PREVALENCE OF *SALMONELLA SPP* AND *ESCHERICHIA COLI* IN FRESH-CUT FRUITS SOLD IN THE OPEN MARKET IN OFADA MOKOLOKI LCDA, OGUN STATE.

BY

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CERTIFICATION

This is to certify that this project was carried out by **ANYASI JOY AWURIN** with matriculation number **15010101003** of the Department of Biological Sciences, College of Basic and Applied Sciences in Mountain Top University, under the supervision of **DR. O. E. FAYEMI**.

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DEDICATION

I dedicate this project report to God Almighty for His grace and guidance.

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First and foremost, I express my gratefulness to the supreme power which God bestowed upon me to carry out this project and submit this report.

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ABSTRACT

Fruits form an essential part of human diet as they are a major source of dietary nutrients of great importance. Consumers demand for fresh, safe, nutritious and inexpensive products. Many seek the opportunity of buying ready to eat fruits and vegetables. Consumption of fresh cut fruits from the local marketplace poses a potential risk of foodborne infection due to microbial contamination. Pathogens may contaminate the fruits during washing, peeling, slicing, trimming, packaging and handling. This study aimed at detecting the presence of *E. coli* and *Salmonella* in the fresh cut fruits sold in the market area in Ofada/Mokoloki Local Council Development Area (LCDA), Ogun State, Nigeria. Samples were randomly collected from the open market area. Subsequently, the samples were screened for Salmonella spp and pathogenic *E. coli* including Shiga toxin producing *E. coli* (STEC) using selective agar media and enrichment procedures. isolated pathogens were subjected to 16S PCR based protocols to detect the presence suspected *Salmonella* spp and STEC. The occurrence of *E. coli* and *Salmonella* contamination in the fresh cut fruits sold in the market area in Ofada/Mokoloki Local Council Development Area (LCDA), highlights the potential health risk of the consumers. There is a need for community awareness programs advocating adoption of good hygiene-based handing and market procedures to prevent any major outbreak associated with contaminated fresh cut fruits.

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CHAPTER ONE

1.0 INTRODUCTION

Fruit is an important component of human diet because it is a significant source of nutritional nutrients. Many chronic diseases, including cancers and cardiovascular diseases, have been countered by fruit consumption (Chukwu et al., 2010). Consumers in developing countries have become more interested about the dietary and sensory elements and the security of the fruits they consume as a result of increasing health awareness (da Costa, Deliza, Rosenthal, Hedderly, 2000). Concurrently, consumers are growing demand for comfort products, and so is the demand for fresh cut fruits. Fresh cut fruits are any fresh fruit that has been physically changed from its initial form but stays in a new state as described by the International Fresh-Cut Produce Association (Garrett et al., 2003). Consumers demand fresh, secure, nutritious and cheap products and many of them pursue the chance to save time by buying Ready-To-Eat fruits and vegetables. While the health and nutritional value of these products are significant characteristics appreciated by customers, comfort and speed are the most significant motive for buying fresh-cut fruits or vegetables. (Devlieghere, Ragaert, Verbeke, & Debevere, 2004).

In many countries, fruits were associated with foodborne disease outbreaks (Chukwu et al, 2010, CDC, 2009). There is a diversity of microorganisms responsible for foodborne diseases, which includes bacteria, viruses, fungi and parasites (Chukwu et al, 2010). Several outbreaks have been involved with fresh cut and whole melons in several countries (Hanning et al., 2009, Lynch et al., 2009). Brazilian watermelon was involved in a multi-country outbreak of *Salmonella* infection in Europe in late 2011-early 2012, including 63 confirmed food poisoning cases (Byrne et al., 2014). *Salmonella spp* outbreaks occurred frequently in both Korea (20.7%) and Japan (14.2%), enteric serovars of *Salmonella Typhimurium* and *Salmonella* enterica serovars enteriditis are the world's most occurring isolated serovars from foodborne outbreaks, with 1,8 million individuals dying from diarrhoeal diseases in 2005 alone (Velusamy et al., 2010).

Fresh products were not regarded an important food matrix for the transmission of *Escherichia. coli* O157:H7 until the mid-1990s, since then, however, a number of important *E. coli* O157:H7 outbreaks have occurred in fresh-cut fruits or vegetables. Enterohemorrhagic *E. coli* infections in the United States of America cost about $1 billion annually (Boyacioglu et al., 2013). The consumption of Ready-To-Eat (RTE) fruits in Nigeria, has risen owing to the fact that they are

readily available, convenient and cheaper than whole fruits, this rise in consumption has resulted to the occurrence of disease risk in which customers can be exposed and this is of huge concern (Oranusi and Olorunfemi 2011). The quality and source of fresh-cut fruits or vegetables washwater impacts the fruit's microbiological quality, waste water has been reported to contaminate fruits with pathogenic microorganisms (Kumar, 2012).

Fields on which livestock or wild animals have grazed are more probable to be contaminated by enteric pathogens, some bacteria can survive for many months in agricultural soils, for instance, *Salmonella* could survive for months in sewage sludge applied as manure to agricultural soils. These pathogens may contaminate farm produce from such contaminated farmland.

In addition, during washing, peeling, slicing, trimming, packaging and handling, pathogens may contaminate fruits. Most importantly, during the process of processing and exposure to the atmosphere, fresh cut fruits or vegetables are mostly contaminated. Cross contamination may occur when clean and unclean fruits or vegetables are mixed together, eventually pathogenic and enteric microbes such as *Salmonella* and pathogenic *E. coli* from infested ones can spread extensively (Oranusi and Olorunfemi, 2011). In the method of processing and exposure to the atmosphere, fresh cut fruits are mostly contaminated (Chukwu et al, 2010, CDC, 2009). Cross contamination happens when there is a mixture of clean and unclean fruits, ultimately pathogenic and enteric microbes, i.e from infested microbes from *Salmonella* and *E. coli* can spread extensively.

This project will deal largely on determining the occurrence and prevalence of *Salmonella* spp and pathogenic *E. coli* in freshly cut fruits (these are fruits which have been cut, sliced and washed) that is being sold at the market area in Ofada/Mokoloki Local Council Development Area (LCDA), Ogun State, Nigeria.

1.1 PROBLEM STATEMENT

Fresh cut fruits are contaminated during the process of handling and exposure to atmospheric microorganisms. Clean and unclean ones are mixed together, leading to eventual cross contamination by pathogenic and enteric microorganisms such as *Salmonella* and pathogenic *E. coli*. Humans are being infected through the consumption of contaminated fresh cut fruits.

1.2 AIMS AND OBJECTIVES TO THE STUDY

This study aimed at the following:

- To determine the occurrence of *Salmonella species* and *E. coli* in street vended freshly cut fruits which is commonly hazardous to public health.
- To Isolate and identify the various species of *Salmonella* and *E. coli* in fresh cut fruits.

CHAPTER TWO

2.0 LITERATURE REVIEW

Around 140 million tonnes of over 3000 tropical fruit kinds are produced annually worldwide (Strawn et al., 2011). China is one of the biggest producing countries of tropical fruit and also the world's biggest importer (FAO, 2009). Banana, mango, pineapple, papaya and dragon fruit are the favourite tropical fruits, often consumed in whole forms, freshly cut and as juice on China's and other developing countries. There are no consistent food security standards in production and post-harvest practices in most countries producing tropical fruits, and there are no outbreak surveillance systems and pathogen detection capabilities, or are very poor; The security of these fruits remains a concern (Strawn et al., 2011).

There have been outbreaks of foodborne disease due to the intake of multiple tropical fruits, including mango, papaya, pineapple, coconut, banana, chicken fruit, avocado and mamey (CDC, 2005; Pui et al., 2010; Strawn et al., 2011). The major bacterial pathogen is *Salmonella* (Strawn et al., 2011). Multiple *Salmonella* serovars have caused foodborne disease from tropical fruits such as Saintpaul, Heidelberg, Litchfield, Typhi, Newport, Paratyphi, Senftenburg (Beatty et al., 2004; Gibbs et al., 2009). *Salmonella* is liable for recorded foodborne disease outbreaks in China and other developing countries as the top bacterial pathogen (NHFPC, 2014). Tropical fruits comprise large amounts of sugar and elevated water activity, providing suitable conditions for microbial growth and survival requirements (Bassett and McClure, 2008). Previous studies have shown that *Salmonella* and *E. coli* O157:H7 can survive or develop on pulp, juice and freshly cut surfacesof tropical fruits such as mango, pineapple, papaya, guava, coconut, avocado, dragon fruit kept at temperatures between 4 ºC and 37 ºC or even at cold temperatures (-20 ºC). (Mutaku *et al.,* 2005; Walter *et al.*, 2009; Strawn and Danyluk, 2010a; El-Safey, 2013; Sim *et al*.,2013; Penteado *et al.*,2014).

Fresh-cut fruits are often exhibited and marketed without refrigeration and adequate packaging in many developing countries in hot and humid environments in Asia and Africa (James and Ngarmsak, 2010). *Salmonella* outbreaks may be more likely during the summer season as the bacteria may survive or develop on contaminated fresh cut fruits or vegetables due to temperature changes during refrigerations (C.Ma et al., 2015).

2.1 Salmonella

Salmonella spp are Gram-negative, facultatively anaerobic and rod-shaped bacteria, with more than 2500 *Salmonella* serovars with typhoid fever by *Salmonella Typhi* and gastroenteritis *Salmonella Typhimurium* being the most prevalent serovars in public health (Park et al., 2009). *Salmonella Typhi* in humans causes enteric fever while *Salmonella Typhimurium* in mice causes systemic disease that strongly resembles typhoid fever in humans (Lin et al., 2007). There are two species of *Salmonella*, bongori and enterica. Both species carry pathogenicity island (SPI)-1 (SPI-1), a separate chromosomal operon which encodes a mechanism of type three (T3SS-1) secretion. All pathogenic *Salmonella* belongs to the enterica species and carries an extra pathogenicity island called SPI-2, encoding a unique T3SS (T3SS-2). Both T3SSs have a role to play in pathogenesis interactions with the host (Tunung et al., 2006). About 40,000 incidence of salmonellosis are recorded annually in the United States, resulting in about 600 fatalities worldwide (Albuferaet al., 2009). Due to the lack of detailed epidemiological studies by the public health and veterinary sector, it is not easy to assess the status of salmonellosis in Malaysia, just as in Africa, however, in 2005, 171 salmonellosis patients with two deaths were reported due to the intake of Ready-To-Eat foods in Kelatan (Tunung et al., 2006). The monitoring program by Ministry of Health Malaysia (Communicable Disease Control Section 2008) indicated that the occurrence of typhoid fever in Malaysia from 1996-2006 was in the range of 0.71-4.50 per 100,000 populations.

Recently, there is a rapid increase in the consumption of fresh produce worldwide due to their nutritional benefits to humans and changes in diet, the reported cases of foodborne outbreaks associated with fresh produce have also increased (Abadiaset al., 2008). Although fresh fruit is less frequently involved as vehicles for salmonellosis, there have been reports of multi-state outbreaks of apple, watermelon, lettuce, melon, fruit and vegetable consumption (Bordiniet al., 2007).

2.1.2 Typhoid Toxin

Typhoid toxins belong to the bacterial AB toxins class composed of the enzymatic subunit "A" and the connecting subunit of the receptor "B." Bacterial AB toxins are secreted virulence factors that often perform an important part for the associated bacterial pathogens in virulence. Typhoid

toxin, however, has several characteristics that differ from other toxins of "AB" (Thomas et al., 2014).

Typhoid toxin was found using genome comparison analysis between *S. Typhi* and *S. Typhimurium* (Marlovits and Stebbins 2010). The small island containing all three parts of typhoid secretion is present in the genome of *S. Typhi* but not in the *S. Typhimurium* equivalent (Troisfontaines and Cornelis 2005). *S. Typhi* reflects effective typhoid toxin, as evidenced by the fact that human cells treated with culture supernatants of *S. Typhi* infected cells induce G2 cell cycle detention due to the method of typhoid toxin intoxication (Marlovits TC and Stebbins CE, 2010).

2.1.2 Infections caused by Salmonella

2.1.2a Gastroenteritis

Salmonella types other than *S. Typhi* and *S. Paratyphi* are known as non-typhoidal *Salmonella* (NTS) infections and are discovered predominantly in reservoirs of animals. NTS diseases are defined by gastroenteritis or' stomach flu,' a gastrointestinal tract inflammatory disorder that is followed by signs such as non-bloody diarrhoea, vomiting, nausea, headache, abdominal cramps and myalgia. In individuals infected with NTS, symptoms such as hepatomegaly and splenomegaly are less frequent (Hohmann, 2001). NTS diseases have a smaller incubation period (6-12 h) compared to typhoid diseases and the symptoms are generally self-limiting and last for 10 days or less. (Crump et al., 2008). Gastrointestinal complications of NTS infections include cholecystitis, pancreatitis, and appendicitis, while terminal ileum perforation is not associated with NTS (Hohmann, 2001). Children, older people and immunocompromised patients are extremely prone to NTS diseases and have more serious diseases than normal people (Scallan et al., 2011).

2.1.2b Salmonellosis

Salmonellosis is a form of gastroenteritis. After a short incubation period of 12 to 72 hours, the disease begins. Most individuals infected with non-typhoidal *Salmonella* acquire fever, vomiting, abdominal cramps, and particularly diarrhoea that may be bloody and may contain mucus. The disease takes 4-7 days in most instances and most individuals recover without therapy. The diarrhea may, however, be so severe in some cases that the patient becomes dangerously dehydrated. Children and individuals with compromised immune systems in particular are prone

to serious diseases of non-typhoidal *Salmonella* species and may acquire typhoid fever like illness (Thomas et al., 2014).

2.1.2c Bacteraemia

Salmonella bacteraemia is a disorder in which the bacteria enter the bloodstream after the intestinal barrier has been invaded. Nearly all salmonella serotypes can trigger bacteraemia, Whereas *S. Dublin* & *S. Cholearaesuis* are two invasive species extremely connected with bacteraemia manifestations (Woods et al., 2008). High fever is the characteristic symptom of bacteraemia, similar to enteric fever, but without the formation of rose spots as seen in enteric fever patients. The immune response triggered by bacteraemia may result in septic shock with a increased fatality rate under severe conditions. Bacteraemia clinical sign is frequently seen in diseases with NTS compared to diseases with typhoid *Salmonella* infections (S. –K. Eng et al., 2015). It is thought that the distinction in the clinical expression is connected with the presence of spv (*Salmonella* plasmid virulence) based on genetic analysis, NTS gene that produces non-typhoidal bacteraemia (Guiney and Fierer, 2011).

2.1.2d Salmonella Typhi and Typhoid Fever

Salmonella Typhi is the etiologic agent of the human systemic infectious disease typhoid fever. *Salmonella ParaTyphi* is the closely related serovar that causes a paratyphoid fever-like disease (Buckle GC et al., 2012). There are human-restricted diseases of both *S. Typhi* and *S. ParaTyphi* (Crump and Mintz 2010; Darton et al. 2014). In comparison, non-typhoidal serovars of *Salmonella* such as *S. Typhimurium* infect humans and animals and cause gastroenteritis recognized as salmonellosis in humans (LaRock DL *et al.,* 2015; Dougan G and Baker S, 2014). About 90 percent of the genomes of *S. Typhi* and *S. Typhimurium* are homologous and few genes are peculiar to the *S. Typhi* genome. A significant large *S. Typhi* specific area is *Salmonella* pathogenicity island 7 (SPI-7) encoding the viaB locus composed of 10 genes linked to the biosynthesis and transfer of Vi polysaccharide capsule involving circumvention of Toll-like receptor-mediated immune surveillance (Raffatellu M et al., 2005; Wangdi T et al., 2014). Another notable small island is the locus encoding five genes, including three parts of typhoid toxins, a homologous bacteriophageal muramidase regulating elements of typhoid toxin secretion, a bacteriophage muramidase homolog controlling typhoid toxin secretion typhoid

toxin components, a bacteriophage muramidase homolog controlling typhoid toxin secretion and a small gene of unknown function (Spano S *et al.*, 2008; Hodak H and Galan JE, 2013).

Acute-phase symptoms of typhoid fever includes fever and abdominal pain, immunologic symptoms such as leukopenia and in some instances neurological complications (Crump JA *et al.,* 2015). Neuropsychiatric manifestations have been observed at a range of 5% to 84% (Chong *et al.,* 2017).

2.1.3 Mechanism and Mode of Salmonella Infection

Salmonella is largely affected by the low pH of the stomach and therefore has a rather elevated infectious dose of approximately 10^5 bacteria that certainly leads to infection, but reduced figures may already trigger infection when *Salmonella* enters the intestine and its infectious process begins (Golubeva, et al., 2012).

The first significant stage is to break into the epithelium of the intestine. *Salmonella* can enter M cells, M cells can then pass *Salmonella* to the inner immune cells, dendritic cells (DC) that transmit their dendrities through the intestinal epithelium can also capture it in the lumen, *Salmonella* may actively invade enterocytes, a mechanism dependent on a separate system of virulence (Fass and Groisman, 2009). Infected cells will be extruded into the intestinal lumen during gastroenteritis, re-infecting and replicating *Salmonella* within enterocytes, enabling *Salmonella* to re-populate but leaving the epithelial layer damaged (Bulmeret al., 2012; Walthers D et al., 2011). *Salmonella* may utilize host-derived ethanolamine as a carbon source under inflammation circumstances (Bustamante et al., 2008). To do so, tetrathionate is needed, which is a by-product of PMN discharge of ROS within the intestinal lumen. *Salmonella* utilizes it as a respiratory electron acceptor, enabling ethanolamine to grow anaerobically (Osborne and Coombes 2011). *Salmonella* produces enterobactin but is also capable of producing an enterobactin glycosylated derivative called salmochelin that is not prone to lipocalin-2 (Martinez et al., 2011).

Salmonella can also partially prevent defensins and other cationic antimicrobials by altering its own LPS charge (Liu et al., 2010). *Salmonella* basically lies inside host cells during the systemic infection stage. Hence, processes were created to withstand adequate protection of the cell and to cut signals to the immune system. The phagocytic mechanism, with phagolysosomes as the

ultimate phase in which the pathogen becomes digested, is a significant cell barrier to incoming bacteria. It is frequently recognized that *Salmonella* could interfere with this defense mechanism by stopping lysosome fusion and encouraging an alternative SCV maturation pathway (Lawley TD et al., 2006). However, others show that fusion with lysosomes occurs and that precise negative selection of anti-bacterial molecules (Golgi derived) allows *Salmonella* to survive in a compartment derived from the phagocytic pathway (Drecktrah et al., 2006; Lara-Tejero M et al., 2009).

Salmonella may also experience oxidative bursts through periplasmic superoxide dismutases or cytoplasmic catalases / peroxidases (Yu et al., 2010; Chakravortty D et al., 2005) instead of excluding the NADPH cluster from the vacuole as earlier suggested (Haraga A et al., 2008). It also interferes with MHC-I and –II antigen presentation to block the activation of an adaptive immune response (Misselwitz B et al., 2011; Wall DM et al., 2007). Lastly, *Salmonella* can kill DCs in a T3SS-1 dependent manner quickly and effectively (Figueira R and Holden DW, 2012).

2.1.4 Detection and Isolation of Salmonella

Usually, the gold standard for detecting *Salmonella spp* from food includes pre-enrichment, selective enrichment, isolation on specific agar cultures and verification of presumptive beneficial cells using biochemical and serological studies, this is a conventional method. As a result, methods for *Salmonella* spp detection based on polymerase chain reaction (PCR) have gained popularity. PCR-based techniques, however, are restricted to qualitative determination. Borowsky and Cardoso (2007) indicated that the quantification of micro-organisms in food specimens is essential for consumer hazard assessment. Hence, the most probable number (MPN) technique that estimates the amount of microorganisms based on the probability integrated for PCR product quantification.

2.2 Escherichia Coli

Escherichia coli is a bacterial commensal of a multitude of animals, including humans, in the intestinal microflora. Not all types of *E. coli* are benign, however, as some can cause disease in humans, mammals and birds. There are two classifications of pathogenic *E. coli* types, those causing intestinal pathologies and those causing extraintestinal pathologies, intestinal pathologies comprise mainly of more or less serious diarrhea induced by various *E. coli* pathotypes such as enterotoxinogenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E.*

coli (EHEC), which may develop into a haemolytic uremic disease (HUS) for EHEC diseases (Kaper et al., 2004). EHEC serotype strains O157.H7 are a developed zoonotic pathogen among these pathotypes. Farm animals, namely cattle, are well known to represent as a reservoir for EHEC O157.H7 (Manning et al., 2008, Friesema et al., 2010). In North America, EHEC is projected to cause fewer than 75,000 human infections and 17 outbreaks annually (Manning et al., 2008). These diseases may develop into an HUS, resulting in mortality in about 5% of fatalities.

2.2.1 Pathotypes of Escherichia Coli

Some *E. coli* isolates have been involved in a wide range of diseases affecting either animals or humans worldwide. There are eight pathovars and there has been wide study of their disease mechanisms. Six prominent pathotypes are: Enteropathogenic *E. coli* (EPEC), Enterohaemorrhagic *E. coli* (EHEC), Enterotoxigenic *E. coli* (ETEC), Enteroinvasive *E. coli* (EIEC; including *Shigella*), Enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC) are diarrhoeagenic and two pathovar uropathogenic *E. coli* (UPEC) and neonatal meningitis *E. coli* (NMEC) are the most prevalent ExPEC isolates (Croxen and Finlay 2010).

2.2.1a Enteropathogenic *Escherichia Coli* (EPEC)

EPEC in emerging countries is a significant source of possibly deadly diarrhoea in infants. They have no colonizing factors and do not genererate from Heat Stable (ST) or Heat Labile (LT) toxins. They generate an intimine labeled non-fimbrial adhesion, an outer membrane protein that mediates the ultimate compliance phases (Kaper et al., 2004). Although, LT or ST toxins are not produced, there are reports that they create an enterotoxin comparable to *Shigella's*. Other virulence factors may be associated with *Shigella*. Adherence of EPEC strains to the intestinal mucosa is a very complex method and generates drastic impacts in the cells ultra-structure leading in actin rearrangements near adherent bacteria. The phenomenon is sometimes referred to as "attaching and effacing'' of cells. EPEC strains are said not to be as invasive as *Shigella* and, unlike ETEC or EAEC, cause an inflammatory response. Diarrhoea and other symptoms of EPEC infections are likely caused by host cell bacterial invasion and interference with normal cellular signal transduction, rather than toxin production. In Mexico and North Africa, they are a major source of traveler's diarrhoea (Kaper et al., 2004).

2.2.1b Shiga Toxin-Producing *E. Coli* (STEC)

Shiga toxin-producing *E. coli* (STEC) is a zoonotic pathogen that colonizes mostly cattle and small ruminants, but, the most commonly known cause of *E. coli* O157 infection is cattle produce particularly beef, other sources include unprocessed fruits and vegetables (Rangel et al., 2005). The mechanism of *E. coli* O157 can be defined in three leaf attachments. In contrast to non-pathogenic *E. coli*, STEC O157:H7 heavily adheres to spinach leaves, tomato skin and alfalfa sprout roots; curli can adhere to these substrates (Jeter and Matthysse, 2005). Curli activity on non-pathogenic *E. coli* surfaces has been shown to be adequate to enable bacterial attachment to alfalfa roots; however, the removal of curli genes in *E. coli* O157 has not nullified adhesion, suggesting that other attachment factors have been involved (Jeter and Mattysse, 2005).

Flagella is also involved in the leaf attachment of *E. coli* O157 as removing *fliC* encoding flagellin decreases the adhesion rate (Xicohtencatl-Cortes et al., 2009). Altogether, this information suggests that *E. coli* O157 has several processes for colonizing crops and is also well adapted to the biosphere, and that *E. coli* O157 also has the capacity to enter the sub-stomatal cavity and spongy mesophyll and survive in the environment (Jablasone et al., 2005; Franz et al., 2007; Xicohtencatl-Cortes et al., 2009).

2.2.1c Enteroaggregative *E. Coli* and Enterotoxigenic *E. Coli*

Enteroaggregative *E. coli* (EAEC) in the United States of America may be the cause of bacterial gastroenteritis, although the source is unknown (Wilson *et al*., 2001; Nataro *et al*., 2006). Recently, however, Berger et al (2009) noted the following two patterns of bacteria distribution after an hour of EAEC incubation with lettuce, They include (i) diffuse epidermis adherence and (ii) localized stomach cell adherence. Research using certain mutants has shown that the AAF pilus, thought to play a part in the colonization of the human intestine, makes it feasible to join the epidermis, but flagellae makes it necessary to aggregate around the stomata (Berger et al., 2009b).

Enterotoxigenic *E. coli* (ETEC) is a major source of diarrheal for children and travelers' (Qadri et al., 2005) and contributes to severe bacterial diarrheal in calves and piglets (Nagy and Fekete, 2005). Flagella is the primary adherence mediating attachment of Enterotoxigenic *E. coli* to the lettuce leaves epidermis (Shaw et al., 2010).

2.2.2 Comparison Of *E. Coli* Isolates

Because of the variation of genome remodeling and horizontal acquisition from other pathogenic bacteria, large-scale comparison of *E. coli* isolates is difficult (Ron 2010). Such a degree of variation can render it difficult to determine the degree to which particular isolates are linked (Manges, 2010). Kauffmann (O, K, H) serotype classification was used to describe the isolates of *E. coli* (Louise et al., 2011). Food is a significant cause of contamination of the human intestinal tract by animal-derived *E. coli* strains (Louise *et al., 2011*). Additional requirements such as migration of outer membrane protein models, plasmid content or virulence-associated features (haemolysin production, haemagglutination, fimbrial adhesive manufacturing and other phenotypes) have been progressively introduced to the comparison of human and animal isolates (Louise et al., 2011). It emerged that clonal populations were not always correlated with serogroups, but with particular variables of virulence. The existence or lack of particular virulence factors has therefore been accepted and has become a significant means of classifying *E. coli* isolates, and latest trials frequently use these requirements in relation to serotyping and new molecular methodologies (Wang et al., 2010).

Virulence factors associated with *E. coli* causing extraintestinal infections, as strains with very different combinations of virulence factors can cause similar pathologies. For instance, very few virulence factors are prevalent among *E. coli* strains derived from septicaemia instances and different species are probable to harbor a range of separate virulence factors that may provide comparable functions at various phases of the disease cycle (Ron, 2010).

Implementation of molecular biology and technological developments have given better techniques for comparing strains, the latest being the full genome analysis of bacteria (Moriel *et al*., 2010). As complete genome sequencing is not yet a practical option for comparing a large number of isolates, most recent studies use a combination of phylogenetic typing, multilocus sequence typing (Wirth *et al*., 2006), pulsed-field gel electrophoresis typing (Ribot *et al*., 2006), plasmid analysis and diagnostic DNA microarrays (Hamelin *et al*., 2006).

2.2.3 Antimicrobial Resistance in E. Coli

In animal husbandry, antimicrobials have been widely used for disease prevention, infection reduction, and animal growth. The use of antimicrobials as growth promoters in the European Union is no longer allowed, but is still practiced in other parts of the world (Bonnet et al., 2009).

The use of antimicrobials has led in the commensal microbiota choice of anti-microbial resistant *E. coli* that could contaminate people by consuming contaminated meat. Studies of broiler chickens have shown an elevated level of multi-resistant *E. coli* antimicrobial (Diarra *et al*., 2007). Despite the antimicrobial prohibition in Europe, in broiler buildings, antibiotic-resistant strains persist. The use of antimicrobials does not contribute to the choice of virulent species, but rather to the distribution among commensal types of resistant genes. Virulence plasmids and plasmids with multidrug resistance have not been discovered in the same species in a latest research (Bonnet et al., 2009). On the other side, in some APEC species, hybrid resistance plasmids containing various antimicrobial resistance and virulence-related genes have been discovered and these species have been known to infect human cells and trigger meningitis in rodents (Johnson et al., 2010).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Experimental

Samplings

Fresh cut fruits, pineapple and watermelon were collected from various locations of Magboro marketplace, Ogun State, Nigeria. Samples were collected in sterile plastic bags and taken immediately to the laboratory for analysis. Sampling was repeated three times.

Materials

Petri dishes (glass and plastic), conical flasks, beakers, measuring cylinder, micropipette, glass spreader, cork borer, sterile tips, bunsen burner, inoculating loop, cotton wool, 70% ethanol, hand gloves, wash brush, microwave, oven, distiller, autoclave, laminar air flow, waterbath, weighing balance, hot plate, microscope, immersion oil, eppendorf tubes , sterile tips, test tubes, test tube racks, foil paper, glass slide, distilled water, centrifuge, scotch bottles, gram staining kit, oxidase test strip, incubator.

Sterilization of materials

All glassware were thoroughly washed in detergent and properly rinsed with distilled water. The laboratory work bench was swabbed with 70% ethanol to kill all microorganisms and spores before the commencement of work. The glass petri dishes, conical flasks, beakers, measuring cylinders used were sterilized in an oven at 160ºC for 1 h.

Agar Media

Peptone water

Peptone water is a microbial growth medium composed of peptic digest of animal tissue and sodium chloride, also, rich in tryptophan. The pH of the medium is 7.2 ± 0.2 at 25ºC. Peptone water is a non-selective medium which can be used as a primary enrichment medium for the growth of bacteria.

Procedure

Based on the manufacturer's instructions, an amount of the medium is measured and dissolved using distilled water into a conical flask and mixed thoroughly. The conical flask is covered using a cork in the form of foil paper containing cotton wool. The suspension was then heated very quickly to dissolve the medium and then autoclaved at 121ºC for 15 minutes to make it sterile. It was then dispensed into appropriate tubes such as using it for serial dilution and conical flasks such as using it for primary enrichment.

Culture Media

The culture media used for the isolation of *E. coli* includes MacConkey agar, Nutrient agar and sorbitol-MacConkey Agar (SMAC). The culture media used for the isolation of *Salmonella* includes Selenite F and Selenite Z broth, also, *Salmonella-Shigella* agar.

Nutrient agar

Nutrient agar is a multi-purpose medium supporting the growth of a wide range of non-fastidious organisms. It contains the required nutrient for bacterial growth.

Procedure

Based on the manufacturer's instructions, 28g of the medium in 1000 ml of distilled water in a conical flask and mixed thoroughly. The conical flask was then corked, and the suspension heated very quickly to dissolve the medium and autoclaved at 121ºC for 15 minutes for sterility. The medium was allowed to cool in a waterbath at 45ºC-50ºC and poured aseptically into sterile petri dishes and allowed to solidify.

MacConkey agar

MacConkey agar is used for gram negative enteric bacteria isolation and lactose fermentation differentiation from non-lactose fermenting bacteria and lactose fermenting bacteria.

Procedure

Based on the manufacturer's instructions, 48.5g of the medium in 1000 ml of distilled water in a conical flask and mixed thoroughly. The conical flask was then corked, and the suspension heated very quickly to dissolve the medium and autoclaved at 121ºC for 15 minutes for sterility.

The medium was allowed to cool in a waterbath at 45ºC-50ºC and poured aseptically into sterile petri dishes and allowed to solidify.

Sorbitol-MacConkey agar

SMAC is a variant of traditional MacConkey agar used in the detection of *E. coli* O157:H7. It can be used as a selective and differential medium.

Procedure

Based on the manufacturer's instructions, 51.5g of the medium in 1000 ml of distilled water in a conical flask and mixed thoroughly. The conical flask was then corked, and the suspension heated very quickly to dissolve the medium and autoclaved at 121ºC for 15 minutes for sterility. The medium was allowed to cool in a waterbath at 45ºC-50ºC and poured aseptically into sterile petri dishes and allowed to solidify.

Salmonella-Shigella agar

SS agar is moderately selective and differential for the isolation, cultivation and differentiation of *Salmonella* spp and some strains of *Shigella* spp. Sodium thiosulfate is added to the medium as a source of hydrogen sulphide and ferric citrate is added as an indicator for hydrogen sulphide production. The inclusion of bile salts, sodium citrate and brilliant green serve to inhibit gram positive and coliform organisms.

Procedure

Based on the manufacturer's instructions, 63g of the medium in 1000 ml of distilled water in a conical flask and mixed thoroughly. The conical flask was then corked, and the suspension heated very quickly to dissolve the medium and autoclaved at 121ºC for 15 minutes for sterility. The medium was allowed to cool in a waterbath at 45ºC-50ºC and poured aseptically into sterile petri dishes and allowed to solidify.

Selenite F and Z broth

This is the medium used for the selective enrichment of *Salmonella* spp. It is a buffered lactose peptone broth to which Sodium Biselenite is added as the selective agent.

Procedure

19g of the Selenite F is dissolved in 750 ml distilled water in a sterile conical flask as the part A. 4 g of Sodium Biselenite is dissolved in another sterile conical flask as the Selenite Z which is the part B. Both part A and B are mixed together, the medium is then boiled using a hot plate, and then allowed to cool in the waterbath at 50ºC. Then, it is poured into various scotch bottles (90 ml). This media does not require autoclaving.

Sample analysis

Twenty-five gram each of Pineapple and Watermelon were weighed and aseptically transferred in 225 ml of sterile buffered peptone water. The samples were homogenized for 2 mins in a stomacher. Nutrient agar and MacConkey agar was used for the enumeration of mesophiles incubated at 37ºC for 24 h. For the enumeration of *E. coli* O157 and non-O157 sorbitol-MacConkey Agar (SMAC) was used, incubated at 37ºC for 24 h. For *Salmonella* spp enumeration, primary enrichment in buffered peptone incubated at 37ºC for 24 h, followed by secondary enrichment in Selenite F and Selenite Z broth at 37ºC for 24 h, 1 ml of the secondary enrichment was transferred to Salmonella-Shigella agar for presumptive confirmation of *Salmonella* spp.

3.2 Biochemical Tests

Catalase test

This test demonstrates the presence of enzyme catalase in the organism. The enzyme catalase mediates the breakdown of hydrogen peroxide into Oxygen and Water. The presence of enzyme in a bacterial isolate is evident when a small inoculum is introduced into hydrogen peroxide and the rapid effervescence of oxygen bubbles occur. The lack of catalase is indicated by a lack of bubble production.

Procedure

A smear of the isolate was made on a grease free glass slide, after which a loopful of hydrogen peroxide was dropped on the slide, then mixed together. The absence of gas bubbles indicates a negative reaction.

Oxidase test

This test is used to identify bacteria that produces cytochrome c oxidase, an enzyme of the bacterial electron transport chain. When present, the cytochrome c oxidase oxidizes the reagent (tetramethyl-p-phenylenediamine) to (indophenols) purple color end product. When the enzyme is not present the reagent remains reduced and colorless.

Gram staining

This was carried out to differentiate gram negative from gram positive organisms. A wire loop was sterilized in Bunsen burner and allowed to cool then a loopful of growth was collected from the agar plate and applied on a clean grease-free slide. Then a drop of sterile distilled water was added, emulsified and heat fixed by passing over a flame three times. The smear was flooded with crystal flooded violet (primary stain) for 30-60 seconds then washed with water. Then it was flooded with iodine for 30-60 seconds and then washed with water. It was then decolorized with acetone until no color runs off the slide and rinsed immediately. The slide was then covered with safranin (counter stain) for 60 seconds and then washed off with clean water. The slide was then kept in a rack to air dry after wiping the back with cotton wool. The stained smear was then examined microscopically under oil immersion at ×100 objective lens. Gram positive bacteria appeared purple while gram negative appeared pink.

3.3 Preservation of Isolates

For long term preservation, a loopful of each isolate was inoculated into 5 ml buffered peptone water (BPS) containing 10% glucose and then incubated for 18 hours, then the suspension was added to eppendorf tubes containing sterile 20% glycerol as cryoprotectant and homogenized for 10 s using a vortex, then it was stored at -20ºC.

3.3.1 Genotypic Characterizations of The Isolates

DNA extraction

Each isolate were streaked out on Nutrient agar and incubated overnight at 37ºC. the loopfuls actively dividing cells were emulsified in 500 ml double distilled water until it was turbid. The cell suspension were then kept in a boiling water bath for 10 minutes. After cooling for 20 minutes, the suspension was centrifuged at 15,000 rpm for 15 minutes and the supernatant was used as template DNA.

PCR PROTOCOL

16S rRNA amplification

Partial 16S rRNA gene amplification using forward primer fD1 (5'-AGA GTT TGATCC TGG CTC AG-3') and reverse primer rD1 (5'-AAG GAG GTG ATC CAG CCG CA-3') (Weisburg *et al*., 1991). The components of the PCR and constituent mixes were summarized in Table 222 below. The PCR was carried with initial denaturation at 95ºC for 5 min; 35 cycles of 95ºC for 2 min; 42ºC for 30 s and 72ºC for 4 min; and a final elongation step at 72ºC for 10 min. The PCR products were confirmed by electrophoresis and visualized under UV light with a Gel Doc system (Cleaver Scientific Ltd, United Kingdom).

Table 3.1 - PCR reaction components used for 16S rRNA amplification

No.	Code		
1		**Component**	**1 Reaction**
2		**Mastermix**	**5**
3		**FDl**	**0.4**
4		**rDl**	**0.4**
5		**DNA**	**1**
6		**RNaseFreeH_2O**	**3.2**
7		**Total**	**10**

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

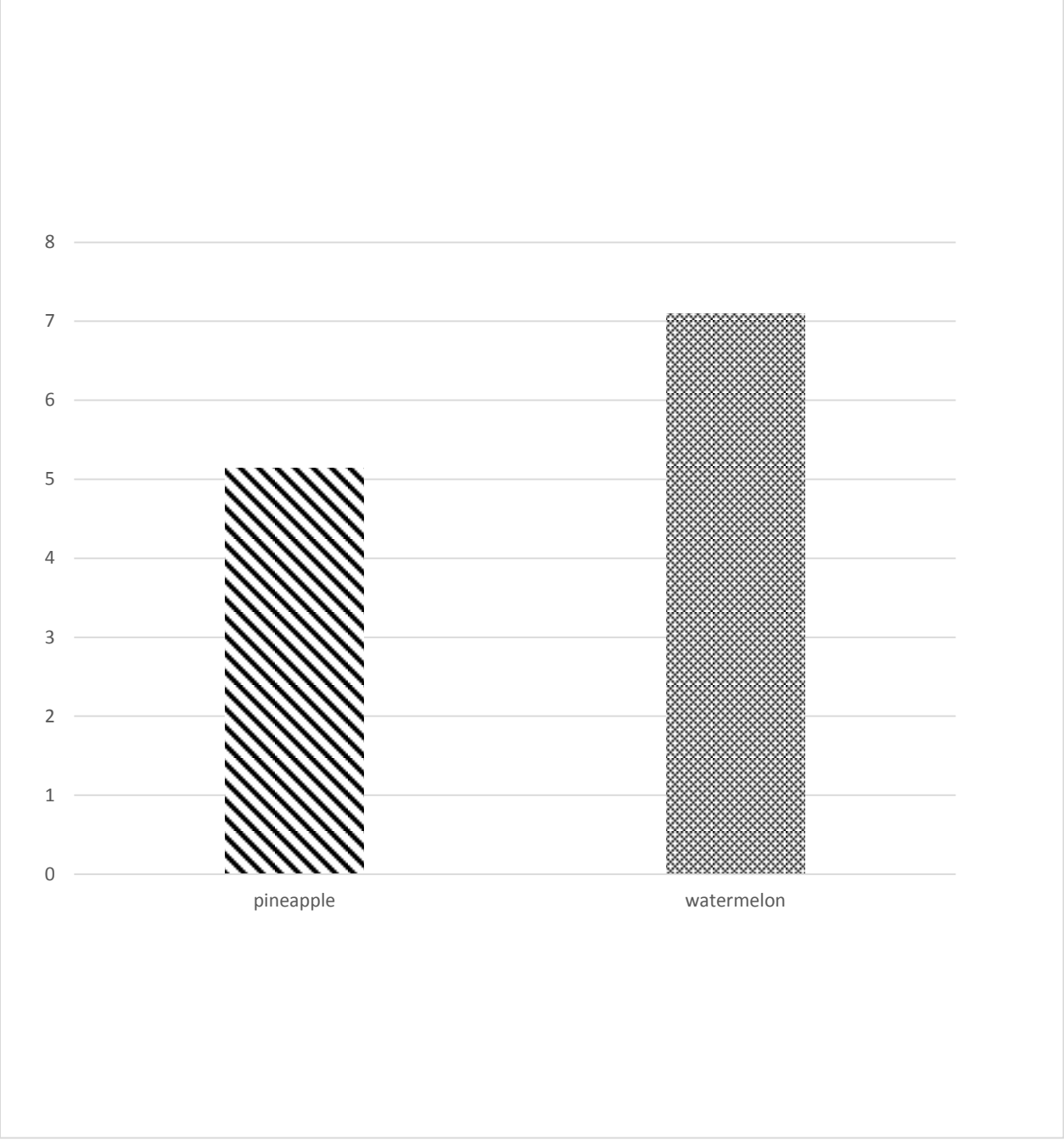

Figure 4.1: Occurrence of *E. coli* in fresh cut pineapple and watermelon

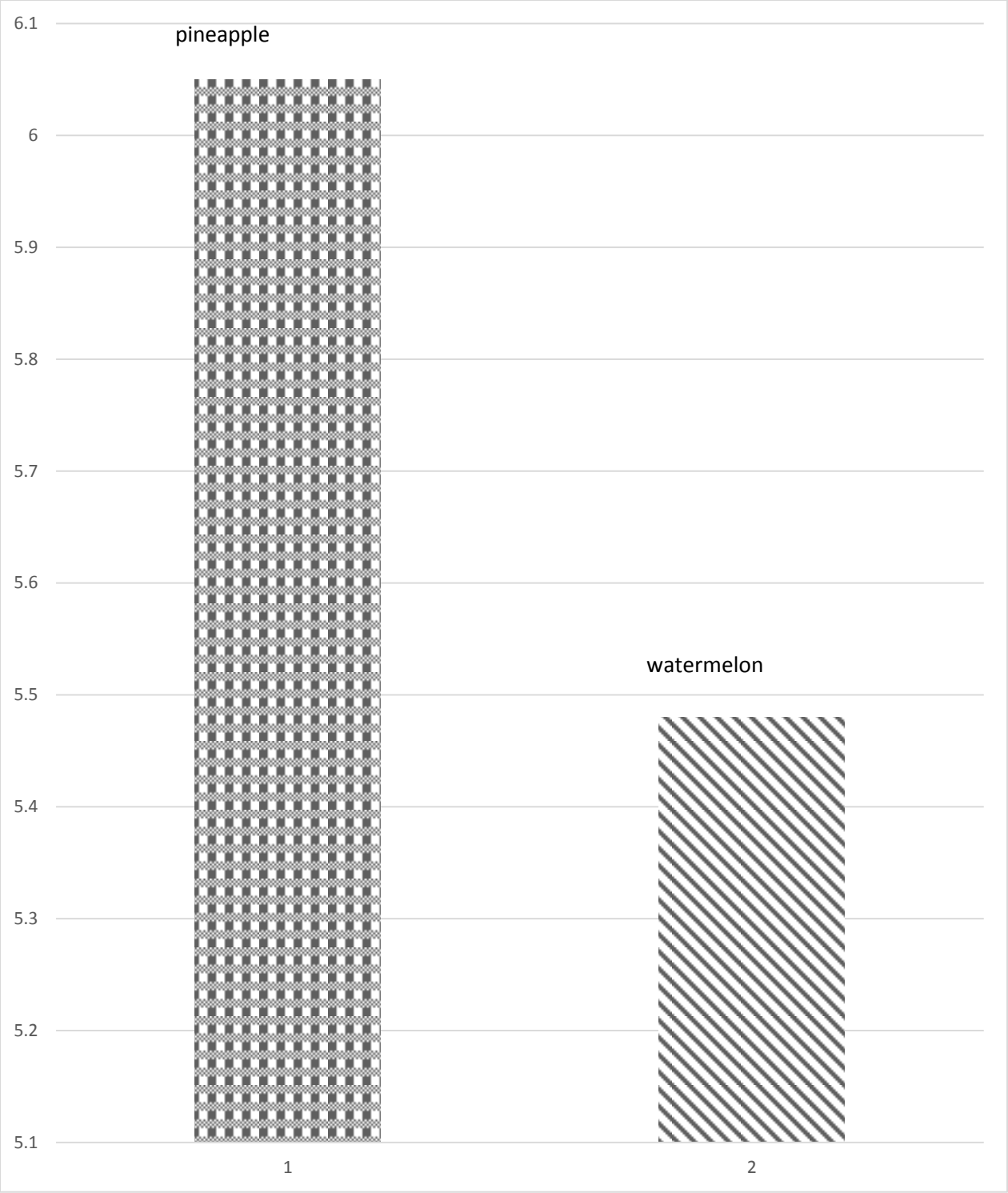

Figure 4.2: Occurrence of shiga toxin-producing *Escherichia coli* in fresh cut pineapple and watermelon.

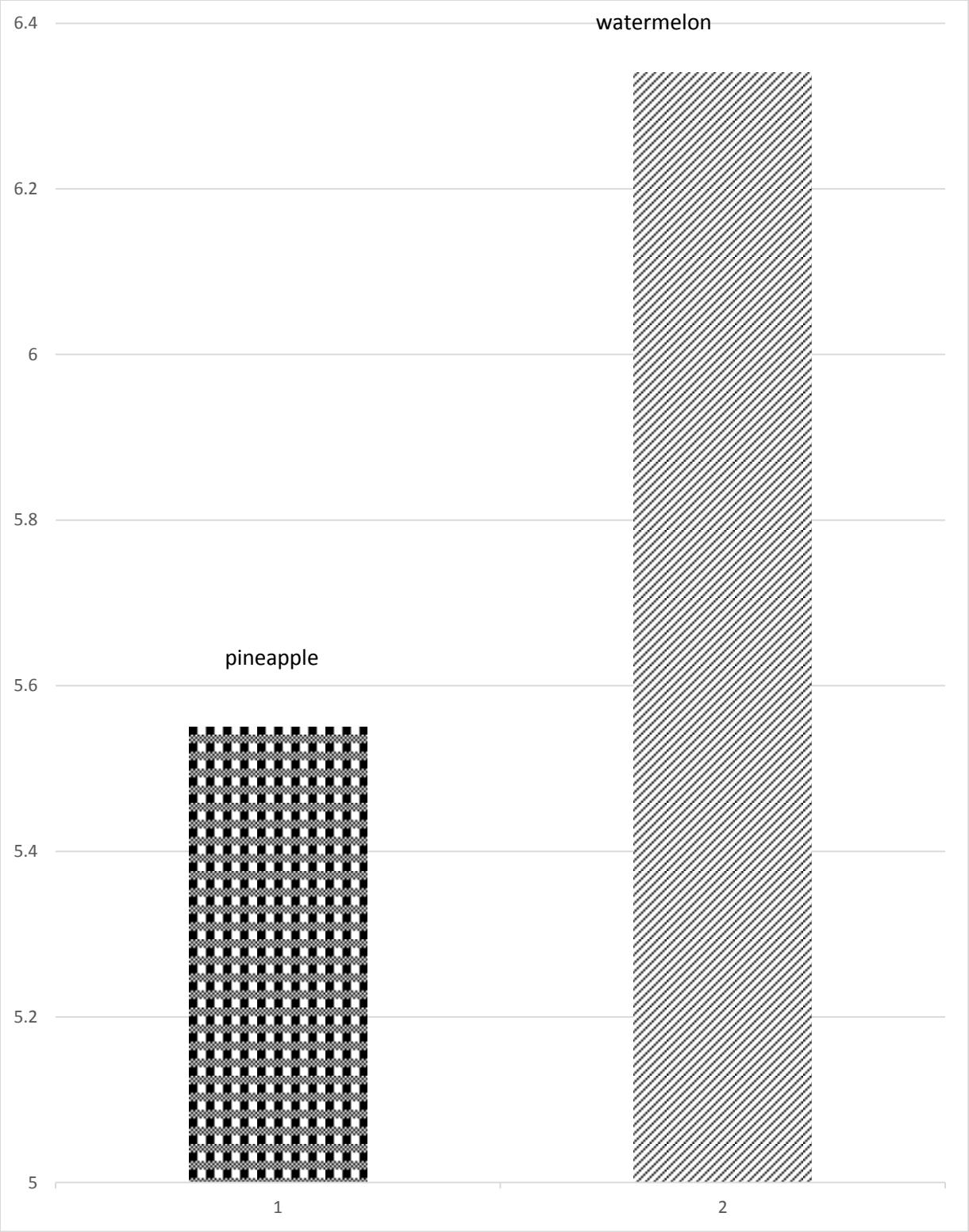

Figure 4.3: Total viable counts (TVC) in fresh cut pineapple and watermelon

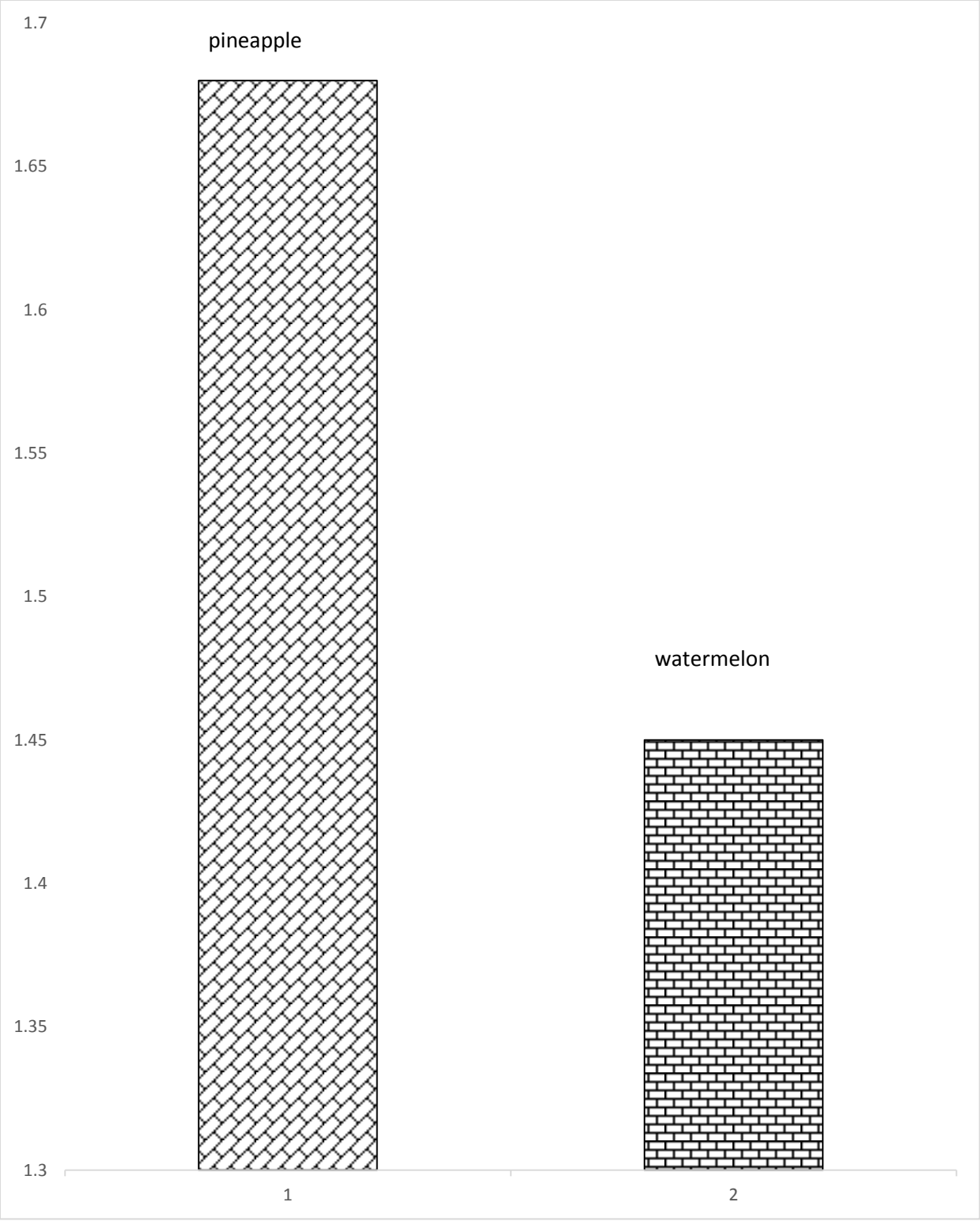

Figure 4.4: Occurrence of *Salmonella* in fresh cut pineapple and watermelon.

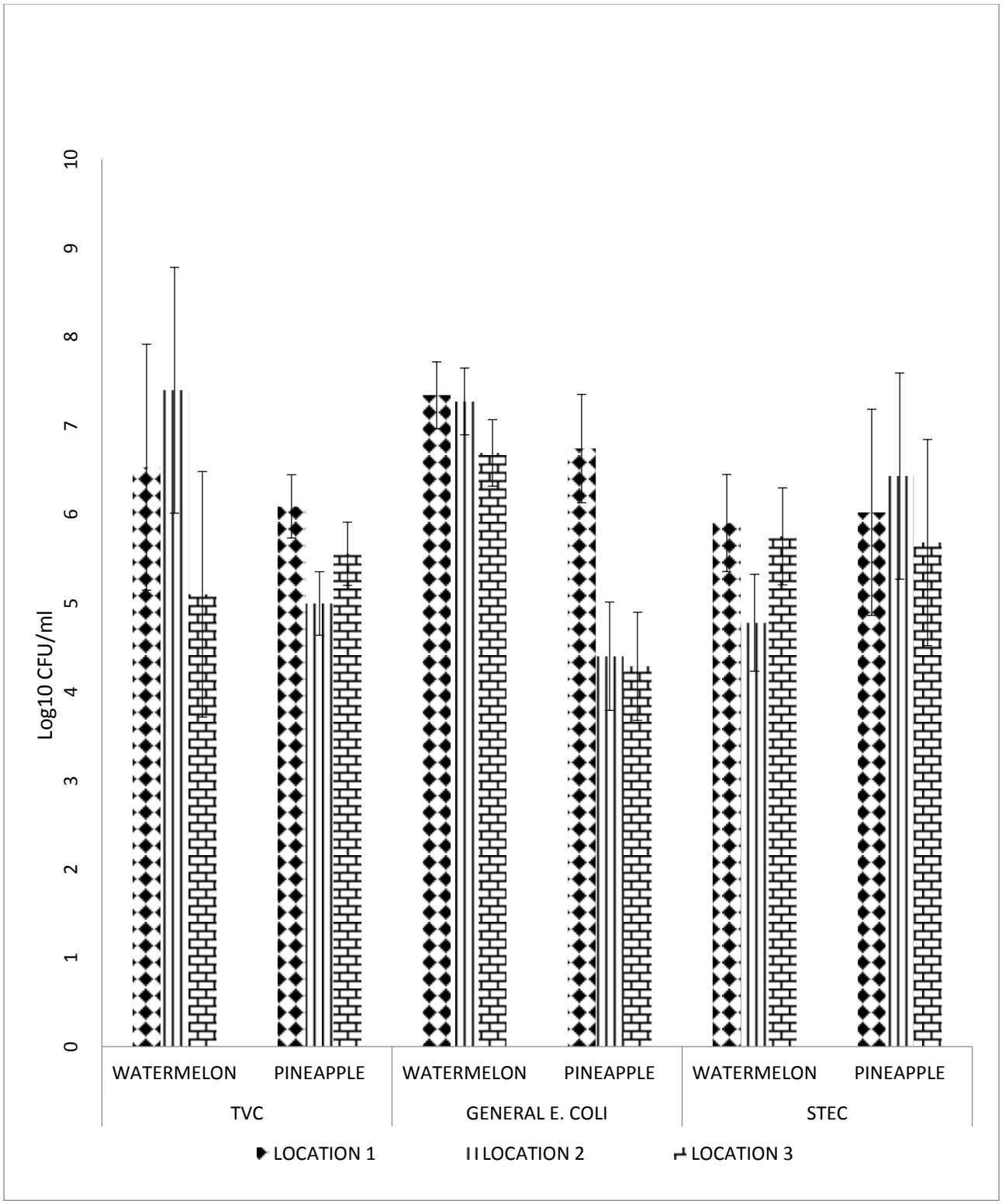

Figure 4.5: Occurrence of *E. coli,* total viable counts (TVC) and shiga toxin producing *E. coli* from the three different locations.

4.1 Biochemical Identification of Isolates

Isolate code	Colour	Shape	Size	Elevation	Appearance
PA01	Pink	Circular	Puntiform	Raised	Shiny
PA02	Pink	Circular	Puntiform	Raised	Shiny
PA03	Pink	Circular	Puntiform	Raised	Rough
PA04	Pink	Circular	Puntiform	Convex	Shiny
PA05	Pink	Circular	Puntiform	Convex	Shiny
PA06	Pink	Circular	Medium	Raised	Rough
PA07	Pink	Circular	Puntiform	Convex	Smooth
PA08	Pink	Circular	Puntiform	Raised	Shiny
PA09	Pink	Circular	Medium	Raised	Smooth
WM01	Pink	Circular	Medium	Convex	Smooth
WM02	Pink	Circular	Puntiform	Raised	Rough
WM03	White	Circular	Puntiform	Raised	Smooth
WM04	White	Circular	Puntiform	Raised	Smooth
WM05	Pink, white	Circular	Puntiform	Raised	Smooth
WM06	Pink	Circular	Puntiform	Raised	Smooth
WM07	Pink	Circular	Medium	Convex	Shiny
WM08	Pink	Circular	Medium	Raised	Shiny
WM09	Pink	Circular	Medium	Raised	Rough

Table 4.1: Morphological characteristics of isolates on sorbitol-MacConkey agar

Isolate code	Colour	Shape	Size	Elevation	Appearance
PA01	Black, yellow	Circular	Medium	Raised	Shiny
PA02	Black	Circular	Medium	Raised	Shiny
PA03	Black, yellow	Circular	Puntiform	Convex	Rough
PA04	Yellow	Circular	Puntiform	Convex	Shiny
PA05	Yellow	Circular	Puntiform	Convex	Smooth
PA06	Yellow	Circular	Medium	Raised	Rough
PA07	Yellow	Circular	Puntiform	Convex	Shiny
PA08	Yellow	Circular	Puntiform	Raised	Shiny
PA09	Yellow	Circular	Medium	Convex	Smooth
WM01	Yellow	Circular	Medium	Convex	Smooth
WM02	Yellow	Circular	Medium	Raised	Rough
WM03	Black, yellow	Circular	Medium	Raised	Smooth
WM04	Yellow	Circular	Puntiform	Raised	Smooth
WM05	Yellow	Circular	Puntiform	Raised	Smooth
WM06	Black	Circular	Puntiform	Raised	Rough
WM07	Yellow	Circular	Medium	Raised	Rough
WM08	Yellow	Circular	Medium	Raised	Rough
WM09	Yellow	Circular	Medium	Raised	Rough

Table 4.2: Morphological characteristics of isolates Salmonella-Shigella agar

AGAR (MacConkey)	ISOLATE CODE	CATALASE TEST	OXIDASE TEST	GRAM REACTION
LOCATION	PA01	+	-	-
1-3	PA02	+	-	-
	PA03	+	-	-
	PA04	+	-	-
	PA05	+	-	-
	PA06	+	-	-
	PA07	+	-	-
	PA08	+	-	-
	WM01	+	-	-
	WM02	+	-	-
	WM03	+	-	-
	WM04	+	-	-
	WM05	+	-	-
	WM06	+	-	-
	WM07	+	-	-
	WM08	+	-	-

Table 4.3: Biochemical Identification of Isolates

AGAR (SMAC)	ISOLATE CODE	CATALASE TEST	OXIDASE TEST	GRAM REACTION
LOCATION	PA01	+	-	-
1-3	PA02	+	-	-
	PA03	+	-	-
	PA04	+	-	-
	PA05	+	-	-
	PA06	+	-	-
	PA07	+	-	-
	PA08	+	-	-
	PA09	+	-	-
	PA10	+	-	-
	PA11	+	-	-
	PA12	+	-	-
	WM01	+	-	-
	WM02	+	-	-
	WM03	+	-	-
	WM04	+	-	-
	WM05	+	-	-
	WM06	+	-	-
	WM07	+	-	-
	WM08	+	-	-
	WM09	+	-	-
	WM10	+	-	-
	WM11	+	-	-
	WM12	+	-	-

Table 4.4: Biochemical identification of isolates

AGAR (NA)	ISOLATE CODE	CATALASE TEST	OXIDASE TEST	GRAM REACTION
LOCATION	PA01	+	-	-
1-3	PA02	+	-	-
	PA03	+	-	-
	PA04	+	-	-
	PA05	+	-	-
	PA06	+	-	-
	PA07	+	-	-
	PA08	+	-	-
	PA09	+	-	-
	PA10	+	-	-
	PA11	+	-	-
	PA12	+	-	-
	WM01	+	-	-
	WM02	+	-	-
	WM03	+	-	-
	WM04	+	-	-
	WM05	+	-	-
	WM06	+	-	-
	WM07	+	-	-
	WM08	+	-	-
	WM09	+	-	-
	WM10	+	-	-
	WM11	+	-	-
	WM12	+	-	-

Table 4.5: Biochemical identification of isolates

AGAR (Salmonella-Shigella)	ISOLATE CODE	CATALASE TEST	OXIDASE TEST	GRAM REACTION
LOCATION 1-3	PA01	+	-	-
	PA02	+	-	-
	PA03	+	-	-
	PA04	+	-	-
	PA05	+	-	-
	PA06	+	-	-
	PA07	+	-	-
	PA08	+	-	-
	PA09	+	-	-
	PA10	+	-	-
	PA11	+	-	-
	PA12	+	-	-
	WM01	+	-	-
	WM02	+	-	-
	WM03	+	-	-
	WM04	+	-	-
	WM05	+	-	-
	WM06	+	-	-
	WM07	+	-	-
	WM08	+	-	-
	WM09	+	-	-
	WM10	+	-	-
	WM11	+	-	-
	WM12	+	-	-

Table 4.6: Biochemical identification of isolates

FOOD	PRESENCE OF *SALMONELLA*		
SAMPLE CODE	**LOCATION 1**	**LOCATION 2**	**LOCATION 3**
PA01SS	-	-	-
¤			
PA02SS	+	_	+
PA03SS	+	+	_
PA04SS	_	+	_
¤			
WM01SS	+	+	_
¤			
WM02SS	_	_	_
WM03SS	+	+	_
WM04SS	+	+	+

Table 4.7: Detection of *Salmonella*

Figure 4.6: Detection of *Salmonella* on Salmonella-Shigella agar plate

16S PCR AMPLIFICATION

The genetic characterization shows DNA band on agarose gel of the presumptive Salmonella spp and pathogenic E. coli after PCR amplification. This confirms the results from the morphological and biochemical test. The DNA ladder and DNA bands for Salmonella and E coli are shown in figure 4.7

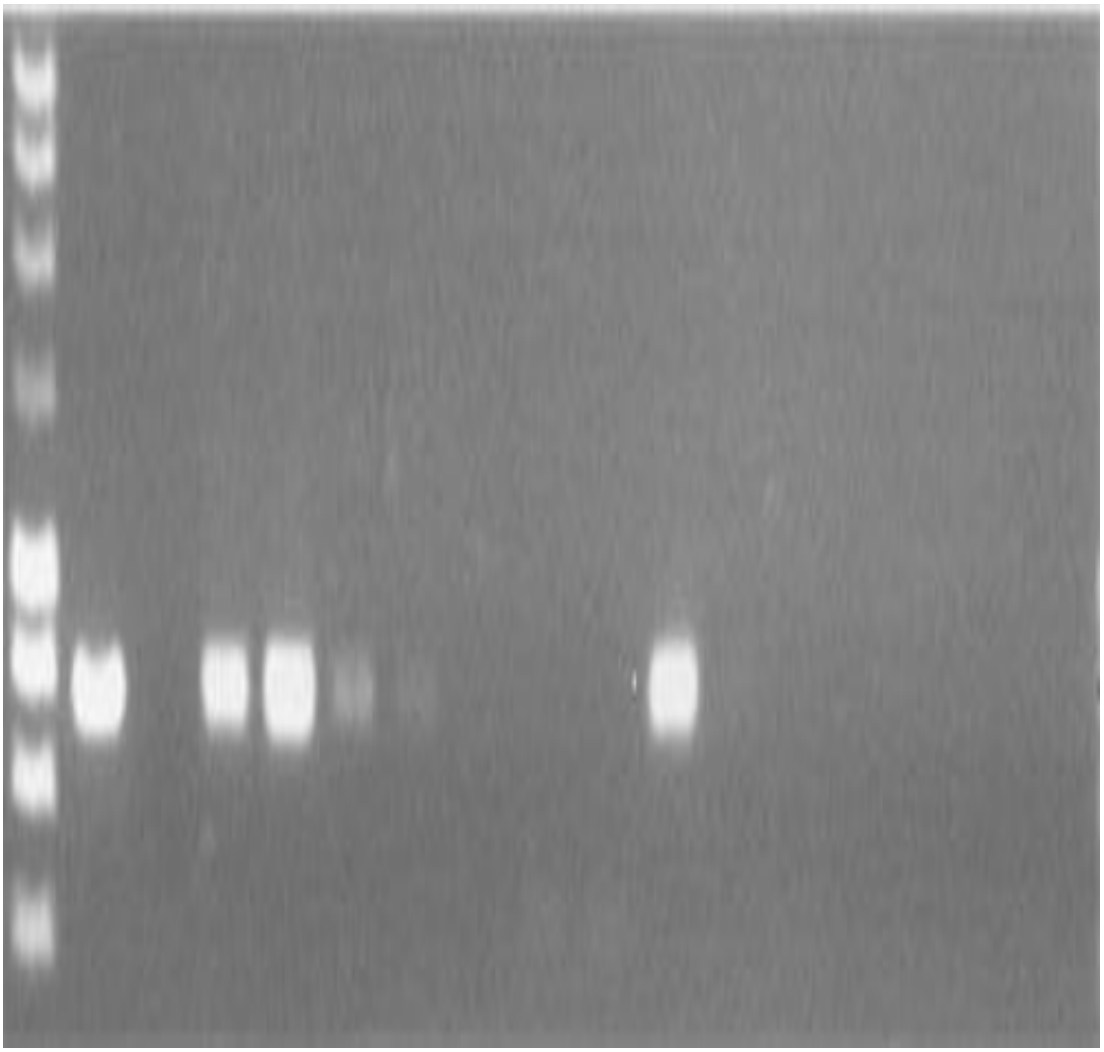

Figure 4.7: DNA ladder

4.1 DISCUSSION

The microbial analysis of the samples obtained from Mokoloki LCDA, Ogun State, was carried out for total viable counts, general *E. coli,* shiga toxin-producing *E.coli* and *Salmonella* species. The results of the findings were summarized in chapter four, figure 4.1 shows that the occurrence of general *E. coli* in fresh cut pineapple was lower than that in fresh cut watermelon, 5.14 log10 CFU/g and 7.1 log10 CFU/g respectively. Figure 4.2 shows the occurrence of shiga toxin-producing *E. coli* in fresh cut pineapple 6.1 log10 CFU/g and 5.5 log10 CFU/g was higher than that in watermelon. Figure 4.3 shows the total viable counts of fresh cut watermelon was higher than that in pineapple, 6.3 log10 CFU/g and 5.6 log10 CFU/g respectively,figure 4.4 shows the occurrence of *Salmonella* in fresh cut pineapple was higher than that in watermelon, 1.7 log10 CFU/g and 1.5 log10 CFU/g. Table 4.1 shows the morphological characteristics of samples cultured on sorbitol-MacConkey agar and Table 4.2 shows the morphological characteristics of isolates on Salmonella-Shigella agar collected from food samplings from three locations in Mokoloki, LCDA, Ogun State. While Table 4.3, 4.4, 4.5 and 4.6 shows the results of the biochemical characterization of the 16 isolates each that were suspected to be *E. coli* and *Salmonella* based on the selective media used for identification such as MacConkey agar, sorbitol-MacConkey agar, Nutrient agar and Salmonella Shigella agar, using catalase and oxidase test alongside gram stain results from using spread plate method with bacterial isolates collected from food samplings from three locations. All the isolates were catalase positive and oxidase positive, they were also identified as gram negative bacteria. Table 4.7 shows the identification of isolates with black colonies *Salmonella*. Figure 4.6 shows the detection of *Salmonella* in Salmonella Shigella agar as black colonies. Figure 4.7 shows the DNA ladder from the 16S PCR amplification which further confirms the results from the morphological and biochemical test of the isolates.

According to international regulations (Gilbert et al., 2000), *Salmonella* species should be absent in 25g of food samples. The presence of *Salmonella* in 25g of pineapple and watermelon will pose a threat to consumers thereby resulting to illness. The presence of pathogenic *E.coli* is also a major health concern.

CHAPTER FIVE

5.1 CONCLUSIONS

This project aimed at isolating *E. coli* and *Salmonella* from fresh cut fruits by using selective enrichment broth and selective media. For this purpose, fresh cut fruit samples such as pineapple and watermelon were tested. In all the samples analysed, 45 isolates were isolated. The prevalence rate of shiga toxin-producing *E. coli* (STEC) in pineapple was found to be higher than that in watermelon while the detection level of *Salmonella* in pineapple was also higher than that in watermelon. All the isolates were catalase positive and oxidase negative. The gram stain reaction indicated that the isolates are gram negative pathogenic bacteria. The results of the 16S rRNA amplification further confirmed the identity of *Salmonella spp* and STEC. Based on the risk of the presence of *E. coli* and *Salmonella* from fresh cut fruits, contamination might be due to watering these fruits with contaminated water, lack hygiene while transport. Prevention strategies may include the use of hand washing sanitizer, controlled and supervised handling of fresh cut fruits and the separation of the fruits to avoid cross-contamination. All these may help to reduce the risk of transmission of this pathogens and prevent public health hazard.

5.2 RECOMMENDATION

Considering the low infective dose of these organisms and the fact that these fresh cut fruits can be consumed raw, there should be proper health awareness for the general public in which they are educated on the risk involved with the consumption of contaminated fresh cut fruits. Also, the market people should be educated on personal hygiene, proper handwashing and the use of clean wash water for the fruits to avoid cross contamination.

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