonies of interest were obtained from the various agar plates by repeated subculturing and subjected to standard cultural, morphological and biochemical tests according to descriptions of Holt *et al* (1994), Harley and Prescott (2002), and Cheesbrough (2010)

3.4.1 Gram Staining

Gram staining differentiates bacteria into Gram-positive and Gram-negative groups based on the thickness of the peptidoglycan layer in the cell wall. Gram-negative bacteria possess a thinner peptidoglycan layer and an outer lipopolysaccharide membrane, which prevents retention of the primary stain (crystal violet-iodine complex) after decolorization with alcohol. They counterstain red/pink with safranin (supply reference). To Gram stain, a smear of the test bacterial culture was made on a grease free glass slide, moistened with a drop of distilled water and heat fixed by passing over a flame. The heat fixed smear was flooded with crystal violet and allowed to stand for 1 min; it was washed off under a slow running tap water and immediately flooded with Lugol's Iodine (which served as a mordant), and held for 1 min. The iodine was washed off under slow running tap water. Ethanol (95% vol) was used to flood the stained smear and immediately washed off. Finally, Safranin was used to flood the smear and left for 60 secs before washing off under a slow running tap water. The slide was allowed to air dry in a slanted position. A drop of immersion oil was added to the stained smear and viewed

under a binocular compound light microscope using the ×100 magnification (Harley and Prescott, 2002). Gram-positive bacteria appeared purple due to retention of the crystal violet–iodine complex, while Gram-negative bacteria appeared pink after taking up the safranin counterstain (Cheesbrough, 2010).

3.4.2 Motility Test

The aim of this test was to ascertain the ability of bacterial isolates to move by means of propeller-like flagella or through the means of special fibrils that create a gliding motion (Tille *et al*., 2014). This procedure was conducted by preparing half strength nutrient agar medium. Six millilitre (6 ml) of the half strength nutrient agar was dispensed into test tubes, autoclaved at 121°C for 15 mins and allowed to gel. Using a flame sterilised straight needle, each test isolate was inoculated by stabbing to half the depth of the agar and the culture incubated at 37°C for 24 hrs. Growth away from the inoculation line was recorded as evidence of motility while non-motile strains were indicated by growth along the line of stab (Harley and Prescott, 2002).

3.4.3 Indole Test

This test evaluates the ability of bacteria to produce the enzyme tryptophanase, which hydrolyses tryptophan to indole, pyruvate, and ammonia (Cheesbrough, 2010. The indole test detects an organism's ability to produce indole from tryptophan via the enzyme tryptophanase. Sterile tryptone water broth was inoculated with the test organism and incubated at 37 °C for 24–48 hrs. After incubation, 0.5 mL of Kovac's reagent was added gently down the side of the test tube. The tube was shaken slightly and observed for color change. Formation of a red ring at the surface of the medium indicated an indole-positive reaction. No color change or a yellow ring indicated an indole-negative reaction (Cheesbrough, 2010).

3.4.4 Citrate Utilisation Test

This test determines the ability of an organism to use citrate as its sole carbon source and ammonium salts as the sole nitrogen source. The procedure involves, preparation of Simmon Citrate agar by boiling. Five millilitre (5ml) of the medium was dispensed into sterile test tubes and autoclaved at 121°C for 15 mins. The sterile medium was allowed to cool and solidify in a slant position. Simmons citrate agar slants were inoculated by streaking the surface with a light inoculum. The tubes were incubated at 37 °C for 24-48 hrs. A blue coloration of the medium indicated citrate utilization (positive result). No colour change (green) indicated a negative reaction (Cheesbrough, 2010).

3.4.5 Urease Test

The urease test detects the enzyme urease, which hydrolyses urea to ammonia and carbon (iv) oxide, raising the pH of the culture medium. Urea agar slants were prepared according to manufacturer's instructions and inoculated with the test organism by streaking onto the surface of the agar. The tubes were incubated at 37 °C for 18–24 hrs (Cheesbrough, 2010). A colour change from yellow-orange to pink-red after incubation indicated a positive test.

3.4.6 Triple Sugar Iron (TSI) Agar Test

This test differentiates Enterobacteriaceae based on carbohydrate fermentation (glucose, lactose, sucrose), gas production, and hydrogen sulphide (H_2S) production. The TSI test assesses bacterial ability to ferment glucose, lactose, and sucrose, as well as produce hydrogen sulphide (H_2S) and gas. TSI agar slants were prepared according to manufacturer's instructions and inoculated by stabbing the butt with a sterile needle and streaking the slant surface with the test organism. The tubes were incubated at 37 °C for 18-24 hrs. Yellow (acidic) slant and butt indicated fermentation of glucose, lactose, and/or sucrose. Red (alkaline) slant / yellow butt indicated fermentation of glucose only. Black precipitate indicated H_2S production while

gas bubbles or cracks in the agar indicated gas production. Red slant and butt indicated no sugar fermentation (Cheesbrough, 2010).

3.4.7 Oxidase Test

This test is used to demonstrate the production of the enzyme cytochrome C oxidase which is known to catalyse the transportation of electrons between electron donors present in bacteria and a redox dye, tetra methyl-p-phenylenediamine hydrochloride (oxidase reagent). A smear of 24 hr test isolate was made on Whatman No. 1 filter paper. This was flooded with 1% solution of tetramethyl-p-phenylenediamine hydrochloride. The formation of dark purple colour by the test isolate within 5 -10 secs. was indicative of a positive oxidase reaction. Negative oxidase reaction was indicated by no colour change (Harley and Prescott, 2002).

3.4.8 Catalase Test

The catalase test detects catalase . A clean glass slide was placed on a Petri dish. A drop of 3% hydrogen peroxide (H_2O_2) was added to the slide. Using a sterile glass rod, a small amount of the bacterial colony was transferred onto the drop. Immediate effervescence (bubbling) indicated a positive catalase reaction, due to oxygen release. No bubbling indicated a negative reaction (Cheesbrough, 2010).

3.5 Antibiotic Susceptibility Testing

Antibiotics susceptibility profile of the selected isolates was determined using the Kirby Bauer disk diffusion method on Mueller Hinton agar according to CLSI (2021) guidelines. The tested antibiotics included: ertapenem (10 µg), meropenem (10 µg), gentamicin (10 µg), cefotaxime (30 µg) and ceftazidime (30 µg). The inhibition zone diameters were measured in millimetres and interpretation as sensitive, intermediate or resistance according to the description of CLSI (2021). Multidrug resistance (MDR) was defined as resistance to ≥3 antibiotic classes (Umeh *et al.*, 2022). Multiple Antibiotic Resistance (MAR) indices were computed following Krumperman (1983) using the formula:

where:

- **a** = the number of antibiotics to which a particular isolate is resistant
- **b** = the total number of antibiotics tested against that isolate

3.6 Molecular Characterisation

3.6.1 DNA Extraction

Genomic DNA (g-DNA) extraction from bacterial isolates was carried out using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, USA; Catalogue No. D6005) following the manufacturer's protocol (Zymo Research, 2024). The kit utilizes both mechanical and chemical disruption to lyse cells and purify high-quality DNA suitable for molecular applications. Briefly, a loopful of freshly cultured bacterial colonies (18–24 hr old) was suspended in 200 µL of sterile phosphate-buffered saline (PBS) and transferred into a 2.0 mL BashingBead™ tube containing 750 µL of Lysis Solution. The mixture was vortexed at maximum speed for 5–10 mins to ensure effective cell disruption and then centrifuged at 10,000 × g for 1 min to pellet cell debris. About 400 µL of the supernatant was transferred into a new tube, mixed with 1,200 µL of Genomic Lysis Buffer, and loaded onto a Zymo-Spin™ IIIC Column fitted in a collection tube. The column was centrifuged at 10,000 × g for 1 min, and the flow-through was discarded. To remove residual contaminants, 200 µL of DNA Pre-Wash Buffer was added, followed by centrifugation for 1 min, and then 500 µL of g-DNA Wash Buffer was applied and centrifuged again. After discarding the flow-through, the column was centrifuged once more for 30 secs to eliminate wash buffer traces. DNA was eluted by adding 100 µL of DNA Elution Buffer directly to the column matrix, allowing it to stand for 1–2 mins , and centrifuging at 10,000 × g for 30 secs. The eluted DNA was collected in a clean micro-centrifuge tube and stored at −20 °C until further use. DNA purity and concentration were assessed using a NanoDrop spectrophotometer at A_{260}/A_{280}nm ratio, while integrity was verified by electrophoresis on a 1% agarose gel, where intact high-molecular-weight bands

indicated successful DNA extraction. All centrifugations were carried out at room temperature, and sterile, aerosol-resistant pipette tips were used throughout to avoid contamination. The entire procedure was performed under aseptic conditions within a Class II biosafety cabinet to ensure sample integrity and operator safety (Zymo Research, 2024).

3.6.2 PCR amplification and sequencing of 16S rRNA genes

The 16S rRNA target region was amplified using OneTaq® Quick-Load® 2× Master Mix (New England Biolabs, Catalogue No. M0486) with universal primers 27F- AGAGTTTGATCMTGGCTCAG and 1492R- TACGGYTACCTTGTTACGACTT . Each 25 µL reaction mixture contained 12.5 µL of the master mix, 0.5 µL each of forward and reverse primers (10 µM), 1–2 µL of template DNA (approximately 10–50 ng), and nuclease-free water to make up the final volume. Amplification was carried out in a thermocycler under the following conditions: initial denaturation at 95°C for 2 mins, followed by 30 cycles of denaturation at 95°C for 30 secs, annealing at 52°C for 30 secs, and extension at 68°C for 90 secs, with a final extension at 68°C for 5 mins and a hold at 4°C. The resulting amplicons were analyzed by agarose gel electrophoresis using 1.0% agarose in 1× TAE buffer, stained with ethidium bromide, and visualized under UV illumination. A 1 kb DNA ladder was included as a molecular size marker, and bands of approximately 1500 bp (for full-length 16S) were confirmed. PCR products showing single, specific bands were purified enzymatically using the ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fisher Scientific, 2020) by adding 2 µL of reagent to 5 µL of PCR product, incubating at 37°C for 15 mins to degrade residual primers and dNTPs, and subsequently inactivating the enzymes at 80°C for 15 mins (Werle *et al.*, 1994). The cleaned PCR products were then subjected to cycle sequencing in both forward and reverse directions using the Nimagen BrilliantDye™ Terminator Cycle Sequencing Kit v3.1 (Catalogue No. BRD3-100/1000). Each 10 µL sequencing reaction contained 0.5–1.0 µL of BrilliantDye Terminator mix, 2 µL of sequencing buffer, 1 µL of primer (3.2 µM), 1–3 µL

of purified PCR product, and nuclease-free water to the final volume. The cycle sequencing conditions included an initial denaturation at 96°C for 1 min, followed by 25 cycles of 96°C for 10 secs, 50°C for 5 secs, and 60°C for 4 mins, with a final hold at 4°C. Sequencing reaction products were purified using the ZR-96 DNA Sequencing Clean-up Kit™ (Zymo Research, Catalogue No. D4050) following the manufacturer's instructions, and the purified fragments were analyzed on the ABI 3500xl Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific). BioEditSequence Alignment Editor version 7.2.5 was used to analyse the. ab1 files generated by the ABI 3500 xL Genetic Analyzer and results were obtained by a BLAST search (NCBI).

3.6.3 Blast analysis

The blast analysis of the sequenced DNA was done on the National Centre for Biotechnology Information (NCBI) website (http:/blast.ncbLnlm.nih.gov/). DNA sequences of each of the selected test organisms were copied in fasta format into the nucleotide sequence search engine and used to query the NCBI data base in search of sequences producing significant alignments with a view to determining the best fit identity for each of the test organisms. The 16S rRNA partial sequence of the test isolates were submitted to GenBank (NCBI) with receipt corresponding GenBank accession numbers

3.7 Statistical Analysis

Statistical analysis (mean ± SD) was performed using Microsoft Excel and antibiotics resistance data were expressed as percentages.

CHAPTER FOUR

4.1 Total Bacterial Counts

Table 4.1 shows the bacterial counts for the RTE salad samples. The highest heterotrophic bacterial count was observed in Sample 1 (152 ± 82.1 10^6 CFU/ml) and the second lowest heterotrophic bacterial count was observed in Sample 22 (1.0 ± 1.0 $\times 10^6$ CFU/ml) whilst a total of 6 samples showed no growth recorded as 0. The table also shows the highest coliform count was observed in Sample 1 (192 ± 107.9 $\times 10^6$ CFU/ml) and the second lowest coliform count was observed in Sample 27 (4.0 ± 14.2 $\times 10^6$ CFU/ml) whilst sample 10 showed no growth recorded as 0. The highest *E. coli* count was observed in Sample 6 (38 ± 11.6 $\times$ 10^6 CFU/ml) and the second lowest *E. coli* count observed in Sample 23 (0 ± 14.5 $\times$ 10^6 CFU/ml) whilst a total of 17 samples recorded no growth.

4.2 Presumptive Identity of Isolates

Table 4.2 revealed the cultural, morphological and biochemical attributes of the presumptively identified bacterial isolates. The probable identities of the isolates include *Citrobacter* spp. (12), *Proteus* spp. (9) *Enterobacter* spp. (4)., *Escherichia coli* (3), *Providencia* spp. (1), *Alcaligenes* spp. (1), *Klebseilla* spp. (7), *Pseudomonas* spp. (1) and *Serratia* spp.(1).

4.3 PCR amplification and sequence analysis of 16S rRNA genes

Table 4.3 shows the identity of selected isolates using 16S rRNA sequencing analysis. The selected isolates were identified as *Klebsiella spallanzanii, Citrobacter freundii and Proteus mirabilis.*

Table 4.1: Total Bacterial Counts of RTE salad samples

Sample Code	**Total Heterotrophic Bacterial Count** (Mean ± SD × 10^6 CFU/ml)	**Total Coliform Count** (Mean ± SD × 10^6 CFU/ml)	**Total *E.coil* Count** (Mean ± SD × 10^6 CFU/ml)
1	152 ± 82.1	192 ± 107.9	0 ± 0
2	64 ± 39.4	58 ± 37.5	6.0 ± 1.5
3	52 ± 23.4	104 ± 87.4	0 ± 0
4	34 ± 60.1	13 ± 10.6	0 ± 0
5	30 ± 37.6	59 ± 25.5	21 ± 20.0
6	3.0 ± 8.7	9 ± 11.9	38 ± 11.6
7	0 ± 0	28 ± 1.5	5 ± 17.6
8	0 ± 0	5 ± 7.1	19 ± 6.7
9	48 ± 28.9	12 ± 7.1	0 ± 0
10	33 ± 11.0	0 ± 0	6.0 ± 2.6
11	26 ± 11.0	5 ± 2.6	19 ± 15.7
12	13 ± 24.8	14 ± 10.4	0 ± 0
13	37 ± 20.3	12 ± 26.4	0 ± 0
14	12 ± 12.3	19 ± 3.1	29 ± 7.5
15	8 ± 18.5	11 ± 7.2	8 ± 10.5
16	13 ± 18.2	12 ± 17.5	0 ± 0
17	45 ± 42.7	7 ± 8.1	0 ± 0
18	0 ± 0	12 ± 6.8	0 ± 0
19	0 ± 0	36 ± 32.1	0 ± 0
20	21 ± 44.2	54 ± 13.7	0 ± 0
21	71 ± 37.2	73 ± 35.2	0 ± 0
22	1.0 ± 1.0	89 ± 86.0	0 ± 0
23	0 ± 0	6.0 ± 11.4	0 ± 14.5
24	0 ± 0	0 ± 0	0 ± 0
25	25 ± 10.5	23 ± 17.0	0 ± 0
26	12 ± 5.3	42 ± 17.1	14 ± 1.5
27	7 ± 13.6	4.0 ± 14.2	8 ± 2.1
28	54 ± 18.5	11 ± 6.6	0 ± 0
29	98 ± 96.7	21 ± 41.6	9 ± 12.3
30	68 ± 29.1	21 ± 13.1	0 ± 0

Table 4.2. Cultural, morphological and biochemical characteristics of selected isolates from RTE vegetable salad and water samples

S/N(SC)	*C/M on MA*		*C/M on EMB*		*BIOCHEMICAL TESTS*											
	Colour	Size	Colour	Size	Catalase	Oxidase	Indole	TSI	Citrate	Urease	H2S	Motility	Gram reaction	Probable Identity	No. of Isolates	Percentage Frequency
1(F10)	Pink	Large	Black	Large	+VE	-VE	+VE	YYG	+VE	-VE	-VE	+VE	-VE	*E. coli*	3	7.7
2(F3)	Pink	Small	Black	Large	+VE	+VE	-VE	RR	+VE	-VE	-VE	+VE	-VE	*Pseudomonas* spp.	1	2.6
3(F27)	Amber	Small	Black	Small	+VE	+VE	-VE	RB	+VE	-VE	+VE	-VE	-VE	*Proteus* spp.	9	23.1
4(F8)	Pink	Small	Black	Small	+VE	-VE	+VE	YYG	+VE	-VE	-VE	-VE	-VE	*Klebsiella* spp.	7	17.9
5(F13)	Pink	Large	Black	Large	+VE	-VE	+VE	YB	+VE	-VE	+VE	+VE	-VE	*Citrobacter* spp.	12	30.8
6(F24)	Red	Small	Pink	Small	+VE	-VE	-VE	RYG	+VE	-VE	-VE	+VE	-VE	*Serratia* spp.	1	2.6
7(F29)	Pink	Small	Black	Small	+VE	-VE	-VE	YYG	+VE	-VE	-VE	+VE	-VE	*Enterobacter* spp.	4	10.3
8(W4)	Pale	Large	Amber	Small	+VE	+VE	-VE	RR	-VE	-VE	-VE	+VE	-VE	*Alcalígenes* spp.	1	2.6
9(W5)	Pale	Small	Pink	Large	+VE	-VE	+VE	RY	+VE	-VE	-VE	+VE	-VE	*Providencia* spp.	1	2.6

Key: S/N= Serial Number, SC= Sample Code (F= food[salad], W=water) MA= MacConkey Agar, EMB= Eosin Methylene Blue agar, -VE= Negative, +VE= Positive, (TSI reading in order; slope, butt, gas production), spp.= species, C/M = Cultural/Morphological, YYG = Yellow, Yellow, Gas

RR = Red,Red RB = Red, Black RYG = Red, Yellow, Gas YB = Yellow, Black RY = Red, Yellow

Table 4.3. Identity of selected isolates after 16S rRNA sequence analysis

S/N	SAMPLE CODE	PERCENTAGE ID (%)	ORGANISM NAME	GENBANK ACCESSION NUMBER	E-VALUE
1	8	98.98	*Klebsiella spallanzanii*	CP125310.1	0.0
2	13	99.32	*Citrobacter freundii*	CP125310.1	0.0
3	27	97.50	*Proteus mirabilis*	CP045538.2	0.0

4.4 Antibiotic Susceptibility Test

Table 4.4 shows the antibiotics susceptibility profiles of the selected isolates. The isolates were generally resistant to cefotaxime (*Citrobacter* spp. (83.4%), *Proteus* spp. (100%), *Klebsiella* spp. (100%), *Enterobacter* spp. (50%), *Escherichia* spp. (66.7%), *Serretia* sp. (100%), *Providencia* sp. (100%) and *Alcaligenes* sp. (100%); and ertapenem (*Citrobacter* spp. (58.4%), *Proteus* spp. (100%), *Klebsiella* spp. (85.7%), *Enterobacter* spp. (50%), *Escherichia* spp. (66.7%), *Serretia* sp. (100%), and *Alcaligenes* sp. (100%). *Proteus* spp. was resistant to ceftazidime (55.6%) and meropenem (55.6%) while *Alcaligenes* sp. demonstrated complete resistance (100%) to ceftazidime and meropenem. *Pseudomonas* sp. was sensitive to all the tested antibiotics. Overall, gentamicin was observed to be the most effective antibiotics demonstrating high sensitivity ranging from 50% to 100% against the test isolates. MAR index results varied largely amongst the 39 isolates ranging from 0 to 1. MAR index of 0 was recorded in 4 isolates (C10,C12, ET3 and PD1) and MAR index of 1 was seen in 6 isolates (C1,C2,C4,C9, PT8 and EC2).

Table 4.4. Antibiotic susceptibility profile of the selected bacteria isolates

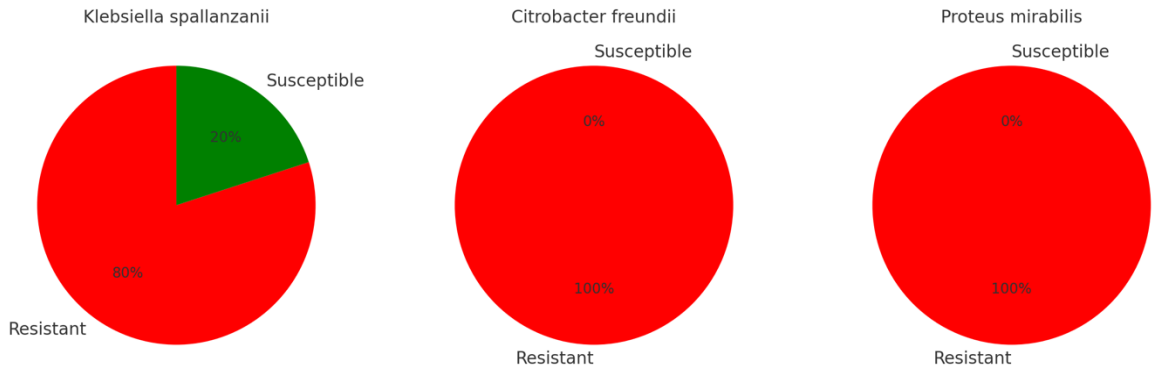
Antibiotics	**Interpretive category**	***Citrobacter* spp. *n=12 (%)***	***Proteus* spp. *n=9(%)***	***Klebsiella* spp. *n=7(%)***	***Enterobacter* spp. *n=4(%)***	***Escherichia* spp. *n= 3(%)***	***Serratia* sp. *n=1 (%)***	***Providencia* sp. n= 1(%)**	***Alcaligenes* spp. n=1(%)**	***Pseudomonas* sp. n=1(%)**
Gentamicin	Sensitive	8 (66.7)	6 (66.7)	4 (57.1)	2 (50)	2 (66.7)	1 (100)	1 (100)	1 (100)	1 (100)
	Intermediate	0 (0)	1 (11.1)	2 (28.6)	2 (50)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Resistant	4 (33.3)	2 (22.2)	1 (14.3)	0 (0)	1 (33.3)	0 (0)	0 (0)	0 (0)	0 (0)
Ceftazidime	Sensitive	2 (16.6)	1 (11.1)	0 (0)	1 (25)	2 (66.7)	0 (0)	1 (100)	0 (0)	0 (0)
	Intermediate	5 (41.7)	3 (33.3)	1 (14.3)	2 (50)	0 (0)	1 (100)	0 (0)	0 (0)	1 (100)
	Resistant	5 (41.7)	5 (55.6)	6 (85.7)	1 (25)	1 (33.3)	0 (0)	0 (0)	1 (100)	0 (0)
Cefotaxime	Sensitive	2 (16.6)	0 (0)	0 (0)	2 (50)	1 (33.3)	0 (0)	0 (0)	0 (0)	0 (0)
	Intermediate	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)
	Resistant	10(83.4)	9 (100)	7 (100)	2 (50)	2 (66.7)	1 (100)	1 (100)	1 (100)	0 (0)
Meropenem	Sensitive	4 (33.3)	2 (22.2)	3 (42.85)	3 (75)	2 (66.7)	1 (100)	1 (100)	0 (0)	0 (0)
	Intermediate	3 (25)	2 (22.2)	1 (14.3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)
	Resistant	5 (41.7)	5 (55.6)	3 (42.85)	1 (25)	1 (33.3)	0 (0)	0 (0)	1 (100)	0 (0)
Ertapenem	Sensitive	2 (16.6)	0 (0)	0 (0)	1 (25)	1 (33.3)	0 (0)	1 (100)	0 (0)	0 (0)
	Intermediate	3 (25)	0 (0)	1 (14.3)	1 (25)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)
	Resistant	7 (58.4)	9 (100)	6 (85.7)	2 (50)	2 (66.7)	1 (100)	0 (0)	1 (100)	0 (0)
MAR index		0.52	0.67	0.66	0.30	0.46	0.40	0.20	0.80	0.00

Table 4.5: Multiple Antibiotic Resistance (MAR) Index of bacteria isolates

Isolate Code	No. of Antibiotics Resisted(a)	Total No. Of Antibiotics Tested (b)	MAR Index: a/b
C1	5	5	1
C2	5	5	1
C3	1	5	0.2
C4	5	5	1
C5	2	5	0.4
C6	2	5	0.4
C7	2	5	0.4
C8	1	5	0.2
C9	5	5	1
C10	0	5	0
C11	1	5	0.2
C12	0	5	0
PT1	4	5	0.8
PT2	3	5	0.6
PT3	2	5	0.4
PT4	2	5	0.4
PT5	2	5	0.4
PT6	2	5	0.4
PT7	4	5	0.8
PT8	5	5	1
PT9	4	5	0.8
K1	3	5	0.6
K2	2	5	0.4
K3	4	5	0.8
K4	3	5	0.6
K5	3	5	0.6
K6	3	5	0.6
K7	3	5	0.6
ET1	2	5	0.4
ET2	1	5	0.2
ET3	0	5	0
EC1	4	5	0.8
EC2	5	5	1
EC3	4	5	0.8
S1	4	5	0.8
PD1	0	5	0
A1	4	5	0.8
PA1	2	5	0.4

Key: C= *Citrobacter* spp; PT=*Proteus* spp; K=*Klebseilla* spp; ET=*Enterobacter* spp; EC= *E. coli*; PD= *Providencia* spp; A=*Alcaligenes*, PA=*Pseudomonas* spp

Plate 4.1 Antibiotic Resistance Pattern of 16s rRNA sequence analysis Identified Organisms



Key: Red (Resistant); Green (Susceptible);

CHAPTER FIVE

DISCUSSION AND CONCLUSION

5.1 Discussion

The investigation of multidrug-resistant Gram-negative bacteria associated with ready to eat vegetables was studied in one of the Nigeria states (Benin City). The mean total heterotrophic bacterial counts ranged from 0.0 to 152 ± 82.1 × 10^6 CFU/ml, while the total coliform counts varied between 0.0 and 192 ± 107.9 ×10^6 CFU/ml, and *E. coli* counts was from 0.0 to 38 ± 11.6 × 10^6 CFU/ml (Table 4.1). The observations were within the range of values reported by Feroz *et al*. (2016) in their study on vegetables in Bangladesh and that reported by Amoah *et al*. (2006) in Ghana. The high mean bacterial load may suggest improper handling of vegetables from harvest through transportation and handling within the market space (Mritunjay and Kumar, 2017). The presence of *E. coli* suggests the possibility of fecal contamination in samples (Mensah and Englund 2025).

Citrobacter spp. (12), *Proteus* spp. (9) *Enterobacter* spp. (4), *Escherichia coli* (3), *Providencia* spp. (1), *Alcaligenes* spp. (1), *Klebseilla* spp. (7), *Pseudomonas* spp. (1) and *Serratia* spp.(1) were all isolated in this study. In agreement with the observation of this study, Adekanmbi and Falodun (2015) reported the isolation of *Klebsiella pneumoniae* in Nigerian street-vended lettuce and cabbage.

Kalpana *et al.* (2024) in a comprehensive review of produce and salads in Nigeria reported the isolation *E. coli*, *Klebsiella* spp. and *Pseudomonas* species from fresh vegetables and RTE salads. Okafor-Elewon and Imade (2020) asserted that vegetables frequently harbor potentially pathogenic bacteria when good hygienic practices are not upheld during preparation). *Escherichia coli and Klebsiella* spp., are known opportunistic pathogens implicated in pneumonia, urinary tract infections, and bloodstream infections, particularly in

immunocompromised individuals (Pitout *et al*., 2015). Their presence in ready-to-eat food therefore raises serious public health concerns.

Proteus spp, particularly *Proteus mirabilis*, were also frequently detected in this study. Known for their strong urease activity, *Proteus* species have been implicated in urinary tract infections, septicaemia, and wound infections (O'Hara *et al*., 2000). Their presence in vegetables underscores fecal contamination, possibly linked to the use of untreated wastewater for irrigation or unhygienic handling during food preparation (supply reference). Similar patterns have been reported in Ghana, where Addo-Tham *et al*. (2020) found *Proteus* among bacteria contaminating street-vended salads.

Citrobacter spp. were also recovered from samples, corroborating findings by Barro *et al*. (2006) in Burkina Faso, where *Citrobacter freundii* was prevalent in lettuce salads. Though often considered opportunistic, *Citrobacter* can cause neonatal meningitis, septicaemia, and gastroenteritis (Liu *et al.*, 2018). Their recovery highlights poor sanitary conditions and cross-contamination pathways from soil, water, or handlers' hands (Oluwafemi *et al*., 2013; Nwankwo *et al*., 2015)

The recovery of *Serratia marcescens* aligns with a study by Okafor *et al.* (2020) on RTE vegetable salads sold by restaurants in Nigeria which documented the isolation of *Proteus vulgaris, Citrobacter freundii,* and *Serratia marcescens* among the multidrug-resistant isolates recovered from salads.

Water, especially when untreated or contaminated with animal or human waste is a known reservoir for the aforementioned bacteria spp. The presence in both vegetable and water samples in this study suggests that the water used in preparing the RTE vegetable salad could be a source of contamination of the food product. Post-harvest handling by vendors and or intermediaries, have been reported to be important factors in the bacterial contamination of vegetables (supply reference). The findings echo calls in the literature to consider fresh produce

and its agricultural environment as part of a "One Health" contamination network connecting environment, food and human health (Pintor-Cora *et al.,* 2023, Kläui *et al*., 2024)

The detection of multidrug resistant isolates resistant to gentamicin (an aminoglycoside); cefotaxime, ceftazidime (third-generation cephalosporins); and the carbapenems (meropenem, ertapenem) in this study is deeply concerning. Aligning with this study, a 2024 Swiss study demonstrated that fresh produce harbors cephalosporin resistant Enterobacteriacea and in contrast to this study carbapenem resistant *Pseudomonas aeruginosa*, with 95% of samples carrying at least one antimicrobial resistance gene (ARG) (Kläui *et al.,* 2024). The observation suggests that produce may serve as a reservoir of antimicrobial resistant bacteria that could disseminate resistance through the human food chain (Pintor-Cora *et al.,* 2021).

Additionally, a 2023-2024 study from Nigeria (Edo State) recovered ESBL-producing *E. coli* from vegetables and open-market salads; 85.9% of isolates were resistant to ≥ 3 antibiotic classes, confirming multidrug resistance in produce-associated strains in a context similar this study where *E. coli* demonstrated an average 66.7% resistance to carbapenems tested (Igbinosa *et al.,* 2023). These findings show that antimicrobial resistant bacteria on vegetables is a real and present threat and not just a theoretical concern.

From a public-health perspective, consumption of RTE vegetables contaminated with MDR bacteria could lead to hard to treat infections especially in vulnerable populations (children and immunocompromised individuals). Furthermore, such produce may act as a vector for horizontal gene transfer of antimicrobial resistant genes to the human gut flora or pathogens, promoting the spread of resistance at the community level, consistent with "One Health" risk models (environment ↔ food ↔ human) described in recent literature (Rahman *et* al., 2021, Pinta Cora *et al.,* 2023).

Resistance to carbapenems was common, paralleling findings by Iwu *et al*. (2020) in Nigerian fresh produce. Such resistance patterns may reflect indiscriminate antibiotic use in agriculture,

poultry, and human medicine, leading to the spread of resistant strains into the food chain (Van Boeckel *et al*., 2019). This is particularly worrisome since MDR Gram-negative bacteria represent a critical threat group according to WHO's priority pathogen list (Tacconelli *et al*., 2018).

Accordingly, most isolates had MAR(multiple antibiotic resistance) index greater than 0.2 indicating significant exposure to antimicrobial agents, such high range in this study is seen across *Klesbseilla, Citrobacter, Proteus, Enterobacter, Echerichia, Alcaligenes and Pseudomonas* species . These findings align with a study conducted in Enugu, Nigeria with similar isolate profiles recording MAR indexes of 0.5 to 1. (Afunwa *et al.* 2020). These MAR index figures also invites curiosity into the vegetable farming pathways and choice of manure/organic fertiliser used by farmers.

From a public health perspective, the presence of potentially pathogenic and multidrug resistant Gram-negative bacteria in unregulated ready to eat salads represents a significant risk for foodborne illness outbreaks in Benin City and beyond. This aligns with WHO estimates that foodborne diseases cause over 91 million illnesses and 137,000 deaths annually in Africa (WHO, 2015). Street vended foods, while economically important, therefore pose a dual challenge of livelihood sustenance and health risk management. The presence of multidrug-resistant bacteria in food destined for immediate consumption is particularly concerning, as it not only increases the risk of infections but also limits available therapeutic options

5.2 Conclusion

This study demonstrated a high prevalence of Gram-negative bacterial contaminants particularly *Klebsiella, Proteus,* and *Citrobacter* in ready to eat salads sold by street food vendors in Benin City, Nigeria. The isolates demonstrated high resistance to Cefotaxime and Ertapenem and to a lesser degree to Meropenem, Ceftazidime and Gentamicin respectively. The MAR indeces of the isolates ranged from 0.0 to 1.0 (with over 89% having index of 0.2 or

above) suggesting sources of contamination where antibiotics are often over used or abused. The detection of these potentially pathogenic organisms, coupled with their multidrug resistance profiles, underscores the urgent need for improved food safety practices and stricter regulatory oversight of informal food vending in Nigeria. The presence of multidrug-resistant bacteria in food destined for immediate consumption is particularly concerning, as it not only increases the risk of infections but also limits available therapeutic options. . It underscores the urgent need for public health interventions, including routine microbial monitoring of ready-to-eat vegetables , enforcement of hygienic food handling standards, and targeted health education campaigns for street food vendors and consumers.

5.3 Contribution to Knowledge

The study highlights the critical role of street food as a potential avenue for the dissemination of multidrug resistant Gram-negative bacteria within the Benin Metropolis of Nigeria. The baseline information generated in this study will be very helpful to researchers, clinicians and public health practitioners and stakeholders to formulate and implement policies aimed at mitigating the role of RTE vegetables in the public health of Nigerians. For further research, a larger sample size taking into account seasonal variations, virulence factors and integration with agricultural/environmental data should be considered

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APPENDIX

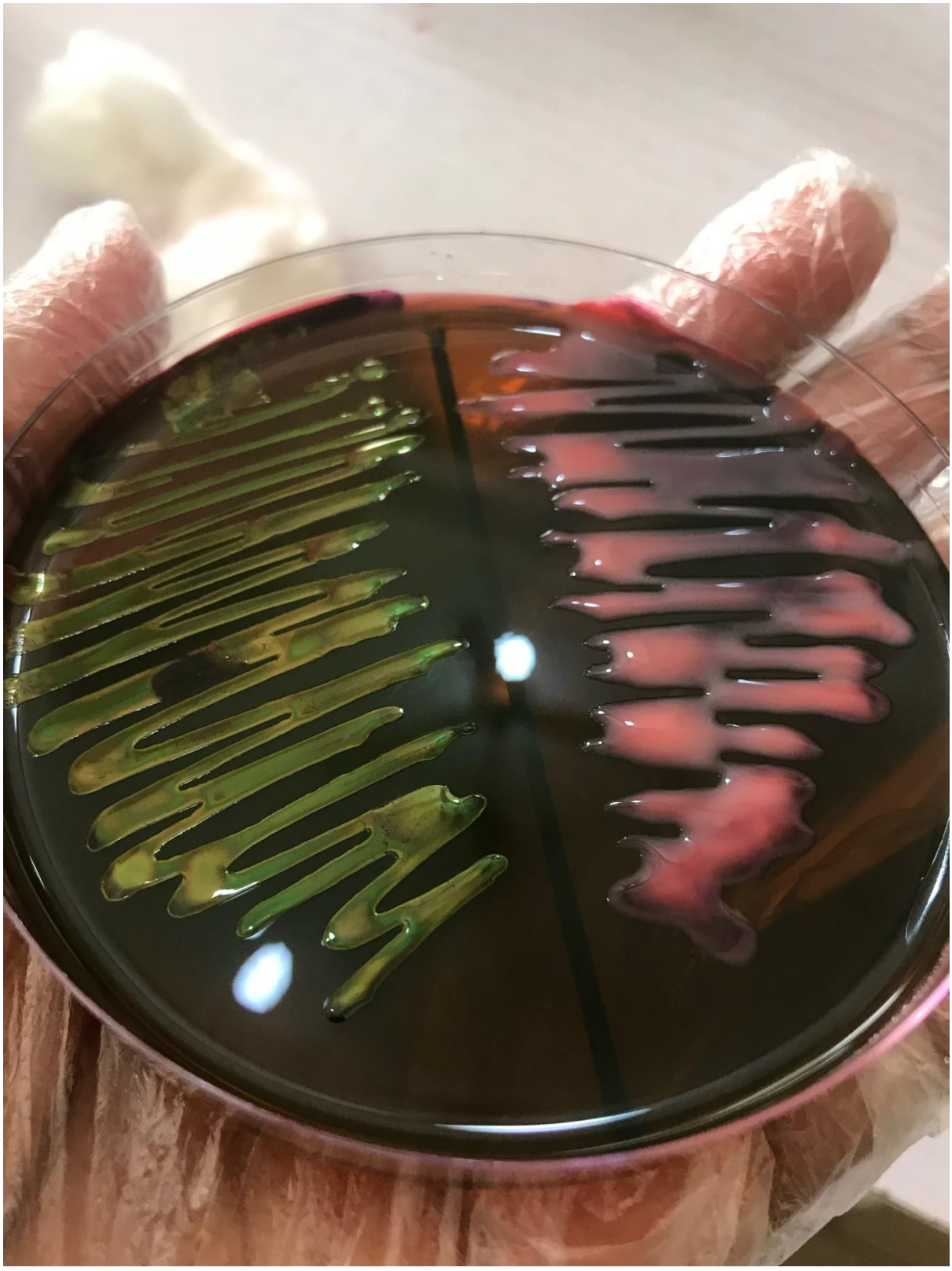


Plate 1: Bacterial Isolates on Eosin Methylene Blue Agar



Plate 2: Antibiotic susceptibility test plates using the Kirby Bauer disk diffusion method on Muller Hilton Agar



Plate 3: Motility test tubes

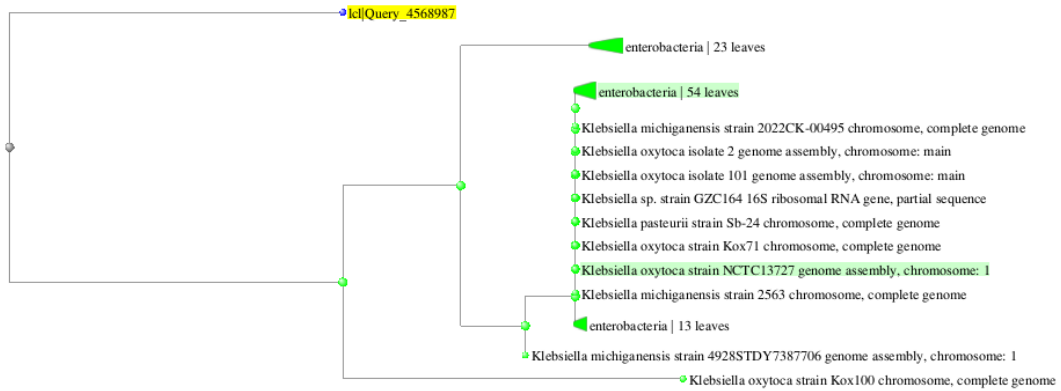


Fig 4.1: Phylogenetic tree showing sample 8 in comparison with isolates in the NCBI database

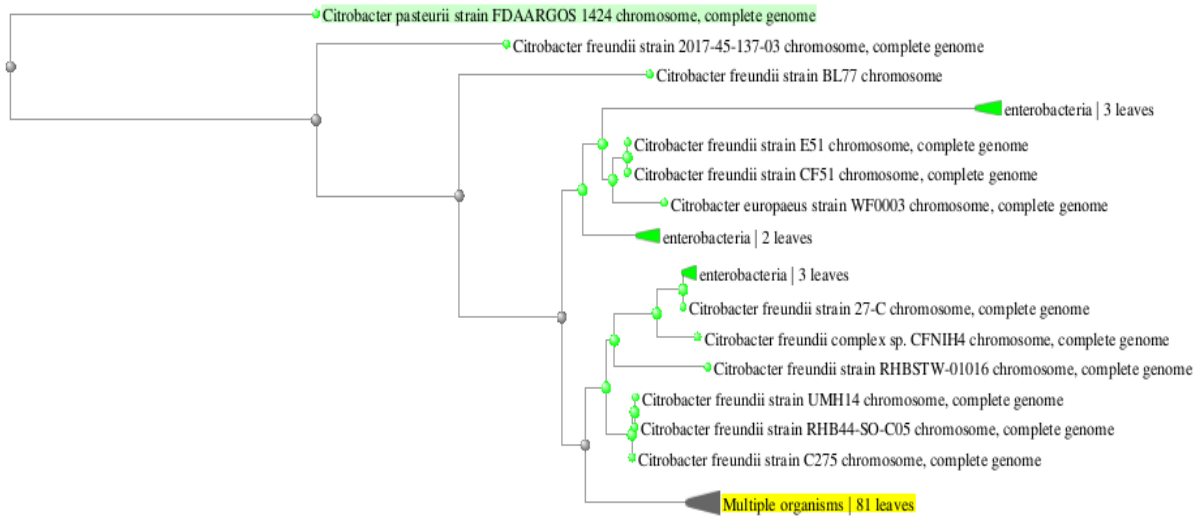


Fig 4.2: Phylogenetic tree showing sample 13 in comparison with isolates in the NCBI database

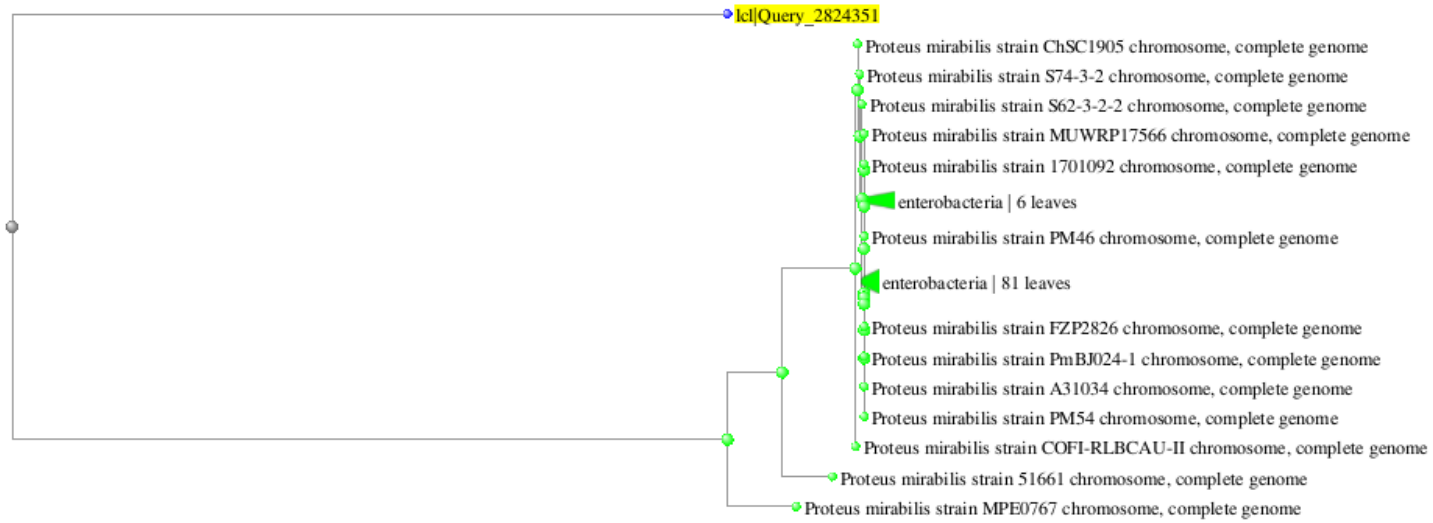


Fig 4.3: Phylogenetic tree showing sample 27 in comparison with isolates in the NCBI da